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Abbreviations list

Aβ – Amyloid beta peptide
AC – Adenylate cyclase
AD – Alzheimer’s disease
ADA – Adenosine deaminase
ADAC – Adenosine amine congenere
ADO – Adenosine
AK – Adenosine kinase
AMP – Adenosine 5’-monophosphate
AP5 – DL-2-amino-5-phosphonopentanoate
ATP – Adenosine 5’-triphosphate
BDNF – Brain-derived neurotrophic factor
Bmax – Maximal number of binding sites
cAMP – cyclic adenosine monophosphate
CGS 21680 – 4-{2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuroanamidosyl)-9H-purin-2-yl]amino]ethyl}benzenepropanoic acid
CNS – Central nervous system
CR – Cysteine-repeated
CREB – cAMP response element binding protein
DAG – Diacylglycerol
DbcAMP – Dibutyril cyclic adenosine monophosphate
DG – Dentate Gyrus
DMEM – Dulbecco’s modified Eagle’s medium
DMSO – Dimethylsulphoxide
DPCPX- 1,3-Dipropyl-8- cyclopentylxanthine
DTT– 1,4-Dithiothreitol
EC – Entorhinal cortex
EDTA – Ethylenediaminetetraacetic acid
EGTA – Ethylene glycol-bis(2-aminoethyl ether)-N,N –tetraacetic acid
ER – Endoplasmic reticulum
ERK– extracellular signal-regulated protein kinases
FCT – Fundação para a ciência e a tecnologia
fEPSP – Field excitatory post-synaptic potential
GABA – γ-aminobutyric acid
GFAP – Glial fibrillary acidic protein
HBSS – Hank’s balanced salt solution
HD – Huntington’s disease
IP3 – Inositol 1,4,5-triphosphate
ITU – 5-Iodotubercidin
JNK – c-jun N-terminal kinases
KAc – Potassium acetate
Kd – Equilibrium dissociation constant
Ki – Equilibrium dissociation constant of the competitor
LDH – Lactate dehydrogenase
L-DOPA – (L-3,4-dihydroxyphenylalanine)
LRR– Leucine-rich repeats
LTP – Long-term potentiation
MAPK – mitogen-activated protein kinase
NF-KB – Nuclear factor KB
NGF – Nerve growth factor
NMDA– N-Methyl-D-aspartate
3-NP – 3-nitropropionic acid
NT – Neurotrophin
PBS – Phosphate buffered saline
PI3K – Phosphatidylinositol-3-kinase
PIP2 – Phosphatidylinositol-4,5-biphosphate
PKA – Protein kinase A
PKC – Protein kinase C
PLC – Phospholipase C
PLD – Phospholipase D
pNA – p-nitroanilide
SAH – S-adenosylhomocysteine
SAPK – Stress-activated protein kinases
SCH 58261 – 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo
[1,5-c]pyrimidine
SDS - PAGE – Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis
SEM – Standard error of the mean
TGN – Trans-golgi network
Tris – Tris-hydroxymethyl-aminomethane
Trk – Tropomyosin-related kinase
XAC – 1,3-dipropyl-8-phenylxanthine amine congener
ZM 241385 – 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol
Abstract

The brain-derived neurotrophic factor (BDNF) belongs to a group of signalling factors that are essential for neuronal survival and differentiation, inducing modulation of cell death events such as apoptosis. This neurotrophin also has synaptic regulatory actions on basal synaptic transmission, including effects on plasticity such as long-term potentiation (LTP).

The hippocampus is under neuromodulatory control of adenosine, which through activation of inhibitory (A1) and excitatory (A2A) receptors fine-tunes the action of neurotransmitters and neuromodulators. The hippocampus is a brain area where both A2A and TrkB receptors are expressed. Since activation of A2A receptors can acutely induce transactivation of BDNF TrkB receptors in cell culture, in the present work it was evaluated how the activation of adenosine A2A receptors could influence the actions of BDNF. The first aim of the present study was to investigate the influence of adenosine A2A receptors on BDNF effects on hippocampal synaptic transmission and the possible mechanisms involved. Secondly, the influence of age on BDNF modulation of hippocampal synaptic transmission, as well as the interplay between adenosine A2A receptors and BDNF TrKB receptors, was examined. As a third objective, the BDNF actions on LTP during aging and the influence of adenosine A2A receptors on these actions was also evaluated. Finally, the influence of the A2A receptor activation on the neuroprotective action of BDNF on apoptosis was investigated.

The acute excitatory action of BDNF on synaptic transmission in the hippocampus of infant rats was found to be inducible by a depolarisation that is dependent on adenosine A2A receptor activation, through a mechanism that requires cyclic adenosine monophosphate (cAMP) formation and protein kinase A (PKA)
activity. Subsequently, for the first time a relationship between age-related changes in the density of TrkB and A2A receptors as it concerns BDNF-induced enhancement of synaptic transmission in the hippocampus was elucidated. In LTP studies, it was observed that BDNF increases the magnitude of θ-Burst stimuli-induced LTP and that this excitatory action is also dependent on A2A adenosine receptor activation. Moreover, it was found that BDNF loses the ability to increase LTP in aged animals. Finally, BDNF protected neurons from apoptosis induced by amyloid beta peptide (Aβ) 25-35 and this protection was more evident when adenosine A2A receptors are activated.

In conclusion, the results now presented demonstrate that activation of adenosine A2A receptors facilitates the synaptic and neuroprotective actions of BDNF.
Resumo

O factor neurotrófico derivado do cérebro (BDNF, brain-derived neurotrophic factor) pertence a um grupo de factores de sinalização essenciais para a sobrevivência e diferenciação neuronal, modulando a morte celular por apoptose. Esta neurotrofina regula quer a transmissão sináptica quer a plasticidade sináptica como é o caso da potenciação de longa duração (LTP, long term potentiation).

O hipocampo encontra-se sob controlo da adenosina, que através da activação dos seus receptores inibitórios (A1) e excitatórios (A2A) regula a acção de neurotransmissores e neuromoduladores. Uma vez que a activação dos receptores A2A pode induzir transactivação dos receptores TrkB do BDNF em células em cultura, no presente estudo avaliou-se se a activação dos receptores A2A poderia influenciar a função do BDNF no hipocampo, área cerebral que expressa quer receptores A2A da adenosina quer receptores TrkB para o BDNF. Primeiro avaliou-se a influência dos receptores A2A na acção do BDNF sobre a transmissão sináptica no hipocampo e possíveis mecanismos envolvidos. Seguidamente, foi avaliada a influência da idade na modulação da transmissão sináptica induzida pelo BDNF, e a sua relação com os receptores A2A da adenosina. Posteriormente estudaram-se as acções do BDNF na LTP em função do envelhecimento e a influência exercida pelos receptores A2A da adenosina. Finalmente, estudou-se a consequência da activação dos receptores A2A da adenosina no efeito neuroprotector do BDNF, ou seja sobre a sua influência na morte celular por apoptose.

Os resultados obtidos mostraram que a acção excitatória do BDNF aplicado agudamente na transmissão sinaptica em hipocampo de ratos jovens pode ser induzida por uma despolarização, e que este efeito é dependente da activação dos receptores A2A da adenosina, através de um mecanismo que requer a formação de monofosfato ciclico de adenosina (cAMP, cyclic adenosine monophosphate) e a
ativação da cinase de proteínas tipo A (PKA, protein kinase A). Observou-se pela primeira vez que, ao longo da idade, existe uma relação entre a alteração do efeito do BDNF na transmissão sináptica e modificações nas densidades dos receptores A<sub>2A</sub> da adenosina e TrkB de BDNF no hipocampo. Verificou-se também que o BDNF aumenta a magnitude da LTP induzida por um estímulo θ-Burst, e que perde esta acção em animais idosos. Estes efeitos são, igualmente, dependentes da activação dos receptores A<sub>2A</sub> da adenosina. Finalmente, demonstrou-se que o BDNF protege os neurónios da morte celular induzida pelo peptídio beta amiloide (Aβ, amyloid beta peptide) 25-35 e que esta protecção é mais evidente quando os receptores A<sub>2A</sub> se encontram activados, deixando de se observar quando estes receptores são bloqueados.

Em conclusão, os resultados apresentados na presente dissertação demonstram que a activação dos receptores A<sub>2A</sub> da adenosina facilita a acção sináptica e neuroprotectora do BDNF quando administrado agudamente.
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The scientific content of the present thesis has been included in the publication of the following original articles:


The following chapters of this thesis are in preparation to be submitted as manuscripts to international journals:

**Chapter 4.3** “Influence of age on the BDNF modulation of long-term potentiation: interplay with adenosine A$_{2A}$ receptors.”

**Chapter 4.4** “Influence of the activation of adenosine A$_{2A}$ receptors on the protective BDNF action against apoptosis induced by amyloid beta peptide.”

Other papers where the author of this thesis participate during her doctoral studies:


1. INTRODUCTION

1.1 NEUROTROPINS

In 1953, Rita Levi-Montalcini and Victor Hamburger discovered that a mouse sarcoma tumor implanted close to the spinal cord of developing chicks in ovo secreted a soluble factor that induced the hypertrophy and fiber outgrowth of sympathetic neurons. Later, this factor was isolated and named as nerve growth factor (NGF) (Levi-Montalcini and Cohen, 1956). The discovery of this molecule opened a new field in neurobiology research. After NGF identification, Barde and collaborators (1982) isolated a different neurotrophin (NT), from a pig brain, designated as brain-derived neurotrophic factor (BDNF). Since then other members of the family of NTs have been identified in mammals namely, NT-3 and NT-4, and in fish NT-6 e NT-7 (Gotz et al., 1994; Lai et al., 1998).

1.1.1 Neurotrophin synthesis

All NTs are generated first as precursor proteins, pre-pro-neurotrophins (approximately 240-260 amino acids long, Figure 1.1.1), which then are cleaved intracellularly to mature proteins of 118-120 amino acids. The pre-mRNA sequence directs the synthesis of the nascent protein in the endoplasmic reticulum (ER) attached ribosomes, leading to the sequestration of the newly formed polypeptide chain into the ER (see Lessmann et al., 2003). The signal peptide is cleaved off immediately after sequestration in the ER. The resulting pro-neurotrophins can
spontaneously form non-covalently-linked homodimers in the ER. The pro-neurotrophins in the ER then transit the Golgi apparatus most likely via intermediate non-clathrin-coated transport vesicles and finally accumulate in the membrane stacks of the trans-golgi network (TGN). There are three fates for intracellular pro-neurotrophins: 1) intracellular cleavage followed by secretion, 2) secretion followed by extracellular cleavage, or 3) secretion without subsequent cleavage. All of these products serve as signalling molecules (see Lee et al., 2001).

Two different types of secretory vesicles may be generated (Figure 1.1.2), filled with one or a combination of different neuropeptides. Thus, BDNF release can occur through two different pathways: 1) a regulated pathway, where neurotrophins are secreted in response to a stimuli or 2) a constitutive pathway, where these molecules are spontaneously secreted. The secretory granules of the constitutive pathway are smaller (50-100 nm in diameter) and independent of intracellular Ca$^{2+}$ concentration elevation. Vesicles can fuse with the plasma membrane to release their content in the absence of any specific triggering mechanisms. In the regulated pathway, vesicles are large (100-300 nm in diameter) and their release depends on intracellular Ca$^{2+}$ concentration elevation. Secretion of neurotrophins can be regulated by neuronal activity, potassium, and glutamate; this release is also dependent on the stimulus-frequency (Lim et al., 2003). Interestingly, constitutive and regulated secretion of BDNF can coexist in the same cell (see Lessman et al., 2003)

1.1.2 Neurotrophin receptors

Neurotrophins function by activating two distinct classes of transmembrane receptors: the p75 neurotrophin receptor (p75NTR) and the Trk family of receptor tyrosine kinases that includes TrkA, TrkB and TrkC. Unlike the non-selective p75NTR, which
has a similar affinity for all neurotrophins, each Trk receptor selectively binds a different neurotrophin.

**Figure 1.1.1- Structure of the rat BDNF gene and protein.** (A) Each of the four 5'-exons (I–IV) includes its own promotor, and is combined with the 3'exon (V) to yield an mRNA (B) coding for pre-pro-BDNF. The short white stretch in exon V represents an alternative splice site, giving rise to two differentially spliced mRNA variants for each of the four transcripts. Each of these mRNAs is expressed in a tissue-specific and developmentally regulated manner. The gray portion in exon V codes for the pre-pro-BDNF protein. (C) Primary structure and sequence of the BDNF protein. The pre-sequence (18 aa, black) is cleaved off immediately after sequestration of the nascent protein into the ER. The mature BDNF protein (black) is excised from the pro-BDNF precursor by virtue of specific protein convertases residing either in the TGN or in immature secretory granules. (Adapted from Lessmann et al., 2003.)
1.1.2.1 p75 receptor

p75NTR is a member of the tumor necrosis receptor family with an extracellular domain that includes four cysteine-rich motifs, a single transmembrane domain and a cytoplasmic domain, that includes a ‘death’ domain as represented in Figure 1.1.3 (Liepinsh et al., 1997; He and Garcia 2004).

All neurotrophins, including the pro-neurotrophins, can bind to p75NTR (Figure1.1.3) (see Lu et al., 2005). This receptor can interact with several proteins and forms multimeric receptor complexes that transmit important signals for regulating neuronal survival and differentiation as well as synaptic plasticity (see e.g. Reichardt, 2006). P75NTR also regulates the responsiveness of Trk receptors to neurotrophins. The presence of p75NTR enhances the specificity of TrkA and TrkB for their primary ligands, NGF and BDNF, respectively (see Reichardt, 2006).
1.1.2.2 Trk receptors

The name TrK, from tropomyosin-related kinase, derives from the oncogene that resulted in its discovery (Barbacid, 1991). The oncogene was discovered in a carcinoma and it was found to consist in the first seven of eight exons of nonmuscle tropomyosin fused to the transmembrane and cytoplasmic domains of a novel tyrosine kinase. Consequently, the protooncogene was named tropomyosin-related kinase (Trk), commonly referred as TrkA. The TrkB and TrkC genes were identified because of their homology with TrkA. Seventeen years ago, it was demonstrated that NGF binds to TrkA and that it activates the tyrosine kinase activity of the receptor (Kaplan et al., 1991; Klein et al., 1990). Subsequently, TrkB was identified as the receptor for BDNF and NT-4, and TrkC as the receptor for NT-3 (see for review Huang and Reichardt, 2001; Bibel and Barde, 2000). Although NT-4 and BDNF bind to the same receptor, several observations indicate that the biological effects mediated by these neurotrophins are not identical, which may mean that neurons can discriminate between these two molecules (see e.g. Lewin and Barde, 1996). The extracellular domain of each Trk receptor consists of a cysteine-rich cluster, followed by three leucine-rich repeats, another cysteine-rich cluster and two immunoglobulin-like domains. Each receptor terminates with a cytoplasmic domain consisting of a tyrosine kinase domain surrounded by several tyrosines that serve as phosphorylation-dependent docking sites for cytoplasmic adaptors and enzymes. The major site at which neurotrophins interact with these receptors is in the membrane-proximal immunoglobulin-like domain (Figure 1.1.3).

Splicing generates isoforms of TrkB and TrkC that include comparatively short cytoplasmic motifs without a tyrosine kinase domain; these receptors are designated as truncated receptors. Different TrkB isoforms can be generated: T1, T2 and T-Shc. Expression of these non-kinase-containing isoforms has been shown to inhibit productive dimerization of kinase-containing Trk receptors, thereby inhibiting
responses to neurotrophins (Eide et al. 1996). Moreover, recent data indicates that
some truncated isoforms help to regulate the expression of full-length TrkB receptors
(Haapasalo et al., 2002).

Figure 1.1.3- Representation of the domain structures of the Trk and p75
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which CR2 and CR3 are involved in the neurotrophin-binding interaction. (Adapted from
Reichardt, 2006.)
1.1.2.3 Trk-mediated signalling pathways

The Trk receptors are activated specifically by the mature and not by the pro-forms of the neurotrophin gene products (Lee et al., 2001). Binding of mature neurotrophins to their specific Trk receptors induces dimerization and autophosphorylation at specific tyrosine residues within intracellular domains. The generation of phosphotyrosine residues in turn catalyses the formation of large signalling complexes. (Segal and Greenberg, 1996). Phosphorylation at Y515 (in the case of TrkB receptors) creates the binding site for Shc (Src homology 2/α-collagen-related protein), whereas phosphorylation at Y816 (in case of TrkB receptors) forms the adaptor site for Phospholipase C (PLC)γ as represented in Figure 1.1.4. The major signalling pathways activated by the Trk receptors are; (1) phosphatidylinositol-3 kinase (PI3K); (2) Ras-MAPK pathway and (3) PLCγ pathway, and their downstream effectors. These include PI3K stimulation of Akt kinase, Ras stimulation of mitogen-activated protein MAPK cascades and PLC-γ dependent generation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), which results in mobilization of Ca^{2+} stores and activation of Ca^{2+} and DAG-regulated protein kinase (Segal and Greenberg, 1996). These intracellular signalling pathways modulate gene expression in a cell type-specific manner and are responsible for most of the effects of neurotrophins related to neuronal growth, survival and differentiation, along with synaptic transmission and plasticity.

Neurotrophins and activated Trk receptors are transported together in endocytic vesicles (see e.g. Lessman et al., 2003). Internalization and transport of Trk receptors serve two functions: 1) to bring activated Trk receptors into proximity of cell compartments, such as the nucleus, where signalling is required to enable specific cellular responses, such as gene transcription and 2) to transport activated Trk receptors to membrane compartments, where signalling effectors are concentrated (see Reichardt, 2006). The classic retrograde route is destined to exert the long-
lasting effects of neurotrophins, which are, for example, mediated by new gene transcription; but, there is also an additional anterograde targeting that occurs in the axon towards synaptic terminals which is involved in the fast actions of neurotrophins (see e.g. Lessmann et al., 2003). Mechanisms underlying the fast action of neurotrophins include intracellular Ca\(^{2+}\) signalling, neuronal excitation, augmentation of synaptic excitation by modulation of N-methyl-D-aspartate (NMDA) receptor activity and control of synaptic inhibition through the regulation of the K\(^+\)/Cl\(^-\) cotransporter KCC2. The fastest action of brain-derived neurotrophic factor and neurotrophin-4/5 occurs within milliseconds, and involves activation of TrkB and the opening of the Na\(^+\) channel Na\(_{\text{v}1.9}\). Through these rapid actions, neurotrophins orchestrate neuronal activity, modulate synaptic transmission and produce instructive signals for the induction of long-term changes in the efficacy of synaptic transmission (see Kovalchuk et al., 2004).
Figure 1.1.4- TrK-mediated neurotrophin signalling. The activated signalling pathways mediate effects of neurotrophins on neuronal survival, differentiation, and gene expression as well as acute effects on synaptic transmission and plasticity, as indicated. Abbreviations represent the following: P, phosphorylation; Y, tyrosine residue; PI3K, phosphatidylinositol 3-kinase; AKT, AKT kinase; Ras, small GTP-binding protein Ras; PIP2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate. (Adapted from Blom and Konnerth, 2005).
1.1.3 Pathophysiologica implications of BDNF actions

The first suggestion to use a neurotrophin for therapeutic purposes appeared in the 1980s, when NGF was the only known member of the NT family. This proposal was based on the knowledge that in the CNS, NGF promotes the survival and function of cholinergic neurons in the basal forebrain (Fisher et al., 1987). These neurons project to the hippocampus and are believed to be important in the memory process, which is specifically affected in patients with Alzheimer's disease. Thus, in 1993, a group of investigators from the Institute of Karolinska developed a way to administer NGF directly to the brain of Alzheimer's patients (Seiger et al., 1993). This method consisted in a canule placed in the lateral ventricles connected to an infusion pump, enabling administration of NGF to patients (Figure 1.1.5). However this route of administration is troublesome since it requires neurosurgery.

*Figure 1.1.5- NGF administration directly to the brain.* NGF was delivered through a canule implanted in the lateral ventricle connected to an infusion pump. (Adapted from Seiger et al., 1993.)
The discovery of other members of the neurotrophin family, in particular the discovery of BDNF and the knowledge of its physiological functions, contributed to the large number of studies correlating the pathogenesis of human neurodegenerative disorders with alteration in BDNF actions. These changes can be a result of genetic polymorphisms in the BDNF gene or in the genes for BDNF receptors (TrKB full length, truncated, and P75 receptors), alterations in the levels of mRNA for BDNF, modifications in levels of BDNF protein and changes in BDNF receptor densities.

Regarding modifications in the BDNF gene, mRNA for BDNF and protein levels, a polymorphism in BDNF gene leading to a different BDNF protein (BDNF<sub>met</sub>) appears to alter susceptibility to neuropsychiatric disorders, such as Alzheimer’s disease (AD) (Ventriglia <i>et al.</i>, 2002), Parkinson’s disease (Momose <i>et al.</i>, 2002), depression (Sen, 2003), and bipolar disorders (Sklar <i>et al.</i>, 2002; Neves-Pereira <i>et al.</i>, 2002). In 1991, Phillips and colleagues reported a selective reduction of BDNF mRNA expression in the hippocampus of individuals with Alzheimer’s disease, which was later confirmed by others (Murray <i>et al.</i>, 1994). Moreover, a decrease in BDNF protein levels in the hippocampus has been associated with depression (Russo-Neustadt <i>et al.</i>, 2001; Chen <i>et al.</i>, 2001a). Furthermore, a decrease in BDNF protein levels has been reported in nigrostriatal dopamine regions in the brains of Parkinson’s patients (Mogi <i>et al.</i>, 1999, Parai <i>et al.</i>, 1999). A decrease in the expression of the BDNF protein in the hippocampus of people with dementia exhibiting Lewy bodies (Immamura <i>et al.</i>, 2005) in patients with diabetic brain neuropathies (Nitta <i>et al.</i>, 2002) and in the striatum of individuals with Huntington’s disease (see Zuccato and Cattaneo, 2007) was also reported. There is also evidence that brain BDNF content is diminished in the cortex and the hippocampus of AD patients (Connor <i>et al.</i>, 1997, Ferrer <i>et al.</i>, 1999).

Concerning modifications in TrkB receptors, a study demonstrated that a mutation in the kinase domain of TrkB, that leads to impaired intracellular signalling is
associated with obesity and developmental delay (Yeo et al., 2004). On the other hand, the expression of different TrkB splice variants is associated with cognitive capacity, while over-expression of the kinase-containing form enhances memory and learning in transgenic mice (Koponen et al., 2004); conversely, over-expression of the TrkBT1 isoform in adult neurons impairs long-term memory (Saarelainen et al., 2000). Interestingly, increased content of the TrkBT1 isoform and decreased TrkB expression has been observed in AD patients (Ferrer et al., 1999).

Finally, genetic modification in the gene encoding P75NTR can be involved in pathology. Polymorphisms in this gene can lead to an increased susceptibility to depressive disorders (Kunugi et al., 2004).

The regulatory actions of neurotrophic factors in neuronal functions and in response to neuronal injury, together with the evidence that changes in BDNF signalling are associated with a wide variety of pathologies, led to the working hypothesis that the delivery of BDNF to the brain could ameliorate those diseases. A number of clinical trials involving the use of BDNF for neurodegenerative disorders such as amyotrophic lateral sclerosis and diabetic neuropathy has been carried out in the past decade. However, BDNF had minimal beneficial effects and produced side effects such as pain and gastrointestinal symptoms (Thoenen and Sendtner, 2002). The lack of promising results can be attributed to a short half-life for BDNF, a non-controlled delivery of the neurotrophin, lack of controlled levels of BDNF at the site of action and the presence of endogenous compensative processes that may regulate BDNF levels. Therefore further basic studies are needed in order to better understand BDNF actions in neurons, which may help in a better design of future clinical trials.
1.2 ADENOSINE

Adenosine (Figure 1.2.1) is a nucleoside formed when N9 of adenine (a purine) is covalently bound to the C1’ position of ribose.

Adenosine is an ubiquitous homeostatic substance present in all cells, and it is released from apparently all cells, including neurons and glia (see Ribeiro et al., 2003). Adenosine plays a major role in the cardiovascular system, in the central nervous system, in the gastrointestinal tract, in the immune system, in mast cell degranulation, and asthma, as well as cell growth, proliferation and apoptosis (see Schultle and Fredholm, 2003).

![Figure 1.2.1 – Adenosine chemical structure. Adenosine contains adenine (in the blue square) and ribose (in the red square) attached by a C1'-N9 glycosideic bond.](image)

1.2.1 Adenosine synthesis

Adenosine can be formed by the action of the enzyme endo-5’-nucleotidase on adenosine monophosphate (AMP) (Phillis and Newsholme, 1979) and by the hydrolysis of S-adenosylhomocysteine (SAH) (Nagata et al., 1984) catalysed by SAH hydrolase. The intracellular concentration of adenosine at equilibrium is about 100 nM (Meghji, 1992). This level is controlled by adenosine kinase (AK), which
phosphorylates adenosine to produce AMP, and by adenosine deaminase (ADA), which catalyzes the formation of inosine (Figure 1.2.2). Adenosine is neither stored nor released as a classical neurotransmitter since it does not accumulate in synaptic vesicles, but is instead released from the cytoplasm into extracellular space through a nucleoside transporter (see e.g. Ribeiro et al., 2003). Adenosine is released from the presynaptic component, from the activated postsynaptic component or from non-synaptic regions of neurons (see Cunha, 1997). The extracellular adenosine concentration in all body fluids is rather constant under basal conditions (30-300 nM) (see Schultle and Fredholm, 2003).

Figure 1.2.2- Pathways of adenosine production, metabolism and transport
Abbreviations are as follows: ADA, adenosine deaminase; AK, adenosine kinase; es, equilibrative-sensitive nucleoside transporters; ei, equilibrative-insensitive nucleoside transporters; SAH, S-adenosyl homocysteine. (Adapted from Latini and Pedata, 2001.)
1.2.2 Adenosine receptors

Adenosine receptors have been intensively studied, and to the present date, four different receptors have been cloned and designated as A$_1$, A$_{2A}$, A$_{2B}$ and A$_3$ receptors. These receptors are classified as seven transmembrane domain G-protein-coupled receptors. The original receptor classification was based on the effect on adenosine binding to the receptor affecting the cyclic AMP (cAMP) levels in different tissues, with A$_1$ decreasing and A$_2$ increasing cAMP levels (van Calker et al., 1979; Londos et al., 1980).

The adenosine A$_1$ receptors are highly expressed in brain cortex, cerebellum, hippocampus, and the dorsal horn of spinal cord (Figure 1.2.3) (see e.g. Fredholm et al., 2001). These receptors are present at pre-, post- and non-synaptic sites. When they are located pre-synaptically, they inhibit neurotransmitter release (Dunwiddie and Hass, 1985) whereas post-synaptically and in neuronal cell bodies they inhibit calcium influx through voltage-sensitive calcium channels, inhibit NMDA receptor and inhibit potassium currents, leading to membrane hyperpolarization (reviewed in Fredholm et al., 2005). A$_1$ receptors are coupled to inhibitory G-proteins (G$_i$/G$_o$) that lead to an inhibition of the activity of the enzyme adenylate cyclase (Figure 1.2.4) (see Linden, 2001).

The A$_{2A}$ receptors are highly expressed postsynaptically in the striato-pallidal GABAergic neurons and olfactory bulb (see e.g. Fredholm et al., 2001); they are also expressed in hippocampus and cortex (Figure 1.2.3) (see e.g. Ribeiro et al., 2003), where they have a predominant presynaptic localization (Rebola et al., 2005b). Adenosine A$_{2A}$ receptors are mostly coupled to stimulatory G-proteins (G$_s$), which consequently increase intracellular cAMP (Figure 1.2.4). In striatum, they are also coupled to Golf (Corvol et al., 2001); and, in the hippocampus, there is evidence that this receptor can be coupled to G$_s$/G$_o$ (Cunha et al., 1999), in addition to its usual
coupling to Gs proteins. Moreover, adenosine A2A receptors overexpressed in COS-7 cells were shown to couple to G15/16, but there is no evidence that this interaction occurs in vivo (Offermanns and Simon, 1995). Investigating endogenously expressed adenosine A2A receptors in human endothelial cells, Sexl and collaborators cautiously proposed signalling via G12/13 proteins without providing direct experimental evidence for this hypothesis (Sexl et al., 1997). Activation of the adenosine A2A receptors also induces formation of inositol phosphates in COS-7 cells via pertuxis toxin-insensitive Ga15 and Ga16 proteins (see Jacobson and Gao, 2006).

The A2B receptors display low levels of expression in the brain (Dixon et al., 1996), however their expression is noted in astrocytes (van Calker et al., 1979). A2B receptors are coupled to Gs proteins (Figure 1.2.4).

The A3 receptor is found frequently in peripheral tissues, in particular in mast cells and testis (Schubert et al., 1994); and, it has apparent intermediate levels of expression in the human cerebellum and hippocampus (Figure 1.2.3) and low levels in the rest of the brain (see Fredholm et al., 2001). The affinity of this receptor for adenosine (K_i=1000 nM) is considerably lower than the adenosine affinity of A1 receptors (K_i=10 nM) and A2A (K_i=30 nM). This receptor is usually coupled to inhibitory G-proteins (G_i and G_o) (Figure 1.2.4)(see Linden, 2001).
Figure 1.2.3 Distribution of adenosine receptors ($A_1$, $A_{2A}$ and human $A_3$) in brain regions. High levels of expression are indicated by larger font. (From Ribeiro et al., 2003.)
Figure 1.2.4- Adenosine receptor-signalling pathways. Activation of the A₁ and A₃ adenosine receptors inhibits adenylyl cyclase activity through activation of pertussis toxin-sensitive Gᵢ proteins resulting in increased activity of phospholipase C (PLC). Activation of the A₂A and A₂B adenosine receptors increases adenylyl cyclase activity through activation of Gₛ proteins. Activation of the A₂A receptor induces formation of inositol phosphates. A₂B receptor-induced activation of PLC occurs via Gₓ proteins. All four subtypes of adenosine receptors can couple to mitogen-activated protein kinase (MAPK), giving them a role in cell growth, survival, death and differentiation. Abbreviations represent the following: CREB, cAMP response element binding protein; DAG, diacylglycerol; IP₃, inositol 1,4,5-trisphosphate; PI3K, phosphatidylinositol 3-kinase; PIP₂, phosphatidylinositol-4,5-bisphosphate; PK, protein kinase; PLD, phospholipase D; NF-kB, nuclear factor-kB. (Adapted from Jacobson and Gao, 2006.)
1.2.3 Pathophysiological implications of adenosine actions

Adenosine has the ability to modulate the release of neurotransmitters and neuromodulators. The adenosine receptors have a crucial function in the regulation of the activity of other receptors that affect several biological functions such as differentiation, synaptic transmission, plasticity and apoptosis. Thus, adenosine receptors regulate the function of neuropeptide receptors, nicotinic autofacilitatory receptors, metabotropic glutamate receptors, NMDA receptors and neurotrophic factor receptors (see e.g Ribeiro et al., 2003).

Adenosine is apparently involved in many functions that can play a role in the pathology of the nervous system. Therefore, the modifications of extracellular adenosine levels or the pharmacological or molecular manipulation of adenosine receptors will interfere with the action of other important molecules that regulate brain functions. This may prove relevant in the treatment of several diseases, where activation or inhibition of adenosine receptors through modulation of certain pathways may change the fate of the diseases.

The pharmacological manipulation of adenosine has been suggested for the treatment of several health conditions (see Ribeiro et al., 2003). Adenosine functions as a natural sleep-promoting agent mostly through activation of $A_1$ receptors (Benington et al., 1995; Porkka-Heiskanen et al., 1997). It was suggested that adenosine participates in resetting of the circadian clock by manipulation of behavioral stats (Antle et al., 2001). Thus, it emerges that there exists a potential role for adenosine-related compounds and of $A_1$ receptor agonists as sleep promoters and adenosine receptor antagonists as arousal stimulators. In addition, adenosine $A_1$ receptor agonists have anxiolytic activity suggesting that drugs that facilitate adenosine $A_1$ receptor-mediated actions may be effective for the treatment of anxiety (Jain et al., 1995; Florio et al., 1998). Adenosine $A_1$ receptor antagonists have also
been proposed for the treatment of memory disorders (see Ribeiro et al., 2003). Caffeine, which is an antagonist of adenosine receptors, has cognitive effects mostly due to its ability to antagonize adenosine A<sub>1</sub> receptors in the hippocampus and cortex, the brain areas actively involved in cognition. Positive actions of caffeine on information processing and performance might also be attributed to improvement of behavioral routines, arousal enhancement and sensorimotor gating (Fredholm et al., 1999). This is further supported by the observation that theophylline, an adenosine receptor antagonist, enhances spatial memory performance only during the light period, which is the time of sleepiness in rats (Hauber and Bareiß, 2001). However, some cognitive actions of caffeine were also described in relation to A<sub>2A</sub> receptors antagonism (Chen et al., 2007).

In vivo and in vitro studies have demonstrated the neuroprotective role of adenosine A<sub>1</sub> receptor activation. Evaluating the action of hypoxia on synaptic transmission in hippocampal slices, it has been shown that substances that are released during hypoxia, such as GABA, acetylcholine, and even glutamate through metabotropic receptors, may have a neuroprotective role; however, their action is evident only when activation of adenosine A<sub>1</sub> receptors is impaired, leading to the proposal that adenosine A<sub>1</sub> receptors play a pivotal role in response to hypoxia (Sebastião et al., 2001).

The pharmacological manipulation of adenosine receptors has also been evaluated in the context of Alzheimer's disease treatment. Caffeine intake has been associated with a significantly lower risk for AD (Maia and de Mendonça, 2002). In recent studies with a model of AD in mice, chronic caffeine protected against cognitive impairment and resulted in reduced brain levels of amyloid beta protein (Arendash et al., 2006). The most widely used drugs to treat AD patients increase the availability of acetylcholine in central cholinergic pathways by inhibiting the enzyme acetylcholinesterase (Doody et al., 2001). Another strategy to promote cholinergic
transmission might be to activate adenosine A\textsubscript{2A} receptors, which facilitate acetylcholine release, or to block adenosine A\textsubscript{1} receptors, which inhibit acetylcholine release (see Sebastião and Ribeiro, 1996). Thus, either adenosine A\textsubscript{2A} receptor agonists or adenosine A\textsubscript{1} receptor antagonists (or the combination of both) might be useful as cognitive enhancers. However, the adenosine A\textsubscript{2A} receptor antagonist has also been studied to improve cognition of AD patients (see Chen et al., 2007).

Furthermore, the pharmacologic manipulation of A\textsubscript{2B}-adenosine receptor have been proposed as possible therapeutic agents for Alzheimer’s disease patients (Rosi et al., 2003). AD is associated with glial activation and increased levels of pro-inflammatory cytokines. Epidemiological results suggest that anti-inflammatory therapies can slow the onset of AD (see Rojo et al., 2008). Adenosine, acting at A\textsubscript{2A} receptors, is an effective endogenous anti-inflammatory agent that can modulate inflammation both in the periphery and the brain (Mayne et al., 2001).

Moreover, antagonists of adenosine A\textsubscript{2A} receptors emerged as potential anti-parkinsonian agents, based, in part, on the CNS distribution of the A\textsubscript{2A} receptors. A key finding in this process is the co-localization and reciprocal antagonistic interactions between A\textsubscript{2A} and D2 receptors in the striatum, initially observed in rats (Ferré et al., 1999), but also described in humans (Díaz-Cabiale et al., 2001). A\textsubscript{2A} receptor antagonism not only diminishes Parkinsonian-like muscle rigidity in rats but also potentiates the effect of L-DOPA (Wardas et al., 2001), which may allow the use of lower doses of L-DOPA and hence minimize or retard side effects and tachyphylaxis caused by L-DOPA treatment in Parkinson’s disease.

In Huntington’s disease (HD) both adenosine A\textsubscript{1} receptor agonists and adenosine A\textsubscript{2A} receptor antagonists appear to exert neuroprotective actions. The adenosine amine congener (ADAC), an adenosine A\textsubscript{1} receptor agonist, attenuates the striatal lesion, as well as the dystonia, induced in a rat model of HD by the administration of the mitochondrial toxin 3-nitropropionic acid (3-NP) (Blum et al.,
Since $A_{2A}$ receptors are mainly localized on the neurons, which degenerate early in HD, and given their ability to stimulate glutamate outflow and inflammatory gliosis, it was hypothesized that $A_{2A}$ receptors could be involved in the pathogenesis of HD, thus $A_{2A}$ receptors antagonists could be neuroprotective.

In addition, other pathologies such as schizophrenia, epilepsy, drug addiction, pain, and control of ventilation can also be improved through the use of adenosine receptor agonists and antagonists (see Ribeiro et al., 2003).
1.3 HIPPOCAMPUS

Buried deep within the medial temporal lobe of the human brain lies a group of many million of neurons organized into a network quite different from that found anywhere else in the nervous system. The hippocampal formation is a brain area consisting of the dentate gyrus, hippocampus, subiculum, presubiculum, parasubiculum, and entorhinal cortex (Andersen et al., 2007).

The hippocampus has played a central role in brain investigations. There are several reasons for the interest in this specific brain area. It has a relatively simple organization of principal cell layers coupled with the highly-organized laminar distribution of its inputs. There is also increasing information concerning the involvement of the hippocampus in several physiological processes, such as, memory and in pathological processes, such as, epilepsy or AD. In addition, the hippocampus is a brain area extremely susceptible to aging (see e.g., Smith, 1996), which can contribute to several age related changes observed in older subjects. Moreover, the basic layout of cells and fiber pathways is much the same in all mammals (Figure 1.3.1), although the volume of the hippocampus is about 10 times larger in monkeys than in rats and 100 times larger in humans than in rats. Since the basic hippocampal architecture is common in mammalian species (Andersen et al., 2007), the study of hippocampal functions in rat models allows correlation to human hippocampal processes.

The anatomist Giulio Cesare Aranzi was the first to coin the name “hippocampus”, undoubtedly because of its similarity to the tropical fish Hippocampus leria (Figure 1.3.2) (Andersen et al., 2007).

After the advent of microscopy, the hipocampus was deemed even more impressive, with its characteristic, neatly regimented cellular arrangement. The entorhinal cortex can be considered the first step in the intrinsic hippocampal circuit.
because much of the neocortical input reaching the hippocampal formation does so through the entorhinal cortex. Cells in the superficial layers of entorhinal cortex give rise to axons that project, among other destinations, to the dentate gyrus and CA3 area. These projections from the entorhinal cortex to the dentate gyrus and CA3 form part of the major hippocampal input pathway called the perforant path. These pathways are not reciprocal since dentate gyrus and CA3 do not project back to entorhinal cortex. Neurons of entorhinal cortex also project to the CA1 field and to the subiculum via the perforant and alvear pathways. Both the CA1 and the subiculum project back to the deep layers of the entorhinal cortex (see Amaral and Lavenex, 2007 and Figure 1.3.3).

The granule cells, which are the principal cells of the dentate gyrus, give rise to axons called mossy fibers that connect with pyramidal cells of the CA3 field of the hippocampus. However, CA3 cells do not project back to the granule cells. The pyramidal cells of the CA3 field are in turn the source of major input to the CA1 hippocampal field (the Schaffer collateral axons). Again, CA1 neurons do not project back to the CA3 field. The CA1 field projects to the subiculum, providing its major excitatory input. Subiculum does not project back to CA1 field of the hippocampus (see Amaral and Lavenex, 2007 and Figure 1.3.3).
Figure 1.3.1- Hippocampus in rat and human. Although there are some differences in certain regions, the most striking feature is the general similarity of hippocampus structure across phylogeny. A- Brain and hippocampus of the rat (adapted from Squire and Kandel, 2000). B- Brain and hippocampus of humans (adapted from Amaral and Lavenex, 2007).

Figure 1.3.2 Human hippocampus dissected free (left) compared to a specimen of *Hippocampus loria* (right). (Adapted from Andersen et al., 2007.)
Figure 1.3.3 The hippocampal formation anatomy. The hippocampus forms a principally uni-directional network with input from the entorhinal cortex that forms connections with the dentate gyrus and CA3 pyramidal neurons via the perforant path. CA3 neurons also receive input from the DG via the mossy fibers. The dendrites of pyramidal cells in the CA1 regions form a thick band (stratum radiatum), where they receive synapses from Shaffer collaterals, the axons of pyramidal cells in the CA3 region (see Lopes da Silva and Arnold, 1978). CA1 neurons also receive input directly from the perforant path and project axons into the subiculum. These neurons, in turn, send the main hippocampal output back to the entorhinal cortex, forming a loop. Abbreviations: EC, entorhinal cortex; DG, dentate gyrus; Pre, presubiculum; Para, parasubiculum and Sub, subiculum. (Adapted from Amaral and Lavenex, 2007.)
As briefly reviewed in the Introduction, adenosine is a modulator of modulators; the activation of its specific receptors can modify the action of other molecules interfering with several biologic functions. In particular, adenosine A$_{2A}$ receptor activation induces phosphorylation of TrkB receptors (Lee and Chao, 2001), the specific receptor for the neurotrophin BDNF, which, is lacking in several neurodegenerative diseases (see introduction 1.1.3). However the clinical use of BDNF is difficult and it remains necessary to clarify if it can be administrated peripherally. Thus, it is crucial to identify small molecules that can potentiate BDNF actions in the brain. Adenosine, through A$_{2A}$ receptor activation, was considered as a good candidate. Therefore, the first aim of this work was to understand if activation of adenosine A$_{2A}$ receptors modulates TrkB receptor mediated actions in the hippocampus and to characterize the possible mechanisms that underlie this cross talk.

Aging is an inevitable life event and the hippocampus is a brain area extremely susceptible to aging (see e.g. Smith, 1996). Besides functional evidence for age-related dysfunctions in this area (Lynch, 1998; Barnes, 2003; Gooney et al., 2004), cellular and molecular mechanisms are also modified. A decrease in the expression of brain-derived neurotrophic factor (BDNF) has been implicated in neuronal death occurring with aging, as well as in some neurological disorders (Murer et al., 2001). It is also known that the expression of the high-affinity receptor for BDNF, TrkB, as well as TrkB mRNA expression, is decreased in aged rats (Silhol et al., 2005) and in old humans (Webster et al., 2006). The neuromodulatory action of adenosine and the expression of its membrane receptors also change with age (Sebastião et al., 2000, Lopes et al., 1999a; Rebola et al., 2003).
Considering that the therapeutic potential for BDNF-based strategies is greater in aged subjects, that the hippocampus is particularly vulnerable to aging, and that A$_{2A}$ receptor-mediated actions are more evident in older subjects, it was considered of interest to investigate how the BDNF actions in the hippocampus change with age, as well as whether these actions may depend on adenosine A$_{2A}$ receptors activation.

During normal aging, the brain and in particular the hippocampus suffers changes that might contribute to age-related memory deficits. On the other hand, the use of BDNF has been attempted in treating patients with AD, where memory is dramatically affected. Since, both adenosine and BDNF can modulate long-term potentiation (LTP), and LTP is considered the neuropsychological correlate of synaptic plasticity and memory it was considered important to evaluate the influence of A$_{2A}$/TrkB receptor cross talk in a model of synaptic plasticity during the aging process.

Finally, apoptosis is a type of cellular death present in the brain of Alzheimer's patients as a result of abnormal amyloid-beta deposition. It is currently accepted that BDNF, acting through TrkB, protects neurons from apoptosis induced by numerous agents. Moreover, adenosine can also modulate apoptosis in several tissues including the brain. Thus, it is of vital importance to understand if the activation of adenosine A$_{2A}$ receptors modulates the anti-apoptotic neuroprotective role of BDNF.
In summary, the work now reported aims to identify and to understand the functional consequences of the cross talk between adenosine A_2A and TrkB receptors, having the following specific objectives:

1- To understand whether activation of adenosine A_2A receptors can modulate BDNF action on synaptic transmission.

2- To evaluate age-related changes in synaptic transmission in the density of A_2A and TrkB receptors and in the cross talk between A_2A and TrkB receptors.

3- To evaluate the age-related changes that could result from changes in the cross talk between A_2A and TrkB receptors, using an in vitro synaptic plasticity model, LTP.

4- To evaluate the effect of the activation of A_2A receptors on the neuroprotective action of BDNF against apoptosis.
3. METHODS

3.1 BIOLOGIC SAMPLE PREPARATIONS

3.1.1 Hippocampal slices

3.1.1.1 Hippocampus

McIlwain and collaborators were pioneers in developing methods for ex vivo CNS preparations to perform biochemistry studies. In 1957, Li and McIlwain published the first electrophysiological study performed in cortex slices. In spite of these early reports, these biological preparations were believed to maintain their normal physiological properties only after the studies by Yamamoto and McIlwain (1966) and Richards and McIlwain (1967), showing that hippocampal slices, sectioned perpendicularly to the long axis of the hippocampus, maintained synaptic activity and that the evoked responses were similar to those recorded in vivo. Since then, slices of CNS are commonly used as experimental models in pharmacological, biochemical and neurophysiological studies. The hippocampus is used extensively in these techniques because of the unique laminated organization of neuronal pathways in this area (see e.g. Andersen and Colingridge, 1971 and previous Chapter 1.3). The arrangement of neurons in this brain structure allows it to be sectioned such that most of the relevant circuitry is left intact. In this preparation, the cell bodies of the pyramidal neurons lie in a single packed layer that is easily visualized.

Thus, the regular sequential arrangement of hippocampal neurons that facilitates electrophysiological studies, the prominence of adenosine and BDNF receptors in the hippocampus and the impact of aging and neurodegenerative
diseases on the hippocampus prompted us to choose this structure to perform the studies here reported.

3.1.1.2 Hippocampus isolation and slice preparation
Male Wistar rats were decapitated after halothane anesthesia. The skull was exposed by cutting the skin at the top of the head. The brain was removed as illustrated in Figure 3.1.1 A,B as already described by others (e.g. Palkovits et al., 1983), and placed into ice-cold Krebs’ solution (124 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 1 mM MgSO₄, 2 mM CaCl₂, and 10 mM glucose) previously gassed with 95% O₂ and 5% CO₂, pH 7.4. The cerebellum was cut off and discarded and the cerebrum was bisected along the midline, separating the two hemispheres. The hemispheres were placed with the medial surface facing up, as illustrated in Figure 3.1.1 C. The neocortex was peeled off toward the caudal surface, and the midbrain was pulled away ventrally by pulling in opposite directions at the location marked by the arrows, using small spatulas. At this point, the dentate surface of the hippocampus was revealed. Care was taken not to touch the hippocampus with the spatulas. The fornix was cut by pushing the point of the spatula into the brain at the point indicated by the dotted line. Next, one spatula was inserted gently under the fimbria and then further under the hippocampus (area of insertion indicated by the large arrows). The hippocampus was flipped out of the brain by lifting and pushing on the spatula, and then rotating the spatula tip around the long axis of the hippocampus. Once the hippocampus was isolated (Figure 3.1.1 D), it was trimmed at the line indicated by the two arrows, and the rest of the brain was pulled away from the hippocampus. Slices (400 μm thick) were cut perpendicularly to the long axis of the hippocampus (Figure 3.1.1 E,F) with a McIlwain tissue chopper and allowed to
recover for at least one hour in a resting chamber (Figure 3.1.2) in Krebs’ solution at room temperature.

Figure 3.1.1- Hippocampal slices preparation. (A) Scissor introduction into the foramen magnum to remove the occipital bone. (B) Brain extraction. (C) Separation of the hemispheres and hippocampus isolation. (D) Isolated hippocampus. (E) Orientation used to prepare hippocampal slices for electrophysiological recordings. (F) Hippocampal slices. (Adapted from Palkovits and Brownstein, 1983).
3.1.2 Hippocampal homogenates

After isolation, the hippocampus was disrupted with a Teflon pestle in 0.32 M sucrose-Tris pH 7.5 and supplemented with protease inhibitors (Complete; Roche Applied Science, Mannheim, Germany).

3.1.3 Neuronal cell cultures

Primary cultures of rat hippocampal or cortical neurons were prepared from 18- to 19-day-old fetuses of Wistar rats as previously described (Brewer et al., 1993). Pregnant rats were anesthetized with halothane and then decapitated. The fetuses were collected in Hanks’ balanced salt solution (HBSS-1; Invitrogen, Grand Island, NY, USA) and rapidly decapitated. After removal of meninges and white matter, the brain

Figure 3.1.2- Resting chamber for hippocampal slices. 1 is a support with a Teflon net to hold the slices; 2 is the inlet tub through which the gas mixture composed by 95% O₂ and 5% CO₂ is delivered and 3 represents the place where the support with a Teflon net (1) is located. (Adapted from Harvard apparatus catalog).
cortex or hippocampus were collected in HBSS without Ca\(^{2+}\) and Mg\(^{2+}\) (HBSS-2; Invitrogen, Grand Island, NY, USA). The cortex or hippocampus were then mechanically fragmented, transferred to HBSS-2 solution containing 0.025\% trypsin, and incubated for 15 minutes at 37\(^\circ\)C. Following trypsinization, the cells were washed twice in HBSS-2 with 10\% fetal bovine serum and resuspended in Neurobasal medium (Invitrogen, Grand Island, NY, USA), supplemented with 0.5 mM L-glutamine, 25 \(\mu\)M L-glutamic acid, and 2\% B-27 supplement (Invitrogen, Grand Island, NY, USA), and 12 mg/ml gentamicin. The cells were then plated on tissue culture plates (2 x 10\(^6\) cells/ml) precoated with poly-D-lysine and maintained at 37\(^\circ\)C in a humidified atmosphere of 5\% CO\(_2\). On the third day, the medium was removed and replaced by medium without B-27 supplement until the end of the experiment. All assays were performed on cells cultured for 5 days. Cells were characterized by immunofluorescence labelling, confirming that neuronal cultures contained < 5\% of glia.

3.1.3.1 Immunofluorescence characterization of cultured neuronal cells

The media was removed from the culture and cells were rinsed with phosphate buffered saline (PBS) and the cells were then fixed for 30 minutes in paraformaldehyde (4\% in PBS). Cells were subsequently rinsed with PBS and permeabilized with 0.2\% Triton X-100 in PBS for 15 minutes. After washing with PBS, blocking with 3\% BSA in PBS for 30 minutes was performed. Cells were then incubated overnight at 4\(^\circ\)C with primary antibodies: mouse monoclonal antibody to glial fibrillary acid protein (GFAP) (1:200; see table 3.3) and/or rabbit polyclonal antibody to neurofilament (1:200; see table 3.3), with subsequent treatment with secondary antibodies (anti rabbit IgG AlexaF488 and/or anti mouse IgG AlexaF568; see table 3.3). Fluorescence was visualized with a 40 x (0.45 NA) using an inverted
fluorescence microscope Axiovert 135 TV (Zeiss) as previously described by others (Rodrigues et al., 2002).

**3.1.4 Cytosolic protein isolation**

Cells were homogenized in buffer A (composed by: 10 mM Tris pH 7.6, 5 mM MgCl, 1.5 mM KAc) supplemented with protease inhibitors and 2 mM 1,4-dithiothreitol (DTT) as previously described with some modifications (Rodrigues et al., 2002). Then, cells were centrifuged at 500 g for 10 minutes at 4°C. Supernatant was recovered and the pellet was again resuspended in buffer A and centrifuged at 500 g for 10 minutes at 4°C. The supernatants were combined and centrifuged at 3160 g for 10 minutes at 4°C. Pellets were discarded and supernatants containing cytosolic proteins were rapidly frozen and stored at -20°C until used for experimentation.
### 3.2 Drugs and Antibodies

#### 3.2.1 Drugs

**Table 3.2-** Drugs used in the experimental work.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Designation</th>
<th>Function</th>
<th>Supplier</th>
<th>Stock solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADA</td>
<td>Adenosine deaminase (EC 3.5.4.4)</td>
<td>Adenosine deamination promoting enzyme</td>
<td>Roche Diagnostics Corporation (Germany)</td>
<td></td>
</tr>
<tr>
<td>AP5</td>
<td>DL-2-aminos-5-phosphonopentanoate</td>
<td>NMDA receptor antagonist</td>
<td>Tocris (Bristol, UK)</td>
<td></td>
</tr>
<tr>
<td>Aβ 25-35</td>
<td>Amyloid beta fragment 25-35</td>
<td>Apoptosis inducer</td>
<td>Bachem AG (Bubendorf, Switzerland)</td>
<td>100 mM in water</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
<td>Neurotrophin</td>
<td>Regeneron Pharmaceuticals (Tarrytown, NY)</td>
<td>Supplied in 1 mg/ml solution in 150 mM NaCl, 10 mM Na2PO4 buffer, 0.004% Tween 20</td>
</tr>
<tr>
<td>CGS 21680</td>
<td>4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofururonamido)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride</td>
<td>Adenosine A2A receptor agonist</td>
<td>Tocris (Bristol, UK)</td>
<td>5 mM stock solutions in DMSO*1</td>
</tr>
<tr>
<td>DbcAMP</td>
<td>5'-cAMP Tris salt, N6, 2'-o-dibutyryladenosine-3':5'-cAMP</td>
<td>cAMP analogue</td>
<td>Sigma (ST Louis, USA)</td>
<td>5 mM stock solutions in DMSO*1</td>
</tr>
<tr>
<td>DPCPX</td>
<td>1,3-Dipropyl-8-cyclopentylxanthine</td>
<td>Adenosine A1A receptor antagonist</td>
<td>Tocris (Bristol, UK)</td>
<td>5 mM stock solution in DMSO*1</td>
</tr>
<tr>
<td>H-89</td>
<td>N-[2-[p-bromocinnamylamino]ethyl]-5-isouquinolinesulfonamide hydrochloride</td>
<td>Protein kinase A inhibitor</td>
<td>Sigma (ST Louis, USA)</td>
<td>5 mM stock solutions in DMSO*1</td>
</tr>
<tr>
<td>ITU</td>
<td>5-iodotubercidin</td>
<td>Adenosine kinase inhibitor</td>
<td>Sigma (ST Louis, USA)</td>
<td>5mM stock solution in DMSO*1</td>
</tr>
<tr>
<td>[3H]ZM 241385</td>
<td>[H]-4-[2-[5-amino-2-[2-furyl]-[1,2,3]triazolo[2,3-a][1,3,5]triazinylamino]ethyl]phenol</td>
<td>Tritiated adenosine A2A receptor antagonist</td>
<td>American Radiolabeled Chemicals, Inc. (ST Louis, USA)</td>
<td>Supplied as a 36.5µM solution in ethanol</td>
</tr>
<tr>
<td>K-252a</td>
<td>8[R29,9S11S*]-[6,7,8,9-hydroxy-9-methyloxycarboxyl]8-methyl-2,3,8,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazolobenzo(a)cycloocta(cde)trindene n-1-one</td>
<td>Inhibitor of tyrosine protein kinase activity</td>
<td>Calbiochem (La Jolla, CA)</td>
<td>1 mM stock solution in DMSO*1</td>
</tr>
<tr>
<td>TrkB-Fc</td>
<td>Recombinant human TrkB/Fc chimera</td>
<td>BDNF scavenger</td>
<td>R&amp;D Systems (Minneapolis, USA)</td>
<td>50 µg/ml stock solution in PBS with BSA 0.1%*</td>
</tr>
<tr>
<td>SCH 58261</td>
<td>7-[2-(phenylethyl)-5-amino-2-(2-furyl)-pyrazolo[4,3-e]-1 ,2,4-triazolo[1,5-c]pyrimidine</td>
<td>Adenosine A2A receptor antagonist</td>
<td>Tocris (Bristol, UK)</td>
<td>5 mM stock solution in DMSO*1</td>
</tr>
<tr>
<td>ZM 241385</td>
<td>4-[2-(7-Amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yamino]ethyl]phenol</td>
<td>Adenosine A2A receptor antagonist</td>
<td>Tocris (Bristol, UK)</td>
<td>5 mM stock solutions in DMSO*1</td>
</tr>
</tbody>
</table>

* Aliquots of stock solutions were kept frozen at ~20°C until used. † The concentration of DMSO added to the slices (0.001% v/v) was well below the concentration that influences glutamatergic synaptic transmission (0.02% v/v, Tavyetynska et al., 2005).
### 3.2.2 Antibodies

**Table 3.3- Antibodies used in the experimental work.**

<table>
<thead>
<tr>
<th>Description</th>
<th>Supplier</th>
<th>Dilution</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse monoclonal antibody to Adenosine A&lt;sub&gt;2A&lt;/sub&gt; receptor</td>
<td>Upstate Biotechnology (Lake Placid, NY, USA)</td>
<td>1:500</td>
<td>Western Blotting</td>
</tr>
<tr>
<td>Mouse monoclonal antibody to TrKB receptor</td>
<td>BD Transduction Laboratories (Mississauga, ON Canada)</td>
<td>1:1000</td>
<td>Western Blotting</td>
</tr>
<tr>
<td>Rabbit polyclonal antibody to beta-actin</td>
<td>Abcam (Cambridge, UK)</td>
<td>1:10000</td>
<td>Western Blotting</td>
</tr>
<tr>
<td>Mouse monoclonal antibody to GFAP</td>
<td>Abcam (Cambridge, UK)</td>
<td>1:200</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>Rabbit polyclonal antibody to neurofilament 200 kD</td>
<td>Chemicon (Temecula, CA, USA)</td>
<td>1:200</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>Rabbit polyclonal antibody to caspase 3</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA, USA)</td>
<td>1:1000</td>
<td>Western Blotting</td>
</tr>
<tr>
<td><strong>Secondary antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-mouse HRP</td>
<td>Biorad laboratories (Hercules, CA, USA)</td>
<td>1:5000</td>
<td>Western Blotting</td>
</tr>
<tr>
<td>Anti-rabbit HRP</td>
<td>Biorad laboratories (Hercules, CA, USA)</td>
<td>1:5000</td>
<td>Western Blotting</td>
</tr>
<tr>
<td>Anti-rabbit IgG AlexaF488</td>
<td>Invitrogen (Grand Island, NY, USA)</td>
<td>1:400</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>Anti-mouse IgG AlexaF568</td>
<td>Invitrogen (Grand Island, NY, USA)</td>
<td>1:400</td>
<td>Immunofluorescence</td>
</tr>
</tbody>
</table>
3.3 TECHNIQUES

3.3.1 Microelectrophysiological recordings

After 1 hour of functional and energetic recovery, slices were transferred to a recording chamber for submerged slices and continuously superfused at 3 ml/min with bathing solution gassed with 5% CO$_2$ and 95% O$_2$ at 31-32°C (Figure 3.3.1). The drugs were added to this superfusion solution for experiments.

Recordings were obtained with an Axoclamp 2B amplifier and digitized (Axon Instruments, Foster City, CA). Individual responses were monitored, and averages of eight consecutive responses were continuously stored on a personal computer with the LTP program (Anderson and Collingridge, 2001).

![Figure 3.3.1- Setup for extra-cellular microelectrophysiology recordings. 1-reference electrode; 2-stimulation electrode; 3-recording electrode and 4-temperature sensor.](image-url)
Field excitatory post-synaptic potentials (fEPSPs) were recorded (Figure 3.3.2C) through an extracellular microelectrode (4 M NaCl, 2–6 MΩ resistance) placed in the stratum radiatum of the CA1 area (Figure 3.3.2A). Stimulation (rectangular 0.1 ms pulses, once every 15 seconds) was delivered through a concentric electrode placed on the Schaffer collateral-commissural fibers, in the stratum radiatum near the CA3–CA1 border except as otherwise indicated. The intensity of stimulus (80–200 µA) was initially adjusted to obtain a large fEPSP slope with a minimum population spike contamination.

Alteration on synaptic transmission was evaluated as the % change in the average slope of the fEPSP in relation to the average slope of the fEPSP measured during the 10 minutes that preceded the addition of drugs as previously described (Diógenes et al., 2004).

3.3.1.1 LTP induction and quantification
In LTP experiments, stimulation (rectangular 0.1 ms pulses, once every 10 seconds) was delivered alternatively to two independent pathways through two bipolar concentric electrodes placed on the Shaffer collateral/commissural fibers in the stratum radiatum (Figure 3.3.2B). LTP was induced by θ-Burst with two or three trains of 100 Hz, 3 stimuli, separated by 200 ms (Figure 3.3.2D).

The intensity of the stimulus was never changed during these induction protocols. LTP was quantified as the % change in the average slope of the fEPSP taken from 46 to 60 minutes after LTP induction in relation to the average slope of the fEPSP measured during the 14 minutes that preceded the induction of LTP. In each individual experiment the same LTP-inducing paradigm was delivered to each pathway. One hour after LTP induction in one of the pathways, BDNF was added to
the superfusion solution and LTP was induced in the second pathway, no less than 30 minutes after BDNF perfusion and only after stability of fEPSP slope values was observed for at least 10 minutes. The effect of BDNF upon LTP was evaluated by comparing the magnitude of LTP in the first pathway in the absence of BDNF (control pathway), with the magnitude of LTP in the second pathway in the presence of BDNF (test pathway); each pathway was used as control or test on alternate days. To test the modification of the effect of BDNF upon LTP, the modulatory drugs (agonist and antagonist of adenosine A2A receptors, or the inhibitor of Trk receptors) were added to the superfusing bath at least 30 minutes before induction of LTP in the first pathway and remained in the bath up to the end of the experiment. BDNF was added, as usual, 60 minutes after induction of LTP in the first pathway. Thus, modulatory drugs were present during both LTP-inducing periods, whereas BDNF was only present during the second induction of LTP. This protocol allows the comparison between the effect of BDNF upon LTP under different experimental conditions, keeping the magnitude of LTP under the same drug condition, without BDNF in the same slice as an internal control. When testing the effect of a drug upon LTP (rather than the modulation of the BDNF effect on LTP), the drug was added to the bath 30 minutes before induction of LTP in the second pathway and the magnitude of the resulting LTP was compared with that previously obtained (first pathway) in the absence of the drug as previously described (Fontinha et al., 2008).
Figure 3.3.2- Extracellular recordings in hippocampal slices. Representation of the stimulation of one pathway (A) or two pathways (B) in a hippocampal slice to record field excitatory postsynaptic potentials (fEPSP) (C). Traces obtained after stimulation are composed of the stimulus artifact (1), followed by the presynaptic volley (2) and the fEPSP (3). A schematic representation of the stimulation paradigms used in plasticity experiments is represented in D.

3.3.2 Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate the level of the receptor TrKB immunolabeling as previously described (Diógenes et al., 2007). Hippocampi were disrupted in sucrose-tris solution with a
Teflon pestle. Hippocampal proteins (20 µg) were separated on 8% SDS-polyacrylamide electrophoresis gels, and then transferred onto nitrocellulose membranes. Immunoblots were incubated with 15% H$_2$O$_2$ for 15 min at room temperature. After blocking with 5% milk solution, the blots were than incubated overnight at 4°C with primary mouse monoclonal antibodies recognizing TrkB receptors, adenosine A$_2A$ receptors, caspase-3 or β-actin; and, finally the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase for 3 hours at room temperature. The proteins of interest were detected using Super Signal™ substrate (Pierce, Rockford, IL, USA). β-actin was used as a loading control. Protein concentrations were quantified using the Bio-Rad protein assay according to Bradford (Bradford, 1976).

3.3.3 Binding assays

$[^3]$H$\text{ZM 241385}$ binding studies were performed as previously described (Palmer et al., 1995), with some modifications. The experiments were performed with hippocampal homogenates (90 µg protein per assay). After incubation with 2 U/mL adenosine deaminase (ADA) for 30 minutes at 37°C, $[^3]$H$\text{ZM 241385}$ binding was carried out in an incubation solution containing 50 mM Tris-HCl buffer and 10 mM MgCl$_2$ (pH 7.4) for 60 minutes at room temperature, in a final volume of 200 µL. Specific binding was calculated by subtraction of the nonspecific binding, defined in the presence of 2 µM XAC. The reaction was stopped by addition of cold incubation buffer and vacuum filtration through glass fiber filters (FilterMAT for receptor binding, Skatron Instruments, Lier, Norway) using a semi-automatic cell harvester from Skatron Instruments. The samples were transferred to scintillation vials and the radioactivity of each sample measured using a liquid scintillation analyser (Tri Carb...
Membrane protein content was quantified with the Bio-Rad protein assay according to Bradford (Bradford, 1976).

3.3.4 Apoptosis detection

To induce apoptosis, rat neurons cultured for 4 days, were incubated with amyloid-β peptide (Aβ) 25-35 (25 µM) one hour after the administration of all drugs. 24 hours after the induction of apoptosis by Aβ 25-35, cells were analyzed with the following assays: hoescht staining for morphologic analysis, caspase activity assay and caspase-3 cleavage detection as described previously by others (Rodrigues et al., 2002).

3.3.4.1 Morphologic Analysis

Analysis of nuclear morphology was performed as described previously (Rodrigues et al., 2002). Cell culture medium was gently removed from the cultured plates at the end of the incubation period. Cells were fixed with 4% formaldehyde in PBS (pH 7.4) for 10 minutes at room temperature, incubated with Hoechst dye 33258 (Sigma, St. Luis, USA) at 5 µg/mL in PBS for 5 minutes, washed with PBS, and mounted using an aqueous mounting medium (Sigma, St. Luis, USA). Fluorescence was visualized at 40x magnification (0.45NA) with an inverted fluorescence microscope Axiovert 135 TV (Zeiss). Fluorescent nuclei were independently scored by different observers and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed noncondensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. Five random
microscopic fields per sample were counted and mean values expressed as the percentage of apoptotic nuclei in the counted cell population.

3.3.4.2 Pro-caspase-3 cleavage
To determine caspase-3 processing, cytosolic proteins were separated by 15% SDS-PAGE. Blots were probed with primary rabbit polyclonal antibodies to caspase-3 at a dilution of 1:1000, and subsequently incubated with horseradish peroxidase–conjugated secondary anti-rabbit antibody. Finally, membranes were processed for caspase-3 cleavage detection (32 KDa and 20 KDa) (Rodrigues et al., 2002).

3.3.4.3 Caspase activity assay
Caspase activation was evaluated in cytosolic protein extract as described previously (Solá et al., 2003). Cells were harvested and homogenized in buffer A (previously described in 3.1.4). General caspase-3-like activity was evaluated by enzymatic cleavage of the chromophore p-nitroanilide (pNA) from the substrate N-acetyl-Asp-Glu-Val-Asp (DEVD)-pNA. The proteolytic reaction was preceded in isolation buffer containing 50 µg of cytosolic protein and 50 µM DEVD-pNA. The reaction mixtures were incubated at 37°C for 1 hour, and the liberation of pNA determined by measuring absorbance at 405 nm using a 96-well plate reader.
3.4 STATISTICS

The values presented are mean ± SEM of \( n \) experiments. To test the significance of the effect of a drug versus control, a paired Student’s \( t \)-test was used. When making comparisons from different set of experiments with control, a one-way variance analysis (ANOVA) was used, followed by a Dunnett’s test. One-way variance analysis (ANOVA) followed by a Bonferroni test was used when different sets of experiments were compared between them. Values of \( p < 0.05 \) were considered to be statistically significant.
RESULTS

4.1 INTERPLAY BETWEEN TrkB AND ADENOSINE A2A RECEPTORS IN INFANT RATS

4.1.1 Rationale
Adenosine and adenosine agonists can induce TrkB phosphorylation through a mechanism involving the adenosine A2A receptor (Lee and Chao, 2001). These data together with the finding that adenosine A2A receptors and neurotrophin receptors have a considerable overlap in their distribution (Lewin and Barde, 1996; Fredholm et al., 2001), prompted the author to evaluate how endogenous activation of adenosine A2A receptor could influence the action of BDNF on hippocampal synaptic transmission. Because the excitatory action of BDNF on synaptic transmission at the developing neuromuscular junction is facilitated by depolarisation (Boulanger and Poo, 1999a) and depolarising conditions are able to induce adenosine release (Pazzagli et al., 1993), it was also investigated how depolarising conditions influence BDNF actions and how this could be related to adenosine receptor activation. Since the actions of neurotrophins are expected to be more relevant at young ages, 3-week-old animals were used.

4.1.2 Pre-depolarisation induced by high K+ facilitates BDNF excitatory action on hippocampal synaptic transmission through TrkB receptors

As illustrated in Figure 4.1.1A, BDNF (20 ng/ml) when applied alone to the hippocampal slices did not significantly influence the slope of fEPSP (n=7). Even at a higher concentration (100 ng/ml), BDNF could not enhance synaptic transmission
(n=2) (Figure 4.1.1B). However, when applied to slices that had been shortly depolarised (Figure 4.1.2A) with a pulse of high-K\(^+\) (10 mM) for 2 minutes, 46 minutes before application of the neurotrophin, BDNF (20 ng/ml) caused a significant increase in fEPSP slope (41 ± 9.8\% increase; n=9; p<0.05). The depolarising pulse of high-K\(^+\) (10 mM) for 2 minutes, caused a transient enhancement of fEPSP slope, which returned to the basal level, within 35 minutes after returning to the normal K\(^+\) concentration in the bath; in slices where BDNF was not applied (n=3), the fEPSP slope remained within those basal levels for at least 60 minutes. To evaluate the type of receptor involved in the excitatory action of BDNF on synaptic transmission, we studied the effect of this neurotrophin in the presence of K252a, an inhibitor of Trk receptors phosphorylation (Berg et al., 1992). This inhibitor was added to the perfusion solution 30 minutes before the pulse of high-K\(^+\) and remained in the bath up to the end of the experiment. The slices were therefore perfused with K252a (200 nM) for 76 minutes before BDNF application. K252a (200 nM) was virtually devoid of effect on synaptic transmission; and, as shown in Figure 4.1.2B, it abolished the excitatory effect of BDNF (n=3; p<0.05).
Figure 4.1.1- The averaged time course of changes in fEPSP slope induced by application of BDNF alone 20 ng/ml (A) or 100 ng/ml (B). Values obtained in individual experiments at time 0 and 60 minutes are shown as a scatter representation in (A). The horizontal bars represent the application of BDNF. All values are mean ± SEM; 100% (averaged fEPSP slopes obtained during 10 minutes immediately before BDNF application): -0.64 ± 0.05 mV/ms, n=7 (A), -0.70±0.03 mV/ms, n=2 (B).
Figure 4.1.2- Pre-depolarisation induced by high K⁺ facilitates BDNF excitatory action on hippocampal synaptic transmission through TrkB receptors. (Aa) illustrates the averaged time course of changes in fEPSP slope under pre-depolarisation (n=9). Slices were superfused with BDNF (20 ng/ml, corresponding to approximately 0.8 nM BDNF) 46 minutes after treatment with high-K⁺ (10 mM) for 2 minutes. In (Ab) are shown traces obtained in a representative experiment in (a); each trace is the average of eight consecutive responses obtained immediately before (1) and during (2) BDNF application, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. In (B) is shown the averaged time course of the effect of BDNF (20 ng/ml) 46 minutes after treatment with high-K⁺ (10 mM) for 2 minutes in the presence of an inhibitor of TrkB receptors, K252a (200 nM), which was added to the slices 30 minutes before the pulse of high-K⁺ (n=3). Values obtained in individual experiments at time 0 and 60 minutes are shown as a scatter representation in (Aa). The arrows represent the beginning of the 2 minutes high-K⁺ application and the horizontal bars represent the application of the different drugs. All values are mean ± SEM; 100% (averaged fEPSP slopes obtained during 10 minutes immediately before BDNF application): -0.57 ± 0.03 mV/ms (A), and -0.50 ± 0.10 mV/ms (B). Note that BDNF increased fEPSP slope only in pre-depolarised slices, an action prevented by the treatment with K252a.
4.1.3 Theta Burst stimulation paired with BDNF can elicit synaptic potentiation

The K⁺ depolarising stimulus could still be effective if applied after BDNF administration. Indeed, when BDNF (20 ng/ml) was added to the slice at least 30 minutes before the K⁺ depolarising pulse (10 mM, 2 minutes) and remained in the bath up to the end of the experiment, an increase (81 ± 2.7%; n= 2) in fEPSP slope was observed (Figure 4.1.3), which is clearly different from what occurred when the BDNF (20 ng/ml) was applied without the K⁺ pulse, or when the K⁺ pulse was applied without BDNF (see above). Because the K⁺ pulse causes a broad depolarisation in all cells, including glial cells and interneurons, we evaluated whether a focal depolarisation delivered through the stimulation electrode placed at the synaptic afferents (Schaffer collaterals) was able to elicit the BDNF effect. Weak theta Burst stimulation (three Bursts of three pulses each at 100 Hz, delivered 100 ms apart) was applied to slices that had been previously perfused with BDNF (20 ng/ml) for at least 10 minutes, and fEPSPs were increased by 91 ± 9.5% at 60 minutes after stimulus (n=3, p<0.05 as compared with values before stimulation) (Figure. 4.1.3B); as expected (de Mendonça and Ribeiro, 2000), the same stimulation applied in the absence of BDNF caused a smaller and non-sustained increase in fEPSP slope (20 ± 16.1% increase 60 minutes after stimulation; n=3; p< 0.05).

4.1.4 Adenosine A₂A receptors activation facilitates BDNF excitatory action on synaptic transmission

To evaluate if adenosine A₂A receptors activation could influence the action of BDNF on synaptic transmission, the effect of a selective agonist of the adenosine A₂A receptor CGS 21680 (Jarvis et al., 1989) upon BDNF action was tested. CGS 21680 (10 nM) was added to the slices at least 30 minutes before BDNF application and was
virtually devoid of effect on fEPSP slope (Figure 4.1.4C). In the presence of CGS 21680 (10 nM), BDNF increased (Figure 4.1.4D) the slope of fEPSPs by 43 ± 6.7% (n=6; p<0.05) in slices that had not been predepolarised by the high-K⁺ pulse. Blockade of adenosine A₂A receptors with the selective antagonist (Poucher et al., 1995), ZM 241385 (50 nM), which was added 30 min before CGS 21680 (10 nM), prevented this excitatory effect of BDNF (n=3; p<0.05 as compared with absence of ZM 241385) (Figure 4.1.4D). Indeed, in the presence of ZM 241385 (50 nM), BDNF (20 ng/ml) even decreased (26 ± 6.5%), rather than increased, fEPSP slope (Figure 4.1.4B). When added alone, ZM 241385 (50 nM) was virtually devoid of effect on fEPSP slope (Figure 4.1.4A). To know whether the adenosine A₂A receptor could play a role in the excitatory action of BDNF observed after K⁺ depolarisation, the influence of adenosine A₂A receptor blockade upon the effect of BDNF was evaluated. In the presence of the adenosine A₂A receptor antagonist, ZM 241385 (50 nM), which was applied for at least 30 minutes before the K⁺ pulse, the excitatory action of BDNF (20 ng/ml) was completely prevented (n=3; p<0.05) (Figure 4.1.4B). Under these conditions BDNF (20 ng/ml) even decreased (17 ± 8.0%) fEPSP slope as it occurred in the presence of CGS 21680 (10 nM) plus ZM 241385 (50 nM).
Figure 4.1.3-Theta-Burst stimulation paired with BDNF can elicit potentiation effect. (A) Shows the averaged time course of changes in fEPSP slope induced by BDNF when the high-K⁺ pulse was applied in the presence of the neurotrophin. In (Ba) is illustrated the averaged time course of changes in fEPSP slope induced by BDNF (20 ng/ml) after a theta-Burst stimulus (3 Bursts at 5 Hz, each composed of three pulses at 100 Hz) (n=3) (●) and the changes of fEPSP slope in the same conditions of stimulation but without administration of the neurotrophin (n=3) (○). Slices were superfused with BDNF (20 ng/ml) at least 10 min before electrical stimulation. In (Bb) are shown traces obtained in representative experiments in (Ba); each trace is the average of eight consecutive responses obtained immediately before (1) and after (2) theta-Burst stimulation in the absence (n=3) (○) or in the presence (n=3) (●) of BDNF, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. The arrow represents the beginning of stimulation and the horizontal bars represent the application of the BDNF. All values are mean ± SEM; 100% (averaged fEPSP slopes obtained during 10 minutes immediately before stimulation): -0.61 ± 0.07 mV/ms, n=2 (A), -0.70± 0.02 mV/ms, n=3 (Ba,●), -0.66 ± 0.01 mV/ms, n=3 (Ba,○).
Figure 4.1.4- Adenosine A<sub>2A</sub> receptors activation facilitates BDNF excitatory action on synaptic transmission. (A) illustrates the averaged time course of changes in fEPSP slope induced by application of 50 nM ZM 241385 alone (n=3). (B) represents the averaged time course of the effect of BDNF after a depolarising stimulus of potassium in the presence of an A<sub>2A</sub> adenosine receptor antagonist (ZM 241385). Slices were superfused with BDNF (20ng/ml) 46 min after treatment with high-K<sup>+</sup> (10 mM) for 2 min and in the presence of ZM 241385 (50 nM) which was added for at least 30 min before the potassium pulse. In (C) averaged time course of changes in fEPSP slope induced by application of 10 nM CGS 21680 alone (n=3) is illustrated. In (D<sub>a</sub>), the averaged time course of the effect of BDNF (20 ng/ml) in the presence of the A<sub>2A</sub> receptor agonist, CGS 21680 (10 nM) (●) or in the presence of both CGS 21680 (10 nM) and the A<sub>2A</sub> receptor antagonist, ZM 241385 (50 nM) (○) are shown. CGS 21680 was applied at least 30 minutes before BDNF application (○ and ●) and ZM 241385 was applied 30 minutes before CGS 21680 application (○). In (D<sub>b</sub>) are shown traces obtained in a representative experiment in (D<sub>a</sub>, ●), each trace is the average of eight consecutive responses obtained immediately before (1) and during (2) BDNF application, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. The arrow represents the beginning of the 2 minutes high-K<sup>+</sup> application and the horizontal bars represent drug application. All values are mean ± SEM; 100% (averaged fEPSP slopes obtained during 10 min immediately before ZM 241385 application in (A), before BDNF application in (B and D<sub>a</sub>), or before CGS 21860 application in (C): -0.54 ± 0.090 mV/ms (A), -0.58 ± 0.06 mV/ms (B), -0.60 ± 0.09 mV/ms (C), -0.61 ± 0.01 mV/ms (D<sub>a</sub>, ●) and -0.53 ± 0.14 mV/ms (D<sub>b</sub>, ○). Note that activation of adenosine A<sub>2A</sub> receptors facilitates the excitatory action of BDNF on synaptic transmission, an action prevented by A<sub>2A</sub> receptor blockade.
The excitatory action of BDNF is facilitated by the selective adenosine kinase inhibitor 5-iodotubercidin

The findings that a brief depolarisation by a pulse of high-K⁺ (10 mM) and subsequent application of BDNF resulted in a significant increase in fEPSP slope, together with previous findings that treatment of slices with high-K⁺ Krebs' solution results in an increase of adenosine release (Pazzagli et al., 1993), prompted the next question: does an enhancement of the extracellular adenosine levels mimic the action of the pulse of high-K⁺? A selective adenosine kinase inhibitor, 5-iodotubercidin (ITU), which enhances the concentration of endogenous extracellular adenosine in hippocampal slices (Pak et al., 1994) was used. The application of ITU (100 nM) caused a decrease in synaptic transmission, which may be attributed to activation of adenosine A₁ receptors (the predominant adenosine receptors in the hippocampus) because it was prevented by the DPCPX (50 nM), a specific adenosine A₁ receptor antagonist (Lohse et al., 1987), (Figure 4.1.5A).

In the experiments where the action of BDNF in the presence of ITU was tested, the neurotrophin was applied when the full effect of ITU was achieved, and the slope values of the fEPSPs recorded under these conditions were taken as 100%. As shown in Figure 4.1.5B, perfusion of BDNF (20 ng/ml) under these conditions resulted in a significant increase in fEPSP slope (44 ± 8.7% increase; n =3; p<0.05); this did not occur when A₂A receptors were blocked with ZM 241385 (50 nM) before ITU application (n=3) (Figure 4.1.5B). When ZM 241385 (50 nM) was added only 50 minutes after ITU, BDNF (20 ng/ml) caused the expected enhancement (51%) of synaptic transmission (one experiment). This may suggest that A₂A receptors are required to trigger the action of BDNF, but they do not need to remain activated during the BDNF action to enhance synaptic transmission.
4.1.6 The activation of the cAMP–PKA transducing system is a critical step in the excitatory action of BDNF

Because ITU and the pulse of high K⁺ mimicked the action of the adenosine A₂A receptor agonist, CGS 21680, in respect to its ability to trigger BDNF actions, and because the activation of A₂A receptors stimulates the formation of cAMP–PKA transducing system (Fredholm et al., 2001), the next series of experiments were designed to evaluate if a selective PKA inhibitor H-89 (Chijiwa et al., 1990) could modify the action of BDNF. H-89 (1 µM) was added for at least 30 minutes before K⁺ pulse application (Figure 4.1.6A), CGS 21680 perfusion (Figure 4.1.6B), or ITU administration (Figure 4.1.6C) and remained in the bath up to the end of the experiments. In all cases, H-89 abolished (p<0.05) the excitatory action of BDNF on fEPSPs (n=3 for each experimental condition), but by itself H-89 was virtually devoid of effect on fEPSPs (Figure 4.1.6D). To further evaluate the involvement of cAMP-dependent PKA activity on the synaptic action of BDNF, experiments were designed to test if a membrane-permeable cAMP analog, dibutyryl cAMP (dbcAMP) (Henion et al., 1967), influences BDNF action. By itself dbcAMP (0.5 mM) was virtually devoid of effect on fEPSP slope (Figure 4.1.7A), and it was added to the slices at least 30 minutes before BDNF application. In the presence of dbcAMP (0.5 mM), BDNF caused a significant increase (49 ± 13.6%; n=4; p<0.05) in fEPSP slope (Figure 4.1.7B), an effect fully blocked by H-89 (1 µM; n=2; p<0.05) (Figure 4.1.7B). In slices predepolarised by a pulse of high-K⁺ (10 mM), dbcAMP caused only a small increase in the fEPSP slope (17 ± 1.4%; n=2; p<0.05 as compared with dbcAMP alone), suggesting that cAMP-mediated actions are slightly influenced by predepolarisation.
Figure 4.1.5- The excitatory action of BDNF is facilitated by a selective adenosine kinase inhibitor, 5-iodotubercidin. (A) Represents the averaged time courses of changes of fEPSP slope in the presence of 5-iodotubercidin (ITU, 100 nM) (○) and in the presence of both ITU (100 nM) plus DPCPX (50 nM) (●), an A₁ adenosine receptor agonist. Note that the application of DPCPX inhibited the decrease of synaptic transmission induced by ITU (n=3). (Bₐ, ●) shows the averaged time courses of changes in fEPSP slope induced by BDNF (20 ng/ml) in the presence of ITU (100 nM), (n=3) and (Bₐ, ○) in the presence of both ITU (100 nM) plus ZM 241385 (50 nM), an A₂A adenosine receptor antagonist. The horizontal bars represent drug application. In (Bₐ) are shown traces obtained in a representative experiment in (Bₐ, ●). Each trace is the average of eight consecutive responses obtained immediately before (1) and during (2) BDNF application, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. All values are mean ± SEM; 100% (averaged fEPSP slopes obtained during 10 minutes immediately before the ITU application in (A) or before BDNF application in (Bₐ)): -0.65 ± 0.06 mV/ms, n=3 (A, ●), -0.57 ± 0.03 mV/ms, n=3 (A, ○), -0.52 ± 0.02 mV/ms, n=3 (Bₐ, ○) and -0.50 ± 0.10 mV/ms, n=3 (Bₐ, ●).
Figure 4.1.6- The activation of the cAMP–PKA transducing system is a critical step for the excitatory action of BDNF. Averaged time course of changes in fEPSP slope induced by BDNF in the absence (●) and in the presence (○) of the PKA inhibitor H-89 (1 µM) are represented under the following experimental conditions: (A) after a pre-depolarising pulse of high-K⁺ (10 mM, 2 min); (B) in the presence of CGS 21680 (10 nM); (C) in the presence of 5-iodotubercidin (ITU; 100 nM). H-89 (1 µM) was added to the slices 30 minutes before the application of the K⁺ pulse (A), CGS 21680 (B), or ITU (C) and alone was virtually devoid of effect on fEPSP slope, as shown in D. In all panels the horizontal bars represent drug application, and the arrow in A represents the beginning of the 2 minutes high-K⁺ application. All values are mean ± SEM; 100% (averaged fEPSP slopes obtained at times -10–0): -0.57 ± 0.03 mV/ms, n=9 (A ●), -0.48 ± 0.23 mV/ms, n=3 (A ○), -0.61 ± 0.01 mV/ms, n=6 (B ●), -0.47 ± 0.06 mV/ms, n=3 (B ○), -0.50 ± 0.10 mV/ms, n=3 (C ●), -0.43 ± 0.17 mV/ms, n=3 (C ○), -0.59 ± 0.08 mV/ms, n=3 (D). Note that the PKA inhibition prevented the action of BDNF in all experimental conditions.
Figure 4.1.7- Presynaptic cAMP mimics the effect of high-K+, ITU, or CGS 21680. A shows the averaged time course of changes in fEPSP slope induced by application of dbcAMP (0.5 mM), and B_a shows the averaged time course of changes in fEPSP slope induced by BDNF (20 ng/ml) in the presence of dbcAMP (0.5 mM), either in the absence (○) and or in the presence (●) of the PKA inhibitor H-89 (1 µM). Slices were superfused with dbcAMP 30 minutes before BDNF application. B_b shows traces obtained in a representative experiment in B_a; each trace is the average of eight consecutive responses obtained immediately before (1) and during (2) BDNF application, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. In all panels the horizontal bar represents the application of the different drugs. All values are mean ± SEM; 100% in A and B (averaged IEPSP slopes obtained at times -10–0: -0.70 ± 0.2, n=2 (A), -0.56 ± 1.60 mV/ms, n=4 (B_a ●), and -0.64 ± 0.04 mV/ms, n=2 (B_a ○).
The present results show that the excitatory action of BDNF on hippocampal synaptic transmission is facilitated by presynaptic depolarisation and that this effect is dependent on adenosine $A_{2A}$ receptor activation. These actions were revealed by using a BDNF concentration that on its own did not significantly modify the slope of fEPSPs and was mediated by a receptor of the tyrosine kinase receptor family, because pretreatment with the Trk receptor inhibitor K252a (Berg et al., 1992), prevented the excitatory effect of this neurotrophin on fEPSPs. BDNF possesses a greater affinity for TrkB receptors than for TrkA or TrkC receptors (Lewin and Barde, 1996) and, therefore, the observed actions of BDNF are probably caused by activation of TrkB receptors, which are present in the hippocampus (Klein et al., 1990; Lewin and Barde, 1996).

The findings that after the brief K$^+$ depolarising pulse, BDNF could facilitate synaptic transmission are in accordance with the results of Boulanger and Poo (1999a) at developing neuromuscular synapses showing that presynaptic depolarisation is a critical factor in causing the synaptic action of neurotrophins. This could result from the following mechanisms: (1) the prepulse of K$^+$ triggers secretion of endogenous neurotrophin that could act synergistically with applied BDNF to potentiate synapses; (2) potassium treatment could induce the expression of more TrkB receptors, and (3) treatment with high-K$^+$ induces release of adenosine (Pazzagli et al., 1993), which could gate the excitatory action of BDNF on synaptic transmission. The results obtained with the adenosine $A_{2A}$ selective agonist, CGS 21680 and with ITU, a selective inhibitor of adenosine kinase that enhances the extracellular amount of adenosine (Pak et al., 1994), support this last hypothesis. Thus, in the presence of CGS 21680, BDNF increased the slope of fEPSP, even in slices that had not been presynaptically depolarised by the high-K$^+$ pulse. Moreover the adenosine
A2A receptor antagonist ZM 241385 prevented the excitatory effects of BDNF in conditions of predepolarisation, as well as in the presence of CGS 21680. Finally, ITU was also able to unmask the BDNF excitatory action, as did CGS 21680 and the brief K+ pulse, an action also prevented by the A2A antagonist ZM 241385.

There are two sources of extracellular adenosine: release of adenosine by facilitated diffusion, through membrane adenosine transporters that are equilibrative and bidirectional (Gu et al., 1995), and extracellular conversion of released adenine nucleotides into adenosine through a series of ectoenzymes, the last one in the cascade and the rate-limiting step for adenosine formation being ecto-5'-nucleotidase (Zimmermann and Braun, 1999). ATP is co-stored with most neurotransmitters, and therefore neuronal activity causes the release of ATP and extracellular formation of adenosine in most brain areas, including the hippocampus (Wieraszko et al., 1989; Cunha et al., 1996). Depolarisation by K+ predominantly induces the release of adenosine as such (see Latini and Pedata, 2001). The intracellular adenosine levels are kept low mainly because of the activity of adenosine kinase (see Arch and Newsholme, 1978) and when this enzyme is inhibited (e.g., by ITU) there is a marked increase in the release of adenosine (Pak et al., 1994). One may question why K+-induced adenosine release did not cause an A1-receptor mediated decrease in synaptic transmission, as ITU did. This most probably results from the time course of A1-receptor mediated actions (Sebastião et al., 1990; Lupica et al., 1992): any fast inhibitory actions, because of adenosine released during the 2 minutes application of K+, should be masked below the transient excitation induced by depolarisation. In contrast, activation of A2A receptors for a similar period of time might lead to long lasting enhancement in intracellular cAMP caused by adenylate cyclase activation (Fredholm et al., 2001), which could gate BDNF actions (Boulanger and Poo, 1999b). In line with this possibility are the observations that the PKA inhibitor, H-89, prevented the excitatory action of BDNF in the presence of K+ pulse, CGS 21680, or ITU.
Moreover in the presence of the cAMP analog dbcAMP, BDNF enhanced synaptic transmission, an effect blocked by H-89.

Figure 4.1.8- Mechanism of the facilitatory action of adenosine A₂A receptor activation on the BDNF hippocampal synaptic transmission effects on infant rats. The activation of adenosine A₂A receptors by the selective agonist, CGS 21680 or through the increase of endogenous adenosine by the addition of the inhibitor of adenosine kinase, ITU or by the pulse of K⁺, facilitates the excitatory action of BDNF on hippocampal synaptic transmission. This mechanism is dependent on the transducing system cAMP/ PKA since its inhibition by H-89 blocks the BDNF excitatory action. The addition of an analogue of cAMP, dbcAMP, also facilitates the BDNF excitatory action. K252a, the inhibitor of Trk phosphorylation totally prevents BDNF effect on synaptic transmission.

It is known that cAMP can induce a variety of cellular processes in different systems, including expression of mRNA for Trk receptors and neurotrophins in primary astroglial cultures (Condorelli et al., 1994) and recruitment of TrkB receptors to the plasma membrane of CNS neurons (Meyer-Franke et al., 1998). Cytosolic cAMP can be positively modulated by depolarisation and synaptic activity (Ferrendelli et al., 1980), and activation of cAMP signalling has been shown to potentiating
facilitatory actions of BDNF at the developing neuromuscular junction (Boulanger and Poo, 1999b). A cAMP—PKA—CREB-dependent pathway is also involved in the ability of adenosine A$_{2A}$ receptor agonists to potentiate nerve growth factor-induced neurite outgrowth in cultured PC12 cells (Chen et al., 2002). The results now described show that the interactions between neurotrophins and adenosine A$_{2A}$ receptors can occur in CNS neurons that are not in culture, therefore not at developing stage. The finding that blockade of adenosine A$_{2A}$ receptors prevents the action of BDNF after a K$^+$ predepolarising pulse, suggests that the predepolarisation pulse per se is not an essential requisite to unmask BDNF excitatory effects, eventually by a K$^+$-induced increase in cAMP levels (Ferrendelli et al., 1980). In contrast, activation of adenosine A$_{2A}$ receptors either by released adenosine or by a selective agonist (CGS 21680) appears to be an essential step to trigger BDNF excitatory action on synaptic transmission.

Clear excitatory actions of BDNF on hippocampal synaptic transmission have been observed by some authors (Kang and Schuman, 1995, 1996), whereas others reported minimal or no effects of this neurotrophin in the same brain area (Figurov et al., 1996; Gottschalk et al., 1998). Whether this discrepancy results from different levels of endogenous activation of adenosine A$_{2A}$ receptors, awaits further investigation.

Neurotrophins have been suggested to have an important role in protecting mature neurons from neuronal atrophy in the degenerating human brain. A decrease in BDNF levels might be involved in neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease (Connor and Draganow, 1998), or diabetic neuropathies (Nitta et al., 2002), making the use of the naturally occurring neurotrophic factors very promising for treatment of these disorders. However, until now the pharmacological administration of in vivo BDNF has not been easy. One of the reasons is that these molecules are unable to cross the blood–brain barrier,
making invasive application strategies like intracerebroventricular infusion necessary. Intravenous administration of BDNF has been attempted, but it involves a complex molecular reformulation of the neurotrophin (Wu and Pardridge, 1999). The results described in this chapter open a new prospective to potentiate BDNF actions on CNS neurons, i.e., by co-activation of a specific type of adenosine receptors, the $A_{2A}$ receptors. This possibility expands the pathophysiological implications of adenosine receptor functioning in the brain (Ribeiro et al., 2003) and points toward new strategies to interfere with neurotrophic factors in the therapeutics of some neurodegenerative disease.

In summary, the results described in this chapter clearly indicate a way to enhance the excitatory action of BDNF on synaptic transmission via activation of adenosine $A_{2A}$ receptors, which can be achieved either by enhancing adenosine release or by pharmacological manipulation of the $A_{2A}$ receptors.
4.2 INFLUENCE OF AGE ON BDNF MODULATION OF HIPPOCAMPAL SYNAPTIC TRANSMISSION: INTERPLAY WITH ADENOSINE A2A RECEPTORS

4.2.1 Rationale

As reported in chapter 4.1 (see also Diógenes et al., 2004), adenosine, through A2A receptor activation, potentiates synaptic actions of brain-derived neurotrophic factor (BDNF) in the hippocampus of infant (3-4 weeks) rats. Since A2A receptor mediated actions are more relevant in older than in young rats and since the therapeutic potential for BDNF-based strategies is greater in old subjects, the next step was focused upon 1) the synaptic actions of BDNF, 2) the levels of TrkB receptors, and 3) the levels of adenosine A2A receptors, in the hippocampus of three groups of adult rats: young adult rats (10-16 weeks), an age where reproductive behavior is fully established (Havenaar et al., 1993), old adult rats (36-38 weeks), the time where rats get the third part of life expectancy (Havenaar et al., 1993), and aged rats (70-80 weeks old); a group of infant (3-4 weeks) rats was also used again for comparison.

4.2.2 BDNF facilitates synaptic transmission in young adult rats in a “LTP-like” process

As previously observed (see chapter 4.1), BDNF (20 ng/ml) did not significantly influence the slope of fEPSP when applied alone to hippocampal slices from infant rats (3-4 weeks old) (n=7, Figure 4.2.1A and 4.2.2). However, in hippocampal slices from young adult (10-16 week-old) rats, BDNF (20 ng/ml) significantly increased fEPSP slope by 32 ± 5.3% (n=8, P<0.05, Figure 4.2.1B and 4.2.2). This excitatory action of BDNF (20 ng/ml) was lost (n=9) in old adults (36-38 weeks, Figure 4.2.1C
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and 4.2.2), though a higher BDNF concentration (100 ng/ml, Figure 4.2.3) increased synaptic transmission by 18 ± 2.5%, n=5. Finally, in the aged group of rats (70-80 weeks), hippocampal synaptic transmission was enhanced by approximately 18 ± 4.4% (n=5; P<0.05, Figure 4.2.1D and 4.2.2) by BDNF (20 ng/ml).

The excitatory action of BDNF (20 ng/ml) on synaptic transmission of young adult rats (10-16 week-old) can be attributed to Trk receptor activation since it was prevented (n=3; p<0.05, Figure 4.2.1B) by K252a (200 nM), an inhibitor of Trk receptors phosphorylation (Berg et al., 1992). K252a (200 nM) was added to the perfusion solution 30 minutes before BDNF administration and K252a alone was devoid of effect on synaptic transmission (n=3).

Neurotrophins, and in particular BDNF, modulate long-term potentiation (LTP) in the CA1 area of the hippocampus by presynaptically enhancing synaptic responses to tetanic stimulation (Figurov et al., 1996; Gottschalk et al., 1998). N-methyl-D-aspartate (NMDA) glutamate receptors play a key role in LTP induction but are not required for LTP maintenance (Bliss and Collingridge, 1993). To evaluate if the increase of synaptic transmission induced by BDNF in young adult rats was dependent on NMDA receptor activation we studied the effect of BDNF in the presence of AP5, an antagonist of NMDA receptors, at a concentration (50 µM) known to prevent NMDA responses in the hippocampus (e.g. Sebastião et al., 2001). As shown on Figure 4.2.4A, when AP5 (50 µM) was applied to hippocampal slices 30 minutes before BDNF (20 ng/ml), the neurotrophin failed to increase fEPSP slope (n=4, p<0.05 as compared with absence of AP5). When AP5 (50 µM) was added 60 minutes after BDNF (20 ng/ml) perfusion, therefore, after the full effect of BDNF on synaptic transmission, it did not revert the action of the neurotrophin (n=3, Figure 4.2.4B). Therefore, as it occurs with LTP (Collingridge et al., 1983), NMDA receptors are required for the induction of the excitatory action of BDNF on synaptic
transmission but they do not need to remain activated during BDNF action. The AP5 (50 µM) by itself, did not significantly change synaptic transmission (n=4).

Figure 4.2.1- The action of BDNF on excitatory synaptic transmission varies in rats from different age groups. In A-D are shown the averaged time courses of changes in fEPSP slope induced by application of 20 ng/ml (corresponding to ≈ 0.8 nM) BDNF to hippocampal slices taken from 3-4 week-old rats (A), 10-16 week-old rats (B), 36-38 week-old rats (C) and 70-80 week-old rats (D) in the absence (●) or in the presence (○) of the inhibitor of Trk phosphorylation, K252a (200 nM), which was applied at least 30 minutes before BDNF superfusion. The horizontal bars represent the application of the BDNF. Averaged fEPSPs (Bs and Ds) obtained in a representative experiment in B and D are also shown below the respective panel; each trace is the average of eight consecutive responses obtained immediately before (1) and during (2) BDNF application, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. In all panels the values are mean ± SEM; 100% represents the averaged fEPSP slopes recorded for 10 minutes immediately before BDNF, and were: -0.64 ± 0.05 mV/ms, n=7 (A), -0.73 ± 0.06 mV/ms, n=8 (B●), -0.62 ± 0.04 mV/ms, n=3 (B○), -0.62 ± 0.04 mV/ms, n=9 (C) and -0.52 ± 0.05 mV/ms, n=5 (D).

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Figure 4.2.2- Comparison between the averaged effects of BDNF in rats from different age groups. The ordinates shows the % change of fEPSP slope induced by BDNF (20 ng/ml) 50-60 minutes after its application to hippocampal slices taken from infant (3-4 weeks), young adult (10-16 weeks), old adult (36-38 weeks) and aged (70-80 weeks) rats, as indicated below each bar *p<0.05, **p<0.001 versus infants (one way ANOVA with the Dunnett's correction).
Figure 4.2.3- A high concentration (100 ng/ml) of BDNF increases synaptic transmission in slices taken from old adult rats. In A the averaged time courses of changes in fEPSP slope induced by application of 100 ng/ml BDNF to hippocampal slices taken from 36-38 week-old rats are shown. Averaged fEPSPs obtained in a representative experiment in A are shown in B; each trace is the average of eight consecutive responses obtained immediately before (1) and during (2) BDNF application, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. The values in A are mean ± SEM; 100% represents the averaged fEPSP slopes recorded for 10 minutes immediately before BDNF, and were: -0.52 ± 0.05 mV/ms, n=5 (A).
**Figure 4.2.4-** BDNF facilitates synaptic transmission in young adult rats in a “LTP-like” process. A and B show the averaged time course of changes in fEPSP slope induced by BDNF (20 ng/ml) in hippocampal slices taken from young adult (10-16 week-old) rats; in the experiments shown in (A), the antagonist of NMDA receptors, AP5 (50 µM), was applied at least 30 minutes before BDNF application; whereas in the experiments shown in (B), AP5 was applied 60 minutes after BDNF, as indicated by the horizontal bars. All values are mean ± SEM; 100% (averaged fEPSP slopes recorded for 10 minutes immediately before BDNF: -0.55 ± 0.01 mV/ms, n=4 (A) and -0.52 ± 0.03 mV/ms, n=3 (B). Note that AP5 inhibited the induction but not the maintenance of the effect of BDNF.
4.2.3 Adenosine $A_{2A}$ receptors are involved in the excitatory action of BDNF in the hippocampus of young adult rats and aged rats.

As shown previously (Chapter 4.1), to trigger an excitatory action of BDNF in infant rats (3-4 weeks) it is necessary to co-activate adenosine $A_{2A}$ receptors. It was therefore evaluated if the excitatory action of BDNF observed in young rats could also be under the influence of adenosine $A_{2A}$ receptors. As illustrated in Figure 4.2.5A and Figure 4.2.6, the $A_{2A}$ receptor selective antagonist, ZM 241385 (50 nM, added 30 minutes before BDNF) prevented the excitatory effect of BDNF (20 ng/ml) in hippocampal slices taken from young adult (10-16 weeks old) rats ($n=5$; $p<0.05$ as compared with the absence of ZM 241385), suggesting that in this group of animals the action of BDNF upon hippocampal synaptic transmission requires active adenosine $A_{2A}$ receptors.

In the oldest group of rats (70-80 weeks) the small increase of the fEPSP induced by application of the neurotrophin alone was also prevented by ZM 241385 (50 nM, $n=3$, Figure 4.2.5B and Figure 4.2.6, $p<0.05$ as compared with absence of ZM 241385), which was applied 30 minutes before BDNF.

Considering that a pre-depolarisation pulse, by inducing adenosine release and $A_{2A}$ receptor activation, can trigger a facilitatory action on synaptic transmission in infant (3-4 weeks old) rats (Chapter 4.1), and that pre-depolarisation also triggers BDNF actions in other preparations (Boulanger and Poo, 1999a; Pousinha et al., 2006), it was now evaluated whether a similar facilitation of BDNF actions could occur in the old adult (36-38 weeks) rats. As before, the slices were shortly depolarised by a pulse of high-$K^+$ (10 mM) for 2 minutes and BDNF was applied 46 minutes after the pulse. As shown in Figure 4.2.7A, the depolarising pulse of high-$K^+$ caused a transient enhancement of fEPSP slope, which returned to the basal level, within 35 minutes after returning to the normal $K^+$ concentration in the bath. When BDNF was applied to
the pre-depolarised slices, it enhanced fEPSP slope by 18 ± 2.4% (n=4) (Figure 4.2.7A). This facilitatory action of BDNF upon synaptic transmission in slices from old adult (36-38 weeks) rats was dependent on A_{2A} receptor co-activation since it was prevented by the adenosine A_{2A} receptor selective antagonist, ZM 241385 (50 nM, Figure 4.2.7B) (n= 3; p< 0.05).

In all age groups, ZM 241385 (50 nM), on its own, was virtually devoid of effect on the EPSPs (n=3-5).
Figure 4.2.5- Involvement of adenosine A2A receptors in the excitatory action of BDNF in synaptic transmission. In (A,B) are shown the averaged time course of changes in synaptic transmission induced by application of BDNF (20 ng/ml) to hippocampal slices taken from young adult (10-16 weeks) and aged rats (70-80 weeks) in the absence (•), or the presence of the selective A2A receptor antagonist, ZM 241385 (50 nM) (○), which was applied at least 30 minutes before BDNF. The horizontal bars represent time of BDNF application; 100% (averaged IEPSP slopes recorded for 10 minutes immediately before BDNF): \(-0.73 \pm 0.06\) mV/ms, \(n=8\) (A,•), \(-0.51 \pm 0.04\) mV/ms, \(n=5\) (A,○), \(-0.52 \pm 0.05\) mV/ms, \(n=5\) (B,•), \(-0.62 \pm 0.08\) mV/ms, \(n=3\) (B,○). The data in (A,•) is the same as shown in Figure 4.2.1B(•) and the data in (B,•) is the same as in Figure 4.2.1D(•).
Figure 4.2.6 - Involvement of adenosine A<sub>2A</sub> receptors in the excitatory action of BDNF in synaptic transmission. The percentage of change of fEPSP slope (ordinates) induced by BDNF (20 ng/ml) 50-60 minutes after its application to the young adult and aged rats in the absence (filled bars) and presence (open bars) of ZM 241385 (50 nM) is shown. *p<0.05 versus the same age group in the absence of the A<sub>2A</sub> receptor antagonist.
Figure 4.2.7- Pre-depolarisation induced by high K⁺ facilitates BDNF excitatory action on hippocampal synaptic transmission in slices taken from old adult (36-38 weeks old) rats. In A are shown the averaged time courses of changes in fEPSP slope induced by application of BDNF (20 ng/ml), which was perfused 46 minutes after treatment with high-K⁺ (10 mM) for 2 minutes. Averaged iEPSPs (Aₐ) obtained in a representative experiment in A are shown; each trace is the average of eight consecutive responses obtained immediately before (1) and during (2) BDNF application, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. B shows the averaged time course of the effect of BDNF (20 ng/ml) applied 46 minutes after treatment with high-K⁺(10 mM) for 2 minutes in the presence of the selective antagonist of adenosine A₂A receptor, ZM 241385 (50 nM), which was added to the slices 30 minutes before the pulse of high-K⁺. In all panels, the arrows represent the beginning of the 2 minutes high-K⁺ application, and the horizontal bars represent the application of the different drugs. All values are mean ± SEM; 100% (averaged iEPSP slopes recorded for 10 minutes immediately before BDNF): -0.57 ± 0.04 mV/ms, n=4 (A), -0.55 ± 0.02 mV/ms, n=3 (B).
4.2.4 The density of TrkB full length receptors and adenosine A\textsubscript{2A} receptors is altered in older rats

To evaluate if the changes in the BDNF excitatory effect on hippocampal slices taken from animals with different ages could be related to changes in the number of TrkB full-length receptors, the levels of this receptor in hippocampal homogenates taken from the same animals used in electrophysiology were assessed by western blot. As illustrated in Figure 4.2.8A and B, there was a small, though non-significant, increase in TrkB receptor density in the hippocampus of young adult (10-16 weeks old) rats as compared with TrkB receptor density in infant (3-4 weeks old, which was taken as 100% reference level) rats. TrkB receptor levels in the hippocampus of old adult (36-38 weeks old) rats were significantly \( (p<0.05) \) reduced, and the decrease was even more pronounced in the hippocampus from aged (70-80 weeks old) rats.

Since the excitatory action of BDNF on synaptic transmission is dependent on adenosine A\textsubscript{2A} receptors activation, the density of A\textsubscript{2A} adenosine receptors in the same animals was also evaluated. The existence of commercially available tritiated ligands for A\textsubscript{2A} receptors allowed receptor binding assays, which are frequently preferred for quantitative analysis of receptors. The A\textsubscript{2A} receptor antagonist \[^{3}H\]ZM 241385 was used as ligand for A\textsubscript{2A} receptors (Palmer \textit{et al.}, 1995). Figure 4.2.9 shows the saturation isotherms for the binding of \[^{3}H\]ZM 241385 to hippocampal homogenates taken from the same hippocampi as those used in Western blot analysis of TrkB receptors. It is evident that the specific \[^{3}H\]ZM41385 binding is higher in aged (70–80 weeks old) than in young adult (10–16 weeks old) rats, the difference being more pronounced for higher ligand concentrations. The Bmax values obtained by nonlinear regression analysis were 28 ± 7.7 fmol mg/protein \((n = 5)\) for the 10–16-week-old rats, and 67 ± 16 fmol mg/protein \((n = 4)\) for the 70–80-week-old rats \((p<0.05)\). No significant differences in the Kd values were found (young adult: 1.7
± 1.1 nM, n = 5; aged: 2.4 ± 1.2 nM, n = 4, p>0.05) and a ZM 241385 concentration near the Kd (2 nM) was therefore used in subsequent experiments. Binding of 2 nM [3H]ZM 241385 was significantly higher in aged (70–80 weeks old, p<0.01) and old adult (36–38 weeks, p<0.05) rats than in infant (3–4 weeks old) rats (Figure 4.2.9B).

Figure 4.2.8- Changes in the density of TrkB full length receptors in different age groups. In (A) are shown the Western blots of TrkB full length (FL) (145 KDa) receptors and for control purposes, β-actin protein (43 KDa), in homogenates of rat hippocampus taken from infant (3 weeks), young adult (10-16 weeks), old adult (36-38 weeks) and aged (70-80 weeks) rats, as indicated above each lane. In (B) are shown the averaged of TrkB FL receptor density (n=5-6). All values are mean ± SEM. 100% was taken as the density of TrkB (B) receptors in infant rats. *p<0.05 and **p<0.01 versus infants (one way ANOVA with Bonferroni correction). The hippocampus homogenates were taken from the same animals that were used in electrophysiology experiments.
Figure 4.2.9- Specific binding to adenosine A\(_{2A}\) receptor receptors in the hippocampus of rats from different age groups. In (A) are shown saturation isotherms for the binding of the selective A\(_{2A}\) receptor antagonist [\(^{3}\)H]ZM 241385 to the hippocampal homogenates of young adult (○, n=5) and aged (●, n=4) rats. In (B) is shown the comparison between the specific binding of 2 nM [\(^{3}\)H]ZM 241385 to the hippocampal homogenates of infant (3-4 weeks), young adult (10-16 weeks), old adults (36-38 weeks) and aged (70-80 weeks) rats (n=3-5). In both panels the ordinates represent the specific binding of [\(^{3}\)H]ZM 241385 obtained upon subtraction of the non-specific binding (determined in the presence of 2 µM XAC) from total binding. The specific binding of 2 nM [\(^{3}\)H]ZM 241385 corresponded to 18 ± 1.9% of total binding for all age groups; no appreciable differences in the proportion of specific binding over total binding occurred among the different groups. All values are mean ± SEM. *p<0.05 versus infant rats; **p<0.01 versus infant rats; §p<0.01 versus young adults (one way ANOVA with Bonferroni correction). The hippocampus homogenates were taken from the same animals used in electrophysiology experiments, and from the same hippocampi as those used in western blot analysis.
4.2.5 Discussion

The present results show that the effect of BDNF on rat hippocampal synaptic transmission changes according to the age of the animals and that this could be related to changes induced by age in the density of TrkB and of adenosine A<sub>2A</sub> receptors.

BDNF when applied alone to hippocampal slices from infant rats (3-4 weeks) does not affect synaptic transmission, but it is able to do so when adenosine A<sub>2A</sub> receptors are exogenously activated or when extracellular adenosine levels are increased (see Chapter 4.1). This ability of A<sub>2A</sub> receptors to trigger a BDNF action in infant rats requires cAMP formation and protein kinase A (PKA) activity (Chapter 4.1). Moreover, in A<sub>2A</sub> receptor knockout mice BDNF is unable to facilitate hippocampal synaptic transmission, reinforcing the idea that BDNF-induced modulation of synaptic transmission requires functional adenosine A<sub>2A</sub> receptors (Tebano et al., 2008). These new results show that when BDNF is applied to hippocampal slices taken from young adult rats (10-16 weeks), it induces a significant increase in fEPSPs. This effect is mediated by a tyrosine kinase receptor, since it was prevented by K252a, and it also requires co-activation of adenosine A<sub>2A</sub> receptors, since it was abolished in the presence of the selective A<sub>2A</sub> receptor antagonist, ZM 241385. Therefore, in spite of not being necessary to exogenously activate A<sub>2A</sub> receptors to trigger a BDNF action in young adult rats, A<sub>2A</sub> receptors are still required for the effect of BDNF on synaptic transmission.

BDNF enhances glutamate release (Canas et al., 2004) and increases activation (Levine et al., 1998) and phosphorylation of glutamate NMDA receptors (Suen et al., 1997) in the hippocampus. By operating pre- and postsynaptic mechanisms, BDNF also facilitates LTP induction (Figurov et al., 1996; Gottschalk et al., 1998; Kang and Schuman, 1995; Xu et al., 2000). In the present work it was
observed that induction (but not the maintenance) of the excitatory action of BDNF upon synaptic transmission is abolished by the NMDA receptor antagonist, AP5. It thus appears that BDNF can by itself induce a long-lasting enhancement of synaptic transmission that does not require high-frequency stimulation but involves, as LTP does, NMDA receptor activation. This BDNF-induced “LTP-like” phenomenon is clearly observed in young adult rats (10-16 weeks old) but disappears in old adult rats. At this age (36-38 weeks old) animals have clearly lower density of TrkB receptors and this may account for the loss of effect of BDNF on synaptic transmission. Interestingly, after a pre-depolarising potassium pulse, a condition known to increase extracellular adenosine levels (Pazzagli et al., 1993), the neurotrophin increased synaptic transmission in an adenosine A2A receptor-dependent manner, suggesting that in this age group an increase in the A2A receptor-mediated adenosinergic tonus is able to trigger a BDNF action upon hippocampal synaptic transmission.

A decrease in the amount of TrkB receptor with aging fits to previously reported data (Silhol et al., 2005). The decrease was evident in old adult animals but was much more pronounced in aged animals. Surprisingly, BDNF could enhance synaptic transmission in aged (70-80 weeks old) animals. In this age group we could observe: 1) a marked increase in Bmax value for A2A receptor binding, indicating higher density in A2A receptors and 2) that the effect of BDNF on synaptic transmission requires A2A receptors since it was prevented in the presence of the selective antagonist of these receptors, ZM 241385. Taken together, these data indicate that the ability of BDNF to enhance synaptic transmission in aged rats is probably due to an increase in A2A receptor levels that partially compensate for the marked loss of TrkB receptors. It is possible that critical levels of A2A and TrkB receptors need to be present together to allow the facilitatory effect of BDNF on synaptic transmission. However, concomitant changes in the transducing pathways
operated by A2A (Rebola et al., 2003) or TrkB receptors (Gooney et al., 2004), or in the levels of endogenous extracellular adenosine (Cunha et al., 2001) or BDNF (Narisawa-Saito and Nawa, 1996; Katoh-Semba et al., 1997; Croll et al., 1998) may also account for a change in BDNF effects throughout age. Indeed, the changes in TrkB and A2A receptors density in the hippocampus from infant (3-4 weeks old) to young adult (10-16 weeks old) animals are very mild and probably do not account entirely for the quantitative changes of the BDNF effect in the two age groups. Unfortunately, the complexity of the transducing pathways operated by TrkB receptors (Huang and Reihardt, 2003), or even A2A receptors (e.g. Sebastião and Ribeiro, 2000) makes it difficult to directly address this issue.

The tonic action of adenosine results from a balance between inhibitory A1 and facilitatory A2A receptor-mediated actions. It is known that in the limbic cortex there is a lower density of A1 receptors and a higher density of A2A receptors (Cunha et al., 1995). These changes result in a decreased ability of A1 receptor agonists to inhibit (Sebastião et al., 2000) and an enhanced efficiency of A2A receptors to facilitate (Rebola et al., 2003) hippocampal synaptic transmission. Furthermore, the A1/A2A cross talk also changes with age (Lopes et al., 1999b). How this A1/A2A adenosine receptors interplay contributes to the observed age related changes in the facilitatory effect of BDNF on synaptic transmission deserves further investigation.

After the pioneer publication by Kang and Schuman (1995) showing that BDNF enhances synaptic transmission in the rat hippocampus, several papers reported the absence of an effect of the neurotrophin on evoked EPSPs induced by low frequency stimulation (e.g. Figurov et al., 1996; Gottschalk et al., 1998; Tanaka et al., 1997). Methodological differences (e.g. flow rate, temperature, type of chamber) may not be the sole reason for this discrepancy. Thus, Kang and Schumman (1995) used young adult (≅ 50 days old) rats whereas the other studies used hippocampus from developing (12-18 postnatal days old) rats. From the data now presented, where
the effect of BDNF was compared in the same experimental conditions in animals from different ages, it is clear that at least two physiological variables that are not fully independent may contribute to the variability of the effect of BDNF on fast excitatory transmission in the hippocampus: age and adenosine through $A_{2A}$ receptor activation.

In summary, the present results show, for the first time, a relationship between age-related changes in the density of TrkB receptors and adenosine $A_{2A}$ receptors BDNF-induced enhancement of synaptic transmission in the hippocampus. This interplay should be taken into consideration whenever evaluating BDNF actions in nerve cells, and may prove relevant in the design of BDNF-based therapeutic strategies.
4.3 INFLUENCE OF AGE ON THE BDNF MODULATION OF LONG TERM POTENTIATION: INTERPLAY WITH ADENOSINE A2A RECEPTORS

4.3.1 Rationale

During normal aging and in some pathologies such as Alzheimer’s disease, the whole brain, and in particular the hippocampus, suffers changes that might contribute to memory deficits. It is commonly accepted that the neurophysiological basis for learning and memory involve modifications in the efficiency of synapses. Using specific patterns of stimulation, it is possible to experimentally induce long-term modifications in synaptic strength, for instance long-term potentiation (LTP) (Bliss and Collingridge, 1993). Neurotrophins have a key role in LTP, in particular through activation of TrkB receptors (Poo, 2001).

Besides functional evidence of dysfunctions in the hippocampus (Lynch, 1998; Barnes, 2003; Gooney et al., 2004), cellular and molecular mechanisms are also modified during aging. A decrease in the expression of BDNF high-affinity receptor, TrkB, in rats (Silhol et al., 2005, Diógenes et al., 2007, chapter 4.2) and also in humans (Webster et al., 2006) has been reported. Furthermore, the neuromodulatory action of adenosine and the expression of its receptors also change with age. Thus, the inhibitory adenosine A1 receptor is less efficient in aged animals (Sebastião et al., 2000), whereas the functioning and number of the adenosine A2A receptors in the hippocampus is higher in aged rats (Lopes et al., 1999a; Rebola et al., 2003, Diógenes et al., 2007, chapter 4.2 of this thesis).

Considering the relevance of the action of BDNF upon synaptic plasticity phenomena and considering the change of TrkB and A2A receptors density upon aging, the next step was developed to study the influence of BDNF upon LTP during aging and the influence of adenosine A2A receptors on the effect of BDNF.
4.3.2 BDNF effect on LTP induced by θ-Burst stimulation (3 Bursts, 100Hz, 3 stimuli, separated by 200 ms).

As illustrated in Figure 4.3.1, when a θ-Burst (3 Burst of 3 stimuli each, 3x3) was applied to hippocampal slices taken from 4 week-old rats, a very small LTP was observed (12 ± 1.0% increasing in fEPSP slope, n=4). In contrast, when BDNF (20 ng/ml) was present, the same θ-Burst stimuli elicited a robust LTP (50 ± 5.1 % increase in fEPSP slope, n=4, p<0.05). This facilitation of LTP induced by BDNF was totally abolished when the adenosine A2A receptors were antagonized by the selective antagonist, SCH 58261 (100 nM) (n=3, p<0.05, Figure 4.3.2). In these experiments SCH 58261 was added to the superfusing bath at least 30 minutes before induction of LTP in the first pathway and remained in the bath up to the end of the experiment, whereas BDNF was added 60 minutes after induction of LTP in the first pathway and at least 30 minutes before induction of LTP in the second pathway. No significant differences in the magnitude of the first LTP were found in the presence or in the absence of SCH 58261 (100 nM, compare first and third columns of the Figure 4.3.2).
Figure 4.3.1- Effect of the BDNF (20 ng/ml) on θ-Burst (3x3) induced LTP in slices taken from 4 week-old rats. A_a shows averaged time courses changes of fEPSP slope induced by a θ-Burst (3x3) stimulation in the absence (○) or in the presence (●) of BDNF (20 ng/mL). The neurotrophin (●) was applied 60 minutes after induction of LTP in the first pathway (○). The ordinates represents normalized fEPSP slopes where 0% corresponds to the averaged slopes recorded for 14 minutes before θ-Burst stimulation (-0.61 ± 0.06 mV/ms, n= 4 (○) and -0.52 ± 0.06 mV/ms, n= 4 (●)) and the abscissa represents the time of every recordings. The recordings obtained in representative experiments are shown in A_b; each recording is the average of eight consecutive responses obtained before (1) and 46-60 minutes after (2) LTP induction in the presence (upper panel) or in the absence of BDNF (lower panel), and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. All values are mean ± SEM.
Figure 4.3.2- Effect of the BDNF (20 ng/ml) on θ-Burst (3x3) induced LTP in slices taken from 4 week-old rats is dependent on adenosine A2A receptors. A shows averaged time course changes of fEPSP slope induced by the θ-Burst (3x3) stimulation in the absence (○) or in the presence (●) of BDNF (20 ng/mL). The adenosine A2A receptor antagonist, SCH 58261 (100 nM), was applied 30 minutes before the LTP induction in the first pathway (○) and remained in the bath up to the end of the experiment. BDNF (20 ng/ml) was applied 60 minutes (●) after the induction of LTP in the first pathway (○). The ordinates represents normalized fEPSP slopes where 0% corresponds to the averaged slopes recorded for 14 minutes before θ-Burst stimulation (-0.52 ± 0.03 mV/ms, n=3 (○), -0.55 ± 0.08 mV/ms, n=3 (●)) and the abscissa represents the time of every recordings. All values are mean ± SEM. Panel B depicts the magnitude of LTP (change in fEPSP slope at 46-60 minutes) induced by θ-Burst (3x3) stimulation in relation to pre-θ-Burst values (0%) in the absence of any drugs (control), in the presence of BDNF (20 ng/mL) alone, in the presence of SCH 58261 (100 nM) alone or BDNF (20 ng/mL) together with SCH 58261 (100 nM, n=3) as indicated below each column. *p<0.05 (one-way ANOVA with the Bonferroni’s correction).
A similar result was observed in slices taken from 10-16 week-old rats (Figure 4.3.3). Thus, the same θ-Burst stimulus (3x3) only induced a moderate LTP, (13 ± 1.8% increase in fEPSP slope, n=5) which in the presence of the BDNF (20 ng/ml) was significantly higher (33 ± 3.2 % increase, n=5, p<0.05). In the presence of SCH 58261 (100 nM) the facilitation of LTP induced by the neurotrophin was prevented (n=5 p<0.05, Figure 4.3.4). As before, SCH 58261 was added to the superfusing bath at least 30 minutes before induction of LTP in the first pathway and remained in the bath up to the end of the experiment. No significant differences in the magnitude of the first LTP were found in the presence or in the absence of SCH 58261 (100 nM, compare first and third columns of the Figure 4.3.4).

![Figure 4.3.3- Effect of BDNF (20 ng/ml) on θ-Burst (3x3) induced LTP in slices taken from 10-16 week-old rats.](image)

Averaged time course changes of fEPSP slope induced by a θ-Burst (3x3) stimulation in the absence (○) or in the presence (●) of BDNF (20 ng/mL). The neurotrophin (●) was applied 60 minutes after induction of LTP in the first pathway (○). The ordinates represents normalized fEPSP slopes where 0% corresponds to the averaged slopes recorded for 14 minutes before θ-Burst stimulation (-0.58 ± 0.06 mV/ms, n= 4 (○) and -0.57 ± 0.06 mV/ms, n= 4 (●)) and the abscissa represents the time of every recordings. All values are mean ± SEM.
Figure 4.3.4- Effect of the BDNF (20 ng/ml) on θ-Burst (3x3) induced LTP in slices taken from 10-16 week-old rats is dependent on adenosine A2A receptors. A shows averaged time course changes of fEPSP slope induced by the θ-Burst (3x3) stimulation in the absence (○) or in the presence (●) of BDNF (20 ng/mL). The adenosine A2A receptor antagonist, SCH 58261 (100 nM), was applied 30 minutes before the LTP induction in the first pathway (○) and remained in the bath up to the end of the experiment. BDNF (20 ng/ml) was applied 60 minutes (●) after the induction of LTP in the first pathway (○). The ordinates represents normalized fEPSP slopes where 0% corresponds to the averaged slopes recorded for 14 minutes before θ-Burst stimulation (-0.54 ± 0.03 mV/ms, n=5 (○), -0.63 ± 0.06 mV/ms, n=3 (●)) and the abscissa represents the time of every recordings. All values are mean ± SEM. Panel B depicts the magnitude of LTP (change in fEPSP slope at 46-60 minutes) induced θ-Burst (3x3) stimulation in relation to pre-θ-Burst values (0%) in the absence of any drugs (control), in the presence of BDNF (20 ng/mL) alone, in the presence of SCH 58261 (100 nM) alone or BDNF (20 ng/mL) together with SCH 58261 (100 nM, n=5) as indicated below each column. *p<0.05 (one-way ANOVA with the Bonferroni’s correction).
As illustrated in Figure 4.3.5A, in slices taken from 36-38 week-old rats, the θ-Burst stimulation (3x3) was able to induce LTP (37 ± 7.4 % increase in fEPSP slope, n=4). In parallel, in slices from the same animals, BDNF (20 ng/ml) did not cause a further significant enhancement of LTP (42 ± 7.0 %, n=4, p>0.05 as compared with the absence of BDNF). Since the synaptic effects of BDNF are potentiated by adenosine A2A receptors activation (Chapters 4.1 and 4.2), it was next studied if the agonist of this receptor also potentiates the effect of BDNF on LTP. The presence of a selective agonist of the adenosine A2A receptor, CGS 21680 (10 nM), did not trigger an effect of the neurotrophin (20 ng/ml) upon LTP (n=3, Figure 4.3.5B). CGS 21680 was added to the superfusing bath at least 30 minutes before induction of LTP in the first pathway and remained in the bath up to the end of the experiment.

Finally, in the oldest group of rats (70-80 week old rats), θ-Burst stimulation was able to induce LTP (35± 3.5 % increase in fEPSP slope, n=4, Figure 4.3.6), but BDNF (20 ng/ml) did not induce any further significant increase in the magnitude of LTP (37 ± 1.4% increase of fEPSP slope, n=4 p>0.05, when compared with the absence of BDNF Figure 4.3.6).

In Figure 4.3.7 are summarized the results from the experiments with all age groups, where it is clear that BDNF gradually loses the ability to facilitate LTP but it also emerge that LTP is progressively larger with aging.
Figure 4.3.5- Absence of effect of the BDNF (20 ng/ml) on θ-Burst (3x3) induced LTP in slices taken from 36-38 week-old rats. A and B show the averaged time courses changes of fEPSP slope induced by a θ-Burst (3x3) stimulation in the absence (○) or in the presence (●) of BDNF (20 ng/mL). In panel B, the adenosine A2A receptor agonist, CGS 21680 (10 nM), was applied 30 minutes before the LTP induction in the first pathway (○) and remain in the bath up to the end of the experiment. BDNF (20 ng/ml) was applied 60 minutes (●) after the induction of LTP in the first pathway (○). The ordinates represent normalized fEPSP slopes where 0% corresponds to the averaged slopes recorded for 14 minutes before θ-Burst stimulation (-0.69 ± 0.07 mV/ms, n=4 (A, ○), -0.68 ± 0.05 mV/ms, n=4 (A, ●), -0.59 ± 0.07 mV/ms, n=3 (B, ●) and -0.59 ± 0.05 mV/ms, n=3 (B, ○)) and the abscissa represent the time of every recordings. All values are mean ± SEM.
Figure 4.3.6- Absence of effect of the BDNF (20 ng/ml) on θ-Burst (3x3) induced LTP in slices taken from 70-80 week-old rats. Averaged time course changes of fEPSP slope induced by a θ-Burst (3x3) stimulation in the absence (○) or in the presence (●) of BDNF (20 ng/mL). The neurotrophin (●) was applied 60 minutes after induction of LTP in the first pathway (○). The ordinates represents normalized fEPSP slopes where 0% corresponds to the averaged slopes recorded for 14 minutes before θ-Burst stimulation (-0.69 ± 0.07 mV/ms, n= 4 (○) and -0.68 ± 0.07 mV/ms, n= 4 (●)) and the abscissa represent the time of every recordings. All values are mean ± SEM.
Figure 4.3.7- Age-related modulation of long-term potentiation (LTP) by BDNF.
The bars represent the % change of fEPSP slope 46-60 minutes after LTP induction in
the presence (black bars) and the absence (white bars) of BDNF (20 ng/ml). BDNF (20
ng/ml) significantly increases LTP magnitude in hippocampi taken from 4 (n=4) and 10-
16 (n=5) week old rats (first and second black bars respectively) when compared to
controls (first and second white bars). In slices taken from 36-38 (n=4) and 70-80 (n=4)
week old, BDNF was not able to increase LTP (fourth and fifth black bars) magnitude
compared to the control LTPs (fourth and fifth white bars). *P<0.05 (paired Student’s t
 test) as compared with absence of BDNF in the same experiments (adjacent white bar
to the left).
4.3.3 BDNF effect on LTP induced by a weak θ-Burst stimulation (2 Bursts, 100Hz, 3 stimuli, separated by 200 ms).

The observed reduction in the effect of BDNF upon LTP in aged animals could be due to the age-related increase in the magnitude of LTP in the absence of BDNF, which would therefore contribute to masking the effect of BDNF. Thus, the effect of BDNF in LTP induced by a weaker θ-Burst stimulation (2 Bursts, 100Hz, 3 stimuli, separated by 200 ms - 2x3) was tested. In these conditions, the magnitude of LTP (without BDNF) attained in hippocampal slices taken from both 36-38 week-old and 70-80 week-old rats was lower, (14 ± 3.5 % and 31 ± 2.7 %, increase in fEPSP respectively, Figure 4.3.8) as compared with LTP observed in the animals of the same age but after the stronger θ-Burst (3x3) stimulation protocol (see 4.3.2).

BDNF was not able to increase LTP induced by the weak θ-Burst (2x3) in slices taken from 36-38 week-old rats (Figure 4.3.8A) even when adenosine A_{2A} receptors were pharmacologically activated by CGS 21680 (10 nM) (n=5, P>0.05, Figure 4.3.8.B). In slices taken from 70-80 week-old rats, BDNF was also unable to increase the magnitude of LTP (31 ± 7.1% increase, n=3, Figure 4.3.8C).
Figure 4.3.8- Absence of effect of the BDNF (20 ng/ml) on θ-Burst (2x3) induced LTP in slices taken from 36-38 week-old rats and 70-80 week-old rats. Averaged time course changes of fEPSP slope induced by a θ-Burst (2x3) stimulation in the absence (○) or in the presence (●) of BDNF (20 ng/mL) in hippocampal slices taken from 36-38 week-old rats (A and B) or taken from 70-80 week-old rats (C). The neurotrophin (●) was applied 60 minutes after induction of LTP in the first pathway (○) in the absence (A and C) or in the presence (B) of CGS 21680 (10 nM). CGS 21680 was added to slice 30 minutes before induction of LTP in the first pathway and remained in the bath up to the end of the experiment. The ordinates represents normalized fEPSP slopes where 0% corresponds to the averaged slopes recorded for 14 minutes before θ-Burst stimulation: -0.51 ± 0.03 mV/ms, n=3 (A,○), -0.50 ± 0.18 mV/ms, n=3 (A,●), -0.58 ± 0.03 mV/ms, n=5 (B,○), -0.54 ± 0.02 mV/ms, n=5 (B,●), -0.66 ± 0.14 mV/ms, n=3 (C,○), -0.56 ± 0.07 mV/ms, n=3 (C,●) and the abscissa represent the time of every recordings. All values are mean ± SEM.
4.3.4 Effects of a BDNF scavenger on LTP in hippocampal slices

In slices taken from 10-16 week-old rats, the magnitude of LTP after a θ-Burst (3x3) in the presence of BDNF was similar to that observed on hippocampal slices taken from 36-38 week-old rats in the absence of the exogenous BDNF administration (Figure 4.3.7). This could suggest that the higher LTP in control conditions in older rats was due to higher endogenous levels of BDNF. To test this hypothesis, hippocampal slices from 36-38 week-old rats were treated with a BDNF scavenger, TrkB-Fc. Slices were incubated as described by Patterson and collaborators (2001), submerged in either gassed Krebs’ solution alone or in gassed Krebs’ solution containing 2 µg/ml TrkB-Fc at room temperature for 1–1.5 hours prior to use for electrophysiological experiments. Slices were then moved to the interface-recording chamber and were stimulated using the θ-Burst stimuli. The TrkB-Fc fusion protein (the ligand binding domain of the native TrkB receptor coupled to the Fc fragment of human immunoglobulin) scavenges unbound TrkB ligands (Shelton et al., 1995), such as the BDNF.

If extracellular BDNF levels in 36-38 week-old rats were higher and this was the cause for a higher LTP, a lower LTP magnitude in the slices pre-treated with TrkB-Fc would be expected. As shown in Figure 4.3.9, LTP magnitude in the presence of Trk-Fc (2 µg/ml) was similar (n=3, p>0.05) to that observed without the scavenger, suggesting that the age related increase of LTP magnitude may not be a result of an increase of endogenous BDNF levels acting via TrkB receptors. However, the slices were, as mentioned before, pretreated with TrkB-Fc for 1–1.5 hours prior to use for electrophysiological experiments allowing the scavenging of the BDNF available in that time. The slices were placed in the recording chamber continuously perfused with Krebs’ solution, which probably removed the complex (BDNF)-(TrkB-Fc) from the hippocampal slice. On the other hand, during the θ-Burst stimulation a higher amount of BDNF can be released (Aicardi et al., 2004); since more TrkB-Fc
was not administered, the activation of TrkB receptors could not be prevented because of excess of BDNF. Thus, it was next tested the effect of the K252a, the inhibitor of Trk phosphorylation, on LTP magnitude of hippocampal slices taken from 36-38 week-old rats.

Figure 4.3.9- Effect of Trk-Fc (2 µg/ml) on θ-Burst (3x3) induced LTP in slices taken from 38-38 week-old rats. Averaged time course changes of fEPSP slope induced by a θ-Burst (3x3) stimulation. In the pathway represented as (●), slices were pre-incubated for 1.5 hr with Trk-Fc (2 µg/ml). In the pathway represented by (○), no drug was added to slices. The arrow represents θ-Burst stimulation. All values are mean ± SEM; 0% (averaged fEPSP slopes recorded for 14 minutes immediately before θ-Burst stimulation: -0.58 ± 0.06 mV/ms, n=5 (○) and –0.53 ± 0.02 mV/ms, n=3 (●)).
K252a was applied to slices 60 minutes after the induction of the LTP in the first pathway and 30 minutes before LTP induction in second pathway. As illustrated in Figure 4.3.10, in slices taken from 36-38 week-old rats, K252a (200 nM) reduced significantly the magnitude of the LTP (22 ± 2.8 %, n=3, p<0.05). K252a did not influence the basal synaptic transmission as well as does not influence LTP in infant rats (Fontinha et al., 2008). This suggests that the increase of LTP observed in older rats comparing to the younger can be in part attributed to the activation of Trk receptors as a result of the increase in BDNF levels during the θ-Burst stimulation. Further experiments using animals for the different age groups are required for further clarification of the ages related increase in LTP due to an age related enhancement of BDNF release.

Figure 4.3.10- Effect of the K252a (200nM) on θ-Burst (3x3) induced LTP in slices taken from 38-38 week-old rats. A shows averaged time course changes of fEPSP slope induced by the θ-Burst (3x3) stimulation in the absence (○) or in the presence (●) of K252a (200 nM). K252a was applied 60 minutes (●) after the induction of LTP in the first pathway (○). 0 % in the ordinates: -0.60 ± 0.03 mV/ms, n=3 (○), -0.82 ± 0.02 mV/ms, n=3 (●). All values are mean ± SEM. Panel B depicts the magnitude of LTP (change in fEPSP slope at 46-60 minutes) induced θ-Burst (3x3) stimulation in relation to pre-θ-Burst values (0%) in the absence or in the presence of K252a (200 nM). *p<0.05 (paired Student’s t-test).
4.3.5 Discussion

The aim of the work described in this chapter was to study the effect of BDNF on LTP induced by θ-Burst stimulus during aging and the influence of adenosine A$_{2A}$ receptor activation on the BDNF effects.

In previous Chapters (4.1 and 4.2) it was observed that activation of adenosine A$_{2A}$ receptors is required for the excitatory action of BDNF on hippocampal synaptic transmission of young and aged rats; but the reduced ability of BDNF to increase synaptic transmission in aged rats even when adenosine receptors are activated, could be related to reduced TrkB receptor levels (Diógenes et al., 2007). These observations lead to the question that was addressed in this chapter: Does the effect of BDNF on LTP change upon aging and what is the involvement of adenosine A$_{2A}$ receptors in this process?

In experiments performed using hippocampal slices from the two youngest groups of rats (4 week-old and 10-16 week-old rats), a marked increase on LTP induced by BDNF was observed. This effect was totally prevented when adenosine A$_{2A}$ receptors were blocked. These results reinforce the conclusions from previous chapters that the activation of adenosine A$_{2A}$ receptors is required for BDNF synaptic excitatory actions, extending it to synaptic plasticity phenomena.

In slices taken from 36-38 and 70-80 weeks old rats, BDNF no longer increased the magnitude of LTP even when adenosine A$_{2A}$ receptors were pharmacologically activated. On the other hand, a marked increase was observed in LTP magnitude in slices from 36-38 and 70-80 weeks old rats when compared with LTP magnitude obtained in slices from young rats (4 or 10-16 week-old). To understand if the effect of BDNF was masked by the increase in LTP magnitude, the effect of BDNF upon LTP induced by a weaker θ-Burst stimulation was evaluated. However, under these weak LTP induction conditions, BDNF was still devoid of effect.
In slices taken from 10-16 week-old rats, the magnitude of LTP induced by θ-Burst in the presence of BDNF was similar to that observed in hippocampal slices taken from 36-38 week-old rats in the absence of exogenous BDNF. This could suggest that the higher LTP in control conditions in older rats was due to higher endogenous levels of BDNF and a consequent increase in TrkB activation. The BDNF scavenger, Trk-Fc, did not reduce LTP observed in older animals, suggesting that the high LTP in aged animals is not due to an increase of extracellular BDNF levels. However, it is important to be aware of some technical aspects of these experiments. When Trk-Fc was tested, the slices were pre-incubated with this scavenger during 1.5 hours immediately before electrophysiological recordings. Thus, Trk-Fc scavenges the available endogenous BDNF during that time and any molecules of Trk-Fc that became loosely bound to the tissue would be expected to scavenge BDNF released afterwards. The slices were then continuously perfused for electrophysiological recordings. For economical reasons and in accordance with a previously reported protocol (Patterson et al., 2001), no Trk-Fc was added to the perfusion solution. However, it is known that θ-Burst induces BDNF release (Aicardi et al., 2004), which facilitates LTP (Figurov and et al., 1996). If the amount of BDNF released during θ-Burst stimulus is higher in aged then in young animals, the remaining Trk-Fc molecules may not be sufficiently able to scavenge it. Thus, experiments were performed using the inhibitor of Trk phosphorylation, K252a. In the presence of this inhibitor, the LTP magnitude was reduced in aged animals, which may suggest that the increase of LTP magnitude observed in aged rats may indeed be a consequence of higher BDNF levels in these animals.

Figurov and collaborators (1996), studied θ-Burst induced LTP in the hippocampus from Sprague-Dawley rats with ages between one to 2.5 week-old postnatal days and a group of adult rats. They also found that exogenous BDNF enhanced LTP in the neonatal hippocampus where endogenous BDNF level is low;
but, in the adult hippocampus, where endogenous BDNF is higher, the Trk-IgG decreased LTP, suggesting that endogenous levels of this neurotrophin strongly influenced LTP.

It is commonly accepted that in normal aging the hippocampus suffers changes, which might contribute to memory deficits. Since LTP is related to learning and memory (Lynch, 2004), the effect of aging on LTP is of great interest to understand the age-related decline in cognitive function. However, the results relating LTP and aging are controversial. The first reports indicated intact hippocampal LTP induction at the Schaffer collateral-CA1 synapses in old rats (Landfield and Lynch, 1977; Landfield et al., 1978). On the other hand, further studies showed that age-related deficits in cognitive function are accompanied by an impaired ability to sustain LTP (see Barnes, 2003). An increase in LTP in slices from aged rats has been previously reported (Costenla et al., 1999).

What contributes to the changes here described in LTP, is a fundamental question. It is known that 1) there is no loss of hippocampal CA1 pyramidal cells in old rats (Rapp and Gallangher, 1996; Rasmussen et al., 1996), 2) most biophysical properties of old pyramidal cells do not differ from those in young cells, and 3) there is no change in spontaneous firing rates of single cells in the hippocampus of freely behaving old rats comparing with young rats (see Barnes, 2003). However, 4) a deficit in calcium regulation upon aging has been reported (Landfield and Lynch, 1977). There are also results suggesting that an increase in microglia activation could be the key factor for plasticity changes observed during aging (Griffin et al., 2006). Additionally, changes in the density of NMDA subtype of glutamate receptors have been discussed. With aging, declines in the expression of mRNA for different NMDA receptors subunits in rats (Magnusson, 2000) and in konkeys (Bai et al., 2004) were already observed, as well as, a decrease in the expression of several NMDA subunits (Sonntag et al., 2000; Clayton et al., 2002). Yet, these changes do not
explain the now observed increase in LTP upon aging. But there are other points which can, in fact, contribute to this increase in LTP: 1) a lower GABAergic inhibition observed in aged rat hippocampus (Potier et al., 1992; Billard et al., 1995); 2) a possible increase in BDNF release or the release of other facilitatory substances upon θ-Burst stimulation in aged animals; and 3) the increase of the levels of adenosine A2A excitatory receptors in aged animals (Chapter 4.2). According to the results observed with K252a, the second possibility seems to hold true, but this does not exclude any of the others.

During the present work it was also observed that BDNF loses the ability to increase LTP in old rats. The facilitatory effects of BDNF on plasticity have been related to the activation of TrkB receptors (Figurov et al., 1996), and, TrkB receptor density decreases with age (Chapter 4.2). Thus, it was postulated that BDNF would be unable to increase LTP in aged rats. Since BDNF can activate both TrkB and p75 receptors and p75 receptor activation frequently has opposite effects to TrkB receptor activation (Lu et al., 2005), further investigation needs to be done to understand the role of p75 receptors in the age-related changes in LTP.

In summary, the work described here shows that BDNF increases θ-Burst induced-LTP in young and adult rats, that this facilitatory effect is dependent on adenosine A2A receptor activation, that LTP magnitude increases with the age of these animals and that this could be due to an increase of the release of BDNF upon LTP induction, during aging and consequent increase in TrkB receptor activation.
4.4 INFLUENCE OF ADENOSINE A\textsubscript{2A} RECEPTORS ON THE PROTECTIVE ACTION OF BDNF AGAINST APOPTOSIS INDUCED BY THE AMYLOID BETA PEPTIDE

4.4.1 Rational

Neurotrophins have been suggested to have an important role in protecting neurons from neuronal atrophy in the degenerating human brain. A decrease in BDNF levels might be involved in neurodegenerative disorders such as Alzheimer’s disease (AD) (Connor and Dragunow, 1998), making the use of this molecule very promising for treatment of these disorders. However, as discussed in Chapter 1.1.3, the pharmacological administration of \textit{in vivo} BDNF has not been easy since these molecules are unable to cross the blood–brain barrier, making invasive application strategies like intracerebroventricular infusion necessary. Intravenous administration of BDNF has been attempted, but it involves a complex molecular reformulation of the neurotrophin (Wu and Pardridge, 1999).

As showed in previous chapters, a way to potentiate BDNF actions on hippocampal neurons is the co-activation of a specific type of adenosine receptors, the A\textsubscript{2A} receptors. This could open a new strategy for the treatment of neurodegenerative diseases by the use of drugs that can induce activation of A\textsubscript{2A} receptors and consequently potentiate the neuroprotective effect of the endogenous BDNF. However, this type of adenosine receptors can also interfere with apoptosis. Thus, it was observed that the blockade rather than the activation of A\textsubscript{2A} receptors is involved in neuroprotection (Dall'lgna \textit{et al}., 2007). However, adenosine A\textsubscript{2A} receptor stimulation also has been associated with decreased apoptosis in the amygdala (Boucher \textit{et al}., 2006) and it is known that endogenous adenosine via the A\textsubscript{2A}
receptor subtype may be important in mediating protection of sympathetic neurons from apoptosis (Ramirez et al., 2004).

Taking into account that adenosine A$_{2A}$ receptors activation facilitates BDNF actions on synaptic transmission (Chapter 4.1), that the contribution of this adenosine receptor in apoptosis is controversial and that apoptosis can be a key factor for some neurodegenerative diseases where BDNF may play a role, it was decided to evaluate: 1) the effect of the adenosine A$_{2A}$ receptors on apoptosis in cultured neurons, and 2) the function of the A$_{2A}$ receptor activation on the neuroprotective action of BDNF. The amyloid beta peptide (Aß) was used as an apoptotic stimulus since Aß accumulates in the brain of AD patients (Finder and Glockshuber, 2007), where it seems to induce apoptosis (Loo et al, 1993), which has been implicated as a potential cause for the neuronal loss in AD (Shimohama, 2000).

**4.4.2 Apoptosis induced by amyloid beta in primary cultures of rat neurons is prevented by BDNF and this protection is potentiated by adenosine A$_{2A}$ receptors activation.**

Since all the work described in previous chapters of this thesis was carried out in hippocampus, the initial apoptosis studies were performed separately in hippocampal and cortical cultured neurons. The results obtained were similar in the two brain regions and therefore all remaining experiments were performed in cortical neuron cultures to increase the amount of available biological material and thus decrease the number of animals required.

Neuronal cells were incubated in the presence of BDNF (20 ng/ml), of the adenosine A$_{2A}$ receptor agonist CGS 21680 (10 nM), or both CGS 21680 (10 nM) and the neurotrophin (20 ng/ml) for 24 hours. Whenever both drugs were added, CGS
21680 was applied 30 minutes before BDNF. The characteristic morphologic nuclear changes associated with apoptosis were observed using Hoechst staining (Figure 4.4.1). As expected, few apoptotic cells were detected in control cultures as well as when BDNF, CGS 21680 or both were present. In contrast, almost 31 ± 4.4% of neurons exhibited condensed chromatin and nuclear fragmentation with formation of apoptotic bodies after treatment with 25 µM of amyloid beta (Aβ) 23-35 for 24 hours (p<0.05 as compared with control, Figure 4.4.2). To evaluate if increased cell death could be prevented by BDNF and/or CGS 21680, neurons were pre-exposed to these drugs for 1 hour before Aβ application (Figure 4.4.3). BDNF, added prior to Aβ 25-35, reduced the percentage of apoptotic cells to 19 ± 1.6% (n=5). CGS 21680 reduced apoptosis to 24 ± 2.1% (n=7, p>0.05, as compared with Aβ 25-35 alone). When added together, BDNF and CGS 21680 fully prevented Aβ 25-35-induced apoptosis reducing apoptotic cells to a value of 11 ± 1.2% (n=7, P<0.05 as compared with Aβ 25-35 alone), similar to that obtained in the absence of Aβ 25-35 or any other drug.

The effect of the selective antagonist of A2A receptors, SCH 58261 (100 nM) was tested in two cultures. SCH 58261 (100 nM) applied 1 hour before Aβ 25-35 (25 µM) did not appreciably change the percentage of apoptotic cells when compared with Aβ 25-35 alone (n=3, p>0.05, Figure 4.4.4). This antagonist of adenosine A2A receptors, added 30 minutes before BDNF completely prevented the neuroprotective effect of the neurotrophin (Figure 4.4.4A). As expected when applied 30 minutes before CGS 21680 (10 nM), SCH 58261 (100 nM) also reverted the slight decrease of apoptosis induced by the A2A receptor agonist (Figure 4.4.4B). Moreover, in experiments where BDNF and CGS 21680 were added together, SCH 58268 fully reverted the marked neuroprotection already observed (Figure 4.4.4C).
Figure 4.4.1- Morphologic analysis of cells. Cortical neurons were isolated and cultured for 4 days, subsequently they were incubated with no drugs (control), BDNF (20 ng/ml), CGS 21680 (10 nM) or both BDNF and CGS 21680 as indicated. In co-incubation experiments CGS 21680 was added 30 minutes before BDNF application. 24 hours after drugs administration, cells were fixed and then assessed for nuclear morphologic alterations characterized by condensed chromatin, fragmentation, and formation of apoptotic bodies using fluorescence microscopy with Hoechst staining as shown in A. In B the mean ± SEM values obtained in five to eight experiments are illustrated. Dotted line the value obtained without any drug.

Figure 4.4.2 Amyloid-beta (Aβ)-induced apoptosis in primary rat neurons. Cortical neurons were isolated and cultured for 4 days. When Aβ 25-35 (25 µM) was added alone it remain in the cell cultures for 24h, afterward cells were fixed and then assessed for nuclear morphologic alterations characterized by condensed chromatin, fragmentation, and formation of apoptotic bodies using fluorescence microscopy with Hoechst staining as shown in A. In B the mean ± SEM values obtained in eight experiments are illustrated. *P<0.05 (paired Student’s t-test).
Figure 4.4.3 Apoptosis induced by amyloid-beta (Aβ) in primary rat neurons is prevented by BDNF and CGS 21680. Cortical neurons were isolated and cultured for 4 days; subsequently, they were incubated with BDNF (20 ng/ml), CGS 21680 (10 nM) or both BDNF and CGS 21680 as indicated. In co-incubation experiments CGS 21680 was added 30 minutes before BDNF application. Aβ 25-35 (25 µM) was added 1 hour after BDNF and/or CGS 21680 and it remained in the cell cultures for 24 hours. Cells were then fixed and assessed for nuclear morphologic alterations characterized by condensed chromatin, fragmentation, and formation of apoptotic bodies using fluorescence microscopy with Hoechst staining as shown in A. Arrows indicate apoptotic cells. In B, the mean ± SEM values obtained in four to eight experiments are illustrated. *P<0.05 (one-way ANOVA with the Dunnett correction). Dotted line the value obtained without any drug.
Figure 4.4.4- SCH 58261 reverts the neuroprotection induced by BDNF. In panel A, black bars illustrate the neuroprotective effect of BDNF upon Aβ 25-35-induced apoptosis (already showed in Figure 4.4.3), grey bars show the effect of SCH 58261, an antagonist of adenosine A2A receptors, in the presence of Aβ 25-35 (first grey bar, n=3) and on the effect of BDNF with Aβ 25-35 treatment (second gray bar, n=2). In panel B, black bars illustrate the effect of CGS 21680 upon Aβ 25-35-induced apoptosis (already showed in Figure 4.4.3), grey bars show the effect of SCH 58261, in the presence of Aβ 25-35 (first grey bar, n=3) and on the effect of CGS 21680 (second gray bar, n=2). In panel C, black bars illustrate the neuroprotective effect of BDNF in the presence of CGS 21680 upon Aβ 25-35-induced apoptosis (already showed in Figure 4.4.3), grey bars show the effect of SCH 58261 in the presence of Aβ 25-35 (first grey bar, n=3) and on the effect of BDNF plus CGS 21680 (second gray bar, n=2). *p<0.05 (one-way ANOVA with the Dunnett correction). Dotted lines represent the control values (without drugs).
4.4.3 Amyloid beta induced fragmentation of pro-caspase-3 is prevented by BDNF in the presence of adenosine A\textsubscript{2A} receptor activation

Mitochondria are central life and death regulator, which release cytochrome c, leading to the activation of caspase-3, which subsequently cleaves downstream substrates. Processed caspase-3 in the cytosol of neuronal cells, assessed by immunoblot analysis, was therefore used as measure of the degree of apoptosis in cultured cells. Proteolytically activated caspase-3 (20 KDa) was low in cultured neurons after incubation without drugs (control), BDNF (20 ng/ml) and or CGS 21680 (10 nM) (Figure 4.4.5). However, when Aβ 25-35 (25 µM) was added, a significant increase (more than 2-fold) in caspase-3 formation (p<0.05 when compared with control cells) was observed (Figure 4.4.6). The pre-incubation with BDNF (20 ng/ml) reduced the formation of caspase-3 induced by Aβ 25-35, however the decrease did not reveal statistical significance. A similar trend occurred when cells were incubated with the A\textsubscript{2A} receptor agonist (CGS 21680, 10 nM, Figure 4.4.7). However, when cells were incubated with BDNF (20 ng/ml) in the presence of CGS 21680 (10 nM), the caspase-3 formation from pro-caspase-3 was significantly reduced to levels similar to the control (without drugs) (P>0.05 when compared to control cells, Figure 4.4.7).
Figure 4.4.5 Processing of caspase-3 in primary rat neurons in the absence of amyloid-beta. Cortical neurons were isolated and cultured for 4 days before incubation as described in Methods. Cells were incubated either with vehicle (control, no added drugs), BDNF (20 ng/ml), CGS 21680 (10 nM) or both BDNF and CGS 21680. In coinoculation experiments CGS 21680 was added 30 minutes before BDNF application. Procaspase-3 (32 KDa) and active caspase-3 (20 KDa) expression were analysed using western blotting. In A representative Western blots of protein expression are shown for cytosolic protein fractions. The histogram B show mean ± SEM values of the ratio caspase 3/ pro-caspase- 3 for 3-5 independent experiments. Dotted line represents the control values (without drugs).
Figure 4.4.6 Amyloid-beta induced processing of caspase-3 in primary rat neurons. Cortical neurons were isolated and cultured for 4 days before incubation as described in Methods. Cells were incubated either with vehicle (control) or with Aβ 25-35 (25 µM) for 24 hours. Pro-caspase-3 (32 KDa) and active caspase-3 (20 KDa), expression were analysed using Western blotting. Representative western blots of protein expression are shown for cytosolic protein fractions in A. The histogram B show mean ± SEM values of the ratio caspase 3/ pro-caspase-3 for 3 independent experiments. *p<0.05 (paired Student’s t-test). Dotted line represents the control values (without drugs).
Figure 4.4.7: Amyloid-beta induced processing of caspase-3 in primary rat neurons, which is reduced by BDNF in the presence of adenosine A2A receptors activation. Cortical neurons were isolated and cultured for 4 days before incubation as described in Methods. Cells were incubated with vehicle (values represented as the dotted line) or with Aβ 25-35 (25 µM) alone or together with BDNF (20 ng/ml), with CGS 21680 (10 nM) or with both BDNF and CGS 21680. In coculture experiments CGS 21680 was added 30 minutes before BDNF application. Aβ 25-35 (25 µM) was added 1 hour after BDNF and/or CGS 21680 and it remained in the cell cultures for 24 hours. Procaspase-3 (32 KDa) and active caspase-3 (20 KDa) expression was measured using western blotting. Representative Western blots of protein expression are shown for cytosolic protein fractions in A. The histogram B shows mean ± SEM values of the ratio caspase 3/ pro-caspase-3 for 3 independent experiments. *p<0.05 (one-way ANOVA with the Dunnett correction).
4.4.4 Amyloid beta induced increase in caspase-3 activity is prevented by BDNF in the presence of adenosine A<sub>2A</sub> receptors activation

Caspase-3-like activity was evaluated by enzymatic cleavage of the chromophore p-nitroanilide (pNA) from the substrate N-acetyl-Asp-Glu-Val-Asp (DEVD)-pNA and the formation of pNA was measured at 405 nm, as mentioned in Methods (Chapter 3.4).

Neither BDNF nor CGS 21680 alone or together influenced significantly the activity of caspase 3 (Figure 4.4.8). Aβ 25-35 (25 µM) significantly increased caspase-3 activity (p<0.05, n=4, Figure 4.4.8B). When either BDNF (20 ng/ml) or CGS 21680 (10 nM) were added alone, a slight reduction in the increase in caspase-3 activity induced by Aβ 25-35 (25µM) was noted (Figure 4.4.9). The increase in caspase-3 activity caused by Aβ 25-35 (25 µM) was fully prevented when cells were incubated with BDNF (20 ng/ml) together with CGS 21680 (10 nM) (Figure 4.4.8C).

![Figure 4.4.8- Activity of caspase-3 in primary rat neurons.](image-url)

Cortical neurons were isolated and cultured for 4 days before incubation as described in Methods. Cells were incubated with either vehicle (control), BDNF (20 ng/ml), CGS 21680 (10 nM) or both BDNF and CGS 21680 as indicated. In coinoculation experiments CGS 21680 was added 30 minutes before BDNF application (A). When Aβ 25-35 (25 µM) was added alone it remained in the cell cultures for 24 hours (B). Caspase activity was measured in cytosolic protein fractions as described in Methods. The histograms show mean ± SEM values of caspase-3 activity for 3-4 experiments are represented. *p<0.05 (paired Student’s t-test). Dotted line represents the control values (without drugs).
Aβ 25-35 (25 µM)
BDNF (20 ng/ml)
CGS 21680 (10 nM)

Figure 4.4.9- Aβ-induced enhancement of caspase-3 activity in primary rat neurons is reduced by BDNF in the presence of adenosine A2A receptor activation. Cortical neurons were isolated and cultured for 4 days before incubation as described in Methods. Cells were incubated with either vehicle (represented as the dotted line), or with Aβ 25-35 (25µM) alone or together with BDNF (20 ng/ml), CGS 21680 (10 nM) or both BDNF and CGS 21680. In coinubation experiments CGS 21680 was added 30 minutes before BDNF application. Aβ 25-35 (25µM) was added 1 hour after BDNF and/or CGS 21680 and it remained in the cell cultures for 24 hours. Caspase activity was measured in cytosolic protein fractions as described in Methods. These results are the mean ± SEM values of caspase-3 activity for 3 independent experiments. *p<0.05 (one-way ANOVA with the Dunnett correction).
4.4.5 Discussion

Treatment of rat cortical neurons with Aβ 25-35 resulted in high levels of apoptosis showing the consequent elevated levels of pro-caspase-3 cleavage into caspase-3 and also an increase in its proteolytic activity. The main finding in the work described in this chapter was that the molecular modifications resulting from an increase in neuronal death by apoptosis were totally prevented when cells were incubated with BDNF in the presence of a selective agonist of adenosine A2A receptors, CGS 21680. In addition, when the antagonist of adenosine A2A receptors, SCH 58261, was added to cells prior to the other drugs, it completely reverted the protective affects of BDNF, CGS 21680 or both BDNF and CGS 21680 added together. Therefore, A2A adenosine receptors need to be activated to allow BDNF neuroprotection against apoptosis induced by Aβ.

The accumulation of Aβ in the brain has been implicated as a potential cause for the neuronal loss that occurs in AD (Shimohama, 2000). Cell death from Aβ-induced toxicity is a complex process that is thought to involve a number of different pathways, including oxidative stress, perturbation of calcium homeostasis, mitochondrial dysfunction, and activation of caspases (Selkoe, 2001). It is generally believed that Aβ peptides contribute significantly to the pathogenesis of AD, although the mechanisms for this pathogenesis are not yet fully understood. Accordingly, it was reported that central administration of Aβ 25-35 caused a memory impairment in mice, assessed by a decrease in performance in both inhibitory avoidance and spontaneous alteration tasks (Maurice et al., 1996; Dall'Igna et al., 2007).

Neurotrophins, after binding to their Trk receptors, activate two main signal transduction pathways that regulate the cell death machinery (Segal and Greenberg, 1996, Kaplan and Miler, 2000). The primary pathway is the phosphatidylinositol 3-kinase (PI3K)–Akt pathway, which suppresses cell death by inhibiting the apoptotic
activities of forkhead (Brunet et al., 1999) and BCL2 (B-cell leukaemia/ lymphoma 2)-associated death protein (BAD) (del Pesos et al., 1997; Datta et al., 1997). The other pathway is the mitogen-activated protein kinase (MAPK)–MEK (MAPK/ERK extracellular signal regulated kinase) signalling cascade, which stimulates the activity and/or expression of antiapoptotic proteins, including BCL2 and the transcription factor cyclic AMP response element binding protein (CREB) (Riccio et al., 1999).

Adenosine is an ubiquitous homeostatic substance present in all cells, which modulates several functions in the brain, including neuronal survival. However, the actions of adenosine can be diverse depending on the cell type, the aggressiveness of stimuli and the age of animals or cells. In neurons, Lee and Chao (2001) observed by measuring lactate dehydrogenase (LDH) release, that activation of adenosine A2A receptors rescues neurons from death induced by the absence of BDNF, in a TRK receptor dependent way. In PC12 cell cultures, adenosine A2A receptor activation antagonizes apoptosis due to serum deprivation (Huang et al., 2001) and also rescues the impairment of neurite outgrowth when the NGF-evoked MAPK cascade is suppressed (Chen et al., 2002). Recently it was shown that the adenosine A2A receptor contributes to motoneuron survival by transactivating the TrkB receptors (Wiese et al., 2007). In primary glial cultures from cerebral cortices, the activation of adenosine A2A receptors increases NGF expression (Heese et al., 1997), which in turn can exert its neuroprotective effects. Adenosine also has important roles in peripheral nervous system where it has neuroprotective effects on neurotrophin-deprived sympathetic neurons through A2A receptor activation, via a pathway that requires ERK signalling (Ramirez et al., 2004).

During hypoxia or ischemic conditions, adenosine is released in large amounts and can mediate cellular protection. This neuroprotection against ischemia or hypoxia is mainly due to A1 receptor activation (Ribeiro et al., 2003). However, in liver, administration of A2A receptor agonists before induction of ischemia can
attenuate post-ischemic apoptotic hepatic injury and thereby minimize liver injury (Ben-Ari et al., 2005). On the other hand, A2A antagonists have been reported to reduce hypoxic-ischemic neuronal injury (von Lubitz et al., 1995; Phillis, 1995) as well as to block the neurotoxicity induced by Aβ in primary cultures of cerebellar granule cells prepared from 8-day-old Wistar rats (Dall'Igna et al., 2003). In fact, the ability of A2A agonists to enhance glutamate release (see Sebastião and Ribeiro, 1996) together with the frequent finding that these receptors operate in an opposite way to the A1 receptors, which are widely accepted as neuroprotective receptors, leads to a frequent assumption that A2A receptors enhance cell death. As shown in the work now reported, and in line with data mentioned above, A2A receptors enhance the neuroprotective action of neurotrophins and, even more importantly, the neuroprotective actions of neurotrophins are fully lost when A2A receptors are blocked. This clearly point towards a putative neuroprotective role of A2A receptors whenever neurotrophic actions are required. Therefore, studies are required on the consequences of therapy using specific A2A receptor antagonists in neurodegenerative diseases, where neurotrophic factors play a beneficial role (see e.g. Muller and Ferré, 2007).

In summary, as shown in the work presented in this chapter, activation of adenosine A2A receptors did not intensify the apoptosis induced by Aβ. On the contrary, adenosine A2A receptor activation enhanced the ability of BDNF to decrease cell death, strengthening the idea that adenosine A2A receptor agonists may be useful in potentiating the BDNF therapeutic actions on AD.
The goal of the present study was to investigate functional aspects in the cross talk between adenosine $A_{2A}$ receptors and the brain-derived neurotrophic factor (BDNF).

This study was started using hippocampal slices taken from infant rats (3-4 week-old rats) to understand if the activation of adenosine $A_{2A}$ receptors modulates BDNF action actions in basal synaptic transmission and the possible mechanisms that underlie this cross talk. We found that adenosine $A_{2A}$ receptor activation facilitates the excitatory effect of BDNF on synaptic transmission and that this facilitatory action of adenosine $A_{2A}$ receptor activation is due to the activation of the cAMP/PKA transducing system (Chapter 4.1).

Since BDNF has been pointed out as a promising drug to treat neurodegenerative diseases, such as Alzheimer’s disease, a pathology more frequent in old people, it was imperative to study the influence of adenosine receptors activation upon the effect of BDNF on synaptic transmission during aging. With the present work it became clear that BDNF has distinct actions throughout the lifetime of animals, that the effects of BDNF are also dependent on adenosine $A_{2A}$ receptors activation and that these changes can be in part due to a decrease of TrkB receptor levels and an increase of $A_{2A}$ receptor densities (Chapter 4.2).

In addition, it was considered essential to evaluate the influence of adenosine $A_{2A}$ receptors upon the BDNF effect on LTP, a commonly accepted way to study the neurophysiological basis of learning and memory, and to understand if $A_{2A}$ receptors would influence the neuroprotection induced by BDNF. The main reason for this approach was that in neurodegenerative pathologies, such as Alzheimer’s disease, there is both a memory deficit and neuronal loss probably due to enhanced apoptosis as a consequence of amyloid beta peptide deposition. It was observed that BDNF
increases LTP in young and adult rats, and that this facilitatory effect is dependent on adenosine $A_{2A}$ receptor activation. It was also observed that LTP magnitude increases with age and this may be due, in part, to an increase in BDNF release upon stimulation. Finally, BDNF loses the capacity to increase LTP in aged animals, which can be attributed to a decrease of TrkB receptors density.

As stated earlier, BDNF has been proposed to be used in the treatment of Alzheimer's disease patients, a pathology characterized by the deposition of amyloid plaques containing the amyloid beta peptide, the formation of neurofibrillary tangles with hyperphosphorylated tau protein and cell death. Thus we studied the neuroprotective effect of BDNF against cellular death induced by amyloid beta peptide and the involvement of adenosine $A_{2A}$ receptors in this neuroprotective action. This study showed that activation of adenosine $A_{2A}$ receptors enhances the ability of BDNF to prevent apoptosis cellular death in cultured neurons.

The chronic actions of neurotrophins, such as differentiation, growth and maturation of CNS cells, are already well established. A new area of investigation was recently opened with the finding that these molecules also have important acute actions upon synaptic transmission. The work presented in this thesis contributes to a more elucidated understanding of these acute neuronal actions of BDNF and describes a way to potentiate these actions through the activation of adenosine $A_{2A}$ receptors.
The importance of administering neurotrophic factors, in particularly BDNF, to Alzheimer’s patients has been pointed out several times throughout this thesis. The discovery of a molecule that crosses the blood-brain barrier and that could potentiate BDNF actions would be a great therapeutic breakthrough. The first hint of the potential benefits of adenosine or adenosine $A_{2A}$ receptor agonists as drugs to potentiate BDNF action obtained in 2001 by Lee and Chao (2001), who found that a selective adenosine $A_{2A}$ agonist induced phosphorylation of the BDNF receptors, TrkB. But it remained to be known if this molecular interplay would be relevant to the actions of BDNF. Answering this question was the global objective of this work.

As reported here, $A_{2A}$ receptor activation is an obligatory step for the synaptic and neuroprotective function of BDNF, and this $A_{2A}$ facilitatory action occurs in a cAMP-PKA transduction signal-dependent way. In addition to the possibilities already discussed about the manner in which the cAMP-PKA transducing system can modulate TrkB actions, one could envision that this cross talk could also occur between the signalling transduction pathways initiated by adenosine receptors and Trk receptors. Thus, the work here reported opens new perspectives for the investigation of common players involved in this interplay between adenosine and BDNF receptors.

A schematic representation of the relationship between pathways initiated by activation of both $A_{2A}$ and TrkB receptors is presented in Figure 5.1. As mentioned in detail in the introduction of this thesis, adenosine $A_{2A}$ receptors generally couple to Gs proteins. In addition, in the striatum they are also coupled to $G_{oll}$ proteins (Corvol et al., 2001) and, in the hippocampus, they can be coupled to $G_i/G_o$ proteins (Cunha et al., 1999). Furthermore, adenosine $A_{2A}$ receptors, when overexpressed in COS-7 cells, were shown to couple to $G_{15/16}$ proteins (Offermanns and Simon, 1995). In human endothelial cells, the signalling via $G_{12/13}$ proteins has been hypothesized (Sexl
et al., 1997). There is also evidence that adenosine $A_{2A}$ receptors can induce formation of inositol phosphates via pertussis toxin-insensitive $G_{\alpha 15}$ and $G_{\alpha 16}$ (see Jacobson and Gao, 2006). Therefore, the adenosine $A_{2A}$ receptor couple, via $G$ proteins, to an intricate network of signalling pathways, which enables adenosine to communicate with cells in a complex manner. Initially, signalling pathways were thought to be linear, starting with activation of receptors in the cellular membrane and a subsequent activation of different steps including small signalling molecules, called second messengers, protein kinases and transcription factors that regulate gene expression. In the particular case of $A_{2A}$ receptors, this involves the cAMP and cAMP-dependent protein kinase (PKA) pathway (in blue in the Figure 5.1). However, adenosine $A_{2A}$ receptors can also be involved in the MAPK signalling and as well as in PI3K signalling (Figure 5.1 blue dotted line).

Regarding to Trk receptors, as described in the introduction, there are three major pathways involved in the signalling mediated by these receptors: 1) phosphotydilinositol-3 kinase (PI3K); 2) Ras-MAPK pathway and 3) PLCγ pathway, and their downstream effectors (Figure 5.1 brown dotted line).

Therefore, signalling cascades resulting from the activation of $A_{2A}$ and TrkB receptors have common steps (Figure 5.1 in green), the Ras-MAPK and PI3K pathways. These are involved in survival, growth and differentiation as well as in synaptic transmission and in plasticity (Blom and Konnerth, 2005). Since adenosine $A_{2A}$ receptors activation potentiates BDNF actions on synaptic transmission, plasticity and neuroprotection, I advance the hypothesis that one or both of these pathways are involved in the cross talk between $A_{2A}$ and TrkB receptors.
In conclusion, the key finding in this thesis is that adenosine A<sub>2A</sub> receptors activation potentiates BDNF actions. Furthermore, as clearly shown throughout this work, the actions of this neurotrophin upon both synaptic transmission and neuronal death are fully lost when A<sub>2A</sub> receptors are blocked. Therefore, agonists of adenosine A<sub>2A</sub> receptors may prove to be of high therapeutic interest where there is the need to increase BDNF actions in neurodegenerative diseases. In other words, in the early stages of neurodegenerative diseases, where an enhancement of neurotrophic factors is highly desirable, A<sub>2A</sub> receptor agonists can be an important therapeutic option and antagonists should be avoided in order to allow beneficial neurotrophic influences. On the other hand, in the late stages of neurodegenerative diseases, A<sub>2A</sub> receptor antagonists can be advantageous to inhibit excitatory processes.
In the future, investigation of how to promote the expression of TrkB receptors in aged subjects and how to avoid eventual deleterious consequences of the upregulation of neurotrophic actions (e.g. tumor promotion) deserves to be investigated.
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