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# Regulation of GABA transporter GAT-1 in neuronal cells: role of Brain-Derived Neurotrophic Factor and Adenosine

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Tese orientada pela Professora Doutora Ana Maria Sebastião

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**Cover illustration:**

"Neuroglia of the pyramidal layer and stratum radiatum of the Ammon horn. Adult man autopsied three hours after death. Chloride of gold. (*A*) big astrocyte embracing a pyramidal neuron. (*B*) twin astrocytes forming a nest around a cell (*C*), while one of them sends two branches forming another nest (*D*). (*E*) cell with signs of autolysis" (drawing by Ramón y Cajal, 1899)

## PUBLICATIONS

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

- **Vaz SH**, Rasmussen TN, Cristóvão-Ferreira S, Duflot S, Ribeiro JA, Gether U, Sebastião AM. (2011) Brain-derived neurotrophic factor modulates trafficking of GAT-1 to the plasma membrane of rat cortical astrocytes, enhancing GAT-1-mediated GABA transport. J Biol Chem. (*In press*).
- **Vaz SH**, Cristóvão-Ferreira S, Ribeiro JA, Sebastião AM. (2008) Brain-derived neurotrophic factor inhibits GABA uptake by the rat hippocampall nerve terminals. Brain Res. 1219:19-25.

**Other publications closely related to the content of this thesis:**

- 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. (2011) Adenosine A1R-A2AR heteromers modulate GAT-1- and GAT-3-mediated GABA uptake by astrocytes. J. Neurosci. (*In press*).
- Sebastião AM, Assaife-Lopes N, Diógenes MJ, **Vaz SH**, Ribeiro JA. (2011) Modulation of brain-derived neurotrophic factor (BDNF) actions in the nervous system by adenosine A(2A) receptors and the role of lipid rafts. Biochim Biophys Acta., 1808:1340-1349.

- Cristóvão-Ferreira S, **Vaz SH**, Ribeiro JA, Sebastião AM. (2009). Adenosine A2A receptors enhance GABA transport into nerve terminals by restraining PKC inhibition of GAT-1. J Neurochem., 109:336-347.

**Other publications from the author:**

- Moreno E*, **Vaz SH***, Cai N, Ferrada C, Quiroz-Molina C, Barodia S, Kabbani N, Canela E, McCormick P, Lluis C, Franco R, Ribeiro JA, Sebastião AM, Ferré S. (2011). Dopamine-galanin receptor heteromers modulate cholinergic neurotransmission in the rat ventral hippocampus. J Neurosci. 31:7412-7423. *Co-first authors
- Gomes CARV, **Vaz SH**, Sebastião AM, Ribeiro JA. (2006). Glial Cell Line-Derived Neurotrophic Factor enhances dopamine release from striatal nerve endings in an adenosine $A_{2A}$ receptor dependent manner. Brain Res., 1113:129-136.

Aos meus pais e ao Rui

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## ABBREVIATION LIST

**AC**, adenylate cyclise

**ADA**, adenosine deaminase

**AK**, adenosine kinase

**Akt**, protin kinase B

**AMPA**, α-Amino-2,3-duhydro-1H-indene-1,5-methyl-4-isoxazolepropionic acid

**AMP**, adenosine 5'-monophosphate

**AmpR**, β-lactamase expression cassette for ampicilin resistance

**ANOVA**, analysis of variance

**ATP**, adenosine 5'-triphosphate

**BDNF**, brain-derived neurotrophic factor

**BGT-1**, betaine-GABA transporter 1

**BSA**, bovine serum albumin

**cAMP**, 3',5'-cyclic AMP; adenosine 3',5'-cyclophosphate

**cDNA**, complementary DNA

**CGS 21680**, 4-[2-[[6-amino-9-(N-ethyl-b-D-ribofuranuro-namidosyl)-9H-purinyl]amino]ethyl] benzene-propanoic acid hydrochloride

**CMV**, cytomegalovirus promoter

**CNS**, central nervous system

**DMEM**, Dulbecco's Modified Eagles Medium

**DMSO**, dimethylsulfoxide

**DNA**, deoxyribonucleic acid

**Ecto-5'-NT**, Ecto-5'-nucleotidades

**EDTA**, ethylenediaminetetraacetic acid

**ELISA**, Enzyme-Linked Immunosorbent Assay

**ENT**, equilibrative nucleosidade transporter

**ER**, endoplasmatic reticulum

**ERK**, extracellular signal-regulated kinases

**EPSP**, excitatory postsynaptic potentials

**FBS**, fetal bovine serum

**GABA**, gamma-aminobutyric acid

**[$^3$H]GABA**, 4-amino-n-[2,3-$^3$H]butyric acid

**GABA-T**, GABA transaminase

**GAD**, glutamate decarboxylase

**GATs**, GABA transporters

**GAT-1**, GABA transporter-1

**GAT-2**, GABA transporter-2

**GAT-3**, GABA transporter-3

**GPCRs**, G-protein coupled receptors

**GS**, glutamine synthetase

**H-89**, N-[2-(p-bromocinnamylamino) ethyl]-5 isoqui-nolinesulfonamide dihydrochloride

**HA**, hemagglutinin epitope

**HEK293**, human embryonic kidney 293 cells

**HEK293T**, variant of the human embryonic kidney 293 cells

**HEPES**, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

**HRP**, horseradish peroxidase

**InsP3,** inositol 1,4,5-trisphosphate

**IPSP**, inhibitory postsynaptic potentials

**Kd**, equilibrium dissociation constant

**α-KG**, α-ketoglutarate

**KHR solution**, Krebs-Henseleit-Ringer Solution

$\mathbf{K_m}$, affinity constant (Michaelis-Menton Constant)

**LTP**, long term potentiation

**3'LTR**, 3' long terminal repeat with partial U3 deletion that results in the self-inactivation of these vector

**5'LTR**, 5' long terminal repeat

**LY294002**, 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one

**MAPK**, mitogen-activated protein kinase

**MCS**, multiple cloning site

**mGluRs**, metabotropic glutamate receptors

**NCX**, $Na^+/Ca^{2+}$ exchanger

**NGF**, nerve groth factor

**NT-3**, neurotrophin-3

**NT-4**, neurotrophin-4

**NF-kB**, Nuclear Factor-KappaB

**NSF**, N-ethylmaleimide-sensitive fisuion protein

**PAC1**, cyclase-activating polypeptide receptor 1

**PAG**, phosphate-activated glutaminase

**PBS**, phosphate buffer saline

**PCR**, polymerase chain reaction

**PI3-K**, phosphatidylinositol 3-kinases

**PKA**, protein kinase A

**PKC**, protein kinase C

**PKC-δ**, protein kinase C-delta

**PLC-γ**, phospholipase C-gamma

**POL**, polymerase

**PVDF**, polyvinylidene fluoride

**pUC18 ori**, pUC18 vector-derived replication origin;

**RAS**, RAt Sarcoma

**REV**, Regulator of Virion

**RNA**, ribonucleic acid

**RRE**, REV response element

**RTKs**, tyrosine kinase receptors

**SA**, splice acceptor site

**SAH**, S-adenosil-homocistein

**SD**, splice donor site

**SEM**, standard error of the mean

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

**SDS**, sodium dodecyl sulphate

**Shc**, collagen-like adaptor protein

**SKF 89976A hydrochloride**, 1-(4,4-Diphenyl-3-butenyl)-3-piperidine-carboxyliacidhydro-chloride)

**SNAP**, soluble NSF attachment protein

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

**SNARE**, Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment Protein Receptor

**SSADH**, succinate semialdehyde dehydrogenase

**T7**, T7 promoter recognition site

**TAT**, transactivator (it is a regulatory gene which accelerates production of more HIV virus)

**TCA**, tricarboxilic acid cycle

**TEMED**, 1,2-bis(dimethylamino)ethane

**TM**, transmembrane domain

**Tris**, tris-hydroxymethyl-aminomethane

**TrkB**, tyrosine kinase B receptor

**TrkB-fl**, full-length tyrosine kinase B receptor isoform

**TrkB-t**, truncated tyrosine kinase B receptor isoform

**U012**6, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene

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

**3'UTR**, 3' untranslated region

**VGAT**, vesicular GABA transporter

$\mathbf{V_{max}}$, Maximal velocity of transport

**VSV-G**, G glycoprotein of vesicular stomatitis virus

**WPRE**, woodchuck postregulatory response element

## ABSTRACT

Gamma-aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the central nervous system. Its activity at the synapse is terminated by re-uptake into nerve terminals and astrocytes, through membrane located specific GABA transporters (GATs), which therefore shape GABAergic transmission. There are three main high affinity subtypes of GATs, GAT-1, GAT-2 and GAT-3, and a low affinity one, the betaine transporter. GAT-1 is the predominant GABA transporter in the brain and is expressed in neurons and astrocytes.

Several factors can regulate the continuous traffic of GATs to and from the neuronal plasma membrane. For instance surface expression of GAT-1 in cultured neurons and isolated nerve terminals is decreased by protein kinase C (PKC)-dependent phosphorylation. In contrast, surface expression of GAT-1 in neurons is enhanced by brain derived neurotrophic factor (BDNF)-mediated tyrosine kinase-dependent phosphorylation. Though reuptake of GABA might occur at different places of the neuronal membrane, its reuptake by the nerve terminal is the process that allows quick refilling of the released stores. On the other hand, uptake by astrocytes contributes to a fast removal of GABA from the synapse and delays its delivery to the neuronal release stores. To understand how GABAergic transmission can be shaped, it is therefore important to know how a single modulator can affect both processes of GABA removal from synapse.

Thus, in the work presented on this thesis I evaluated the influence of BDNF upon GAT-1 transporters on presynaptic nerve terminals and cortical primary astrocyte culture.

BDNF decreased GAT-1 mediated GABA uptake by isolated hippocampal rat nerve terminals (synaptosomes), an effect that occurred within 1 min of

incubation with BDNF through activation of TrkB receptor. In contrast with what has been observed for other synaptic actions of BDNF, the inhibition of GABA transport by BDNF does not require tonic activation of adenosine $A_{2A}$ receptors, nevertheless is facilitated by activation of $A_{2A}$ receptors.

On the other hand, BDNF enhances GAT-1 mediated GABA transport in cultured astrocytes, an effect mostly due to an increase in $V_{max}$ kinetic constant. This effect involves the truncated form of TrkB receptors (TrkB-t) coupled to a non-classic PLC-γ/PKC-δ and Erk/MAP kinase pathway and requires active adenosine $A_{2A}$ receptors. To elucidate the trafficking of GAT-1 when astrocytes were treated with BDNF, a functional mutant of the rat GAT-1 was generated in which hemagglutinin epitope (HA) was incorporated into the second extracellular loop. By ELISA experiments, performed with astrocytes expressing HA-rGAT-1 transporter, it was possible to observe an exocytosis of HA-GAT-1 to plasma membrane when cells were treated with BDNF. In addition, cell surface biotinylation experiments, performed with astrocytes overexpressing the wild type rat GAT-1 (rGAT-1), also demonstrate an increase of GAT-1 transporter at plasma membrane when astrocytes were treated with BDNF. Results from experiments using selective inhibitors of endocytosis or selective inhibitors of recycling of molecules back to the plasma membrane allowed concluding that BDNF enhances GAT-1 expression at surface astrocytic membrane by slowing down exocytosis.

A new role for BDNF is proposed whereby the effect of BDNF on GAT-1 transporter differs between pre-synaptic nerve terminals and astrocytes, suggesting that this neurotrophin operates in a much localized way, so that it may retard GABA uptake by the nerve terminal, enhancing synaptic actions of GABA, and accelerate its reuptake at extracellular neuronal areas allowing replenishment of neuronal pools of GABA. The results suggest that BDNF plays an active role

in the regulation of GABAergic synaptic signalling, contributing to information processing.

## RESUMO

O ácido γ-aminobutírico (GABA) é o principal neurotransmissor inibitório do sistema nervoso central. A rápida remoção do GABA presente na fenda sináptica, por transportadores de alta afinidade para o GABA, que se localizam quer a nível do terminal pré-sináptico dos neurónios, quer a nível das células da glia, nomeadamenrte dos astrócitos (Gether et al., 2006), é essencial para uma sinalização eficaz mediada por este neurotransmissor. Até ao momento quatro transportadores foram identificados para o GABA, três destes de alta afinidade, denominados de GAT-1, GAT-2 and GAT-3 e um quarto, de baixa afinidade, denominado *betaine transporter*. O transportador GAT-1 é o transportador de GABA predominante no sistema nervoso central e encontra-se expresso preferencialmente em neurónios, sendo, no entanto, também expresso em astrócitos. Relativamente ao transportador GAT-3, sabe-se que este é maioritariamente expresso em astrócitos, onde tem um predomínio de transporte de GABA relativamente ao transportador GAT-1. Assim a recaptação de GABA pode ocorrer em diferentes localizações celulares. Quando ocorre para o terminal nervoso pre-sináptico, tem como consequência uma rápida reposição do nível de GABA nas vesículas sinápticas. A ocorrência para os astrócitos contribui para uma remoção mais rápida do GABA da fenda sináptica, diminuindo assim a velocidade de reposição de GABA nas vesículas sinápticas. Para se entender como é que a transmissão GABAérgica é regulada, torna-se pois extremamente relevante compreeender como pode apenas uma molécula modular os dois locais onde ocorre transporte de GABA, nomeadamente o pré-sináptico e o astrocítico. Salienta-se também a importância dos transportadores para o controlo da excitabilidade e o seu eventual envolvimento em situação patológica, nomeadamente em doentes com epilepsia do lobo temporal que apresentam uma aumento da expressão dos transportadores de GABA nos astrócitos.

Os transportadores de GABA são regulados de diversos modos, estando envolvidos diferentes factores e várias cascatas de transdução de sinal. Esta modulação pode ocorrer de dois modos distintos: por alteração do $K_m$ ou da $V_{max}$ do transportador. A regulação do tráfego dos transportadores de GABA de, e para a membrana plasmática neuronal, pode ocorrer por variações da velocidade de endocitose e exocitose e/ou por alteração da quantidade de transportadores disponíveis neste processo de tráfego contínuo.

Uma molécula já identificada como reguladora do transportador GAT-1 é o *Brain derived neurotrophic factor* (BDNF). O BDNF é um factor neurotrófico com importantes funções na diferenciação, maturação e sobrevivência neuronal, levando a modificações estruturais e moleculares a longo-prazo que são cruciais para o desenvolvimento, mas também para a função e plasticidade sináptica no indivíduo adulto (Vicario-Abejon et al., 2002). O BDNF exerce a sua acção através da activação de receptores tirosina cinase B (TrkB), que se apresentam em diferentes isoformas: uma isoforma "completa" (TrkB-fl) que apresenta domínios tirosina cinase e uma isoforma truncada (TrkB-t) que não apresenta estes domínios. O BDNF favorece a recaptação de GABA devido a um aumento da expressão de GAT-1 a nível da membrana plasmática em culturas primárias de neurónios, não se sabendo até ao início deste trabalho qual a função do BDNF no controlo da actividade do GAT-1 local a nível de terminais nervosos.

Os astrócitos são a maior classe de células da glia encontrada no cérebro dos mamíferos e têm um papel extremamente relevante na transmissão sináptica, contribuindo para o processamento de informação a nível sináptico ao controlar quer a composição do meio extracelular, quer a quantidade de neurotransmissores presentes na fenda sináptica. Os astrócitos são assim células fundamentais a nível da comunicação existente entre astrócitos ou entre astrócitos-neurónios. No que diz respeito à regulação dos níveis extracelulares de GABA, estas células têm um

papel muito importante uma vez que expressam transportadores específicos de GABA, que permitem, como foi anteriormente referido, o controlo dos níveis deste neurotransmissor na fenda sináptica. Todavia, pouco tem sido descrito em relação à regulação dos transportadores de GABA nos astrócitos.

O trabalho que aqui se apresenta teve como objectivo estudar o efeito do BDNF sobre o transportador de GABA, em terminais nervosos pré-sinápticos e em astrócitos, bem como estudar os mecanismos subjacentes ao efeito do BDNF. Foi também abordado o possível envolvimento dos receptores $A_{2A}$ da adenosina, uma vez que a interacção entre o receptor do BDNF, TrkB e o receptor de adenosina $A_{2A}$, tem sido descrita em vários sistemas biológicos.

Verificou-se que em terminais nervosos pré-sinápticos o BDNF tem uma acção inibitória sobre o transportador exclusivo de GABA (GAT-1) nesta estrutura, levando a uma diminuição da recaptação de GABA através deste transportador. Este efeito depende da concentração de BDNF e ocorre num intervalo de tempo extremamente curto (1 minuto). O efeito do BDNF no transportador GAT-1 ocorre através da activação do receptor TrkB e, contrariamente a outros efeitos mediados pela activação deste receptor, não requer a activação tónica dos receptores $A_{2A}$ da adenosina.

Em culturas primárias de astrócitos o BDNF aumentou a recaptação de GABA mediada pelo transportador GAT-1, não tendo qualquer efeito no transportador GAT-3, também presente nos astrócitos. Este efeito ocorre devido a um aumento da velocidade máxima do transportador. O efeito do BDNF envolve a forma truncada do receptor TrkB, estando esta acoplada a uma via não clássica da PLC-$\gamma$/PKC-$\delta$ e da Erk/MAP cinases. O efeito descrito requer que os receptores $A_{2A}$ da adenosina estejam activos, sendo que os níveis endógenos de adenosina extracelular são suficientes para desencadear o efeito do BDNF.

Uma vez que um aumento do $V_{max}$ se correlaciona com um aumento do número de transportadores na membrana plasmática, procedeu-se seguidamente à avaliação de um possível aumento da expressão do transportador GAT-1 quando as células eram tratadas com BDNF. Para avaliar se o efeito do BDNF se correlacionava com o tráfego de GAT-1 de, e para a membrana celular, foi gerado um mutante funcional do transportador GAT-1 de rato (rGAT-1), no qual foi introduzido o epítopo hemaglutinina (HA) no segundo *loop* extracelular do transportador, procedendo-se à infecção dos astrócitos com o referido mutante. Após o tratamento das células com BDNF observou-se um aumento da expressão de HA-rGAT-1 na membrana plasmática. Também através de experiências de biotinilação, realizadas com astrócitos que sobreexpressavam rGAT-1, se pôde concluir que o BDNF aumenta a expressão de rGAT-1 na membrana plasmática. Estudos onde se usou um inibidor da endocitose (*dynasore*) ou um inibidor da reciclagem de moléculas internalizadas de volta para a membrana plasmática (*monensin*), permitiram concluir que o efeito do BDNF envolve inibição da internalização de GAT-1 nos astrócitos, tendo esta acção consequências na expressão do GAT-1 e na velocidade de transporte de GABA.

Os resultados apresentados nesta tese mostram que o BDNF exerce a sua acção de um modo muito localizado, levando a uma diminuição da recaptação de GABA no terminal nervoso que favorece eventualmente as suas acções sinápticas, e a uma aceleração da recaptação de GABA em regiões extra-sinápticas, que contribui para uma redução da acção tónica deste neurotransmissor. Em última instância, este efeito do BDNF deverá determinar uma diminuição da velocidade de reposição de GABA nas vesículas sinápticas, conduzindo desta forma a um aumento da excitabilidade neuronal.

xxx

# 1. INTRODUCTION

The complex architecture of adult brain rises from integration of genetic information, cellular interactions and eventually interactions between developing brain and the outside world. Nevertheless, the adult brain is able to keep part of these interactions, since it is continuously changing (neuronal plasticity), allowing learning, establishing new memories and even responding after an injury. The brain is constituted by a large number of different types of neurons, as well as several types of non neuronal cells, that form complex neuronal circuits. Although these circuits have the inherent complexity associated with biological systems, they can be simplified and, as a consequence, the different functional units can be individualized. The balance between different circuits is highly relevant for the correct function of brain; an example is the balance between excitatory (glutamatergic system) and inhibitory (GABAergic systems) circuits.

## 1.1. GABA and the GABAergic system

Gamma-aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the adult brain, being discovered by three different groups in 1950 (Awapara et al., 1950; Roberts and Frankel, 1950; Udenfriend, 1950).

GABA biosynthesis in neurons mainly involves decarboxylation of glutamate yielding GABA and $CO_2$ via the enzyme glutamate decarboxylase (GAD) (Roberts and Kuriyama, 1968). The glutamate can be obtain by neurons from two different sources, namely from glutamine that comes from tricarboxilic acid (TCA) cycle in glia cells and from glutamine obtain in nerve terminals (Figure 1.1.1). There are two isoforms of GAD, namely GAD65 and GAD67, being GAD67 found ubiquitously in GABAergic neurons, whereas GAD65 is

preferentially located in the nerve terminals. Based on these findings it has been suggested that GAD65 is specialized to readily synthesize GABA under short-term demand (Martin and Rimvall, 1993). The synthesized GABA is loaded into synaptic vesicle through vesicular GABA transporter (VGAT) (McIntire et al., 1997) and the complex formed by VGAT with GAD65 appears to be the necessary for efficient GABA synthesis and packaging into synaptic vesicles (Jin et al., 2003).

In the nerve terminals GABA can be released to the synaptic cleft mainly by two different pathways; i.e., via a $Ca^{2+}$ dependent vesicular release or a $Ca^{2+}$ independent release through transporter reversal (Belhage et al., 1993; Kirmse and Kirischuk, 2006; Wu et al., 2007; Ade et al., 2008). However, other less characterized mechanisms might also contribute, namely non-transporter-mediated $Ca^{2+}$- and SNARE-independent release (Demarque et al., 2002), transmitter leakage that ocours through damaged plasma membrane (Phillis et al., 1996), release via P2X7 receptor-activated unidentified channels (Wang et al., 2002; Duan and Neary, 2006). Upon release to the synaptic cleft GABA interacts with GABA receptors located pre and postsynaptically. Presynaptic metabotropic GABA receptors ($GABA_B$) mediate a negative feedback mechanism on GABA release whereas, postsynaptic GABA receptors, that can be both metabotropic ($GABA_B$) and ionotropic ($GABA_A$), mediate usually hyperpolarization of the cell (Watanabe et al., 2002).

After dissociation from the receptor complex, GABA is transported back into the presynaptic nerve terminal or into surrounding astrocytes via a high affinity GABA transport system thereby terminating GABA's inhibitory action (Iversen and Neal, 1968) and keeping the extracellular GABA concentrations under physiological levels. The GABA taken up by neurons can be directly loaded into

synaptic vesicle, while the GABA taken up by astrocytes is first catabolised to the TCA cycle intermediate succianate via the concerted action of GABA transaminase (GABA-T) and succinate semialdehyde dehydrogenase (SSADH), and next succianate originates α-ketoglutarate via TCA cycle that will be funnelled out of TCA cycle and converted into glutamate by the enzyme glutamate dehydrogenase (Figure 1.1.1B). This TCA by-pass reaction is known as GABA *shunt*. The glutamate is converted to glutamine by glutamine synthetase (GS), which is afterward transported to neurons where it is converted into glutamate by phosphate-activated glutaminase (PAG) (Bak et al., 2006). GABA-T is located both on neurons and astrocytes presenting a highest activity in astrocytes (Madsen et al., 2008), indicating that GABA transport to neurons or to astrocytes have different functional consequences. Indeed it has been estimated that neuronal GABA transport system is three- to six-fold more efficient than the astrocytic GABA transport mechanisms (Hertz & Schousboe, 1987) which is correlated with the existence of different GABA transporters and with the differential expression and density of this transporter in neurons and astrocytes.

Demonstration of a physiological role for GABA transporters comes from experiments involving specific GABA uptake inhibitors; these inhibitors prolonged the decay phase of the "fast" $GABA_A$ receptor-mediated post-synaptic potentials (Isaacson et al., 1993) and increase both the decay phase and the magnitude of responses mediated by G-protein coupled $GABA_B$ receptors (Dingledine and Korn, 1985; Solis and Nicoll, 1992; Isaacson et al., 1993; Bernstein and Quick, 1999).

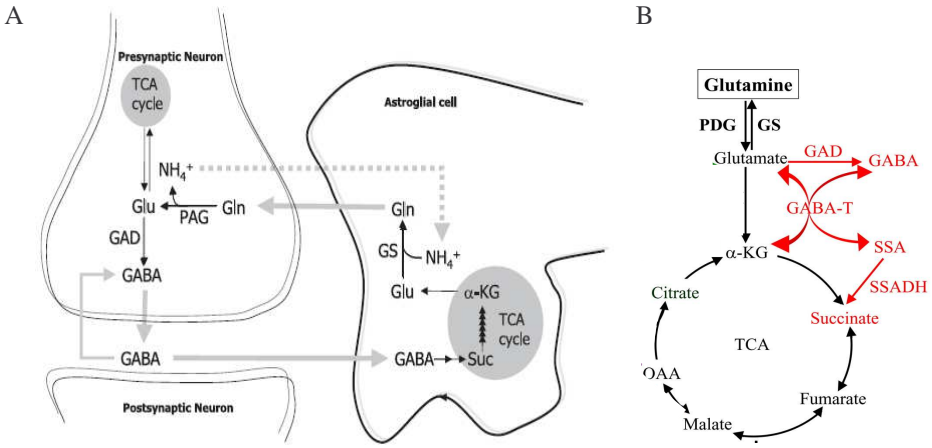



**Figure 1.1.1. Schematic representation of GABA metabolism and uptake.** In GABAergic synapse (A), after an appropriate stimulus GABA is released from the presynaptic terminal activating specific GABA receptors. GABA mediated effects are finished by the uptake of GABA through specific GABA transporters into both the surrounding astrocytes and the pre-synaptic terminal. In the astrocytes, GABA is metabolized to glutamine and glutamine return to the neurons. In neurons the enzyme glutamic acid decarboxylase (GAD) forms GABA from glutamate, being this glutamate obtain from two different sources: glutamine from TCA cycle in glia cells and glutamine in nerve terminals. The GABA shunt is shown in red (B). GAD is the entry point for glutamate into the GABA shunt with succinate as end product. GABA transaminase (GABA-T) is a mitochondrial enzyme which converts GABA into glutamate by reaction with α-ketoglutarate (α-KG). (Adapted from Bak et al., 2006; Li et al., 2008 with small modifications).

### 1.1.1. GABA receptors and phasic/tonic inhibition

There are two distinct types of GABA receptors: the ionotropic $GABA_A$ (widespread in CNS) and $GABA_C$ (mostly found in retina) receptors and the metabotropic $GABA_B$ receptors. For ionotropic receptors, the ligand binding is followed by a conformational change in the channel protein that allows a net

inward flow or outward flow of ions through the membrane-spanning pore of the channel, depending on the electrochemical gradient of the particular permeant ion (Figure 1.1.2).

$GABA_A$ receptors are pentameric and consist of several subunits (e.g. α, β, γ, δ, ε, θ, π and ρ). To date nineteen subunits have been cloned (six α subunits, three β subunits, three γ subunits, one δ subunit, one ε subunit, one θ subunit, one π subunit and three ρ subunits) from the mammalian CNS, with further variation resulting from alternative splicing (Farrant and Nusser, 2005). A great variety of $GABA_A$ receptors with distinct pharmacology could be assembled by combining these subunits. The most common combination is triplet α1/β2/γ2, which is detected in various cell types in the CNS (McKernan and Whiting, 1996). These receptors encompass the chloride channels and can be specifically blocked by bicuculline. The $GABA_A$ receptor has modulatory binding sites for benzodiazepines, barbiturates, ethanol and neurosteroids (Macdonald and Olsen, 1994).

$GABA_B$ receptors consist of a single peptide that is coupled to G-proteins through second messenger pathways, which change the activity of post-synaptic $K^+$ channels and/or currents mediated by pre- and post-synaptic voltage-dependent $Ca^{2+}$ channels (Marshall et al., 1999). Until now two subtypes of the receptor have been identified, the $GABA_BR$-1 and $GABA_BR$-2, existing two isoforms of the $GABA_BR$-1 (R1a and R1b) (Kaupmann et al., 1998; Pierce et al., 2002). This receptor is an obligate heterodimer that is only functional when both the $GABA_BR$-1 and $GABA_BR$-2 are co-expressed in the same cell. When the $GABA_BR$-1 is expressed alone, it is trapped in vesicles within the cell, whereas the $GABA_BR$-2 alone is expressed on the cell surface, but cannot bind GABA or activate G proteins. When both receptor subunits are expressed in the same cell,

the receptors interact through coiled-coil domains in their carboxyl tails. They are then expressed on the cell surface, bind GABA and activate G proteins. The $GABA_B$ receptor is activated by baclofen and antagonized by saclofen and phaclofen, but is insensitive to bicuculline.

A third GABA receptor, $GABA_C$ receptor, which is pharmacologically and structurally distinct from $GABA_A$ and $GABA_B$ receptors, has been identified and occurs predominantly in the vertebrate retina (Feigenspan et al., 1993; Qian and Dowling, 1993; Bormann and Feigenspan, 1995; Lukasiewicz and Shields, 1998; Bormann, 2000). $GABA_C$ receptors are $Cl^-$ pores, which can be blocked by picrotoxin, a non-selective chloride channel blocker, but they are insensitive to bicuculline and baclofen. Structually, $GABA_C$ receptors are exclusively composed of a single or multiple ρ-subunits. These subunits are heterologously expressed and form homoligomeric channels with the characteristic pharmacology of $GABA_C$ receptors (Bormann, 2000). In addition to the differences in pharamacology and structure, several lines of evidence indicate that $GABA_A$ and $GABA_C$ receptor-mediated responses have different kinetics (Bormann and Feigenspan, 1995; Djamgoz et al., 1995; Lukasiewicz, 1996; Han et al., 2000). Moreover, the $GABA_C$ receptor is more sensitive to GABA than the $GABA_A$ receptor. The GABA concentration, producing half-maximal response ($EC_{50}$), is 1–5 μM for $GABA_C$ receptors, whereas the $EC_{50}$ is 10–100 μM for $GABA_A$ receptors (Polenzani et al., 1991; Qian and Dowling, 1993; Feigenspan and Bormann, 1994; Qian and Dowling, 1994; Han et al., 1997).

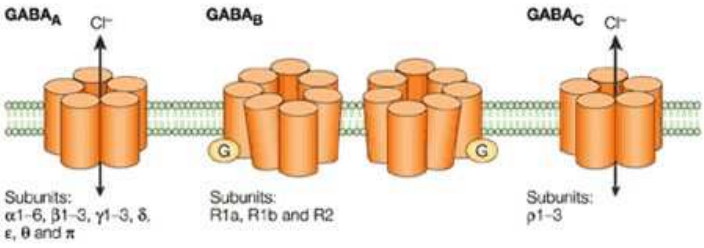



**Figure 1.1.2. Subunit composition and assembly of GABA receptors.** $GABA_A$ and $GABA_C$ receptors are closely related pentameric receptors that carry chloride; however, whereas $GABA_A$ receptors are composed of combinations of several subunit types, $GABA_C$ receptors are composed of only single or multiple ρ-subunits. $GABA_B$ receptors are metabotropic receptors that exist as R1a, R1b and R2 isoforms, and are associated with G proteins. Native $GABA_B$ receptors are dimmers composed of one R1 subunit and the R2 subunit (Adapted from Owens and Kriegstein, 2002).

Two different types of GABAergic inhibition can be activated by GABA receptors, the phasic and tonic inhibition. Phasic inhibition, which regulates point-to-point interneuronal communication, is related with $GABA_A$ receptors facing pre-synaptic release sites and is activated by a high concentration of GABA released by synaptic vesicle exocytosis; and tonic inhibition, which regulates membrane potential and network excitability (Semyanov et al. 2004; Farrant and Nusser 2005; Mody, 2005), is related with the exposure of extrasynaptic distant $GABA_A$ receptors to a persistently low concentration of "ambient" GABA (Lindquist and Birnir 2006). The peak concentration of synaptic GABA (1.5 – 3 mM) differs considerably from the "ambient" GABA concentration found in extrasynaptic regions (0.2 – 0.8 μM) (Lerma et al., 1986; Mozrzymas et al., 2003). Interesting the concentration of GABA in extrasynaptic regions is sufficient to activate extrasynaptic $GABA_A$ receptors (Figure 1.1.3) but not

$GABA_A$ receptors within the synaptic cleft, since the subunit composition of synaptic and extrasynaptic $GABA_A$ receptors differs, so that extrasynaptic receptors have much higher sensitivity to GABA and much slower desensitisation kinetics.

The extrasynaptic GABA concentration is maintained by GABA that escapes from the synaptic cleft (GABA spillover) or that is released via non-vesicular mechanism by neurons and glia (Isaacson et al., 1993; Mitchell and Silver, 2000; Scanziani, 2000; Volknandt, 2002; Ruiz et al., 2003; Semyanov et al., 2003).

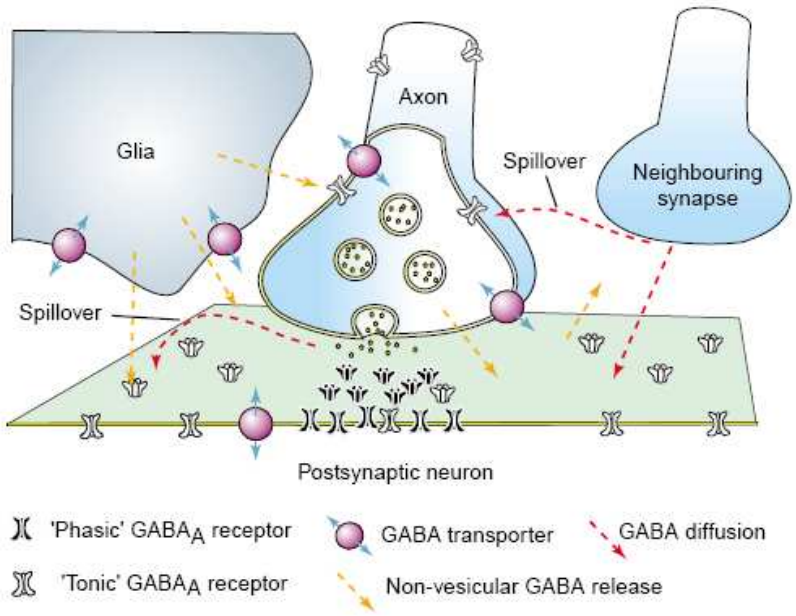



**Figure. 1.1.3. Sources and targets of extrasynaptic GABA.** Extracellular GABA concentration is regulated by release, diffusion and uptake mechanisms. GABA can escape from synaptic cleft (GABA spillover) and can be released via non-vesicular mechanism by neurons and glia. Once released, GABA can reach target receptors on presynaptic terminals, axon and somatodendritic compartments (Adapted from Semyanov et al., 2004).

## 1.2. GABA transporters

The concept that after release the neurotransmitter is inactivated by uptake into the nerve terminal from which it had been released or into adjacent cells is less than 50 years old. In 1958 was first described that GABA in the incubating medium could accumulate into slices of cerebral cortex (Elliott and Van Gelder, 1958); however the discovery of a high affinity transport system for GABA in neurons and astrocytes was only achieved 10 years later (Iversen and Neal, 1968; Iversen and Kelly, 1975). Until now, four GABA transporters have been characterized and cloned: there are three high affinity subtypes of GABA transporter, GAT-1-3, and a low affinity one, betaine-GABA transporter 1 (BGT-1).

Radian and collaborators (1986) isolated for the first time a $Na^+$ and $Cl^-$ dependent GABA transporter from rat. Afterwards this transporter was cloned and designated GAT-1 transporter (Guastella et al., 1990). This revealed a 599 amino acid content and a $K_m$ of 7μM for GABA (Guastella et al., 1990). The GAT-1 transporter was later on cloned from human (Nelson et al., 1990).

Three other GABA transporters were identified. Two rat GABA transporters designated rGAT-2 and rGAT-3 with a $K_m$ of 8 and 12 μM for GABA, respectively, and an amino acid sequence of 602 and 627, respectively, have also been cloned (Borden et al., 1992). Both human GAT-2 and GAT-3 have been identified and cloned (Borden et al., 1994b; Christiansen et al., 2007). Finally a transport protein capable of transporting both GABA and the osmolyte betaine with an apparent $K_m$ of 93 and 398 mM, respectively, was isolated from rat kidney and named betaine-GABA transporter 1 (BGT-1) (Yamauchi et al., 1992). It

encodes a 614 amino acid protein also with a dependence on $Na^+$ and $Cl^-$ for transport. The human BGT-1 has also been cloned (Borden et al., 1995).

Four mouse GABA transporters displaying a $Na^+$ and $Cl^-$ dependence for transport have been also cloned and characterized pharmacologically. The mouse GABA transporters are termed GAT1, GAT2, GAT3, and GAT4 (without hyphen) and are composed of 598, 614, 602, and 627 amino acids, respectively (Liu et al., 1993). Mouse GAT3 and GAT4 correspond to the rat GAT-2 and GAT-3, respectively. This is due to the fact that mouse GAT2 in reality is a betaine transporter being homologous to the human and rat BGT-1 transporter (Schousboe et al., 2004).

### 1.2.1. Subcellular location of GABA transporters

GAT-1 is widely distributed in the central nervous system, being the most copiously expressed GAT in the cerebral cortex. Indeed specific GAT-1 immunoreactivity is present in all cortical layers as well as in hippocampal formation (Minelli et al., 1995; Conti et al., 2004). GAT-1 is found both in neurons and astrocytes, and the majority of neurons expressing GAT-1 mRNA contain GAD67 immunoreactivity. However, a few pyramidal cells also express GAT-1 mRNA (Minelli et al., 1995). GAT-1 is also expressed in cortical astrocytes (Minelli et al., 1995), retinal Muller cells (Brecha and Weigmann, 1994; Johnson et al., 1996) and in hippocampal astrocytic processes (Ribak et al., 1996). It is important to mention that GAT-1 is located closely to GABAergic synapses, and it can also be found in glutamatergic neurons (Minelli et al., 1995).

GAT-2 transporter is primarily located in the extrasynaptic region, and it can be found both in neuronal and non-neuronal cells (Conti et al., 1999; Conti et al., 2004).

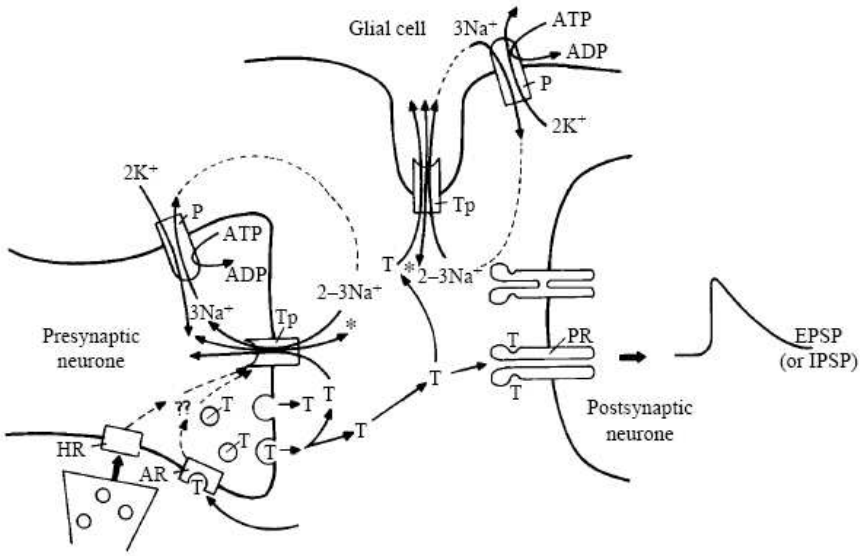
GAT-3 is localized to both neurons and astrocytes; however, it is primarily localized to the latter cell type (Durkin et al., 1995; Minelli et al., 1996; Conti et al., 2004). GAT-3 positive astrocytic processes are adjacent to axon terminals making synaptic contacts with cell bodies or dentrites, or are close to neuronal profiles that do not form synaptic contacts (Conti et al., 2004).

BGT-1 can be found in dendritic process and cell body of hippocampal neurons (Zhu and Ong, 2004), being preferential located at extrasynaptic regions (Borden et al., 1995; Zhu and Ong, 2004). BGT-1 is also found on glia cells and in primary cultures of astrocytes (Zhu and Ong, 2004; Olsen et al., 2005).

### 1.2.2. Structure and functioning of GABA transporters

As it was previously mention, all GABA transporters are $Na^+/Cl^-$ dependent transporters and belong to the SLC6 gene family. The SLC6 gene family also includes transporters of dopamine, 5-HT, norepinephrine and glycine (Gether et al., 2006). These transporters mediate-GABA transport together with sodium ion and chloride ion in an electrogenic process since they use the electrochemical gradient of sodium (generated by $(Na^+ + K^+)$-ATPase) between the outside and inside surfaces of the cell membrane to provide the thermodynamic energy required to pump neurotransmitters from low concentrations outside the cell to the much higher concentrations inside the cell (reviewed in Kanner, 2006). Under normal conditions, the stoichiometry for transport is 2 sodium ions : 1 chloride ion : 1 GABA molecule (Keynan and Kanner, 1988; Kavanaugh et al., 1992; Mager et al., 1993; Lu and Hilgemann, 1999) and they are able to generate a gradient in the order of $10^5$ between the intra- and extracellular GABA concentration (Figure 1.2.1) (Beleboni et al., 2004).

The primary sequence of GAT-1, like of most SLC6 members, predicts twelve transmembrane domains (TM) connected by hydrophilic loops with the amino and carboxyl termini residing inside the cell (Guastella et al., 1990; Nelson et al., 1990). GAT-1 is modified by asparagine-linked glycosylation (Radian et al., 1986; Kanner et al., 1989; Keynan et al., 1992) and the model predicts a large extracellular loop between transmembrane helices 3 and 4 containning three N-linked glycosylation sites (Guastella et al., 1990), that were later on confirmed (Figure 1.2.2) (Yamashita et al., 2005).




**1.2.1. The role of plasma membrane neurotransmitter transporters in synaptic transmission.** Neurotransmitter (T), stored in synaptic vesicles, is released by fusion of the vesicles with the synaptic plasma membrane. After its diffusion across the synaptic cleft, it binds to postsynaptic receptors (PR), resulting in the opening of channels often present in the same structure as the receptor. Channel opening may lead to excitatory or inhibitory postsynaptic potentials (EPSP or

IPSP). Transmitter is removed from the cleft by re-uptake mediated by electrogenic sodium-coupled transporters (Tp), which also translocates other ions (*). In the case of the transporters of GABA, * is chloride, which moves in the same direction as sodium and the neurotransmitter. The main driving force for this process is the electrochemical gradient of sodium ions, which is maintained by the sodium pump ($Na^+/K^+$-ATPase, P). The transporters are located in the synaptic plasma membrane and also in the processes of glial cells, which are in close contact with the synapse. The activity of these transporters may be the subject of physiological regulation. This may be mediated by receptors for the same neurotransmitter (autoreceptors, AR) or by those of others (heteroreceptors, HR) (Adapted from Kanner, 1994).

Several amino acids residues have a relevant role in GABA transporters functioning. For instance arginine R69 that has a positive charge is essential for transport, since its substitution with other amino acids including charged ones does not recover transport activity (Pantanowitz et al., 1993). Tryptophan W68, W222, and W230 when substituted with either serine or leucine resulted in a 90% reduction in transport activity. It appears that W68 and W222 are required for intrinsic activity, while W230 has been reported to be involved in plasma membrane targeting (Kleinberger-Doron and Kanner, 1994). Tyrosine Y140 replacement is not tolerated even with the aromatic amino acids phenylalanine or tryptophan (Bismuth et al., 1997). Cysteine C74 located in EL1 is also suggested to be involved in the pore formation or GABA translocation (Figure 1.2.2) (Yu et al., 1998).

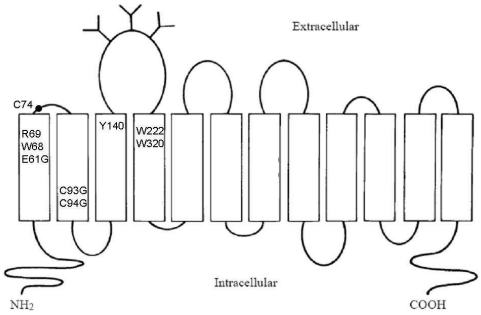



**Figure 1.2.2. Schematic representation of the GABA transporter GAT-1 and the location of critical amino acids.** Putative transmembrane segments are shown as rectangles. The potential glycosylation sites are indicated by branched lines. Critical residues of GAT-1 are indicated using the one-letter code and their positions are also given (Adapted from Kanner, 1994 with small modifications).

One of the properties of neurotransmitter transporters, namely of GABA transporters, that was not taken into account in traditional models of synaptic function is that they can reverse and release neurotransmitter (Attwell et al., 1993; Levi and Raiteri, 1993). This reversal is a direct and expected consequence of the dependence of transporters on the transmembrane ion gradients (Figure 1.2.3). For a long time it was accepted that the reversion of GABA transporter would not occur under physiological condition, since it was only observed by using strong stimuli. For instance it was observed that, after removal of extracellular $Ca^{2+}$, GABA release still occurs from cortical slices in response to an increase in $K^+$ to 50 mM, to exposure to 50 μM veratridine, or to high-frequency electrical stimulation (Szerb, 1979). This idea was confirmed later on when it was observed that GABA release still occurs in the absence of extracellular $Ca^{2+}$ from cultured striatal neurons (Pin and Bockaert, 1989) and from cultured cortical neurons

(Belhage et al., 1993) in response to 55–56 mM $K^+$ or 100 μM glutamate. However, the reversion of the GABA transporter can occur under physiological condition, and the initial idea that this reversal was only observed by using strong stimuli was probably a result of the relative insensitivity of the biochemical assays used to measure GABA release. As calculated (Richerson and Wu, 2003) and experimentally demonstrated (Gaspary et al., 1998; Wu et al., 2001; Wu et al., 2007) the reversal potential for GAT-1 is close to the normal resting potential of neurons, therefor a small level of depolarization can cause reversal of transport direction. GABA transporters can also reverse in presence of nipecotic acid, gabapentine and vigabatrin, being the last two anticonvulsants drugs (Wu et al., 2001; Richerson and Wu, 2003; Wu et al., 2003).

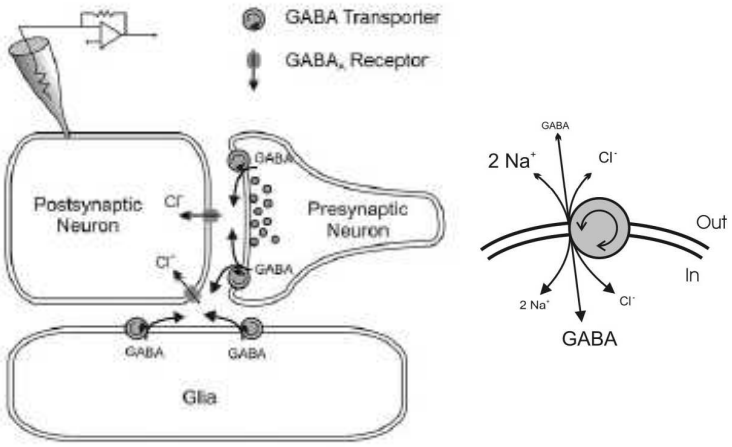



**Figure 1.2.3. GABA transporters can reverse.** *Left panel*, **Schematic representation of the performed experiments in order to asses the reversion of GABA transporters**. Patch-clamp recordings of $GABA_A$ receptor mediated currents were used to assay GABA release. Experiments were performed in 0 $Ca^{2+}$ bath solution. When the GABA transporter reversed, by increasing $[K^-]_o$, GABA release was detected as a change in holding current in the recorded neuron. *Right panel,*

**Stoichiometry of GAT-1**. A thermodynamic reaction cycle involves coupled translocation of 2 $Na^+$ ions, one $Cl^-$ ion and 1 GABA molecule. Thus GABA is normally driven up its concentration gradient using the energy stored in the $Na^+$ and electrical gradients (Adapted from Richerson and Wu, 2003).

### 1.2.3. Regulation of synaptic transmission by GABA transporters

At first glance, the presence of transporters at or near the synapse, coupled with their role in transmitter removal, suggests that transporters are ideally situated to affect the magnitude and the time course of synaptic signalling. However, one of the difficulties of this hypothesis of transport-mediated influence on synaptic signalling comes from kinetic studies revealing that transport rates are slow, since the unitary transport rates are on the order of 10/sec for the $Na^+/Cl^-$-dependent transporter subfamily, including the GABA (Mager et al., 1993) and norepinephrine (NE) transporters (Galli et al., 1995), and 100–1000/sec for the $Na^+/K^+$-dependent glutamate transporter subfamily (Schwartz and Tachibana, 1990). This slow turnover rates induces a transport that might affect normal synaptic signalling only at ‘‘slow’’ synapses; i.e., synapses in which responses are mediated through G protein-coupled receptors and signalling occurs on a time scale of hundreds of milliseconds. This led to the idea that uptake of transmitter could not be the only mechanism of shutting down neurotransmitter through transporters action. Presently some evidence suggests that transporters can alter synaptic responses on a millisecond time scale by sequestering transmitter at its binding sites within the transporter (Diamond and Jahr, 1997), effectively serving as a diffusion sink. Ample number of sites for GABA sequestration is provided by GAT-1 transporter present at nerve terminals calculated as high as 1000-2000 per square micron (Wang et al., 2002). This mechanism would allow for transporter-

mediated signalling effects at "fast", ligand-gated ion-channel synapses as well (Beckman and Quick, 1998). Indeed, upon blockade of GABA transporters there is an alteration of both the amplitude and decay of the response mediated through slow, "$GABA_B$" receptors (Isaacson et al., 1993), and of the decay phase of the "fast" $GABA_A$ receptor-mediated response (Isaacson et al., 1993). If the number of GABA transporter binding sites is a crucial determinant of GABAergic transmission, then modulating the number of these sites via transporter trafficking will be important physiologically, not only by controlling synaptic GABA signalling but also potentially by regulating spillover GABA onto neighbouring synapses.

More interesting is that recent reports continue to point to an important role of GABA transporters in regulating GABAergic inhibition. GAT-1 influences both phasic and tonic activation of GABAergic inhibition in striatal output neurons (Ade et al., 2008; Kirmse et al., 2008), demonstrating that GAT-1 is critically involved in the maintenance of low ambient [GABA] in this brain structure, as well as in maintaining phasic inhibition when there is failure of vesicular neurotransmitter release (Wu et al., 2007). Also GAT-3 has a relevant role in GABAergic transmission since an acute blocked of GAT-3 under resting conditions is fully compensated by GAT-1. This indicates that GAT-3 might provide an additional uptake capacity when neuronal activity and GABA release are increased (Kirmse et al., 2009).

GABA transporters look like to be involved in the GABAergic signaling between neurons and glial cells (Doengi et al., 2009). Activation of GABA uptake increases the intracellular [$Na^+$], that afterwards reduce $Na^+/Ca^{2+}$ exchange, thereby leading to a $Ca^{2+}$ increase sufficient to trigger $Ca^{2+}$ induced $Ca^{2+}$ release

via inositol 1,4,5-trisphosphate ($InsP_3$) receptors (Doengi et al., 2009). This role of GABA transporters, point out GABA as a mediator for neuron-glia signalling.

### 1.2.4. Functional regulation of GABA transporters

It is well established that GAT-1 functioning can be altered by a variety of initiating factors and signal transduction cascades, namely this functional modulation occurs through a variety of second messengers, such as kinases and phosphatases (Corey et al., 1994; Quick et al., 1997), transporter agonists and antagonists (Quick, 2002) and interacting proteins (Deken et al., 2000; Fan et al., 2006). This modulation occurs in two ways: by changing the rate of transmitter flux through the transporter (the Michaelis constant, $K_m$, for the transporter) or changing of functional transporters on the plasma membrane through the redistribution of transporters between intracellular locations and the plasma membrane.

The first studies concerning the regulation of GAT-1 transporter were related with the activation of protein Kinase C (PKC). Gomeza and collaborators (1991) showed that PKC activation reduces GABA transport in glial cells; later on by using Xenopus oocytes cloned with GAT-1 transporter it was demonstrated that PKC modulates the activity of this transporter by regulating its subcellular redistribution (Corey et al., 1994). This regulation by continuously traffic of GAT-1 to and from the plasma membrane (Deken et al., 2003) can occur through changes in the endocytosis and exocytosis rates, and/or the number of transporters available for recycling. Indeed, PKC decreases surface expression of GAT-1 in cortical neuronal cultures by increasing the endocytosis rate (Wang and Quick, 2005; Cristovão-Ferreira et al., 2009). More interesting is that PKC modulation of GABA transport in oocytes coexpressing GAT-1 and total rat brain mRNA can be

eliminated by injecting antisense synaptophysin and syntaxin oligonucleotides, being the antisense nucleotides strings of oligonucleotides that are complementary to "sense" strands of regions of synaptophysin and syntaxin, and that bind to and inactivate synaptophysin and syntaxin; injection of botulinum toxins, which inactivate proteins involved in vesicle release and recycling, such as SNAP-25, syntaxin and synaptobrevin (Schiavo et al., 1992; Blasi et al., 1993b; Blasi et al., 1993a), also eliminates PKC-mediated modulation; and coexpression of GAT-1 and syntaxin 1A cRNA is sufficient to permit PKC-mediated modulation (Quick et al., 1997). Indeed it was later on shown that syntaxin-1 interaction with GAT-1 results in a decrease of transport rates (Beckman et al., 1998; Lin et al., 1998; Deken et al., 2000), and that SNAP-25 efficiently inhibits GAT-1 reuptake function in the presence of syntaxin 1A (Fan et al., 2006).

Tyrosine kinases also modulate GAT-1 since their inhibition induces an intracellular accumulation of the transporter that is correlated with an increase of the internalization rate (Law et al., 2000; Whitworth and Quick, 2001). Thus, the translocation o GABA transporter from/to the plasma membrane is related with the phosphorylation states of the transporter (Quick et al., 2004). GAT-1 is phosphorylated on serine residues in a PKC-dependent manner, however this state is only revealed when GAT1 tyrosine phosphorylation is eliminated or greatly reduced. The relative levels of serine phosphorylation and tyrosine phosphorylation are negatively correlated, which indicates that the amount of serine phosphorylation is regulated by agents that affect tyrosine phosphorylation, and *vice versa* (Quick et al., 2004). So, GAT-1 can exist in either of two mutually exclusive phosphorylation states and the relative abundance of these states determines in part the relative subcellular distribution of the transporter (Quick et al., 2004): an increase in the relative amount of GAT-1 that is tyrosine

phosphorylated correlates with a relative increase in GAT-1 surface expression; an increase in the relative amount of GAT-1 that is serine-phosphorylated correlates with an accumulation of intracellular GAT-1.

Extracellular GABA can also regulate GABA transport (Bernstein and Quick, 1999) and this regulation depends on syntaxin-1 (Quick, 2002), providing a feedback mechanism for the control of neurotransmitter level at the synapse, since GABA transporter fine-tunes its function in response to extracellular GABA. Extracellular GABA induces an up-regulation of the transport that is correlated with an increase in surface expression due to a slowing of GAT-1 internalization rate, while transporter inhibitors down-regulate this transport (Bernstein and Quick, 1999). The described mechanism occurs on a time scale of minutes and requires in part direct tyrosine phosphorylation of the transporter (Whitworth and Quick, 2001). In contrast the presence of GABA on a longer time scale causes a net decrease in GAT surface expression, which suggests that multiple pathways, perhaps converging upon mechanisms involving protein phosphorylation, act to regulate GAT-1 expression in neurons (Hu and Quick, 2008).

### 1.2.5. Pathological implication of GABA transporters

In order to maintain the brain function at all levels, it is fundamental to tightly regulate the synthesis, release, and removal of synaptically released GABA (Madsen et al., 2010). Thus, there is considerable medical interest on GABA transporters, since they function to regulate neurotransmitter activity by removing GABA from the synaptic cleft, and specific transporter inhibitors can be potentially used as novel drugs for neurological disease.

One of the most studied disorders concerning GABA transporters is epilepsy. Epilepsy is a heterogeneous neurological disorder characterized by the onset of

spontaneous convulsive and non-convulsive seizures. A common feature in epilepsy seems to involve hyperexcitable neurons which discharge in a highly synchronized manner to produce a seizure, being suggested that seizure activity is a consequence of an imbalance between the inhibitory and excitatory neurotransmission (Dalby and Mody, 2001). Interestingly in patients with temporal lobe epilepsy there is an increase in GAT-3 expression in astrocytes and a decrease of GAT-1 expression in nerve terminals (Lee et al., 2006), suggesting that transport regulation may differ in different cell types. Nevertheless, attenuation of GABA removal, by inhibiting GABA transporters, will prolong the effect of this inhibitory transmitter, leading to an attenuation of seizure activity. This led to the development of inhibitors of GABA uptake, and the first GABA uptake inhibitor to be marketed as a novel antiepileptic was tiagabine (Schachter, 1999; Iversen, 2006). It is worthwhile to note that tiagabine, launched initially for use in epilepsy, is now being investigated for other possible indications, in the treatment of psychosis, generalized anxiety, sleep in the elderly and drug addiction (Iversen, 2004).

It is therefore highly likely that drugs acting on GABA transporters may become potential therapeutic candidates for the treatment of other neurologic and psychiatric conditions associated with dysfunction of the GABAergic system; e.g., anxiety, sleep disorders, chronic pain, post-traumatic stress disorder, migraine, and others.

### 1.3. Neuron-Astrocyte communication and the tripartite synapse

The concept of tripartite synapse rises from the recognition of a bidirectional communication between neurons and astrocytes at the synapse, in which the astrocyte, in addition to pre- and postsynaptic compartments, is a functional

component of the synapse (Agulhon et al., 2008). ATP, glutamate and GABA are considered important molecules involved in this bidirectional communication between neurons and astrocytes, since they are stored in, and released from, synaptic vesicles and glial cells at mM concentrations, thereby affecting neurotransmitter release and rising glial calcium concentration, respectively (Neary and Zhu, 1994; Lechner et al., 2004; Hamilton and Attwell, 2010). The calcium increase in astrocytes promotes the release of gliotransmitters, such as classical transmitters, chemokines, cytokines and peptides (Halassa and Haydon, 2010), including ATP (Newman, 2001; Pryazhnikov and Khiroug, 2008), glutamate (Nedergaard, 1994; Parpura et al., 1994), D-serine (Mothet et al., 2005) and BDNF (Bergami et al., 2008). All these gliotransmiters can directly activate neuronal receptors and modulate synaptic transmission (Figure 1.3.1).

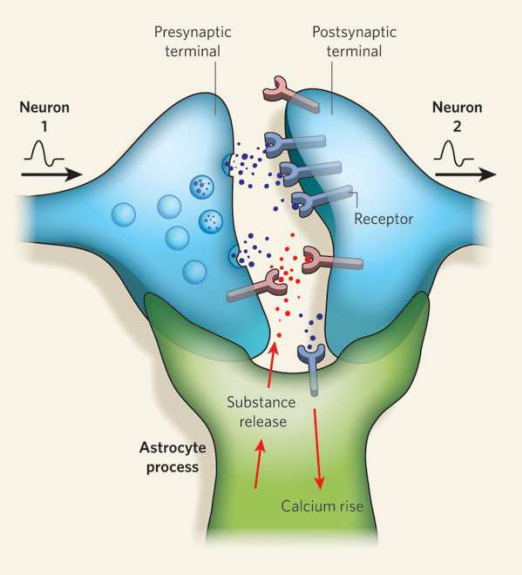


**Figure 1.3.1. Schematic representation of the tripartite synapse**. When neurotransmitters are released from the presynaptic terminal of a neuron, astrocytic receptors are thought to be activated, leading to a rise in calcium ions in the astrocyte and the release of various active substances (gliotransmitters), such as ATP, which act back on neurons to either inhibit or enhance neuronal activity. Astrocytes also release proteins, which control synapse formation, regulate presynaptic function and modulate the response of the postsynaptic neuron to neurotransmitters (Adapted from Allen and Barres, 2009).

Until now two mechanisms have been proposed where GABA has a preponderant role in neuron/astrocyte communication. The evoked release of GABA by repetitive firing of interneurons activates $GABA_B$ receptors on astrocytes, which increase their $[Ca^{2+}]_i$. This leads to the release of glutamate from astrocytes, which acts on presynaptic ionotropic receptors to increase the probability of GABA release onto pyramidal cells (Kang et al., 1998). On the other hand astrocytes may also have a role in heterosynaptic depression in the hippocampus. The released glutamate from excitatory afferents to CA1 pyramidal cells activates inhibitory interneurons to release GABA. GABA inhibits postsynaptic neurons but also activates astrocyte $GABA_B$ receptors, raising astrocytic $[Ca^{2+}]_i$, which triggers the release of glutamate from the astrocytes and thus depresses transmitter release from other afferents (Andersson et al., 2007). This heterosynaptic depression is mediated by group II and III mGluRs on the presynaptic terminals of the afferents (Figure 1.3.2). A third mechanism was described in astrocytes of olfactory bulb slices. In this case GABA evoked $Ca^{2+}$ transients in astrocytes through GABA transporter, since GABA uptake mediates a $Na^+$ rise in the cells that reduce $Na^+/Ca^{2+}$ exchange, thereby leading to a $Ca^{2+}$ increase sufficient to trigger $Ca^{2+}$-induced $Ca^{2+}$ release via InsP3 receptors (Figure 1.3.3) (Doengi et al., 2009).

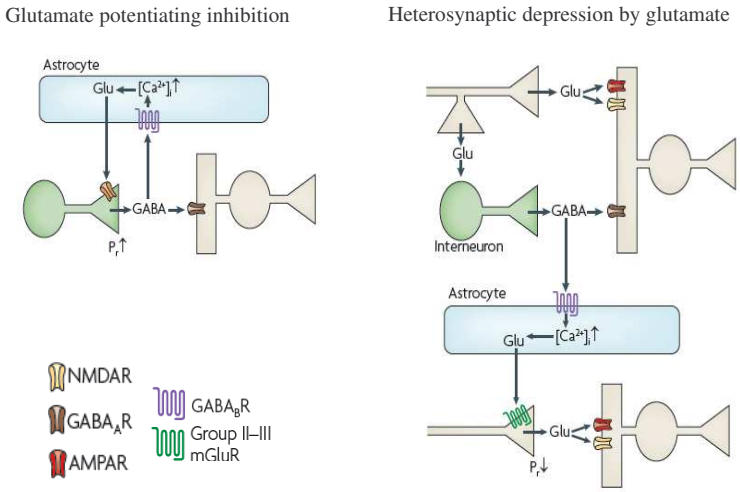



**Figure 1.3.2. Proposed mechanisms where GABA is implicated in functional effects of glutamate, d-serine and ATP release from astrocytes.** *Left panel*, *Glutamate potentiating inhibition.* Glutamate release from astrocytes, triggered by GABA activating astrocyte $GABA_B$ receptors, increases presynaptic GABA release. *Right panel*, *Heterosynaptic depression by glutamate*. Stimulating the Schaffer collaterals evokes GABA release from hippocampal interneurons, which activates $GABA_B$ receptors on astrocytes. The resulting $[Ca^{2+}]_i$ increase releases glutamate, which acts on presynaptic group II–III mGluRs to suppress glutamate release from other afferents (Adapted from Hamilton and Attwell, 2010).

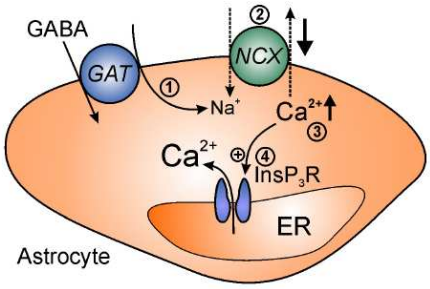



**Figure 1.3.3. Scheme of the mechanism of GABA uptake-mediated $Ca^{2+}$ signaling in astrocytes.** GABA uptake mediated by glial GABA transporter (GAT) is coupled with an intracellular $Na^+$ rise (1). The increased $[Na^+]_i$ reduces the inwardly directed electrochemical $Na^+$ gradient and, hence, the efficacy of the $Na^+/Ca^{2+}$ exchanger (NCX) (2). $Ca^{2+}$ accumulates in the astrocyte due to reduced $Ca^{2+}$ extrusion by NCX (3). The increase in intracellular $Ca^{2+}$ triggers $Ca^{2+}$ -induced $Ca^{2+}$ release from the endoplasmic reticulum (ER) via InsP3 receptors (4) (Adapted from Doengi et al., 2009).

### 1.3.1. Astrocytes

Glia refers to a diverse set of cell types phenotypically diverse that are likely to carry out distinct functions in neurophysiology, and were first described in mid-19th century by Virchow, Golgi, Müller, Deiters and others (Kettenmann and Ransom, 2005). Glial cells are electrically unexcitable cells in the nervous system and have long been considered to be supporting cells with little direct impact on neuronal performance. Indeed when glia was first described there was considerable debate as to whether glia was a connective tissue or a true population of cells (see Somjen, 1988). This idea began to change when it was found that glial cells express a large number of G protein-coupled receptors that activate a diverse array of intracellular signalling cascades (McCarthy and de Vellis, 1978; Van Calker et al., 1978; Porter and McCarthy, 1997). Later on, it was recognized that astrocytes, the major class of glial cells in the mammalian brain, participate in

synaptic transmission and contribute to information processing, since they can control the ionic environment of the neuropil and control the supply of neurotransmitters to the synapses (Haydon and Carmignoto, 2006; Halassa et al., 2007a; Halassa and Haydon, 2010), leading to a stabilization of cell-to-cell communication. Astrocytes due to their ability to release neurotrophic factors and gliotransmitters, among others, are also involved in the active control of synaptogenesis and plasticity, the regulation of blood flow (Koehler et al., 2009; Carmignoto and Gomez-Gonzalo, 2010) and the promotion of myelination (Barres, 2008).

There are four major groups of glial cells in the nervous system: (1) Schwann cells and oligodendrocytes, which produce and wrap layers of myelin around axons in the peripheral and central nervous systems, respectively; (2) microglia, the immune cell type of the nervous system, which participate in inflammatory responses; (3) nerve/glial antigen 2 (NG2)-positive glia, which include oligodendrocyte and astrocyte progenitor cells as well as NG2+ cells that persist in the mature brain; and (4) astrocytes (Agulhon et al., 2008).

In 1893 Lenhossek introduced the term astrocyte to refer to starshaped glial cells (Kettenmann and Ransom, 2005). Astrocytes can be found in CNS in a contiguous and nonoverlapping manner and are divided into several groups (Reichenback and Wolburg, 2005): protoplasmic astrocytes (type-I), fibrous astrocytes (type-II), velate astrocytes, perivascular astrocytes, radial astrocytes, Bergmann glia, Müller glia, marginal glia, tanycytes and various forms of ependymal glia.

Protoplasmic astrocytes are found in gray matter and wrap neuronal cell bodies and synapses (estimates suggest a single human astrocyte wraps more than 270,000 synapses) (Ventura and Harris, 1999; Bushong et al., 2002; Halassa et

al., 2007b); whereas fibrous astrocytes, which are found in white matter, in optic nerve and in the nerve fiber layer of mammalian vascularise retina, have processes that wrap nodes of Ranvier (Waxman, 1986; Butt et al., 1994; Sofroniew and Vinters, 2010). Therefore protoplasmic astrocytes are intimately associated with neuronal cell bodies and synapses, whereas fibrous astrocytes are associated with neuronal axons.

Radial astrocytes are common in spinal cord and brain of lower vertebrates, Bergmann glia are the radial astrocytes of the cerebellum in all vertebrates, Müller glia are the radial astrocytes of the retina, velate astrocytes were described in the granule layer of the cerebellum, perivascular astrocytes and marginal glia are near pia matter and may form several layers of endfeet, tanycytes are the most commom glia in lower vertebrates (Reichenback and Wolburg, 2005).

## 1.4. Neurotrophins

The history of neurotrophins started in 1950s when Levi-Montalchini, Cohen and Hamburger isolated the nerve growth factor (NGF) from the snake venom (Cohen et al., 1954). Only 30 years later was identified other neurothopin, the brain-derived neurotrophic factor (BDNF) (Barde et al., 1982) and for now there are four neurotrophins characterized in mammals: NGF, BDNF, neurotrophin-3 (NT-3) , and neurotrophin-4 (NT-4), which are derived from a common ancestral gene and are similar in sequence and structure (e.g. Hallbook, 1999). Since their discovery neurotrophins were extensively studied and they were primarily seen as key regulators of growth, survival and differentiation of neurons (Chao, 2003; Lu et al., 2008). More recent studies demonstrated that neurotrophins cause long-term structural and molecular changes at synapses, which are crucial not only to development but also to synaptic function and plasticity in the adult brain

(Vicario-Abejon et al., 2002; Lu et al., 2008). On the other hand, neuronal activity plays a crucial role in the synthesis, release and effects of neurotrophins (for example see (Zafra et al., 1990; Figurov et al., 1996; Nagappan and Lu, 2005).

Of the four neurotrophins, BDNF actions on central neurons have been best characterized. The synthesis of BDNF is highly regulated since, for instance, the *Bdnf* gene is comprised of at least eight distinct promoters that initiate transcription of multiple distinct mRNA transcripts (Aid et al., 2007). Through the use of alternative promoters, splicing and polyadenylation sites, at least 18 transcripts can be produced, but remarkably, each encodes an identical initial BDNF protein product (Greenberg et al., 2009). Furthermore, the *Bdnf* transcripts are polyadenylated in two differet sites: those with a short 3'UTR and those with a long 3'UTR (Timmusk et al., 1993), having these two type of *Bdnf* mRNAs distinct subcellular compartments on neurons. The short 3'UTR *Bdnf* mRNA is restricted to the soma, whereas the long 3'UTR *Bdnf* mRNA is also targeted to dendrites where BDNF can be locally translated (Figure 1.4.1) (An et al., 2008; Greenberg et al., 2009).

Although BDNF action can be altered through mechanisms of regulation of *Bdnf* mRNA, it can also be modified at the protein level. BDNF is initially synthesized as a precursor protein (preproBDNF) in endoplasmic reticulum. Following cleavage of the signal peptide, proBDNF is transported to the Golgi for sorting into either constitutive or regulated secretory vesicles. ProBDNF can be cleaved to mature BDNF (mBDNF) by furin within the Golgi and by proconvertases within the secretory vesicles (Mowla et al., 1999). It has long been thought that only secreted mBDNF is biologically active, and proBDNF is exclusively localized intracellularly, serving as an inactive precursor. However, recent observations of proBDNF secretion and its conversion to mBDNF *in vitro* by

plasmin and matrix metalloproteases suggest that proBDNF may be biologically active (Pang et al., 2004; Yang et al., 2009), although the efficiency of intracellular cleavage is controversial (Matsumoto et al., 2008) and may vary among neuronal cell types.

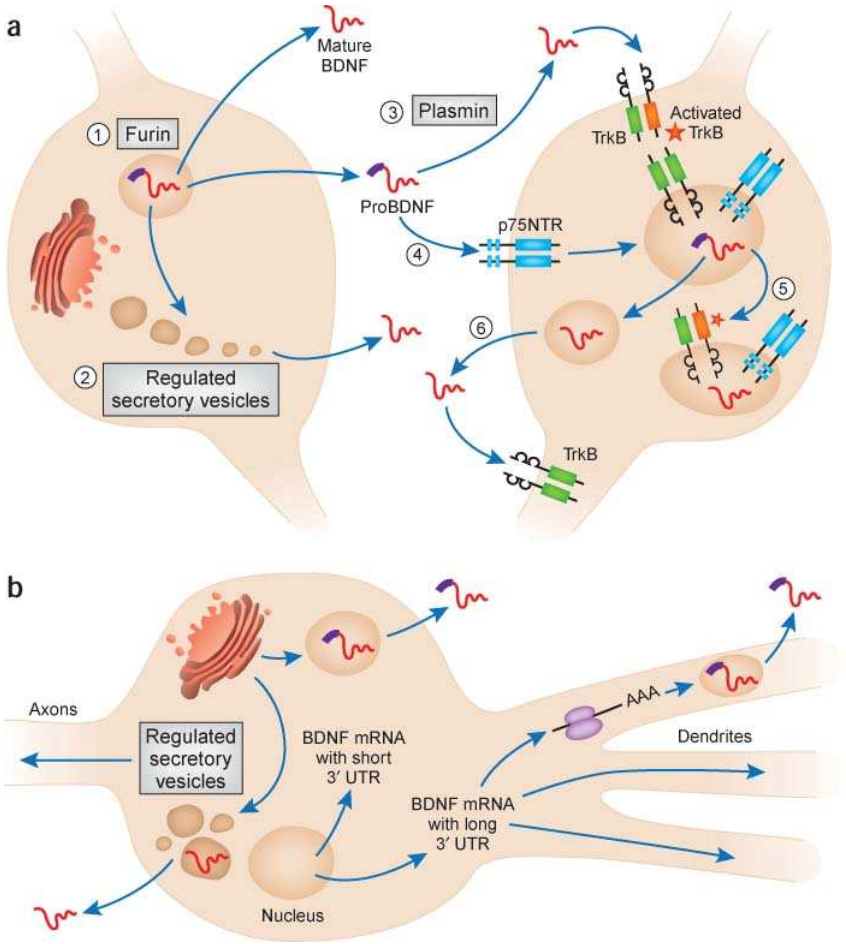


**Figure 1.4.1. Production and processing of BDNF in the central nervous system.** (**a**) ProBDNF may be processed to mature BDNF by several cellular mechanisms. ProBDNF can be cleaved within the endoplasmic reticulum by furin (1) and in regulated secretory vesicles by proconvertase enzymes (2). If proBDNF reaches the extracellular milieu, it can be processed by plasmin, and the mature BDNF produced can then activate cell surface TrkB receptors (3). Alternatively, extracellular proBDNF can bind p75NTR and become endocytosed and then cleaved to produce mature BDNF that either activates TrkB within endosomes (5) or is recycled to the cell surface (6). (**b**) The site of BDNF translation within the neuron may determine the form of BDNF released. BDNF mRNA with a short 3′ UTR accumulates in the neuronal soma, whereas BDNF mRNA with a long 3′ UTR is trafficked to dendrites. (Adapted from Barker, 2009).

### 1.4.1. Neurotrophin receptors

Neurotrophins mediate their effects through binding to two different families of receptors (Figure 1.4.2):

1. the high-affinity tropomyosin-related kinase (Trk) receptors, which belong to the tyrosine kinase family of receptors. There are three types of Trk receptors, namely TrkA that specifically bind NGF, TrkB that binds BDNF and NT-4 and TrkC that recognize NT-3.
2. the p75 neurotrophin receptors ($p75^{NTR}$), which are single transmembrane proteins of the superfamily of tumor necrosis factor (TNF) (Arevalo and Wu, 2006). All neurotrophins bind $p75^{NTR}$ with equal affinity, however neurotrophins are recognized with low affinity (100-fold less that the Trk receptors) and proneurotrophins with high affinity (Lee and Chao, 2001).

BDNF binds as dimmer to TrkB receptor triggering the dimerization of the receptor (Figure 1.4.2). The activation of TrkB leads to the autophosphorylation of the receptor, at multiple tyrosine residues in the cytoplasmic domain of the

receptor, and the activation of intracellular signaling cascades. The Src homologous and collagen-like (Shc) adaptor protein links the activated TrkB receptor to two separate intracellular signaling pathways (Figure 1.4.2.): neuronal survival requires Shr binding to the TrkB receptor, which results in increases in phosphatidylinositol 3-kinase (PI3K) and Akt activities, whereas neuronal differentiation requires the phosphorylation of Shr that leads to increases in the activity of RAS and the extracellular signal-regulated kinase (ERK). In addition, phospholipase C-γ (PLC-γ) binds to activated TrkB receptor and initiates an intracellular cascade that to a subsequent activation of protein kinase C-δ (PKC-δ) (Chao, 2003; Huang and Reichardt, 2003; Reichardt, 2006).

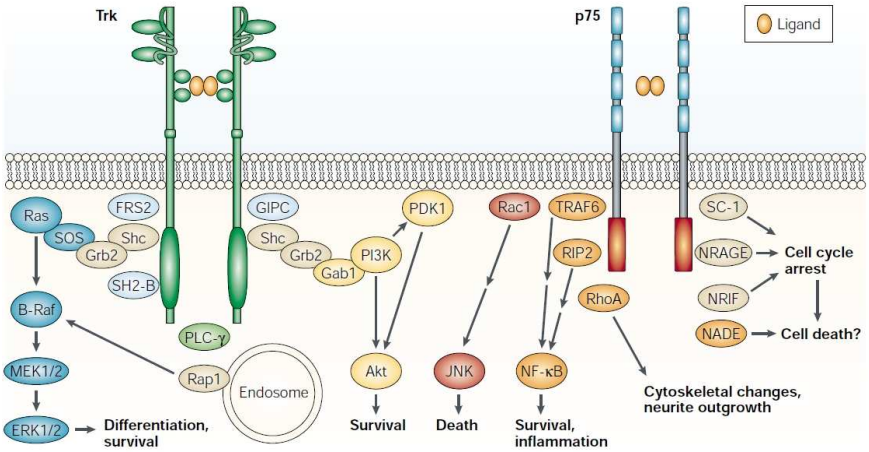



**Figure 1.4.2. Neurotrophin receptors and intracellular cascade activated in neurotrophin signalling.** Trk receptors mediate differentiation and survaival siganling through extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and phospholipase C-γ (PLC-γ) pathways. The p75 receptor predominantly signals to activate NF-κB and Jun N-terminal kinase (JNK) (Adapted from Chao, 2003).

Different TrkB receptor isoforms are generated by alternative splicing, namely one full-length form of TrkB (TrkB-fl) with an extracellular ligand binding domain, a single transmembrane domain, and a typical tyrosine kinase-containing intracellular domain (Berkemeier et al., 1991; Klein et al., 1991; Middlemas et al., 1991; Soppet et al., 1991; Squinto et al., 1991) and three truncated TrkB (TrkB-t1, TrkB-t2 and TrkB-t-Shr) isoforms with the same extracellular and transmembrane domains as TrkB-fl but with "truncated" intracellular domains lacking the kinase domain (Figure 1.4.3) (Klein et al., 1990; Middlemas et al., 1991; Shelton et al., 1995; Stoilov et al., 2002). The TrkB-t1 and TrkB-t2 have a "truncated" intracellular domain with 23 and 21 amino acids, respectively, lacking the kinase domain (Klein et al., 1990; Middlemas et al., 1991) and being the last 11 and 9 residues isoform-specific, respectively. The TrkB-t-Shc contains a Shc-binding site in the juxtamembrane domain similar to TrkB-fl, but it lacks the kinase domain and has a unique truncated C terminus (Stoilov et al., 2002).

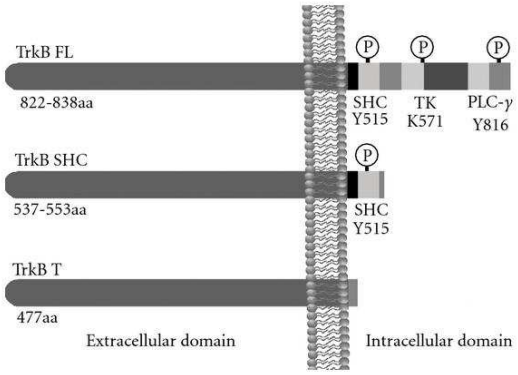



**Figure 1.4.3. TrkB isoforms structure and functional domains.** The extracellular portion of the receptor is conserved, however the intracellular domain differs among the isoforms. TrkB-fl contains a tyrosine kinase domain, a SHC-binding domain, and a PLC-$\gamma$-binding domain, TrkB SHC contains a SHC-binding domain and TrkB-t (TrkB-t1 and TrkB-t2) does not have any functional domain (Adapted from Ansaloni et al., 2011).

### 1.4.2. Fast BDNF actions at synapse

Most of the trophic and plastic actions of BDNF are mediated by TrkB receptors which trigger a complex signalling cascade leading to changes in transcription and in synthesis of key molecules involved in growth, survival and differentiation of neurons (Vicario-Abejon et al., 2002). It is commonly wide accepted nowadays that activation of TrkB receptor can also modulate neuronal activity since neurotrophins are able to induce structural and molecular changes at synaptic level that influence not only the neuronal development but also the synaptic function and plasticity of the adult brain.

Thus it has been shown that BDNF also has fast actions on synapses that occur in a time scale of less than 1 h and lead to facilitation of synaptic transmission (Kang and Schuman, 1995b; Diógenes et al., 2004; Pousinha et al., 2006; Diogenes et al., 2007; Fontinha et al., 2008; Assaife-Lopes et al., 2010), facilitation of end plate potential (Pousinha et al., 2006) and log-term potentiation (LTP) (Fontinha et al., 2008). These relatively fast synaptic actions of BDNF may also require de novo protein synthesis, as it has been shown to occur in relation to the BDNF-induced increases in AMPA (Caldeira et al., 2007b) and NMDA (Caldeira et al., 2007a) receptor levels in the cytoplasmic membrane of cultured neurons or require the translocation of these receptor to lipid rafts (Assaife-Lopes et al., 2010). On the other hand some synaptic BDNF actions result from a local and very fast action at synapses; indeed, this neurotrophin is able to facilitate glutamate release from synaptosomes (Sala et al., 1998; Canas et al., 2004; Pereira et al., 2006; Assaife-Lopes et al., 2010), which lack the somatic machinery for modulation at the gene transcription level. All the different action of BDNF can be related with the fact that cellular responses to BDNF differed markedly depending on how BDNF is delivered, since in cultured rat hippocampal neurons acute or gradual increases in BDNF elicited transient or sustained activation of TrkB receptor and its downstream signaling, respectively (Ji et al., 2010). Indeed transient TrkB activation promoted neurite elongation and spine head enlargement, whereas sustained TrkB activation facilitated neurite branch and spine neck elongation. On the other hand, also in hippocampal slices, fast and slow increases in BDNF enhanced basal synaptic transmission and LTP, respectively (Ji et al., 2010).

The above described BDNF effects are mediated by TrkB-fl receptors; nevertheless accumulating evidence suggest that truncated isoforms of TrkB

receptors, even not having the characteristic catalytic domain of the TrkB-fl receptor, may be coupled to an intracellular signaling pathway(s). TrkB-t1 isoform is the most studied of the truncated receptors. Initially it was thought that due to the lack of a kinase domain Trkb-t1 could not signal to the cytoplasm, working this receptors as a buffer for BDNF since TrkB-t1 form could bind to BDNF, sequestering it, so that it cannot bind TrkB-fl (Biffo et al., 1995; Bothwell, 1995) or by acting as a dominant negative inhibitor of TrkB-fl signaling (Eide et al., 1996; Ninkina et al., 1996; Haapasalo et al., 2001). This idea started to change when it was shown that, for instance, a short cytoplasmic domain of TrkB-t is required for BDNF-induced signal transduction (Baxter et al., 1997). It has also been shown that in cultured astrocytes, TrkB-t mediates rapid calcium transient in response to brief application of BDNF that results from the activation of phospholipase C and release of calcium from inositol trisphosphate-sensitive stores (Rose et al., 2003). This conclusion came from the observation that the calcium transient was insensitive to a tyrosine kinase inhibitor and was present in mutant mice lacking TrkB-fl (Rose et al., 2003). Signaling via TrkB-t may also play roles in the formation of dendritic filopodia in hippocampal neurons (Hartmann et al., 2004). Truncated TrkB-t1 regulates the morphology of neocortical layer I astrocytes in adult rat brain slices (Ohira et al., 2007) and at least in primary astrocyte cultures TrkB-t1 regulates astrocytic morphology via Rho GTPases (Ohira et al., 2005). The importance of these two TrkB isoforms in the present study concerns with the fact that some authors defend that astrocytes only express TrkB-t form (Rose et al., 2003; Bergami et al., 2008).

Concerning the BDNF effects upon GABA transporters it is known that BDNF increases GABA uptake in hippocampal neuron cultures, resulting this increase from a redistribution of the transporter from intracellular locations to the cell

surface (Law et al., 2000). On the other hand BDNF inhibits release of GABA from isolated nerve terminals, and this effect occurs through reversal of GABA transporters (Canas et al., 2004).

## 1.5. Adenosine

Adenosine is an ubiquitous molecule present in all cells and with important modulatory functions within the CNS, where it is involved in the inhibitory tone of neurotransmission and in neuroprotective action in pathological conditions. This molecule is a nucleoside composed of a molecule of adenine attached to a ribose sugar molecule (ribofuranose) through a a β-$N_9$-glycosidic bond.

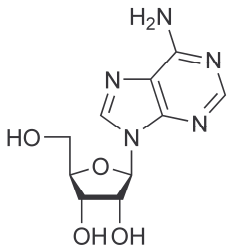



**Figure 1.5.1. Molecular structure of adenosine molecule.**

### 1.5.1. Adenosine synthesis

Adenosine is continuously formed at extra and intracellular level being extracellular adenosine obtained in three ways: (1) release of adenosine by facilitated diffusion though nucleoside transporters after an increase of the intracellular levels of adenosine or a reversal of sodium gradient, (2) extracellular

conversion of released adenine nucleotides (especially adenosine triphosphate, ATP) into adenosine through the ectonucleotidade pathway (Zimmermann and Braun, 1999) and (3) extracellular formation of adenosine after release of cyclic adenosine monophosphate (cAMP) and its breakdown by ecto-phosphodiesterase pathway. (1) and (2) above are the predominant ones and the relative importance of each of them depends on the metabolic state of the cells and, in the case of neuronal cells, their excitability status. Thus, high frequency neuronal firing and astrocytic stimulation leads to predominance of pathway (2) for extracellular adenosine formation (Dunwiddie and Masino, 2001; Latini and Pedata, 2001; Fredholm et al., 2005).

The intracellular production of adenosine is mediated by the cytosolic enzyme 5'-nucleotidase that desphosphorylates AMP or by hydrolyses of S-adenosil-homocistein (SAH) mediated by the enzyme SAH hydrolase (Figure 1.5.2). The synthesized adenosine is afterward transported for the extra cellular medium through bidirectional specific transporters that can therefore regulate extracellular concentration of adenosine. There are two main categories of nucleoside transporters: (1) equilibrative nucleoside transporters, which carry both purine and pyrimidine nucleosides across cell membranes in either direction and following their concentration gradient; (2) concentrative nucleoside transporters that mediate the influx of nucleosides coupled under the force of transmembrane sodium gradient. In the CNS the equilibrative type of nuleoside transporter appears to dominate (for a review see Thorn and Jarvis, 1996). On the other hand, the enzyme responsible for the extracellular formation of adenosine is the ecto-5'-nucleotidase, which is linked to the plasma membrane with its active site exposed in the extracellular space. The activity of ecto-5'-nucleotidase is associated with both neuronal and astrocyte cells , several works demonstrated that it is mainly

associated with astrocytes, oligodendrocytes and microglia (for a review see Latini and Pedata, 2001).

In basal condition extracellular adenosine concentration is in the range of 25-250 nM (Dunwiddie and Masino, 2001), and this concentration is sufficient to tonically activate a substantial fraction of $A_1$ and $A_{2A}$ receptors. Indeed, in normal physiological conditions adenosine has several roles, which include regulation of sleeping, regulation of general state of arousal as well as local neuronal excitability and coupling cerebral blood flow to energy demand. However in pathological conditions extracellular brain concentrations of adenosine are markedly elevated by several types of stimuli, namely hypoxia, ischemia and epilepsy. Many of the effects of adenosine that are observed to a minor extent under normal conditions (e.g. presynaptic inhibition of glutamate release) are greatly augmented during pathological events and are neuroprotective in that context. In addition to having acute protective effects, transient activation of adenosine receptors offers protection against damage induced by a subsequent hypoxic or ischemic event (Newby et al., 1985; de Mendonça et al., 2000; Fredholm et al., 2005). Thus, the actions mediated by adenosine are related with it capacity to modulate direct release of neurotransmiters, synaptic transmission and plasticity, as well as, to modulate other receptor and modulator molecules, such as neurotrophins, dopamine and cannabinoids (Sebastião and Ribeiro, 2009).

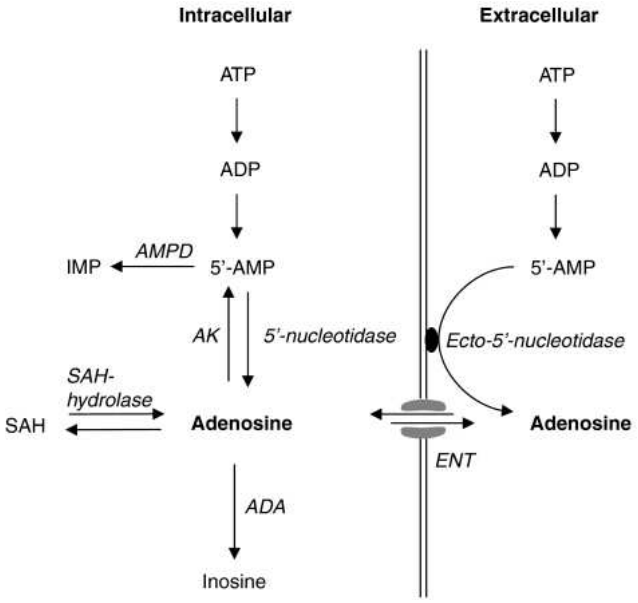



**Figure 1.5.2 Pathways of adenosine production, metabolism and transport.** Adenosine is produced intra- and extracellular by nucleotidase enzymes activity. Bidirectional specific transporters, namely equilibrative nucleoside transporters or concentrative nucleoside transporters, regulate extracellular concentration of adenosine. Abbreviations are as follow: ADA, adenosine deaminase; AK, adenosine kinase; cAMP, cyclic adenosine monophosphate; SAH, S-adenosyl homocysteine; ENT, equilibrative nucleoside transporters (Adapted from Meijer et al., 2008).

### 1.5.2. Adenosine receptors

Until now four different adenosine receptors have been identified and cloned, namely adenosine $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ receptors (Fredholm et al., 2001). All four receptors are G protein coupled receptors: $A_1$ and $A_3$ are negatively coupled to adenylyl cyclase through the $G_{i/o}$ protein α-subunits, whereas $A_{2A}$ and $A_{2B}$ are

positively coupled to adenylyl cyclase through Gs proteins (Figure 1.5.3). Adenosine receptors also have different affinities for adenosine: $A_1$ and the $A_{2A}$ exhibit a higher affinity than $A_{2B}$ and $A_3$, which are considered of low affinity (Dunwiddie and Masino, 2001). The $A_3$ receptor is a high affinity receptor in humans but has a low density in most tissues (Ribeiro et al., 2002).

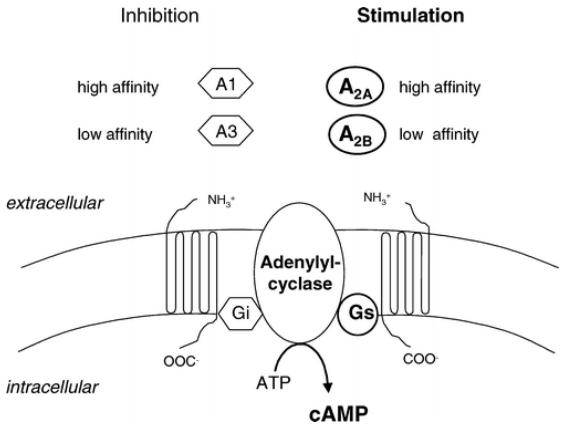



**Figure 1.5.3 Classification of adenosine receptors and their coupling to the enzyme adenylyl cyclase.** $A_1$ and $A_3$ receptors are mainly coupled to Gi proteins, while $A_{2A}$ and $A_{2B}$ are mainly coupled to Gs proteins, either inhibiting or stimulating the enzyme adenylyl cyclase, respectively. Adenosine receptors bind adenosine with different affinities (Adapted from Sitkovsky et al., 2004).

The four adenosine receptors are differentially expressed in different areas of CNS. Adenosine $A_1$ receptor is highly expressed in brain cortex, cerebellum, hippocampus, and dorsal horn of spinal cord (Figure 1.5.4), whereas $A_{2A}$ receptor is highly expressed in the striato-pallidal GABAergic neurones and olfactory bulb, being expressed in lower levels in other brain regions (see Ribeiro et al., 2002). Although $A_{2A}$ receptors are expressed in much low density in the

hippocampus (Cunha et al., 1994; Cunha et al., 1996) they can be activated by extracellularly generated adenosine in order to facilitate neurotransmitter release (Cunha et al., 1999). The $A_{2B}$ receptor has a low level of expression in the brain (Dixon et al., 1996). The $A_3$ receptor has apparently intermediate levels of expression in the human cerebellum and hippocampus and low levels in most of the brain (see Fredholm et al., 2001). All four adenosine receptors are detected in astrocytes (Bjorklund et al., 2008), and all have been reported to be expressed in microglial cells or microglial cell lines (Hammarberg et al., 2003; van Calker and Biber, 2005; Dare et al., 2007). In the hippocampal neurons both $A_1$ and $A_{2A}$ receptors are predominantly, but not exclusively, located presynaptically (Rebola et al., 2003; Rebola et al., 2005a; Rebola et al., 2005b), while in striatum the $A_{2A}$ receptors are located presynatically. In hippocampal presynaptic nerve terminals has been shown that $A_1$ receptors do not directly modulate GABA release (Cunha and Ribeiro, 2000) but influence the action of other neuromodulators upon GABA release (Cunha-Reis et al., 2008). On the other hand a facilitation of GABA release was observed when $A_{2A}$ receptors were activated (Cunha and Ribeiro, 2000). Adenosine receptos expressed in nerve terminals also modulate GABA transporters: $A_{2A}$ receptors, but not $A_1$ or $A_{2B}$ receptors, enhance GABA uptake and this occurs through activation of the adenylyl cyclase/cAMP/protein kinase A transducing system (Cristovão-Ferreira et al., 2009).

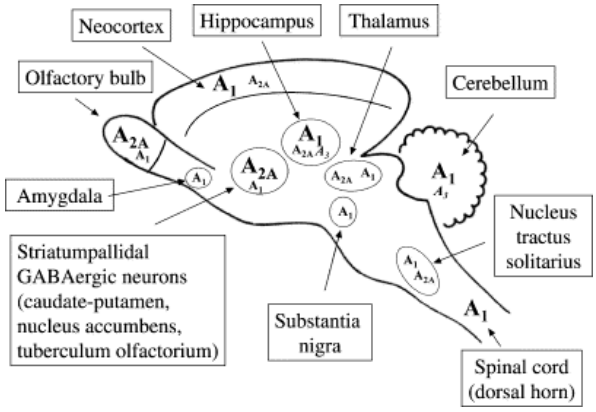



**Figure 1.5.4 Distribution of adenosine receptors in the main regions of the central nervous system.** Adenosine receptors are differentially expressed in different areas of the central nervous system (Adapted from Ribeiro et al., 2002).

### 1.5.3. Interaction between $A_{2A}$ receptors and TrkB receptors

Activation of G-protein coupled receptors (GPCRs) and tyrosine kinase receptors (RTKs) generate independent responses with different biological effects as well as different signaling pathways, nevertheless their actions are tightly related (Pyne et al., 2007). So the action of GPCR agonist on RTKs usually facilitate/sensitize RTKs for their cognate ligands, increase the synthesis of the endogenous RTK ligand and activate RTKs in the absence of ligand, a phenomenon called transactivation (for a review see Sebastião et al., 2010).

Concerning interactions between $A_{2A}$ receptors and TrkB receptor it has been shown that activation of $A_{2A}$R or pituitary adenylyl cyclase-activating polypeptide receptor 1 (PAC1) were able to directly activate TrkA and TrkB receptors in the absence of NGF or BDNF (transactivation), respectively (Lee and Chao, 2001;

Lee et al., 2002). This transactivation requires long-term incubation (3h) with $A_{2A}$ receptor agonistand involves mostly immature, intracellular Trk receptors located in Golgi-associated membranes (Lee and Chao, 2001; Lee et al., 2002). Furthermore, direct activation of Trk receptors by adenosine requires cAMP, increases in intracellular calcium, protein synthesis and activation of Fyn, a member of the Src-family kinases. In fact, Fyn is able to directly phosphorylate TrkB receptors in response to adenosine, and is colocalized with intracellular TrkB receptors (Rajagopal and Chao, 2006). It has also been recently demonstrated that activation of $A_{2A}$ receptor induces translocation of TrkB receptor to lipd rafts (Assaife-Lopes, 2010), through a mechanism most probably independent of TrkB transactivation.

Besides inducing TrkB transactivation, $A_{2A}$R activation also increase the biological effects of a RTK ligand, i.e., activation of $A_{2A}$Rs is able to trigger various TrkB-mediated BDNF actions in the nervous system. It is known that presynaptic depolarization increases extracellular adenosine levels, as well as enhances intracellular cyclic AMP, the canonical $A_{2A}$ receptor transducing pathway, triggering synaptic actions of BDNF (Boulanger and Poo, 1999a; Boulanger and Poo, 1999b). Indeed, several works demonstrated that adenosine $A_{2A}$ receptor activation is a crucial requisite for the functioning of TrkB receptor at synapse. This has been shown for the actions of BDNF on synaptic transmission (Diógenes et al., 2004; Diógenes et al., 2007; Tebano et al., 2008), and LTP (Fontinha et al., 2008) at the CA1 area of the hippocampus as well as at glutamatergic nerve ending where the release of glutamate is modulated by BDNF and adenosine (Canas et al., 2004; Pousinha et al., 2006; Assaife-Lopes et al., 2010). An interection between $A_{2A}$ and TrkB receptors has also been demonstrated at the neuromuscular junction (Pousinha et al., 2006), a single

nerve ending synapse model. The ability of BDNF to facilitate synaptic transmission on LTP (Diógenes et al., 2007) is dependent of the age of the animals (Diógenes et al., 2011) and this may be related to the degree of activation of adenosine $A_{2A}$ receptors by endogenous adenosine at different ages. In all cases the actions of BDNF are lost by blocking $A_{2A}$ receptors with selective antagonists. This requirement of $A_{2A}$ receptors acticatition to trigger a BDNF action was further confirmed by using $A_{2A}$ receptor knockout mice (Tebano et al., 2008).

## 2. AIM

The main objective of the present work was to understand how BDNF could modulate GABA uptake at tripartite synapse level, namely to investigate the effect of BDNF on GABA transporter in the nerve terminal as well as in astrocytes from a functional and mechanistic point of view. Thus the following specific objectives were persued:

1. to know the effect of BDNF on GABA transport mediated by GAT-1 in presynaptic nerve terminal and a possible modulation of this effect through activation of adenosien $A_{2A}$ receptors.
2. to study the action of BDNF upon the GAT-1 and GAT-3 mediated uptake of GABA by astrocytes and the underlying mechanisms for this BDNF action. At this point I have studied the possible involvement of adenosine $A_{2A}$ receptors on BDNF effect.

# 3. TECHNIQUES

The methods used in the presented thesis were the following: neurotransmiter uptake in nerve terminals (synaptosomes), neurotransmiter uptake in primary astrocyte cultures, molecular biology techniques (PCRs, plasmid manipulation, cell transfection), lentivirus production and cell transduction, biotinylation method and western blot analysis and affinity screening by Enzyme-Linked Immunosorbent Assay (ELISA).

In this section, will be given an introduction to the techniques and preparations most used to help the comprehension of results.

## 3.1.1. Neurotransmitter uptake from synaptosomes

Synaptosomes are sealed presynaptic nerve terminals that were first isolated by Whittaker in 1958 (Whittaker, 1993) and identified as such by electron microscopy in 1962 (Gray and Whittaker, 1962). Synaptosomes contain all the components necessary to store, release and retain neurotransmitters. When oxygenated, synaptosomes maintain a plasma membrane of -60 to -70 mV in medium containing low concentration of $K^+$ (Scott and Nicholls, 1980). The plasma membrane $Ca^{2+}$-ATPase is primarily responsible for extruding $Ca^{2+}$ from the synaptosomes (Snelling and Nicholls, 1985) and for maintaining the intarcellular calcium concentration in polarized synaptosomes in the range 0.1-0.3 μM (Richards et al., 1984). In addition, essentially all synaptosomes contain functioning mitochondria (Kauppinen and Nicholls, 1986) and unstimulated synaptosomes respire in the absence of glucose or in presence of a glycolytic inhibitor. Synaptosomal energy state is highly sensitive to "ischaemia-like" conditions, since inhibition of oxidative phosphorylation is followed by a drop of ATP and membrane potential (Kauppinen and Nicholls, 1986). Despite all

bioenergetic parameters that affect neurotransmission that can be measured by using synaptosomes, this biological preparation is quite usefull for quantifying release and uptake of neurotransmitters.

In uptake experiments synaptosomes are incubated with fixed volume of medium before separation by filtration or centrifugation. This methodology is very usefull in the sense that uptake of neurotransmitters can be followed, as well as the bioenergetic parameters that affect neurotransmission, such as plasma and mitochondrial membrane potential, calcium fluxes, respiration, ATP/ADP ratios and translocation of receptors or transporter to plasma membrane.

### 3.1.2. Primary astrocyte cell cultures

The use of tissue culture, and its application to the problems of neurobiology, started more that 100 years ago, when Ross Granville Harrison observed the outhgrowth of fibers from fragments of frog and chick neural tube cultured in drops of clotted lymph or plasma (Harrison, 1907, 1910, 1912, 1914). The demonstration that tissue could survive and grow outside the body crated widespreade interest and soon were followed by those of other (for review, see Murray, 1965).

About 45 years ago cell cultures began to gain a more prominant and important position in neurobiology: in 1969, two papers described the development of clonal lines of neuroblastoma cells obtained from a neural tumor (Augusti-Tocco and Sato, 1969; Schubert et al., 1969). When maintained in culture, these cells continued to proliferate but could be induced to stop division and acquire properties characteristic of differentiated neurons. On the other hand, two other papers described cultures of dissociated neurons from autonomic and sensory ganglia under conditions that led to the possibility of studing this type of cells as

individual living nerve cells (Bray, 1970; Yamada et al., 1970). The described types of cultures are referred to as cell lines and primary cultures, respectively.

Primary cultures are prepared from cells taken directely from the animal. The cells divide or not, depending on their type, acquire differentiated characteristics of their tissue of origin and ultimately die. For a new culture is necessary to use other animal, in order to obtain new tissue from which new cells are dissociated. Same cells are still able to divide after being dissociated from the tissue while other never divide after dissociation. One exemple of cells that are able to divide in culture are glial cells, while neurons do not have this capacity.

Cell culture is particulary valuable approach for studying glial development and function. It has been difficult to study the function of glia in situ because of the inherent heterogeneity of nervous tissue, hovewer it is relatively easy to obtain pure preparations of astrocytes, oligodendrocytes and Schwann cells, free of contaminating neurons. Astroglia precursor cells are particularly hard and gow readily in primary cultures. Usually cells are prepared from neonatal brain and divide relatively rapidly, forming a confluent monolayer of astroglia within a week or two. The condition of culture preparation is chosen to preclude the survival of neurons but, in some cases, addicional steps are needed to remove other nonneuronal contaminants. Using this approach, it is possible to produce amounts of cells suitable for biochemical as well as morphological or physiological analysis (Banker and Goslin, 1998).

### 3.1.3. Viral particles and infection of primary cell cultures

Lentiviral vectors have been widely used in experimental gene therapy paradigms, because they are capable of transducing nondividing cells such as stem cells (e.g., hematopoetic stem cells) and terminally differentiated cells (e.g., neurons)

(Naldini et al., 1996a; Naldini et al., 1996b; Johansen et al., 2005). The most extensively used and best-characterized lentiviral vectors are based on the human immunodeficiency virus type 1 (HIV-1) genome, since the properties of HIV-1 life cycle are consistent to the purpose of viral-mediated gene transfer, such as host cell attachment, receptor-mediated entry into host cells, viral mediated reverse transcription, and integration of the viral genome into the host-cell chromatin (Debyser, 2003; Kafri, 2004). Another feature of HIV-1 that makes it an ideal gene therapy vector and that is its ability to escape from cellular immune responses (Wodarz and Nowak, 1999). Following HIV-1 infection, neutralizing antibodies are rarely generated *in vivo* (Stebbing et al., 2003) and lentiviral vectors similarly integrate their genome into that of target cells without an inflammatory response (Kafri, 2004). Early generations of HIV-1-based vectors were unsuitable for gene therapy applications due to serious biosafety concerns. In recent years, however, the biosafety of lentivectors has been considerably improved by eliminating viral components and the main HIV transactivator, and by separating trans- and cis-acting viral components on different plasmids. Furthermore, the range of target cells has been vastly broadened by virus particle pseudotyping with the vesicular stomatitis virus glycoprotein (VSVG) envelope (for review, see Johansen et al., 2005).

In lentiviral production a three-plasmid expression system is used to generate HIV-derived retroviral vector particles by transient transfection, as described for other vectors (Weinberg et al., 1991). Packaging cells are used as the viral factory where the three-plasmid are brought together to produce the vector particles. The packaging cells that are used to produce lentiviral vectors are 293T cells (ATCC#CRL-11268), a highly transfectable derivative of the 293 human fetal

kidney cell line, into which the temperature sensitive gene for simian virus 40 (SV40) large T antigen has been inserted (Pear et al., 1993).

The three plasmids involved in viral particles production are: a packaging construct, an envelope plasmid and a vector construct (transducing vector). In the work now presented the plasmid pCMVΔR8.91, used as packaging construct, contains the human cytomegalovirus (hCMV) immediate early promoter and is defective for the production of the viral envelope as well as four accessory protein of HIV-1, Vif, Vpr, Vpu and Nef (Zufferey et al., 1997). Since cis-acting sequences were eliminated, this plasmid is not able of packaging, reverse transcription, and integration of transcripts derived from the packaging plasmid (Lever et al., 1989; Aldovini and Young, 1990). To broaden the tropism of the vector, a second plasmid (pMD.G) that encodes a heterologous envelope protein is used (Naldini et al., 1996a). It was used a variant that encodes the G glycoprotein of vesicular stomatitis virus (VSV-G), which offers the additional advantage of high stability, allowing for particle concentration by ultracentrifugation (Burns et al., 1993). The third plasmid is the pHsCXW lentiviral transfer vector (Johansen et al., 2005), which is modified from a lentiviral transfer vector (pHR) originally generated by Naldini and collaborators (Naldini et al., 1996b). The transducing vector contains cis-acting sequences of HIV required for packaging, reverse transcription, and integration, as well as unique restriction sites for the cloning of heterologous complementary DNAs (cDNAs). pHsCXW vector was obtained from pHR vector by including a polylinker and replacing the entire vector backbone with a pUC18 vector-derived backbone. These modifications allowed for easier cloning and higher DNA yields without compromising the fundamental ability of this vector system to transduce cells in vitro and in vivo (Johansen et al., 2005).

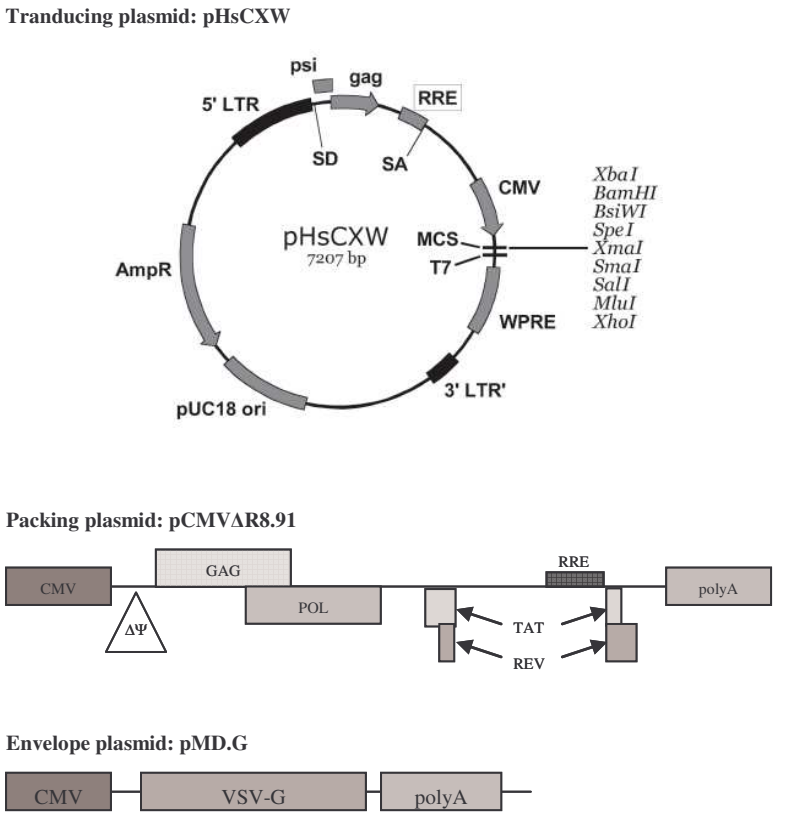



**Figure 3.1.1. Schematic representation of the three-plasmid expression system used for generating viral particles by transient transfection.** pHsCXW vector is the transducing plasmid, pCMVΔR8.91 is the packing vector and pMD.G is the envelope vector. 5'LTR: 5' long terminal repeat; Psi (Ψ): packaging signal; gag (truncated): structural protein; RRE: REV response element; SD: splice donor site; SA: splice acceptor site; CMV: cytomegalovirus promoter; MCS: multiple cloning site; T7: T7 promoter recognition site; WPRE: woodchuck postregulatory response element; 3'LTR: 3' long terminal repeat with partial U3 deletion that results in the self-inactivation of these vector; AmpR: β-lactamase expression cassette for ampicilin resistance; pUC18 ori: pUC18 vector-derived replication origin; VSV-G: G glycoprotein of vesicular stomatitis virus; CMV: cytomegalovirus; REV: Regulator of Virion; TAT: transactivator (it is a regulatory gene which accelerates production of more HIV virus); POL: polymerase (Adapted from Naldini et al., 1996b; Zufferey et al., 1997; Johansen et al., 2005)

### 3.1.4. Biotinylation

The extraordinarily stable, non-covalent interaction between avidin and biotin is one of the most commonly exploited tools in chemistry and biology, being this technique known by biotinylation.

Biotin, whose properties and structure are identical to Vitamin H, establish covalently bonds to protein lysine groups at temperatures low enough to inhibit endocytosis. The egg-white protein avidin has a high affinity for biotin (Boas, 1927; Du Vigneaud et al., 1940; Gyorgy et al., 1940; Gyorgy et al., 1941), and hence biotinylated proteins can be efficiently purified from lysates with avidin conjugated to silica beads. Indeed, the interaction between biotin and avidin is one of the tightest known with a Kd of ~$10^{-15}$ M (Green, 1975), and the robustness of this binding permits purification of biotinylated proteins from solutions containing denaturing agents such as SDS. Sulfo-NHS-biotin (N-hydroxysulfosuccinimidobiotin) is a biotin derivative that is commonly used in neuronal receptor trafficking experiments as it contains a negatively charged sulphate group which confers membrane impermeablity, thus minimizing labeling of intracellular proteins; furthermore it has low cytotoxicity, being suitable for experiments lasting many hours.

Although biotinylation is a biochemical technique that does not permit visualization of receptor location, it has many advantages over techniques that label surface receptors with specific antibodies. It is particularly useful if antibodies to the protein in question are not suitable for microscopy, or are raised to intracellular and therefore inaccessible domains. Biotinylation also affords the luxury of being able to study the trafficking of multiple different receptors/membrane proteins within the same experiment. During biotinylation experiments, the receptors on millions of cells are assayed simultaneously, with

greater quantitative sensitivity than that obtained while using immunofluoresence techniques, and so subtle differences in endocytosis and trafficking rates can be evaluated (for review, see Arancibia-Carcamo et al., 2006).

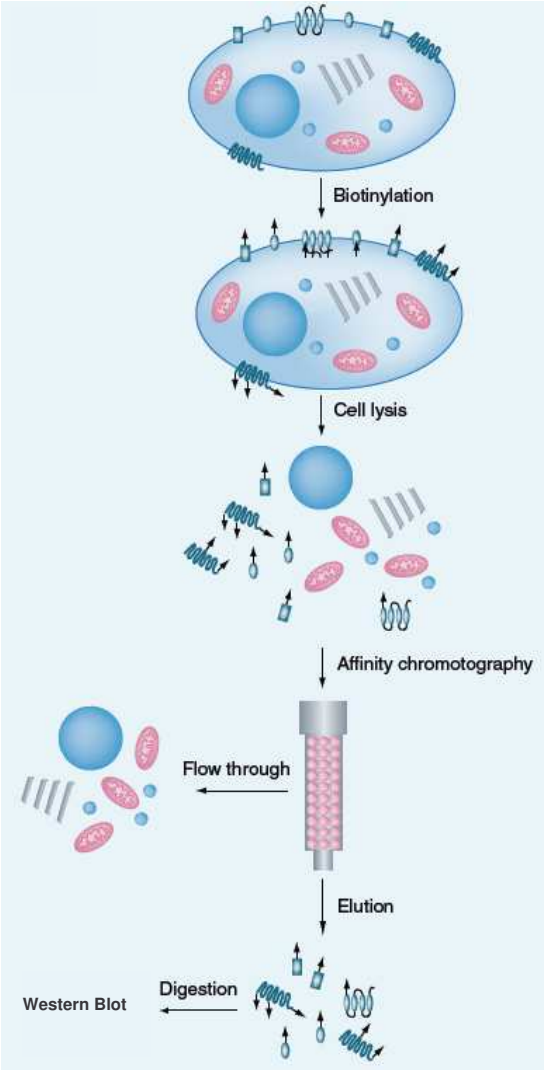


**Figure 3.1.2. Cell surface biotinylation (Adapted from Elschenbroich et al., 2010)**

### 3.1.5. ELISA (Enzyme-Linked Immunosorbant Assay)

The ELISA was developed in 1970 by Eva Engvall and Peter Perlmann (Engvall and Perlmann, 1971). The purpose of an ELISA assay is to determine if a particular protein is present in a sample and if so, how much. There are two main variations on this method: it is possible to determinate how much antibody is in a sample, or to determinate how much protein is bound by an antibody. The distinction is whether trying to quantify an antibody or some other protein.

In the simplest system, bound antigen is probed with antibodies which carry covalently attached enzyme molecules. Antibody binding immobilizes enzyme in the vicinity of the bound antigen, allowing detection of the antigen. Variations include competitive ELISA in which sample antigen is used to titrate the antibody from bound antigen. In this system, comparison of signal with signals from known antigen standards allows very accurate quantitation. In sandwich (capture) ELISAs, the antibody is bound to a surface, and used to capture the antigen for detection by a second antibody. Sandwich ELISAs are extremely specific, because the antigen must react with 2 antibodies to be detected. ELISA signals may be chromogenic and interpreted by eye or spectrophotometer, or luminogenic and detected by a luminometer. Typically, ELISA's are run in 96 well plates.

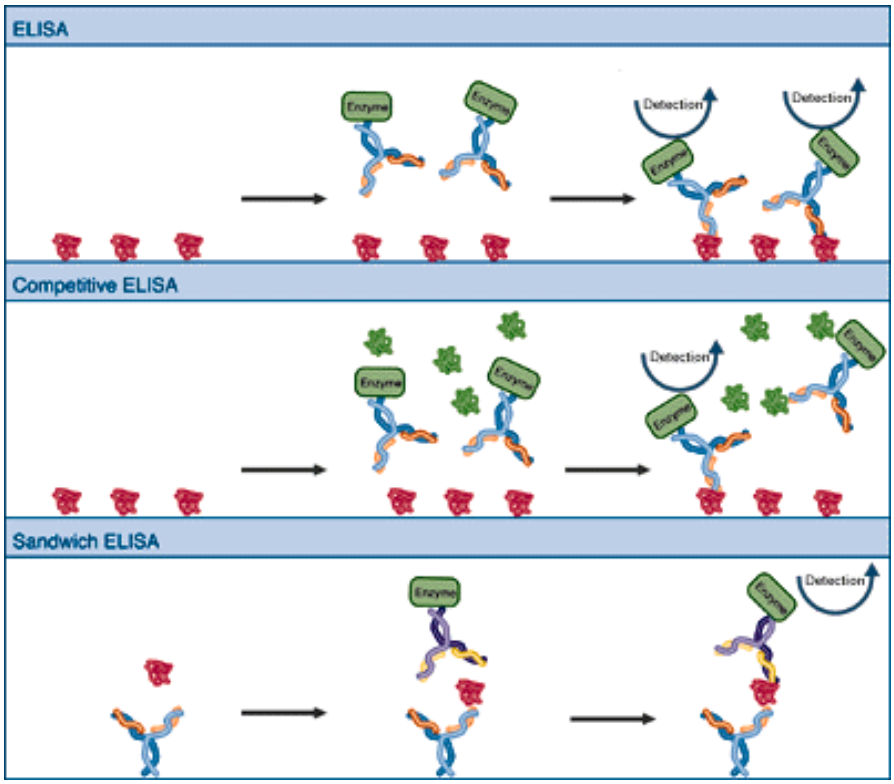



**Figure 3.1.3. The variations of ELISA (Enzyme Linked Immunosorbent Assay).** Top: Basic ELISA, in which antigen is bound to a surface and probed with enzyme linked antibody, providing semi-quantitative information. Middle: Competitive ELISA. Purified antigen is bound to the surface. Probing is carried out in the presence of samples or dilutions of free antigen (standards). The free antigen competes with the bound antigen, reducing the amount of antibody bound. Comparison between samples and standards yields quantitative information. Bottom: Sandwich ELISA, using one antibody to capture the antigen and another to detect it, resulting in increased specificity (Adapted from http://www.nationaldiagnostics.com/article_info.php/articles_id/90).

# 4. METHODS AND MATERIAL

## 4.1. Biological sample preparation

### 4.1.1. Synaptosomes

The synaptosomal fraction from Wistar rat hippocampus (3-4 weeks old) was prepared as routinely in our laboratory (Pinto-Duarte et al., 2005), according to the European guidelines (86/609/EEC). Briefly, the animals were anaesthetized with halothane before decapitation, the brain was quickly removed, the hippocampi were dissected out and added to 5 ml of a chilled sucrose solution (in mM: 320 sucrose, 1 EDTA, 10 HEPES, 1 mg/ml BSA, pH 7.40); after homogenization at 4ºC, the volume was completed to 15 ml with ice-cold sucrose solution. After a first centrifugation at 3000 x g for 10 minutes (Heraeus sepatech – Biofuge 28RS centrifuge, refrigerated at 4ºC), the supernatant was collected, centrifuged at 14000 x g for 12 minutes and the pellet resuspended in 3 ml Percoll solution, which contained Percoll 45% (v/v) in KHR solution (in mM: 140 NaCl, 1 EDTA, 10 HEPES, 5 KCl and 5 glucose, pH 7.40), adjusted to pH 7.4 with NaOH. The mixture was centrifuged again at 14000 x g for 2 minutes and the top layer, which corresponds to the synaptosomal fraction, was removed, washed with 2 ml KHR solution and centrifuged again at 14000 x g for 2 minutes.

The synaptosomal fraction was resuspended in 1 ml of chilled Krebs-HEPES solution (in mM: 10 glucose, 125 NaCl, 3 KCl, 1.2 $MgSO_4$, 1 $NaH_2PO_4$, 1.5 $CaCl_2$, 0.1 A.O.A.A. and 10 HEPES, pH 7.40) and kept at 4ºC until use. The synaptosomal protein concentration was assayed according to the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

### 4.1.2. Rat astrocytes cell cultures

Cultures of astrocytes from Wistar rat cerebral cortex were prepared as previous reported (Biber et al., 1997). In brief, rat brains were dissected out of newborn Wistar rats pups (0–2 days old). After the brains were dissected, the olfactory bulbs, hippocampal formations, basal ganglia and meninges were carefully removed in cold PBS solution (in mM: 140 NaCl, 2.7 KCl, 1.5 $KH_2PO_4$ and 8.1 $NaHPO_4$, pH 7.40). Cortex tissue was dissociated gently by trituration in 4.5 g/l glucose Dulbecco's Modified Eagles Medium (DMEM, Gibco, Paisley, UK) and filtered through meshes of 230 μm and centrifuged at 200 g for 10 minutes (at room temperature). The pellet was resuspended in 4.5 g/l glucose DMEM medium and passed through meshes of 70 μm (BD Falcon, Erembodegem, Belgium) and centrifuged at 200 g for 10 minutes (at room temperature). Cells were seeded into 24-well plates for uptake experiments, into 6-well plates for biotinylation experiments and 96-well plates for ELISA experiments. Cultures were maintained for 3 weeks in 4.5 g/l glucose DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco Paisley, UK) and 0.01% antibiotic/antimycotic (Sigma, Steinheim, Germany) in a humidified atmosphere (5% CO2) at 37°C, however during the first 2 days cells were maintained in 4.5 g/l glucose DMEM medium containing 20% FBS and 0.01% antibiotic/antimycotic.

## 4.2. Methods

### 4.2.1. GABA uptake mediated by GABA transporters in rat synaptosomes

The protocol for [$^3$H]GABA uptake was adapted from Santos and collaborators (Santos et al., 1990). Briefly, the synaptosomal suspension (0.5 mg protein ml$^{-1}$) in Krebs-HEPES solution was preincubated at 37ºC for 20-35 minutes in a total volume of 300 µl, in the presence or absence of testing drugs, and the transport was initiated by addition of 5 µM [$^3$H]GABA (specific activity 0.133 Ci/mmol), unless otherwise specified. Transport was terminated after 40 seconds by the addition of 5 ml ice-cold Krebs-HEPES solution followed by low-pressure filtration through 1.2 µm filters (Millipore, Glass Fibre Prefilters) and a second wash with 10 ml of the same solution. The 40 seconds incubation time was chosen since we observed a linear correlation between the uptake of GABA vs. incubation times ranging from 0 to 80 s (correlation coeficent = 0.95). The filters were analysed by liquid scintillation counting for determination of tritium retained by synaptosomes after addition of 5 ml of scintillation cocktail (OptiPhase "HiSafe" 2, Perkin-Elmer, Foster City, CA, USA). GAT-1 mediated GABA uptake was calculated as the difference between the total radioactivity retained in the filters and the non GAT-1 mediated component of [$^3$H]GABA uptake, which was determined by preincubation with SKF89976a (20 µM), an inhibitor of GAT-1 transporter (Borden et al., 1994a).

BDNF was added to the synaptosomes 5 min before addition of [$^3$H]GABA and the effect of BDNF was calculated taking as 100% the uptake of GABA in the absence of BDNF in the same experiments and under the same experimental conditions. Whenever the influence of any drug over the effect of BDNF was tested, that drug was incubated with the synaptosomes for at least 15 min before

addition of BDNF; the effect of BDNF in the presence of these drugs was calculated taking as 100% the uptake of GABA in the absence of BDNF but in the presence of the same drugs. Whenever removal of endogenous adenosine was required, adenosine deaminase (ADA, 1 U/ml) was added 30 min before BDNF.

### 4.2.2. GABA uptake mediated by GABA transporters in rat astrocytes

For determination of GABA uptake, astrocytes were preincubated for 3 h at 37ºC in serum-free 1 g/l glucose DMEM (Gibco, Paisley, UK). Following preincubation, cells were rinsed one time in 1 g/l glucose DMEM free serum and allowed to equilibrate for 10 min in this medium. Buffer was then exchanged with control DMEM or drug containing DMEM. The transport was initiated by addition of 30 µM [$^3$H]GABA (specific activity 0.141 Ci/mmol) (PerkenElmer, Boston, MA, USA) in a transport buffer (KHR) composed of (in mM): 137 NaCl 137, 5.4 KCl, 1.8 $CaCl_2 \cdot 2H_2O$, 1.2 $MgSO_4$ and 10 HEPES, pH adjusted with NaOH to 7.40. Transport was stopped 1 min after [$^3$H]GABA addition by rapidly washing the cells twice with ice-cold stop buffer (in mM: 137 NaCl and 10 HEPES, pH adjusted with Tris-base to 7.40) followed by solubilization with 250 µl of lyses buffer (in mM: 100 NaOH and 0.1% SDS) at 37ºC for 1 hour. The amount of [$^3$H]GABA taken up by astrocytes was quantified by liquid scintillation counting. GAT-1 and GAT-3-mediated GABA uptake was taken as the difference between the [$^3$H]GABA uptake in the absence and in the presence of the GAT-1 blocker, SKF 89976a (20 µM) and the GAT-3 blocker, SNAP 5114 (20 µM), respectively.

BDNF was added to astrocytes 10 min before addition of [$^3$H]GABA and the effect of BDNF was calculated taking as 100% the uptake of GABA in the absence of BDNF in the same experiments and under the same experimental

conditions. Whenever the influence of any drug over the effect of BDNF was tested, that drug was incubated with the cells for 20 min before addition of BDNF; the effect of BDNF in the presence of these drugs was calculated taking as 100% the uptake of GABA in the absence of BDNF but in the presence of the same drugs. GAT-1 and GAT-3 blockers were added to astrocytes at the same time that other used drugs.

Statistical analyses of the uptake data were performed using GraphPad (San Diego, CA, USA) Prism software. Two-sample comparisons were made using t tests; multiple comparisons were made using one-way analysis of variances (ANOVAs) followed by Bonferroni correction post-test.

### 4.2.3. Plasmid construction

To generate HA tagged GAT-1 the sequence of YPYDVPDYA (HA epitope) was inserted in the extracellular loop 2 (EL2) of rat GAT-1-pRc/CMV as previously described (Sorkina et al., 2006). The HA tag insertion was made using a Stratagene (La Jolla, CA, USA) Quick-change mutagenesis kit according to the manufacture's protocol.

First, it were generated four different HA tagged GAT-1 that differ in the location of the HA tag insertion in EL2 of the transporter. Thus four forward and four reverse primers were design and named has HA1, HA2, HA3 and HA4. The designed primers for the several HA tags insertion in EL2 of rat GAT-1 were: HA1 - Forward - CTCCAACTATAGCTACCCCTACGACGTCCCCGATTACG CCTCACTGGTCAACACCAC and HA1-Reverse-GTGGTGTTGACCAGTGA GGCGTAATCGGGGACGTCGTAGGGGTAGCTATAGTTGGAG; HA2-Forward- CTGGTCAACACCACCTACCCCTACGACGTCCCCGATTACGC CTCACTCAACATGACCAGTGCC and HA2-Reverse – GGCACTCATGTTGA

GTGAGGCGTAATCGGGGACGTCGTAGGGTAGGTGGTGTTGACCAG; HA3 - Forward - CCACCAACATGACCTACCCCTACGACGTCCCCGATTAC GCCAGTCTCGTGGTGGAATTC and HA3-Reverse-GAATTCCACCACCAG ACTGGCGTAATCGGGGACGTCGTAGGGGTAGGTCATGTTGGTGG; HA4 –Forward- CTGGTCAACACCTACCCCTACGACGTCCCCGATTACGCCTCA CTCAACATGACCAGTGCC and HA4-Reverse-GGCACTGGTCATGTTGAGT GAGGCGTAATCGGGGACGTCGTAGGGGTAGGTGGTGTTGACCAG. The HA tag insertion was verified by automatic dideoxynucleotide sequencing.

After kinetic analysis of all HAs-rGAT-1 in HEK293T cells (see 3.3.8. Kinetic analysis of rGAT-1 and HA-rGAT-1 in HEK cells), the construct with more similar kinectic characteristics to rGAT-1 wild type, namely HA2, was insert on a lentiviral transfer vector. Thus, to insert GAT-1 and HA tagged GAT-1 (the previously named HA2) on the lentiviral transfer vector pHsCXW a forward primer (GCATAAGCTTCTAGACATGGCGACTGA CAACAGC) containing a XbaI digestion site and a reverse primer (CAACTAGAAGGCACAGTCGAG) for a region of pRc/CMV vector localized after the XbaI restriction site were used to amplify the rat GAT-1 sequence by PCR using Phusion polymerase. Both DNA fragments, GAT-1 and HA-GAT-1, were cloned into pHsCXW vector, using XbaI restriction enzyme.

### 4.2.4. Lentivirus production and transduction

Lentiviral vectors were produced according to procedures modified from (Naldini et al., 1996b). HEK293T packaging cells (ATCC number CRL-11268) were plated on poly-lysine-coated 175 $cm^2$ flaks and transiently triple transfected with the following: (1) 18 µg of a packaging plasmid encoding viral structure proteins (pBRΔ8.91) (Zufferey et al., 1997); (2) 12 µg of an envelope plasmid encoding

the envelope protein VSV-G (pMD.G) (Naldini et al., 1996a); and (3) 18 µg of the transfer plasmid containing the gene of interest (pHsCXW-rGAT-1 or pHsCXW-HA-rGAT-1). Transfection was performed in DMEM (Gibco) supplemented with 10% FBS (Invitrogen) using calcium phosphate precipitation. Medium was replaced with fresh medium after 5 h. Approximately 48 and 72 h after transfection, media containing lentivirus was collected, centrifuged at 900 x g for 5 min to remove cellular debris, filtered through a 0.45 µm filter, and concentrated by ultracentrifugation at 50,000 x g for 1.5 h at 4°C. The virus-containing pellet was resuspended in MEM (Sigma) at 1/280 of the original volume and stored in aliquots at –80°C. The astrocyte cultures were incubated with concentrated lentivirus on days 14-21 in vitro and experiments were performed 6–8 d after infection.

### 4.2.5. HEK293 cell culturing and transfection

HEK293 cells (ATCC, number CRL-1573) were grown in DMEM supplemented with 10% FBS and gentamicin (10 µg/ml) at 37 °C in a humidified incubator with 5% CO2. Transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbed, CA, USA). For uptake experiments, 1 µg plasmid encoding the cDNA of interest was used for transient transfection of cells in a 75 cm2 culture flask. Cells were assayed 48–72 h after transfection.

### 4.2.6. Kinetic analysis of rGAT-1 and HA-rGAT-1 in HEK cells

The cells were grown in 24-well plates for 2d and tranfected with rGAT-1 or HA-rGAT-1 using Lipofectamine 2000. Cells were assayed in the transport buffer (KRH; in mM: 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 10 HEPES, pH adjusted with NaOH to 7.40.). Assays included 8.62 nM [$^3$H]GABA and increasing concentrations of unlabeled GABA (1, 2.5, 4, 5, 7.5, 15, 25 and 50

μM). Nonspecific [$^3$H]GABA accumulation was determined in the presence of 20 μM SKF89976a. After 1 min of incubation with GABA containing buffer at RT, uptake was terminated by quickly washing the cells two times with 1 ml of ice-cold stop solution (in mM: 137 NaCl and 10 HEPES, pH 7.4). Cells were then solubilized in 0.250 ml of 1% SDS for 60 min with gentle shaking. Accumulated [$^3$H]GABA was determined by liquid scintillation counting. $K_m$ and $V_{max}$ values for [$^3$H]GABA uptake were calculated with nonlinear regression fitting using GraphPad (San Diego, CA, USA) Prism software.

### 4.2.7. Biotinylation experiments

Astrocytes were grown in 6 well plates and transduced with rGAT-1 lentivirus on days 13-15 *in vitro* and experiments were performed 6–8 d after transduction. After a starvation period of 3 h cells were treated with BDNF for 10 min. The cells were rinsed twice with 4°C phosphate-buffered saline (PBS)/ $Ca^{2+}$/$Mg^{2+}$ (138 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 9.6mM $Na_2HPO_4$, 1 mM $MgCl_2$, 0.1mM $CaCl_2$, pH 7.3). The cells were next incubated with a solution containing 1.2 mg/ml sulfo-NHS biotin (Pierce, Rockford, IL, USA) in PBS/$Ca^{2+}$/$Mg^{2+}$ for 40 min at 4°C. The biotinylation solution was removed by two washes in PBS/$Ca^{2+}$/$Mg^{2+}$ plus 100 mM glycine. The cells were rinsed twice with 4°C PBS/$Ca^{2+}$/$Mg^{2+}$. The cells were solubilised with lysis buffer (25 mM Tris-base, pH 7.5, 150 mM NaCl, 5 mM *N*-Ethylmaleimide (NEM), 1 mM EDTA, 1% Triton X-100, 0.2 mM phenylmethanesulfonyl fluoride (PMSF) and protease inhibitor cocktail from Roche (Mannheim, Germany)) at 4°C and incubated with rotation for 20 min at 4ºC. The cell lysates were centrifuged at 16,000 x *g* at 4°C for 15 min. The supernatant fractions (300 μg of protein) were incubated with Avidin agarose resine (Pierce, Rockford, IL, USA) at room temperature (RT) for

60 min. The beads were washed four times with lysis buffer, and adsorbed proteins were eluted with 5x SDS sample buffer (50 mM Tris- Cl, pH 6.8, 2% SDS, 100 mM Dithiothreitol (DTT), 0.1% bromophenol blue, 10% glycerol) at 37ºC for 30 min. The supernatant was collected (surface membrane expression) for western blot analysis. Astrocytes lysates were also denaturated with 5x SDS sample buffer and an equal quantity (30 μg) were used for western blot analysis. Protein determination was made using Bio-Rad Dc Protein Assay.

### 4.2.8. Western blot assays

After denaturation (by SDS sample buffer at 37ºC for 30 minutes), both the extracts from biotinylated fractions and the astrocytes lysates were run on a 7.5% SDS-PAGE gel. Protein was transferred to a PDVF membrane (Millipore, Bedford, MA, USA) by electroblotting and blocked for 1 h at room temperature with 5% non-fat milk in PBS with 0.05% Tween-20 (PBS-T). Incubations with primary antibodies were performed overnight at 4ºC, all of them diluted in 3% BSA in PBS-T and 0.02% sodium azide. HRP-coupled secondary antibodies were diluted in blocking buffer and incubated for 1 h at RT. Detection of proteins was made with ECL plus Western blotting detection (Amersham Biosciences, Buckinghamshire, UK).

### 4.2.9. Affinity Screening by Enzyme-Linked Immunosorbent Assay (ELISA)

For ELISA experiments astrocytes were grown in 96 well plates coated with poli-ornythn and transduced with HA-rGAT-1 lentivirus on days 14-21 in vitro, in order to perform the experiment 6–8 d after transduction. Before the experiment, astrocytes were treated or not with different concentrations of BDNF for 10

minutes, as indicated. After BDNF incubation, cells were fixed in 4% PFA in phosphate-buffered saline/$Ca^{2+}$/$Mg^{2+}$ (138 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 9.6mM $Na_2HPO_4$, 1 mM $MgCl_2$, 0.1mM $CaCl_2$, pH 7.3) for 20 minutes on ice, washed twice in PBS/$Ca^{2+}$/$Mg^{2+}$, blocked in 5% goat serum in PBS/$Ca^{2+}$/$Mg^{2+}$ for 30 minutes and incubated with HA.11 antibody (1:1000) in 5% goat serum/PBS/$Ca^{2+}$/$Mg^{2+}$ for 60 minutes. Following 4 washes in PBS/$Ca^{2+}$/$Mg^{2+}$, cells were then incubated with HRP-conjugated goat anti-mouse antibody (1:1000; Pierce, Rockford, IL, USA) for 30 minutes and subsequently washed 4 additional times in PBS/$Ca^{2+}$/$Mg^{2+}$. The HRP activity was detected and quantified instantaneously by chemiluminescence using Supersignal ELISA femto maximum sensitivity substrate (Pierce) and a Wallac Victor 2 luminescence counter (PerkinElmer Life Science, Boston, MA, USA).

### 4.2.10. Reagents

GABA was purchased from Sigma (St. Louis, USA) and [$^3$H]GABA (4-amino-n-[2,3-3H]butyric acid, specific activity 92.0 Ci/mmol) from PerkenElmer Life Sciences (Boston, MA, USA). Stock solutions of BDNF (kindly supplied by Regeneron Pharmaceuticals, Tarrytown, New York) were in phosphate-buffer saline (PBS) at a final concentration of 1 mg/ml. Adenosine deaminase (E.C. 3.5.4.4, 200 U/mg in 50% glycerol (v/v), 10 mM potassium phosphate) was purchase from Roche. CGS21680 (4-[2-[[6-amino-9-(N-ethyl-b-D-ribofuranu-ronamidosyl)-9H-purinyl]amino]ethyl]benzene-propanoic acid hydrochloride), SCH58261 (2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazo-lo[1,5-c]pyrimidin-5-amine), U73122 (1-[6-[[(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione), SKF 89976A hydrochloride (1-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic acidhydro-chloride), SNAP5114

(1-[2-[tris(4-methoxyphenyl)methoxy]ethyl]-(S)-3-piperid inecarboxylic acid) were purchsed from Tocris (Bristol, UK). K252a was acquired Calbiochem (Darmstadt, Germany). LY 294002 (2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one) and U0126 (1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene) were acquired from Ascent (Weston-Super-Mare, UK). H-89 dihydrochloride hydrate (N-[2-(p-bromocinnamylamino) ethyl]-5 isoqui-nolinesulfonamide dihydrochloride), forskolin, dynasore hydrate and monensin sodium salt were obtained from Sigma.

The following stock solutions were prepared in dimethylsulfoxide: CGS 21680 (5 mM), SCH 58261 (5 mM), U73122 (5 mM), H-89 (5 mM), forskolin (5 mM), K252a (1 mM), LY 294002 (10 mM), U0126 (10 mM). SKF 89976A (10 mM) and SNAP 5114 (10 mM) were prepared in water. All aliquots were kept frozen at -20ºC until used; appropriate dilutions in incubation buffer were daily prepared.

Antibodies were purchase from the following sources: rabbit polyclonal antibody to GAT-1 (AB1570W) from Millipore (Bedford, MA, USA); rabbit anti-phospho-Trk (pTyr-490), rabbit anti-phospho-Akt, rabbit anti-PLCγ1, rabbit anti-phospho-p44/p42 MAPK and rabbit anti-p44/p42 MAPK from Cell Signaling (Boston, MA, USA); mouse monoclonal antibody to Akt1 and mouse monoclonal antibody to PLCγ1 from Santa Cruz (Santa Cruz, CA, USA); mouse monoclonal antibody HA11 from Covance (Princeton, NJ, USA); rabbit polyclonal antibody to beta-actin from Abcam (Cambridge, MA, USA); mouse IgG1 antibody to TrkB from BD Bioscience (San José, CA, USA); goat anti-mouse antibody conjugated with HRP from Pierce (Rockford, IL, USA); goat anti-mouse antibody conjugated with HRP from Santa Cruz (Santa Cruz, CA, USA); goat anti-rabbit antibody conjugated with HRP from Santa Cruz (Santa Cruz, CA, USA).

# 5. RESULTS

## 5.1. BDNF induces modulation of GABA transport into nerve terminals

### 5.1.1. Rationale

As detailed in the introduction GABA activity at synapses is terminated by rapid reuptake through specific high-affinity $Na^+/Cl^-$ dependent transporters (GATs), which are located on the plasma membrane of neurons and astrocytes, in close apposition to the synapse. The activity of GATs regulates GABA levels at synapses and therefore influences neuronal excitability. The most copiously expressed GABA transporter in the brain is GAT-1 (see Conti et al., 2004) and it is regulated by several signalling cascades that include kinases and phosphatases (Quick et al., 2004) as well as direct interaction with synaptic proteins (Quick, 2006). The number of functional GAT-1 in cultured neurons is increased by direct tyrosine phosphorylation, an action mimicked by BDNF making it a plausible candidate as the physiological trigger of the tyrosine phosphorylation signalling cascade (Law et al., 2000). BDNF also has fast actions on synapses that occur in a time scale of less than 1 h and lead to facilitation of synaptic transmission (Kang and Schuman, 1995a; Diógenes et al., 2004). However, some synaptic BDNF actions result from a local and very fast action at synapses; indeed, this neurotrophin is able to facilitate glutamate release from isolated nerve endings (synaptosomes) (Sala et al., 1998; Canas et al., 2004; Pereira et al., 2006; Assaife-Lopes et al., 2010), which therefore lack the somatic machinery for modulation at the gene transcription level. Interestingly, BDNF inhibits carrier mediated release of GABA from hippocampal synaptosomes (Canas et al., 2004), suggesting an inhibitory action upon GABA transporters. Since GABA transporters operating at

presynaptic level are physiologically designed to remove GABA from the synapses, it was decided to first evaluated the influence of BDNF on GAT-1-mediated GABA uptake by isolated nerve terminals. The involment of adenosine $A_{2A}$ receptor upon BDNF effect was also studied.

### 5.1.2. Determination of the $K_m$ and $V_{max}$ for GAT-1 transporter in rat hippocampal synaptosomes

An initial experiment was performed to determine the $K_m$ value of GAT-1 for GABA in nerve terminals. The concentrations of GABA ranged from 0.3 to 10 μM and the $K_m$ obtained was 5.05 μM (95% confidence intervals from 3.04 to 7.04 μM, Figure 5.1.1), a value similar to that already reported by other authors (Wood and Sidhu, 1986). The remaining experiments were therefore performed with 5 μM GABA, since the use of this concentration of GABA will allow to observe enhancement or inhibition of transport in presence of tested drugs. On the other hand, it was also observed that the uptake of GABA was almost completely blocked by the selective GAT-1 inhibitor, SKF89976a (20 μM), an evidence that GAT-1 transporter is by far the predominant GABA transporter in the synaptosomal preparation (Figure 5.1.2). This indicates that in the present experimental conditions, any contamination with astrocytic membranes, which predominantly possess GAT-3 (Schousboe et al., 2004), does not appreciably contribute to GABA transport values and that the observed effects are indeed mediated by GAT-1 transporter located presynaptically.

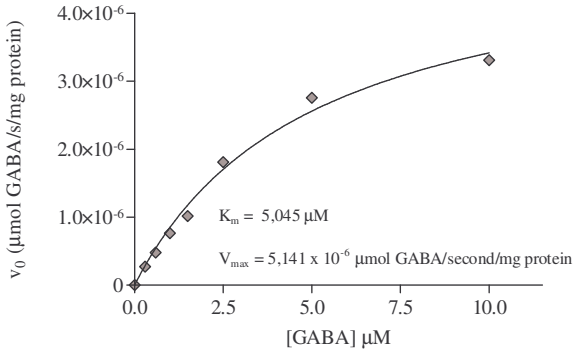



**Figure 5.1.1. Saturation analysis of GAT-1 mediated GABA transport.** Data shown in the graph was obtained from one experiment. The averaged $K_m$ (Michaelis constant) and $V_{max}$ (maximal velocity) values are shown in the graph.

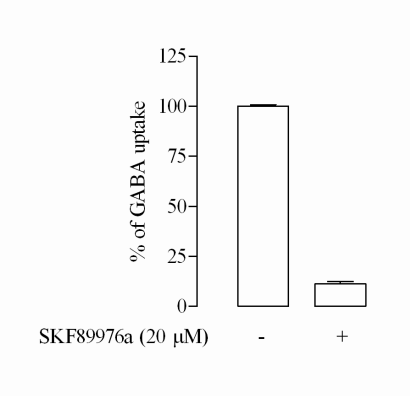



**Figure 5.1.2. SKF89976a, a high affinity antagonist of GAT-1, blocks a majority of GABA uptake in nerve terminals.** Drug concentration (in µM) is shown below the abscissa.

### 5.1.3. BDNF decreases GABA uptake from rat hippocampal synaptosomes

Next it was evaluated the effect of BDNF on GABA transporter mediated by GAT-1 in synaptosomes. As shown in Figure 5.1.3.A, BDNF (10 - 200 ng/ml) caused a concentration dependent decrease in GAT-1 mediated GABA uptake, maximum effects being already observed with 100 ng/ml. An intermediate concentration of BDNF (30 ng/ml) was used in the remaining experiments. The effect of BDNF was very fast since a full effect could be observed after 1 min incubation and it was kept constant up to 20 min incubation (Figure 5.1.3.B). In the remaining experiments, a 5 min incubation period with BDNF was used.

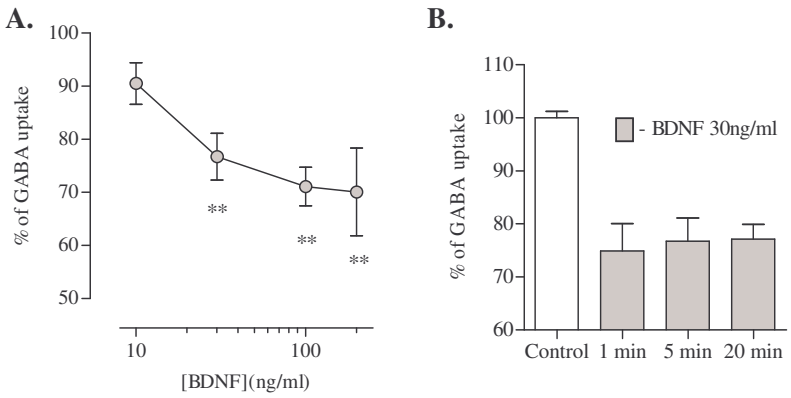



**Figure 5.1.3. Brain-derived neurotrophic factor inhibits GABA uptake in hippocampal synaptosomes.** ***A***. concentration-response curve for the effect of BDNF (n=6); ***B***. time-course curve of BDNF effect (30 ng/ml, n=4). In each experiment, the effect of BDNF in the absence and in the presence of the enzyme inhibitors was always tested using the same synaptosomal batch. BDNF was incubated with the synaptosomes for 5 min, except for the time-course experiments (B.), where the BDNF incubation times are indicated below each filled column. The ordinates represent [$^3$H]GABA uptake as percentage of the control value in the same experiments, which was taken as 100%. The results are mean ± S.E.M.;**p<0.01, as compared with absence of BDNF in the same conditions.

### 5.1.4. BDNF effect upon GAT-1 is mediated through activation of TrkB receptor

BDNF operates through the high-affinity receptor tyrosine kinase, TrkB, and the p75 receptor, which lacks catalytic activity (see e.g. Chao, 2003). Although different TrkB receptor isoforms are generated by alternative splicing, namely one full-length form of TrkB (TrkB-fl) (Berkemeier et al., 1991; Klein et al., 1991; Middlemas et al., 1991; Soppet et al., 1991; Squinto et al., 1991) and three truncated TrkB (TrkB-t1, TrkB-t2 and TrkB-t-Shr) isoforms with the same extracellular and transmembrane domains as TrkB-fl but with "truncated" intracellular domains lacking the kinase domain (see chapter 1.4.1) (Klein et al., 1990; Middlemas et al., 1991; Shelton et al., 1995; Stoilov et al., 2002), neurons mainly express the TrkB-fl receptor and this isoform has been described as responsible for BDNF fast actions that lead to facilitation of synaptic transmission (Kang and Schuman, 1995b; Diógenes et al., 2004; Pousinha et al., 2006; Diogenes et al., 2007; Fontinha et al., 2008; Assaife-Lopes et al., 2010), facilitation of end plate potential (Pousinha et al., 2006) and log-term potentiation (LTP) (Fontinha et al., 2008). Thus, the described inhibitory effect of BDNF can be attributed to activation of TrkB-fl. To test this hypothesis the tyrosine kinase inhibitor, K252a (100 nM, Tapley et al., 1992), was used simultaneously with BDNF. K252a by itself was devoid of effect upon GABA uptake, but prevented the inhibitory action of BDNF (30 ng/ml) on GABA uptake (Figure 5.1.4.A).

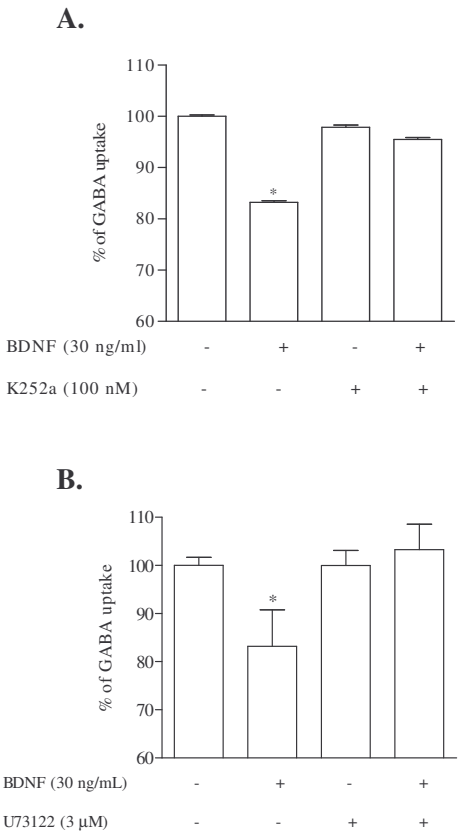



**Figure 5.1.4. Brain-derived neurotrophic factor effect on GABA transport is mediated by TrkB receptor, being a PLCγ-dependent mechanism.** ***A.*** the blockade of the effect of BDNF by the inhibitor of tyrosine kinase autophosphorylation, K252a (n=3); ***B.*** the ability of the PLC inhibitor, U73122 (n=5) to prevent the effect of BDNF. In each experiment, the effect of BDNF in the absence and in the presence of the inhibitors was always tested using the same synaptosomal batch. Synaptosomes were incubated with BDNF for 5 min. All other drugs were added 15 min before BDNF as indicated below each bar in A and B. The ordinates represent [$^3$H]GABA uptake as percentage of the control value in the same experiments, which was taken as 100%. The results are mean ± S.E.M.; *$p < 0.05$, as compared with absence of BDNF in the same conditions.

BDNF, through TrkB receptors, induces activation of different signalling pathways, namely ERK, phosphatidylinositol 3-kinase (PI3-K) and phospholipase Cγ (PLC-γ) (Chao, 2003; Huang and Reichardt, 2003), the last one being frequently involved in synaptic actions of BDNF (eg Pousinha et al., 2006). In rat hippocampal synaptosomes, BDNF induces PLC-γ activation but does not affect ERK or PI3-K/Akt signaling pathways (Pereira et al., 2006). Therefore, we only evaluated the requirement of the PLC-γ signalling pathway. The synaptosomes were previously incubated for 20 min with the PLC inhibitor, U73122, which was used at a supramaximal concentration (3 mM, Bleasdale et al., 1990; Smith et al., 1990). As shown in Figure 5.1.3.B, U73122 (3 μM) fully prevented the inhibitory action of BDNF (30 ng/ml) upon GABA uptake by the synaptosomes, indicating that this effect of BDNF requires PLC activity.

### 5.1.5. Endogenous activation of $A_{2A}$ receptors is not required for the inhibitory action of BDNF upon GABA uptake

It was previously observed that blockade of adenosine $A_{2A}$ receptors fully prevents the facilitatory action of BDNF upon synaptic transmission and plasticity at the hippocampus (Diogenes et al., 2004; Diogenes et al., 2007; Fontinha et al., 2008) as well as at the neuromuscular junction (Pousinha et al., 2006). Therefore, the influence of $A_{2A}$ receptors upon the inhibitory action of BDNF on GABA transport was evaluated. The $A_{2A}$ receptor antagonist, SCH 58261, at a concentration (50 nM) nearly 70 times its $K_i$ value for $A_{2A}$ receptors (Zocchi et al., 1996a) and below its $K_i$ value for other rat adenosine receptor subtypes, was incubated with the synaptosomes for 15 min before addition of BDNF (30 ng/ml) and the amount of GABA transported under these conditions was taken as 100%. As illustrated in Figure 5.1.5A, the inhibition of GABA uptake induced by BDNF (30 ng/ml) in the presence of SCH 58261 was not significantly different ($p>0.05$)

from that observed with the same synaptosomal batch in the absence of SCH 58261. This indicates that tonic activation of adenosine $A_{2A}$ receptors is not required to trigger a BDNF effect upon GABA uptake. $A_{2A}$ receptor activation with a selective agonist, CGS 21680 (30 nM, Jarvis et al., 1989) also did not significantly ($p>0.05$) influence the inhibitory effect of BDNF upon GABA transport (Figure 5.1.5A).

By itself SCH 58261 (50 nM) inhibited GAT-1 mediated GABA uptake by 26 ± 4.5% ($n=5$, $p<0.05$), suggesting that the adenosine released by synaptosomes is tonically activating $A_{2A}$ receptors to enhance GAT-1 mediated GABA transport. It is therefore possible that membrane $A_{2A}$ receptors were already occupied with the endogenous ligand, which would occlude any further action of an exogenously added agonist, such as CGS 21680. To evaluate the influence of $A_{2A}$ receptor activation in the absence of endogenous adenosine, synaptosomes were incubated with adenosine deaminase (ADA, 1 U/ml, added 30 min before GABA uptake assay) to inactivate external adenosine, and the effect of BDNF in the absence or presence of the $A_{2A}$ receptor agonist was compared. As illustrated in Figure 5.1.5.B., activation of $A_{2A}$ receptors with GCS 21680 (30 nM) in an external adenosine depleted background caused a significant ($p<0.05$) potentiation of the inhibitory effect of BDNF (30 ng/ml) upon of GAT-1 mediated GABA transport. This potentiation of the BDNF action by the adenosine $A_{2A}$ receptor agonist was prevented by the selective $A_{2A}$ receptor antagonist, SCH 58261 (50 nM), since in the simultaneous presence of the $A_{2A}$ receptor agonist and antagonist BDNF (30 ng/ml) inhibited GABA uptake by a similar magnitude as in the absence of any $A_{2A}$ receptor ligand (Figure 5.1.5).

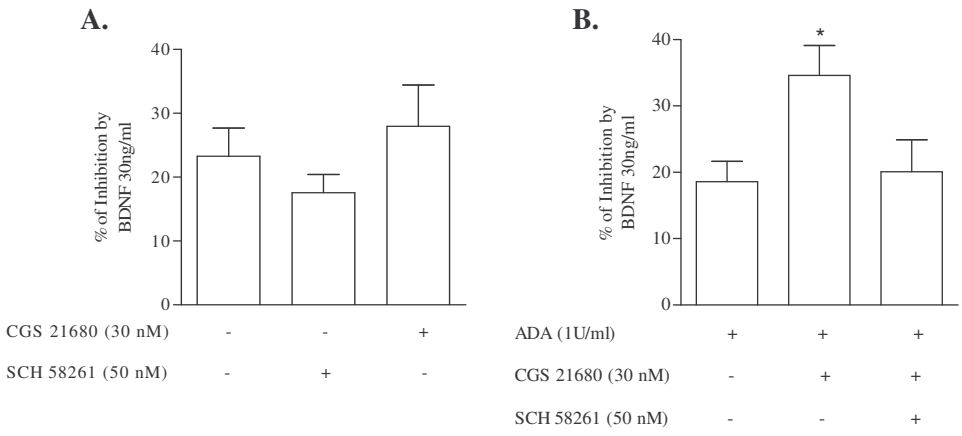



**Figure 5.1.5. Modulation of the effect of BDNF upon synaptosomal GABA uptake by adenosine $A_{2A}$ receptors.** The $A_{2A}$ receptor ligands were added to the synaptosomes either in the absence (***A***, n=6) or in the presence (***B***, n=5) of adenosine deaminase (ADA, 1U/ml) to remove external endogenous adenosine. The ordinates represent the inhibition caused by BDNF (30 ng/ml), where 0% corresponds to absence of effect and 100% to a complete inhibition of GAT-1 mediated GABA transport. In each experiment, the effects of BDNF under the drug conditions indicated within each panel were always tested using the same synaptosomal batch. BDNF was incubated with the synaptosomes for 5 min. All other drugs were added 15 min before BDNF and their presence is indicated below each column. ADA (1U/ml) was added 30 min before BDNF. The results are mean ± S.E.M.;*$p<0.05$ as compared with the effect of BDNF in the same experiments but absence of CGS 21680 (first column).

### 5.1.6. Discussion

The experiments described in this chapter were designed to evaluate the effect of BDNF on GAT-1 transporter in rat presynaptic nerve terminal. The main findings that were achived are that BDNF, through TrkB and PLCγ signalling inhibits GAT-1-mediated GABA transport by nerve endings, and that this action of BDNF is not dependent on, but can be enhanced by, cross talk with adenosine $A_{2A}$ receptors. The present results contrast with a previous report that BDNF enhances GAT-1-mediated GABA transport in serum deprived cultured neurons (Law et al., 2000). In isolated nerve endings (present work) as in neuronal cultures (Law et al., 2000) the effect of BDNF might result from TrkB receptor autophosphorylation since it is prevented by a tyrosine kinase inhibitor, K252a. The difference may reside in the use of intact cells (Law et al., 2000) versus a subcellular fraction specialized in synaptic signalling; the isolated nerve endings (present work). Indeed, the intact cells may allow BDNF-induced changes in gene expression and protein synthesis, whereas at the nerve endings most of the machinery for protein synthesis is lacking. The time course of the BDNF actions at the nerve endings and in intact neurons (Law et al., 2000) may also be different, since the inhibition of GABA transport at the nerve endings is very fast, being already seen after 1 min incubation with BDNF and the used 30 minuts incubation time of Law and collaboraters (2000) may be in whole neuron enough to allow BDNF-induced changes in protein synthesis (see Caldeira et al., 2007b; Caldeira et al., 2007a) for BDNF induced changes in receptor expression). Interestingly, the two opposite actions of BDNF upon GAT-1, potentiation of GABA uptake by hippocampal neurons (Law et al., 2000) and fast inhibition of GABA uptake now demonstrated, may have a coherent physiological goal. Thus, at a nerve ending, an inhibition of GABA transport may lead to an increase in the amounts of

synaptic GABA, therefore to an increase in GABAergic signalling. On the other hand, an increase in GABA transport in other neuronal membrane compartments may rescue GABA to replenish the releasable pool. It is also of interest that the BDNF-induced inhibition of GABA transport at nerve endings appears to be similar when transport is either in the inward direction (present work) or is reversed, releasing GABA (Canas et al., 2004). This suggests a common mechanism used by BDNF to inhibit GAT-1 at nerve endings, independently of the concentration gradient of GABA across the cell membrane.

The molecular mechanisms that underlie the different ways BDNF uses to differentially modulate GAT-1 at nerve endings or entire neurons require further studies, but the evidence so far available points towards the involvement of different signalling cascades (Osawa et al., 1994; Law et al., 2000). Indeed, TrkB receptor signalling includes the activation of the Akt pathway, the activation of MAP kinases and the activation of PLC-γ (see e.g. Chao, 2003). TrkB receptor activation by BDNF in hippocampal nerve endings enhances PLC-γ activity, leaving the ERK and Akt phosphorylation pathways unaffected (Pereira et al., 2006). We observed that the inhibitor of PLC, U73122, prevented the effect of BDNF upon GABA transport in isolated nerve endings, but the effect of BDNF on GABA transport in the cultured neurons was not affected by PKC inhibition (Law et al., 2000). Activation of PLC leads to diacylglycerol formation and subsequent PKC activation, which is known to decrease GABA transport (Osawa et al., 1994) due to phosphorylation of GAT-1 serine residues (Quick et al., 2004). It is therefore not surprising that BDNF, through PLCγ-mediated signalling, decreases GAT-1-mediated GABA transport. However, through promotion of phosphorylation of tyrosine residues, BDNF may enhance GABA transport (Law et al., 2000). To reconcile these two observations Quick and collaboraters (Quick et al., 2004) proposed that the relative abundance of the two mutually exclusive

phosphorylation states of GAT-1, one phosphorylated in serine residues and another phosphorylated in tyrosine residues, determines the relative subcellular distribution of the transporter. However, it is hard to anticipate how the action of BDNF, which induces phosphorylation of tyrosine and serine residues, will ultimately influence GAT-1. The present data showing that BDNF decreases GAT-1-mediated GABA transport at the nerve endings in a PLC-dependent way, together with the previous report that BDNF increases GAT-1-mediated transport in cultured neurons in a PLC independent way (Law et al., 2000) suggest that the subcellular distribution of the transducing systems operated by TrkB receptors may decide the fate of BDNF action upon GABA transport.

The effect of BDNF on synaptosomal GABA uptake was not appreciably affected by $A_{2A}$ receptor blockade or removal of endogenous adenosine with adenosine deaminase, suggesting that adenosine $A_{2A}$ receptor co-activation is not an essential step for this action of BDNF. This contrasts with what has been observed by us in what concerns the facilitatory action of BDNF on excitatory synaptic transmission (Pousinha et al., 2006; Diógenes et al., 2007) where $A_{2A}$ receptor blockade fully prevents the action of BDNF. However, $A_{2A}$ receptors, in spite of not being essential, may influence the action of BDNF upon GAT-1 at nerve endings since activation of adenosine $A_{2A}$ receptors when they were not already occupied by the endogenous ligand, enhanced the BDNF-induced inhibition of GABA transport, an enhancement that was antagonized by the $A_{2A}$ receptor antagonist. On the light of these results, one could expect that when $A_{2A}$ receptors were occupied by the endogenous ligand, i.e., in the absence of adenosine deaminase, the $A_{2A}$ antagonist *per se* would induce at least a slight attenuation of the effect of BDNF. The lack of appreciable effect of $A_{2A}$ receptor blockade under these conditions may suggest partial $A_{2A}$ receptor desensitization

by endogenous adenosine that adds to a low efficacy of $A_{2A}$ receptors to modulate the influence of BDNF upon GABA transport.

BDNF has been shown to quickly modulate GABAergic transmission in the hippocampus through pre- and postsynaptic mechanisms. Postsynaptically, BDNF decreases GABAergic transmission to pyramidal neurones (Tanaka et al., 1997), inducing a rapid downregulation of $GABA_A$ receptor surface expression (Brunig et al., 2001). Presynaptically, a decrease in GABAergic input to glutamatergic neurons has been reported (Frerking et al., 1998). A detailed analysis of the action of BDNF at different types of GABAergic synapses revealed that they are synapse specific (Wardle and Poo, 2003) but the trend is towards an inhibition of GABAergic transmission. The present results showing that BDNF inhibits GABA uptake by nerve endings add a new role of BDNF at synapses, which may lead to an increase in the lifespan of GABA at GABAergic synapses, counteracting the inhibition of GABAergic transmission caused by the neurotrophin. Interestingly, in immature neurons BDNF enhances, rather than inhibits, GABA release, and this is part of a positive feedback loop between GABA and BDNF expression (Obrietan et al., 2002). Since GAT-1-mediated transport in nerve terminals appears to contribute to the maturation of point-to-point GABAergic synapses (see Conti et al., 1999; Conti et al., 2004), a fine control of GABAergic transmission that simultaneously involves BDNF and GAT-1 may be particularly relevant in the shaping GABAergic synapses under maturation. Further studies are, therefore, required to evaluate how BDNF influences GAT-1 during development.

## 5.2. Effects mediated by BDNF on rat astrocyte cultures

### 5.2.1. Rationale

In the previously chapter was shown that BDNF inhibits GABA transport mediated by GAT-1 in presynaptic nerve terminal, which therefore shape GABAergic transmission. However GABA transporters are also expressed in astrocytes, and BDNF at this level can also shape GABAergic transmission. Indeed, as mention in detailed at the Introduction, astrocytes, the major class of glial cells in the mammalian brain, play a relevant role in synaptic transmission and contribute to information processing, since they can control the ionic environment of the neuropil and control the supply of several neurotransmitters to synapses (Haydon and Carmignoto, 2006; Halassa et al., 2007a; Halassa and Haydon, 2010), as well as modulate cell-to-cell communication (Perea et al., 2009). Astrocytes predominantly express GAT-3 but GAT-1 is also found in this type of cells. Although astrocytes play an important role in the regulation of extracellular GABA levels (Kirmse et al., 2009), surprisingly little is known on how GABA transporters are controlled in these cells. On the other hand, the observation that BDNF inhibits GAT-1 mediated GABA transport at the isolated nerve endings (see chapter 5.1) but facilitates it in whole neurons (Law et al., 2000), suggests that this neurotrophin operates in a much localized way and eventually in a cell specific manner. Thus in this second part of the work it was decided to study the effect of BDNF on GABA transporters, namely GAT-1 and GAT-3, in astrocytes.

The mechanisms underlying the action of BDNF were them investigated taking into consideration that regulation of the continuous traffic of GATs to and from the neuronal plasma membrane can occur through changes in the endocytosis and

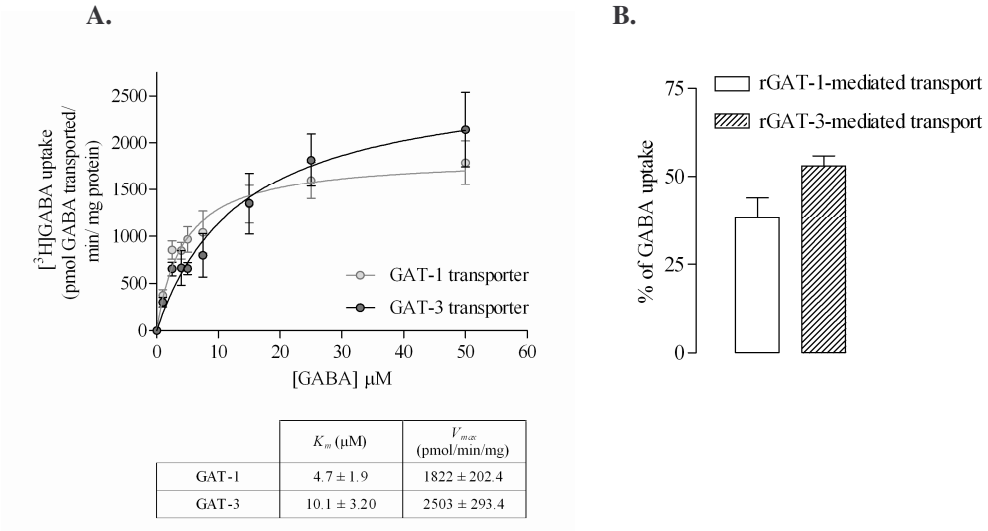
exocytosis rates, and/or the number of transporters available for recycling (Deken et al., 2003). For instance surface expression of GAT-1 in cultured neurons (Beckman et al., 1999; Wang and Quick, 2005) and isolated nerve terminals (Cristovão-Ferreira et al., 2009) is decreased by protein kinase C (PKC)-dependent phosphorylation. In contrast, surface expression of GAT-1 in neurons is enhanced by brain derived neurotrophic factor (BDNF)-mediated tyrosine kinase-dependent phosphorylation (Law et al., 2000; Whitworth and Quick, 2001).

Finally, since in nerve terminals the regulation of GAT-1 by BDNF is modulated by activation of adenosine $A_{2A}$ receptors (see chapter 5.1 and Vaz et al., 2008), as it was observed for other fast BDNF actions (for a review see Sebastião et al., 2010), it was next evaluated how adenosine could modulate BDNF effects upon GABA transporters in astrocytes.

### 5.2.2. BDNF increases GAT-1-mediated GABA uptake by increasing $V_{max}$ constant of the transporter on astrocyte cultures

GABA transport into the astrocytic culture was first characterized by evaluating the maximum velocity ($V_{max}$) and affinity constant ($K_m$) for GAT-1 transport, and the relative contribution of GAT-1 and GAT-3 for total GABA transport. The $K_m$ value obtained for GABA uptake in astrocytes was around 30 μM, a value similar to the one already reported by others (Schousboe et al., 2004). When we isolated GAT-1 and GAT-3 mediated GABA transport (see methods, Figure 5.2.1A), the obtained $K_m$ value was 4.7 ± 1.89 μM (n=6, open circles) and 10.1 ± 3.20 μM (n=4, filled circles), for GAT-1 and GAT-3 transporters respectively, values also of the same magnitude to previously reported in relation to these transporters (Wood and Sidhu, 1986; Schousboe et al., 2004).

The uptake of GABA in the presence of the selective GAT-1 inhibitor, SKF89976a (20 μM), was reduced by 38 ± 5.8 % (n=5) of total uptake; when GAT-3 was blocked with SNAP 5114 (20 μM), a selective inhibitor of GAT-3 transporter, GABA transport was reduced by 53 ± 2.8 % (n=5). These data indicates that near 55 % total GABA transport into astrocytes occurs through GAT-3, the remaining 40% being through GAT-1 (Figure 5.1.2.B). From now on, while refering to GAT-1 mediated GABA transport I am reporting data from experiments where GAT-1 had been blocked with a supramaximal concentration (20 μM) of SKF 89976a (Borden et al., 1994a), being the transport mediated by GAT-1 calculated by the difference between total transport (absence of GAT-1 blocker) and the transport measured in presence of GAT-1 blocker in the same experiment. Conversely, when we refer to GAT-3 mediated GABA transport we are referring to experiments where GAT-3 had been blocked with a supramaximal concentration (20 μM) of SNAP 5114 (Borden et al., 1994b), the GAT-3 mediated transport being calculated by the difference between total transport and transport in the presence of the GAT-3 blocker in the same experiment.



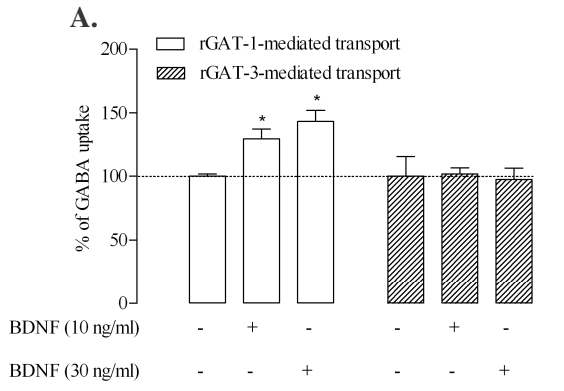

| | $K_m$ (µM) | $V_{max}$ (pmol/min/mg) |
|---|---|---|
| GAT-1 | 4.7 ± 1.9 | 1822 ± 202.4 |
| GAT-3 | 10.1 ± 3.20 | 2503 ± 293.4 |

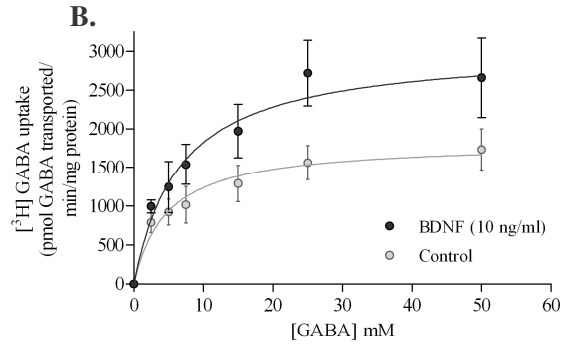
**Figure 5.2.1. Characterization of GABA transport in astrocytes.** ***A***. Saturation analysis of GAT-1 (open circles, n=6) and GAT-3 (filled circles, n=4) transporters; data shown in the upper panel are the mean values from four experiments performed in quadruplicate (four wells per GABA concentration); the averaged $K_m$ (Michaelis constant) and $V_{max}$ (maximal velocity) values being shown in the lower panel. ***B***. Percentage of GABA uptake that occurs through GAT-1 (open bar, n=5) and GAT-3 (filled bar, n=5) GABA transporters. The GAT-1 transporter was isolated by using GAT-1 transporter inhibitor SKF89976a (20 µM), while GAT-3 transporter was isolated by using GAT-3 inhibitor SNAP5114 (20 µM). The results are expressed as mean ± SEM.

As it can be observed in Figure 5.2.2A, BDNF (10-30 ng/ml) caused a concentration-dependent increase in GAT-1 mediated GABA transport in astrocytes, the effect of 10 ng/ml BDNF being of 30 ± 8.0% (n=7, $p<0.05$) increase. GAT-3 mediated transport remained, however, unaffected.

Changes in the uptake of neurotransmitters induced by transporter-interacting compounds can result from alterations in the turnover rate of transporters that reflects in the number of functional transporters at the cytoplasmatic membrane, or from changes in the transport capacity of individual transporters. Data obtained from saturation experiments are often used to distinguish between these two possibilities, since changes in the maximum velocity of transport ($V_{max}$) are indicative of changes in the number of transporter binding sites, that are correlated with translocation of transporter to or from plasma membrane, and changes in affinity ($K_m$) are indicative of changes in the function of individual transporters. Michaelis-Menton fitting of saturation curves for GAT-1 mediated GABA transport into astrocytes reveal a significant ($p<0.05$, $n=6$) increase in $V_{max}$ in the presence of BDNF (10 ng/ml) compared to untreated cells from the same culture (Figure 5.2.2B). $K_m$ values were not significantly ($p>0.05$) affected by BDNF (Figure 5.2.2B)

From the time course of the effect of BDNF (10 ng/ml) upon GAT-1 mediated GABA transport (Figure 5.2.3) it is clear that the effect of BDNF occurs within minutes after its application, with the half maximal effect being observed after about 2 min and the maximal effect after 10 minutes of its application. Increasing the incubation time up to 30 min did not alter the effect of BDNF, as compared with 10 min incubation. Wash-out experiments performed after a 30 min incubation period showed that the effect of BDNF was reversible (Figure 5.2.3), with GABA uptake values being back to control levels 30 min after removing the neurotrophin from the incubation medium. In the remaining experiments, a 10 min incubation period with BDNF was used.




| | $K_m$ (μM) | $V_{max}$ (pmol /min/mg protein) |
|---|---|---|
| Control | 4.7 ± 1.9 | 1822 ± 202.4 |
| BDNF (10 ng/ml) | 6.7 ± 2.5 | 3048 ± 372.2* |

**Figure 5.2.2. BDNF enhances GAT-1 GABA transport in astrocyte primary cultures.** ***A***. Saturation analysis of GAT-1 mediated GABA transport in presence of BDNF; cells were incubated in the absence (open circles) or presence of 10 ng/ml BDNF (filled circles) before addition of [$^3$H]GABA at the concentrations indicated in the abscissa; data shown in the upper panel were the mean values from six experiments performed in quadruplicate (four wells per GABA concentration); the averaged $K_m$ (Michaelis constant) and $V_{max}$ (maximal velocity) values being shown in the lower panel; *$P < 0.05$ (Student's *t* test, as compared with control). ***B***. Influence of BDNF upon GAT-1 (open bars) or GAT-3 (filled bars)-mediated GABA transport; *$P < 0.05$ (one-way ANOVA followed by the Bonferroni's post-test, as compared with control). The results are expressed as mean ± SEM from 6 (*A*) or 7 (*B*) independent experiments. BDNF was incubated with astrocytes for 10 min.

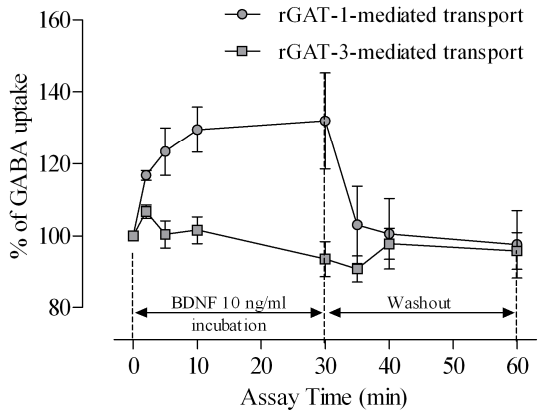



**Figure 5.2.3. Time-course of BDNF (10 ng/ml) effect.** The effect of BDNF is reversible upon drug washout. The ordinates represent the [$^3$H]GABA uptake relative to uptake in the absence of BDNF (100% ) in the same experiments. The results are expressed as mean ± SEM from 5 independent experiments. BDNF was incubated with astrocytes as indicated below each time point.

### 5.2.3. Modulation of GAT-1 by BDNF occurs through the truncated TrkB receptor isoform.

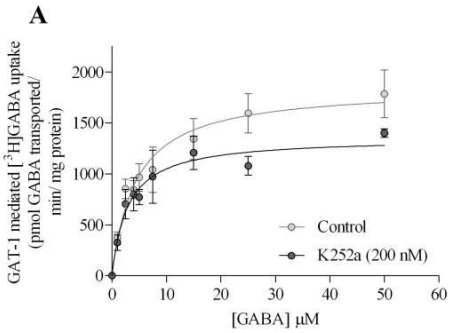
BDNF operates through high-affinity receptor tyrosine kinase B, TrkB, which exists in at least two isoforms, a full length isoform (TrkB-fl) and a truncated isoform (TrkB-t) (see e.g. Chao, 2003). Only the TrkB-fl possesses the catalytic kinase domain and tyrosine kinase inhibitors, such as K252a (Tapley et al., 1992) can thus be used to evaluate if a given effect of BDNF occurs through TrkB-fl or through another receptor isoform. We first evaluated whether tyrosine phosphorylation of GAT molecules could play a role in the functional regulation of GABA transport in astrocytes. For this purpose, astrocytic cultures were treated with K252a and changes in $K_m$ and $V_{max}$ values were calculated from Michaelis-

Menton fitting of saturation curves. K252a induced a significant ($p<0.05$, $n=5$) decrease in $V_{max}$ of GAT-1 (Figure 5.2.4A) and GAT-3 (Figure 5.2.4B) mediated transport, without significant ($p>0.05$) changes in $K_m$ values. It is worthwhile to note that data obtained with K252a somehow contrasts with that obtained with BDNF since while the neurotrophin affects GAT-1 but not GAT-3 mediated transport, the inhibitior of tyrosine phosphorylation affects both transporters.

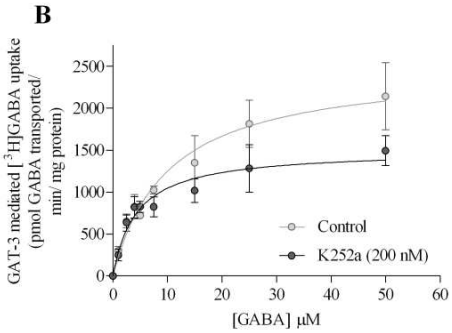
To evaluate if the effect of BDNF requires a tyrosine phosphorylation signalling cascade, it was decided to test first if K252a could prevent the effect of BDNF. Given the marked inhibitory effect of K252a per se, the strategy was to compare, within the same astrocytic culture, GABA transport under the following 4 different conditions: no drug added, only BDNF added, only K252a added and BDNF added in the presence of K252a, therefore allowing the comparison of the effect of BDNF under similar conditions, in the absence and in the presence of K252a. From data shown in Figure 5.2.4C, it is evident that in spite of the presence of K252, BDNF was able to enhance GABA transport. Indeed, upon calculation of the effect of BDNF as % increase, i.e. taking as control the transport of GABA in the same drug conditions but absence of BDNF, it became clear that BDNF increased GABA transport by 31 ± 4.4 % ($n=6$, $p<0.05$) in the absence of K252a (value calculated by considering the control condition as 100%) and by 32 ± 4.9 % ($n=6$, $p<0.05$) in the presence of K252a (value calculated by considering the K252a treated astrocytes condition as 100%) with no statistically significant differences ($n=6$, $p>0.05$) between both BDNF effects. These results indicate that tyrosine kinase activity is not required for the effect of BDNF, therefore are highly suggestive of a non TrkB-fl mediated effect.

In accordance with the results obtained with K252a, data from Western Blot analysis showed that cultured astrocytes express TrkB-t receptors but not TrkB-fl receptors. Indeed, a band corresponding to the molecular weight of TrkB-fl

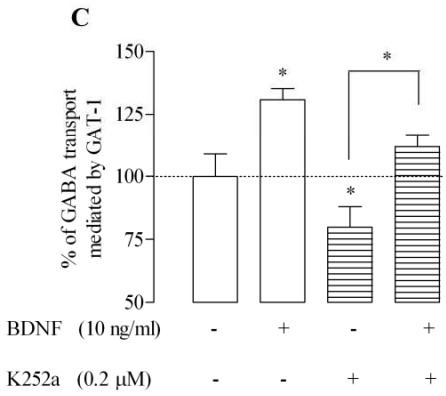
isoform (145 kDa) was never detected in the Western Blots of homogenates of astrocytic cultures, while the TrkB antibody clearly recognized a 95 KDa protein in the samples, a molecular weight compatible with the one of the TrkB-t isoform (Figure 5.2.4D). As a control for the antibody used, homogenates from cultured hippocampal neurons, non-treated or treated with 10 ng/ml BDNF, were also assayed and, as illustrated in Figure 5.2.4D, a 145 KDa protein corresponding to the molecular weight of the TrkB-fl was clearly detected in the neurons. Since activation of TrkB-fl by BDNF leads to receptor autophosphorylation, we assessed the levels of pTrkB in astrocyte and neurons treated with BDNF (10 ng/ml) by using an antibody against pTrkB. It became clear that pTrkB staining could be detected in neurons but not astrocytes (Figure 5.2.4D). Altogether, this data strongly suggest that astrocytes express the truncated form but virtually not the full length form of the TrkB receptor. Interestingly, exposure of astrocytes to different concentrations of BDNF (10 - 30 ng/ml) for 10 minutes, leads to a concentration-dependent increase ($p<0.05$, $n=4$) in the expression of TrkB-t receptor on the surface membrane, as assessed by Western Blot analysis of the biotinylated astrocyte membrane fractions (Figure 5.2.4E).

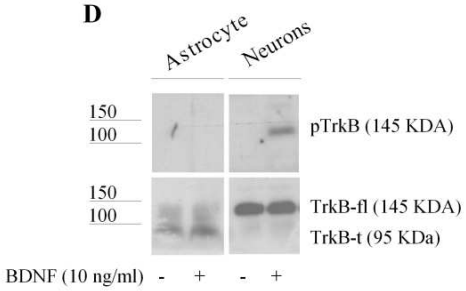


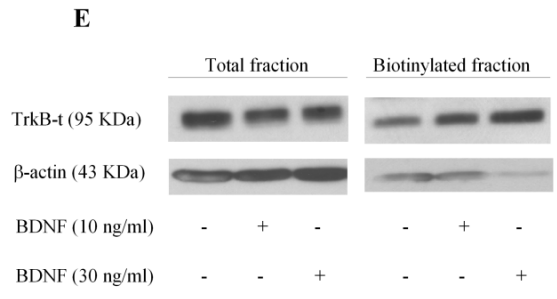

| | $K_m$ (µM) | $V_{max}$ (pmol/min/mg) |
|---|---|---|
| Control | 4.7 ± 1.9 | 1822 ± 202.4 |
| K252a (200nM) | 2.9 ± 0.9 | 1354 ± 113.2 * |




| | $K_m$ (µM) | $V_{max}$ (pmol/min/mg ) |
|---|---|---|
| Control | 10.1 ± 3.20 | 2503 ± 293.4 |
| K252a (200nM) | 4.4 ± 1.3 | 1501 ± 130.7* |



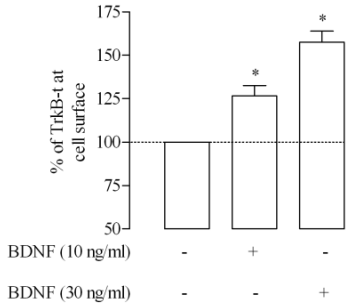



**Figure. 5.2.4. BDNF modulates GAT-1 through activation of TrkB-t receptor.** Saturation analysis of GAT-1 (***A***) and GAT-3 (***B***) transport; cells were incubated in the absence (*open circles*) or presence of the non-selective tyrosine kinase inhibitor, 200nM K252a (*filled circles*) before addition of [$^3$H]GABA at the concentrations indicated in the abscissa; data shown in the upper panel are the mean values from five experiments performed in quadruplicate (four wells per GABA concentration); the averaged $K_m$ (Michaelis constant) and $V_{max}$ (maximal velocity) values being shown in the lower panel; *p<0.05 (Student's *t* test, as compared with control). ***C***. Effect of BDNF upon [$^3$H]GABA uptake in the presence or absence of K252a; *p<0.05 (one-way ANOVA followed by the Bonferroni's post-test, as compared with control conditions (1$^{st}$ bar in the left) except where otherwise indicated by the connecting lines above the bars. ***D***. Analysis of TrkB and pTrk staining in total lysates of astrocytes or neurons treated with/without 10 ng/ml BDNF, as indicated. ***E***. Western Blot analysis of TrkB-t immunoreactivity in cell lysates and biotinylated (surface) fractions of astrocytes. In the left panel is shown a representative immunoblot and in the right panel depicts the quantitative densitometric analysis of the immunoreactivity in the biotinylated fraction; blots were probed with anti-TrkB (1:1000); β-actin immunolabelling (1:10000, 43 kDa band) was used as a loading control. In *C* 100% in the ordinates correspond to the amount of [$^3$H]GABA taken up by astrocytes in the same experiments in the absence of any drug; in *E* 100% in the ordinate corresponds to TrkB-t staining in the absence of BDNF, after normalization for β-actin immunoreactivity. The results are expressed as mean ± SEM from 5 (*A* and *B*), 6 (*C*) or 4 (*E*) independent experiments.

Experiments were then designed to assess signalling pathways involved in the modulatory action of BDNF upon GAT-1. The effect of BDNF in absence and in presence of inhibitors of known TrkB-fl signalling cascades were compared in the same astrocytic cultures. As shown in Figure 5.2.5A, the PI3-K inhibitor LY294002 (10 μM, Ding et al., 1995) had no significant inhibitory effect on GAT-1 mediated GABA uptake (p>0.05, n=4); also, it did not prevent the facilitatory action of BDNF, which remained virtually unchanged in the presence of this inhibitor. On the other hand, inhibition of the ERK/MAP kinase signalling

pathway with the selective MEK1 and 2 inhibitor U0126 (10 µM, Favata et al., 1998) had no effect upon GABA transport in rat astrocytes when tested alone, but fully prevented the facilitatory effect of BDNF on GAT-1 mediated GABA transport. Indeed, GAT-1 mediated GABA uptake in astrocytes in the presence of U0126 (10 µM) was not significantly ($n=4$, $p>0.05$) altered by BDNF (10 ng/ml) (Figure 5.2.5A). Likewise, the PLC-γ inhibitor U73122 (3 µM, Smith et al., 1990) also prevented the effect of BDNF while being by itself devoid of effect ($p>0.05$, $n=4$, Figure 5.2.5A) upon GABA transport. These results suggest that the BDNF-induced increase in GAT-1 mediated GABA uptake involves the activity of at least two transduction pathways, namely the PLC-γ and the Erk/MAP kinase pathways. Since TrkB receptor activation coupled to PLC-γ phosphorylation leads to a subsequent activation of PKC-δ (Patapoutian and Reichardt, 2001), and in order to assess if PKC-δ is involved in the BDNF-induced GAT-1 modulation, I tested the influence of the PKC-δ inhibitor, GF109203x (1 µM, Toullec et al., 1991). This inhibitor *per se* did not affect GAT-1 mediated GABA transport, but abolished the effect of BDNF, also implicating PKC-δ in the signalling cascade. BDNF (10 ng/ml) induced a significant increase of the phosphorylation state of PLC and MAP kinase ($p<0.05$, $n=3$), but no alteration for the phosphorylation state of Akt (Figure 5.2.5B), which fits to the results obtained in uptake experiments with PI3-K, ERK/MAP kinase or PLC-δ blockers. Summarizing, the results so far suggest that BDNF-induced modulation of GAT-1 is not mediated by TrkB-fl, since it does not involve phosphorylation at tyrosine residues, and that it is most likely mediated through a TrkB-t isoform coupled to a non classic PLC and Erk/MAP kinase mechanism, involving PKC-δ.

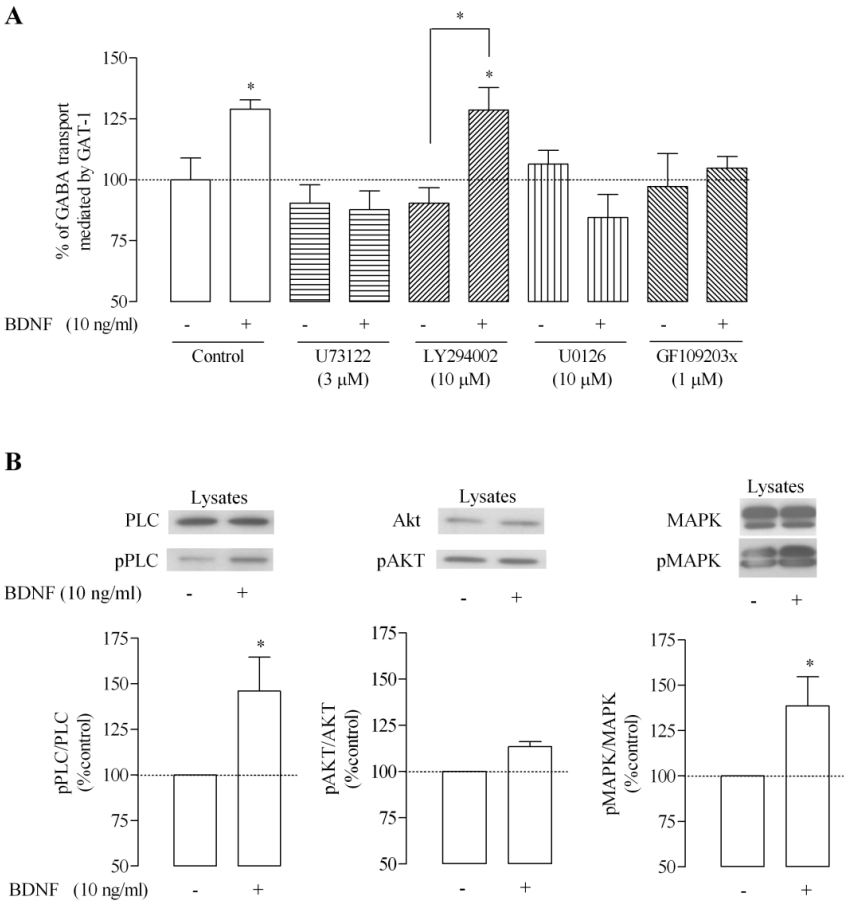


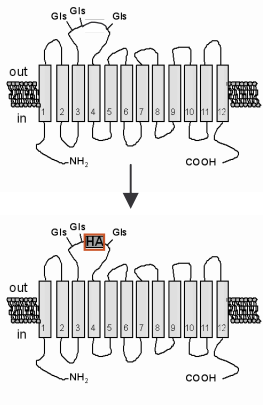

**Figure 5.2.5. Transduction pathways involved in GAT-1 modulation by BDNF**. ***A***. Effect of BDNF upon [$^3$H]GABA uptake in the presence of inhibitors of the different signal transducing pathways of TrkB receptor, namely, the PLC-δ inhibitor, U73122, the PI3-K inhibitor, LY294002, the MEK Kinase inhibitor, U0126, and the PKC-δ inhibitor, GF109203x (see text for references) as indicated below the bars. ***B***. Western blot analysis of PLC/pPLC, Akt/pAkt and MAPK/pMAPK staining in total lysates of cells treated with 10 ng/ml BDNF, as indicated. In the upper panels are shown representative immunoblots and the lower panels depicts the quantitative densitometric analysis of the immunoreactivity of phosphorylated proteins; blots were probed with anti-PLC

(1:750), pPLC (1:250), Akt (1:1500), pAkt (1:500), MAPK (1:6000) and pMAPK (1:500). In *A* 100% in the ordinates correspond to the amount of [$^3$H]GABA taken up by astrocytes in the same experiments in the absence of any drug; in *B* 100% in the ordinate corresponds to PLC/pPLC, Akt/pAkt or MAPK/pMAPK staining in the absence of BDNF. All enzyme inhibitors were added 20 minutes before BDNF, which was added to cells 10 minutes before [$^3$H]GABA. The results are expressed as mean ± SEM from 4 (*A*) or 3 (*B*) independent experiments. *$p<0.05$ (one-way ANOVA followed by the Bonferroni's post-test, as compared with no drug (1$^{st}$ bar in the left) or, whenever indicated, with the closest bar in the left (absence of BDNF under same drug conditions).

### 5.2.4. Incorporation of an HA epitope into EL2 of GAT-1 does not affect GAT-1 affinity for GABA neither sensitivity to BDNF

To test the hypothesis that BDNF increases GABA uptake through GAT-1 by increasing the density of transporters at cell surface, the stretagy was to generate a functional rat GAT-1 transporter that has an antibody accessible extracellular epitope enabling the measurement of changes in rGAT-1 surface expression by ELISA. For the closely related dopamine transporter, an HA-epitope has previously been inserted into the extracellular loop 2 (EL2) without altering the function of the transporter (Sorkina et al., 2006). Accordingly, an HA tag of nine amino acid residues (YPYDVPDYA) was introduced in the EL2 loop of rGAT-1 in different locations in order to obtain a construct kinectically similar to wild type rGAT-1 transporter (Figure 5.2.6A). Thus four different constructs containing the HA tag were designed: HA1-rGAT-1 where the tag was inserted between the residues 693 and 694, HA2-rGAT-1 where the tag was inserted between the residues 698 and 699, HA3-rGAT-1 where the tag was inserted between the residues 706 and 706 and HA3-rGAT-1 where the tag was also inserted following the residue 706 but where the following 11 residues were removed (Figure 5.2.6A).

**A.**

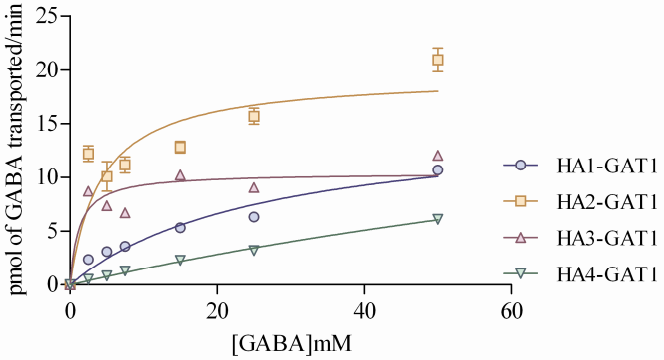



HA epitope: ***YPYDVPDYA*****SL** (previously described for the homoge DAT by Sorkina et al., 2006)

Sequence in rGAT surrounding the glycosylation sites:
-Asn-Tyr-Ser-Leu-Val-Asn-Thr-Thr-Asn-Met-Thr-Ser-Ala-Val-
Blue = glycosylation site (Asn-X-Ser/Thr)

HA1: Asn-Tyr-Ser-**HA**-Val-Asn-Thr-Thr-Asn-Met-Thr-Ser-Ala-Val
HA2: Asn-Tyr-Ser-Leu-Val-Asn-Thr-Thr-**HA**-Asn-Met-Thr-Ser-Ala-Val
HA3: Asn-Tyr-Ser-Leu-Val-Asn-Thr-Thr-Asn-Met-Thr-**HA**-Val
HA4: Asn-Tyr-Ser-Leu-Val-Asn-Thr-Thr-Asn-Met-Thr-**HA** - (the following 11 amino acids deleted)

**B.**




| | $K_m$ (µM) | $V_{max}$ (pmol/min) |
|---|---|---|
| HA1-rGAT-1 | 26.7 ± 9.9 | 15.46 ± 2.9 |
| HA2-rGAT-1 | 4.2 ± 1.4 | 19.54 ± 1.2 |
| HA3-rGAT-1 | 1.2 ± 1.0 | 10.42 ± 1.2 |
| HA4-rGAT-1 | 159.6 ± 50.5 | 25.34 ± 6.5 |

**Figure 5.2.6. The schematic structure and characterization of HAs-GAT-1.** ***A***. The HA tag was placed in the second extracellular loop of rGAT-1. Predicted *N*-glycosylation sites in the EL2 are indicated as well as the location of HA tag in the different inserts ***B***. Saturation kinetics of GAT-1-mediated GABA uptake in HEK293 cells stably expressing HAs-GAT-1 as indicated. The $K_m$ (Michaelis constant) and $V_{max}$ (maximal velocity) values calculated by non-linear regression analysis (GrapPad software) are shown bellow the graph.

Kinetic measurements of [$^3$H]GABA uptake into HEK293 cells expressing the different HA-rGAT-1 constructs (Figure 5.2.6B) yielded different $K_m$ values, being the HA2-GAT-1 (4.2 ± 1.35 μM, n=3) the construct that did not differ from the $K_m$ value obtained in HEK293 cells expressing wild type rGAT-1 (7.70 ± 2.90 μM, n=3) (Figures 5.2.7A and 5.2.7C). Notably, the $K_m$ values obtained in HEK293 cells expressing rGAT-1 or HA2-rGAT-1 were similar to the $K_m$ value for rGAT-1 endogenously expressed in astrocyte primary cultures (Figure 5.2.7A, 5.2.7C and 4.2.1A). The $V_{max}$ values for all HAs-rGAT-1 expressed in HEK293 (Figure 5.2.6B) were significantly different from the value obtained in a parallel experiments on cells expressing wild type rGAT-1 (Figure 5.2.7C). Nevertheless, since the HA2-GAT-1 was the construct with more similar Km value to wild type rGAT-1, it possible to deduce with these uptake experiments, that introduction of the HA-epitope in EL2, between the residues 698 and 699, yielded a functional transporter that was expressed at the cell surface (although to a lower extent than the wild type transporter) and had an unaltered apparent affinity for GABA. Thus the HA2-GAT-1 was the selected contruct to proceed with the following experiments and from now on HA2-GAT-1 will be referring as HA-GAT-1.

Next step was to evluate the expression of HA-rGAT-1 in rat astrocytes. Following transduction of astrocytes with lentivirus encoding HA-rGAT1, GABA uptake was increased more than twofold compared to non-transduced astrocytes

(Figure 5.2.7B). Treatment of astrocytes that were transduced with HA-rGAT-1 with BDNF (10 and 30 ng/ml) revealed an increase of GABA uptake that was similar to BDNF effect upon astrocytes that were not expressing HA-rGAT-1 (Figure 5.2.7D). The effect of BDNF on GABA uptake in HA-rGAT-1 transduced astrocytes was maximal already with a 10 ng/ml BDNF concentration; indeed for the 10 ng/ml BDNF concentration it was observed an increase of 45.0 ± 12.14 % of GABA uptake through GAT-1 transporter. It is important to mention that results of BDNF action on GABA transport presented in Figure 5.2.7D, with both non-transduced and transduced astrocytes, were obtained simultaneously and with the same batch of cells, indicating that HA-rGAT-1 and endogenous rGAT-1 have similar sensitivity to BDNF.

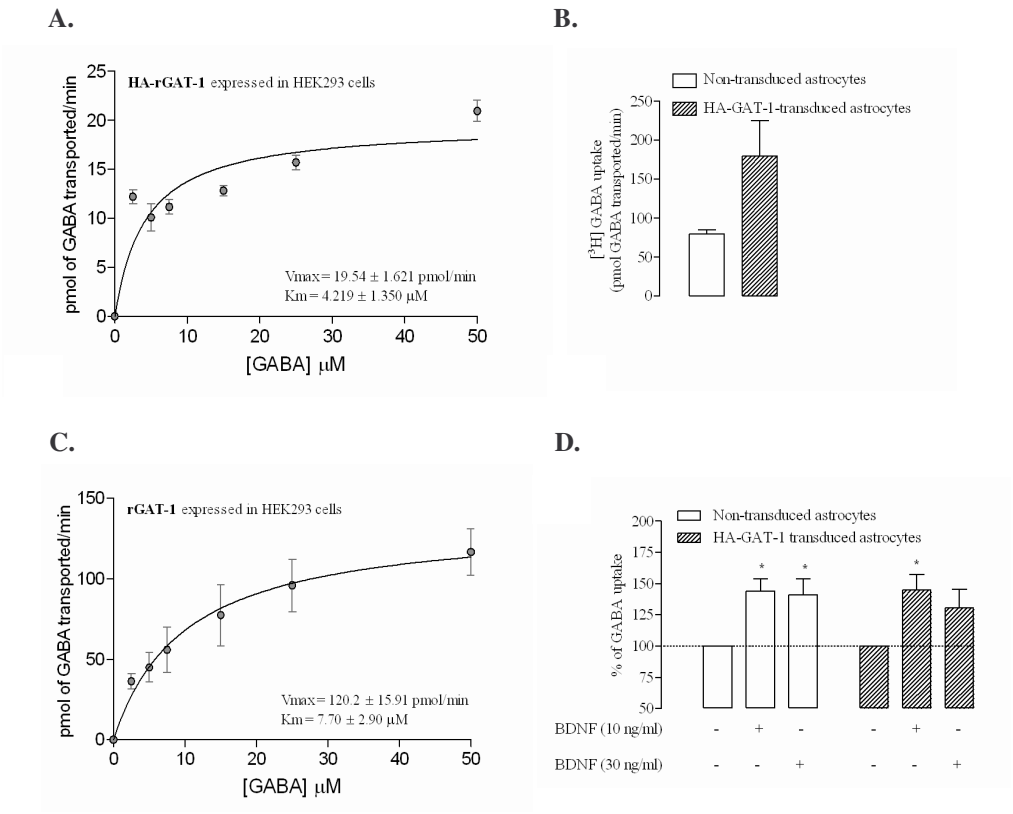



**Figure 5.2.7. Characterization of HA-GAT-1 mediated GABA transport.** ***A****. and* ***C****.* Saturation kinetics of GAT-1-mediated GABA uptake in HEK293 cells stably expressing HA2-GAT-1 (***B***, n=3) or rGAT-1 (***C***, n=3) The $K_m$ (Michaelis constant) and $V_{max}$ (maximal velocity) values calculated by non-linear regression analysis (GrapPad software) are shown as an inset in the corresponding graph. ***B***. [$^3$H]GABA uptake trough GAT-1 in HA-GAT-1 transduced (n=2) and non-transduced (n=2) astrocytes. The ordinates represent the [$^3$H]GABA uptake as pmol of GABA transported *per* min, mediated by GAT-1 transporter; values are mean ± S.E.M values (n=2); note that the transduction of astrocytes with HA-rGAT-1 increased GABA uptake. ***D***. BDNF effects on GABA uptake in HA-rGAT-1 transduced and non-transduced astrocytes from the same cell batch (n=5). Note that BDNF increased GABA uptake in both non-transduced and HA-rGAT-1 transduced astrocytes. The ordinates represent the [$^3$H]GABA uptake as percentage of the control value (no BDNF added) in the same experiment and in the same cell batch in similar conditions. GABA uptake assays were performed for 1 min at 37°C using 8.62 nM [$^3$H]GABA and increasing

amounts of unlabeled GABA (***A*** and ***C***; final concentrations, 1 to 50 µM), or a mixture of 8.62 nM [$^3$H]GABA with unlabeled GABA to a final concentration of 30 µM GABA (***B*** and ***D***) . Non-GAT-1-mediated uptake was determined in the presence of 20 µM SKF89976a. In all panels the results are mean ± S.E.M. *p<0.05 (one-way ANOVA followed by the Bonferroni's post-test) as compared with control (no drug added, first column in the left) in the same group of cells.

### 5.2.5. BDNF enhances translocation of rGAT-1 to plasma membrane of astrocytes

The influence of BDNF upon GAT-1 trafficking in rat astrocytes was first assessed by cell surface ELISA using an antibody against the HA tag. HA-rGAT-1 transduced astrocytes were incubated with BDNF (10-100 ng/ml) for 10 min before the assay, inducing a significant increase (20.1 ± 12.90 %, 32.2 ± 8.97 %, and 39.9 ± 6.68 % for 10, 30 and 100 ng/ml of BNDF, respectively, n=4, p<0.05) in immunodetected HA tagged surface transporter as compared to astrocytes that were not incubated with the neurotrophin (Figure 5.2.8A).

To rule out the possibility that the effect of BDNF upon trafficking was related to the HA tag, biotinylation experiments using astrocytes not transfected with the HA tagged GAT-1 were performed. Since GAT-1 levels in biotinylated astrocytic membranes are below the detection limit by Western Blot (data not shown), rGAT-1 was overexpressed in the astrocytes by transduction with lentivirus encoding wild type rGAT-1. Cells were then incubated in the absence or presence of BDNF before biotinylation of surface proteins. As shown in the representative immunoblot (Figure 5.2.8B, upper panel) and in summary plot (Figure 5.2.8B, lower panel), incubation of cells with BDNF for 10 min leads to an increase (17.7 ± 5.38 and 20.9 ± 4,76 %, for 10 or 30 ng/ml BDNF, respectively, n=5, p<0.05,)

in GAT-1 immunoreactivity in the biotinylated fraction, without appreciable change in total GAT-1 immunoreactivity.

Altogether, the data above clearly shows that BDNF enhances the expression of GAT-1 transporters at the plasma membrane. To assess whether BDNF affects the rate of internalization of GAT-1 or its recycling back to the plasma membrane, we tested the influence of dynasore, a dynamin inhibitor therefore blocking dynamin/clathrin dependent endocytosis (Macia et al., 2006) as well as of the cation ionophore monensin, which blocks protein recycling back to the membrane without influencing protein internalization (Mollenhauer et al., 1990). Monensin, used in conditions (25µM, 1h) previously shown shown to inhibit recycling of dopamine transporters in neuronal cell lines (Eriksen et al., 2010) slightly decreased GABA uptake by around 10% (n=5, Figure 5.2.8C), suggestive of constitutive recycling of GAT-1 in astrocytes. Similarly, biotinylation assays showed that monensin slightly decreases GAT-1 expression at surface membranes (n=3, Figure 5.2.8D). In the presence of monensin, however, BDNF still increased GAT-1 mediated GABA uptake by 26 ± 10.2 % (n=5, $p<0.05$, Figure 5.2.8C) as well as GAT-1 expression at surface membranes (n=3, $P<0.05$, Figure 5.2.8D). This suggests that BDNF does not operate by enhancing GAT-1 recycling back to the plasma membrane. The dynamin inhibitor, dynasore (70 µM) increased GAT-1 mediated GABA uptake by 24 ± 6.1 % (n=5, $p<0.05$, Figure 5.2.8C) as well as increased GAT-1 expression at surface membranes (Figure 5.2.8D) indicating that in astrocytes, as it occurs in neurons (5), GAT-1 internalization involves the dynamin/clathin-dependent mechanism, rather than the $Ca^{2+}$ regulated dynamin-independent endocytotic pathway, which is also present in cortical astrocytes (Jiang and Chen, 2009). Dynasore (70 µM) fully abrogates the facilitatory effect of BDNF upon GAT-1 mediated GABA transport (n=5, Figure 5.2.8C) as well as upon GAT-1 expression at surface membranes (n=3, Figure 5.2.8D) therefore

indicating that BDNF inhibits constitutive internalization of GAT-1 rather than enhances the insertion/recycling pathway.

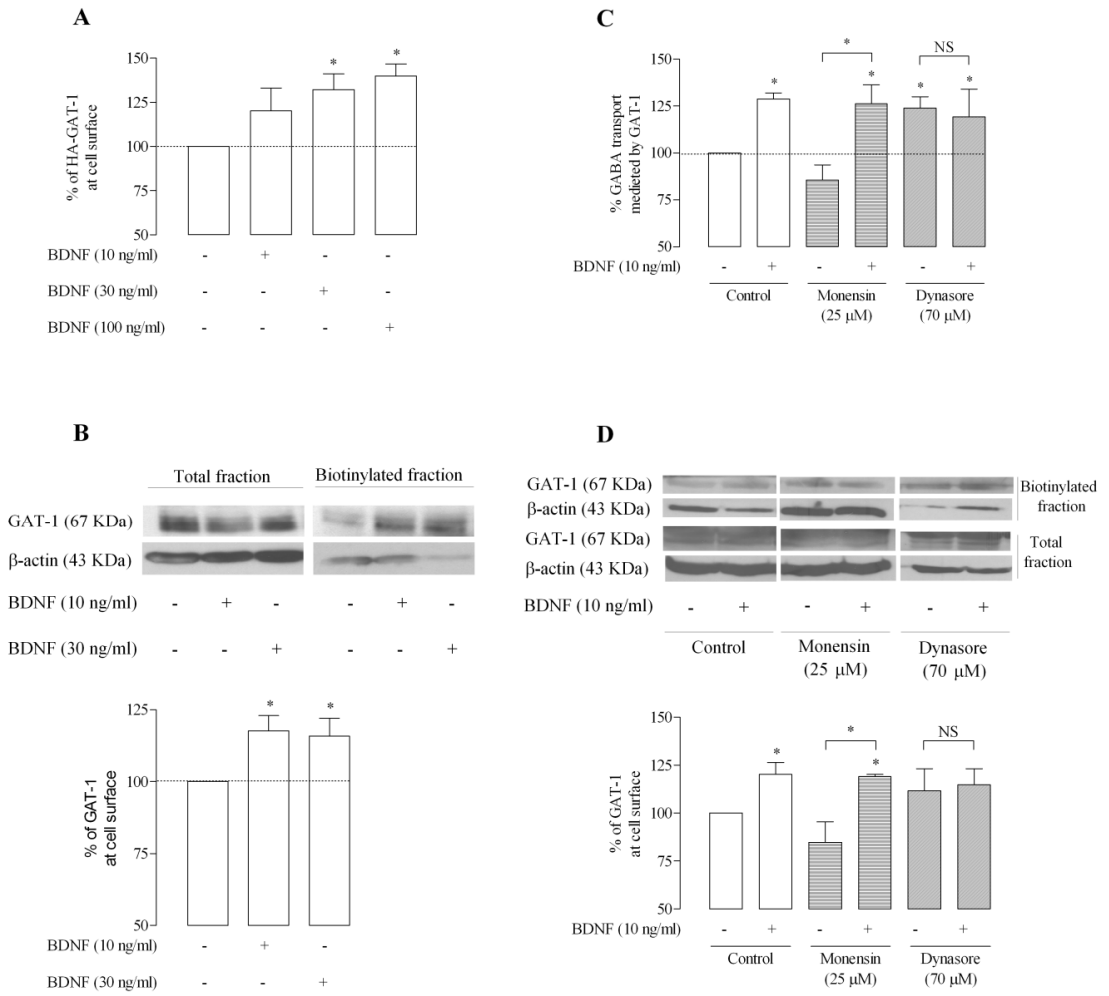


**Figure 5.2.8. BDNF enhances surface expression of GAT-1 in astrocytes**. ***A***, HA-rGAT-1 transduced astrocytes were incubated for 10 min with (+) or without (-) BDNF (10-30 ng/ml) as indicated below each bar and then assayed by ELISA. 100% in the ordinates represent normalized HA-GAT-1 expression in plasma membrane of astrocytes in control situation (absence of BDNF). ***B***. rGAT-1 transduced astrocytes were incubated as in ***A***, but changes in surface GAT-1 immunoreactivity were assessed by surface biotinylation. In the upper panel, is shown a representative immunoblot from total lysate and biotinylated (surface membrane) astrocyte fractions; blots were probed with anti-GAT-1 (1:500, 67 kDa band); β-actin (1:10000, 43 kDa band) immunoreactivity was used as loading control; in the lower panel is shown the average densitometric analysis, where 100% in the ordinates represent normalized rGAT-1 expression in the biotinylated fraction in the absence of BDNF. ***C*** and ***D****:* Influence of monensin, an inhibitor of insertion/recycling pathway, and dynasore, a dynamin inhibitor (see text for references) upon the effect of BDNF on GABA uptake (***C***) or surface expression of rGAT-1 (***D***); in the upper panel in (***D***) is shown a representative immunoblot from total lysate and biotinylated (surface membrane) astrocyte fractions, and in lower panel is shown the average densitometric analysis, where 100% in the ordinates represent normalized rGAT-1 expression in the biotinylated fraction in the absence of BDNF. The results are expressed as mean ± SEM from 4 (**A**), 5 (**B** and **C**) or 3 (**D**) individual experiments. *p<0.05 (one-way ANOVA followed by the Bonferroni's post-test), as compared with control conditions (1$^{st}$ column in the left) except where otherwise indicated by the connecting lines above the bars; NS: non-statistically significant (p>0.05).

## 5.2.6. Tonic levels of extracellular adenosine are enough to trigger the effect of BDNF.

It has been repeatedly observed that actions of BDNF at synapses require co-activation of adenosine $A_{2A}$ receptors, a mechanism that involves activation of PKA and TrkB translocation to lipid rafts (Diógenes et al., 2007; Assaife-Lopes et al., 2010). An exception is BDNF-induced inhibition of GABA transport into nerve terminals, since the effect of BDNF on GAT-1 in nerve terminals is not prevented by removal of extracellular adenosine or by blockade of adenosine $A_{2A}$

receptors (Vaz et al., 2008). In order to evaluate if adenosine $A_{2A}$ receptors could influence the facilitatory action of BDNF on GAT-1 mediated GABA transport into astrocytes we tested whether $A_{2A}$ receptor blockade with a selective antagonist, SCH 58261 (50 nM, Zocchi et al., 1996b), or $A_{2A}$ receptor activation with a selective agonist, CGS 21680 (30 nM, Jarvis et al., 1989) affected the action of BDNF. *Per se*, these drugs did not significantly (N=4, $p>0.05$) affect GABA transport, though there was a tendency for a facilitation by the $A_{2A}$ receptor agonist and an inhibition by the $A_{2A}$ receptor antagonist (Figure 5.2.9A). Similarly, adenosine deaminase (ADA, 1U/ml), an enzyme that catabolises adenosine into inosine, therefore removing extracellular adenosine caused a slight but non-significant decrease in GAT-1 mediated GABA transport (n=4, $p>0.05$). To evaluate the effect of BDNF in the presence or absence of the $A_{2A}$ receptor ligands, experiments were designed so that GABA transport in the presence of each $A_{2A}$ receptor ligand was taken as control for the effect of BDNF, which was tested in the same astrocytic culture in the absence or in the presence of the ligand. A summary of the results is shown in Figure 5.2.9C. The facilitatory effect of BDNF was fully lost upon incubation of astrocytes with the $A_{2A}$ receptor antagonist, SCH 58261 (50 nM, n=8). The same occurred when extracellular endogenous adenosine was removed by incubating the cells with ADA (1 U/ml, n=4, Figure 5.2.9C). Activation of $A_{2A}$ receptors with CGS 21680 (30 nM) caused a slight but non-significant (n=4, $p>0.05$, Figure 5.2.9C) enhancement of the facilitatory action of BDNF, suggesting that tonic activation of $A_{2A}$ receptors by endogenous adenosine is enough to fully trigger the facilitatory effect of BDNF on GAT-1 mediated GABA transport into astrocytes. A similar conclusion can be drawn from evaluating the facilitatory action of BDNF upon surface expression of GAT-1 (Figure 5.2.9D) or BDNF-induced enhancement of TrkB-t surface

expression (Figure 5.2.9E), since in no case a further activation of $A_{2A}$ receptors with GCS 21680 induce a further effect of BDNF.

Activation or inhibition of the canonical signal transducing pathway of adenosine $A_{2A}$ receptors should influence the effect of BDNF in a way similar to what is observed when $A_{2A}$ receptor activity is manipulated by receptor ligands. Accordingly, the adenylate cyclase activator, forskolin (5 µM, Awad et al., 1983), did not cause a further enhancement of the effect of BDNF (n=6, Figure 5.2.9B), whereas the protein kinase A inhibitor, H-89 (10 µM, Murray, 2008), fully prevented the effect of BDNF on GAT-1 mediated GABA transport (n=6, Figure 5.2.9B). Upon activation of adenylate cyclase with forskolin, the $A_{2A}$ receptor antagonist was no longer able to block the effect of BDNF (n=4, Figure 5.2.9B) strongly indicating that blockade of the action of BDNF by $A_{2A}$ receptor antagonism is upstream of adenylate cyclase, therefore reinforcing the conclusion that $A_{2A}$ receptors operate through this transducing pathway to allow BDNF actions upon GABA transport into astrocytes.

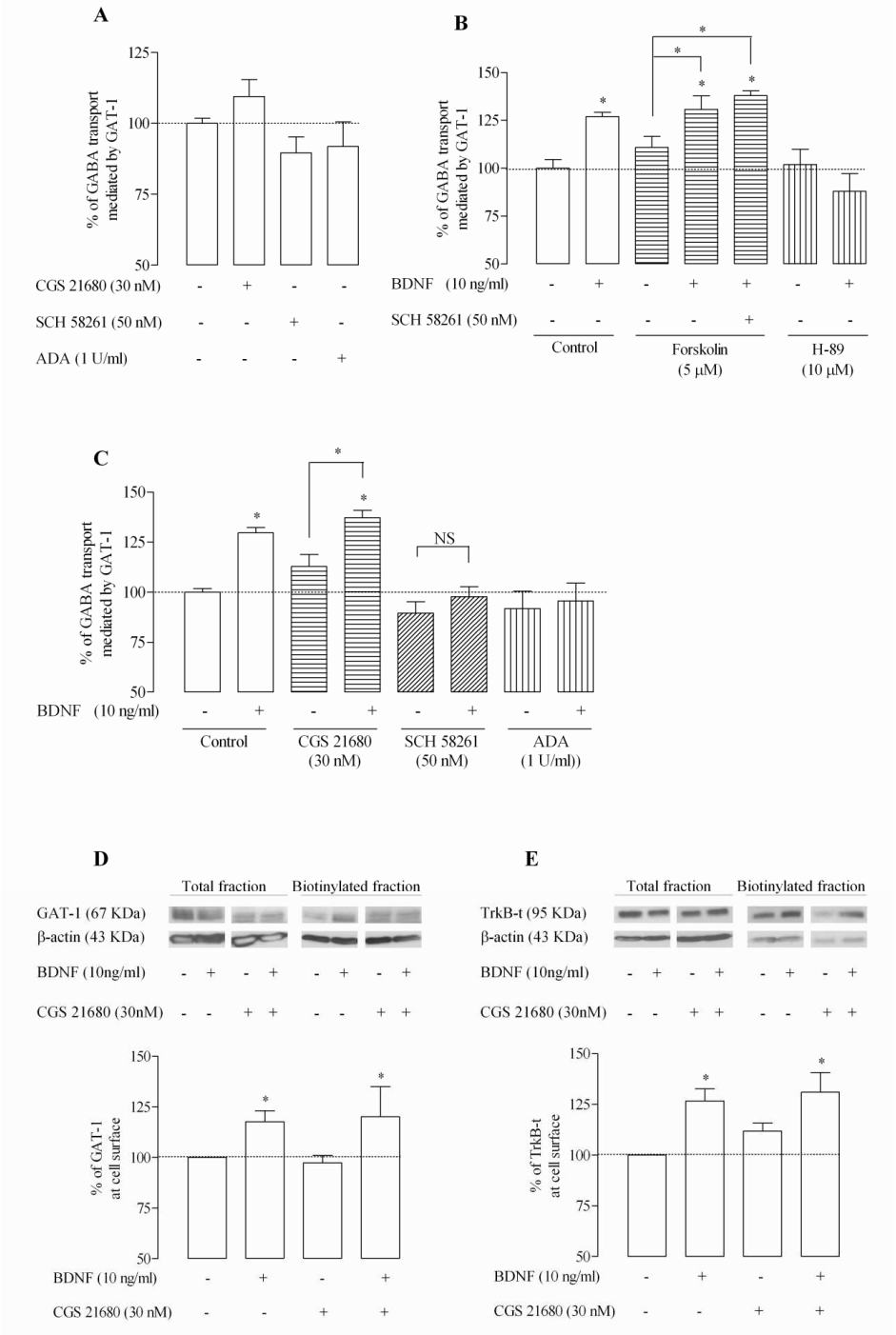

A
% of GABA transport mediated by GAT-1
125
100
75
50
CGS 21680 (30 nM) - + - -
SCH 58261 (50 nM) - - + -
ADA (1 U/ml) - - - +
B
% of GABA transport mediated by GAT-1
150
125
100
75
50
BDNF (10 ng/ml) - + - + + - +
SCH 58261 (50 nM) - - - - + - -
Control
Forskolin (5 µM)
H-89 (10 µM)
C
% of GABA transport mediated by GAT-1
150
125
100
75
50
NS
BDNF (10 ng/ml) - + - + - + - +
Control
CGS 21680 (30 nM)
SCH 58261 (50 nM)
ADA (1 U/ml))
D
Total fraction
Biotinylated fraction
GAT-1 (67 KDa)
β-actin (43 KDa)
BDNF (10ng/ml) - + - + - + - +
CGS 21680 (30nM) - - + + - - + +
% of GAT-1 at cell surface
150
125
100
75
50
BDNF (10 ng/ml) - + - +
CGS 21680 (30 nM) - - + +
E
Total fraction
Biotinylated fraction
TrkB-t (95 KDa)
β-actin (43 KDa)
BDNF (10ng/ml) - + - + - + - +
CGS 21680 (30nM) - - + + - - + +
% of TrkB-t at cell surface
150
125
100
75
50
BDNF (10 ng/ml) - + - +
CGS 21680 (30 nM) - - + +

**Figure 5.2.9. Modulation of the effect of BDNF by adenosine $A_{2A}$ receptors**. ***A***. Influence of $A_{2A}$ receptor activation with a selective agonist, CGS 21680, or blockade with a selective antagonist, SCH 58261, or removal of extracellular adenosine with adenosine deaminase (ADA) on [$^3$H]GABA uptake mediated by GAT-1. ***B***. Influence of drugs that affect cAMP signaling upon the effect of BDNF on [$^3$H]GABA uptake; forskolin was used as an activator of adenylate cyclase and H-89 as an inhibitor of PKA (see text for references). ***C***. Influence of CGS 21680, SCH 58261 and ADA on the effect of BDNF upon [$^3$H]GABA uptake. ***D*** and ***E***. $A_{2A}$ receptor agonist, CGS 21680, affects on the BDNF-induced increase in surface expression of GAT-1 (***D***) or TrkB-t (***E***). In the upper panels are shown, representative immunoblots for GAT-1 (***D***) and TrkB-t (***E***) immunoreactivity in cell lysate and biotinylated fractions of astrocytes; blots were probed with anti-GAT-1 or anti-TrkB antibodies; β-actin immunoreactivity was used as loading control; in the lower panel is shown the average densitometric analysis, where 100% in the ordinates represent rGAT-1 expression or TrkB-t levels (both normalized for β-actin in the same lane) in the biotinylated fraction in the absence of BDNF. For ***A***, ***B*** and ***C***, 100% in the ordinates correspond to the amount of GABA taken up in the absence of any drug. In all panels data is expressed as mean ± S.E.M from 4-8 (***A***, ***B*** and ***C***) or 6 (***D*** and ***E***) individual experiments. Drug presence (+) or absence (-) is indicated below each bar. *P<0.05 (one-way ANOVA followed by the Bonferroni's post-test), as compared with control conditions (no BDNF added) except where otherwise indicated by the connecting lines above the bars; NS: non-statistically significant (p>0.05).

Finally, it was evaluated whether the BDNF-induced enhancement of the phosphorylation state of PLC and MAP kinase (Figure 5.2.5B) was also under control of adenosine $A_{2A}$ receptors. As shown in Figure 5.2.10, BDNF-induced phosphorylation of PLC and MAP kinase was also fully blocked by the $A_{2A}$ receptor antagonist, SCH 58261 (50 nM) but not significantly affected by a further activation of $A_{2A}$ receptors with the agonist, CGS 21680 (30 nM), therefore mimicking what we observed while measuring GABA transport. *Per se*, neither the $A_{2A}$ receptor agonist (CGS 21680, 30 nM) nor the antagonist (SCH 58261, 50 nM) significantly affected the phosphorylation state of PLC and MAP kinase in astrocytes.

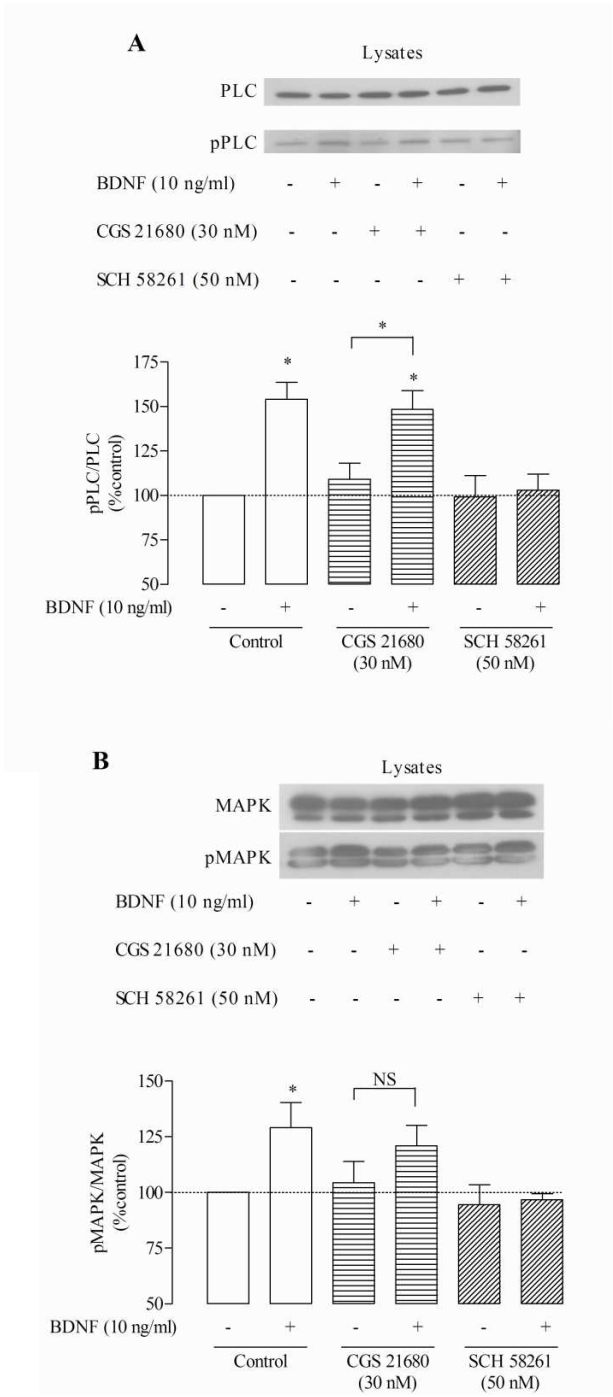

A
Lysates
PLC
pPLC
BDNF (10 ng/ml) - + - + - +
CGS 21680 (30 nM) - - + + - -
SCH 58261 (50 nM) - - - - + +
pPLC/PLC (%control)
175
150
125
100
75
50
*
*
*
BDNF (10 ng/ml) - + - + - +
Control
CGS 21680 (30 nM)
SCH 58261 (50 nM)
B
Lysates
MAPK
pMAPK
BDNF (10 ng/ml) - + - + - +
CGS 21680 (30 nM) - - + + - -
SCH 58261 (50 nM) - - - - + +
pMAPK/MAPK (%control)
150
125
100
75
50
*
NS
BDNF (10 ng/ml) - + - + - +
Control
CGS 21680 (30 nM)
SCH 58261 (50 nM)

**Figure 5.2.10. $A_{2A}$ receptor mediated modulation of the signalling pathways activated by BDNF**. Western blot analysis of PLC/pPLC (***A***) and MAPK/pMAPK (***B***) staining in total lysates of cells treated with BDNF, CGS 21680 and SCH 58261, as indicated. In the upper panels are shown representative immunoblots for PLC/pPLC (***A***) and MAPK/pMAPK (***B***) in cell lysate; in lower panels are shown the average densitometric analysis, where the ordinates represent quantitative densitometric analysis of immunoreactivity of phosphorylated proteins; data is expressed as mean ± S.E.M from 6 (***A***) or 4 (***B***) individual experiments. *p<0.05 (one-way ANOVA followed by the Bonferroni's post-test), as compared with no drug conditions (1st column in the left) except where otherwise indicated by the connecting lines above the bars; NS: non-statistically significant (p>0.05).

## 5.2.7. Discussion

The main finding of the present work is that BDNF increases GAT-1 mediated GABA transport into astrocytes through a mechanism that involves the TrkB-t isoform of TrkB receptors and a non classic PLC-γ/PKC-δ and Erk/MAP kinase pathway, leading to enhanced expression of GAT-1 at the cell surface due to a reduced internalization.

BDNF facilitates the maximum velocity of GAT-1 mediated transport ($V_{max}$) without a significant change in $K_m$ value, suggestive of an increase in the number of transporters at the membrane. This was directly confirmed by cell surface biotinylation and surface ELISA in astrocytes over-expressing wild type GAT-1 or HA-tagged GAT-1, respectively. Constitutive recycling of neurotransmitter transporters is known to occur in neurons (Wang and Quick, 2005; Jiang and Chen, 2009). Therefore, and if this would apply to astrocytes, enhanced expression of GAT-1 on astrocytic surface membranes could result either from

inhibition of endocytosis or from enhancement of its recycling back to the membrane. The finding that monensin, which inhibits protein recycling back to the membrane without affecting endocytosis (Mollenhauer et al., 1990), does not prevent the effect of BDNF upon surface expression of GAT-1 molecules rules out an influence of BDNF upon membrane reinsertion of the transporters. Endocytosis frequently occurs through clathrin-dependent coated vesicle formation, a process that also depends on dynamin and, in neurons, controls synaptic vesicle turnover. Astrocytes, however, also possess a $Ca^{2+}$-dependent/dynamin independent endocytic pathway (Jiang and Chen, 2009), but since the dynamin inhibitor, dynasore, mimics the effect of BDNF and abrogates its influence upon surface expression of GAT-1, it seems likely that the effect of BDNF results from inhibition of GAT-1 internalization through a dynamin-dependent process.

BDNF has a complex pattern of modulation of GABAergic transmission, which is time-dependent and cell- and synapse-specific. In a time frame from minutes to a few hours, BDNF inhibits $GABA_A$ receptor mediated responses (Tanaka et al., 1997) by downregulation of $GABA_A$ receptor surface expression (Brunig et al., 2001). These fast BDNF actions are synapse specific, but the trend is towards a postsynaptic inhibition of GABAergic transmission, in particular towards an inhibition of inhibitory inputs to interneurons (Wardle and Poo, 2003). The inhibitory action of BDNF disappears after prolonged exposure to BDNF (Brunig et al., 2001), turning into a long-lasting facilitatory action of $GABA_A$ responses, associated with an increase in $GABA_A$ receptor clusters (Elmariah et al., 2005). These long-lasting effects of BDNF are more related to trophic than to fast signalling actions since they were shown to involve establishment of functional inhibitory synapses between interneurons (Elmariah et al., 2005). I now report

another fast and easily reversible mechanism operated by BDNF that influences GABAergic transmission. Thus, the enhanced GABA transport into astrocytes most probably contributes to transiently fasten the shut-down of GABAergic responses, a process that if added to the fast decrease in postsynaptic sensitivity to GABA (Tanaka et al., 1997; Brunig et al., 2001; Wardle and Poo, 2003) and to a facilitation of GABA transport into neurons (Law et al., 2000) will lead to a marked inhibition of inhibitory signalling. At the synaptic level this action of BDNF may, however, be slightly compensated by an inhibition of GAT-1 mediated GABA uptake by the nerve endings (Vaz et al., 2008) as well as by an enhanced pre-synaptic release of GABA (Wardle and Poo, 2003). Therefore, the facilitatory action of BDNF upon GAT-1 might predominantly contribute to decrease tonic inhibition, that is to say, to a decreased exposure of extrasynaptic distant $GABA_A$ receptors (Lindquist and Birnir, 2006) to a persistently low concentration of 'ambient' GABA. It is also worthwhile to note that tonic inhibition is predominantly influenced by GAT-1, rather than GAT-3 (Kirmse et al., 2009) and that BDNF affected GAT-1, but not GAT-3 mediated transport in astrocytes.

In accordance with previous reports (Rose et al., 2003; Bergami et al., 2008), the now reported action of BDNF in astrocytes might not involve TrkB-fl receptors since it was still evident when tyrosine kinase activity was blocked with K252a. Accordingly, no TrkB-fl or pTrkB immunoreactivity was detected in astrocytes, which were clearly immunopositive for the TrkB-t isoform. The TrkB-t receptor is characterized by lacking the tyrosine kinase domain, which is present in the full length TrkB receptor. Since it lacks the tyrosine kinase domain, the TrkB-t receptor does not directly activate the classical transduction pathways that characterize TrkB-fl receptor, namely the activation of ERK, PI3-K and PLC-γ pathway (Chao, 2003; Huang and Reichardt, 2003). TrkB-t isoforms can,

however, operate intracellular signalling pathway(s) leading to phosphorylation and changes in kinase activity (Baxter et al., 1997; Ohira et al., 2007). When testing which pathways activated by BDNF are related to its ability to modulate GAT-1, we found that the inhibition of PLC-γ, as well as the inhibition of MAP kinase pathways, prevented the BDNF action upon GAT-1 transporter, implicating these pathways in the action of the neurotrophin in the astrocytes. BDNF-induced activation of PLC-γ is often associated to PKC-δ activation (Patapoutian and Reichardt, 2001). Accordingly, blockade of PKC prevented the BDNF effect upon GAT-1 in astrocytes. An inhibitor of PI3-K did not prevent the effect of BDNF, suggesting the absence of involvement of PI3-K/Akt pathway on the action of BDNF upon GABA transport into astrocyes. Likewise, after a brief incubation with BDNF, there was an enhanced phosphorylation of PLC and of MAP kinase, but not of Akt. The signalling pathway operated by BDNF to quicky modulate GAT-1 in astrocytes is consistent with its time-frame of action, since PLC-γ/PKC-δ is associated with fast signalling, whereas the PI3-K/Akt pathway is mostly related to long lasting survival-related influences of BDNF (Blum and Konnerth, 2005). Nevertheless the blockade of tyrosine kinase by K252a diminished the $V_{max}$ of both GAT-1 and GAT-3 modulating GABA transporters in a similar way to what was already described in neurons for GAT-1 (Law et al., 2000; Quick et al., 2004).

Interestingly, astrocytes treated for a few minutes with BDNF had increased TrkB-t receptor levels in the cell membrane, suggesting a BDNF-induced translocation of TrkB-t receptor to the plasma membrane. At least in neurons, where membrane translocation of TrkB receptors has been mostly studied, acute exposure to BDNF rapidly (within seconds) increases TrkB surface expression, whereas long-lasting (within hours) treatment with BDNF leads to decreased

surface TrkB levels (Quick et al., 2004). A similar process might therefore occur in relation to surface expression of TrkB-t receptors in astrocytes. This process may result into a localized positive feed-back loop between neurons and astrocytes, where fast neuronal spiking leads to release of the neurotrophin, which can be taken up by astrocytes endowing these with the ability to resecrete it upon stimulation (Lindquist and Birnir, 2006), a mechanism that when coupled to a quick and BDNF-dependent overexpression of TrkB receptors at neuronal (Haapasalo et al., 2002) and astrocytic (present work) membranes further restricts neurotrophin actions to neuron–astrocyte contacts.

Several studies demonstrated that the excitatory action of BDNF on synaptic transmission is fully dependent on adenosine $A_{2A}$ receptors activation, since it is absent when $A_{2A}$ receptors are blocked (Diógenes et al., 2004; Pousinha et al., 2006; Diógenes et al., 2007; Fontinha et al., 2008), knocked-down (Tebano et al., 2008) or upon removal of extracellular endogenous adenosine (Fontinha et al., 2008; Assaife-Lopes et al., 2010). Activation of adenosine $A_{2A}$ receptors transactivate TrkB (Lee and Chao, 2001) and induce TrkB translocation to lipid rafts (Assaife-Lopes et al., 2010), which probably underlies the mechanisms behind the $A_{2A}$/TrkB receptor facilitatory interaction. In the case of the inhibitory action of BDNF on presynaptic GAT-1 activity, it is modulated by $A_{2A}$ receptor activation but remains present either in the presence of $A_{2A}$ receptor antagonists or upon extracellular adenosine removal (Vaz et al., 2008). The now reported facilitatory action of BDNF upon GAT-1 in astrocytes was lost upon blockade of adenosine $A_{2A}$ receptors or extracellular adenosine removal. It therefore appears that fast excitatory actions of BDNF are those that require co-activation of adenosine $A_{2A}$ receptors by endogenously released adenosine. Interestingly, the present results allow to extend the adenosine/BDNF cross talk to astrocytes and to the truncated form of the TrkB receptor (TrkB-t). Noteworthy, the reported results

show for the first time a functional consequence of the crosstalk between TrkB-t and adenosine $A_{2A}$ receptors, indicating that the catalytic domain of the TrkB receptor is not involved in the cross-talk with $A_{2A}$ receptors.

GABA has the ability to increase intracellular calcium levels in astrocytes and this action triggers the release of ATP from the astrocytes (Serrano et al., 2006). On the other hand, ATP is released with several neurotransmitters and triggers astrocytic calcium waves, leading to further release of ATP as well as of gliotransmitters (Fields and Burnstock, 2006; Hamilton and Attwell, 2010). Released ATP can be extracellularly catabolised by a cascade of ectoenzymes leading to adenosine formation. Therefore, it is expected that the extracellular adenosine levels at the tripartite synapse are enough to activate high affinity adenosine receptors, namely $A_{2A}$ receptors, which will gate BDNF actions in astrocytes. BDNF itself is able to trigger calcium responses in astrocytes (Rose et al., 2003) therefore most probably further reinforcing the cycle of astrocyte-to-neuron communication involving purines. In our experimental conditions, extracellular levels of adenosine were probably already high enough to maximally activate $A_{2A}$ receptors since further activation of these receptors with a selective $A_{2A}$ receptor agonist did not cause an enhancement of the facilitatory action of BDNF either upon GAT-1 mediated GABA transport or surface expression of GAT-1 transporters, or even upon surface expression of TrkB-t receptors.

The present results allow to conclude that gating of TrkB-t receptors by $A_{2A}$ receptor activation is upstream of adenylate cyclase activation, since the effect of BDNF could be observed when $A_{2A}$ receptors were blocked but adenylate cyclase activated. Receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs), by sharing protein signalling components specific for each receptor that are in close proximity, can form platforms, producing an integrate response upon

engagement of ligands (Pyne and Pyne, 2011). Examples of such platforms are lipid rafts, and $A_{2A}$ receptors are known to promote translocation of TrkB receptors to lipid rafts in a cyclic AMP-dependent manner (Assaife-Lopes et al., 2010). Such proximity interaction may also occur with $A_{2A}$ receptors, adenylate cyclase and TrkB-t receptors in astrocytes, having an impact upon GAT-1 mediated GABA transport.

In conclusion, the data now reported highlight a new role for TrkB-t receptor in astrocytes, namely the modulation of activity and trafficking of GAT-1. This action of BDNF may impact upon synaptic transmission, namely decreasing tonic inhibition, and in such way adding to the plethora of mechanisms operated by the neurotrophin to reinforce synaptic activity.

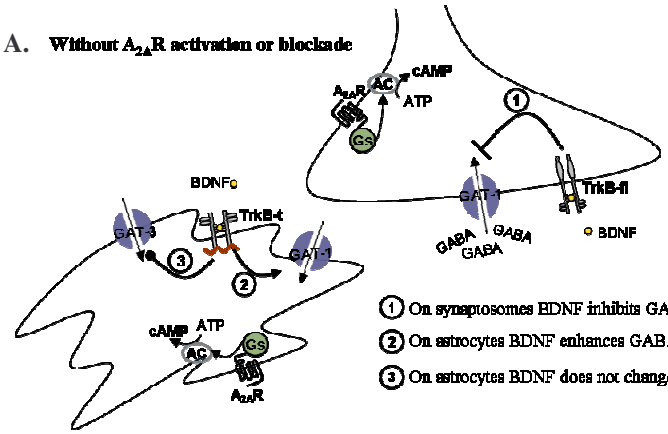
## 6. GENERAL CONCLUSIONS

Regulation of GABA transporters has been intensively studied for the last decades due to their ability to control neuronal excitability. Indeed GABA transporters are terapeutic targets for GABAergic regulation in patalogical conditions, such as epilepsy.

The main conclusion of the work now described is that BDNF has opposite actions on GAT-1, depending on the location of the transporter, and this actions result from activation of different BDNF receptors by BDNF, namely TrkB-fl in nerve terminals and TrkB-t in astrocytes.

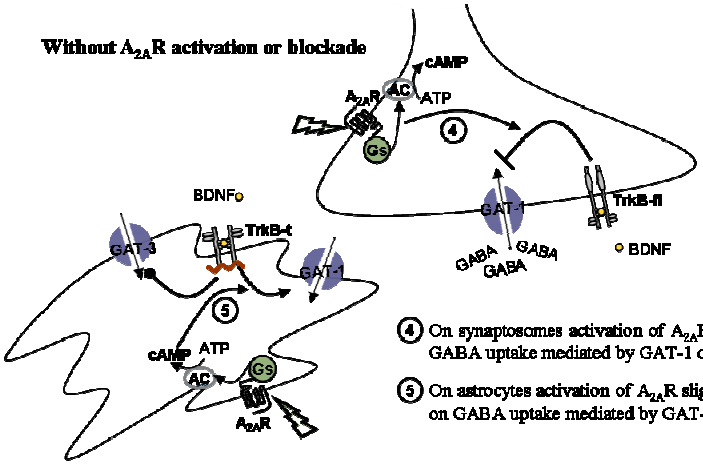
Cell specific differences, not only related with different BDNF receptor subtypes, can also occur. There are previous reports showing differential modulation of GABA transporters according to the cell type. Lee and collaborators (2006) demonstrated that in patients with temporal lobe epilepsy there is an increase of GAT-3 expression in astrocytes and a decrease in GAT-1 expression in nerve terminals. Actually, it looks like there is a parallelism between results described by Lee and collaborators (2006) and the present results, since in nerve terminal, BDNF decreses GAT-1 mediated uptake while in astrocytes this neurotrophin enhances GABA transport (Figure 6.1.1A). The fact that BDNF modulates GAT-1 but not GAT-3 is somehow puzzling, since GAT-1 and GAT-3 have very similar structure although with difference amino acid sequence.

**A. Without $A_{2A}$R activation or blockade**



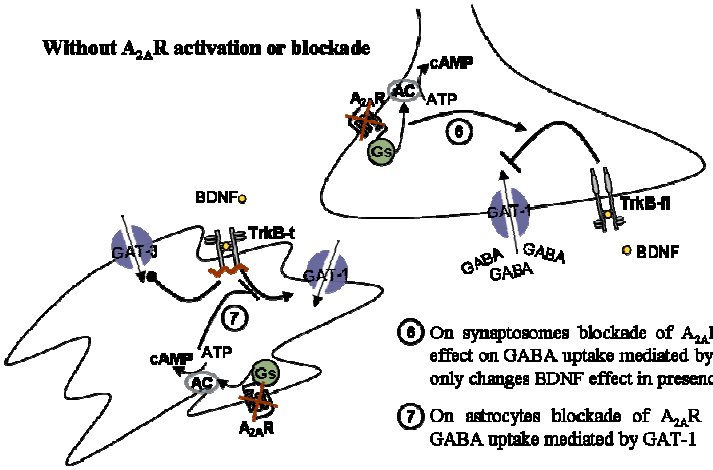

1. On synaptosomes BDNF inhibits GABA uptake mediated by GAT-1
2. On astrocytes BDNF enhances GABA uptake mediated by GAT-1
3. On astrocytes BDNF does not change GABA uptake mediated by GAT-3

**B. Without $A_{2A}$R activation or blockade**




4. On synaptosomes activation of $A_{2A}$R enhances BDNF effect on GABA uptake mediated by GAT-1 only in presence of ADA
5. On astrocytes activation of $A_{2A}$R slightly enhances BDNF effect on GABA uptake mediated by GAT-1

**C. Without $A_{2A}$R activation or blockade**




6. On synaptosomes blockade of $A_{2A}$R does not change BDNF effect on GABA uptake mediated by GAT-1; blockade of $A_{2A}$R only changes BDNF effect in presence of ADA
7. On astrocytes blockade of $A_{2A}$R abolishs BDNF effect on GABA uptake mediated by GAT-1

**Figure 6.1.1. Schematic representation of the influence of BDNF upon GAT-1 mediated GABA transport into nerve endings and astrocytes and modulation by $A_{2A}$ receptors.** Modulation of GAT-1 transporter by BDNF in synaptosomes and astrocytes without activation of $A_{2A}$ receptors (A), with activation of $A_{2A}$ receptor (B) and blockade of $A_{2A}$ receptors (C).

Thinking in the triparte synapse and in tonic and phasic inhibition, it is possible to speculate that the different BDNF effects on nerve terminals and astrocytes produce different responses at GABAergic transmission. GABA transporters located at synaptic level (nerve terminals) are mainly responsible for termination of phasic inhibition while transporters located at astrocytic level are mostly responsible for maintaing tonic inhibiton. Since BDNF decreses GABA uptake mediated by GAT-1 in nerve terminals, this may induce an increase of GABA at synaptic cleft leading to potentiation of GABergic transmission. On the other hand, since BDNF enhances GABA uptake in astrocytes there will be a decrease in GABA concentration in areas surrounding the synapse leading to a decrease in tonic inhibiton. This would imply not only that BDNF decreases the signal-to-noise ratio of GABAergic transmission but also that this neurotrophin is favouring overall excitability preserving the possibility of phasic inhibition.

Another important finding obtained with this work is that the effect of BDNF on GAT-1 transporter in astrocytes occurs through activation of TrkB-t receptor, and not the TrkB-fl receptors. BDNF has different types of effect, depending on the isoform of TrkB receptor that is expressed in cells. TrkB-fl isoform has a tyrosine kinase domain which is responsible for activation of three pathways, the PLC, MAP kinase and PI3-K/Akt pathways while TrkB-t isoform lacks the tyrosine kinase domain, being the transduction pathways for this receptor not completely

clear. As I elucidated, the TrkB-t receptor somehow require coupling to the PLC-γ pathway affecting GAT-1 in astrocytes.

Adenosine $A_{2A}$ receptor has been pointed out as modulator of the action of BDNF or its high-affinity receptors (for a review see Sebastião et al., 2010). In nerve terminals the effect of BDNF on GAT-1 was not modified by blockade of $A_{2A}$ receptors, indicating that this effect is not dependent upon activation of $A_{2A}$ receptor. Nevertheless the effect of BDNF was more pronounced when $A_{2A}$ receptors are activated (Figure 6.1.1B). In astrocytes, $A_{2A}$ receptor blockade fully prevented BDNF actions (Figure 6.1.1C). It is possible that the levels of endougenous adenosine are enough to tonically activate $A_{2A}$ receptors in astrocytes but not in synaptosomes. However, inspite of different endogenous levels of adenosine it is clear that $A_{2A}$ receptors activation is a necessary condition for the effect of BDNF upon GAT-1 in astrocytes but not in nerve ending. Interestingly, and since in astrocytes the effect of BDNF is exercised through TrkB-t it become evident that this TrkB isoform can also be modulated by $A_{2A}$R; the mechanism behind this modulation requires further investigation.

Altogether, the data reported in this thesis strongly suggest that BDNF modulates GABAergic transmission by modulating of GABA transporters located both at synapse and at astrocytes. Depending on the TrkB isoform that is activated, the BDNF effect will depend or not of $A_{2A}$ receptor co-activation. Thus it is likely that BDNF has different effects according to its site of action, which may have different but conserted consequences upon GABAergic transmission. The work I performed opens new aspects of BDNF research highlighting its ability to modulate the GABAergic system at the transporter level.

## 7. FUTURE PERSPECTIVES

GAT-1 transporters located at nerve terminal, in addition to terminating synaptic transmission by clearing released transmitters from the extracelullar space, are the primary mechanism for replenishing transmitter stores and thus regulate presynaptic homeostasis. Thus the effect of BDNF on GAT-1 transporter in nerve terminals should be a target for further evaluation, of the functional consequences of this action of BDNF for the presynaptic GABAergic homeostasis.

In nerve terminals I just analised one of the signalling pathways of TrkB-fl receptor, showing that the PLC pathway is the one that is mainly involved in fast effects of BDNF. Nevertheless MAP kinase and PI3-K/Akt pathways should also be studied since there are other rapid BDNF-mediated signalling cascades described that involve activation of MAP kinase and PI3-K/Akt (Johnson-Farley et al., 2006; Ortega et al., 2010).

In nerve terminal, it is still unknown if the effect of BDNF changes $K_m$ or $V_{max}$ value, and if this is correlated with an increase of GAT-1 transport at synapse level. Law and collaboraters (2000) demonstrated that inhibition of tyrosine kinases decrease GABA uptake in culture neurons by decreasing $V_{max}$ and consequently by decreasing the number of functional transporters on plasma membrane. Therefore I could hypothesise that in synaptosomes BDNF diminished GABA uptake by decreasing the number of functional transporter on plasma membrane. To address this question biotinylation experiments (as in chapter 4.2.4) are required. Finally it would be of particular interest to evaluate how the effect of BDNF modulates tonic and/or phasic inhibition. Changes in the tonic vs phasic $GABA_A$ receptor-mediated transmission can be addressed by patch-clamp mode. Indeed, by measuring variations in the frequency and amplitude of spontaneous miniature inhibitory postsynaptic currents (IPSCs) one can evaluate effects upon phasic GABAergic transmission whilst changes in the holding

current upon fast application of $GABA_A$ antagonists will reflect tonic $GABA_A$ receptor-mediated currents.

The discovery in the last decades that astrocytes are integral functional element of the synapse, responding to neuronal activity and regulating synaptic transmission and plasticity has changed the perception of brain functioning. The effect of BDNF on GAT-1 transporter obtained in astrocytes was opposite of the effect obtained in nerve terminals, and occured on both systems through activation of two different TrkB receptor isoforms. Until now what has been proposed is an interaction between TrkB-fl and $A_{2A}$ receptors, but the results obtained also demonstrated an interaction between TrkB-t and $A_{2A}$ receptors. My hypothesis concerning this issue is that the TrkB domain responsible for this interaction is the transmembrane domain since this portion of TrkB receptor is common to all isoforms of TrkB. Site directed mutagenesis approches can be used to test that hypothesis.

In continuity with the present work I would like to evaluate if this modulatory effect of BDNF on GABA transport in astrocytes is correlated with the BDNF induced calcium wave observed also in astrocytes (Rose et al., 2003). On the other hand, it was also recently demonstrated that GABA induces an increase of intracellular calcium in astrocytes, which is correlated with a reduction of $Na^+/Ca^{2+}$ exchange, thereby leading to a $Ca^{2+}$ increase sufficient to trigger $Ca^{2+}$-induced $Ca^{2+}$ release via InsP3 receptors (Doengi et al., 2009). Is the increase of GABA uptake induced by BDNF sufficient to trigger a calcium wave? This is a question that could be addressed through quantification of the intracellular calcium rise in astrocytes. GAT-1 could be blocked and the consequences for a BDNF-induced calcium wave assessed. If modulation of GAT-1 is a requisite for

the BDNF-induced calcium signaling, the BDNF effect should be lost upon GAT-1 blockade.

Several questions remain unanswered concerning modulation of GABA transporters and all the functional roles that they will have at central nervous system under physiological or phatological conditions. With the work now presented I hope that science has taken one small step forward.

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