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Neuroexcitability control by adenosine and BDNF at the adult hippocampus

Mariana Colino de Oliveira

Orientadora: Prof. Doutora Ana Maria Ferreira de Sousa Sebastião

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Biomédicas, Especialidade em Neurociências

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Table of contents

Figure Index

Table Index

Abbreviation List

A_1R – Adenosine A_1 receptor

$A_{2A}R$ – Adenosine A_{2A} receptor

$A_{2B}R$ – Adenosine A_{2B} receptor

A_3R – Adenosine A_3 receptor

AC – Adenylate cyclase

aCSF – artificial cerebrospinal fluid

ADA – Adenose deaminase

ADK – Adenosine kinase

$Adk^{\Delta brain}$ – Adenosine kinase knockout

AMP – Adenosine-5'-monophosphate

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

AMPAR – AMPA receptor

ATP – Adenosine-5'-triphosphate

BDNF – Brain-derived neurotrophic factor

B_{max} – maximal number of binding sites

BSA – Bovine serum albumin

cAMP – 3',5'- cyclic Adenosine monophosphate

CAMKII – Ca^{2+}/Calmodulin-dependent protein kinase II

CA – Cornu ammonis

CypA – Cyclophilin A

CB1R – Endocannabinoid receptor 1

cDNA – complementary DNA

CPA – N6-cyclopentyladenosine

CGS 21680 – 2-[4-(2-p-carboxyethyl)phenylamino]-50-N-ethylcarboxamidoadenosine

CREB – Cre-response element binding protein

D1R – Dopamine D1 Receptor

D2R – Dopamine D2 Receptor

DAG – Diacylglycerol

DG – Dentate Gyrus

DMSO – Dimethylsulfoxide

DNA – Deoxyribonucleic acid

ENT – Equilibrative nucleoside transporter

DPCPX – 1,3-dipropyl-8-cyclopentylxanthine

ER – Endoplasmic reticulum

GABA – γ-aminobutyric acid

$GABA_AR$ – $GABA_A$ receptor

GPCR – G-protein coupled receptor

fEPSP – field Excitatory post-synaptic potentials

FL-TrkB – Full-length TrkB

IP_3 – Inositol-1,4,5-triphosphate

JNK – c-Jun N terminal kinase

KA – Kainate acid

KCC2 – K-Cl co-transporter

K_d – Equilibrium dissociation constant

Kyn – Kynurenic acid

LTP – Long-term potentiation

mIPSC – miniature Inhibitory post-synaptic current

mRNA – messenger RNA

NF-κB – Nuclear factor kappa B

NKCC1 – Na-K-2Cl co-transporter

NMDA – N-methyl-D-aspartate

NMDAR – NMDA receptor

NPY – Neuropeptide Y

NT – Neurotrophin

$p75^{NTR}$ – p75 neurotrophin receptor

PCR – Polymerase chain reaction

PI3K – Phosphatidylinositol 3-Kinase

PIP2 – Phosphorylates phosphatidylinositol-4,5-biphosphate

PIP3 – Phosphatidylinositol-3,4,5-biphosphate

PKA – Protein kinase A

PKC – Protein kinase C

PLCγ – Phospholipase Cγ

PVDF – Polyvinylidene fluoride

QX-314 – *N*-(2,6-Dimethylphenylcarbamoylmethyl)triethylammonium chloride

Ras-MAPK – ras-Mitogen-Activated Protein Kinase

REX Ab – anti-$p75^{NTR}$ IgG

RNA – Ribonucleic acid

RpL13A – Ribosomal protein L13A

SAM – S-adenosil-homocysteine

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

SDS – Sodium dodecyl sulphate

SEM – Standard error of the mean

TBS – Tris-buffered saline

TGN – *trans*-Golgi network

TrkB – Tropomyosin-related kinase B

TrkB-Tc – TrkB truncated

TTX – Tetrodotoxin

XAC –*N*-(2-Aminoethyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)phenoxy]-acetamide hydrochloride

Resumo

No Sistema Nervoso Central existem duas grandes classes de neurónios: as células piramidais e os interneurónios. As células piramidais libertam glutamato, o principal neurotransmissor excitatório, e por isso facilitam a transmissão sináptica. Os interneurónios controlam a excitabilidade, a velocidade de disparo e a actividade sincronizada das células piramidais, através da libertação de GABA, o principal neurotransmissor inibitório no cérebro. Estes conceitos constituem a visão tradicional de comunicação neuronal. Existem no entanto outros intervenientes que contribuem para a modulação da excitabilidade de um circuito, como é o caso dos neuromoduladores. Estas moléculas não sendo capazes de mediar a comunicação entre dois neurónios, influenciam-na através da modulação dos efeitos do glutamato ou do GABA, por exemplo, por alteração da sua libertação ou pela regulação da actividade dos respectivos receptores. Dois exemplos de neuromoduladores bem caracterizados são a adenosina e o *Brain-Derived Neurotrophic Factor* (BDNF), que foram objecto de estudo deste trabalho. Quer a adenosina, quer o BDNF, representam tal importância na modulação da actividade neuronal que a disfunção na sua sinalização tem sido apontada como causa para o desenvolvimento de actividade epiléptica. Esta é caracterizada por um desequilíbrio da transmissão sináptica em que é favorecida a excitação, em detrimento da inibição, levando a excessivas descargas eléctricas sincronizadas, designadas por convulsões. Deste modo, torna-se uma questão revelante a compreensão dos mecanismos de regulação da actividade neuronal pela adenosina e BDNF, sendo esse o foco do presente trabalho.

A estrutura cerebral utilizada para estudar as implicações funcionais da adenosina e do BDNF no controlo da excitabilidade neuronal foi o hipocampo. O hipocampo está envolvido em processos de aprendizagem, formação de novas memórias e orientação espacial. Tem uma organização que permite facilmente identificar os circuitos, a qual é conservada entre mamíferos. É também uma estrutura que está envolvida no aparecimento de várias doenças neurológicas, entre elas, a epilepsia. Fatias transversais de hipocampo mantêm os circuitos intactos e saudáveis durante longos períodos de tempo, permitindo o seu estudo funcional e tornando-o, por isso, uma estrutura apreciável para estudos electrofisiológicos.

O BDNF é uma proteína pertencente à família das neurotrofinas que exerce as suas acções através da ligação ao seu receptor de alta afinidade TrkB e de baixa afinidade $p75^{NTR}$. O BDNF participa em processos de sobrevivência, diferenciação e crescimento neuronal, no entanto, também é reconhecido pelo seu papel como modulador da transmissão e plasticidade sináptica. O efeito do BDNF na transmissão glutamatérgica está bem caracterizado. Sabe-se que na membrana pré-sináptica facilita a libertação de glutamato por aumento do número de vesículas ancoradas na zona activa da sinapse e que na membrana pós-sináptica, leva ao aumento da fosforilação do receptor de glutamato NMDA, aumentando por sua vez a sua actividade. Apesar do bem documentado efeito do BDNF na transmissão excitatória, a sua influência sobre a transmissão GABAérgica no hipocampo adulto não foi ainda explorada. Deste modo, um dos objectivos propostos deste trabalho foi averiguar uma modulação putativa da sinalização GABAérgica pelo BDNF, bem como os mecanismos envolvidos. Para isso realizaram-se registos intracelulares de células piramidais da região CA1 do hipocampo de ratos adultos (entre 5 a 10 semanas) utilizando a técnica electrofisiológica de *Patch Clamp*. Os registos foram realizados na configuração de *Whole-cell Patch Clamp*, que permite registos que envolvem receptores localizados por toda a membrana.

Começou-se por avaliar o efeito do BDNF na amplitude de correntes GABAérgicas (mediadas pelo receptor ionotrópico de GABA, o receptor $GABA_A$) evocadas por estimulação eléctrica aferente. Dos resultados obtidos, observa-se um efeito claro de facilitação da transmissão inibitória. Em seguida, de modo a caracterizar em mais detalhe os mecanismos envolvidos nesta modulação, testou-se o efeito do BDNF em registos de correntes pós-sinápticas inibitórias miniatura (mIPSCs). Alterações na frequência destes eventos estão relacionados com alterações a nível pré-sináptico e, na amplitude, a nível pós-sináptico. A observação de que o BDNF leva a um aumento da frequência, sem alteração da amplitude, dos mIPSCs, permitiu concluir que o efeito facilitatório do BDNF sobre as correntes GABAérgicas resulta de um efeito pré-sináptico. Este, demonstrou-se ser dependente da activação dos receptores TrkB, bem como dos receptores de adenosina A_{2A}. Tendo isto em consideração, pode-se concluir que, em condições fisiológicas, o BDNF tem um papel na regulação da excitabilidade das redes neuronais por modulação da sinalização inibitória no hipocampo.

A adenosina é um nucleósido de purina que no Sistema Nervoso Central actua como uma molécula sinalizadora extracelular. É libertada como tal ou resulta de sucessivas desfosforilações de ATP. Na fenda sináptica exerce os seus efeitos através da ligação a dois principais receptores de alta afinidade: o receptor predominantemente expresso é o receptor inibitório, o receptor A_1 (A_1R). No entanto, também o receptor facilitatório, A_{2A} ($A_{2A}R$) medeia importantes efeitos da adenosina, nomeadamente a facilitação da sinalização pelo BDNF. Como fino regulador da comunicação neuronal, a concentração extracelular de adenosina tem também que ser bem controlada. O principal interveniente neste processo é o enzima Adenosine cinase (ADK). Este enzima, no cérebro adulto, é maioritariamente expresso nos astrócitos e tem como função fosforilar a adenosina, criando um gradiente electroquímico que permite a entrada de adenosina para o astrócito e consequente remoção da fenda sináptica. A sobre-expressão e sobre-activação do ADK estão normalmente associadas a inferiores concentrações de adenosina extracelular, a uma disfunção da sinalização inibitória de adenosina mediada pelos receptores A_1 e ao aparecimento das características fundamentais da epilepsia, como é o caso da astrogliose e actividade convulsiva. Com o objectivo de tentar compreender as implicações da actividade do ADK na actividade neuronal e abrir novas janelas terapêuticas tendo este enzima como alvo, foi utilizado um modelo de ratinho que não expressa ADK no cérebro ($Adk^{\Delta brain}$). Surpreendentemente, estes ratinhos apresentam um fenótipo epiléptico de forma progressiva e induzida por situações de stress, isto é, a partir dos 5 meses os ratinhos apresentam convulsões epilépticas aquando de uma mudança para um ambiente novo, ou seja, numa situação de stress moderado. No entanto, a partir do primeiro ano de idade, estes episódios epilépticos desaparecem. Tendo isto em consideração, este trabalho teve como objectivo a caracterização funcional e bioquímica dos receptores de adenosina e de BDNF na fase pré-sintomática (2 meses), sintomática (5 a 8 meses) e pós-sintomática (mais de 1 ano de idade) destes animais, na tentativa de relacionar uma possível desregulação da neuromodulação mediada pela adenosina com o fenótipo desenvolvido nos ratinhos ao longo da vida.

As avaliações funcionais foram realizadas através de registos de actividade sináptica de um conjunto de neurónios em resposta a uma estimulação eléctrica aferente, ou seja, através de registos de potenciais excitatórios pós-sinápticos de campo (fEPSPs). A caracterização molecular baseou-se na realização de ensaios de ligação de saturação

utilizando o antagonista selectivo dos receptores A_1 marcado radioactivamente, de ensaios de *Immunoblot* utilizando anticorpos específicos de reconhecimento das proteínas A_{2A}R e TrkB, de ELISA para quantificação de BDNF e de *quantitative Polymerase Chain Reaction* (qPCR), para análise a nível transcricional dos receptores de adenosina.

As observações mais relevantes deste trabalho revelam que o aumento marcado dos níveis de adenosina extracelular, associam-se a uma deficiência na sinalização mediada pelos A_1R, com simultâneo decréscimo da expressão proteica deste receptor nos três estágios fenotípicos dos animais que não expressam ADK no cérebro. Pode pois concluir-se que, embora possa contribuir para uma maior excitabilidade nestes animais, a deficiência na sinalização pelos A_1Rs não é suficiente para justificar o fenótipo epiléptico apenas registados nos animais entre os 5 e os 12 meses de idade. No entanto, no que diz respeito aos receptores A_{2A}, observou-se uma alteração de sinalização e expressão dos receptores dos ratinhos pré- para sintomáticos que levam a adaptações de plasticidade que poderão justificar o aparecimento do fenótipo epiléptico. Ratinhos sintomáticos que não expressam ADK no cérebro apresentam plasticidade sináptica aumentada, a qual é prevenida pelo antagonismo dos receptores A_{2A}, que por sua vez apresentavam níveis de proteína aumentados. Este efeito na magnitude da potenciação de longa duração observou-se ser também dependente da activação dos receptores TrkB, sugerindo um aumento de sinalização pelo BDNF. De acordo com esta observação, ratinhos epilépticos apresentam sobre-expressão desta proteína. Em conclusão, pode-se afirmar que a função do enzima ADK tem implicações intrínsecas na actividade neuronal e que representa um risco genético para o desenvolvimento de epilepsia, o qual, neste modelo, estará relacionado com um exacerbamento da sinalização pelos receptores de adenosina A_{2A} e pelo BDNF. Adicionalmente, este trabalho também oferece novas oportunidades para o desenvolvimento de ferramentas farmacológicas para doenças caracterizadas pelo aparecimento de convulsões epilépticas.

Palavras-chave:

Neuromodulação; Epilepsia; Hipocampo; BDNF; Adenosina; ADK

Abstract

The traditional view of neuronal communication involves a network of neurons communicating through neurotransmitters, which either lead to neuronal depolarization or hyperpolarization. However, the overall output is also determined by the presence of neuromodulators. These are molecules able to alter the function of a circuitry by modifying the properties of synaptic conductance and the intrinsic membrane properties of individual neurons. Neuromodulators are thus critical in the regulation of synaptic transmission and plasticity. Adenosine and Bain-Derived Neurotrophic Factor (BDNF) are two well-known modulators of the central nervous system.

BDNF induces pre- and post-synaptic changes that lead to a facilitation of glutamatergic transmission through its high affinity receptor, TrkB receptor. However, BDNF influence upon GABAergic transmission in the adult brain is poorly understood. I now evaluated whether BDNF modulates GABAergic transmission in the adult hippocampus. Evoked and spontaneous synaptic currents were recorded from CA1 pyramidal cells in acute hippocampal slices from young adult rat brains (6 to 10 weeks-old). BDNF (10-100 ng/mL) increased miniature inhibitory post-synaptic currents (mIPSCs) frequency, but not amplitude, as well as increased the amplitude of inhibitory post-synaptic currents (IPSCs) evoked by afferent stimulation. The facilitatory action of BDNF upon GABAergic transmission was lost in the presence of a Trk inhibitor (K252a, 200 nM), but not upon $p75^{NTR}$ blockade (Anti-$p75^{NTR}$ IgG, 50 μg/mL). Since adenosine A_{2A} receptor is an upstream regulator of TrkB activity, a putative influence of this receptor was also investigated. The facilitatory action of BDNF onto GABAergic transmission was also prevented upon adenosine A_{2A} receptor antagonism (SCH 58261, 50 nM). I thus concluded that BDNF facilitates GABAergic signaling at the adult hippocampus via a pre-synaptic mechanism that depends on TrkB and adenosine A_{2A} receptor activation.

Adenosine net effect is to inhibit synaptic activity through the binding to the adenosine A_1 receptor (A_1R). Despite the low expression of the adenosine A_{2A} receptor ($A_{2A}R$), through its activation, adenosine may also enhance synaptic strength. Astrocytic Adenosine kinase (ADK) accounts as the major contributor for adenosine metabolic clearance from synaptic cleft, determining the intra- and extracellular concentrations of adenosine. Accordingly, ADK up-regulation correlates with decreased adenosine levels,

with consequent impaired A_1R-mediated signaling and increased susceptibility to epileptic seizures. Surprisingly, mice lacking ADK expression in the entire brain develop progressive epilepsy with stress-induced seizures in an age-dependent way. They present spontaneous convulsive seizures by the age of 5 months old, until their first year old, when they become asymptomatic. Functional and biochemical studies were performed in order to characterize adenosine and BDNF receptors in pre-symptomatic (2 months old), symptomatic (5 to 8 months old) and post-symptomatic (over 1 year old) mice. ADK knockout mice at all ages demonstrated enhanced adenosine levels around synapses, but impaired A_1R receptor function of synaptic transmission with concomitant lower expression protein levels of the receptor. Lack of A_1R inhibitory signaling is observed independently of animal age, so it cannot account for the epileptic phenotype, despite it may account for a higher neuronal excitability. However, adenosine $A_{2A}R$ and BDNF signaling undergo a shift from the pre- to the symptomatic stage. In mice lacking ADK, $A_{2A}R$ activation and BDNF signaling is augmented leading to enhanced synaptic plasticity, which may play a role in the triggering of the seizure phenotype. Accordingly, $A_{2A}R$ and BDNF are overexpressed in epileptic mice lacking *Adk* in the brain. This work besides suggesting that $A_{2A}R$ and BDNF exacerbated signaling have pro-epileptic affects, provide evidence that ADK may present a genetic risk for the development of epilepsy since it has intrinsic effects onto brain activity, but also offers new opportunities for the development of pharmacologic tools for seizure disorders.

Key words:

Neuroexcitability; Epilepsy; Hippocampus; BDNF; Adenosine; ADK

1 | INTRODUCTION

1.1 | Homeostatic control of synaptic transmission

In 1897, Sherrington adopted the term synapse (from the greek "hold together") as the junction between neurons that allows their communication through the unidirectional propagation of the action potential. Importantly, Sherrington's work descriptions also suggested the existence of an integration of excitatory and inhibitory signals and thus, different types of synapses (quoted by Bennett 1999; López-Muñoz & Alamo 2009). Since then, great developments on the characterization of synapses and neuronal transmission have been achieved using several methods, including microscopical, electrophysiological, biochemical and pharmacological approaches.

Currently, general assumptions from synaptic transmission rely on the fact that neurotransmitters are released from a specialized pre-synaptic site to the synaptic cleft and subsequently will bind and activate post-synaptic receptors. Those receptors can be either inotropic or metabotropic. Upon neurotransmitter binding, ionotropic receptors allow the flux of ions according to their chemical and electrical gradient, mediating a depolarizing or hyperpolarizing signal, while metabotropic receptors mediate slower responses through the activation of signaling cascades. In the central nervous system, the two main neurotransmitters responsible for neuronal communication are glutamate and GABA (López-Muñoz & Alamo 2009). Glutamate ionotropic receptors allow the influx of Na^+ and Ca^{2+}, thus having excitatory actions, whereas GABA ionotropic receptors allow Cl^- influx, mediating hyperpolarizing responses (Smart & Paoletti 2012).

The modulation of neurotransmission is as important as synaptic transmission itself. The players in this process, the neuromodulators, have a crucial role in maintaining synaptic activity at appropriate levels for a healthy neuronal activity and normal brain function. Some examples of these neuromodulators are ATP, adenosine, cytokines and neurotrophic factors, such as Brain-Derived Neurotrophic Factor (BDNF). They cannot act as a neurotransmitter themselves and generate action potentials, they can however, change neurotransmitter release from pre-synaptic membranes or the activity of their receptors at post-synaptic level and thus, they are able to control the excitability of neuronal circuits (López-Muñoz & Alamo 2009).

1.2 | Epilepsy

Epilepsy is a neurological disease characterized by an imbalance in synaptic transmission, favoring excitatory towards inhibitory signaling. This leads to abnormal, excessive and hypersynchronous electrical discharges of neurons in the brain, denominated seizures, the main phenotypic feature of epilepsy, and eventually neuronal death (Goldenberg 2010).

For a long time, astrocytes were only seen as structural and metabolic supportive cells to neurons. It is now widely accepted that astrocytes regulate neuronal excitability and coordinate neuronal network activity (Fellin 2009). Rather than a neuronal dysfunction, accumulated evidence implies glial dysfunction as a major contributor for seizures. In epileptic brain from human patients and animal mouse models, in the same areas where neuronal loss occur, functional and hypertrophic changes are also observed in astrocytes, a process depicted as astrogliosis (Binder & Steinhauser 2009).

The view that epilepsy is a disorder of neuronal excitability led to the development of therapeutic strategies focused on GABAergic system as the key target. These drugs, however, do not avoid unwanted side effects, such as marked inhibitory effects in cognition, and still fail to control seizures in some patients (Binder & Steinhauser 2009; Goldenberg 2010; Meldrum & Rogawski 2007). It is estimated that over 30% of all patients with epilepsy remain refractory to treatment (Boison 2010). Recently, accumulated evidences have been implying new molecules as important components in epilepsy, such as adenosine and BDNF. The signaling of both neuromodulators is altered in epilepsy and a better understanding of their changes in this pathology may provide breakthroughs in the development of new therapeutic targets (Boison 2010; Scharfman 2005).

1.3 | Brain-Derived Neurotrophic Factor

Brain-Derived Neurotrophic Factor (BDNF) was purified for the first time in 1982 from pig brain and characterized as a protein that could promote the survival and outgrowth of cultured sensory neurons (Barde et al. 1982). BDNF belongs to the neurotrophic family, which also includes Neurotrophin-3, Neurotrophin-4/5, Neurotrophin-6 and Nerve

Growth Factor. It regulates neuronal survival, growth and differentiation but it also has emerged as a key player in modulating synaptic transmission and plasticity (Binder & Scharfman 2004).

1.3.1 BDNF synthesis and release

In the central nervous system, BDNF is synthesized by excitatory, but not inhibitory, neurons from most brain structures, such as hippocampus and cortex (Wetmore et al. 1990; Gorba & Wahle 1999). BDNF expression varies along development, being low at birth, and high during the first and subsequent weeks of development. During lifetime, BDNF expression is positively modulated by neuronal activity (Lessmann et al. 2003; Kuczewski et al. 2009).

BDNF mRNA is initially translated into a precursor protein, the prepro-BDNF, which contains a signal-peptide sequence that targets it into the endoplasmic reticulum (ER). Here, the signal-peptide sequence is immediately cleaved off and the protein is converted into pro-BDNF, which is accumulated in *trans*-Golgi network (TGN). At TGN, pro-BDNF may be sorted to two different secretion pathways: *1)* a constitutive one, in which pro-BDNF is enzymatically cleaved into the mature BDNF and it is incorporated in constitutive releasing vesicles that fuse with the membrane in a spontaneous way; or *2)* pro-BDNF is incorporated in immature secretion vesicles, which also contain enzymes that cleave the pro-neurotrophin, but that only fuse with the membrane upon a specific signaling of regulated secretion, such as an increase in Ca^{2+} levels (Lessmann et al. 2003). It is thus important to note that pro-BDNF can also be released as such. Once in the extracellular space it can either be cleaved to originate the mature form of BDNF or it can also act as a signaling molecule. High frequency stimulation leads to increased pro-BDNF extracellular cleavage and thus mature BDNF signaling, while low frequency stimulation favors pro-BDNF signaling (Lu et al. 2005).

1.3.2 BDNF signaling

Once released onto the extracellular space, BDNF can have autocrine and paracrine actions (Lessmann et al. 2003). BDNF effects are mediated through the activation of two membrane receptors: the high affinity tropomyosin-related kinase receptor B (TrkB) and

the low affinity p75 neurotrophin receptor ($p75^{NTR}$).

A single trans-membrane domain and a cytoplasmic tyrosine kinase domain characterize the TrkB receptor. Upon BDNF binding, the receptor dimerizes, leading to the auto-phosphorylation at specific tyrosines in the intracellular domains. Phosphotyrosine residues will then serve as docking sites for adaptor proteins that will initiate signal transduction pathways (Reichardt 2006), such as:

- Phospholipase Cγ (PLCγ): leads to the formation of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Both support synaptic plasticity phenomena, IP_3 promotes the release of intracellular Ca^{2+} from endoplasmatic reticulum, while DAG activates Protein kinase C (PKC);

- Ras Mitogen-Activated Protein Kinase (Ras-MAPK): activated Ras phosphorylates Raf that subsequently activates Mek1 and/or Mek2, which in turn phosphorylates Erk1 and Erk2, leading to gene transcription activation for cellular differentiation and neurite outgrowth;

- Phosphatidylinositol 3-Kinase (PI3K): phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2) into phosphatidylinositol-3,4,5-biphosphate (PIP3). PIP3, as a secondary messenger, activates Akt, a serine/threonine kinase, which result in transcriptional activation of pro-survival genes and also in the inhibition of pro-apoptotic proteins activity (Figure 1.1).

TrkB mRNA may undergo alternative splicing and originate two other smaller proteins, the truncated TrkB receptors (TrkB.T1 and TrkB.T2), which are also abundantly expressed throughout the brain, both in neurons and glia. By excluding the tyrosine kinase domain coding exons, TrkB truncated (TrkB-Tc) isoforms lack the catalytic domain and hence they were only thought as dominant-negative receptors that would bind to BDNF and indirectly inhibit TrkB function. However, TrkB.T1 and TrkB.T2 do have specific isoform-specific sequences in the intracellular domain that give them ability for signal transduction, participating in processes such as neurite outgrowth, cytoskeletal changes in astrocytes, Rho GTPase activity regulation and PLCγ and MAPK activation (Baxter et al.

1997; Minichiello 2009; Fenner 2012).

p75^{NTR} belongs to the tumor necrosis factor superfamily, it spans the membrane once and it has a characteristic cytoplasmic "death" domain, shared by the other protein family members. Through the interaction with other proteins, p75^{NTR} may lead to transcriptional activation of pro-survival (through NF-κB signaling pathway activation) or pro-apoptotic genes (through JNK signaling pathway activation) (Figure 1.1) and regulate synaptic plasticity (Reichardt 2006). Notably, p75^{NTR} can also be activated by pro-BDNF and its activation was reported to be required for hippocampal NMDAR-dependent Long-Term Depression (Woo et al. 2005), contrary to the BDNF mediated effects on Long-Term Potentiation, via TrkB receptors (Messaoudi et al. 2002).

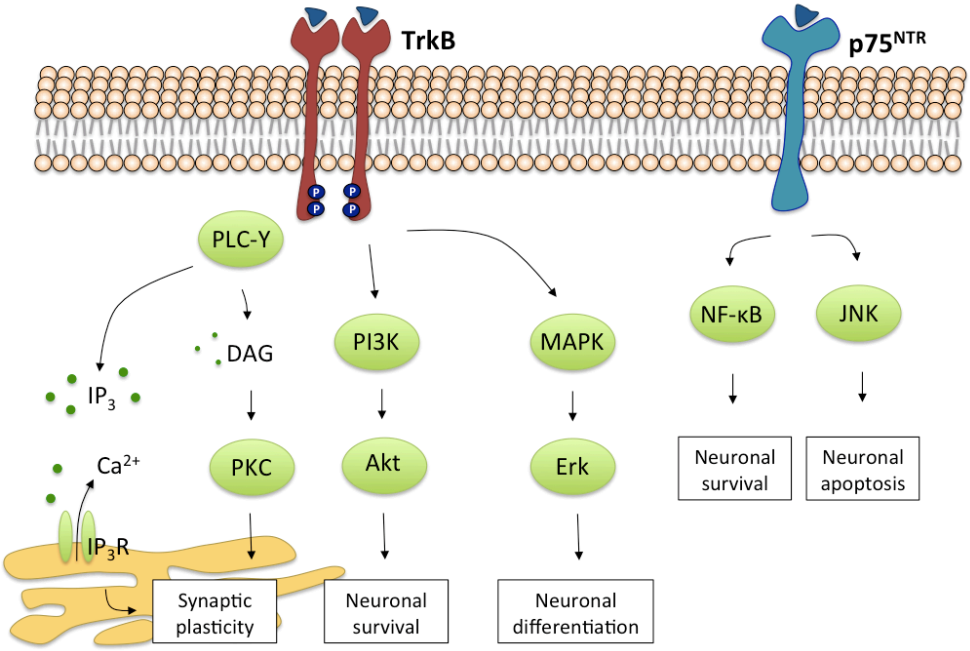

Figure 1.1: Signaling cascades activated by BDNF. BDNF effects are mediated through the activation of TrkB and p75^{NTR} receptors. Upon BDNF binding, TrkB receptor, a tyrosine kinase receptor, dimerizes, auto-phophorylates and creates docking sites for adaptor proteins that will activate three signaling pathways. PLCY activates PKC and, through IP_3, also leads to the release of intracellular calcium from endoplasmatic reticulum, contributing for synaptic plasticity. By activation of gene trancriptin PI3K/Akt and MAPK signaling pathways, promote neuronal survival and differentiation, respectively. P75^{NTR} belongs to the tumor necrosis factor and it may either activate prosurvival or proapoptotic genes, through NF-κB or JNK signaling cascades.

1.3.3 BDNF in synaptic transmission

Much attention has been given to the role of BDNF in neurogenesis, neurite outgrowth and neuronal survival. However, BDNF is also highly involved in the regulation of synaptic transmission and plasticity.

1. Introduction

In 1995, Levine and colleagues unraveled a facilitatory role of BDNF on hippocampal synaptic transmission via activation of TrkB receptors (Levine et al. 1995). This augmentation would result from post-synaptic changes and when evaluated, it was found that BDNF enhances NMDA, but not AMPA, receptor activity (Levine et al. 1998) most probably due to BDNF-induced NMDA phosphorylation (Lin et al. 1998). Additionally, BDNF induces pre-synaptic modifications, namely by increasing glutamate release (Lessmann et al. 1994; Li et al. 1998) through a mechanism involving the increase of the number of docked vesicles at the active zones of excitatory synapses (Tyler & Pozzo-Miller 2001).

The available information about influence of BDNF upon inhibitory transmission mostly refers to developing neurons in culture. BDNF increases GAD65 expression, and thus, GABA synthesis at pre-synaptic terminals (Ohba et al. 2005; Sánchez-Huertas & Rico 2011) and positively modulates GABA release in cultured hippocampal neurons (Baldelli et al. 2005; Bolton et al. 2000). Another important step of inhibitory transmission modulation is the GABA clearance from synaptic cleft mediated by GABA transporters. BDNF inhibits GABA uptake into nerve terminals in a TrkB-dependent way (Vaz et al. 2008) but enhances it in astrocytes in a process involving truncated form of TrkB and $A_{2A}R$ activation (Vaz et al. 2011). In contrast with a well-established influence of BDNF upon excitatory transmission, the influence of BDNF upon inhibitory signaling remains unclear. The available information refers mostly to developing neurons in culture, where BDNF has been shown to cause a post-synaptically mediated fast facilitation of GABAergic currents followed by a prolonged depression (Jovanovic et al. 2004), or to cause a pre-synaptic facilitation of GABAergic inputs to glutamatergic neurons (Wardle & Poo 2003). Prolonged applications of BDNF (>24h) have been shown to facilitate maturation of GABAergic synapses and a pre-synaptically mediated increase in GABAergic transmission (Bolton et al. 2000) or to prevent down scaling of GABAergic responses caused by activity deprivation (Swanwick et al. 2006). In hippocampal slices acutely prepared from P12-P18 rats, BDNF has been reported to cause a post-synaptic inhibition of GABAergic currents recorded from pyramidal neurons (Tanaka et al. 1997). Highly variable actions of BDNF on GABAergic transmission while using hippocampal slices acutely prepared from 2 to 8 weeks old rats have been reported, but data were not discriminated as a function of age (Frerking et al. 1998). Changes in modulation of GABAergic currents by BDNF are

expected to occur during maturation since BDNF affects K^+/Cl^- transport in hippocampal neurons (Wardle & Poo 2003). Indeed, in neurons acutely isolated at the post-natal day 6 (P6), BDNF has been shown to cause a post-synaptically mediated facilitation of GABAergic currents, while causing a long lasting inhibition of GABAergic currents at P14 neurons (Mizoguchi et al. 2003). BDNF-induced inhibition of GABA transport at nerve endings that reflects into reduction of the uptake of GABA (Vaz et al. 2008) as well as in reduction of non-exocytotic release through transport reversal has also been reported either by using young (Canas et al. 2004; Vaz et al. 2008; Vaz et al. 2011) or adult rats (Jerónimo-Santos et al. 2014).

1.3.4 BDNF in synaptic plasticity

Long-term potentiation (LTP) is a cellular model of associative learning and corresponds to a long-lasting enhancement of excitatory transmission upon a high-frequency synaptic stimulation of specific afferents (Bliss & Collingridge 1993). Three phases can be divided in the LTP process: short-term potentiation, early-LTP and late-LTP. Short-term potentiation and early-LTP are the first to occur and are both independent of gene transcription and translation mechanisms. Early-LTP induction depends on an intracellular Ca^{2+} concentration rise, through NMDAR channel opening, with subsequent activation of PKC and Ca^{2+}/Calmodulin-dependent protein kinase II (CAMKII). PKC-mediated phosphorylation of AMPARs increases AMPAR membrane expression, adding a contribution for early-LTP maintenance. Moreover, early-LTP also correlates with enhanced neurotransmitter release from synaptic terminals. Late-LTP in turn depends on local dendritic synthesis and nuclear transcription, in which PKA and MAPK signaling cascades play a central role (Minichiello 2009).

During the LTP process, the increase in synaptic activity leads to an increase in both BDNF expression (Patterson et al. 1992) and release (Goodman et al. 1996). Regarding the evidence that BDNF increases excitatory signaling by simultaneously increasing the release of glutamate and the activity of glutamate NMDAR, BDNF (via TrkB receptor activation) has been proposed as a net regulator of LTP. In fact, mice lacking the *bdnf* or *trkB* gene, display deficits in LTP (Korte et al. 1995; Minichiello et al. 1999), and BDNF treatment of hippocampal slices taken from BDNF knockout mice, rescued LTP (Patterson

et al. 1996). Additionally, BDNF was shown to facilitate LTP in the hippocampus (Figurov et al. 1996; Chen et al. 1999; Fontinha et al. 2008), being both pre- and post-synaptic TrkB receptors, via PLCY activation, involved in this plasticity phenomenon (Gärtner et al. 2006; Gruart et al. 2007). Inhibitors of protein synthesis are able to block late-LTP (Frey et al. 1988; Kang & Schuman 1996) and BDNF contribution for LTP also accounts with protein synthesis dependent mechanisms that sustain the synaptic changes observed in the later phase of this plasticity phenomena. BDNF promotes the phosphorylation of transcriptional factors, as well initiation and elongation translation factors, enhancing their activity and thus, transcription and translation rates, respectively (Takei et al. 2009; Inamura et al. 2005).

1.3.5 BDNF and epilepsy

BDNF increases neuroexcitability playing a fundamental role in synaptic transmission and LTP. However, BDNF excessive signaling may contribute for neuronal excitotoxicity by a positive feedback loop: BDNF increases glutamatergic transmission, enhancing neuronal activity, which in turn increases *bdnf* gene expression (Patterson et al. 1992) and BDNF release (Goodman et al. 1996), propagating BDNF effects. BDNF can thus lead to pathological states where there is an imbalance of excitatory transmission towards inhibitory, such as epilepsy.

In different epileptic models, convulsive activity stimulate BDNF and TrkB receptors expression, namely in the hippocampus, where increased mRNA and protein levels were observed (Isackson et al. 1991; Mudò et al. 1996; Nawa et al. 1995). Interestingly, the increase in BDNF protein levels persisted for longer than the increase in the respective coding mRNA (Nawa et al. 1995), suggesting a post-transcriptional regulation mechanism underlying BDNF up-regulation. TrkB activation relies on BDNF binding to the receptor and subsequent auto-phosphorylation in the tyrosine kinase domain. TrkB activation revealed by phospho-trk immune-reactivity is also increased followed the induction of seizures (He et al. 2002).

Overall, taking in account the ability of BDNF to enhance excitatory transmission and the increased BDNF/TrkB expression and signaling after seizures, BDNF was proposed as a great contributor for epileptogenesis (Figure 1.2). Corroborating this hypothesis, several

genetic manipulation studies relate BDNF/TrkB to epileptogenesis. BDNF knockout (Kokaia et al. 1995) as well reduced BDNF signaling by truncated TrkB overexpression (Lähteinen et al. 2002), prevented the development of epilepsy. On the contrary, the development of epileptic seizures was facilitated by overexpression of BDNF (Croll et al. 1999) and TrkB signaling (Heinrich et al. 2011). Nevertheless, the underlying mechanism by which BDNF contributes for epilepsy is still not fully understood.

In epilepsy, hippocampal circuits undergo anatomical reorganization and particularly mossy fibers branch out to make new excitatory synapses with dendrites of granule cells, a process called mossy fiber sprouting. In a study reported by Koyama and colleagues (2004), BDNF was shown to induce abnormal mossy fiber sprouting, contributing for the formation of these hyperexcitable circuits that are believed to serve as epileptic foci (Koyama et al. 2004). Direct effects of BDNF on synaptic transmission were also observed, since when hippocampal slices are acutely exposed to BDNF, it causes hyperexcitability, in particular upon mossy fiber stimulation (Scharfman 1997). In hippocampal slices taken from epileptic mice, a BDNF-mediated enhancement of synaptic transmission was also observed (Scharfman et al. 1999).

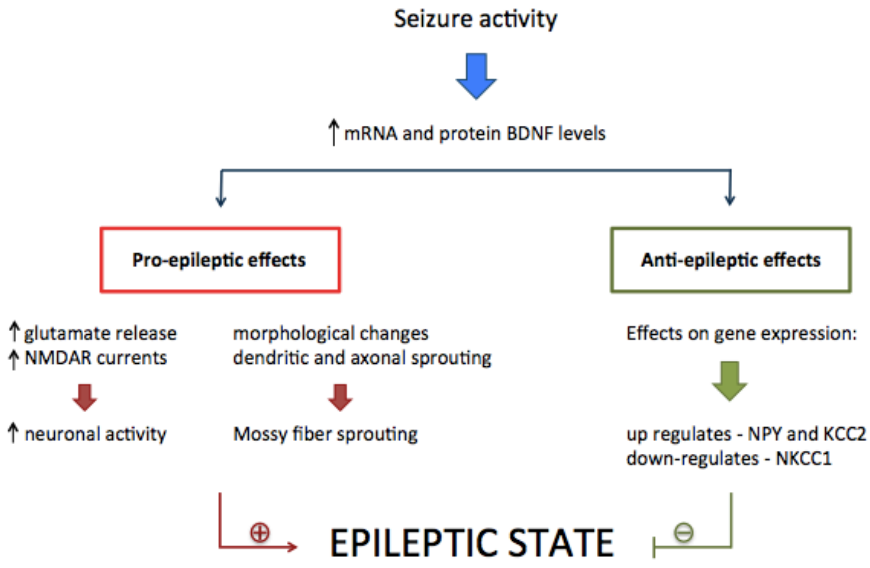

Figure 1.2: Promoting and preventing BDNF effects on epilepsy. There is still a controversy in literature whether BDNF is a pro- or anti-epileptic agent, nonetheless seizure activity is known to increase BDNF expression at mRNA and protein level. Pro-epileptic effects account with BDNF ability to increase glutamatergic transmission, both at pre- and post-synaptic level and to promote aberrant excitatory circuits that operate as epileptic foci. Anti-epileptic effects rely on BDNF modulation of gene expression. BDNF increases NPY expression, which is an anti-epileptic neuromodulator. Cl^- co-transporters expression is also modified by BDNF, being KCC2 positively and NKCC1 negatively modulated, which may allow the reestablishment of hyperpolarizing and thus, inhibitory $GABA_A$-mediated currents.

In opposition to the data previously described, BDNF has also been reported as an anti-epileptic factor (Figure 1.2). Reibel and colleagues performed chronic infusions of BDNF in the hippocampus during the first week of kindling, which strongly suppressed seizures. The proposed molecular mechanism concerning the anti-epileptic BDNF effect is thought to be attributable to the regulation of gene expression (Reibel et al. 2003). BDNF increases Neuropeptide Y (NPY) expression (Croll et al. 1994), which in turn inhibits seizure generation (Baraban et al. 1997).

In the mature brain, upon GABA binding to $GABA_A$ receptors, because of low intracellular level of Cl^-, a passive Cl^- influx that accounts for cell hyperpolarization occurs. However, in epileptic brain, Na-K-2Cl co-transporter (NKCC1, mediates Cl^- influx) expression is up-regulated while K-Cl co-transporter (KCC2, extrudes Cl^-) expression is down-regulated, leading to increased Cl^- intracellular concentration. This increase results on a switch of $GABA_A$ current from hyperpolarizing to depolarizing and thus, from $GABA_A$ inhibitory to $GABA_A$ excitatory actions on synaptic transmission that may contribute for the increased excitability seen in epilepsy. BDNF injections during the latent phase of epilepsy were shown to have anti-epileptic effects by increasing KCC2 and decreasing NKCC1 expression. Concomitantly, hyper-excitability behaviors were ameliorated and spontaneous recurrent seizures were delayed (Eftekhari et al. 2014).

1.4 | Adenosine

Being a purine nucleoside, adenosine (Figure 1.3) is an essential constituent of any living cell, playing a pivotal role in the synthesis of nucleic acids, intracellular energy transfer (as its phosphorylated forms, ATP and AMP) and signal transduction.

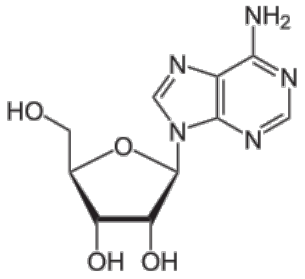

Figure 1.3: Chemical structure of adenosine. Adenosine is composed by adenine, a nucleoside, attached to a ribose sugar molecule.

Mediating its effects through cell membrane receptors, adenosine is involved in important physiological functions, particularly in excitable tissues where it mostly has inhibitory actions, such as in the heart and the brain. For instance, adenosine was first described as having inhibitory actions on the cardiovascular system, by both slowing the heart rate and increasing arterial dilatation (Drury & Szent-Györgyi 1929).

Despite the ubiquitous expression of adenosine and its receptors throughout the body, attention to its regulatory role in the central nervous system was only given in the early 70's. Observations that adenosine increases the accumulation of cAMP in cortical slices (Sattin & Rall 1970), that is released from electrically stimulated cortical preparations (Pull & McIlwain 1972) and that it depresses cortical neurons firing (Phillis et al. 1974), were starting points for an increasing interest about the neuromodulatory role of adenosine.

Adenosine is now widely accepted as a key modulator of neurotransmission. As such, adenosine is neither stored nor released in synaptic vesicles as a classical neurotransmitter and it does not have direct effects on synaptic transmission; it rather influences neuronal communication by influencing neurotransmitter release at pre-synaptic level; post-synaptically, it can modulate the activity of receptors for neurotransmitters and other neuromodulators, and non-synaptically it can also modulate glial cell activity (Sebastião & Ribeiro 2009).

Because adenosine acts as a fine-tuner of synaptic transmission, it has a key role in the homeostatic control of the nervous system activity. When this homeostasis is disrupted, there is an abnormal neuronal communication that can lead to several pathologies of the central nervous system, such as epilepsy, anxiety and Alzheimer's disease. To the re-establishment of neuronal homeostasis in those pathological conditions, adenosine mediated modulation of neurotransmitter receptors or transporters might be used as a useful therapeutic strategy (Lopes et al. 2011).

1.4.1 Adenosine receptors

Adenosine binds specifically to the family of receptors named P1 receptors which is composed of four protein members: the high affinity adenosine A_1 and A_{2A} receptors (A_1R and $A_{2A}R$), and the low affinity adenosine A_{2B} and A_3 receptors ($A_{2B}R$ and A_3R). All the four

proteins have been identified and cloned in several species, such as mouse, rat, rabbit and human (Fredholm et al. 2000; Fredholm et al. 2001). They are classified as G protein-coupled receptors (GPCRs), being characterized by seven trans-membrane domains and by their coupling to a G-protein that can either lead to excitatory (G_s/G_{olf} proteins) or inhibitory effects (G_i/G_o proteins). Adenylate cyclase (AC) is the main effector of the G proteins coupled to the adenosine receptors. It catalyses the conversion of ATP to cyclic AMP (cAMP), which in turn activates PKA, leading to the activation of several signaling cascades. In what concerns adenosine receptor coupling, A_1R and A_3R are mostly coupled to G_i/G_o proteins, which inhibits the activation of AC; while A_{2A}R and A_{2B}R are mostly coupled to G_s/G_{olf} proteins, leading to the activation of AC (Fredholm et al. 2000; Fredholm et al. 2001).

In the brain, the adenosine high affinity receptors, A_1R and A_{2A}R, represent the most relevant receptors for adenosine-meditated effects. Adenosine A_1R is the most highly conserved receptor subtype between species and it is abundantly expressed in cortex, hippocampus and cerebellum. Adenosine A_{2A}Rs are mostly present in the striatum pallidal GABAergic neurons and olfactory bulb, despite its expression in other brain structures at lower levels, such as the hippocampus (Figure 1.4). The A_{2B}R is also widely distributed in the brain but functional studies suggest that these receptors may only be relevant in pathological conditions, when the extracellular adenosine levels are elevated. The A_3Rs are expressed at intermediate level in the human cerebellum and hippocampus and at lower levels in other brain regions (Dixon et al. 1996; Fredholm et al. 2000; Ribeiro et al. 2003).

Aside from differential distribution of adenosine receptors in the brain, changes in its expression levels across ageing have been also suggested in several studies. For instance, with ageing (rats older than 18 months) there is a decrease in A_1R expression in cerebral cortex, hippocampus and cerebellum (Cheng et al. 2000; Pagonopoulou & Angelatou 1992; Cunha et al. 1995) and a decrease in A_1R signaling (Sebastião et al. 2000), even though an increase in G-protein coupling of A_1Rs was described (Cunha 2001). On the contrary, A_{2A}R expression levels increase in cortex and hippocampus along lifetime (Cunha et al. 1995).

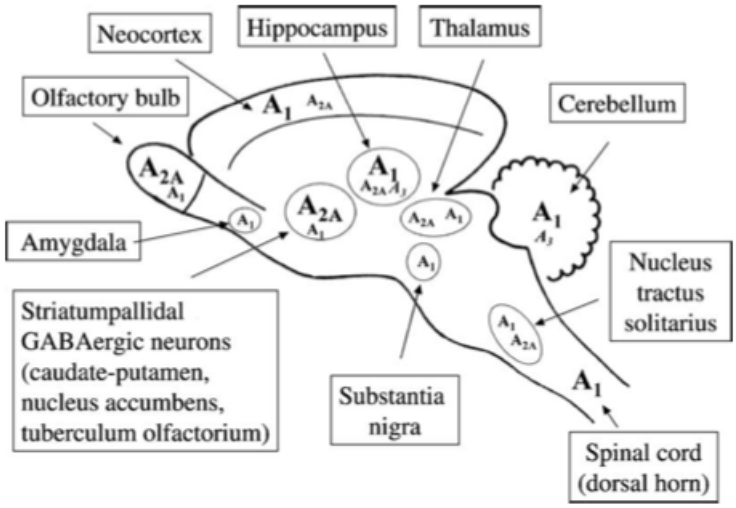

Figure 1.4: Distribution of adenosine receptors in the main regions of central nervous system. Size of alphabets is accordingly to the levels of protein expression (Ribeiro *et al.*, 2003).

Assessment studies on sub-cellular location of adenosine receptors suggest their predominant presence on the synapse. Adenosine A_1R is densely located both in pre- and post-synaptic membranes and adenosine $A_{2A}R$ is mostly a pre-synaptic receptor, apart from the striatum neurons, where it is mostly present at extra- and post-synaptic membrane (Rebola, Canas, et al. 2005). Even though, the primary location of these receptors is neuronal, both are also located in astrocytes and microglia (Cunha 2005).

The existence of two adenosine receptors with opposite effects, the inhibitory A_1R and the excitatory $A_{2A}R$, raised the question about how the cell would sense the needs of the system and decide which receptor should be preferentially activated. Data point towards the conclusion that at lower frequencies stimulation and thus low extracellular adenosine concentrations, inhibitory A_1R is predominantly activated. On the contrary, at higher frequencies stimulation and high levels of extracellular adenosine, excitatory $A_{2A}R$ is preferentially activated (Correia-De-Sá & Ribeiro 1996).

Desensitization of adenosine receptors can either occur by agonist-induced receptor internalization, resulting in a decrease in the expression at membrane level, or through modulation of its activity by upstream molecules, such as kinases of the receptor, that inhibit them. Adenosine A_1Rs are resistant to desensitization, since their internalization occurs in a time scale of hours upon continuous exposure to agonists (Hettinger et al. 1998; Ruiz et al. 1996). They can, however, be modulated by other proteins, such as PKA and PKC, which was shown to attenuate A_1R inhibition of glutamate release and to

decrease the affinity of A_1Rs (Mundell & Kelly 2011). Since A_{2A}Rs are also coupled to both enzymes, it is a potential candidate as an A_1R modulator. Accordingly, activation of A_{2A}R attenuates A_1R inhibition of hippocampal transmission and decreases A_1R binding (Cunha et al. 1994; Lopes et al. 1999; Lopes et al. 2002). In what concerns A_{2A}R desensitization, the mechanism is faster (30 to 45 minutes), without change in receptor number or affinity but maybe in adenylyl cyclase activity (Ramkumar et al. 1991; Chern et al. 1993).

1.4.2 Adenosine control of neurotransmission

The existence of several adenosine receptors with different G-coupled proteins, with different distributions in the brain and synaptic sites may suggest that adenosine can differently modulate synaptic transmission. In the brain, the net effects of adenosine are primarily mediated by its high affinity receptors, A_1R and A_{2A}R.

Due to its most predominant receptor, A_1R, adenosine refrains excitatory signaling and thus, by reducing neuronal firing, it is seen as protective of neuronal networks. A_1Rs are mostly abundantly present in excitatory synapses, where they have a largest effect, rather than inhibitory synapses (Yoon & Rothman 1991). The major mechanism by which A_1R can inhibit synaptic transmission is pre-synaptic, through inhibition of Ca^{2+} channels and a consequent reduction of glutamate probability release (Gundlfinger et al. 2007; Wu & Saggau 1994), the main excitatory neurotransmitter present in the brain. Nevertheless, A_1Rs can also have post-synaptic effects by hyper-polarization of the cell through activation of K^+ channels (Takigawa & Alzheimer 2002), and by inhibiting glutamate NMDA receptor activity (De Mendonça et al. 1995). In a recent study, A_1R was also shown to regulate neuroexcitability in hippocampus through selective inhibition of extrasynaptic $GABA_A$R currents, responsible for tonic cell inhibition, in pyramidal cells and in a specific subpopulation of interneurons, expressing Cannabinoid receptor type 1 (Rombo et al. 2014). Additionally, in the striatum, A_1Rs modulate dopaminergic responses. Dopamine D1 receptor (D1R) facilitates GABA release, however, upon A_1R activation, this effect is attenuated (Mayfield et al. 1999). Therefore, by A_1R/D1R heteromerization (Fuxe et al. 1998), A_1R inhibits D1R and thus, reduces GABAergic transmission.

Adenosine A_{2A}R is generally coupled to a G_s protein, with exception in striatum, where is mainly coupled to G_{olf} (Fredholm et al. 2000). Adenosine binding to the A_{2A}R leads to AC

activation, which in turn activates PKA that will lead to the initiation of several signaling cascades that culminate in the increase of intracellular Ca^{2+} concentration (Fredholm et al. 2001). In addition to PKA activation, $A_{2A}R$-mediated facilitatory effects on neuronal activity may also result from PKC signaling pathway activation, as demonstrated in hippocampal neurons (Cunha & Ribeiro 2000). In opposition to A_1R role, $A_{2A}R$ signaling was shown to increase glutamate release (Rodrigues et al. 2005) and to facilitate NMDA (data not published) and AMPA currents (Dias et al. 2012). $A_{2A}Rs$ also facilitate excitatory transmission by enhancing GABAergic signaling selectively between interneurons, but not on GABAergic synapses to pyramidal cells, contributing to an overall disinhibition of principal neurons (Rombo et al. 2015). $A_{2A}R$ can additionally modulate inhibitory systems. Activation of $A_{2A}R$ is necessary for endocannabinoid CB1 receptor effects (Tebano et al. 2009); moreover, $A_{2A}Rs$ inhibit A_1R actions either through PKC-mediated desensitization (Lopes et al. 2002) or $A_{2A}R$-A_1R heteromerization (Ciruela et al. 2006). By heteromerization with Dopamine D2 receptors (D2Rs), $A_{2A}R$ can also antagonize D2R function (Hillion et al. 2002).

Non-synaptically, $A_{2A}Rs$ also play an important role in neuromodulation by inhibiting glutamate (Matos et al. 2013) and increasing GABA uptake into astrocytes (Cristóvão-Ferreira et al. 2013).

$A_{2A}Rs$ also play an essential role in the control of synaptic plasticity (Costenla et al. 2011) and this may result from the regulation of other neuromodulators activity. Adenosine $A_{2A}Rs$ facilitates BDNF-mediated facilitatory effects on synaptic transmission (Diógenes et al. 2004) and on long-term potentiation at the CA1 area of the hippocampus (Fontinha et al. 2008). $A_{2A}R$ activation increases TrkB translocation to lipid rafts (Assaife-Lopes et al. 2014), membrane microdomains thought to concentrate signaling molecules and facilitate signal transduction (Sebastião et al. 2013); by doing so, $A_{2A}Rs$ potentiate BDNF-induced phosphorylation of TrkB receptors and subsequent modulation of hippocampal plasticity (Assaife-Lopes et al. 2014).

1.4.3 Adenosine metabolism

Adenosine can be formed by two main enzymatic cascades (Figure 1.5): *1)* S-adenosil-homocysteine (SAM) hydrolysis, a reaction catalyzed by SAM Hydrolase (Palmer 1979), a

process that occurs intracellularly and *2)* successive ATP dephosphorylations into adenosine, via intracellular (Endo-5'-) and extracellular (Ecto-5'-) nucleotidases (Meghji 1993)

Neurons act as a source of adenosine, by releasing ATP stored in synaptic vesicles and subsequent catalysis in the synaptic cleft (Di Iorio et al. 1988), but also by direct release of adenosine (Lovatt et al. 2012). Nevertheless, astrocytes were suggested as a major contributor to extracellular adenosine level. Pascual and colleagues (2005) used a transgenic mice model with an inducible astrocyte-selective dominant-negative SNARE domain, a mutation that impairs ATP release from glia cells. Functional studies on these mice suggest that ATP released by astrocytes is crucial for a tonic suppression of glutamatergic transmission and, thus, their importance for synaptic transmission modulation (Pascual et al. 2005).

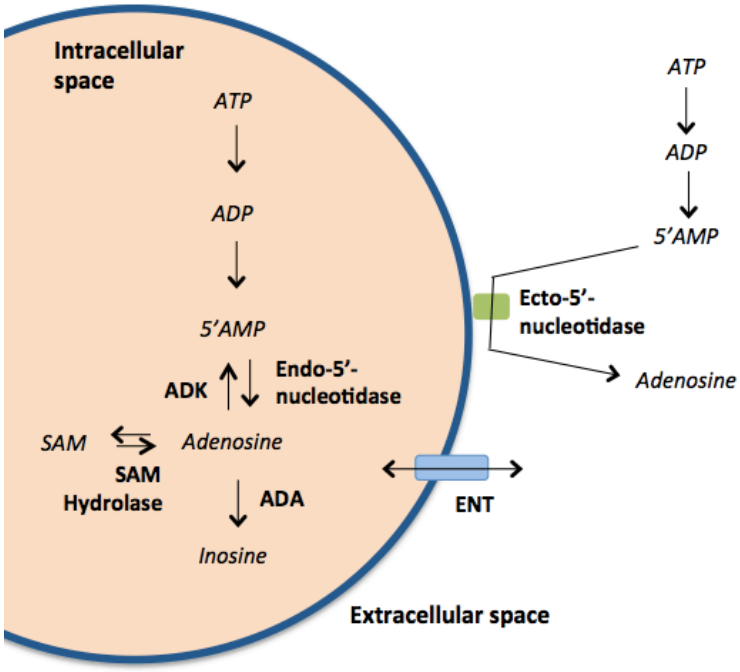

Figure 1.5: Intra and extracellular metabolism pathways of adenosine. Adenosine is synthesized both inside and outside the cell, by ATP catalysis or SAM hydrolysis. Extracellular adenosine concentration is tightly regulated by bi-directional equilibrative nucleoside transporters and by the enzymes, ADK and ADA.

Beside ATP hydrolysis, another extracellular adenosine source can be accounted. Adenosine can be released from neurons and glia by facilitated diffusion. This process is mediated by Equilibrative Nucleoside Transporters (ENTs), that can both transport adenosine into and out of the cell, depending on adenosine concentration gradient across the cell membrane (Latini & Pedata 2001). Usually, a higher concentration of adenosine

can be found in the extracellular space, where it is estimated that the extracellular adenosine concentration is around 140-200 nM (Dunwiddie & Diao 1994), so that the direction of transport occurs from outside into the cell, where it will occur adenosine metabolic clearance. ENTs have a profound effect on adenosine extracellular concentration and thus in adenosine signaling. For instance, ENTs blockers resulted in inhibition of neuronal activity (Motley & Collins 1983) and inhibition of seizures in an amygdala kindling model (Dragunow & Goddard 1984).

Interestingly, different adenosine sources relate with adenosine receptor preferential activation. While adenosine resulting from extracellular ATP breakdown preferentially activates A_{2A}Rs, adenosine transported through ENT will preferentially activate A_1Rs (Cunha et al. 1995; Correia-De-Sá & Ribeiro 1996).

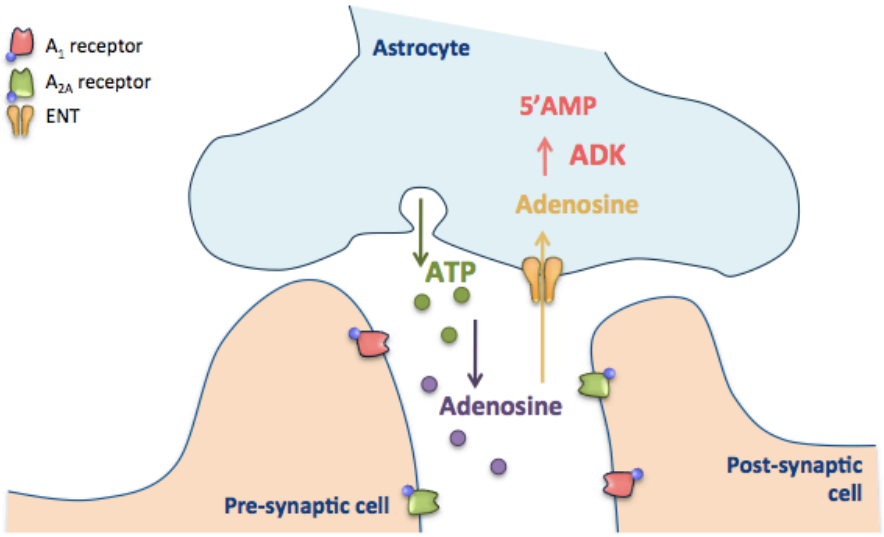

Figure 1.6: Astrocytic-based adenosine cycle. Astrocytes are a major source of adenosine through the ATP vesicular release to the synaptic cleft and, at the same time, is the main responsible for the control of ambient adenosine levels by ADK-mediated phosphorylation. Adenosine phosphorylation creates a concentration gradient that allows adenosine influx to the astrocytes and clearance from the synaptic cleft.

Being such a delicate tuner of synaptic transmission, it is of great importance to ensure a tight control of the extracellular concentration of adenosine. Astrocytes not only have a role as an adenosine source, as they also are greatly involved in the regulation of extracellular adenosine homeostasis, making them crucial regulators of adenosine tonus in synaptic transmission (Figure 1.6). Adenosine kinase (ADK), an enzyme that phosphorylates adenosine into 5'AMP is highly expressed in astrocytes and it is the key regulator of adenosine clearance from the synaptic cleft. Adenosine deaminase (ADA) metabolizes adenosine to inosine, also participating in adenosine degradation, but it is

thought to have a lesser contribution since ADA has a higher K_d than ADK (2 μM *vs.* 14-45 μM). Moreover, inhibitors of ADK increased endogenous adenosine levels and decreased synaptic transmission in hippocampal slices, while no effect of ADA inhibitors were observed (Pak et al. 1994).

1.4.4 Adenosine kinase

From microorganisms to animal cells, *Adk* gene is highly conserved and ubiquitously expressed in rat and human tissues. *Adk* gene encodes two ADK proteins which differ from their N-terminal, being the first 4 residues from the lighter isoform (38.9 kDa) replaced by 21 other residues on the heavier isoform (40.5 kDa) (McNally et al. 1997). Additionally to the sequence, these two isoforms also differ in their function and subcellular location. The long isoform is mainly located at the nucleus and it is thought to act as an epigenetic regulator by modulating methyltransferase reaction, whereas the short isoform is cytoplasmic and it is involved in the maintenance of extracellular adenosine levels (Boison 2013).

The pattern of ADK expression shifts during development from predominant neuronal nuclear ADK, suggesting a supportive role of the enzyme in neuronal development by providing adenosine to acid nucleic synthesis, to primarily astrocytic expression in the cytoplasm, where ADK by metabolizing adenosine, regulates adenosine tonus in synapses (Studer et al. 2006).

ADK activity has profound effects on extracellular adenosine concentration and synaptic inhibitory tonus in an A_1R-dependent manner (Diógenes et al. 2014; Etherington et al. 2009). An *in vivo* study using adenosine microelectrode biosensors actually showed an inverse relation between ADK expression and adenosine levels: where ADK expression was increased, ambient adenosine was significant lower (50%); on the contrary, where ADK expression was decreased, higher adenosine concentration was found (163%) (Shen et al. 2011). Furthermore, ADK overexpression is associated with increased susceptibility to epileptic seizures (Fedele et al. 2005) and injury after stroke (Shen et al. 2011), making it as an interesting pharmacological target for neuroprotective therapies.

1.4.5 ADK hypothesis of epileptogenesis

The major effect of adenosine in the central nervous system is to inhibit excitatory transmission and refrain neuroexcitability, through A_1R activation. Adenosine is thus proposed as a critical endogenous anticonvulsant substance. Administration of an A_1R agonist can effectively suppress and prevent seizures in amygdala-kindled rats (Abdul-Ghani et al. 1997). Taking advantage of a knockout A_1R mice model, a neuroprotective role of A_1R thought to prevent the progression of the epilepticus status was demonstrated (Fedele et al. 2006).

Given the accumulated evidence that ADK protein levels and activity tightly regulate extracellular adenosine concentration and thus, adenosine availability for the control of neuroexcitability, it has been hypothesized that ADK disturbances are related to pathological states. For instance, epilepsy animal models relate with astroliogiosis accompanied by astrocytic ADK up-regulation (Aronica et al. 2011) and adenosine deficient signaling in the hippocampus (Rebola et al. 2003). Additionally, ADK inhibitors, by enhancing adenosine tonus, act as antiepileptic molecules (Wiesner et al. 1999; Gouder et al. 2004).

ADK expression is down-regulated during the first 24 hours after induction of seizures, maybe in an attempt to counteract neuronal excitability (Gouder et al. 2004). However, one week after electrical induction of status epilepticus, there is an up-regulation of ADK, which persisted for 3 to 4 months (Aronica et al. 2011). Whether this up-regulation is associated to epileptic activity was then evaluated. In a kainate acid (KA)-induced mesial temporal lobe epilepsy mouse model, ADK was suggested to directly contribute to an epileptogenic state. Thus, KA-injected mice exhibited astrogliosis in the hippocampus and a great increase in ADK protein expression and enzymatic activity shortly after status epilepticus. Notably, inhibition of adenosine clearance by the administration of an ADK inhibitor blocked seizure activity (Gouder et al. 2004). Further evidence demonstrate that ADK up-regulation and A_1R impaired signaling are both independently capable of inducing spontaneous seizures, since different mice models, either ADK over-expressing or A_1R knockout, showed seizure patterns similar to the KA-injected mice (Li et al. 2007; Fedele et al. 2005). Interestingly, A_1R knockout mice neither develop astrogliosis nor ADK up-regulation. This may suggest that high levels of ADK are the cause of spontaneous

epileptic seizures probably caused by A_1R down-regulation (Li et al. 2007). On the contrary, reduced levels of ADK are translated into reduced KA-induced seizure susceptibility, which can be prevented by prior application of A_1R antagonist was done (Li et al. 2008).

Besides ADK up-regulation, cerebral cortex from epileptic rats demonstrate a decreased A_1R expression and an increased $A_{2A}R$ expression, which also might contribute for a dysfunctional adenosinergic signaling and thus, to a pathological state of excessive neuroexcitabilibity (Rebola, Porciúncula, et al. 2005).

Since astrocytes also express adenosine receptors, alterations in the ADK/adenosinergic system also translate in astrocytic functional consequences. Adenosine A_1R is described as having an inhibitory role on astrocytes proliferation *in vitro* (Ciccarelli et al. 1994), while $A_{2A}R$ has the opposite action, increasing it (Brambilla et al. 2003). Thus, astrocytic adenosine receptors may play an important role in the development of epilepsy, where $A_{2A}R$ up- and A_1R down-regulation may favor astrogliosis.

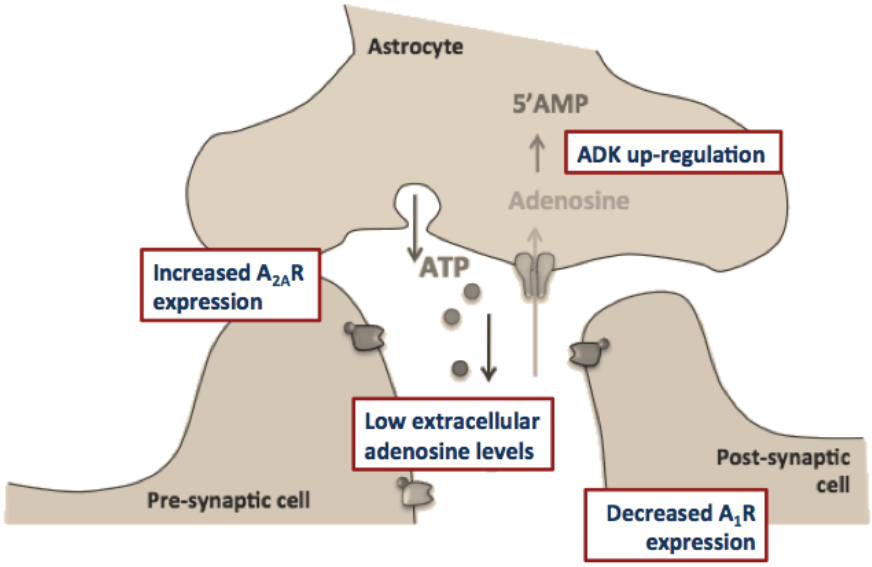

Figure 1.7: Adenosine kinase hypothesis of epileptogenesis. Adenosine kinase is a key regulator of extracellular adenosine levels and, thus, adenosine availability to refrain neuronal excitability. ADK expression thus determines brain susceptibility to seizure generation. In fact, ADK up-regulation in epilepsy is related to a decreased adenosinergic tonus and A_1R-mediated signaling. Additionally, seizure activity decreases A_1R and increases $A_{2A}R$ expression, which may account for the imbalance in synaptic transmission, neuroinflamation and astrogliosis, the major hallmarks of epilepsy.

Summarizing, ADK hypothesis for epileptogenesis relies on the observation that an ADK up-regulation determines lower adenosine extracellular levels and decreased A_1R activity (**Figure 1.7**), with consequences for network activity homeostasis, leading to higher brain

susceptibility for seizures. Recognizing the adenosine system as a major contributor to epileptogenesis, adenosine augmentation has a great potential to be used as a therapeutic approach. For example, studies attempting to restore extracellular adenosine levels by transplantation of biodegradable adenosine loaded silk based polymers (Wilz et al. 2008) or A_1R signaling by applying a ketogenic diet (Masino et al. 2011), showed to be an effective anticonvulsant strategy.

1.5 | Hippocampus

The hippocampus was baptized as such by Giulio Cesare Aranzi due to the resemblance of the shape of this brain structure to the shape of the tropical seahorse fish, *Hippocampus leria*. In the human and rodents brain, there are two hippocampi, one in each hemisphere. It is a limbic structure deep buried within the medial temporal lobe of the brain (Figure 1.8A). Structurally, is characterized by two interlocking C-shaped cell layers, reversed to each other (Figure 1.8B). The Hippocampus proper is constituted by "*Cornu Ammonis"* (CA) and the other "C" constitutes de Dentate Gyrus (DG) (Lopes da Silva et al. 1990). *Cornu Ammonis* is divided into CA1, CA2 and CA3 areas, which in turn are made up of seven layers (Figure 1.8C): *stratum moleculare*, *stratum lacunocum*, *stratum radiatum*, *stratum pyramidale*, *stratum oriens*, *alveus* and *epithelium*. A main excitatory and unidirectional circuit in the hippocampus involving three pathways is found (the so-called trisynaptic circuit). First, the hippocampus receives electrical inputs from cortical areas through entorhinal and perirhinal cortex, which project to DG through the perforant path. The granule cells from DG then project, via mossy fibbers, to CA3 area. Finally, via Schaffer collaterals, CA3 pyramidal cells project to the CA1 area (Andersen et al. 2007).

Concerning the function of the hippocampus, it plays critical roles in cognitive tasks and processing of information. For instance, it is involved in the formation of new memories, learning and spatial memory and orientation. However, it is also greatly involved in epilepsy. The first observations relating epilepsy and the hippocampus were made in the beginning of the XIX century. Hippocampus from patients with chronic epilepsy presented

gliosis and pyramidal cell loss in CA1 region. Whether this is a consequence of or a cause for seizure development is still a matter of debate.

The hippocampus is quite easy to find and dissect intact from the brain. When cut in transverse slices (perpendicular to the septal-temporal axis), the laminar and circuitry structure described above are preserved. Moreover, it is possible to maintain hippocampal acute slices healthy and surviving for several hours. All this features makes the hippocampus a very useful structure to perform functional studies and to give a better comprehension about cellular and network properties (Andersen et al. 2007).

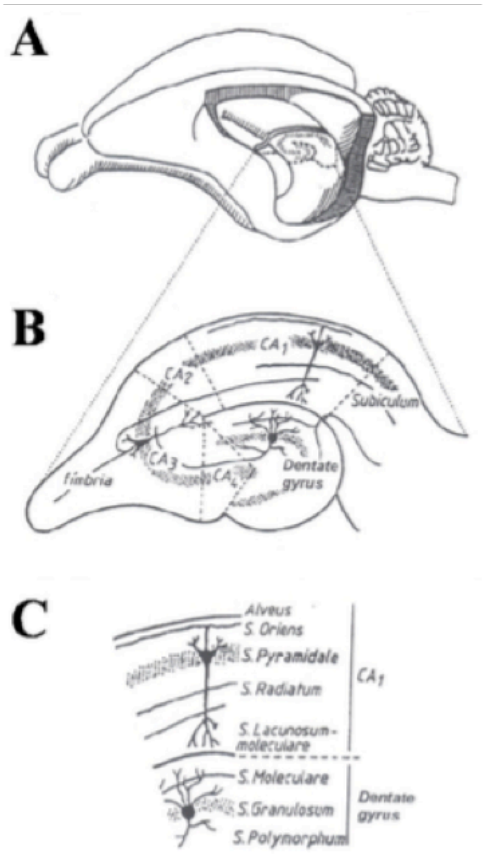

Figure 1.8: The hippocampal formation. ***(A)*** Localization of hippocampus in the brain. ***(B)*** Schematic representation of a transversal slice. ***(C)*** Hippocampal slice section, including CA1 and DG regions (Filipe 1991).

2 | MAIN GOALS

Considering *1)* the relevance of adenosine for epilepsy control, *2)* the ability of adenosine to influence BDNF signaling, *3)* the influence of BDNF in epilepsy and *4)* the role of ADK in the control of adenosine levels, the work described in this thesis aimed to characterize the role of adenosine and BDNF in the control of neuroexcitability at the hippocampus. This work was divided in two chapters, each of one having the specific goals:

- To **address a putative modulation by BDNF of inhibitory GABAergic transmission in the adult hippocampus and to assess whether the activation of adenosine A_{2A} receptors could influence BDNF action onto inhibitory signaling**. The relevance of this work relies on the importance of GABAergic transmission for epilepsy control and the absence of information on the ability of BDNF to modulate GABAergic signaling in the adult brain.

- To **evaluate the impact of ADK deletion in the adenosinergic and BDNF signaling, as well as in the levels of their receptors.** This research line has as hypothesis that an imbalance in ADK function would modify homeostatic control of synaptic transmission and plasticity, leading to the epileptic phenotype in mice lacking ADK in the entire brain.

3 | METHODS

Adult (6 to 10 weeks-old) male Wistar rats, and two different genotypes of C57BL/6 mice were used in these studies: $Adk^{fl/fl}$ and $Adk^{\Delta brain}$ (lacking ADK expression in the entire brain) mice. Our collaborators, from Detlev Boison's lab (Portland, Oregon, USA), generated these mice lines. The *Adk* gene targeting vector to produce global ADK knockout mice ($Adk^{tm1bois}$) has been fully described elsewhere (Boison et al. 2002; Fedele et al. 2004). Briefly, the targeting construct was reengineered and exon 7 of the *Adk* gene was flanked with loxP sites (Figure 3.1). Through homologous recombination in embryonic stem cells an $Adk^{fl/-}$ allele was created, which was used to generate a line of $Adk^{fl/fl}$ mice. $Adk^{fl/fl}$ mice were crossred with *Nestin-Cre* mice (The Jackson Laboratory, stock number: 012906) to generate Nestin-$Cre^{+/-}$:$Adk^{fl/fl}$ mice. Breeding of the experimental animals followed either a Nestin-$Cre^{+/-}$:$Adk^{fl/fl}$ mice X Nestin-$Cre^{-/-}$:$Adk^{fl/fl}$ mating protocol which generated ADK-deficient ($Adk^{\Delta brain}$) and normal mice ($Adk^{fl/fl}$) in a 1:1 ratio as littermates. All mice were generated and propagated on an identical C57Bl/6 background and were genotyped at weaning by PCR.

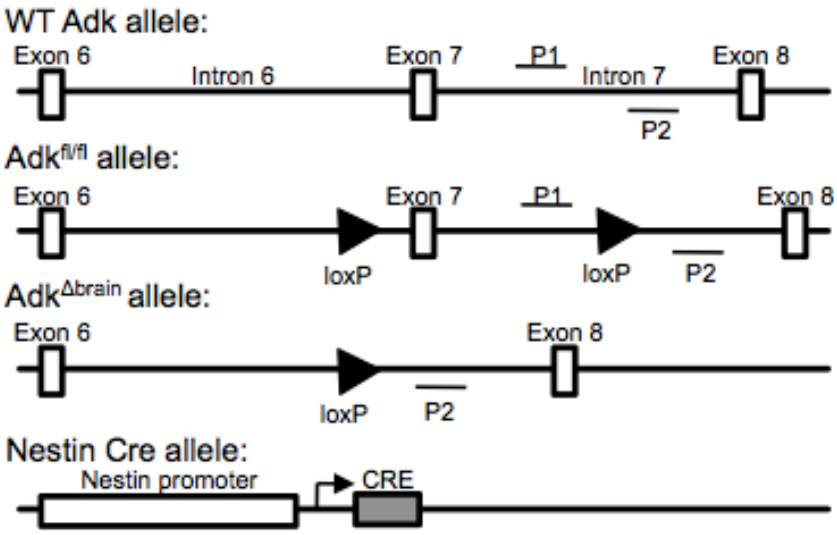

Figure 3.1: Illustration of transgenic strategy. Exon 7 of the Adk allele is flanked with loxP sites in $Adk^{fl/fl}$. CRE recombinase expression is under control of the nestin promoter.

All animals were kept under standardized conditions of light, temperature and humidity, and had access to food and water *ad libitum*. All animal procedures were carried out

according to the Portuguese Law and European Community Guidelines for Animal Care (European Communities Council Directive – 2010/63/EU).

3.1 | Animal Genotyping

Tissue from mice was removed by ear punching and 50 µL of TDB (1 M KCl, 1 M Tris pH 9.0, 10% Triton X-100, 20 ng/mL proteinase K) were added to the tissue sample, followed by overnight incubation at 56ºC. For proteinase K inactivation, samples were heated for 15 minutes at 95ºC and then frozen, until further use.

For DNA sequence amplification, 1 µL of each DNA sample was mixed with 1x Dream Taq Buffer, 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM Mix Primers (Reverse:; Forward:), 1.5 U DreamTaq DNA Polymerase (Thermo Scientific, 5 U/µL) and water to a final volume of 25 µL. The amplification reaction was based on a first heating at 94ºC for 3 minutes for enzyme activation, followed by 30 cycles of 3 discrete temperature steps of 30 seconds: 94ºC (for DNA denaturation), 61ºC (for primer annealing) and 72ºC (for DNA elongation). A final step of elongation of 10 minutes at 72ºC was carried, in order to ensure that all single stranded chains were fully extended. For genotyping Cre DNA sequence amplification with primer 5'- GGACATGTTCAGGGATCGCCAGGCG-3' and 5'- GGACATGTTCAGGGATCGCCAGGCG-3' was performed. For genomic Adk the primer sequences were: 5'-CCTCTATGAGTTGAGATCCTGTCTCC-3' and 5'-ATTTATTAACTTTACATAGATTCAGACAG-3'. The procedure was performed in the BioRad C1000 Thermal Cylcler. Both PCR products and DNA ladder (1 kb gene ruler, Thermo Scientific) were loaded in a 2% agarose gel dyed with RedSafe (Intron Biotechnology) to allow band visualization in a transiluminator (Molecular Imager Gel Doc XR System) and distinguish $Adk^{fl/fl}$ (Cre negative) from $Adk^{\Delta brain}$ (Cre positive) mice. PCR analysis validated the lack of functional *Adk*-alelle in cortex, striatum, hippocampus and cerebellum of $Adk^{\Delta brain}$ mice, whereas the Adk-gene in peripheral organs of $Adk^{fl/fl}$ mice and in all tissues from $Adk^{fl/fl}$ mice was not affected (Figure 3.2).

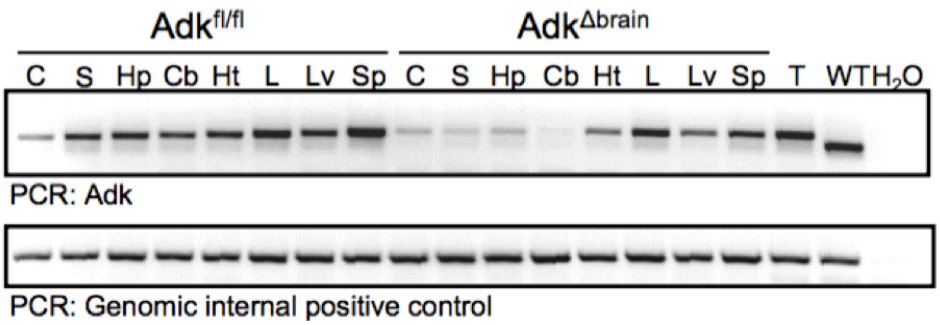

Figure 3.2: Brain specific deletion of ADK in nestin Cre positive Adk loxP transgenic mice. Adk PCR on genomic DNA extracts from the cortex (C), striatum (S), hippocampus (Hp), cerebellum (Cb), heart (Ht), lung (L), liver (LV) and spleen (Sp) from $Adk^{fl/fl}$ and $Adk^{\Delta brain}$ mice. Tail DNA from an $Adk^{fl/fl}$ mouse (T) and wild type (WT) were included as positive controls for the PCR reaction. Adk forward and reverse primer sites are depicted in panel A (P1 and P2). A genomic internal positive control PCR was run on the DNA extracts.

3.2 | Seizure quantification

Our collaborators from Detlev Boison's laboratory at Portland (USA) performed mice phenotype characterization. All mutant animals developed normally without any obvious phenotype until they reached an age of two months. However, from two months onwards and when exposed to novel environments (for instance, when placing mice in a novel environment, a clean mouse cage), $Adk^{\Delta brain}$ mice presented spontaneous convulsive seizures that last at least 5 minutes followed by prolonged partial non-convulsive seizure activity. Seizures were scored for occurrence, latency to onset, and recovery. This characteristic seizure phenotype was age dependent (Figure 3.3A) and progressive (Figure 3.3B) By three months of age, 44% of all mutant animals reacted with a seizure upon being placed in a novel environment, by four months of age the ratio of seizure response was at 78% and continued 100% by 6 months of age (Figure 3.3A). From 6 months of age on, mice loose their epileptic phenotype (and only 38% off all mutants reacted with seizures upon a stress stimuli) (Figure 3.3A).

EEG confirmed behavioral seizures in selected animals with a surface screw electrode surgically implanted above the left cortex while under general anesthesia (2% isofluorane, 100% O_2). Electrical brain activity was amplified (Grass Techonologies, West Warwick, RI) and digitized (PowerLab, AD Instruments, Colorado, CO). EEG seizure activity was defined as high-amplitude rhythmic discharges that clearly represented a new pattern of tracing. A representative cortical electroencephalogram of a confirmed convulsive seizure demonstrates 22 minutes of sustained seizure activity is shown in Figure 3.3C. The seizure

had a distinct onset (Figure 3.3D, upper trace) and spikes with increased amplitude and change in frequency throughout the seizures duration (Figure 3.3D, lower trace).

Seizures in mice born in Lisbon were characterized only by visual inspection.

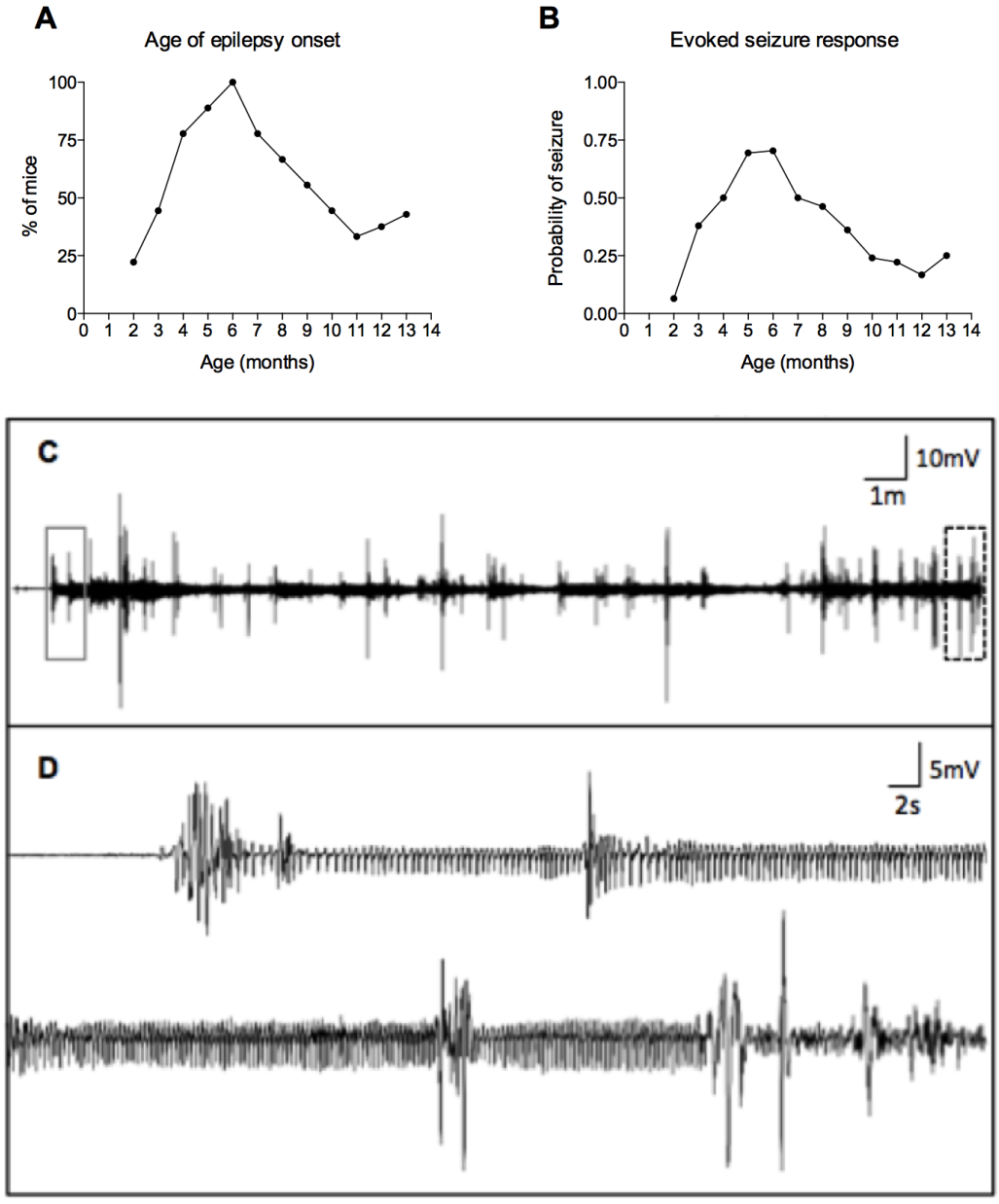

Figure 3.3: Loss of brain ADK results in progressive epilepsy. ***(A)*** Age of epilepsy onset was indexed as the percentage of $Adk^{\Delta brain}$ mice displaying the seizure phenotype in relationship to age (months). ***(B)*** Epilepsy progression in $Adk^{\Delta brain}$ mice indexed as the probability of evoking a seizure with an acute stressor in relationship to age (months). $Adk^{\Delta brain}$ mice (n=9) were tested for evoked seizure response on a weekly basis from 5 to 52 weeks of age for panels A and B. ***(C)*** Representative cortical EEG of a confirmed convulsive seizure in an $Adk^{\Delta brain}$ mouse with electrographic seizure activity that lasts 22 minutes. ***(D)*** EEG trace of the seizure from panel D that depicts the seizure start (upper) and end (lower) at a smaller time scale. The start and end correspond to regions demarcated by grey and dashed boxes in panel D, respectively.

3.3 | Brain dissection

The animals (mice or rats) were sacrificed by decapitation after being anesthetized under isoflurane atmosphere. For Patch Clamp experiments the rat brain was rapidly removed from the skull cavity and the hippocampi were dissected in ice-cold dissecting solution containing (mM): Sucrose 110; KCl 2.5; $CaCl_2$ 0.5; $MgCl_2$ 7; $NaHCO_3$ 25; NaH_2PO_4 1.25 and Glucose 7, pH 7.4, oxygenated with 95% O_2 and 5% CO_2. Each hippocampus was then transversely sliced (300 µm-thick) on a vibratome (VT 1000 S; Leica, Nussloch, Germany) under the same ice-cold dissecting solution. For functional recovery purpose, the slices were first incubated at 35ºC for 20 minutes in artificial cerebrospinal fluid (aCSF) with the following composition (mM: NaCl 124; KCl 3; NaH_2PO_4 1.25; $NaHCO_3$ 26; $MgSO_4$ 1; $CaCl_2$ 2 and Glucose 10, pH 7.4) before being placed at room temperature in the same solution for at least 1 hour before use in patch clamp experiments.

For experiments using $ADK^{fl/fl}$ and $ADK^{\Delta brain}$ mice, the brain was quickly removed and the striata, hippocampi and cortices were dissected free in ice-cold aCSF. While the other brain areas were frozen at -80ºC until further use, one of the hippocampi was sliced (400 µm) perpendicularly to its long axis in the McIlwain tissue chopper. Slices were then immediately transferred to a resting chamber filled with the same solution at room temperature, for 1 hour, to functional recovery before used in extracellular recordings.

3.4 | Patch-Clamp recordings

Individual slices were fixed with a grid in a recording chamber of about 1 mL volume, and were continuously superfused by a gravitational system at 2-3 mL/min with aCSF at room temperature. Drugs were added to this superfusion solution, reaching the recording chamber within approximately 1 minute.

Patch pipettes were made from borosilicate glass capillaries (1.5 mm outer diameter, 0.86 inner diameter, Harvard Apparatus, Holliston, MA, USA) and had a resistance of 4-9 MΩ when filled with an internal solution containing (mM): CsCl 125; NaCl 8; $CaCl_2$ 1; EGTA 10; HEPES 10; Glucose 10; MgATP 5; NaGTP 0.4 and pH 7.2, adjusted with CsOH (50 wt% in H_2O), 280-290 Osm.

Whole-cell recordings were obtained from pyramidal cells located at CA1 *stratum pyramidale*. The cells were visualized with a microscope (Zeiss Axioskop 2FS, Jena, Germany) equipped with infrared video microscopy and differential interference contrast optics. All recordings were performed in voltage-clamp mode (V_H = -70 mV) at room temperature (22-24ºC), with either an EPC-7 (List Biologic) or Axopatch 200B (Axon Instruments) amplifier, under the control of pClamp10 Software (Molecular Devices). Before the giga-seal formation, the offset potentials were nulled. Immediately after having whole-cell access, the membrane potential of the neurons was measured in current-clamp mode ($V_{holding}$ approximately -60 mV). Through all the recordings, the holding current was constantly monitored and if variations of more than 20% were observed, the experiment would be rejected. Data were low-pass filtered using a 3- and 10-kHz three-pole Bessel filter digitized at 5 Hz and registered by the Clampex Software version 10.2 (Molecular Devices, Sunnyvale, CA, USA).

3.4.1 Afferent-evoked inhibitory post-synaptic currents (IPSCs) recordings

Afferent-evoked inhibitory post-synaptic currents were recorded as described elsewhere (Rombo et al. 2014). Briefly, every 15 seconds, stimuli (1-15 μA) was delivered via monopolar stimulation with a patch-type pipette filled with aCSF and positioned in *Stratum radiatum*, 80-120 μm from the recorded cell. Averages of eight consecutive individual recordings were used for analysis. Recordings were performed using the internal solution previously described, to which 1 mM QX-314, a voltage-gated Na^+ channel blocker, was added. During all recordings, the aCSF was supplemented with Kynurenic acid (Kyn, 1 mM), to block glutamate receptors.

3.4.2 Miniature IPSCs recordings (mIPSCs)

Miniature inhibitory post-synaptic currents (mIPSCs) were recorded in aCSF supplemented with Tetrodotoxin (TTX, 0.5 μM) and Kynurenic Acid (Kyn, 1 mM). In some experiments, and only where specified, K252a (200 nM) and SCH 58261 (100 nM) were also added to the superfused aCSF. When testing the dependency on $p75^{NTR}$ activation on the effect of BDNF, the slices were pre-incubated for 2 hours at room temperature with a blocking antibody anti-$p75^{NTR}$ IgG (REX Ab, 50 μg/mL) (Woo et al. 2005). Miniature events

analysis was performed using the MiniAnalysis software (Synaptosoft, GA, USA), with the amplitude threshold for event detection being set at 5x the average RMS noise. mIPSC frequency and amplitude were analyzed for 40 seconds every 2 minutes in order to obtain their time-course variances. Statistical differences were assessed between two different periods: *1)* the 10 minutes prior to BDNF superfusion and *2)* the last 10 minutes in its presence.

3.5 | Extracellular recordings

Slices were transferred to a recording chamber where they were submerged in an oxygenated aCSF solution at 32ºC, continuously superfused at a flow rate of 3 mL/min. When indicated, drugs were added to the superfused solution. Recordings were obtained with an Axoclamp 2B amplifier and digitized (Axon Instruments, Foster City, CA). Evoked field Extracellular Post-Synaptic Potentials (fEPSPs) were recorded extracellularly through a microelectrode filled with 4 M NaCl (2-6 MΩ resistance) placed in the *Stratum radiatum* of the CA1 area. A concentric electrode was placed on the Shaffer collateral-commissural fibers, in *Stratum radiatum* near CA3-CA1 border and delivered the stimulation (rectangular 0.1 ms pulses, once every 15 s). Individual responses were monitored, and averages of eight (Basal synaptic transmission) or six (LTP induction and Input-Output curve) consecutive responses were continuously stored on a personal computer with the WinLTP software (Anderson & Collingridge 2001).

3.5.1 Basal synaptic transmission

The intensity of stimulus (80-200 μA) was initially adjusted to obtain a sub-maximal fEPSP slope (near 0.5 ms) with a minimum population spike contamination. Alteration in synaptic transmission induced by drugs was evaluated as the % change in the average slope of the fEPSP in the presence of the drug in comparison to the average slope of the fEPSP measured during the 10 minutes that preceded its addition.

3.5.2 Input-Output curve

After obtaining a stable baseline for at least 15 minutes, the stimulus delivered to the slice was decreased to 60 μA, with fEPSPs disappearance. The stimulus was delivered to the slice in successively increased steps of 20 μA until a supra-maximal stimulation of 320 μA. For each stimulation condition, data from three consecutive average fEPSP were stored. The Input-Output curve was plotted as the relation between fEPSP slope (ms) *vs.* stimulus intensity (μA).

3.5.3 Long-term potentiation induction

After obtaining a 14-minute stable baseline of fEPSP slope by 0.5 ms, LTP was induced by θ-burst protocol as previously described (Diógenes et al. 2004). This protocol consisted of three trains of 100 Hz, three stimuli, separated by 200 ms, being the intensity of the stimulus maintained during all the induction protocol. LTP was quantified as the % change in the average slope of fEPSP taken from 52 to 60 minutes after LTP induction in relation to the average slope of the fEPSP measured during the baseline period. Regarding to LTP protocols in the presence of drugs, LTP was only induced no less than 30 minutes after the drug perfusion and only after stable fEPSP slope values.

3.6 | Whole tissue homogenates

Frozen cortex was placed in 500 μL of chilled 0.32 M sucrose solution with 50 mM Tris, pH 7.6, complemented with protease inhibitors (Hoffman LaRoche, Inc.). The tissue was homogenized using a Teflon piston (8 to 10 up and down strokes) and then centrifuged (Batalha et al. 2013). Protein was quantified according to Bradford method, using the BioRad Protein assay kit.

3.7 | Saturation binding assay

The saturation binding experiments were adapted from a previously described protocol (Batalha et al. 2013). [^{3}H]DPCPX (radiolabeled A_1 receptor antagonist, specific activity 120 Ci/mmol) was incubated for 2 hours at room temperature with 39-80 μg of protein homogenate and 4 U/mL Adenosine deaminase, in a solution containing 50 mM Tris, 2

mM MgCl2.6H_2O, pH 7.4 with a final volume of 300 μL. Adenosine deaminase (ADA, EC 3.5.4.4) removed endogenous adenosine by catalyzing it to inosine, its inactive metabolite. Saturation curves of [^{3}H]DPCPX were performed at 8 different concentrations, ranging from 0 to 42 nM. Each condition was performed in triplicate. Non-specific binding was measured in the presence of 2 μM XAC, which was subtracted in order to determine the specific binding. Binding reactions were stopped by vacuum filtration with a Skatron semi-automatic cell harvester using chilled incubation solution. Filtermats 1.5 μm (Molecular Devices) were used and placed in scintillation vials to which 3 mL of scintillation cocktail (OptiPhase 'HiSafe' 2, PerkinHelmer) were added. Radioactivity bound to the filters was determined after 12 hours with an efficiency of 55-60% for 2 minutes. The membranes left from the assay were frozen for further protein quantification, using BioRad Protein assay kit.

3.8 | Western Blot

The protein samples were denaturated with 5x Sample buffer (350 mM Tris, 30% glycerol, 10% SDS, 600 mM dithiothreitol, 0.012% bromophenol blue, pH 6.8) at 95ºC for 5 minutes. Total cortical proteins (80-200 μg) and the molecular weight marker were loaded and separated on 10% SDS-poly-acrylamide electrophoresis gels and transferred onto PVDF membranes (Millipore). After 1 hour of incubation with a blocking solution containing 5% non-fat dry milk, membranes were washed (3x, 10 minutes) with TBS-T 0.1% (200 nM Tris, 1.5 M NaCl and 0.1% Tween-20) and probed overnight at 4ºC with primary antibody anti-TrkB, anti-A_{2A}R and anti-α-Tubulin (as internal standard). After 3 washes of 10 minutes with TBS-T 0.1%, the membranes were incubated with secondary antibody anti-mouse or anti-rabbit conjugated with horseradish peroxidase for 1 hour at room temperature (primary and secondary antibody dilution are indicated in Table 3.1.

Table 3.1: Primary and secondary antibodies used in Western Blot for individual proteins. Primary antibodies were diluted in 3% Bovine Serum Albumin, while secondary antibodies in 5% non-fat dry milk.

Protein	Primary antibody	Animal	Dilution	Secondary antibody	Dilution
α-Tubulin	Cell Signaling (#4466)	Rabbit	1:5000	Sta. Cruz Biotechnology (goat anti-mouse; goat anti-rabbit)	1:10000
$A_{2A}R$	Upstate (05-717)	Mouse	1:2000		1:5000
TrkB	BD Biosciences (610101)	Mouse	1:1000		1:10000

The membranes were washed again for 30 minutes with TBS-T and chemoluminescent detection was performed with an ECL-PLUS Western Blot detection reagent (GE Healthcare) using X-Ray films (Fujifilm) or the ChemiDoc XRS (BioRad). Optical density was evaluated using the software Image-J (NIH, MD, USA) and normalized to the respective loading control band density.

3.9 | ELISA

Each hippocampus was lysed in 250 µL of Lysis buffer, which contains 137 mM NaCl, 20 nM Tris-HCl, 10% glycerol, 1 mM PMSF, 1% NP-40, 0.5 sodium vanadate, pH 8.0 plus protease inhibitors (Hoffman LaRoche, Inc.) and homogeneized in a sonicator (Vibra-Cell, Sonics & Materials, Inc). In order to increase the amount of detectable BDNF in hippocampi extracts the samples were processed by acidification followed by neutralization. Samples were acidified to approximately pH 2.6 for 15 to 20 minutes and subsequently neutralized with NaOH to approximately 7.6. For further BDNF quantification, BDNF ImmunoAssay System (G7610, Promega) protocol was followed.

3.10 | mRNA analysis

3.10.1 Total RNA isolation, quantification and quality evaluation

Following cortex sample homogenization in 1 mL of QiAzol Lysis Reagent (Qiagen), using a Teflon piston (8 to 10 up and down strokes), 200 µL of Chloroform (Sigma Aldrich) was added. After 5 minutes incubation at room temperature, the samples were then vortexed, incubated for 3 minutes at room temperature and then, centrifuged for 15

minutes at 13500 rpm, at 4ºC to allow phase separation. The supernatant was collected, to which were added 600 µL 70% Ethanol. For further RNA extraction, RNeasy Lipid Tissue Mini Kit (Qiagen) protocol was followed. RNA concentration and purity were evaluated by spectrometry on the basis of optical density measurements at 260 and 280 nm. Samples showing RNA contamination by DNA or phenols were discarded. The samples were stored at -80ºC until further use.

3.10.2 Reverse Transcription

For first-strand cDNA synthesis, 1.5 µg of total RNA was mixed with 1 µL of dNTPs (each at 10 mM) and 1 µL of Random Hexamer Primers (50 ng/µL), followed by incubation at 65ºC, for 5 minutes, leading to loss of secondary structures that may interfere with the annealing step. The samples were cooled for 2 minutes at 4ºC and mixed with 4 µL of 25 mM $MgCl_2$, 2 µL of 10x RT Buffer, 2 µL of 0.1 M Dithiothreitol and 0.5 µL of SuperScript III Reverse Transcriptase (200 U, Invitrogen Life Technologies). For primer annealing, samples were incubated for 5 minutes at 25ºC prior to the cDNA synthesis reaction, which was performed at 42ºC for 50 minutes. In order to inactivate the enzyme, samples were incubated for 15 minutes at 70ºC. A parallel reaction was run in the absence of enzyme to assess the degree of any genomic DNA contamination. The obtained cDNA samples were stored at -20ºC until use.

3.10.3 Real-Time PCR

For gene expression analysis, 2 µL of 1:10 diluted cDNA were added to 12,5 µL of 2x Power SYBR® Green PCR Master Mix (Life Technologies) and 1 µL of each primer 5 µM (sequences at Table 3.2), in a reaction with a total volume of 25 µL. All reactions were performed in duplicate to reduce confounding variance. Negative control PCR samples were run with no cDNA template. The real-time quantitative PCR reactions were performed on an RT-PCR Rotor Gene 6000 device (Corbett Life Science). Melting curves were analyzed in order to confirm primer specificity and the comparative C_t (threshold cycle) method was used for quantification. C_t corresponds to a relative measure of the concentration of target in the PCR reaction and it is obtained by the intersection of its amplification curve and a threshold line, which was placed in the exponential phase, to

avoid limiting components in the reaction. The genes used to normalize the expression of the target sequences used were PPIA peptidylprolyl isomerase A (cyclophilin A, CypA) and Ribosomal protein L13A (RpL13A). The normalization was done according to the formula: C_t=C_t(target gene)–C_t(reference gene). Data analysis was performed using the Rotor Gene Series Software 1.7 (Corbett Life Science).

Table 3.2: Reverse and Forward Primers used in Real-Time PCR. All primers were purchased from Invitrogen and diluted to a final concentration at 100 µM. Working solutions were at 5 µM.

Gene	Forward Primer	Reverse Primer
CypA	TATCTGCACTGCCAAGACTGAGTG	CTTCTTGCTGGTCTTGCCATTCC
RpL13A	GGATCCCTCCACCCTATGACA	CTGGTACTTCCACCCGACCTC
A_1R	TCGGCTGGCTACCACCCCTTG	CCAGCACCCAAGGTCACACCAAAGC
A_{2A}R	ATTCCACTCCGGTACAATGG	AGTTGTTCCAGCCCAGCAT

3.11 | Drugs

Isofluorane was purchased from Abbot Laboratories (Barcelona, Spain). **BDNF** was generously supplied by Regeneron Pharmaceuticals (Tarrytown, NY), in a 1.0 mg/mL stock solution in 150 mM NaCl, 10 mM sodium phosphate buffer, and 0.004% Tween 20. **REX Ab** (Anti-$p75^{NTR}$ IgG) was a generous gift from Louis Reichardt (Department of Physiology and Neuroscience Program, University of California, San Francisco). **CPA** (N6-cyclopentyladenosine, selective A_1 receptor agonist), **DPCPX** (1,3-dipropyl-8-cyclopentylxanthine, selective A_1 receptor antagonist), **TTX** (Tetrodotoxin, Octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10a*H*-[1,3]dioxocino[6,5-*d*] pyrimidine-4,7,10,11,12-pentol, a sodium-channel blocker) were obtained from Ascent Scientific (Bristol, UK). **K252a** (Tyrosine kinase inhibitor), **SCH 58261** (2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine, selective A_{2A} receptor antagonist), **XAC** (A_1 and A_{2A} receptors antagonist), **QX-314 chloride** (*N*-(2,6-Dimethylphenylcarbamoylmethyl)triethylammonium chloride, blocker of voltage activated Na^+ channel) and **Bicuculline** ([R-(*R**,*S**)]-6-(5,6,7,8-Tetrahydro-6-methyl-1,3-dioxolo[4,5-*g*]isoquinolin-5-yl)furo[3,4-*e*]-1,3-benzodioxol-8(6*H*)-one, selective $GABA_AR$ antagonist) were purchase from Tocris Cookson (Ballwin, MO). **CGS 21680** (2-[p-(2-carboxylethyl)phenethylamino]-50-N-ethylcarboxamido adenosine, selective A_{2A} receptor agonist) was obtained from Research Biochemicals International (RBI, Natick, MA) and **Kynurenic Acid** (4-Hydroxyquinoline-2-carboxylic acid, glutamate receptors antagonist)

from Abcam. **Adenosine deaminase** (ADA, from calf intestine 10 mg/2mL, EC 3.5.4.4) was purchased from ROCHE Diagnostics Corporation and provided in a 50% glycerol (v/v), 10 mM potassium phosphate, pH 6.0. **[^{3}H]DPCPX** (radiolabeled A_1 receptor antagonist, specific activity 120 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc.

CPA, DPCPX, SCH 58261, CGS 21680 and XAC were prepared in a 5 mM, K252a in a 1 mM and Bicuculline in a 100 mM stock solution, all in dimethylsulfoxide (DMSO). TTX (1 mM) and Kynurenic acid (100 mM) were prepared in water. The percentage of DMSO in each experiment did not exceed 0.001% and did not affect either fEPSPs, IPSCs or mIPSCs (Tsvyetlynska et al. 2005; Rombo et al. 2014). Stock solutions were aliquoted and stored at -20ºC until their use. Dilutions of these solutions to the final concentration were made freshly before each experiment.

3.12 | Statistical Analysis

Results are expressed as the mean±SEM of *n* experiments. In Patch Clamp experiments, each *n* corresponds to the number of tested cell from different slices, while in extracellular recprding, each *n* correspond to the number of tested slice from different mice. Measurement values (mIPSC frequency/mIPSC amplitude/ fEPSP slope) were normalized taking into consideration the average values before drug application or LTP induction protocol as 100%. Statistical significance was evaluated by two-tailed paired Student's t-tests when comparing before and after drug perfusion, or by performing one-way ANOVA followed by Bonferroni's post hoc test for comparison between multiple Patch Clamp experimental groups. To assess statistical differences between genotypes, two-tailed unpaired Student's t-tests were used. An F-test ($p<0.05$) was used to determine whether the saturation curves were best fitted by one independent binding site equation and if the parameters obtained from the $Adk^{fl/fl}$ and $Adk^{\Delta brain}$ saturation curves (B_{max} and K_d) were different. An F-test was also used to determine whether input-output curves were best fitted to an log(agonist) *vs*. response equation (four parameters) and if the top value differ between the curves from different genotypes. Statistical significance was assumed if *p* value was inferior to 0.05). Analyses were conducted with the GraphPad Software and specifics pertaining to statistical analysis are in the figures' legends.

4 | RESULTS

The experiments described in this chapter were all performed by myself except for the one described in section 4.1.1, which was performed in collaboration with Diogo M. Rombo.

4.1 | BDNF modulation of GABAergic signaling

Data presented in this chapter was published as Colino-Oliveira M, Rombo DM, Dias RB, Ribeiro JA, Sebastião AM (2016), BDNF-induced pre-synaptic facilitation of GABAergic transmission in the hippocampus of young adults is dependent of TrkB and adenosine A2A receptors, *Purinergic Signalling*, 12, 283-294 (**Appendix 1**).

4.1.1 Rationale

Two main neuronal populations compose neuronal networks: the excitatory principal cells that constitute the majority of the population, and the inhibitory interneurons, the smallest portion. Despite this discrepancy in population size, interneurons are essential players in modulating the excitability of neuronal networks. Indeed, in the rat hippocampus it is estimated that each interneuron has several hundreds of pyramidal cells as target (Sik et al. 1995). Also, interneurons have been shown to control the firing rate of pyramidal cells, to modulate their spike timing and to synchronize their activity, imposing oscillatory activity at different frequencies, often related to corresponding behavioral patterns (Klausberger 2009). Since interneurons determine the network excitability, knowing the mechanisms behind the modulation of GABAergic transmission became a relevant issue.

Most of the present knowledge of the effects of BDNF upon synaptic activity at the hippocampus relies on the facilitatory influence of BDNF upon excitatory synapses (Levine et al. 1995; Lin et al. 1998; Tyler & Pozzo-Miller 2001; Figurov et al. 1996; Chen et al. 1999). BDNF effect onto GABAergic signaling in the adult hippocampus is poorly understood, though. The experimental procedures described in this chapter were designed to address BDNF consequences in synaptic inhibitory signaling. Since most of the actions of BDNF in excitatory synapses are gated by adenosine A_{2A}Rs (Diógenes et al. 2004; Diógenes et al. 2007; Fontinha et al. 2008; Vaz et al. 2008; Pousinha et al. 2006; Rodrigues et al. 2014), the requirement of adenosine A_{2A} receptor activation in a putative influence by BDNF onto inhibitory signaling was also explored.

Patch Clamp experiments were performed to achieve this goal. Patch Clamp is an electrophysiological technique that allows the study of electrical properties of a single cell or even a single channel. Patch Clamp recordings can be made in different configurations; the most common being the whole-cell configuration, which allows the measurement of current changes resulting from the opening of channels in the entire cell membrane. In the present work, whole cell recordings from pyramidal cells from the CA1 region of the hippocampus taken from adult rats (from 5 to 10 weeks old) were performed.

4.1.2 BDNF increases IPSCs in CA1 pyramidal cells

Fast inhibition of synaptic transmission in the hippocampus is mediated by $GABA_A$ receptors ($GABA_ARs$). These are ligand-gated ion channels that allow chloride and bicarbonate flux across the cell membrane, according to their electrochemical gradient. Inhibitory post-synaptic currents (IPSCs) evoked by afferent stimulation were recorded to evaluate the influence of BDNF upon $GABA_AR$-mediated responses at the hippocampus of adult rats. All the recordings were performed in voltage-clamp mode (V_H = -70 mV) in the presence of Kynurenic acid (Kyn, 1 mM), to block glutamate receptors (Robinson et al. 1984) and isolate GABAergic currents from any interference of fast excitatory transmission (Andrásfalvy & Mody 2006). Under such conditions, the recorded currents were completely blocked when applying bicuculline (20 μM), a selective $GABA_AR$ antagonist, thus confirming their GABAergic nature (Figure 4.1A). In addition, QX-314 (1 mM) was added to the intracellular solution avoiding depolarization of the recorded cell and thus, avoiding GABA currents contamination with actions potentials.

Once achieving a stable baseline of electrically evoked IPSCs amplitudes for at least 10 minutes, BDNF was perfused, remaining in the bath for 40 minutes. First we used a moderately high concentration of BDNF (100 ng/mL), since at this concentration BDNF has been shown to post-synaptically affect GABAergic transmission in acute slices prepared from developing hippocampus taken from pre-weaning (P12-18) rats (Tanaka et al. 1997). As shown in Figure 4.1, BDNF induced a progressive increase in the peak amplitude of IPSCs recorded from 5 out of 7 (71%) tested CA1 pyramidal cells. The effect of BDNF on the amplitude of GABergic currents started seen after 15 minutes after its superfusion, being the maximum increase observed between the 30 and 40 minutes of BDNF application (Figure 4.1B). The average increase in peak amplitude of GABA currents caused by BDNF (100 ng/mL) was 39±4.9% (n=5, p<0.01, as compared with baseline values), being this value calculated from IPSCs recorded 30-40 min after starting perfusion of BDNF (100 ng/mL). These data indicate that BDNF facilitates phasic GABAergic transmission in the adult hippocampal neurons.

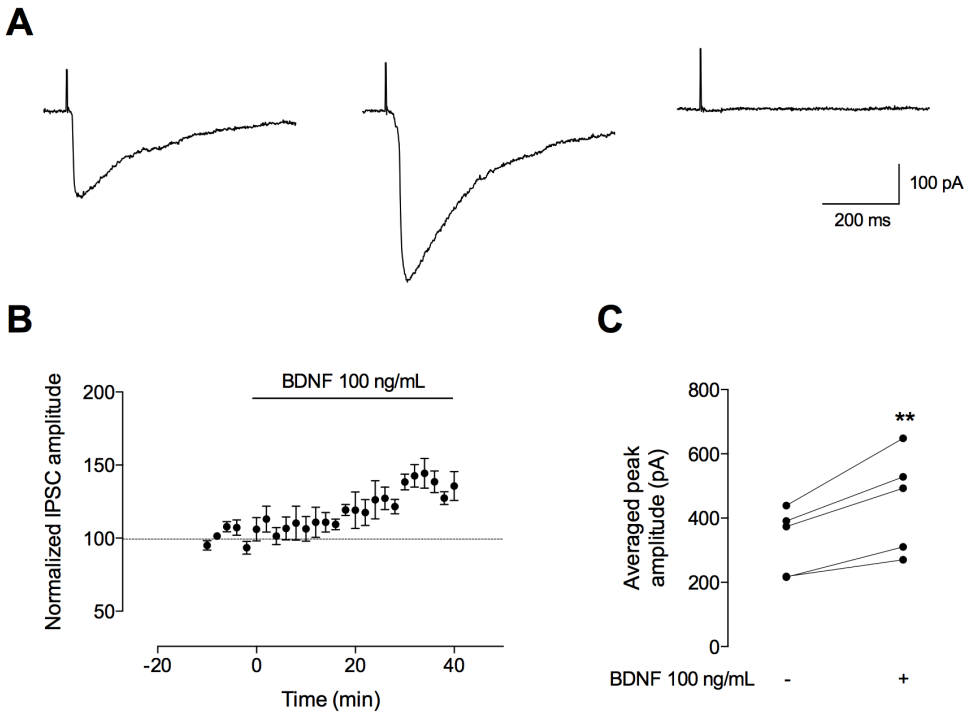

Figure 4.1: BDNF increases the amplitude of evoked IPSCs recorded from CA1 pyramidal neurons. ***(A)*** Representative whole-cell voltage-clamp recordings of inhibitory post-synaptic currents (IPSCs) evoked by afferent stimulation and recorded from a CA1 pyramidal cell in the absence (left panel) and presence (middle panel) of BDNF (100 ng/mL), and in the presence of bicuculline (20 µM) (right panel). ***(B)*** Time-course changes, induced by BDNF (100 ng/mL, n=5), in the peak amplitude of the evoked IPSPs. 100% represents the average peak amplitude of the currents recorded for 10 minutes prior to BDNF application. BDNF effect was quantified by comparing the peak amplitude from the 10 minutes period before BDNF application (baseline) to the 30-40 minutes after BDNF application. ***(C)*** The individual (dots) changes in peak amplitude are shown as individual data obtained in the 10 minutes in the absence (-) of BDNF and 30-40 minutes after (+) BDNF administration (n=5). Values are mean±SEM. ***p*<0.01 (two tailed paired Student's t test).

4.1.3 BDNF increases the frequency, but not the amplitude, of mIPSCs

Upon depolarization of nerve terminals during action potential firing, there is an increase of intracellular Ca^{2+} concentration, which results in vesicle fusion to the membrane and consequent neurotransmitter release to the synaptic cleft. In the absence of an action potential, however, spontaneous neurotransmitter release may also occur. These low-probability, stochastic events are called "miniature" events and correspond to the spontaneous fusion of a single synaptic vesicle (Frerking et al. 1997). As a consequence of neurotransmitter release, post-synaptic receptors are activated and generate miniature post-synaptic currents. In contrast to the neuronal firing rate, which is higher than 10^3 s^{-1}, the spontaneous release rate was estimated to be 10^{-3} s^{-1} (Kaeser & Regehr 2014). Nonetheless, the study of these events is a great tool to gain a better insight into the mechanisms of synaptic transmission, to monitor individual synapses and

to study their modulation and particularly to distinguish pre- from post-synaptic mechanisms.

To evaluate whether the facilitatory action of BDNF upon fast GABAergic transmission results from pre- or post-synaptic mechanisms, recordings of miniature inhibitory post-synaptic currents (mIPSCs) were performed in the presence of tetrodotoxin (TTX, 0.5 μM, that blocks voltage-gated Na^+ channels, preventing action potentials and isolating spontaneous events) and the glutamate receptor antagonist Kyn (1 mM). Changes in frequency of these events are usually interpreted as changes at pre-synaptic level, while changes in the amplitude are thought to relate with a post-synaptic modulation.

As illustrated in Figure 4.2, application of BDNF (100 ng/mL) to the bathing solution caused an increase in the frequency of mIPSCs by 28±7.0% (n=6, $p<0.05$, measured 30-40 minutes after starting BDNF superfusion, (Figre 4.2A,C,D). Baseline frequency of mIPSC was 2.1±0.2 Hz and increased to 2.7±0.3 Hz after BDNF addition. At this concentration, of BDNF, the increase in mIPSC frequency persisted even after washout. BDNF (100 ng/mL) was virtually devoid of effect in mIPSC amplitude (% change as compared with baseline 1±2.3, n=6, $p>0.05$). The average absolute amplitude values were 27±2.3 and 27±2.2 pA, before and 30-40 minutes after BDNF addition, respectively. Additionally, the duration of mIPSCs showed no statistical significance upon BDNF application (Figure 4.2G,H). Indeed, mIPSCs rising time changed to 2±1.9% of baseline (3.0±0.2 ms and 3.1±1.9 ms, before and after BDNF application, respectively) and the decay time to 2±1.7% (from 33±0.7 ms before and 33±0.8 after BDNF perfusion).

A lower concentration of BDNF (10 ng/mL) also increased mIPSC frequency, without affecting mIPSCs amplitude (Figure 4.3), the average increase in mIPSC frequency being 38±10.7% (n=9, $p<0.01$) for the last 10 minutes of its perfusion. Frequency values increased from 1.7±0.4 Hz (before the perfusion of BDNF) to 2.2±0.4 Hz (30-40 minutes after BDNF addition). The effect was not statistically different from that obtained with 100 ng/mL BDNF ($p>0.05$, one-way ANOVA). However, contrary to what was observed for 100 ng/mL BDNF (Figure 4.2C), the effect of 10 ng/mL was fully washed out (Figure 4.3C). BDNF (10 ng/mL) had no effect on mIPSCs amplitude (% change: 3±2.7 as compared to the baseline values; 38±2.2 pA before and 40±2.3 pA after BDNF application, (Figure 4.3E,F) or on mIPSC kinetics (rising time: 7±3.4% of baseline, 3.4±0.2 ms before and

3.6±0.3 after BDNF perfusion; decay time: 2±0.8% of baseline, 35.1±0.8 ms before and 35.7±0.7 ms after BDNF application, Figure 4.3G,H).

These data thus suggest that BDNF positively modulates GABAergic transmission via pre-synaptic changes that lead to increased GABA release.

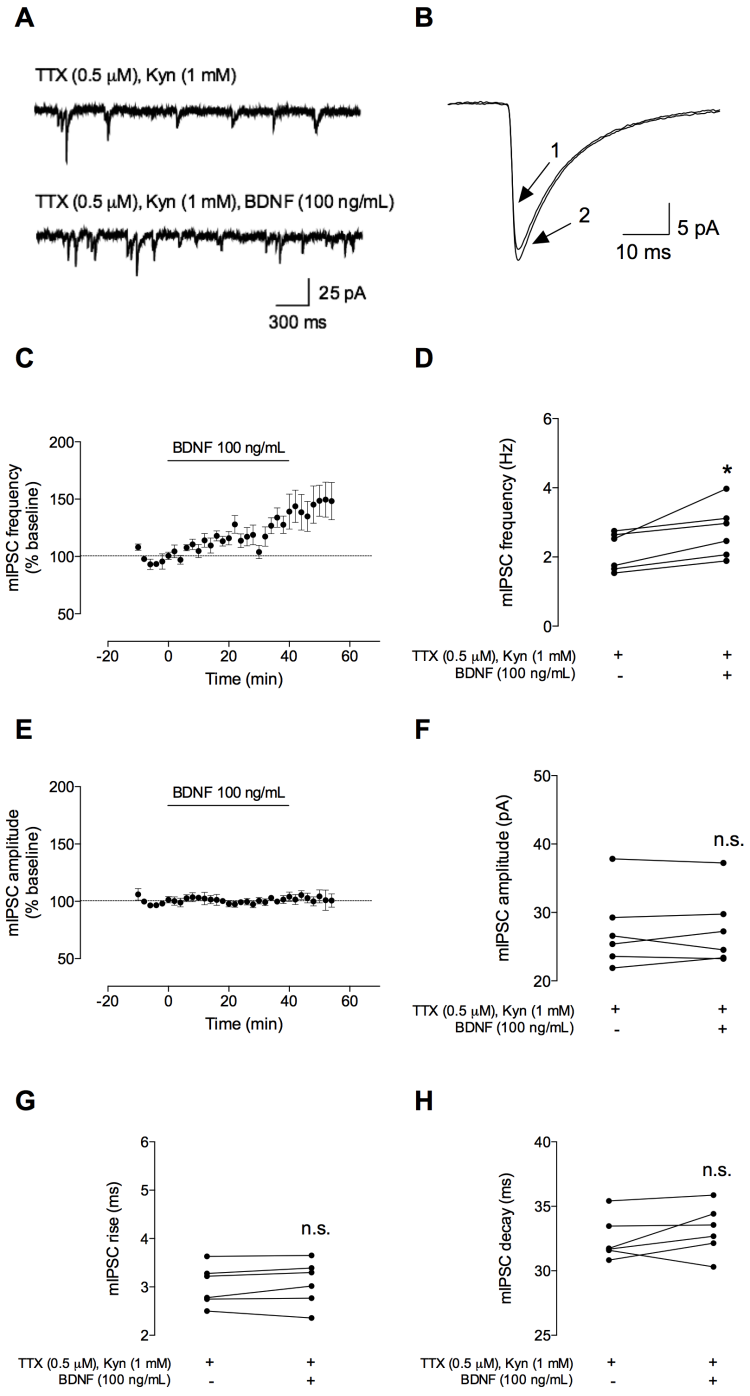

Figure 4.2: BDNF (100 ng/mL) increases the frequency, but not the amplitude, of spontaneous miniature inhibitory post-synaptic currents (mIPSCs). ***(A)*** Representative tracings of mIPSCs recorded in whole-cell configuration from a CA1 pyramidal cell, in the absence (upper trace) and presence (lower trace) of BDNF (100 ng/mL). ***(B)*** Representative average tracings of mIPSCs of two superimposed events in the absence (1) and presence (2) of BDNF (100 ng/mL), from the same cell. During all the recordings, a sodium channel blocker (Tetrodotoxin, TTX, 0.5 μM) and a glutamate receptors antagonist (Kynurenic acid, Kyn, 1 mM) were present. ***(C,E)*** Time-course changes in mIPSCs frequency (C) and amplitude (E) induced by application of BDNF (n=6). 100% represents the average mIPSC frequency or amplitude recorded for 10 minutes prior to BDNF application. mIPSCs frequency and amplitude changes were quantified by comparing the events from the 10 minutes period before BDNF application (baseline) to the final 10 minutes in its presence. ***(D,F)*** Average of absolute values of frequency (D) or amplitude (F) are shown as individual data obtained in the 10 minutes in the absence (-) of BDNF and 30-40 minutes after (+) BDNF administration (n=6). ***(G,H)*** Average of the absolute values of rise (G) or decay (H) times are shown as individual data obtained in the 10 minutes in the absence (-) of BDNF and 30-40 minutes after (+) BDNF administration (n=6). Values are mean±SEM. **p*<0.05 and n.s. *p*>0.05 (two tailed paired Student's t test).

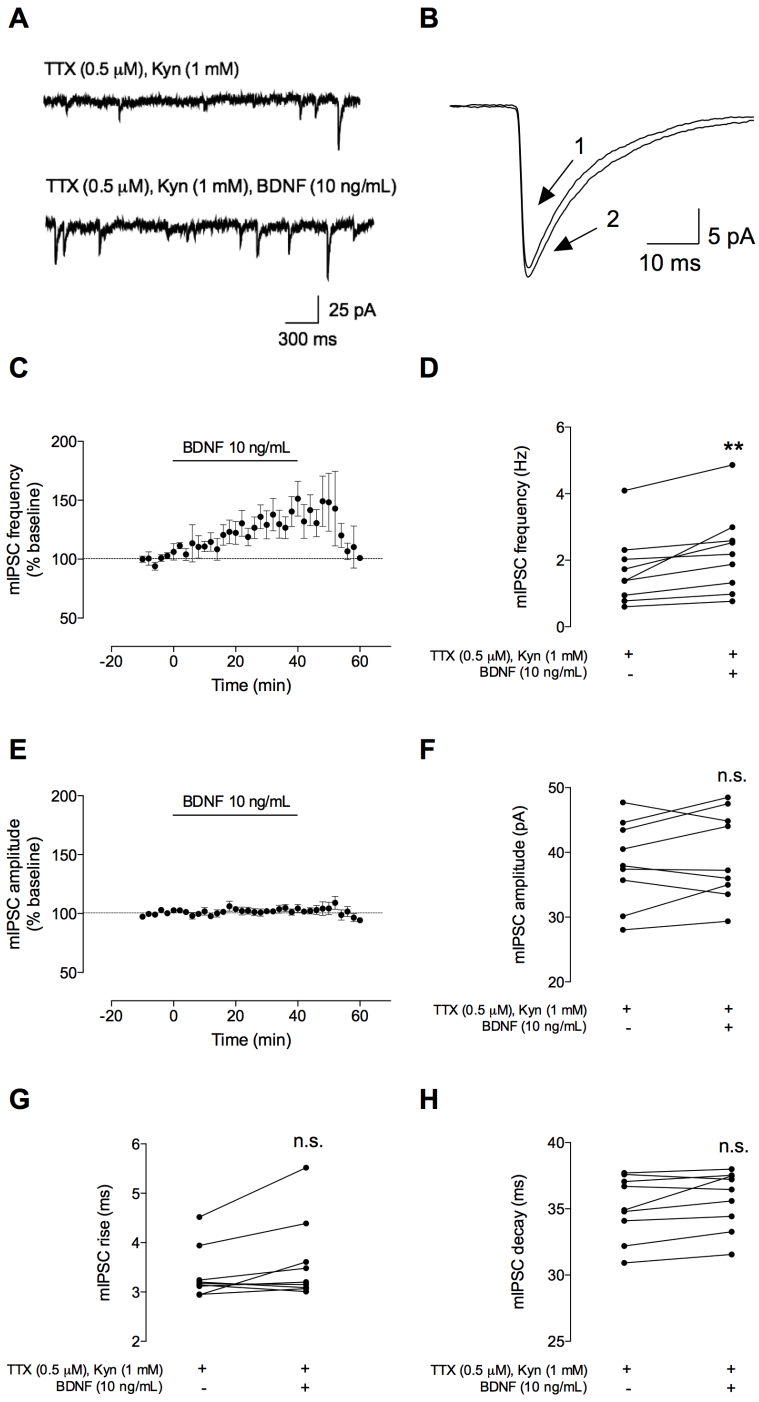

Figure 4.3: BDNF (10 ng/mL) increases the frequency, but not the amplitude, of spontaneous miniature inhibitory post-synaptic currents (mIPSCs). ***(A)*** In the upper panel, a representation of mIPSCs tracings recorded from a CA1 pyramidal cell in whole-cell configuration is shown, in the absence (upper trace) and presence (lower trace) of BDNF (10 ng/mL). ***(B)*** Representative average tracings of mIPSCs of two superimposed events in the absence (1) and presence (2) of BDNF (10 ng/mL), from the same cell. During all the recordings, a sodium channel blocker (tetrodotoxin, TTX, 0.5 μM) and a glutamate receptors antagonist (Kynurenic acid, Kyn, 1 mM) were present. ***(C,E)*** Time-course changes in mIPSCs frequency (C) and amplitude (E) induced by application of BDNF (n=9). 100% represents the average mIPSC frequency or amplitude recorded for 10 minutes prior to BDNF application. mIPSCs frequency and amplitude changes were quantified by comparing the events from the 10 minutes period before BDNF application (baseline) to the final 10 minutes in its presence. ***(D,F)*** Average of the absolute values of frequency (D) or amplitude (F) are shown as individual data obtained in the 10 minutes in the absence (-) of BDNF and 30-40 minutes after (+) BDNF administration (n=9). ***(G,H)*** Average of the absolute values of rise (G) or decay (H) times are shown as individual data obtained in the 10 minutes in the absence (-) of BDNF and 30-40 minutes after (+) BDNF administration (n=9). Values are mean±SEM. ***p*<0.01 and n.s. *p*>0.05 (two tailed paired Student's t test).

4.1.4 The BDNF facilitatory effect onto GABAergic transmission is TrkB, but not p75^{NTR}, dependent

BDNF can activate a high affinity TrkB receptor and a low affinity p75^{NTR} receptor. K252a is a tyrosine kinase inhibitor that prevents Trk receptor activation (Knüsel & Hefti 1992). Thus, to test for the involvement of TrkB receptors on the effect of BDNF, mIPSC recordings in the presence of K252a (200 nM) were performed. To do so, slices were perfused with K252a immediately after going to whole cell configuration and currents allowed to stabilize before applying BDNF in the presence of K252a for at least 20 minutes. In the presence of K252a, 40 minutes of incubation of BDNF did not affect either mIPSC frequency or mIPSC amplitude (Figure 4.4). Indeed, in the last 10 minutes of BDNF application, the percentage of change in mIPSC frequency was -7±5.3% as compared to baseline (n=7, p>0.05), the absolute values being 1.2±0.2 Hz before and 1.1±0.2 Hz after BDNF application. Similarly, for mIPSC amplitude the percentage of change was -1±1.4% (n=7, p>0.05), with absolute values of 31±3.0 pA before and 31±3.3 pA after BDNF application). Perfusion of K252a (200 nM) alone did not alter either frequency or amplitude of mIPSCs (frequency: 1.6±0.4 Hz to 1.7±0.5 Hz; amplitude: 30±7.1 pA to 30±7.2 pA, n=4, p>0.05, Figure 4.4G,H).

To evaluate the involvement of p75^{NTR} activation on the facilitatory action of BDNF on GABAergic currents, the hippocampal slices were pre-incubated for 2 hours with an Anti-p75^{NTR} IgG (50 μg/mL), prior to the whole cell recordings. This antibody binds to the p75^{NTR} receptor and blocks its activation by preventing BDNF binding to the receptor. As shown in Figure 4.5, the excitatory action of BDNF on GABAergic transmission was not affected in slices pre-incubated with the p75^{NTR} antibody. Thus, after BDNF application there was a significant increase in mIPSC frequency (29±7.8%, n=8, p<0.01; 1.8±0.3 Hz before and 2.2±0.3 Hz after BDNF application), without changes in mIPSC amplitude (-1±1.5%, n=8, p>0.05; 35±2.3 pA before and 34±2.0 pA after BDNF application). The effect of BDNF in the presence of the p75^{NTR} antibody was not significantly different to that caused by BDNF (either 10 or 100 ng/mL) alone (p>0.05, one-way ANOVA).

The above data showing that the excitatory effect of BDNF in mIPSC frequency is prevented by the presence of K252a, but not by the Anti-p75^{NTR} IgG, suggesting that the pre-synaptic enhancement of GABAergic transmission caused by BDNF is mediated by Trk receptor activation, and not by p75^{NTR}.

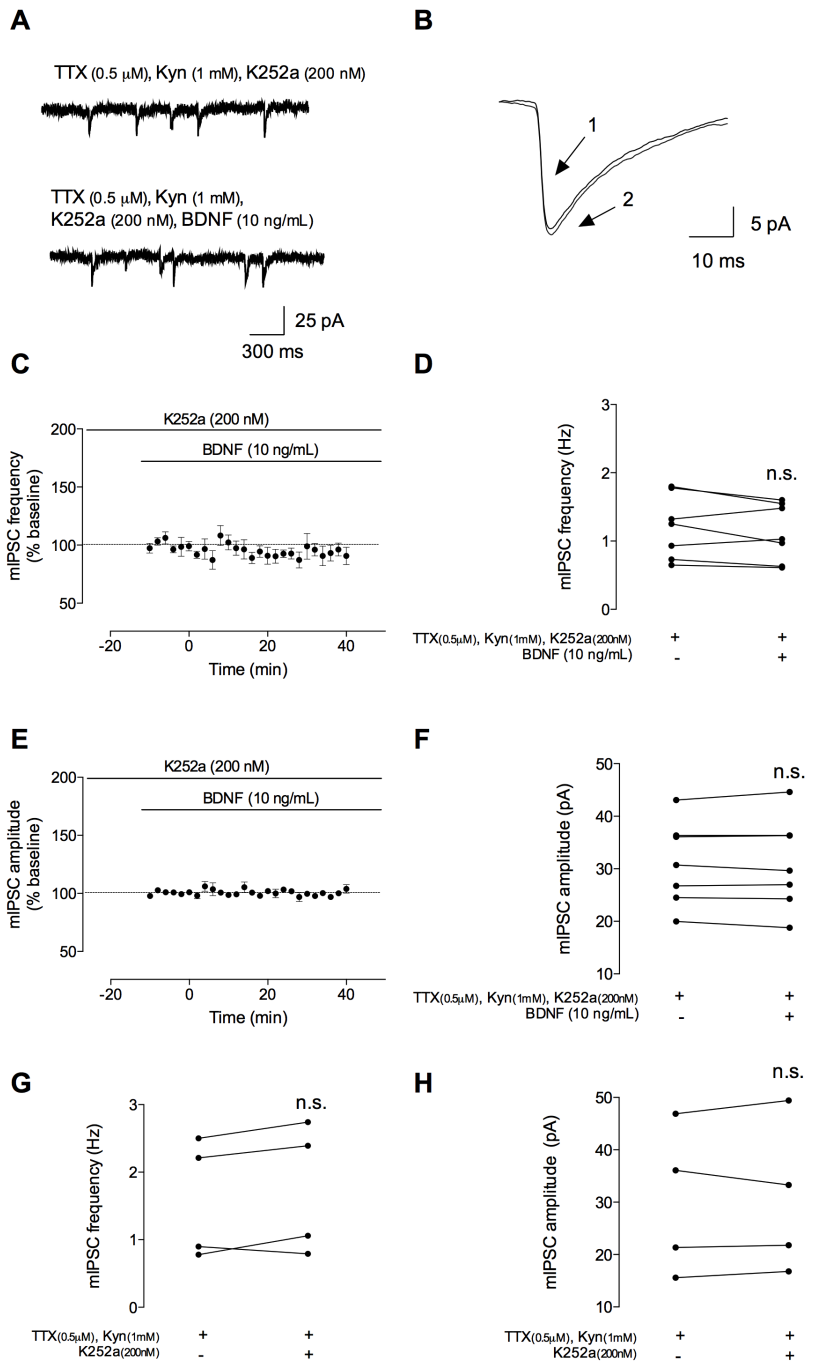

Figure 4.4: The facilitatory effect of BDNF on mIPSCs is dependent on TrkB receptors activation. ***(A)*** In the upper panel, a representation of mIPSCs tracings recorded from a CA1 pyramidal cell in whole-cell configuration is shown, in the absence (upper trace) and presence (lower trace) of BDNF (10 ng/mL)**.** ***(B)*** Representative average tracings of mIPSCs of two superimposed events in the absence (1) and presence (2) of BDNF (10 ng/mL), from the same cell. During all the recordings, a sodium channel blocker (tetrodotoxin, TTX, 0.5 μM), a glutamate receptors antagonist (Kynurenic acid, Kyn, 1 mM) and a tyrosine kynase inhibitor (K252a, 200 nM) were present. ***(C,E)*** Time-course changes in mIPSCs frequency (C) and amplitude (E) induced by application of BDNF (n=7). 100% represents the average mIPSC frequency or amplitude recorded for 10 minutes prior to BDNF application. mIPSCs frequency and amplitude changes were quantified by comparing the events from the 10 minutes period before BDNF application (baseline) to the final 10 minutes in its presence. ***(D,F)*** Average of the absolute values of frequency (D) or amplitude (F) are shown as individual data obtained in the 10 minutes in the absence (-) of BDNF and 30-40 minutes after (+) BDNF administration (n=7). ***(G,H)*** Average of absolute values of frequency (G) or amplitude (H) are shown as individual data obtained in the 10 minutes in the absence (-) of BDNF and 30-40 minutes after (+) K252a administration (n=4). Values are mean±SEM. n.s. $p>0.05$ (two tailed paired Student's t test).

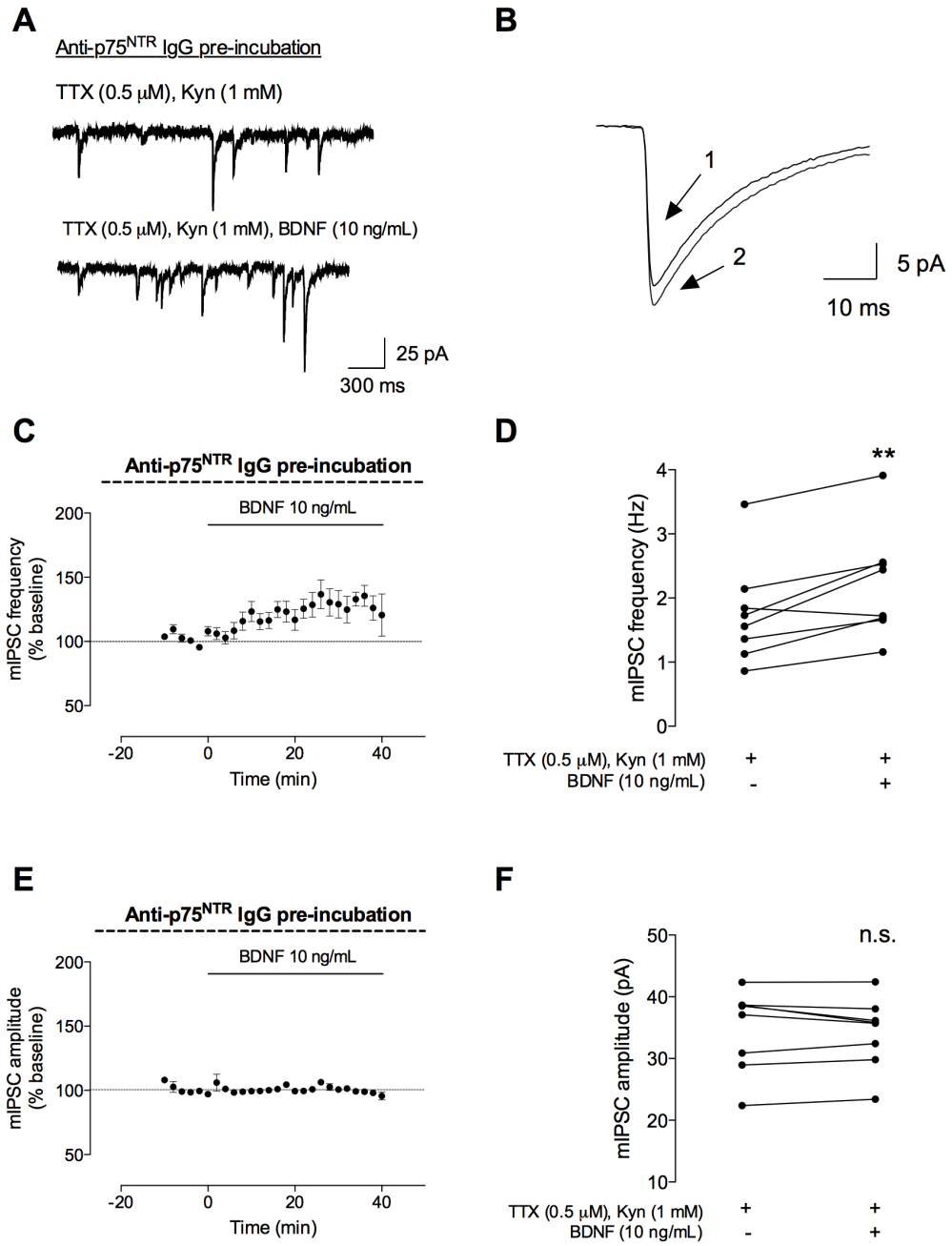

Figure 4.5: The facilitatory effect of BDNF on mIPSCs frequency is not dependent on p75^{NTR} receptors activation. ***(A)*** In the upper panel, a representation of mIPSCs tracings recorded from a CA1 pyramidal cell in whole-cell configuration is shown, in the absence (upper trace) and presence (lower trace) of BDNF (10 ng/mL). ***(B)*** Representative average tracings of mIPSCs of two superimposed events in the absence (1) and presence (2) of BDNF (10 ng/mL), from the same cell. The slices were pre-incubated with an anti-p75^{NTR} IgG (50 mg/mL) for an hour. During all the recordings, a sodium channel blocker (tetrodotoxin, TTX, 0.5 μM) and a glutamate receptors antagonist (Kynurenic acid, Kyn, 1 mM) were present. ***(C,E)*** Time-course changes in mIPSCs frequency (C) and amplitude (E) induced by application of BDNF (n=8). 100% represents the average mIPSC frequency or amplitude recorded for 10 minutes prior to BDNF application. mIPSCs frequency and changes were quantified by comparing the events from the 10 minutes period before BDNF application (baseline) to the final 10 minutes in its presence. ***(D,F)*** Average of the absolute values of frequency (D) or amplitude (F) are shown as individual data obtained in the 10 minutes in the absence (-) of BDNF and 30-40 minutes after (+) BDNF administration (n=8). Values are mean±SEM. ***p*<0.01 and n.s. *p*>0.05 (two tailed paired Student's t test).

4.1.5 Adenosine A_{2A} receptor blockade prevents BDNF effect onto GABAergic transmission

The interplay between TrkB and adenosine A_{2A} receptors has been demonstrated in several contexts, with the activation of A_{2A}R being a requisite for most of the effects of BDNF on excitatory synaptic transmission (Diógenes et al. 2004; Diógenes et al. 2007; Pousinha et al. 2006; Fontinha et al. 2008; Vaz et al. 2008; Rodrigues et al. 2014). I thus hypothesized that A_{2A}R, in spite of disynaptically modulating GABAergic transmission to pyramidal cells (Rombo et al. 2015), could directly affect the monosynaptic action of BDNF to principal cells. I thus tested whether endogenous activation of adenosine A_{2A}R could also influence the action of BDNF on GABAergic transmission. To do so, slices were perfused with the selective adenosine A_{2A}R antagonist, SCH 58261 (100 nM) (Zocchi et al. 1996) immediately after going to cell configuration and currents were allowed to stabilize before applying BDNF in the presence of the A_{2A}R antagonist for at least 20 minutes. However, in contrast with what occurred in the absence of SCH 58261 (Figure 4.3), there was virtually no effect in mIPSC frequency (% change: 0±3.5, n=10, p>0.05, Figure 4.6A,C,D) and amplitude (% change: 0±1.2, n=10, p>0.05, Figure 4.6B,E,F). When analyzing the period between 30 and 40 minutes of BDNF application (in the presence of SCH 58261), the average absolute values were 2.2±0.2 Hz and 37±2.4 pA, while in the 10 minutes prior to BDNF application, they were 2.2±0.2 Hz and 38±2.4 pA.

These data suggest that A_{2A}R activation is required for the BDNF facilitatory effect onto inhibitory transmission.

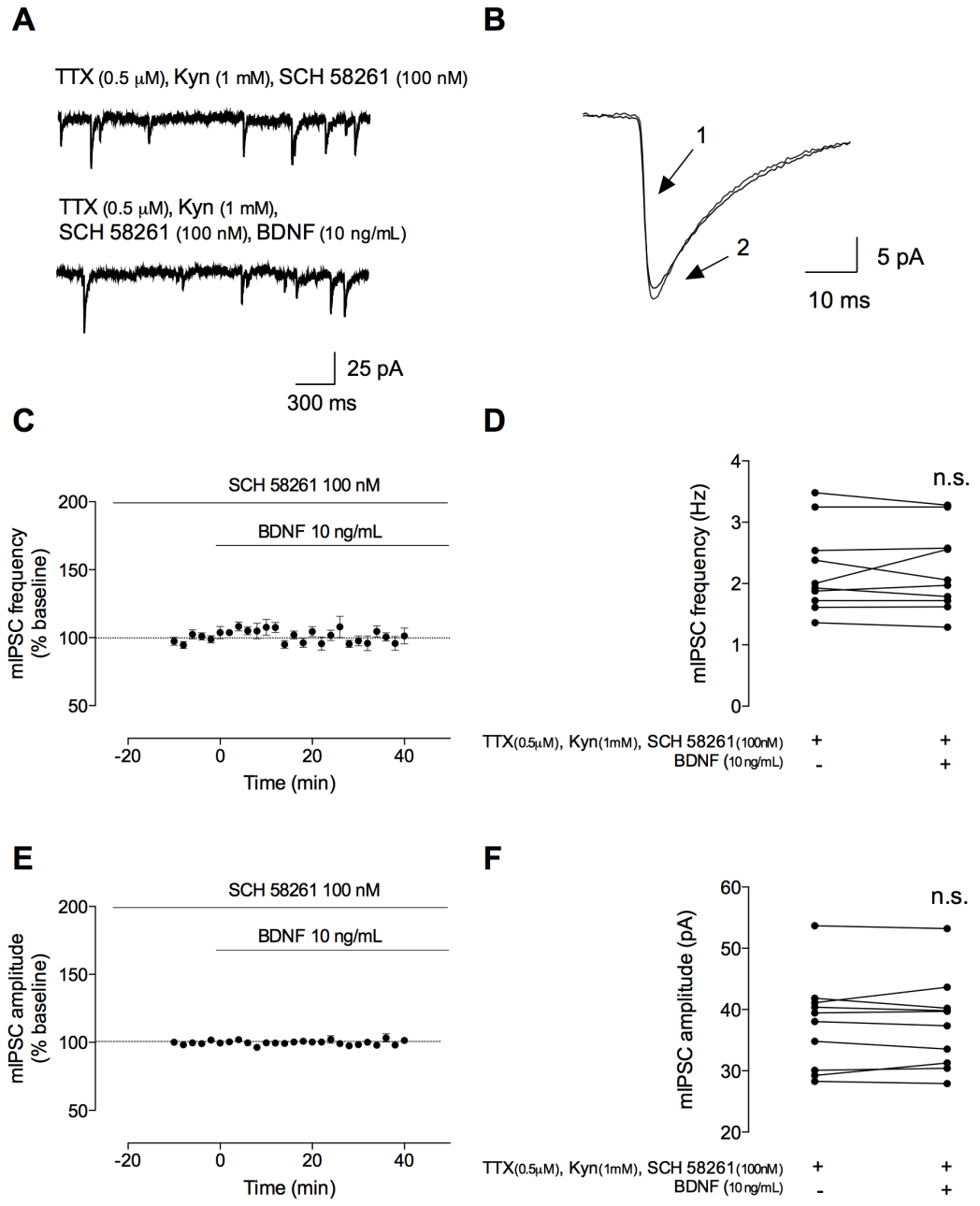

Figure 4.6: BDNF-mediated effect on mIPSCs frequency is dependent on adenosine A_{2A} receptor activation. ***(A)*** In the upper panel, a representation of mIPSCs tracings recorded from a CA1 pyramidal cell in whole-cell configuration is shown, in the absence (upper trace) and presence (lower trace) of BDNF (10 ng/mL). ***(B)*** Representative average tracings of mIPSCs of two superimposed events in the absence (1) and presence (2) of BDNF (10 ng/mL), from the same cell. During all the recordings, a sodium channel blocker (tetrodotoxin, TTX, 0.5 μM), a glutamate receptors antagonist (Kynurenic acid, Kyn, 1 mM) and an adenosine A_{2A} receptor antagonist were present. ***(C,E)*** Time-courses changes in mIPSCs frequency (C) and amplitude (E) induced by application of BDNF (n=10). 100% represents the average mIPSC frequency or amplitude recorded for 10 minutes prior to BDNF application. mIPSCs frequency and changes were quantified by comparing the events from the 10 minutes period before BDNF application (baseline) to the final 10 minutes in its presence. ***(D,F)*** Average of the absolute values of frequency (D) or amplitude (F) are shown as individual data obtained in the 10 minutes in the absence (-) of BDNF and 30-40 minutes after (+) BDNF administration (n=10). Values are mean±SEM. n.s. p>0.05 (two tailed paired Student's t test).

4.1.6 Discussion

The present data herein show that, through TrkB receptor activation, BDNF positively modulates GABAergic transmission by operating a pre-synaptic mechanism that requires adenosine A_{2A} receptor co-activation.

Although only 10-20% of total neurons are GABA-releasing neurons (Peters et al. 1985), they are crucial for a proper regulation of pyramidal cells, controlling their excitability (Klausberger 2009). Impairment of inhibitory signaling has thus been pointed as a possible cause for epileptic seizures (Treiman 2001). Indeed, epileptic tissue relates with interneuron loss (Sloviter 1987; Gorter et al. 2001) and alterations in $GABA_A$ receptors (Baulac et al. 2001; Peng et al. 2004). Accordingly, most anti-epileptic drugs act to enhance GABAergic function (Greenfield 2013). Around 30% of the patients remain refractory to treatment (Boison 2010) being therefore necessary a better understanding of the mechanisms involved in the modulation of GABAergic system to seek for new windows for therapeutic approaches.

BDNF is a critical player in the modulation of neuronal networks. It mediates positive fast actions onto glutamatergic transmission (Levine et al. 1995; Li et al. 1998; Lessmann et al. 1994; Tyler & Pozzo-Miller 2001) and synaptic plasticity (Figurov et al. 1996; Chen et al. 1999). Most of the work regarding BDNF regulation of GABAergic synapses has been focused in developmental stages (Tanaka et al. 1997; Bolton et al. 2000; Mizoguchi et al. 2003; Wardle & Poo 2003; Jovanovic et al. 2004; Swanwick et al. 2006), while no attention to its role in GABAergic synapses of the adult brain being properly given.

To investigate whether BDNF modulates GABAergic transmission, afferent-evoked synaptic IPSCs recordings were performed. These allowed assessing the effect of acute BDNF application on $GABA_AR$ post-synaptic currents. As demonstrated by its blockade with bicuculline, these currents were isolated from any contribution from glutamatergic components. BDNF acute application increased post-synaptic GABAergic currents in 70% of the recorded cells. The lack of response from some cells to the pharmacological treatment may be due to a more difficult BDNF penetration in the slice or to intrinsic cell variability. mIPSC recordings allowed to restrict the observation of BDNF effects to the active synapse, and thus, currents resulting from spontaneous quantal release provided information to dissect whether BDNF increased GABAergic currents through a pre- or

post-synaptic mechanism. The increase in mIPSC frequency caused by BDNF may be interpreted as an increase in the number of release sites or increase in the number of docked vesicles, resulting in increased probability of release or/and an increase in the number of pre-synaptic interneuron terminals. Glutamic acid decarboxylase 65 (GAD65) is the key enzyme for GABA synthesis and a marker for inhibitory terminals, so that immunostaining for GAD65 and synapsin is frequently used to address whether BDNF induces an increase in the inhibitory synapses. Chronic administration of BDNF to organotipic hippocampal slices and hippocampal cultures led to increased GAD expression (Marty et al. 2000; Ohba et al. 2005; Sánchez-Huertas & Rico 2011). Moreover, enhanced GABA probability release due to a BDNF-mediated redistribution of Ca^{2+} channels to vesicle release sites was shown in cultured hippocampal neurons (Baldelli et al. 2005). The herein reported data in slices from adult rats is consistent with these pre-synaptically mediated actions of BDNF, though contrasting with data from slices or acutely isolated neurons around P12-P18 (Tanaka et al. 1997; Mizoguchi et al. 2003) where BDNF has been reported to cause a post-synaptically mediated inhibition of GABAergic signaling. Interestingly, BDNF affects Cl^- transport in hippocampal neurons (Wardle & Poo 2003). The developmental shift from excitatory to inhibitory GABAergic currents occurs around P13, results from changes in Cl^- transport and is regulated by GABA since upon blockade of $GABA_A$ receptors, GABA remains excitatory (Ganguly et al. 2001). One may thus speculate that BDNF shifts from excitatory to inhibitory during a critical period of inhibitory GABAergic synapses maturation, eventually delaying it by inhibiting GABAergic transmission, but returns to its facilitatory role upon phasic transmission once synapses mature. Further studies on the role of BDNF in GABAergic synapses maturation are indeed required to directly assess this possibility.

As described in the introduction of this thesis, adenosine is a ubiquitous molecule released by neurons and glia that, through the activation of its receptors, is able to modulate synaptic transmission (Ribeiro & Sebastião 2010). For instance, adenosine A_{2A} receptors are up-regulators of TrkB receptor signaling, being able to boost BDNF facilitatory effects on synaptic transmission (Diógenes et al. 2004), synaptic plasticity (Fontinha et al. 2008) and neuromuscular junction (Pousinha et al. 2006). Moreover, in hippocampal neurons, adenosine A_{2A}Rs are more abundantly located in nerve terminals (Rebola, Canas, et al. 2005) and are required to translocate TrkB receptors to lipid rafts

during high frequency neuronal firing, allowing a BDNF effect upon glutamate release (Assaife-Lopes et al., 2014). Interestingly, as I now show, the pre-synaptic influence of TrkB receptors upon GABA release at GABAergic synapses is also under control of tonic adenosine $A_{2A}R$ activation since the BDNF-induced increase on mIPSC frequency was prevented in the presence of an adenosine $A_{2A}R$ antagonist. Remarkably, $A_{2A}R$, though present in a subset of GABAergic nerve terminals, do not directly affect GABAergic inputs to pyramidal neurons (Rombo et al. 2015), but as we now show, they can do so in an indirect way by allowing facilitatory BDNF actions in GABAergic nerve terminals.

Homeostatic plasticity is defined by adjustments in synaptic strength and cellular excitability to maintain appropriate levels of activity. For instance, following chronic activity blockade, a weakening of GABAergic signaling occurs, while following chronic increases in network activity there is a strengthening of GABAergic inhibition (Wenner 2011). BDNF is not expressed in interneurons, but only in pyramidal cells (Gorba & Wahle 1999; Swanwick et al. 2004). However, it is generally accepted that interneurons do express TrkB receptors (Gorba & Wahle 1999; Cellerino et al. 1996). Increase in synaptic activity and thus neuroexcitability favors the release of BDNF (Goodman et al. 1996) and ATP, the precursor of adenosine (Wieraszko et al. 1989), and the activation of TrkB and adenosine $A_{2A}Rs$ (Correia-De-Sá & Ribeiro 1996). BDNF is well-known to increase neuronal excitability either directly, by facilitating glutamatergic synapses, or indirectly, by increasing GAT-1 mediated GABA uptake into astrocytes (Amaral and Pozzo-Millers 2012; Vaz et al. 2011) and decreasing non-exocytotic GAT-1 reversal-mediated GABA release from synaptosomes (Amaral and Pozzo-Miller 2012; Vaz el al. 2015). Concerning exocytotic GABA release, we now show that BDNF enhances the activity of interneurons to release GABA, thus initiating a negative feedback mechanism to refrain excitability. Moreover, $A_{2A}R$-dependent BDNF facilitation of GABA release, herein described, together with $A_{2A}R$-dependent BDNF facilitation of GABA uptake into astrocytes (Vaz et al. 2011) will synergistically contribute to increase temporal fidelity of GABA transmission (the time window available for synaptic integration) and synchronize pyramidal cell firing. In fact, deleting TrkB/BDNF signaling in fast-spiking interneurons induces a reduction of GABAergic inputs to pyramidal cells and the disruption of the typical rhythmic neuronal activity observed in neuronal circuits at gamma oscillation frequency (Zheng et al. 2011). Altogether, the available information allows to suggest that BDNF and adenosine $A_{2A}R$

synergistically control synaptic communication by increasing neuronal excitability without losing GABAergic temporal fidelity necessary for proper network functioning and the maintenance of hippocampal oscillations

4.2 | Impact of ADK deletion in adenosinergic and BDNF signaling to control excitability and synaptic plasticity

The work described in this chapter corresponds to the work done by me, as part of a manuscript accepted for publication as:

Sandau U*, Colino-Oliveira M*, Jones A, Saleumvong B, Coffman S, Long L, Miranda-Lourenco C, Palminha C, Batalha V, Xu Y, Huo Y, Diogenes MJ, Sebastiao AM**, Boison D** (2016), Adenosine kinase deficiency in the brain results in maladaptative synaptic plasticity, *Journal of Neuroscience, in the press* (**Appendix 2**). *Co-first authors; **Co-senior authors

This section also includes data yet unpublished, namely, the data referring to pre-symptomatic (2 month old) and to post-symptomatic (> 2 years old) mice, which will be matter of another publication, yet under preparation.

4.2.1 Functional and molecular characterization of adenosine receptors on Adk knockout mice

4.2.1.1 Rationale

Epilepsy is a neurological disease manifested by seizures, with several morphological, functional and cellular changes occurring in epileptic tissue (Avanzini & Franceschetti 2003; Pitkänen & Sutula 2002). The hippocampus and the cerebral cortex are the most susceptible brain regions to epileptic activity (Pitkänen & Sutula 2002). In the hippocampus, characteristic lesions such as neuronal loss and gliosis are observed in the hilus of dentate gyrus, the CA1 and CA3 regions (Pitkänen & Sutula 2002). Moreover, hippocampus is a site of seizure induction (Spencer et al. 1987).

As described in the introduction to this thesis, numerous lines of evidence point to a dysfunction of adenosinergic signaling in epilepsy, namely an up-regulation of Adenosine kinase with consequent adenosine deficient signaling. Adenosine augmentation therapies have shown to be effective in suppressing epileptic activity (Wilz et al. 2008; Masino et al. 2011). Knowing that ADK determines the intra- and extracellular concentrations of adenosine, it may constitute a promising pharmacological target (Boison 2013). Exploiting therapeutic opportunities aiming ADK-regulated activity requires a better understanding of ADK impact on modulation of network activity. Our collaborators thus generated a mice model lacking *Adk* gene in the entire brain. Surprisingly, these mice developed progressive epilepsy with stress-induced seizures from 4-months old until their first year old, when they become asymptomatic. Accordingly, human patients with ADK deficiency also develop epilepsy (Bjursell et al. 2011). We therefore hypothesized that the lack of ADK would cause a dysregulation of adenosinergic signaling that could be related to the epileptic phenotype in these mice. Thus, to provide a mechanics explanation for the epileptic phenotype described above, I performed a detailed biochemical, pharmacological and electrophysiological analysis of hippocampal brain slices derived from pre-symptomatic (2-months old), symptomatic (5 to 8-months old) and post-symptomatic animals (over 1-year old).

The functional analysis was attained through the recording of field Excitatory Post-Synaptic Potentials (fEPSPs) from hippocampal slices. In summary, a stimulation electrode was placed in the Schaffer collaterals, which make excitatory synapses onto the apical

dendrites of the CA1 pyramidal neurons, where the extracellular recording electrode was positioned. The recorded electrical response results from a population of neurons in response to the synaptic stimulation and constitutes the fEPSP. The rising slope of the fEPSP is a reflection of the synaptic current and thus is used as an artificial measure of synaptic transmission strength.

4.2.1.2 Basal synaptic transmission is increased in symptomatic $Adk^{\Delta brain}$ animals

Enhanced excitability reflects into changes in basal synaptic transmission, which can be evaluated through the analysis of the input-output relationship of the synaptic response in hippocampal slices to afferent stimulation. In order to test whether the changes observed in *Adk* knockout mice could lead to enhanced basal synaptic transmission, input-output curves were obtained by recording the fEPSP responses as a function of increased stimulation intensities delivered to hippocampal slices from $Adk^{\Delta brain}$ mice and control littermates.

The maximum fEPSP slope obtained from slices taken from symptomatic $Adk^{\Delta brain}$ mice was significantly higher than that obtained from slices taken from age-matched control mice ($Emax_{Adk}{}^{fl/fl}$: 1.3±0.1 mV/ms, n=5 *vs.* $Emax_{Adk}{}^{\Delta brain}$: 2.7±0.2 mV/ms, n=5, F-test, $p<0.05$, Figure 4.7B). While comparing genotypes at pre- and post-symptomatic stages, however, no differences (F-test, $p>0.05$) of excitability are observed. The Emax value for 2-month old mice was 3.1±0.2 mV/ms in $Adk^{fl/fl}$ mice (n=5) and 3.2±0.2 in $Adk^{\Delta brain}$ mice (n=5) (Figure 4.7A), while for over 1-year old mice was 2.3±0.2 mV/ms in $Adk^{fl/fl}$ mice (n=7) and 2.5±0.2 in $Adk^{\Delta brain}$ mice (n=5) (Figure 4.7C). The higher excitability, which was only observed at hippocampal synapses from 5 to 8-month old animals, matches the epileptic phenotype of $Adk^{\Delta brain}$ mice at that age. Thus, the following experiments aimed to assess whether adenosinergic changes occur that could relate with the higher excitability and the phenotype of $Adk^{\Delta brain}$ mice.

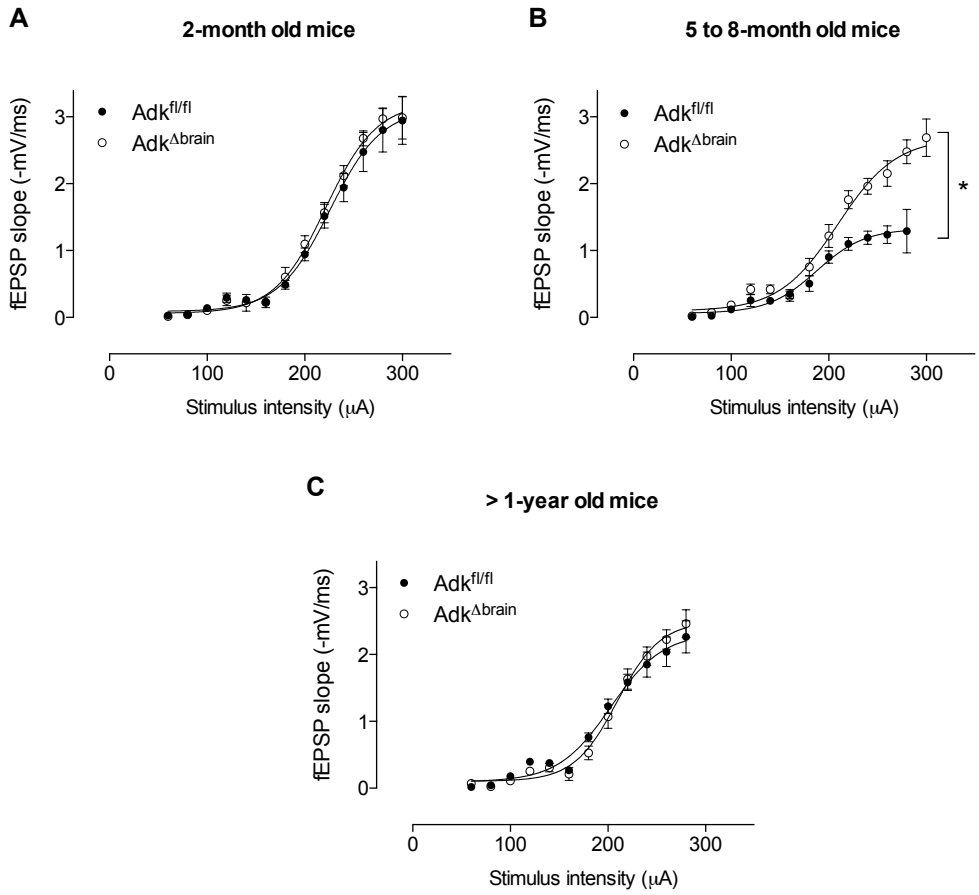

Figure 4.7: Increased excitability in symptomatic Adk$^{\Delta brain}$ mice. Input-Output curves were obtained from hippocampal slices to address changes in synaptic transmission level. The Input-Output curves correspond to responses generated by increasing stimulation intensities (60-320 mA) in Adk$^{fl/fl}$ (●) and Adk$^{\Delta brain}$ (○) mice with ***(A)*** 2-months old (n=5), ***(B)*** 5 to 8-months old (n=5) and ***(C)*** over 1 year old (n=5-7). Results are the mean±SEM of n experiments and statistical analysis was performed using an F-test (*p<0.05).

4.2.1.3 Adk$^{\Delta brain}$ mice have increased adenosine ambient levels around synapses

Adenosine is an important neuromodulator which major net effect is to inhibit synaptic transmission through the activation of the inhibitory adenosine A_1 receptor (Sebastião et al. 1990). As so, disinhibiton of synaptic transmission caused by adenosine A_1R antagonism correlates with the extracellular adenosine levels (Diógenes et al. 2014). Higher concentration of adenosine around synapses with consequent greater A_1R inhibitory signaling is then reflected by a greater disinhibition in synaptic transmission caused by an adenosine A_1R antagonist. Regarding the role of ADK in regulating adenosine tonus in synapses (Diógenes et al. 2014; Etherington et al. 2009), we aimed to evaluate extracellular adenosine levels both in pre-symptomatic, symptomatic and post-symptomatic Adk$^{\Delta brain}$ mice and their age-match controls. To address this question, fEPSPs

recordings were performed during the application of DPCPX, an A_1R antagonist, at a supramaximal concentration (50 nM, 100 times the K_i value for DPCPX at the hippocampus (Sebastião et al. 1990)). Prior to DPCPX application, a period of stable baseline for 10 minutes was obtained.

Figure 4.8A,B,C illustrates the average time course of changes in fEPSP slope induced by application of DPCPX (50 nM). As expected, DPCPX led to an increase in synaptic transmission in hippocampal slices from $Adk^{fl/fl}$ and $Adk^{\Delta brain}$ mice, at all animal ages. As it can be observed in Figure 4.8D,E,F, the DPCPX-induced effect on synaptic transmission was statistical significant (Paired t-test, $p<0.05$), when compared to the value measured before drug perfusion.

In hippocampal slices from pre-symptomatic $Adk^{\Delta brain}$ mice (Figure 4.8A,D), DPCPX induced an increase in fEPSP slope of 93±20.0% (n=5, Paired t-test, $p<0.01$), when compared to the value measured before drug perfusion. This value contrasts with the obtained while using healthy aged-match controls, where DPCPX increased the fEPSP slope by 19±5.4% (n=5, Paired t-test, $p<0.05$). Comparing both genotypes, a significant higher facilitatory action of A_1R antagonist was observed in $Adk^{\Delta brain}$ mice, when comparing to their aged-match controls (Unpaired t-test, $p<0.01$). A similar effect of DPCPX in symptomatic and post-symptomatic $Adk^{\Delta brain}$ and $Adk^{fl/fl}$ mice is observed.

As shown in Figure 4.8B,E, DPCPX-induced hippocampal disinhibition of synaptic transmission was also more pronounced in symptomatic (5 to 8-month old) (Unpaired t-test, $p<0.01$) mice lacking ADK expression in the brain, when comparing to age-matched controls (22±7.8% of increase in fEPSP recorded from $Adk^{fl/fl}$ mice, n=5, Paired t-test, $p<0.05$ *vs.* 118±20.1% in $Adk^{\Delta brain}$ mice, n=5, Paired t-test, $p<0.01$).

In post-symptomatic transgenic animals, DPCPX increased fEPSP slope by 90±15.8% (n=5, Paired t-test, $p<0.01$, Figure 4.8C,F). This increase was significant higher (Unpaired t-test, $p<0.05$, Figure 4.8C,F), when compared to their healthy control animals, in which DPCPX increased fEPSP slope by 30±8.8% (n=5, Paired t-test, $p<0.05$ Figure 4.8C,F).

The above described data show that independently of the age, the higher disinhibition of synaptic transmission caused by the A_1R antagonist in mice not expressing ADK in the brain suggests an enhanced A_1R-mediated tonic inhibitory tonus, pointing to either

increased adenosine ambient levels around synapses or increased A_1R signaling at synapses.

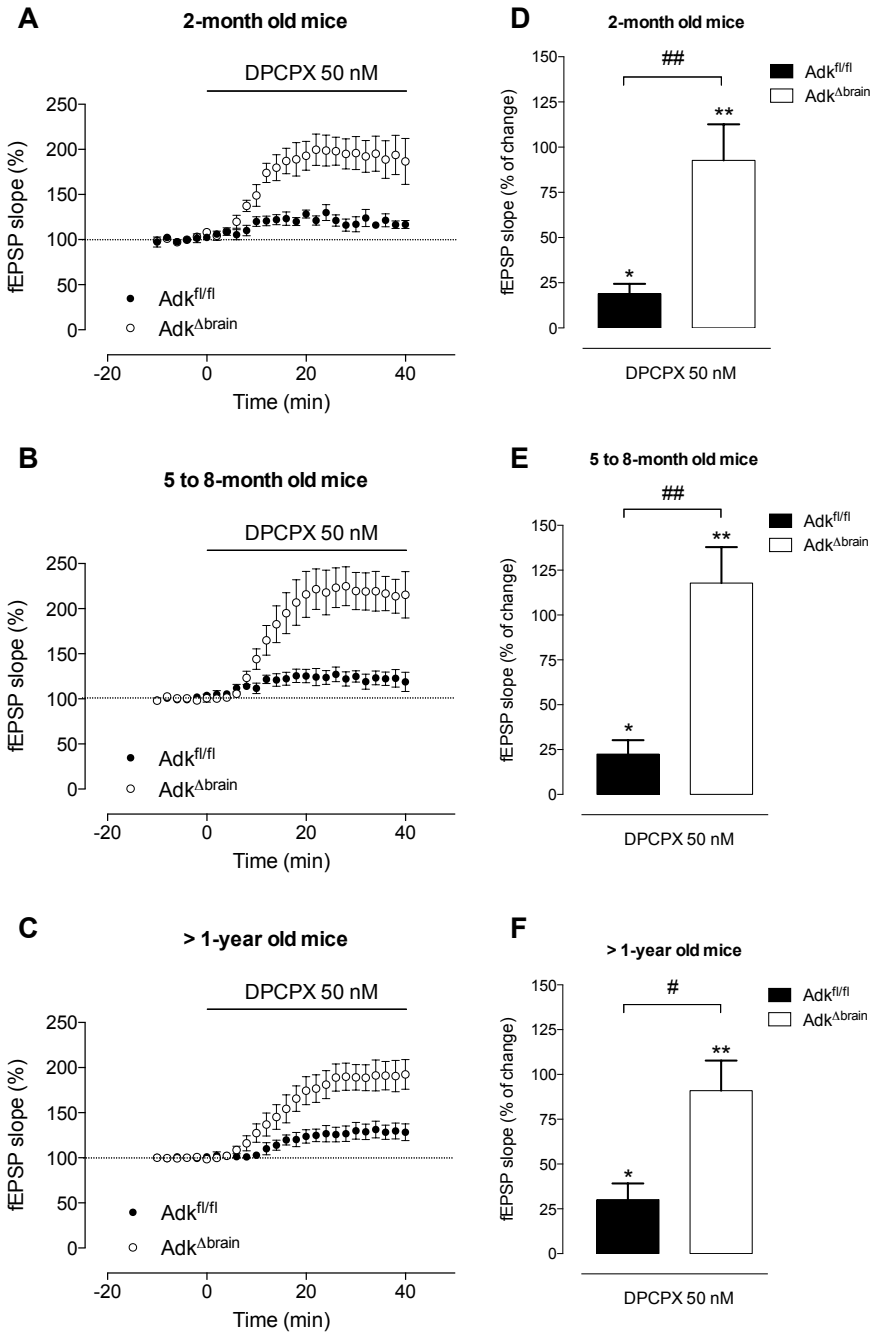

Figure 4.8: Endogenous adenosinergic tonus is influenced by ADK deletion in the brain. The effect of the A_1R antagonist, DPCPX (50 nM), was tested in order to evaluate the degree of disinhibition of synaptic transmission caused by extracellular levels of adenosine in $Adk^{fl/fl}$ (●) and $Adk^{\Delta brain}$ (○) mice. ***(A,B,C)*** Illustrates the average time-course changes in fEPSP slope induced by the application of 50 nM DPCPX in mice of 2 months old (n=5) (A), 5-8 months old (n=5) (B) and over 1 year old (n=5) (C). ***(D,E,F)*** Represents the magnitude of the effect of DPCPX, corresponding to the percentage of change in the fEPSP slopes recorded during the last 10 min in panel A,B,C, respectively. 100% corresponds to the averaged fEPSP recorded for 10 minutes prior to DPCPX perfusion. Results are presented as mean±SEM of n experiments analyzed using a paired t-test when evaluating DPCPX effect in each genotype(*), or an unpaired t-test, when comparing the DPCPX effect between genotypes(#). (*p<0.05, **p<0.01, ##p<0.01).

4.2.1.4 Adenosine A_1 receptor signaling is impaired in $Adk^{\Delta brain}$ mice

To assess A_1R-mediated signaling in hippocampal slices from $Adk^{\Delta brain}$ mice, I evaluated the inhibition of synaptic transmission caused by the A_1R agonist CPA. A dose-response study using 3, 10 and 30 nM of CPA in both $Adk^{\Delta brain}$ mice and their age-matched controls was performed.

Figure 4.9A shows the average time course of changes in fEPSP slope induced by application of increasing CPA concentrations to hippocampal slices taken from 2-month old mice. CPA caused an inhibition of synaptic transmission, reflected by the decrease in fEPSP slope of both $Adk^{\Delta brain}$ and aged-matched $Adk^{fl/fl}$ littermates in a concentration dependent way. As demonstrated in Figure 4.9D, for each genotype, the inhibitory effect of CPA was statistically significant for 30 nM CPA concentration, when compared with the value measured before drug perfusion (n=5, Paired t-test, $p<0.05$). While comparing the effect of the concentration of CPA in different genotypes (Figure 4.9G), I conclude that at 30 nM CPA, the A_1R agonist caused a significantly lower inhibition (Unpaired t-test, $p<0.05$) of synaptic transmission in $Adk^{\Delta brain}$ mice (fEPSP: -18±6.0%, n=5), when comparing to $Adk^{fl/fl}$ (fEPSP: -48±7.3%, n=5).

At the age of 5 to 8-months old, for each genotype, CPA significantly inhibited synaptic transmission for concentrations ≥10 nM (Paired t-test, $p<0.05$, Figure 4.9B,E). As before, the CPA-induced effect was significantly lower in $Adk^{\Delta brain}$ mice, when compared to age-matched control ($fEPSP_{Adk}{}^{fl/fl}$: -53±6.9%, n=5 *vs.* $fEPSP_{Adk}{}^{\Delta brain}$: -28±5.6%, n=7, Unpaired t-test, $p<0.05$, Figure 4.9H).

CPA caused a significant decrease on fEPSP slope in the older $Adk^{fl/fl}$ animals at 10 and 30 nM, when compared to the value measured before the application of the drug (n=5, Paired t-test, $p<0.01$, Figure 4.9C,F). Remarkably, in post-symptomatic mutant mice, CPA did not have any effect on synaptic transmission at any concentration (n=5, Paired t-test, $p>0.05$, Figure 4.9C,F), suggesting a complete absence of activation of the adenosine A_1R by its agonist in $Adk^{\Delta brain}$ mice. While comparing A_1R function in both groups of animals, significant differences at all CPA tested concentrations can be observed between both genotypes, being the greatest at the higher concentration, 30 nM: $fEPSP_{Adk}{}^{fl/fl}$: -72±4.9%, n=5 *vs.* $fEPSP_{Adk}{}^{\Delta brain}$: -1±4.7%, n=5 (Unpaired t-test, $p<0.001$, Figure 4.9I).

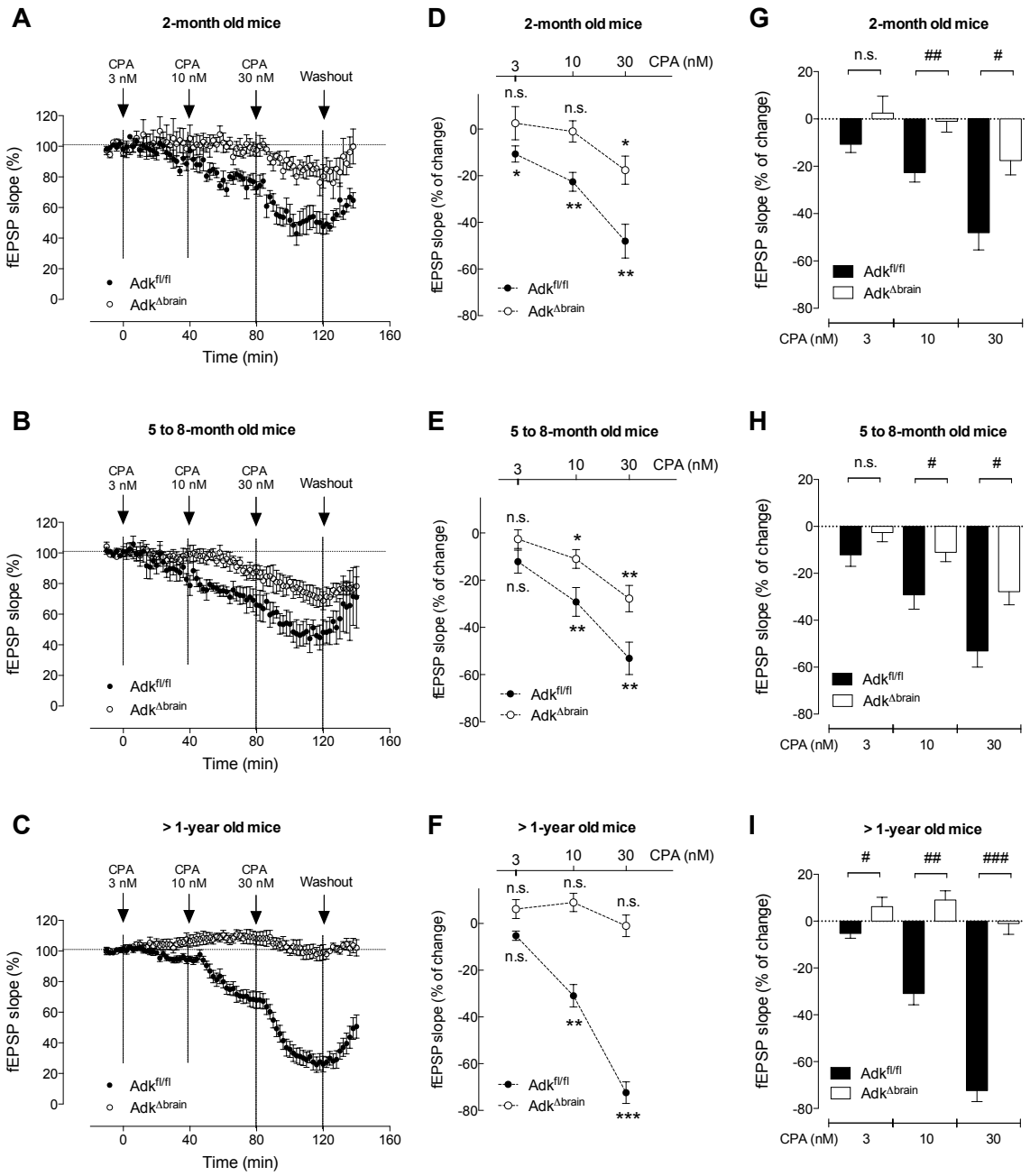

Figure 4.9: Adenosine A_1R signaling is decreased in mice lacking ADK expression. The effect of increasing concentrations of CPA (3, 10 and 30 nM) in synaptic transmission was evaluated to assess A_1R function in $Adk^{fl/fl}$ (●) and $Adk^{\Delta brain}$ (○) mice. ***(A,B,C)*** Illustrates the average time-course changes in fEPSP slope induced by application of increasing concentrations of CPA. ***(D,E,F)*** Represents the logarithmic CPA concentration-response curve. ***(G,H,I)*** Shows the comparison, between $Adk^{fl/fl}$ and $Adk^{\Delta brain}$ mice, at each CPA concentration. **(A,D,G)** represent the data related to 2-month old mice (n=5), **(B,E,H)** to 5 to 8-month old mice (n=5-7) and **(C,F,I)** to over 1-year old mice (n=5). CPA-induced effects correspond to the percentage of change in the fEPSP slopes at 32-40 minutes after CPA superfusion. 100% corresponds to the averaged fEPSP recorded 10 minutes prior to CPA perfusion. Results are presented as mean±SEM of n experiments analyzed using a paired t-test, when evaluating CPA effect in each genotype(*), or an unpaired t-test, when comparing the CPA effect between genotypes(#) (n.s. stands for non significant, *p<0.05, **p<0.01, #p<0.05).

Taken together, these results point to a depressed A_1R-mediated inhibition of synaptic transmission at hippocampal transmission in pre-symptomatic, symptomatic and post-symptomatic $Adk^{\Delta brain}$ mice, being the effect of CPA being virtually lost in post-symptomatic animals. Therefore this allows concluding that $Adk^{\Delta brain}$ mice present an A_1R impaired signaling and that the enhanced disinhibition of synaptic transmission caused by DPCPX in $Adk^{\Delta brain}$ mice results from higher ambient levels of adenosine.

4.2.1.5 Decreased adenosine A_1 receptor expression in $Adk^{\Delta brain}$ mice

To investigate whether the impairment on adenosine A_1R-mediated inhibition of neuronal activity was related to changes in the expression of the receptor, adenosine A_1R protein and mRNA levels were quantified in cortex from $Adk^{fl/fl}$ and $Adk^{\Delta brain}$ animals. Using cortical homogenates, adenosine A_1R protein levels were assessed by saturation binding experiments using [^{3}H]DPCPX as the radioligand. This approach allows the quantification of the maximal number of binding sites (B_{max}) as well as to determine the affinity (K_d) of the receptor for the ligand. Both parameters were obtained by nonlinear regression analysis of the data. A_1R mRNA levels in all animal groups were evaluated through qRT-PCR analysis.

From the binding studies, I observed that transgenic mice A_1R protein levels were significantly decreased at the age of 2 months old (F-test, p<0.001, Figure 4.10A,D). The B_{max} value obtained for $Adk^{fl/fl}$ mice was 237±8.4 fmol/mg protein (n=4) while for $Adk^{\Delta brain}$ animals it was 115±4.7 fmol/mg protein (n=4) (Table 4.1). The dissociation constants presented no significant changes (Figure 4.10E, Table 4.1). A post-transcriptional down-regulation is suggested, since no significant changes were observed at A_1R mRNA level (Figure 4.10F, Table 4.2).

At the age of 5 to 8-months old, the symptomatic age, saturation binding curves also demonstrated a significant decrease in A_1R protein number from $Adk^{\Delta brain}$ mice, when comparing to their age-matched controls (F-test, p<0.001, Figure 4.10B,D). The B_{max} for the transgenic mice was 73±6.5 fmol/mg protein (n=4), while for healthy controls was 239±13.4 fmol/mg protein (n=4) (Table 4.1). No significant differences were found regarding the affinity of the receptor (Figure 4.10E, Table 4.1). Since a significant decrease in A_1R mRNA levels was also found in mice lacking ADK expression at the symptomatic

stage, a transcriptional down-regulation of the receptor is suggested (Figure 4.10F, Table 4.2).

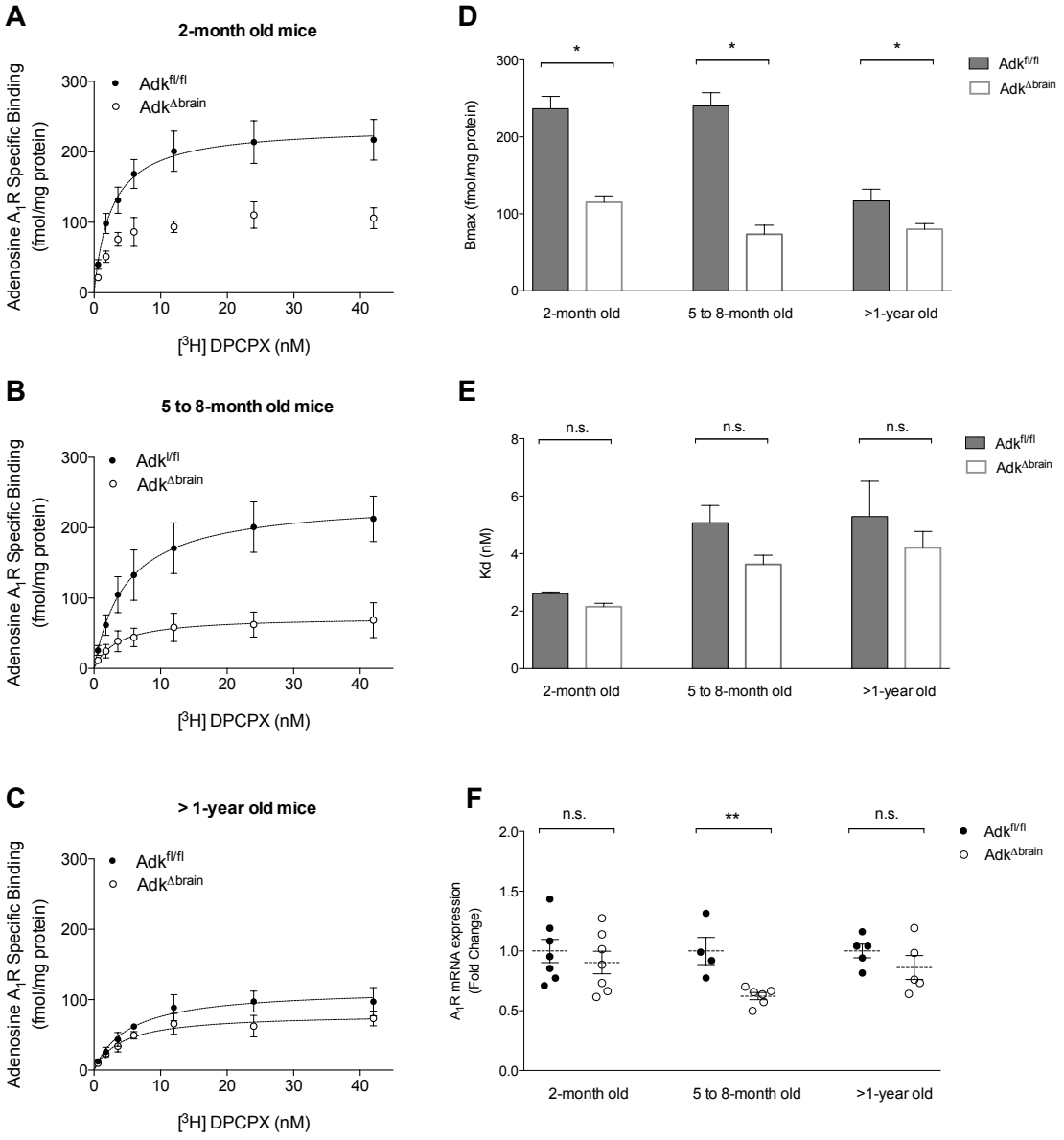

Figure 4.10: Adenosine A_1R expression is down-regulated in Adk$^{\Delta brain}$ mice. Saturation binding assay and qRT-PCR were used to address, respectively, A_1R protein and mRNA levels in Adk$^{fl/fl}$ and Adk$^{\Delta brain}$ mice. ***(A,B,C)*** Illustrates the saturation binding curves for the A_1 receptor selective antagonist [^{3}H]DPCPX using cortical homogenates from Adk$^{fl/fl}$ (●, n=3-4) and Adk$^{\Delta brain}$ (○, n=4) mice of 2-months old (A), 5 to 8-months old (B) and over 1 year old (C). ***(D)*** Shows the average B_{max} and ***(E)*** the average K_d values obtained from the saturation binding curves. ***(F)*** Shows the fold change of A_1 receptor mRNA levels of Adk$^{\Delta brain}$ mice (○, n=5-7), when comparing to healthy Adk$^{fl/fl}$ controls (●, n=4-7). The quantification approach used was the comparative C_t (threshold cycle) method and the genes used to normalize the expression of the target sequences were Cyclophilin A and Ribosomal protein L13A. Results are presented as mean±SEM of n experiments. Saturation binding results were analyzed using an F-test (****p<0.0001), while statistical analysis for mRNA data is an unpaired t-test (**p<0.01).

Table 4.1: Average B_{max} and K_d values obtained for adenosine A_1 receptor. Bmax and K_d were obtained by performing saturation binding curves using [^{3}H]DPCPX (0 to 42 nM) and cortical homogenates from pre-symptomatic, symptomatic and post-symptomatic $Adk^{\Delta brain}$ animals and their age-match healthy controls. Values are mean±SEM of n experiments.

Genotype	Age	B_{max} (fmol/mg protein)	K_d (nM)	Number of experiments
$Adk^{fl/fl}$	2-months old (Symptomatic)	237±8.4	2.6±0.4	4
$Adk^{\Delta brain}$		115±4.7	2.1±0.4	4
$Adk^{fl/fl}$	5 to 8-months old (Symptomatic)	239±13.4	4.8±0.9	4
$Adk^{\Delta brain}$		73±6.5	3.5±1.1	4
$Adk^{fl/fl}$	>1-year old (Post-symptomatic)	116±7.3	5.3±1.1	3
$Adk^{\Delta brain}$		80±4.7	4.2±0.8	4

Table 4.2: $Adk^{fl/fl}$ and $Adk^{\Delta brain}$ A_1R mRNA expression. mRNA quantification was achieved by qRT-PCR analysis, using the comparative C_t method. Cyclophilin A and Ribosomal protein L13A genes were used as normalizers. Values were normalized to respective control ($Adk^{fl/fl}$) and are represented as mean±SEM of n experiments.

Genotype	Age	Normal. A_1R mRNA expression	Number of experiments
$Adk^{fl/fl}$	2-months old (Symptomatic)	1.0±0.10	7
$Adk^{\Delta brain}$		0.9±0.09	7
$Adk^{fl/fl}$	5 to 8-months old (Symptomatic)	1.0±0.11	4
$Adk^{\Delta brain}$		0.6±0.03	6
$Adk^{fl/fl}$	>1-year old (Post-symptomatic)	1.0±0.06	5
$Adk^{\Delta brain}$		0.9±0.10	5

As shown in Figure 4.10C,D,E and Table 4.1 a significant (F-test, p<0.001) decrease of A_1R protein number, without changes in the affinity state, is also revealed in transgenic animals over 1-year old: the B_{max} values were 116±7.3 fmol/mg protein in $Adk^{fl/fl}$ mice (n=3) and 80±4.7 fmol/mg protein in $Adk^{\Delta brain}$ mice (n=4). Accordingly to the data obtained with qRT-PCR, no changes are observed at transcriptional level, since no significant differences were obtained for A_1R mRNA level (Figure 4.10F, Table 4.2).

Altogether, the decreased A_1R protein levels indicates a down-regulation of adenosine A_1R in $Adk^{\Delta brain}$ mice at all ages, which is most probably an adaptation to the extracellular adenosine overload throughout life.

4.2.1.6 Symptomatic and post-symptomatic $Adk^{\Delta brain}$ mice have an increased adenosine A_{2A} receptor tonus in synaptic plasticity

Having found that adenosine overload in $Adk^{\Delta brain}$ mice leads to perturbed A_1R signaling, the next series of experiments were designed to evaluate whether adenosine $A_{2A}R$ function could also be affected. In order to evaluate adenosine $A_{2A}R$ signaling, I first

performed extracellular recordings from hippocampal slices taken from pre-symptomatic, symptomatic and post-symptomatic $Adk^{\Delta brain}$ mice and respective $Adk^{fl/fl}$ littermates in the presence of an adenosine $A_{2A}R$ agonist and of an antagonist and evaluated the effect upon fEPSP recordings. To test whether $A_{2A}R$ activation affected synaptic transmission, the selective $A_{2A}R$ agonist CGS 21680 (Jarvis et al. 1989) was used. As illustrated in Figure 4.11A,B,C, CGS 21680 (10 nM) was virtually devoid of effect (paired t-test, $p>0.05$) on fEPSPs recorded from hippocampal slices taken either from $Adk^{\Delta brain}$ mice (n=4) or $Adk^{fl/fl}$ mice (n=4). To assess for changes in tonic $A_{2A}R$ activation, we then tested the effect of $A_{2A}R$ blockade, by using the selective adenosine $A_{2A}R$ antagonist SCH 58261 (Fredholm et al. 1998). The presence of SCH 58261 at a supramaximal concentration, 50 nM (Lopes et al. 2004), was also virtually devoid of effect on fEPSP slope in all groups of mice (n=4, paired t-test, $p>0.05$, Figure 4.11D,E,F).

The absence of direct effects on synaptic transmission by activating or antagonizing adenosine $A_{2A}R$ may be related with the low expression levels of these receptors in the hippocampal formation (Dixon et al. 1996; Ribeiro et al. 2003). However, $A_{2A}Rs$ may facilitate hippocampal Long-Term Potentiation (LTP). To establish whether *Adk* deletion could affect the tonic influence of $A_{2A}R$ upon LTP, we then tested the effect of the selective $A_{2A}R$ antagonist, SCH 58261 (50 nM), upon LTP. A θ–burst (3 bursts of 3 stimuli each, 3x3) was applied to the hippocampal slices taken from $Adk^{fl/fl}$ and $Adk^{\Delta brain}$, either in the presence or in the absence of 50 nM SCH 58261, this drug being added to the superfusing solution at least 30 minutes before LTP induction.

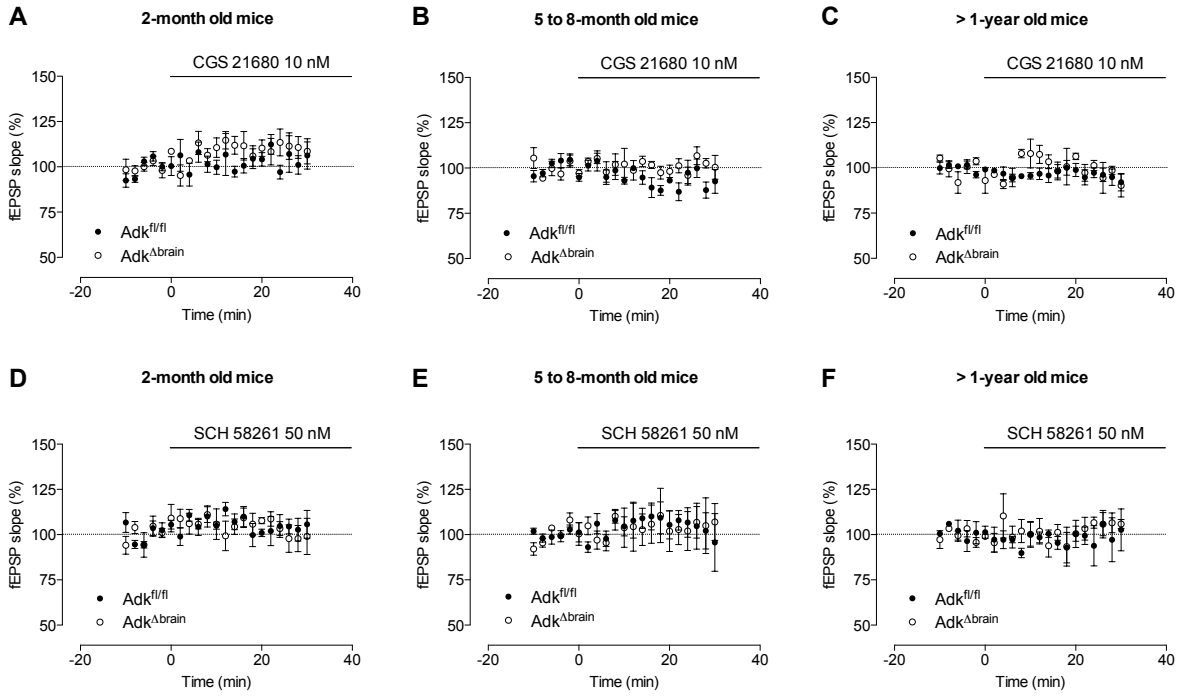

Figure 4.11: Neither adenosine A_{2A} receptor activation nor blockade change basal synaptic transmission in $Adk^{fl/fl}$ and $Adk^{\Delta brain}$ mice. The effect of the $A_{2A}R$ selective agonist CGS 21680 (10 nM) ***(A,B,C)*** and antagonist SCH58261 (50 nM) ***(D,E,F)*** on fEPSP slope recorded from hippocampal slices taken from $Adk^{fl/fl}$ (●, n=4) and $Adk^{\Delta brain}$ (○, n=4) mice at the age of 2 months old (A,D), 5 to 8 months old (B,E) and over 1 year old (C,F) was tested to assess their influence upon synaptic transmission. Results are presented as mean±SEM of n experiments.

In accordance with previous reports using mild LTP induction and slices from young adult rodents (Costenla et al. 2011), adenosine $A_{2A}R$ blockade was virtually devoid of any effects on LTP magnitude in 2-month old $Adk^{fl/fl}$ mice (n=5, Paired t-test, p>0.05, Figure 4.12A,G) since the fEPSP slope after 60 minutes of LTP induction was 128%±3.7 in control conditions and 127%±9.4 in presence of SCH 58261. When comparing LTP magnitude in the absence of any drug in slices from $Adk^{fl/fl}$ mice and from pre-symptomatic $Adk^{\Delta brain}$ mice (Figure 4.12G), no statistical differences were found. Again, in slices taken from 2-months old $Adk^{\Delta brain}$ mice, no differences in LTP magnitude were observed upon $A_{2A}R$ blockade ($fEPSP_{control}$: 147%±9.8 *vs.* $fEPSP_{SCH\ 58261}$: 132%±4.6, n=5, Unpaired t-test, p>0.05, Figure 4.12D,G).

As illustrated in Figure 4.12B,H, the mild LTP obtained upon θ–burst stimuli in hippocampal slices from 5 to 8-months old $Adk^{fl/fl}$ mice was not affected by $A_{2A}R$ blockade ($fEPSP_{control}$: 122%±4.0 *vs.* $fEPSP_{SCH\ 58261}$: 124%±4.5, n=5, Paired t-test, p>0.05). When comparing LTP magnitude in control conditions in slices from $Adk^{fl/fl}$ mice and symptomatic $Adk^{\Delta brain}$ mice (Figure 4.12H), a significant (Unpaired t-test, p<0.01) increase

in LTP was detected ($fEPSP_{Adk}^{fl/fl}$: 122%±4.0, n=5 *vs.* $fEPSP_{Adk}^{\Delta brain}$: 152%±6.9, n=5). Remarkably, the presence of SCH 58261 in symptomatic $Adk^{\Delta brain}$ mice reverted the magnitude of LTP towards values close to the $Adk^{fl/fl}$ mice ($fEPSP_{control}$: 152%±6.9 *vs.* $fEPSP_{SCH\ 58261}$: 113%±5.6, n=5, Paired t-test, $p<0.05$, Figure 4.12E).

Similar results were obtained for the post-symptomatic animals (Figure 4.12C,F,I). No significant differences (Paired t-test, $p>0.05$, Figure 4.12C,I) were found between control and SCH 58261 conditions in aged $Adk^{fl/fl}$ animals ($fEPSP_{control}$: 134%±3.3, n=5 *vs* $fEPSP_{SCH\ 58261}$: 125%±5.1, n=5). Despite no statistically differences (Unpaired t-test, $p>0.05$, Figure 4.11I), there is a tendency to a higher LTP magnitude ($fEPSP_{Adk}^{\Delta brain}$: 150%±10.4, n=5) in *Adk* knockout post-symptomatic mice, when compared to LTP in control conditions in age-matched mice ($fEPSP_{Adk}^{fl/fl}$: 134%±3.3, n=5). As illustrated in Figure 4.12F,I, this increase in LTP magnitude is prevented in the presence of the $A_{2A}R$ antagonist ($fEPSP_{Adk}^{\Delta brain}$: 122%±5.5, n=5, Paired t-test, $p<0.05$).

Taken together, these data suggest that, symptomatic and post-symptomatic, but not pre-symptomatic, $Adk^{\Delta brain}$ mice do possess an enhanced tonic A_{2A} receptor activation that impacts upon exacerbated synaptic plasticity.

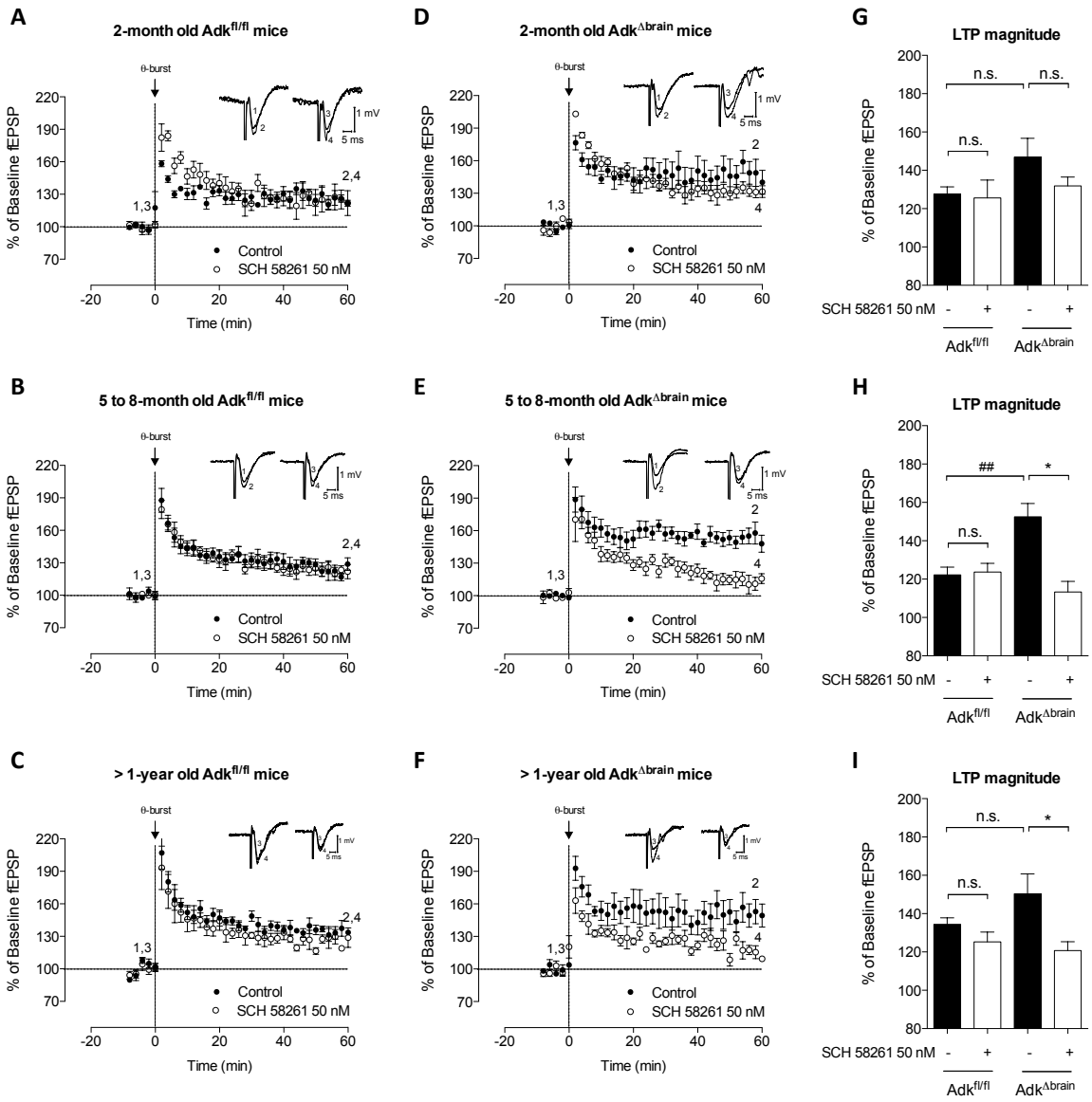

Figure 4.12: Symptomatic and post-symptomatic Adk$^{\Delta brain}$ mice present an enhancement of LTP, which is prevented upon A_{2A} receptor blockade. The effect of the selective A_{2A}R antagonist, SCH58261, in θ-burst (3x3) induced LTP was evaluated in Adk$^{fl/fl}$ and Adk$^{\Delta brain}$ mice across the three phenotypic stages. ***(A-F)*** depict the time courses of the average changes of fEPSP slopes in the absence (●, n=5) or in the presence (○, n=5) of SCH 58261, in Adk$^{fl/fl}$ (A,B,C) and Adk$^{\Delta brain}$ (D,E,F) animals at all ages, as depicted above each graph. The upper panels in (A-F) show traces from representative experiments, which are composed by the stimulus artifact, followed by the pre-synaptic volley and the fEPSP; each trace is the average of six consecutive responses obtained before (1,3) and after (2,4) the θ-burst stimuli, in the absence (1,2) and in the presence of SCH 58261 (3,4). ***(G-I)*** Illustrates the magnitude of LTP, corresponding to the percentage of change in the fEPSP slopes recorded at 52-60 minutes after LTP induction. 100% corresponds to the averaged fEPSP slope recorded 10 minutes prior to LTP induction. Results are presented as mean±SEM of n experiments analyzed using a paired t-test, when comparing LTP magnitudes in the presence or absence of SCH 58261(*), or an unpaired t-test, when comparing LTP magnitudes between genotypes(#). (n.s. stands for non significant, *p<0.05, ##p<0.01).

4.2.1.7 Adaptive changes in A_{2A} receptor expression in $Adk^{\Delta brain}$ mice along ageing

We next investigated whether $Adk^{\Delta brain}$ animals also displayed an altered $A_{2A}R$ expression pattern. $A_{2A}R$ were quantified in the cortex from $Adk^{fl/fl}$ and $Adk^{\Delta brain}$ mice. Saturation binding technique was not technical possible, since $A_{2A}R$ expression in the cortex is low (Dixon et al. 1996; Ribeiro et al. 2003) and high amounts of protein would be required. Therefore, Western Blot analysis to quantify $A_{2A}R$ protein levels in cortical homogenates was performed.

The protein levels of $A_{2A}R$ in pre-symptomatic $Adk^{\Delta brain}$ mice were decreased by 58%±9.9 (n=5), while comparing to 2-month old $Adk^{fl/fl}$ mice (Unpaired t-test, $p<0.01$, Figure 4.13A,B). On the contrary, as shown in Figure 4.13A,B, $A_{2A}R$ immunoreactivity in symptomatic $Adk^{\Delta brain}$ cortical homogenates was significant increased, when compared to their age-matched healthy $Adk^{fl/fl}$ littermates (269%±69.3, n=4, Unpaired t-test, $p<0.05$). Post-symptomatic $Adk^{\Delta brain}$ mice, however, did not present significant different $A_{2A}R$ protein number (112%±31, n=4, Unpaired t-test, $p>0.05$, Figure 4.13A,B) in cortical homogenates compared to their respective $Adk^{fl/fl}$ controls. $A_{2A}R$ mRNA levels were evaluated through qRT-PCR analysis. As illustrated in Figure 4.13C and Table 4.3, no statistical significance (Unpaired t-test, $p>0.05$) was found between genotypes at any phenotypic stage.

Table 4.3: $Adk^{fl/fl}$ and $Adk^{\Delta brain}$ $A_{2A}R$ mRNA expression. mRNA quantification was achieved by qRT-PCR analysis, using the comparative C_t method. Cyclophilin A and Ribosomal protein L13A genes were used as normalizers. Values were normalized to respective $Adk^{fl/fl}$ control and are represented as mean±SEM of n experiments.

Genotype	Age	Normal. $A_{2A}R$ mRNA expression	Number of experiments
$Adk^{fl/fl}$	2-months old (Symptomatic)	1.0±0.36	7
$Adk^{\Delta brain}$		2.4±1.35	5
$Adk^{fl/fl}$	5 to 8-months old (Symptomatic)	1.0±0.08	7
$Adk^{\Delta brain}$		2.0±0.80	7
$Adk^{fl/fl}$	>1-year old (Post-symptomatic)	1.0±0.28	8
$Adk^{\Delta brain}$		1.4±0.33	9

Together, these data, suggest a differential regulation of $A_{2A}R$ expression in mice not expression ADK in the entire brain across ageing, presumably by post-transcriptional mechanisms since no differences at mRNA level were observed. Adaptive down-regulation of $A_{2A}R$ expression in pre-symptomatic $Adk^{\Delta brain}$ mice and up-regulation of $A_{2A}R$

expression in symptomatic Adk$^{\Delta brain}$ mice, while no differences at the post-symptomatic age were found.

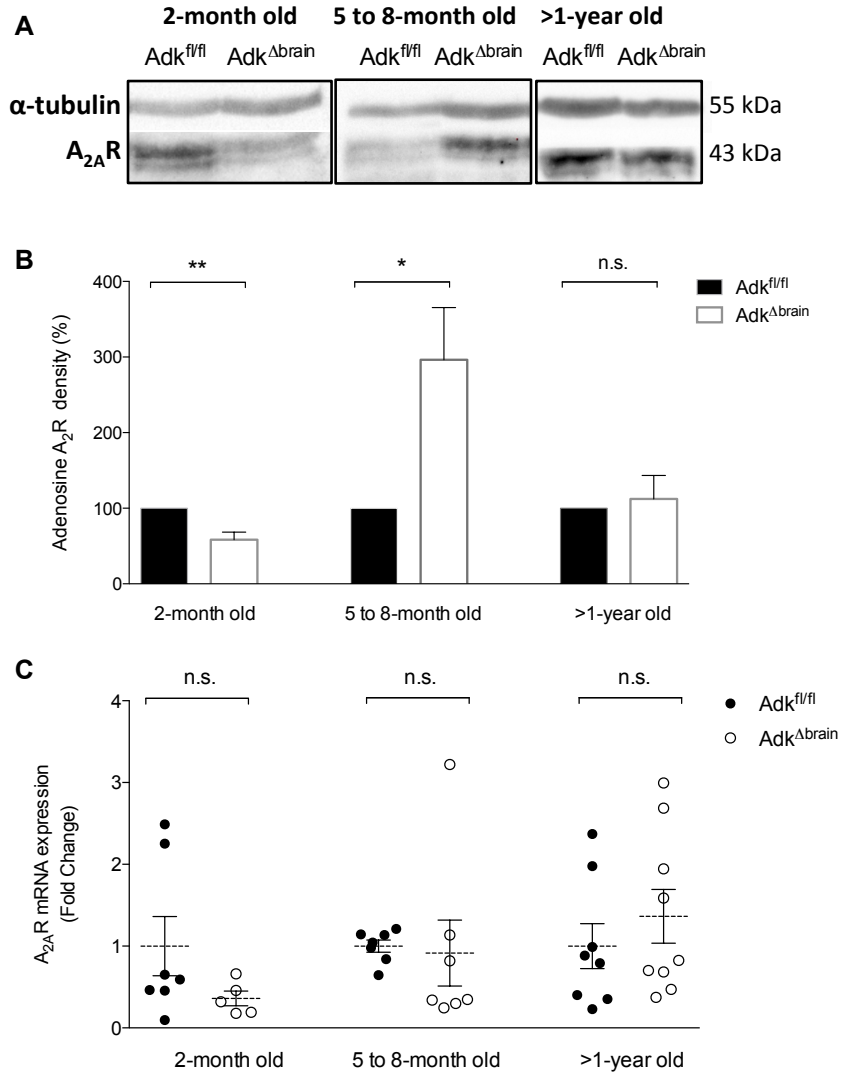

Figure 4.13: Adenosine kinase knockout differently affects adenosine A_{2A}R protein levels along ageing. Western Blot and qRT-PCR were used to address, respectively, A_{2A}R protein and mRNA levels in Adk$^{fl/fl}$ and Adk$^{\Delta brain}$ mice. ***(A)*** A representative image of western blot of adenosine A_{2A}R (43 kDa) and α-Tubulin (55 kDa, loading control) in cortical homogenates from Adk$^{fl/fl}$ (n=4-5) and Adk$^{\Delta brain}$ mice (n=4-5), as indicated above each lane. In ***(B)*** are shown the averaged values of immunoreactivity of A_{2A}R normalized by α-Tubulin. ***(C)*** Illustrates the fold change of A_{2A} receptor mRNA levels of Adk$^{\Delta brain}$ (○, n=5-9), when comparing to healthy Adk$^{fl/fl}$ controls (●, n=7-8). The quantification approach used was the comparative C_t (threshold cycle) method and the genes used to normalize the expression of the target sequences were Cyclophilin A and Ribosomal protein L13A. Results are the mean±SEM of n experiments and statistical analysis was performed using an unpaired t-test (n.s. stands for non significant, *p<0.05).

4.2.2 Influence of Adk *deletion on BDNF modulation of synaptic plasticity*

4.2.2.1 Rationale

In 2001, for the first time, a functional interaction between adenosine $A_{2A}R$ and TrkB receptors was described. When activated and in the absence of BDNF, $A_{2A}Rs$ are able to transactivate the BDNF high affinity receptor, the TrkB receptor, in hippocampal neurons (Lee & Chao 2001). Since then, much attention has been given to the interaction between these two receptors, as well as the impact of its effects on synaptic transmission and plasticity in the hippocampus. Besides triggering TrkB transactivation, adenosine $A_{2A}R$ is also able to facilitate TrkB-mediated actions as first identified in 2004 (Diógenes et al. 2004). Recently, a molecular mechanism for the crosstalk between these two receptors has been proposed. Adenosine $A_{2A}Rs$ activation induces TrkB translocation to lipid rafts, which facilitates receptor activation and signaling (Assaife-Lopes et al. 2014).

In hippocampal slices, $A_{2A}R$ facilitates BDNF facilitatory effect on synaptic transmission. Importantly, while in infant rats BDNF actions were only observed upon adenosine $A_{2A}R$ activation by the application of an agonist; in adult rats, tonic activation of adenosine $A_{2A}Rs$ was enough to trigger BDNF-mediated effects on basal synaptic transmission, which was lost upon $A_{2A}R$ blockade (Diógenes et al. 2007), hence suggesting a higher influence of $A_{2A}Rs$ upon BDNF-mediated actions across ageing and concomitant increase of $A_{2A}Rs$ expression.

Upon high frequency stimulation, the release of BDNF (Goodman et al. 1996) and ATP (Wieraszko et al. 1989), the precursor of adenosine, is increased, therefore favoring the activation of adenosine $A_{2A}R$ (Correia-De-Sá & Ribeiro 1996). Taking this to account, ideal conditions would be then gathered for adenosine $A_{2A}R$ and TrkB crosstalk during plasticity phenomena. Indeed, additionally to the interaction observed at synaptic transmission level, the facilitatory action of BDNF upon synaptic plasticity is also dependent on adenosine $A_{2A}R$ activation. BDNF facilitates θ-burst LTP, however, this effect was fully lost in the presence of an adenosine $A_{2A}R$ antagonist or by removing endogenous extracellular adenosine (Fontinha et al. 2008).

Taking in account: *1)* the increased levels of extracellular adenosine, *2)* the increased LTP magnitude in *Adk* knockout animals, when compared to Wild type and *3)* the prevention of this increase upon $A_{2A}R$ blockade, I next evaluated if the increase on LTP

detected in Adk$^{\Delta brain}$ mice could also result from enhanced BDNF signaling. The next series of experiments were then dedicated to the study of the influence of ADK deletion onto BDNF modulatory role of synaptic plasticity, as well to the characterization of TrkB expression levels.

4.2.2.2 BDNF signaling is increased in the Adk$^{\Delta brain}$ symptomatic mice

Hippocampal slices from symptomatic and age-matched healthy mice were treated with K252a (200 nM). K252a is a serine/threonine protein kinase inhibitor also shown to be a potent inhibitor of tyrosine activity of Trk family (Tapley et al. 1992). During the extracellular recordings, the slices were perfused in either Krebs' solution alone or in Krebs' solution containing 200 nM K252a. Only 30 minutes after drug perfusion and a 10-minute stable baseline, the θ-burst was applied. As previously shown, in the absence of any drug, a large LTP is observed in *Adk* knockout symptomatic mice, when compared to the respective Wild type control. If the larger LTP observed in the epileptic mice was due to increased TrkB signaling, a lower LTP magnitude in the slices treated with K252a would be expected.

In symptomatic Adk$^{\Delta brain}$, the magnitude of LTP in slices in the presence of K252a (200nM) was markedly reduced or even abolished (101%±7.0 increase, n=5, p<0.01, as compared with the magnitude of LTP in the absence of the inhibitor 150%±6.7, n=5, Figure 4.14A,B), thus returning to values similar to those obtained in Adk$^{fl/fl}$ mice. K252a had virtually no effect on LTP magnitude in Adk$^{fl/fl}$ mice (data not shown), in agreement with what has been previously reported for rats (Fontinha et al. 2009). These results thus suggest a higher TrkB receptor activation that accounts for a larger LTP in the epileptic Adk$^{\Delta brain}$ mice.

Due to the low selectivity of K252a, to further assess the possibility of an exacerbated BDNF tonus in Adk$^{\Delta brain}$ mice, I evaluated the influence of the BDNF scavenger TrkB-Fc upon LTP. TrkB-Fc is a chimera protein containing the ligand binding of the native TrkB receptor fused to the Fc fragment of human immunoglobulin and thus has the ability to scavenge unbound BDNF and act as a BDNF buffer, preventing its binding to TrkB (Shelton et al. 1995). As previously described (Figurov et al. 1996), a θ-burst induced in the neonatal hippocampus where endogenous BDNF level is low, no effect of TrkB-Fc was observed; in the adult hippocampus, however, where BDNF extracellular concentration is

higher, the TrkB-Fc decreased the LTP magnitude. So, the decreased LTP magnitude is observed in the symptomatic *Adk* knockout mice in the presence of TrkB-Fc, I may argue these animals have higher levels of endogenous BDNF.

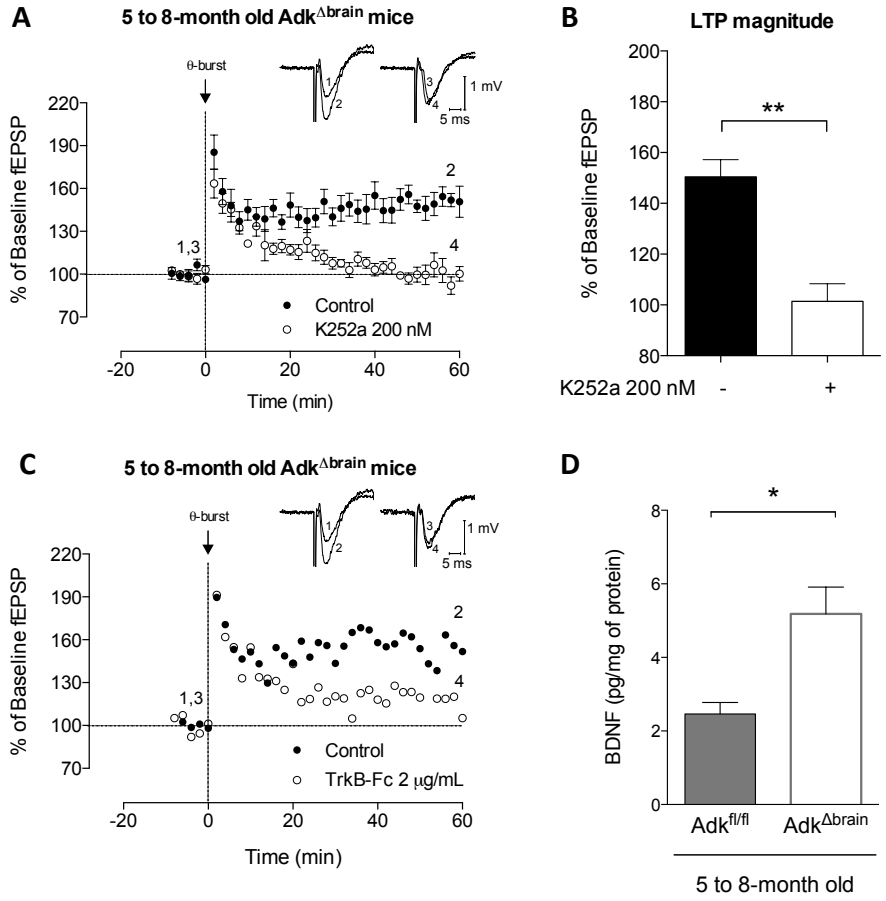

Figure 4.14: Endogenous BDNF signaling upon LTP is enhanced in Adk$^{\Delta brain}$ mice. The effect of the TrkB inhibitor, K252a, and of the BDNF scavenger, TrkB-Fc, upon θ-burst (3x3) induced LTP was evaluated. In ***(A)*** are depicted the time-course changes of fEPSP slopes in the absence (●, n=5) or in the presence (○, n=5) of 200 nM K252a, in slices from Adk$^{\Delta brain}$ mice. ***(B)*** Illustrates the magnitude of LTP, corresponding to the percentage of change in the fEPSP slopes recorded at 52-60 minutes after LTP induction. 100% corresponds to the averaged fEPSP slope recorded 10 minutes prior to LTP induction. Results are presented as mean±SEM and statistical differences of the averaged fEPSP slopes recorded at 52-60 minutes after inducing LTP in the presence or absence of K252a were analyzed using a paired t-test (**p<0.01). ***(C)*** Depicts a representative time-course of the average changes of fEPSP slopes in the absence (●) or in the presence (○) of 2 μg/mL TrkB-Fc in a Adk$^{\Delta brain}$ mice (n=1). The upper panels in (A) and (C) show traces from representative experiments, which are composed by the stimulus artifact, followed by the pre-synaptic volley and the fEPSP; each trace is the average of six consecutive responses obtained before (1,2) and after (3,4) the θ-burst stimuli, in the absence (1,2) and in the presence (3,4) of the test drug. ***(D)*** Shows the average amount of BDNF detected in hippocampal homogenates taken from 5 to 8-month old Adk$^{\Delta brain}$ mice and respective Adk$^{fl/fl}$ littermates. BDNF protein levels quantification was attained by ELISA analysis.

Previous to LTP induction, 2 μg/mL TrkB-Fc was added for 30 minutes. As illustrated in Figure 4.14C, scavenging of BDNF clearly reduced the magnitude of LTP recorded in a representative slice from Adk$^{\Delta brain}$ mouse. While in the absence of TrkB-Fc, the

percentage of fEPSP increase was 150.5% (n=1); in its presence, LTP magnitude was 115.7% (n=1). Taken together, our results support the conclusion that the enhanced LTP in $Adk^{\Delta brain}$ mice can be attributed to a higher influence of endogenous BDNF upon LTP. Accordingly, a marked increase (Unpaired t-test, $p<0.05$) in BDNF protein levels was observed in animals lacking ADK expression in the brain: 2.5±0.3 pg/mg protein in $Adk^{fl/fl}$ mice (n=5) and 4.7±0.7 pg/mg protein in $Adk^{\Delta brain}$ mice (n=6) of BDNF were quantified by ELISA analysis (Figure 4.14D). Interestingly, this marked difference was lost in aged animals, when the epileptic phenotype is lost (data not shown).

4.2.2.3 Adenosine kinase brain knockout affects BDNF high affinity receptor, TrkB, expression along ageing

We next sought to determine whether $Adk^{\Delta brain}$ mice try to compensate for increased BDNF signaling. As before, TrkB expression was assessed by Western Blot analysis of cortical protein extracts derived from $Adk^{\Delta brain}$ mice and their control littermates.

Interestingly, as compared to $Adk^{fl/fl}$ controls, a marked increased expression of the TrkB-FL (148%±15.5, n=6, $p<0.05$) and TrkB-Tc (194%±42.3, n=5, $p>0.05$) receptors in pre-symptomatic (2-month old) $Adk^{\Delta brain}$ mice cortical homogenates was observed (Figure 4.15). However at the symptomatic age, and as previously detected in aged rodents (Diógenes et al. 2011), the exacerbated BDNF tonus was accompanied by a decreased expression of 58%±3.8, n=3 (Unpaired t-test, $p<0.001$) of its high affinity receptor, the full length TrkB receptor (FL-TrkB), without changes in the number of its low affinity receptor $p75^{NTR}$ (Unpaired t-test, $p>0.05$, Figure 4.15). At the post-symptomatic age, no differences in the immunoreactivity of TrkB-FL and TrkB-Tc receptors were found between $Adk^{\Delta brain}$ and $Adk^{fl/fl}$ mice (Figure 4.15).

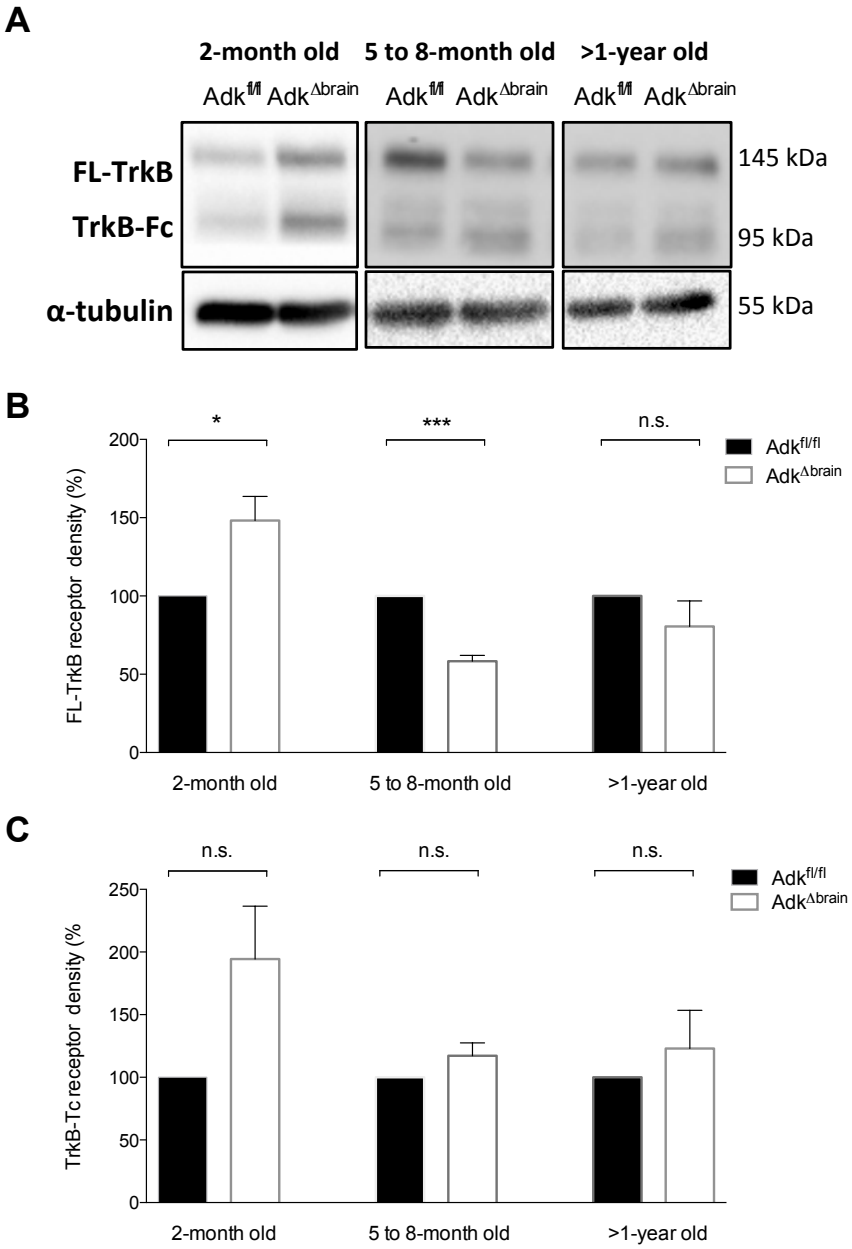

Figure 4.15: TrkB-FL expression is differently affected at each phenotypic stage of Adk$^{\Delta brain}$ mice. Western blot was used to address full length and truncated TrkB (FL-TrkB and TrkB-Tc, respectively) protein levels in pre-symptomatic, symptomatic and post-symptomatic Adk$^{\Delta brain}$ mice and respective Adk$^{fl/fl}$ littermates. ***(A)*** A representative image of immunobloting of FL-TrkB (145 kDa), TrkB-Tc (95 kDa) and α-Tubulin (55kDa, loading control) in cortical homogenates from Adk$^{fl/fl}$ and Adk$^{\Delta brain}$ mice, as indicated above each lane. ***(B)*** and ***(C)*** Show the averaged values of immunoreactivity of FL-TrkB (B) and TrkB-Tc (C) normalized by α-Tubulin. Results are the mean±SEM of 3-6 experiments and statistical analysis was performed using an unpaired t-test (*p<0.05, ***p<0.001).

4.2.3 Discussion

In the present work, I studied a novel mouse line, $Adk^{\Delta brain}$ that, as human patients with mutations in the *Adk* gene (Bjursell et al. 2011), present epileptic seizures. ADK deficiency in the brain leads to neuronal adaptation processes in the mice that develop an age-dependent epileptic phenotype: they start to present seizures from 2-month old, until their first year old, when they become asymptomatic. I demonstrated that ADK deficiency triggers enhanced excitability and epileptic activity, which is related with enhanced $A_{2A}R$ and BDNF-dependent synaptic plasticity. These findings suggest that global ADK deficiency has direct implications on neuronal excitability leading to epileptic activity, which might be amenable to treatment. Moreover, this work highlights the critical role of the homeostatic control of adenosine and BDNF for proper control of synaptic activity and plasticity.

ADK deregulated function consequences. As concerning *Adk* gene mutations leading to gain of function, no mutations in humans are known. However, ADK up-regulation in epileptogenesis is widely described both in hippocampus of patients with epilepsy (Boison 2013; Aronica et al. 2011), as well as in rodent models of epilepsy (Li et al. 2008) and thought to be intrinsically linked to the development of epilepsy (Li et al. 2008; Boison 2010). Both transgenic overexpression of ADK (Li et al. 2008; Fedele et al. 2005) and adeno associated virus-mediated overexpression of ADK in astrocytes (Shen et al. 2014) are sufficient to induce spontaneous recurrent seizures in mice, which are though to be related to deficient adenosine A_1R-mediated signaling (Gouder et al. 2004; Fedele et al. 2006).

Human patients with global ADK deficiency due to homozygosity of point mutations in the coding sequence of the ADK gene are rare with only six patients from three independent families identified to date (Bjursell et al. 2011). Similar features of human patients and a mice genetic model of global *Adk* knockout are found, such as aberrations of liver metabolism and lethal apnea (Boison et al. 2002). Human condition, as a consequence of *Adk* mutations, is also characterized by hepatic encephalopathy, development delay and epilepsy. These features could not be tested for the homozygous $Adk^{-/-}$ mice mutants since within 4 days after birth they would die (Boison et al. 2002).

Brain-wide deletion of *Adk* allowed to specifically investigating the consequences of ADK deficiency on brain physiology were engineered by our collaborators. These mice, $Adk^{\Delta brain}$ mice consistently manifest stress-induced seizures from 5 to 12 months old, when they become asymptomatic. In the presented study, I identified adaptive changes that trigger the pathological phenotype that will be further discussed.

A_1 receptor dependent effects. By using adenosine biosensors, evidence for an inverse relation between ADK expression and adenosine levels has been provided (Shen et al. 2011). The synaptic potential recordings in the present work also revealed a higher adenosinergic tonus in all phenotypic stages of $Adk^{\Delta brain}$ mice when compared to age matched control littermates, since the A_1R antagonist, DPCPX, caused a more pronounced disinhibition of synaptic transmission in $Adk^{\Delta brain}$ mice. In adult brain, astrocytic ADK phosphorylates adenosine, generating a concentration gradient that allows adenosine influx to the astrocytes and clearance from the synaptic cleft (Boison 2010). The lack of expression of ADK in these mice and thus, the lack of an efficient adenosine "buffering" system that clears out extracellular adenosine, may thus lead to the increased adenosinergic tonus observed in *Adk* knockout mice. However, lower expression levels of the A_1R and a lower inhibitory effect of A_1R agonist upon synaptic transmission in all $Adk^{\Delta brain}$ animals was observed. Strong convulsive seizures were already reported to reduce adenosine A_1 receptor protein levels in other epileptic models (Ekonomou et al. 2000; Rebola et al. 2003; Rebola, Porciúncula, et al. 2005) as well as in human patients (Glass et al. 1996). Indeed, seizures trigger an increase in adenosine to levels of up to 65 µM, which is significantly higher that the 20-200 nM baseline levels of adenosine (During & Spencer 1992; Boison 2010). Therefore, in chronic epilepsy, the observed down-regulation of the A_1R is likely a consequence of recurrent seizure activity. Adenosine A_1 receptor activity was shown to suppress epileptic seizures (Gouder et al. 2004) and to keep the epileptic focus localized (Fedele et al. 2006). In this $Adk^{\Delta brain}$ model, where constant adenosine overload is present and seizures start to develop latter in life (after 2-3 months), the decrease of A_1R signaling may be one of the leading causes of increased excitability, revealing ADK as regulator of the homeostatic adenosine tone a critical factor for the regulation of neuronal activity. Though, the leading cause of seizure manifestation

is not only due to A_1R impaired signaling since it is observed at pre-, post- and symptomatic stages.

A_{2A} receptor dependent effects. Besides reducing adenosine A_1 receptor inhibitory signaling, *Adk* knockout also changed adenosine A_{2A} receptor function and expression. A_{2A} receptor protein levels were down-regulated in pre-symptomatic $Adk^{\Delta brain}$ mice, however, in symptomatic mice A_{2A} receptor expression was exacerbated. Interestingly, symptomatic $Adk^{\Delta brain}$ animals expressed a significantly larger LTP magnitude, which was prevented in the presence of an adenosine A_{2A} receptor antagonist, suggesting a higher tonic influence of this receptor in synaptic plasticity modulation. The finding that adenosine $A_{2A}R$ protein expression is up-regulated is consistent with previous findings in the kindling model of epilepsy, in which a long-term increased density of adenosine A_{2A} receptors was observed (Rebola, Porciúncula, et al. 2005). In line with the anti-epileptic effects on adenosine A_{2A} receptor antagonist (Etherington & Frenguelli 2004; Li et al. 2012), adenosine $A_{2A}R$ knockout mice are partially protected from convulsive activity in some experimental models of epilepsy (El Yacoubi et al. 2009). In ex vivo acute models of seizures under conditions where long lasting changes of $A_{2A}R$ expression do not occur, $A_{2A}R$ antagonists also reduce synchronous pyramidal cell firing (Rombo et al. 2015). Thus, a reduction of A_1R expression, together with an enhanced $A_{2A}R$ signaling, thus an $A_1R/A_{2A}R$ imbalance can, *per se,* be pro-epileptogenic.

BDNF dependent effects. In the present work, concomitantly to an enhanced $A_{2A}R$ tonic activation in synaptic plasticity, I also found enhanced BDNF protein levels and enhanced BDNF-dependent plasticity in symptomatic $Adk^{\Delta brain}$ mice. cAMP response element-binding protein (CREB) is a well know regulator of BDNF gene expression (Tao et al. 1998), which in turn is activated by the MAPK pathway and required for BDNF production (Katoh-Semba et al. 2009). Additionally MAPK is one of the classical pathways activated by adenosine $A_{2A}R$, which makes it a suitable upstream regulator of BDNF expression. Indeed, adenosine $A_{2A}R$ induces BDNF expression, presumably via Akt, MAPK and CREB activation (Jeon et al. 2011). Therefore, increased $A_{2A}R$ activation may lead to the increased BDNF protein levels observed in $Adk^{\Delta brain}$ mice. Interestingly, as $Adk^{\Delta brain}$ mice, BDNF overexpressing mice, develop stress-induced seizures associated with placing the

animals into a new cage and this seizure phenotype only develops later in life (at 5 to 6 months after birth) (Papaleo et al. 2011). The authors of that study concluded that life-long overexposure to BDNF triggers detrimental effects that lead to enhanced vulnerability to enhanced seizure susceptibility. Given the knowledge that adenosine, through $A_{2A}R$ activation, is capable of booth BDNF-mediated effects upon synaptic plasticity (Fontinha *et al.*, 2008; Assaife-Lopes *et al.*, 2014; Rodrigues *et al.*, 2014), I thus suggest that the switch to an enhanced $A_{2A}R$ activity in 5-months old animals lacking ADK in the brain may also lead to enhanced tonic activation of TrkB receptors by BDNF, thereby inducing enhanced synaptic plasticity. Through activation of TrkB receptors, BDNF enhances excitatory transmission by increasing NMDA activity (Levine *et al.*, 1995 and 1998) and glutamate release (Lessmann *et al.*, 1994; Li *et al.*, 1998; Tyler and Pozzo-Miller, 2001), therefore causing an imbalance of synaptic transmission. In line with these findings, inhibition of TrkB receptors is sufficient to modulate the evoked stress induced seizure phenotype of $Adk^{\Delta brain}$ mice. Furthermore, BDNF may act as a pro-epileptic factor by inducing abnormal mossy fiber sprouting and thus, hyperexcitable circuits (Kokaia et al. 1995; Koyama et al. 2004; Heinrich et al. 2011). In spite of enhanced BDNF signaling, I concomitantly detected decreased FL-TrkB receptor levels, which may partially compensate the enhanced influence of BDNF upon plasticity and overall excitability and it is in line with previous studies showing that long exposure to BDNF induces FL-TrkB down-regulation (Sommerfield et al. 2000; Haapasalo et al. 2002; Jerónimo-Santos et al. 2014). Interestingly, when animals loose epileptic phenotype, at the age of 1 year old, the differences at $A_{2A}R$, TrkB and BDNF protein levels are also lost. This work allows concluding that up-regulation of BDNF-mediated plasticity phenomena in the $Adk^{\Delta brain}$ mice contributes to the development of epilepsy. Coincident seizure manifestation, and increased $A_{2A}R$ and BDNF-dependent plasticity phenomena thus trigger the epileptic pathology.

Aiming to address the role of increased BDNF and $A_{2A}R$ signaling in the seizure phenotype, our collaborators from Boison Detlev's Lab tested the effect of either SCH 58261 (0.5 mg/kg i.p.) or the TrkB antagonist Ana-12 (0.5 mg/kg, i.p.) in the status epilepticus of $Adk^{\Delta brain}$ mice (5 to 8-month old). $Adk^{\Delta brain}$ mice (n=6) treated with the A_1R antagonist DPCPX (3 mg/kg, i.p.) develop rapid (Figure 4.16A) and lethal (Figure 4.16B)

status epilepticus. Importantly, DPCPX was subconvulsive in $Adk^{fl/fl}$ mice and vehicle injection was not sufficient to evoke a stress-induced seizure in $Adk^{\Delta brain}$ mice.

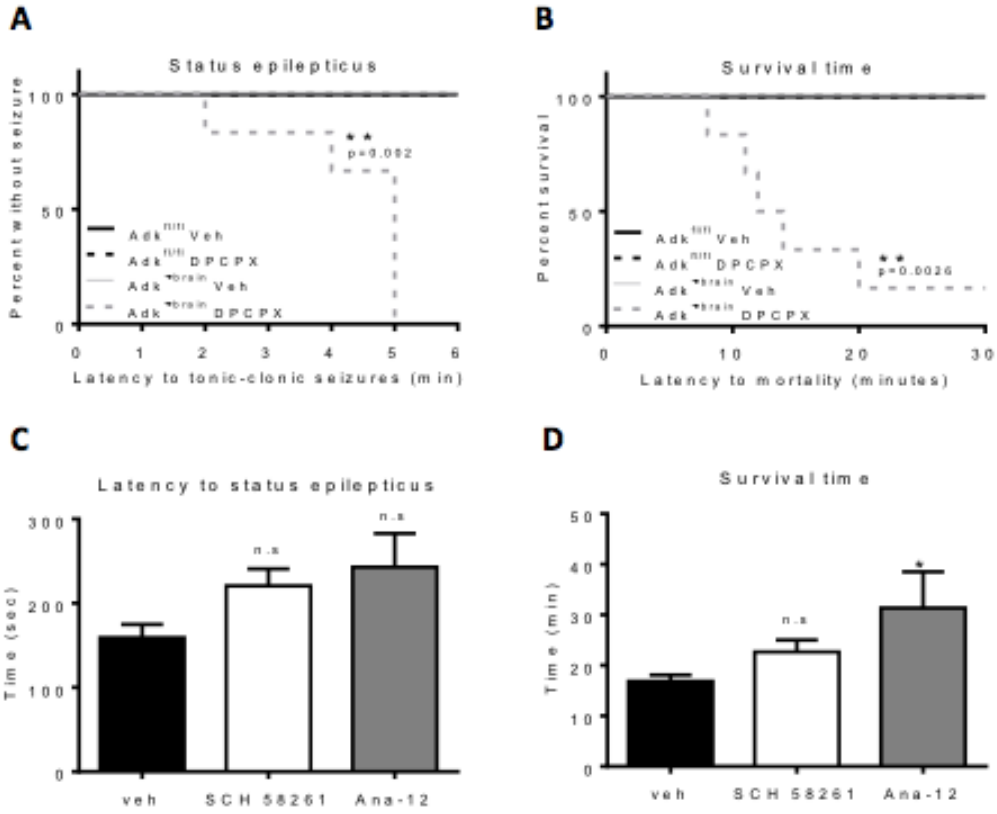

Figure 4.16: TrkB antagonist increases the seizure threshold of $Adk^{\Delta brain}$ mice. ***(A)*** and ***(B)*** DPCPX (3 mg/kg, i.p.) administered to refractory $Adk^{\Delta brain}$ mice (n=6) causes status epilepticus (A) and increased mortality (B) compared to $Adk^{fl/fl}$ DPCPX (n=5), $Adk^{fl/fl}$ vehicle (n=3) and $Adk^{\Delta brain}$ vehicle (n=3). Statistical analysis is a Log-rank (Mantel-Cox) test. The efficacy of $A_{2A}R$ or TrkB antagonists at increasing the seizure threshold was tested in refractory $Adk^{\Delta brain}$ mice using DPCPX to induce status epilepticus. The efficacy of $A_{2A}R$ or TrkB antagonists at increasing the seizure threshold was tested in $Adk^{\Delta brain}$ mice using DPCPX to induce status epilepticus. ***(C)*** Latency to status epilepticus and ***(D)*** survival time of $Adk^{\Delta brain}$ mice pretreated with vehicle (5% DMSO), SCH 56821 (0.5 mg/kg) or Ana-12 (0.5 mg/kg) prior to DPCPX (3mg/kg). Results are the mean±SEM of n=6/treatment group. Statistical analysis was performed using a One-way ANOVA followed by an uncorrected Fischer's LSD posthoc test (*p<0.05, veh vs. Ana-12; n.s. stands for non significant vs. veh).

In order to test for adenosine $A_{2A}R$ and BDNF contribution for the epileptic phenotype, mice received DPCPX (3 mg/kg, i.p.) to induce a seizure 30 minutes after SCH 58261 (adenosine $A_{2A}R$ antagonist) or 4 hours after Ana-12 (TrkB inhibitor) and the latency to status epilepticus onset and survival time were indexed as measure for seizure threshold. The latency to seizure onset in SCH 58261 and Ana-12 treated $Adk^{\Delta brain}$ mice was comparable to $Adk^{\Delta brain}$ mice treated with vehicle prior to DPCPX (Figure 4.16C, n=6/group). In contrast, pretreatment with Ana-12 significantly extended the survival time of $Adk^{\Delta brain}$ mice, compared to the vehicle treated controls (Figure 4.16D, p<0.05).

$Adk^{\Delta brain}$ mice that received vehicle injections in lieu of DPCPX and $Adk^{fl/fl}$ mice that received vehicle, SCH 58261 or Ana-12 ± DPCPX did not have seizures (data not shown). Taken together, these data support the conclusion that the enhanced endogenous BDNF tonus in $Adk^{\Delta brain}$ mice accounts for seizures.

New therapeutic window. Secondary effects of liver dysfunction were pointed as the cause to epileptic events in human patients with ADK deficiency (Bjursell et al. 2011). The present study with $Adk^{\Delta brain}$ mice, however, reveals that seizure generation in human patients results from intrinsically based ADK deficiency in the brain, through elevated levels of brain adenosine. These findings demonstrate that mutations in the *Adk* gene may account for seizure related phenotypes in the human population and may offer new opportunities for rationally drug-designed treatments. Additionally, the provided evidence that $A_{2A}R$ exacerbated function and expression trigger the development of seizures in this model and also that it is often related with other epilepsy models, $A_{2A}R$ antagonists may represent appealing novel anticonvulsive drugs, such as istradefyline (KW-6002) and S-adenosylmethionine (SAM). KW-6002 is a drug that blocks $A_{2A}R$ activation and is already being used in clinical trials for Parkinson Disease (Lopes et al. 2011). SAM, through DNA methylation, was shown to decrease $A_{2A}R$ expression (Buira et al. 2010). Since it has already been used for the treatment of liver diseases and it is known to have few side effects and to cross blood-brain barrier, SAM could also be a plausible drug to test in clinical trials for seizure disorders (Friedel et al. 1989; Bottiglieri et al. 1990).

5 | GENERAL DISCUSSION AND FUTURE PERSPECTIVES

BDNF and adenosine play essential roles for the maintenance of neuronal activity within a regulated range, contributing for enhanced learning and memory and having neuroprotective roles against several insults (Murray & Holmes 2011). BDNF and adenosine dysfunctions are also related with several neurological diseases, which make them interesting potential therapeutic targets for those disorders. Aiming a deeper understanding of their mechanisms of action in particular to control excitability, the this thesis focused on the role of BDNF and adenosine in non-pathological conditions as well as in a mouse model of stress-induced age-dependent epileptic phenotype.

Despite the well-documented BDNF signaling in excitatory synapses, its role in inhibitory synapses was largely unknown. First studies of BDNF effect onto inhibitory signaling at the adult hippocampus are herein presented (Chapter 4.1). The results clearly show that BDNF induces a pre-synaptic facilitation of GABAergic signaling from interneurons to pyramidal cells, through a TrkB- and adenosine A_{2A}R-dependent mechanism. Whether BDNF-induced pre-synaptic effect onto GABAergic signaling is due to an increase in the number of release sites, in the number of docked vesicles or in the number of pre-synaptic interneuron terminals remained to be known and it could be of interest to explore in the future. Neuronal activity promotes BDNF actions, which in turn are known to increase excitatory signaling. The present finding (chapter 4.1) that BDNF enhances GABAergic transmission allows to propose that it concomitantly provides a feedback inhibitory mechanism to refrain excessive activity by facilitating GABAergic inputs to principal cells (Figure 5.1), thus suggesting a role for BDNF in homeostatic plasticity, which is operated though modulation of the GABAergic system. Interneurons, besides directly controlling pyramidal cell excitability, also project to other interneurons. An enhancement of the activity of the interneurons that project to other interneurons could thus contribute to desinhibition of excitatory pyramidal neurons. Therefore, one interesting issue that would add more information about this complex regulation of hippocampal circuits would be the understanding whether (and if so, how) BDNF modulates the synapses between two interneurons (Figure 5.1).

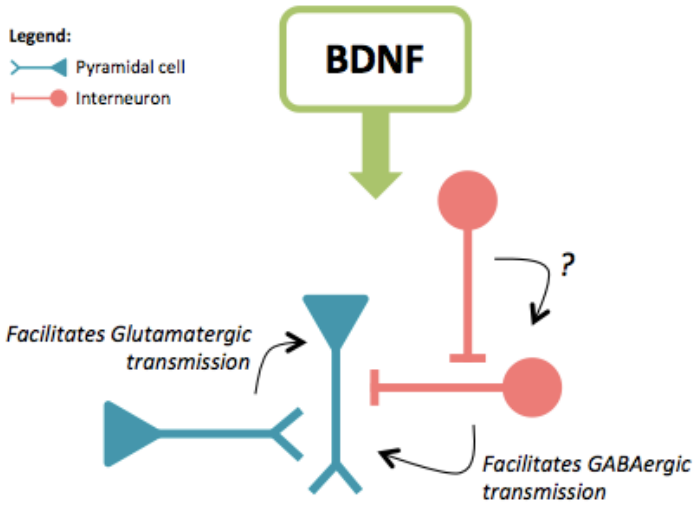

Figure 5.1: Schematic representation of BDNF mediated effects onto synaptic transmission in CA1 hippocampal pyramidal cells and interneurons. BDNF is released in an activity-dependent way. BDNF is generally recognized to increase neuronal excitability, but it is now reported that it can also facilitates GABAergic inputs from interneurons (magenta) to pyramidal cells (blue) in a feedback inhibitory mechanism, probably in an attempted to counteract excessive activity.

Maladaptative regulation of synaptic transmission is generally associated to neurological disorders, as epilepsy (Dias et al. 2013). As the work described in chapter 4.2 strongly suggests, the lack of Adenosine kinase in the entire brain, leads to adaptive changes, which relates to the epileptic phenotype. Thus, high extracellular adenosine levels are associated to a decrease in the expression of adenosine A_1R and impaired synaptic signaling. Seizures were only observed in those age ranges where exacerbated excitability and exacerbated synaptic plasticity were detected. Remarkably, increased protein levels of both BDNF and adenosine $A_{2A}Rs$ do occur at those ages, thus allowing to suggest that seizures may result from reduced A_1R signaling associated to enhanced $A_{2A}R$ signaling (most probably due a lack of control of A_1R over $A_{2A}R$) which determines a deregulated facilitation of excitability and plasticity by BDNF. Interestingly, at pre-symptomatic ages no differences in synaptic plasticity were found despite the lower levels of adenosine $A_{2A}R$ and increased levels of TrkB receptors, suggesting that for seizures to occur it is necessary a pronounced reduction of A_1R-mediated synaptic inhibition together with an over threshold increase in A_2AR-mediated facilitation of BDNF signaling. While reasons for development of stress-induced epilepsy could be clearly identified in the present work, it remained unclear why aged animals loose the epileptic phenotype. Data however points towards the possibility of an adaptative loss of A2AR and BDNF (Table 5.1) Overall, the

work in this thesis suggests that maladaptive regulation of brain function relates with exacerbated adenosine A_{2A}Rs and BDNF signaling, which plays a role in epileptogenesis.

Table 5.1: Characterization of pre-symptomatic, symptomatic and post-symptomatic mice lacking Adenosine Kinase expression in the entire brain.

	Pre-symptomatic (2 months old)	**Symptomatic (5 to 8 months old)**	**Post-symptomatic (> 1 year old)**
Adenosine extracellular levels	↑	↑	↑
A_1R signaling	↓	↓	↓
A_1R protein levels	↓	↓	↓
Excitability	=	↑	=
Synaptic plasticity	=	↑	=
A_{2A}R signaling	=	↑	↑
A_{2A}R protein levels	↓	↑	=
BDNF signaling	?	↑	?
BDNF protein levels	?	↑	=
TrkB protein levels	↑	↓	=

Substantial studies have been showing that adenosine A_{2A}Rs blockade attenuates brain damage, supporting the view that these receptors have detrimental effects in several neurological disorders (Filipe 1991). BDNF, however, has a complex relation with epilepsy and its role as a pro- or anti-epileptic factor is far from being clarified in the literature. On one hand, BDNF triggers neuroprotective effects, suppresses apoptosis, increases protein expression of Neuropeptide Y and KCC2 and decreases NKCC1 expression, which may ameliorate seizure behaviors. On one other hand, given the fact that BDNF increases NMDA activity, promotes neurogenesis, axonal and dendritic sprouting in extreme conditions of excitability, BDNF may spread the toxicity, contributing for the formation of new hyper-excitable circuits and encouraging epileptogenesis (Murray & Holmes 2011).

As the work described in this thesis demonstrates, a tight inter-regulation of different modulators do occur, so that chronic overload of one leads to unbalanced signaling through the others, that could be attributed to changes in the expression of receptors as well as their ability to control synaptic function.

Adenosine, through A_{2A}R, has a broad spectrum of actions in the central nervous system, since A_{2A}R can affect the actions of several other neuromodulators and neurotransmitters. So, it would be interesting to study whether A_{2A}R excessive signaling caused by adenosine overload triggers detrimental actions for brain function by affecting some other major players in excitability as NMDA, AMPA and $GABA_A$ currents.

Knowing that *1)* epileptic ADK knockout mice have increased BDNF expression and signaling (present thesis), *2)* BDNF affects GABAergic transmission (present thesis), *3)* BDNF modifies NKCC1 and KCC2 protein expression (Eftekhari et al. 2014) and that *4)* NKCC1 and KCC2 regulate Cl^- intracellular concentration, defining Cl^- gradient across the membrane through $GABA_A$ receptors (and thus, their hyperpolarizing or depolarizing nature), it would be interesting to evaluate how adenosine overload affects NKCC1 and KCC2 expression and how this could relate to epileptogenesis *vs* epilepsy control.

6 | MAIN CONCLUSIONS

The work presented in this thesis provides evidence that both adenosine and BDNF signaling have relevant roles for the regulation of neuronal networks at the adult hippocampus. Moreover, a deregulation of their function may trigger the development of pathological conditions, such as seizure disorders.

My results show that, **through TrkB receptor activation, BDNF facilitates GABAergic inputs to pyramidal neurons in the adult hippocampus via a pre-synaptic mechanism.** GABAergic modulation by BDNF was furthermore found to be **dependent of adenosine A_{2A} receptor activation**.

Mice lacking Adenosine kinase gene expression in the brain develop seizures from 5 to 12-months old. Attempting to correlate the epileptic phenotype with a dysfunction in adenosinergic and BDNF signaling, I provided evidence that **ADK deficiency in the brain triggers neuronal adaptation processes leading to increased A_{2A}R- and BDNF-dependent plasticity phenomena as rational explanation for the development of epileptic pathology**. Thus, mutations in the human *Adk* gene have intrinsic effects on the brain and may offer new opportunities for rationally designed treatments for inborn seizure disorders.

These findings highlight the role of BDNF and adenosine in the fine-tuning of synapses. Under physiological conditions they may adjust synaptic transmission to maintain homeostasis. However, when their relative balance is disturbed, the actions of these two modulators may aggravate and contribute for pathological states, as herein shown for epilepsy. This knowledge thus opens new possibilities for rationalized drug-designed therapies to fight epileptogenesis or control epilepsy.

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8 | APPENDICES

8.1 | Appendix 1

Purinergic Signalling
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ORIGINAL ARTICLE

BDNF-induced presynaptic facilitation of GABAergic transmission in the hippocampus of young adults is dependent of TrkB and adenosine A_{2A} receptors

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Abstract Brain-derived neurotrophic factor (BDNF) and adenosine are widely recognized as neuromodulators of glutamatergic transmission in the adult brain. Most BDNF actions upon excitatory plasticity phenomena are under control of adenosine A_{2A} receptors (A_{2A}Rs). Concerning gamma-aminobutyric acid (GABA)-mediated transmission, the available information refers to the control of GABA transporters. We now focused on the influence of BDNF and the interplay with adenosine on phasic GABAergic transmission. To assess this, we evaluated evoked and spontaneous synaptic currents recorded from CA1 pyramidal cells in acute hippocampal slices from adult rat brains (6 to 10 weeks old). BDNF (10–100 ng/mL) increased miniature inhibitory postsynaptic current (mIPSC) frequency, but not amplitude, as well as increased the amplitude of inhibitory postsynaptic currents (IPSCs) evoked by afferent stimulation. The facilitatory action of BDNF upon GABAergic transmission was lost in the presence of a Trk inhibitor (K252a, 200 nM), but not upon $p75^{NTR}$ blockade (anti-$p75^{NTR}$ IgG, 50 μg/mL). Moreover, the facilitatory action of BDNF onto GABAergic transmission was also prevented upon A_{2A}R antagonism (SCH 58261, 50 nM). We conclude that BDNF facilitates GABAergic signaling at the adult hippocampus via a presynaptic mechanism that depends on TrkB and adenosine A_{2A}R activation.

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Keywords BDNF · GABAergic transmission · Adenosine A_{2A} receptors · Hippocampus

Introduction

Adenosine and brain-derived neurotrophic factor (BDNF) are two well-known modulators of synaptic maturation, plasticity, and signaling [1, 2]. Most of the present knowledge about BDNF actions upon synaptic signaling at the hippocampus, as well as its modulation by adenosine, relies on its influence upon glutamatergic synaptic transmission. BDNF postsynaptically enhances NMDA receptor activity in cultured neurons [3, 4] and presynaptically enhances glutamate release in developing neurons [4–8] and isolated hippocampal nerve endings from young [9, 10] and adult [11] rats. Furthermore, BDNF is also known to facilitate long-term plasticity of excitatory synapses, such as long-term potentiation (LTP) [12, 13] and long-term depression (LTD) [14] in an A_{2A} receptor (A_{2A}R)-dependent manner [15, 16]. Information on the ability of A_{2A}R to modulate actions of BDNF on GABAergic transmission is restricted to the actions of the neurotrophin on GAT-1-mediated GABA transport in astrocytes [17] or nerve endings, either when transport is in the inward [18] or outward [19] direction.

The action of BDNF upon synaptic GABAergic transmission at the hippocampus is itself poorly known; the information so far available refers mostly to developing neurons in culture, where BDNF has been shown to cause a postsynaptically mediated fast facilitation of GABAergic currents followed by a prolonged depression [20] or to cause a presynaptic facilitation of GABAergic inputs to glutamatergic neurons [21]. Prolonged applications of BDNF (>24 h) have been shown to facilitate maturation of GABAergic synapses and presynaptically increase GABAergic transmission [22] or to

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prevent down scaling of GABAergic responses caused by activity deprivation [23]. Changes in modulation of GABAergic currents by BDNF are expected to occur during maturation since BDNF affects K^+/Cl^- transport in hippocampal neurons [21]. Indeed, in neurons acutely isolated at the postnatal day 6 (P6), BDNF has been shown to cause a postsynaptically mediated facilitation of GABAergic currents, while causing a long-lasting inhibition of GABAergic currents at P12–P18 neurons [24, 25]. On the other hand, highly variable actions of BDNF on GABAergic transmission have been reported for hippocampal slices acutely prepared from 2 to 8 weeks old rats, but data were not discriminated as a function of age [26].

We now evaluated the action of BDNF on synaptic GABAergic transmission at the adult hippocampus as well as its dependence on adenosinergic tuning. We tested the influence of BDNF on evoked and spontaneous inhibitory synaptic currents (inhibitory postsynaptic currents (IPSCs) and miniature inhibitory postsynaptic currents (mIPSCs), respectively) recorded from adult hippocampal pyramidal neurons in acutely isolated slices and assessed the ability of an $A_{2A}R$ antagonist to modify those actions. We report that BDNF facilitates GABAergic signaling at the adult hippocampus via a presynaptic mechanism that depends on TrkB and adenosine $A_{2A}R$ activation.

Materials and methods

Animals

Adult (6 to 10 weeks old) male Wistar rats were used in this study. The animals were kept under standardized conditions of light, temperature, and humidity and had access to food and water ad libitum. All animal procedures were carried out in strict accordance with the ethical recommendations by EU (Directive 210/63/EU), the Portuguese (DL 113/2013) legislation for the protection of animals used for scientific purposes, and the Ethics Committee of the Instituto de Medicina Molecular and of the Faculty of Medicine, University of Lisbon, Lisbon, Portugal, who approved this study. Animals were deeply anesthetized under isoflurane atmosphere before being sacrificed, and all efforts were made to minimize suffering before anesthesia. Deep anesthesia was confirmed by the absence of response to paw pinching while the animals were still breathing.

Hippocampal slice preparation

After decapitation, the brain was rapidly removed from the skull cavity and the hippocampi were dissected in ice-cold dissecting solution containing (mM) the following: sucrose 110; KCl 2.5; $CaCl_2$ 0.5; $MgCl_2$ 7; $NaHCO_3$ 25; NaH_2PO_4 1.25; and glucose 7, pH 7.4, oxygenated with 95 % O_2 and 5 % CO_2. Each hippocampus was then transversely sliced (300 μm thick) on a vibratome (VT 1000 S; Leica, Nussloch, Germany) under the same ice-cold dissecting solution. The slices were then incubated at 35 °C for 20 min in artificial cerebrospinal fluid (aCSF) (mM: NaCl 124; KCl 3; NaH_2PO_4 1.25; $NaHCO_3$ 26; $MgSO_4$ 1; $CaCl_2$ 2; and glucose 10, pH 7.4) before being placed at room temperature in the same solution for at least 1 h before use in patch clamp experiments.

Patch clamp recordings

Individual slices were fixed with a grid in a recording chamber of about 1-mL volume and were continuously superfused by a gravitational system at 2–3 mL/min with aCSF at room temperature. Drugs were added to this superfusion solution and reached the recording chamber within approximately 1 min.

Patch pipettes were made from borosilicate glass capillaries (1.5-mm outer diameter, 0.86 inner diameter, Harvard Apparatus, Holliston, MA, USA) and had a resistance of 4–9 MΩ when filled with an internal solution containing (mM) the following: CsCl 125; NaCl 8; $CaCl_2$ 1; EGTA 10; HEPES 10; glucose 10; MgATP 5; NaGTP 0.4; and pH 7.2, adjusted with CsOH (50 wt% in H_2O), 280–290 Osm.

Whole-cell recordings were obtained from pyramidal cells located at CA1 *stratum pyramidale*. The cells were visualized with a microscope (Zeiss Axioskop 2FS, Jena, Germany) equipped with infrared video microscopy and differential interference contrast optics. All recordings were performed in voltage clamp mode ($V_H = -70$ mV) at room temperature (22–24 °C) with either an EPC-7 (List Biologic) or an Axopatch 200B (Molecular Devices) amplifier, under the control of pClamp10 software (Molecular Devices). Before the giga-seal formation, the offset potentials were nulled. Immediately after having whole-cell access, the membrane potential of the neurons was measured in current clamp mode (V_H approximately −60 mV). Through all the recordings, the holding current was constantly monitored, and if it varied by more than 20 %, the experiment would be rejected. Data were low-pass filtered using a 3- and 10-kHz three-pole Bessel filter, digitized at 5 Hz, and registered by the Clampex Software version 10.2 (Molecular Devices, Sunnyvale, CA, USA).

Afferent-evoked IPSCs were recorded as described elsewhere [27]. Briefly, every 15 s, a stimuli (1–15 μA) was delivered via monopolar stimulation with a patch-type pipette filled with aCSF and positioned in *stratum radiatum*, 80–120 μm from the recorded cell. Averages of eight consecutive individual recordings were used for analysis. Recordings were performed using the internal solution previously described plus 1 mM QX-314, a voltage-gated Na^+ channel blocker.

During all recordings, the aCSF was supplemented with kynurenic acid (Kyn, 1 mM), to block glutamate receptors.

mIPSCs were recorded in aCSF supplemented with tetrodotoxin (TTX, 0.5 μM) and Kyn (1 mM). In some experiments and only where specified, K252a (200 nM) and SCH58261 (100 nM) were also added to the superfused aCSF. When testing the dependency on $p75^{NTR}$ activation for the effect of BDNF, the slices were preincubated for 2 h at room temperature with a blocking antibody anti-$p75^{NTR}$ IgG (REX Ab, 50 μg/mL) [28]. Miniature event analysis was performed using the MiniAnalysis software (Synaptosoft, GA, USA), with the amplitude threshold for event detection being set at 5× the average RMS noise. The rise time was calculated by finding the first data point to the left of the peak that shows 0.5 % of the peak amplitude and subtracting the time at this point from the time at the peak. The decay time was calculated by finding the first data point to the right of the peak that shows 10 % of the peak amplitude and taking a difference between the time at this point and the time at the peak. mIPSC frequency and amplitude were analyzed for 40 s every 2 min in order to obtain their time course variances. Statistical differences were assessed between two different periods: (1) the 10 min prior to BDNF superfusion and (2) the last 10 min in its presence (30 to 40 min after BDNF addition to the bath solution).

Drugs

BDNF was generously supplied by Regeneron Pharmaceuticals (Tarrytown, NY), in a 1.0 mg/mL stock solution in 150 mM NaCl, 10 mM sodium phosphate buffer, and 0.004 % Tween 20; aliquots of this sock solution were kept frozen at −80 °C and diluted in aCSF in the day of the experiment (solvent concentration in the perfusion solution 0.003 % *v*/*v*). REX Ab (anti-$p75^{NTR}$ IgG) was a generous gift from Louis Reichardt. Tetrodotoxin (TTX, octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10a*H*-[1, 3] dioxocino[6,5-*d*] pyrimidine-4,7,10,11,12-pentol, a sodium-channel blocker) was obtained from Ascent Scientific (Bristol, UK). K252a (tyrosine kinase inhibitor) and SCH58261 (2-(2-furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4, 3-*e*] [1, 2, 4] triazolo[1,5-*c*]pyrimidin-5-amine, selective A_{2A} receptor antagonist), QX-314 chloride (*N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium chloride, blocker of voltage-activated Na^+ channel), and bicuculline ([R-(*R**,*S**)]-6-(5,6,7,8-tetrahydro-6-methyl-1,3-dioxolo[4,5-*g*]isoquinolin-5-yl)furo[3,4-*e*]-1,3-benzodioxol-8(6*H*)-one, selective $GABA_AR$ antagonist) were purchased from Tocris Cookson (Ballwin, MO). Kynurenic acid (4-hydroxyquinoline-2-carboxylic acid, glutamate receptor antagonist) was from Abcam. SCH58261 was prepared in a

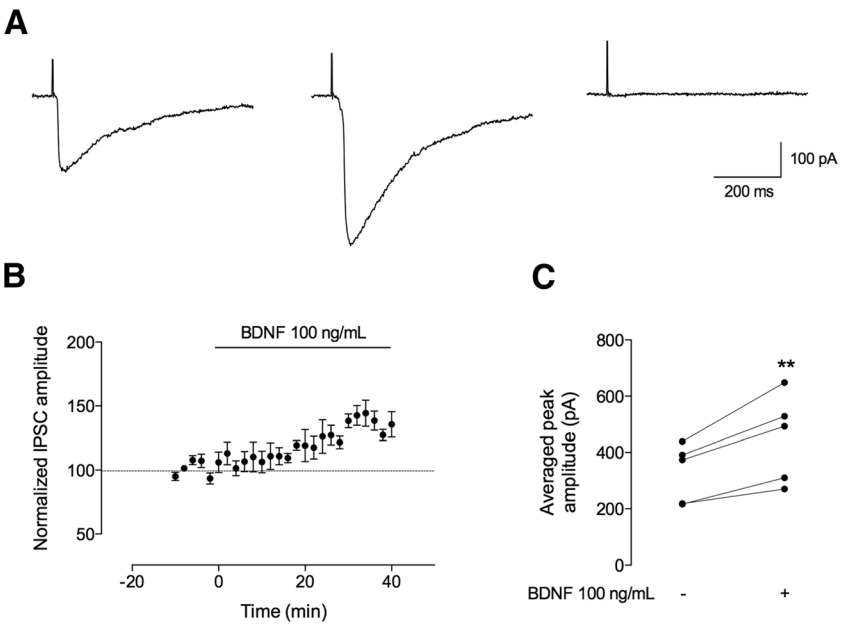


Fig. 1 BDNF increases the amplitude of evoked IPSCs recorded from CA1 pyramidal neurons. The recordings represented here were performed in CA1 pyramidal cells in whole-cell configuration, and inhibitory postsynaptic currents (IPSCs) were evoked by afferent stimulation in the continuous presence of a glutamate receptor antagonist (kynurenic acid, Kyn, 1 mM). **a** Representative IPSC recordings in the absence (*left panel*) and presence (*middle panel*) of BDNF (100 ng/mL) and in the presence of bicuculline (20 μM) (*right panel*). **b** Time course changes, induced by BDNF (100 ng/mL, $n = 5$), in the peak amplitude of the evoked IPSCs. One hundred percent represents the average peak amplitude of the currents recorded for 10 min prior to BDNF application. BDNF effect was quantified by comparing the peak amplitude from the 10-min period before BDNF application (baseline) to the 30–40 min after BDNF application. **c** The individual (*dots*) changes in peak amplitude are shown as individual data obtained in the 10 min in the absence (−) of BDNF and 30–40 min after (+) BDNF administration ($n = 5$). Values are mean ± SEM. $^{**}p < 0.01$ (two-tailed paired Student's *t* test)

5 mM, K252a in a 1 mM, and Bicuculline in a 100-mM stock solution, all in dimethyl sulfoxide (DMSO). TTX (1 mM) and kynurenic acid (100 mM) were prepared in water. Stock solutions were aliquoted and stored at −20 °C until use. Dilutions of these solutions to the final concentration were made freshly before each experiment. The percentage of DMSO in each experiment did not exceed 0.001 % and did not affect neither IPSCs nor mIPSCs [27].

Statistical analysis

Results are expressed as the mean ± SEM of *n* experiments, where *n* corresponds to the number of tested cell from different slices. Statistical significance was evaluated by two-tailed Student's *t* test, when comparing before and after BDNF perfusion or by performing one-way ANOVA followed by Bonferroni's post hoc test for comparison between multiple experimental groups. Statistical significance was assumed if *p* value was inferior to 0.05.

Results

BDNF increases IPSCs in CA1 pyramidal cells

IPSCs evoked by afferent stimulation were recorded to evaluate the influence of BDNF upon $GABA_A$R-mediated responses at the hippocampus of adult rats. All the recordings were performed in voltage clamp mode ($V_H = -70$ mV) in the presence of kynurenic acid (Kyn, 1 mM), to block glutamate receptors [29] and isolate GABAergic currents from interference of fast excitatory transmission [30]. Under such conditions, the recorded currents were completely blocked when applying bicuculline (20 μM), a selective $GABA_A$R antagonist, thus confirming their GABAergic nature (Fig. 1a). In addition, QX-314 (1 mM) was added to the intracellular solution avoiding GABA current contamination with action potentials.

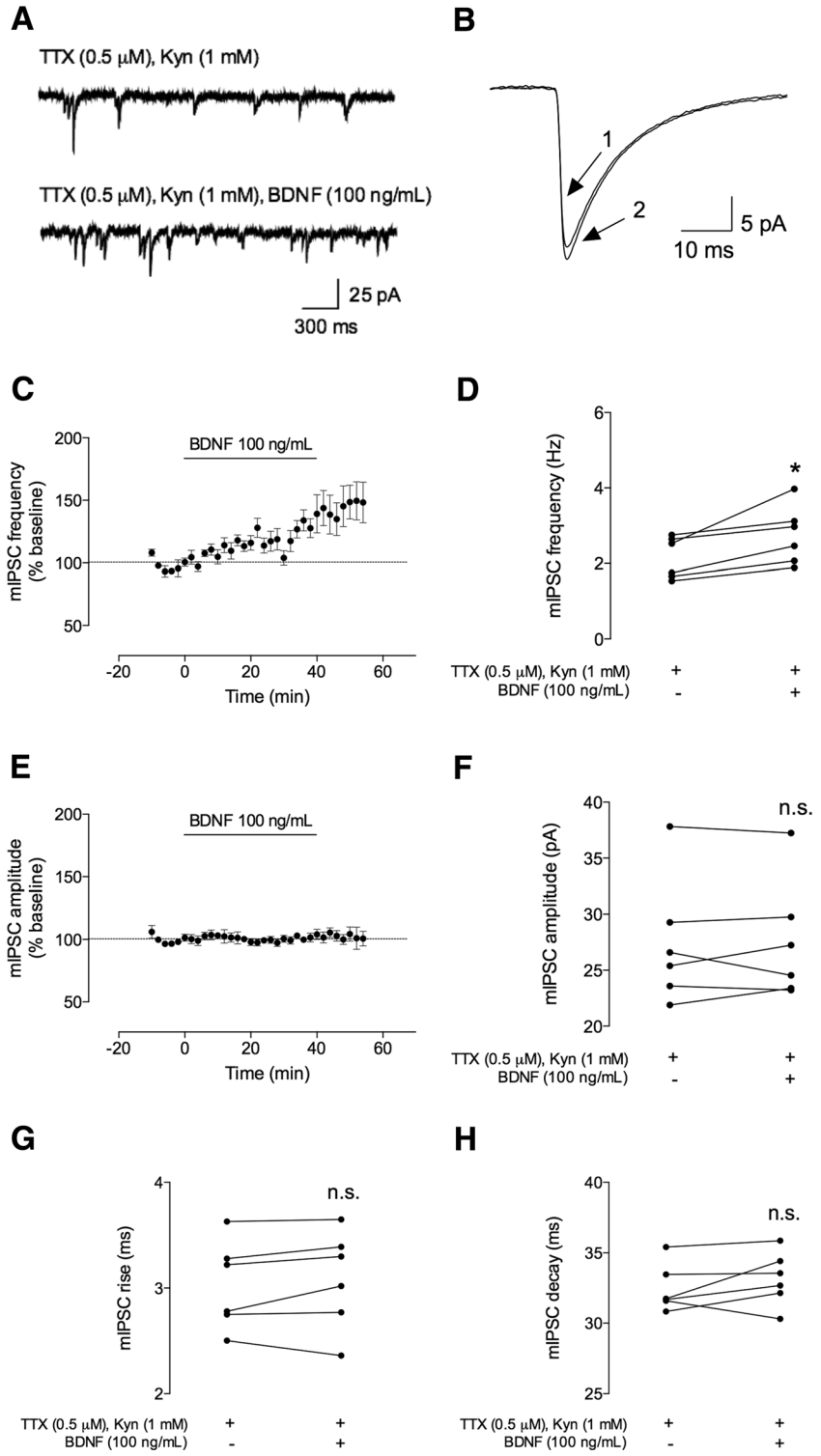
Once achieving a stable baseline of electrically evoked IPSC amplitudes for at least 10 min, BDNF was perfused, remaining in the bath for 40 min. First, we used a moderately high concentration of BDNF (100 ng/mL), since at this concentration, BDNF has been shown to postsynaptically affect GABAergic transmission in acute slices prepared from developing hippocampus taken from preweaning (P12–18) rats (22). As shown in Fig. 1, BDNF (100 ng/mL) induced a progressive increase in the peak amplitude of IPSCs recorded from CA1 pyramidal cells in slices prepared from adult rats. The effect of BDNF on the amplitude of GABAergic currents started 15 min after its perfusion, the maximum increase being observed between the 30 and 40 min of BDNF application (Fig. 1b). The averaged increase in peak amplitude of GABA currents caused by BDNF (100 ng/mL) was 39 ± 4.9 % ($n = 5$, $p < 0.01$, as compared with baseline values), being this value calculated from IPSCs recorded 30–40 min after starting perfusion of BDNF (100 ng/mL). These data indicate that BDNF facilitates phasic GABAergic transmission in the adult hippocampal neurons.

Fig. 2 BDNF (100 ng/mL) increases the frequency, but not the amplitude, of spontaneous miniature inhibitory postsynaptic currents (mIPSCs). The miniature IPSC (mIPSC) recordings represented here were performed in CA1 pyramidal cells in whole-cell configuration and in the presence of sodium channel blocker (tetrodotoxin (TTX) 0.5 μM) and a glutamate receptor antagonist (Kynurenic acid, Kyn, 1 mM). **a** Representative tracings of mIPSCs recorded in the absence (*upper trace*) and presence (*lower trace*) of BDNF (100 ng/mL). **b** Representative average tracings of mIPSCs of two superimposed events in the absence (*1*) and presence (*2*) of BDNF (100 ng/mL), from the same cell. **c**, **e** Time course changes in mIPSC frequency (**c**) and amplitude (**e**) induced by application of BDNF ($n = 6$). One hundred percent represents the average mIPSC frequency or amplitude recorded for 10 min prior to BDNF application. mIPSC frequency and amplitude changes were quantified by comparing the events from the 10-min period before BDNF application (baseline) to the final 10 min in its presence. **d**, **f** The averages of absolute values of frequency (**d**) or amplitude (**f**) are shown as individual data obtained in the 10 min in the absence (−) of BDNF and 30–40 min after (+) BDNF administration ($n = 6$). **g**, **h** Averages of the absolute values of rise (**g**) or decay (**h**) times are shown as individual data obtained in the 10 min in the absence (−) of BDNF and 30–40 min after (+) BDNF administration ($n = 6$). Values are mean ± SEM. *$p < 0.05$ and n.s. $p > 0.05$ (two-tailed paired Student's *t* test)

BDNF increases frequency, but not amplitude, of mIPSCs

To evaluate whether the facilitatory action of BDNF upon fast GABAergic transmission results from presynaptic or postsynaptic mechanisms, recordings of miniature inhibitory postsynaptic currents (mIPSCs) were performed in the presence of tetrodotoxin (TTX, 0.5 μM) and Kyn (1 mM). Changes in frequency of these events are usually interpreted as changes at presynaptic level, while changes in the amplitude are thought to relate with a postsynaptic modulation.

As illustrated in Fig. 2, application of BDNF (100 ng/mL) to the bathing solution consistently increased the frequency of mIPSCs by 28 ± 7.0 % ($n = 6$, $p < 0.05$, measured 30–40 min after starting BDNF superfusion, Fig. 2a, c, d). Baseline frequency of mIPSC was 2.1 ± 0.22 Hz and increased to 2.7 ± 0.31 Hz, 30–40 min after BDNF addition. At this concentration of BDNF (100 ng/mL), the increase in mIPSC frequency persisted even after washout. BDNF (100 ng/mL) was virtually devoid of effect in mIPSC amplitude (% change as compared with baseline 1.0 ± 2.3, $n = 6$, $p > 0.05$). The averaged absolute amplitude values were 27 ± 2.3 and 27 ± 2.2 pA, before and 30–40 min after BDNF addition, respectively. Additionally, the duration of mIPSCs showed no statistical significance upon BDNF application (Fig. 2g, h). Indeed, mIPSC rising time changed to 1.9 ± 1.9 % of



baseline (3.0 ± 0.17 and 3.1 ± 0.19 ms, before and after BDNF application, respectively) and the decay time to 2.2 ± 1.7 % (from 33 ± 0.69 ms before and 33 ± 0.79 ms after BDNF perfusion).

A lower concentration of BDNF (10 ng/mL) also increased mIPSC frequency, without affecting mIPSC amplitude (Fig. 3), the averaged increase in mIPSC frequency being 38 ± 11 % ($n = 9$, $p < 0.01$) for the last 10 min of its perfusion. Frequency values increased from 1.7 ± 0.35 Hz (before the perfusion of BDNF) to 2.2 ± 0.41 Hz (30–40 min after BDNF addition). The effect was not statistically different from that obtained with 100 ng/mL BDNF ($p > 0.05$, one-way ANOVA). However, contrary to what was observed for 100 ng/mL

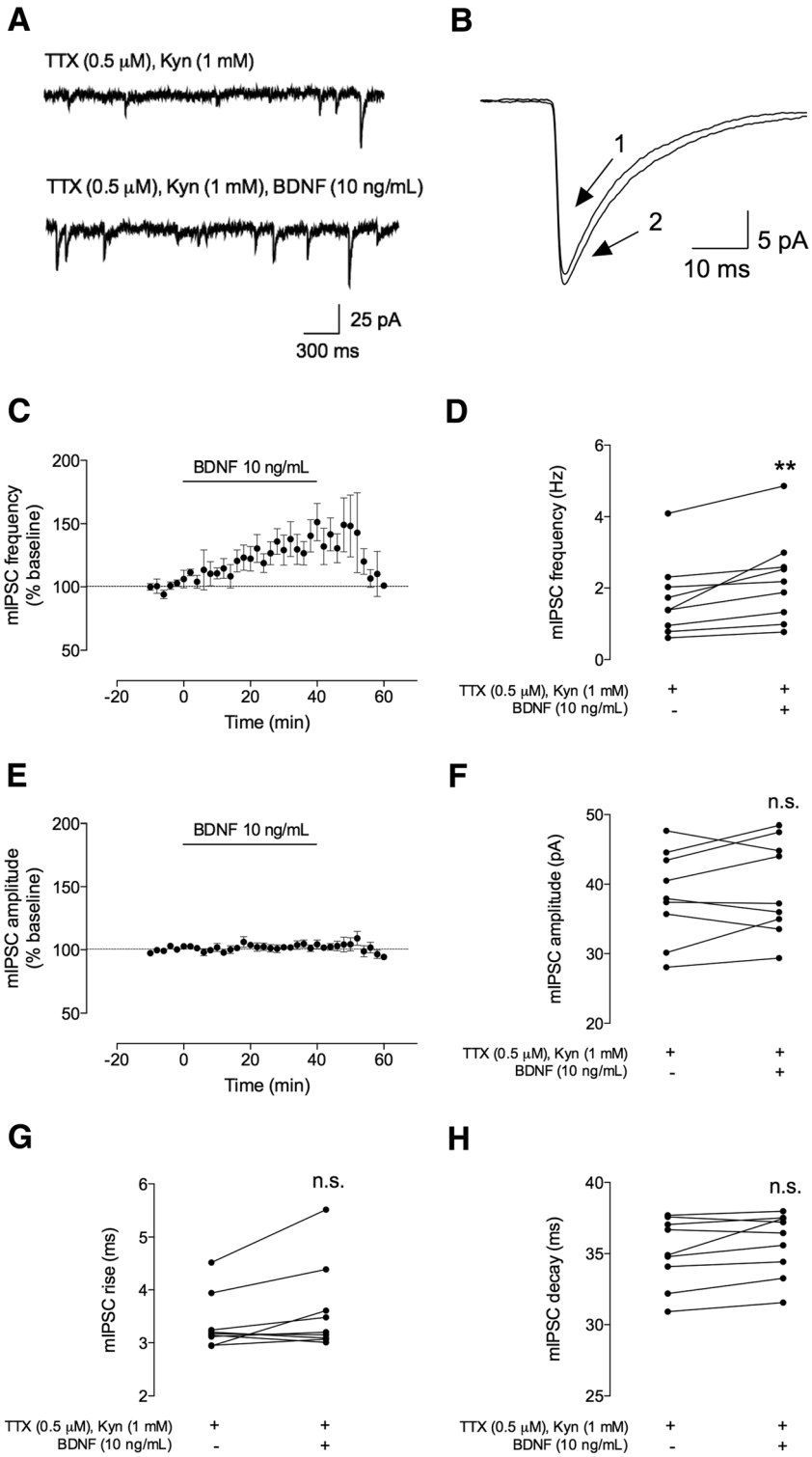


Fig. 3 BDNF (10 ng/mL) increases the frequency, but not the amplitude, of spontaneous miniature inhibitory postsynaptic currents (mIPSCs). The mIPSCs were recorded in the same conditions as described for Fig. 2. **a** The *upper panel* represents mIPSC tracings recorded in the absence (*upper trace*) and presence (*lower trace*) of BDNF (10 ng/mL). **b** Representative average tracings of mIPSCs of two superimposed events in the absence (*1*) and presence (*2*) of BDNF (10 ng/mL), from the same cell. **c**, **e** Time course changes in mIPSC frequency (**c**) and amplitude (**e**) induced by application of BDNF ($n = 9$). **d**, **f** Averages of the absolute values of frequency (**d**) or amplitude (**f**) are shown as individual data points in the absence (−) of BDNF and after (+) BDNF administration ($n = 9$). **g**, **h** Averages of the absolute values of rise (**g**) or decay (**h**) times are shown as individual data obtained in the absence (−) of BDNF and after (+) BDNF administration ($n = 9$). Values are mean ± SEM. $^{**}p < 0.01$ and n.s. $p > 0.05$ (two-tailed paired Student's *t* test

BDNF (Fig. 2c), the effect of 10 ng/mL was fully washed out (Fig. 3c). BDNF (10 ng/mL) had no effect on mIPSC amplitude (% change 3.3 ± 2.7 as compared to the baseline values; 38 ± 2.2 pA before and 40 ± 2.3 pA after BDNF application, Fig. 3e, f) or on mIPSC duration (rising time 6.9 ± 3.4 % of baseline, 3.4 ± 0.17 ms before and 3.6 ± 0.28 ms after BDNF perfusion; decay time 1.8 ± 0.83 % of baseline, 35 ± 0.80 ms before and 36 ± 0.74 ms after BDNF application, Fig. 3g, h).

These data thus suggest that BDNF positively modulates GABAergic transmission via presynaptic changes that lead to increased GABA release.

The BDNF facilitatory effect onto GABAergic transmission is TrkB, but not $p75^{NTR}$, dependent

BDNF can activate a high-affinity TrkB receptor and a low-affinity $p75^{NTR}$ receptor. K252a is a tyrosine kinase inhibitor that prevents Trk receptor activation [31]. Thus, to test for the involvement of TrkB receptors on the effect of BDNF, mIPSC recordings in the presence of K252a (200 nM) were performed. To do so, slices were perfused with K252a immediately after going to whole cell and currents allowed to stabilize before applying BDNF in the presence of K252a for at least 20 min. In the presence of K252a, 40 min of incubation of BDNF did not affect either mIPSC frequency or mIPSC amplitude (Fig. 4). Indeed, in the last 10 min of BDNF application, the percentage of change in mIPSC frequency was −7.3 ± 5.3 % as compared to baseline ($n = 7$, $p > 0.05$), the absolute values being 1.2 ± 0.18 Hz before and 1.1 ± 0.16 Hz after BDNF application. Similarly, for mIPSC amplitude, the percentage of change was −1.0 ± 1.4 % ($n = 7$, $p > 0.05$), with absolute values of 31 ± 3.0 pA before and 31 ± 3.3 pA after

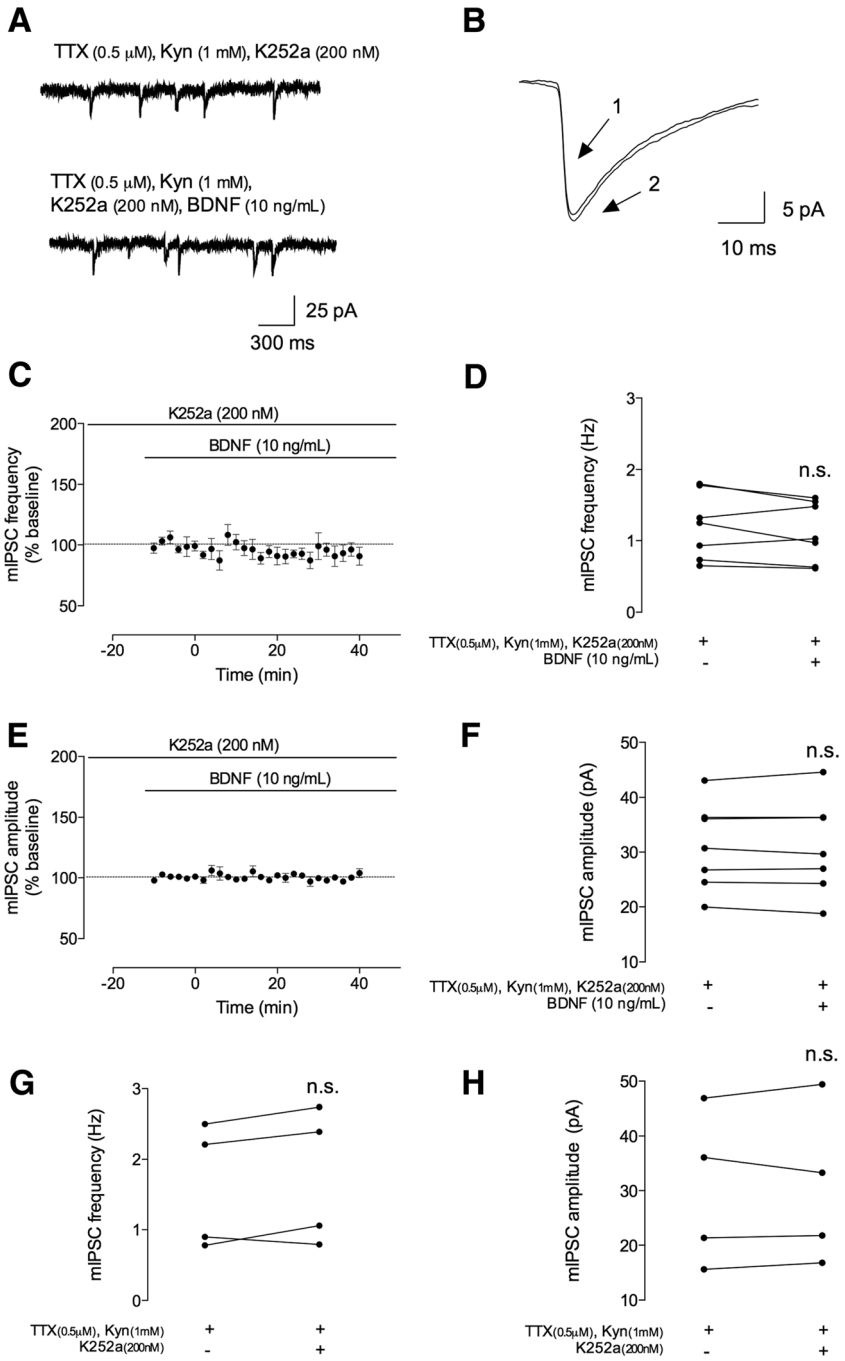


Fig. 4 The facilitatory effect of BDNF on mIPSCs is dependent on TrkB receptor activation. The mIPSCs were recorded in the same conditions as described for Fig. 2. During all the recordings, a tyrosine kinase inhibitor (K252a, 200 nM) was further added to the perfusion. **a** In the *upper panel*, a representation of mIPSC tracings recorded in the absence (*upper trace*) and presence (*lower trace*) of BDNF (10 ng/mL). **b** Representative average tracings of mIPSCs of two superimposed events in the absence (*1*) and presence (*2*) of BDNF (10 ng/mL), from the same cell. **c**, **e** Time course changes in mIPSC frequency (**c**) and amplitude (**e**) induced by application of BDNF ($n = 7$). mIPSC frequency and amplitude changes were quantified by comparing the events before (baseline) and after BDNF application. **d**, **f** Averages of the absolute values of frequency (**d**) or amplitude (**f**) are shown as individual data in the absence (−) and presence (+) of BDNF ($n = 7$). **g**, **h** Averages of the absolute values of frequency (**g**) or amplitude (**h**) are shown as individual data obtained in the absence (−) or presence (+) of K252a ($n = 4$). Values are mean ± SEM. n.s. $p > 0.05$ (two-tailed paired Student's *t* test

BDNF application. Perfusion of K252a (200 nM) alone did not alter neither frequency nor amplitude of mIPSCs (frequency 1.6 ± 0.44 to 1.7 ± 0.48 Hz; amplitude 30 ± 7.1 to 30 ± 7.2 pA, $n = 4$, $p > 0.05$, Fig. 4g, h).

To evaluate the involvement of $p75^{NTR}$ on the facilitatory action of BDNF on GABAergic currents, the hippocampal slices were preincubated for 2 h with an anti-$p75^{NTR}$ IgG (50 μg/mL), prior to whole-cell recordings. This antibody binds to the $p75^{NTR}$ receptor and blocks its activation by preventing BDNF binding to the receptor. As shown in Fig. 5, the excitatory action of BDNF on GABAergic transmission was not affected in slices preincubated with the $p75^{NTR}$ antibody. Thus, after BDNF application, there was a significant increase in mIPSC frequency (29 ± 7.8 %, $n = 8$, $p < 0.01$; 1.8 ± 0.28 Hz before and 2.2 ± 0.30 Hz after BDNF application), without changes in mIPSC amplitude (−0.84 ± 1.5 %, $n = 8$, $p > 0.05$; 35 ± 2.3 pA before and 34 ± 2.0 pA after BDNF application). The effect of BDNF in the presence of $p75^{NTR}$ antibody was not significantly different from to the effect caused by BDNF (either 10 or 100 ng/mL) alone ($p > 0.05$, one-way ANOVA).

The above data show that the excitatory effect of BDNF in mIPSC frequency is prevented by the presence of K252a, but not by the anti-$p75^{NTR}$ IgG, suggesting that the presynaptic enhancement of GABAergic transmission caused by BDNF is mediated by Trk receptor activation, but not by $p75^{NTR}$.

Adenosine A_{2A} receptor blockade prevents BDNF effect onto GABAergic transmission

The interplay between TrkB and adenosine A_{2A} receptors has been demonstrated in several contexts, with the activation of $A_{2A}R$ being a requisite for most of the effects of BDNF on excitatory synaptic transmission [15, 16, 18, 32–34]. We thus hypothesized that $A_{2A}R$, in spite of disynaptically modulating GABAergic transmission to pyramidal cells [35], could directly affect the monosynaptic

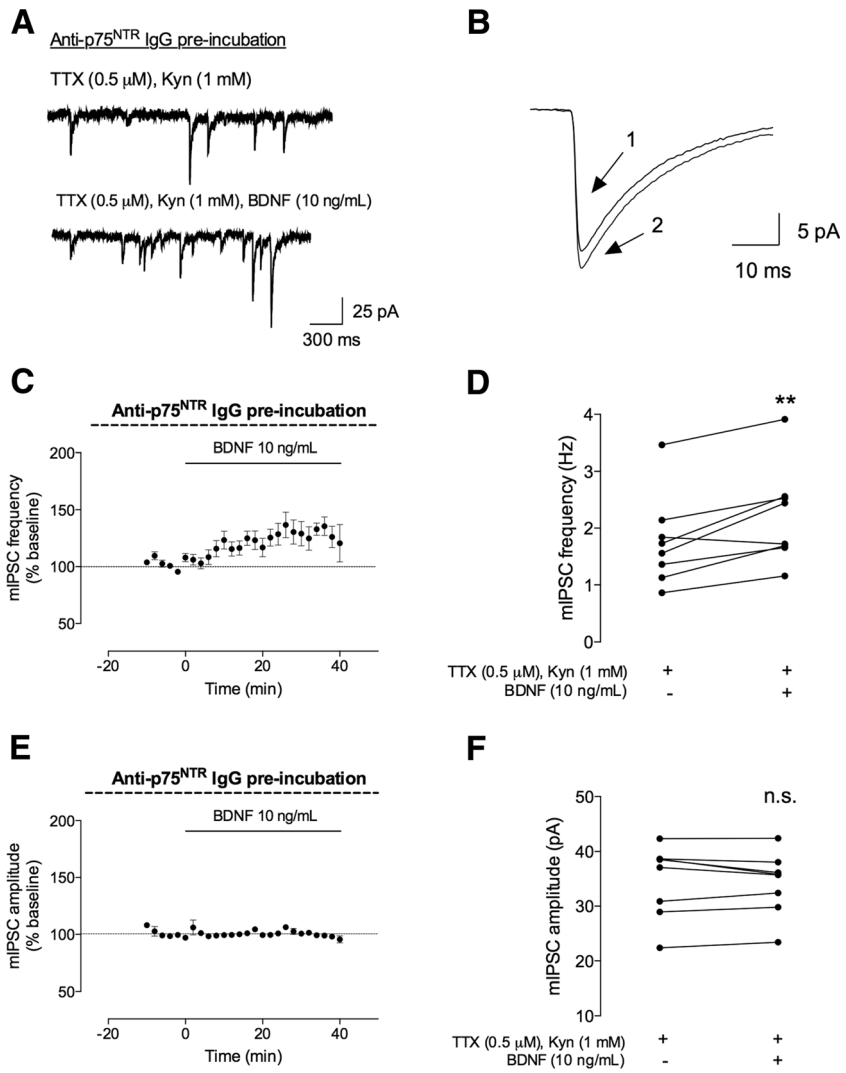


Fig. 5 The facilitatory effect of BDNF on mIPSC frequency is not dependent on $p75^{NTR}$ receptor activation. The mIPSCs were recorded in the same conditions as described for Fig. 2. The slices were preincubated with an anti-$p75^{NTR}$ IgG (50 mg/mL) for 2 h. **a** In the *upper panel*, a representation of mIPSC tracings recorded in the absence (*upper trace*) and presence (*lower trace*) of BDNF (10 ng/mL). **b** Representative average tracings of mIPSCs of two superimposed events in the absence (*1*) and presence (*2*) of BDNF (10 ng/mL), from the same cell. **c**, **e** Time course changes in mIPSC frequency (**c**) and amplitude (**e**) induced by application of BDNF ($n = 8$). mIPSC frequency changes were quantified by comparing the events before (baseline) and after BDNF application. **d**, **f** Averages of the absolute values of frequency (**d**) or amplitude (**f**) are shown as individual data in the absence (−) of BDNF and after (+) BDNF administration ($n = 8$). Values are mean ± SEM. $^{**}p < 0.01$ and n.s. $p > 0.05$ (two-tailed paired Student's *t* test)

action of BDNF to principal cells. We thus tested whether endogenous activation of adenosine $A_{2A}R$ could also influence the action of BDNF on GABAergic transmission. To do so, slices were perfused with the selective $A_{2A}R$ antagonist, SCH 58261 (100 nM) [36], immediately after going to whole cell and currents were allowed to stabilize for at least 20 min before applying BDNF and still in the presence of the $A_{2A}R$ antagonist. However, in contrast with what occurred in the absence of SCH 58261 (Fig. 2), after 40 min of BDNF application in the presence of SCH 58261 (100 nM), there was virtually no effect in mIPSC frequency (% change: -0.38 ± 3.5, $n = 10$, $p > 0.05$, Fig. 6a, c, d) and amplitude (% change 0.16 ± 1.2 of change $n = 10$, $p > 0.05$, Fig. 6b, e, f). When analyzing the period between 30 and 40 min of BDNF application (in the presence of SCH 58261), the averaged absolute values were 2.2 ± 0.22 Hz and 37 ± 2.4 pA, while in the 10 min prior to BDNF application, they were 2.2 ± 0.22 Hz and 38 ± 2.4 pA.

These data thus suggest that $A_{2A}R$ activation is required for the BDNF facilitatory effect onto inhibitory transmission.

Discussion

We herein show that, through TrkB receptor activation, BDNF positively modulates GABAergic transmission in the adult hippocampus by operating a presynaptic mechanism that requires adenosine $A_{2A}R$ co-activation.

GABA-releasing neurons are crucial for a proper regulation of pyramidal cells, controlling their firing rate, spike timing, and synchronized activity [37]. BDNF is a critical player in the modulation of neuronal networks. It mediates positive fast actions onto glutamatergic transmission [3–6] and synaptic plasticity [12, 13]. The increase in mIPSC frequency caused by BDNF may be interpreted as an increase in number of release sites or increase in number of docked vesicles, resulting in increased probability of release and/or an increase in the number of presynaptic interneuron terminals. Glutamic acid decarboxylase 65 (GAD65) is the key enzyme for GABA synthesis, and chronic administration of BDNF to organotypic hippocampal slices and hippocampal cultures led to increased GAD expression [38–40]. Moreover, enhanced GABA release probability due to a BDNF-mediated

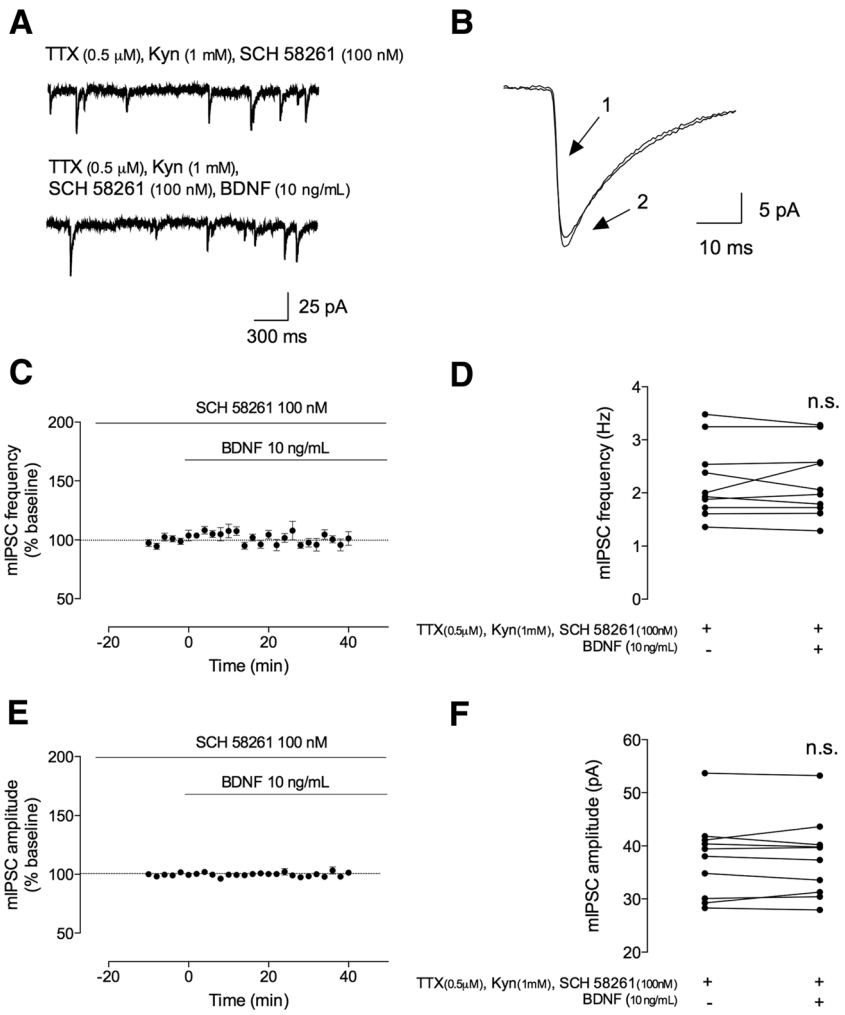


Fig. 6 BDNF-mediated effect on mIPSC frequency is dependent on adenosine A_{2A} receptor activation. The mIPSCs were recorded in the same conditions as described for Fig. 2. During all the recordings, an adenosine A_{2A} receptor antagonist was further added to the perfusion. **a** In the *upper panel*, a representation of mIPSC tracings recorded in the absence (*upper trace*) and presence (*lower trace*) of BDNF (10 ng/mL). **b** Representative average tracings of mIPSCs of two superimposed events in the absence (*1*) and presence (*2*) of BDNF (10 ng/mL), from the same cell. **c**, **e** Time course changes in mIPSC frequency (**c**) and amplitude (**e**) induced by application of BDNF ($n = 10$). mIPSC frequency and changes were quantified by comparing the events before (baseline) and after BDNF application. **d**, **f** Averages of the absolute values of frequency (**d**) or amplitude (**f**) are shown as individual data obtained in the absence (−) and presence (+) of BDNF ($n = 10$). Values are mean ± SEM. n.s. $p > 0.05$ (two-tailed paired Student's *t* test

redistribution of Ca^{2+} channels to vesicle release sites was shown in cultured hippocampal neurons [41]. Our data obtained in slices from adult rats is consistent with these presynaptically mediated actions of BDNF, though contrasting with data from slices or acutely isolated neurons from P12 to P18 [24, 25], where BDNF has been reported to cause a postsynaptically mediated inhibition of GABAergic signaling. Interestingly, BDNF affects Cl^- transport in hippocampal neurons [21]. The developmental shift from excitatory to inhibitory GABAergic currents observed around P13–16 results from changes in Cl^- transport and is regulated by GABA itself, since blockade of $GABA_A$Rs is able to prevent the switch from excitatory to inhibitory GABA [42]. One may thus speculate that BDNF changes the shifts from excitatory to inhibitory during a critical period of inhibitory GABAergic synapse maturation, eventually delaying it by inhibiting GABAergic transmission, but returns to its facilitatory role upon phasic transmission once synapses mature. Further studies on the role of BDNF in GABAergic synapse maturation are indeed required to directly assess this possibility.

Adenosine is a ubiquitous molecule released by neurons and glia that through the activation of its receptors is able to modulate synaptic transmission [43]. For instance, adenosine A_{2A}Rs are up-regulators of TrkB receptor signaling, being able to boost BDNF facilitatory effects on synaptic transmission [32], synaptic plasticity [15], and

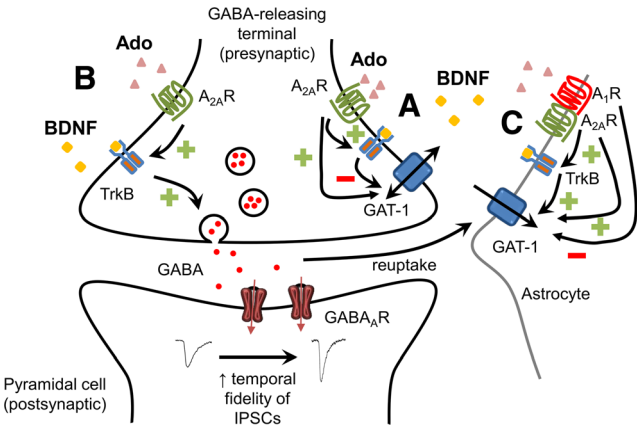


Fig. 7 Summary of the influence of BDNF over GABAergic transmission and its control by adenosine A_{2A}Rs. BDNF inhibits GAT-1-mediated non-exocytotic GABA release [9] and inhibits neuronal GABA reuptake [18] (*A*); A_{2A}Rs facilitate BDNF actions [18, 19]. In addition, BDNF facilitates exocytotic GABA release (*B*) (present work) and astrocytic GAT-1-mediated uptake of GABA (*C*) [17]; in both cases, these actions are gated by A_{2A}R. Direct influences of adenosine over hippocampal GABAergic transmission also occur, and some involving A_{2A}Rs are depicted in this figure (see [2] for details). Thus, A_{2A}Rs also directly facilitate GAT-1-mediated GABA transport into nerve endings (*A*) [52] as well as facilitate GAT-1- and GAT-3-mediated GABA transport into astrocytes (*C*), these actions being counteracted by an inhibitory action mediated by A_1R which is heteromerized with A_{2A}R at the plasma membrane of the astrocytes (*C*) [53]. Altogether, these A_{2A}R-dependent actions of BDNF may contribute to increase neuronal excitability without losing temporal fidelity of GABA transmission

neuromuscular junction [34]. Moreover, in hippocampal neurons, adenosine A_{2A}Rs are more abundantly located in nerve terminals [44] and are required to translocate TrkB receptors to lipid rafts during high-frequency neuronal firing, allowing a BDNF effect upon glutamate release [10]. Interestingly, as we now show, the presynaptic influence of TrkB receptors upon GABA release at GABAergic synapses is also under control of tonic adenosine A_{2A}R activation since the BDNF-induced increase on mIPSC frequency was prevented in the presence of an adenosine A_{2A}R antagonist. Remarkably, A_{2A}Rs, though present in a subset of GABAergic nerve terminals, do not directly affect GABAergic inputs to pyramidal neurons [35], but as we now show, they can do so in an indirect way by allowing facilitatory BDNF actions in GABAergic nerve terminals.

Although BDNF is only expressed in pyramidal cells, but not interneurons [45, 46], it is generally accepted that interneurons do express TrkB receptors [45, 47, 48]. Increase in synaptic activity will favor the release of BDNF [49] and the formation of adenosine from released ATP [50], with consequent activation of TrkB receptors and adenosine A_{2A}Rs [2]. BDNF is well known to increase neuronal excitability either directly, by facilitating glutamatergic synapses [1], or indirectly, by increasing GAT-1-mediated GABA uptake into astrocytes [17] and decreasing non-exocytotic GAT-1 reversal-mediated GABA release from synaptosomes [8, 19]. BDNF-mediated inhibition of GAT-1 activity at nerve endings also occurs when the transporter works in the inward direction [18], which may lead to an attenuation of excitation. Concerning exocytotic GABA release, we now show that BDNF enhances the activity of interneurons to release GABA, thus most probably contributing to a negative feedback mechanism to refrain excitability. Moreover, A_{2A}R-dependent BDNF facilitation of GABA release, herein described, together with A_{2A}R-dependent BDNF facilitation of GABA uptake into astrocytes [17] will synergistically contribute to increase temporal fidelity of GABA transmission (the time window available for synaptic integration) and synchronize pyramidal cell firing. In fact, deleting TrkB/BDNF signaling in fast-spiking interneurons induces a reduction of GABAergic inputs to pyramidal cells and the disruption of the typical rhythmic neuronal activity observed in neuronal circuits at gamma oscillation frequency [51]. Altogether, the available information allows to suggest that BDNF and adenosine A_{2A}R synergistically control synaptic communication by increasing neuronal excitability without losing GABAergic temporal fidelity necessary for proper network functioning and the maintenance of hippocampal oscillations (see Fig. 7). Indeed, by increasing GABA release to principal cells (present work) and by facilitating a major mechanism for GABA clearance from the synapses, GABA uptake into astrocytes [17], BDNF might not only be increasing the power

of GABAergic signaling but also be shortening the time window of GABA action, so increasing, in this way, the temporal fidelity of GABAergic transmission.

In summary, the present work clearly shows that BDNF facilitates GABAergic inputs to pyramidal neurons in the adult hippocampus in an $A_{2A}R$-dependent manner. How the interplay between $A_{2A}R$ and BDNF at the tripartite GABAergic synapse (Fig. 7) impacts in pathological conditions as epilepsy, where GABA, BDNF, and adenosine play a role [54, 55], deserves further investigation.

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Compliance with ethical standards All animal procedures were carried out in strict accordance with the ethical recommendations by EU (Directive 210/63/EU), the Portuguese (DL 113/2013) legislation for the protection of animals used for scientific purposes, and the Ethics Committee of the Instituto de Medicina Molecular and of the Faculty of Medicine, University of Lisbon, Lisbon, Portugal, who approved this study.

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8.2 | Appendix 2

Neurobiology of Disease

Adenosine Kinase Deficiency in the Brain Results in Maladaptive Synaptic Plasticity

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Adenosine kinase (ADK) deficiency in human patients (OMIM:614300) disrupts the methionine cycle and triggers hypermethioninemia, hepatic encephalopathy, cognitive impairment, and seizures. To identify whether this neurological phenotype is intrinsically based on ADK deficiency in the brain or if it is secondary to liver dysfunction, we generated a mouse model with a brain-wide deletion of ADK by introducing a *Nestin-Cre* transgene into a line of conditional ADK deficient $Adk^{fl/fl}$ mice. These $Adk^{\Delta brain}$ mice developed a progressive stress-induced seizure phenotype associated with spontaneous convulsive seizures and profound deficits in hippocampus-dependent learning and memory. Pharmacological, biochemical, and electrophysiological studies suggest enhanced adenosine levels around synapses resulting in an enhanced adenosine A_1 receptor (A_1R)-dependent protective tone despite lower expression levels of the receptor. Theta-burst-induced LTP was enhanced in the mutants and this was dependent on adenosine A_{2A} receptor ($A_{2A}R$) and tropomyosin-related kinase B signaling, suggesting increased activation of these receptors in synaptic plasticity phenomena. Accordingly, reducing adenosine A_{2A} receptor activity in $Adk^{\Delta brain}$ mice restored normal associative learning and contextual memory and attenuated seizure risk. We conclude that ADK deficiency in the brain triggers neuronal adaptation processes that lead to dysregulated synaptic plasticity, cognitive deficits, and increased seizure risk. Therefore, ADK mutations have an intrinsic effect on brain physiology and may present a genetic risk factor for the development of seizures and learning impairments. Furthermore, our data show that blocking $A_{2A}R$ activity therapeutically can attenuate neurological symptoms in ADK deficiency.

Key words: adenosine kinase; epilepsy; gene mutation; human genetic disorder; learning and memory; mouse model

Significance Statement

A novel human genetic condition (OMIM #614300) that is based on mutations in the adenosine kinase (*Adk*) gene has been discovered recently. Affected patients develop hepatic encephalopathy, seizures, and severe cognitive impairment. To model and understand the neurological phenotype of the human mutation, we generated a new conditional knock-out mouse with a brain-specific deletion of *Adk* ($Adk^{\Delta brain}$). Similar to ADK-deficient patients, $Adk^{\Delta brain}$ mice develop seizures and cognitive deficits. We identified increased basal synaptic transmission and enhanced adenosine A_{2A} receptor ($A_{2A}R$)-dependent synaptic plasticity as the underlying mechanisms that govern these phenotypes. Our data show that neurological phenotypes in ADK-deficient patients are intrinsic to ADK deficiency in the brain and that blocking $A_{2A}R$ activity therapeutically can attenuate neurological symptoms in ADK deficiency.

Introduction

Adenosine kinase (ADK) is the key metabolic regulator of the purine ribonucleoside adenosine. In the adult brain, ADK is primarily expressed in astrocytes and determines the availability of adenosine in the synaptic cleft (Boison, 2013). Overexpression of ADK in the brain has been associated with the development of epilepsy and cognitive impairment (Li et al., 2008; Boison et al.,

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2012), whereas therapeutic adenosine augmentation is considered a promising therapeutic strategy for the treatment of epilepsy (Boison et al., 2002a; Pritchard et al., 2010; Boison, 2013). Recently, the first human mutations in the *Adk* gene have been described (OMIM:614300) (Bjursell et al., 2011; Staufner et al., 2016). Consistent with a prominent role of ADK for the maintenance of transmethylation reactions in the liver (Boison et al., 2002b), six patients from three unrelated families displayed a hepatic phenotype composed of disruption of the transmethylation cycle, dysregulation of hepatic metabolites, and hepatic encephalopathy (Bjursell et al., 2011). Affected individuals presented with global psychomotor delay and convulsive seizures commencing between the first and third year of life (Bjursell et al., 2011). Subsequently, 11 additional patients with ADK deficiency from eight families were identified; microvesicular hepatic steatosis and global developmental delay were prominent and most patients developed seizures and cognitive impairment (Staufner et al., 2016). Given the neuroprotective and anticonvulsive properties of adenosine, the neurological phenotype of patients with inborn ADK deficiency is somewhat surprising.

In the brain, adenosine modulates neurotransmission primarily through binding to its two high-affinity G-protein-coupled receptors: the inhibitory adenosine A_1 receptor (A_1R) and the stimulatory A_{2A} receptor ($A_{2A}R$) (Chen et al., 2013). The predominant action of adenosine is the inhibition of synaptic transmission via A_1R-mediated signaling and, in rodent models of epilepsy, this signaling pathway is impaired, accounting for increased excitability and susceptibility to seizures (Rebola et al., 2003). Despite lower expression levels of the $A_{2A}R$ in the hippocampus, activation of $A_{2A}R$s influences the release and uptake of neurotransmitters and also facilitates excitatory tropomyosin-related kinase B (TrkB)-mediated BDNF actions (Diógenes et al., 2004; Fontinha et al., 2008). To determine whether the neurological phenotype in ADK-deficient patients is secondary to hepatic encephalopathy or if it is intrinsic to ADK deficiency in the brain, we generated mice with a brain-wide deletion of ADK. This was achieved by breeding conditional *Adk-flox* ($Adk^{fl/fl}$) with *Nestin-Cre* mice (Burns et al., 2007) to yield Nestin-$Cre^{+/-}$:$Adk^{fl/fl}$ ($Adk^{\Delta brain}$) mice. These mutants completely lacked ADK in the brain and developed progressive stress-induced seizures and deficits in learning and memory. We identified a novel mechanism in which the complete lack of ADK in the brain drives neuronal adaptation processes that lead to enhanced basal synaptic transmission.

Materials and Methods

Transgenic mice. The *Adk* gene targeting vector to produce global ADK knock-out mice ($Adk^{tm1bois}$) has been fully described previously (Boison et al., 2002b; Fedele et al., 2004). Briefly, the targeting construct was reengineered and exon 7 of the *Adk* gene was flanked with loxP sites. Through homologous recombination in embryonic stem cells, an $Adk^{fl/-}$ allele was created, which was used to generate a line of $Adk^{fl/fl}$ mice. $Adk^{fl/fl}$ mice were crossbred with constitutive *Nestin-Cre* mice (Tronche et al., 1999) (The Jackson Laboratory, RRID: IMSR_JAX:003771) to generate Nestin-$Cre^{+/-}$:$Adk^{fl/fl}$ mice. *Nestin-Cre* mice express the Cre driver as early as embryonic day 11, resulting in Cre activation in neuronal and astroglial lineages (Tronche et al., 1999), a strategy chosen to achieve a brain-wide deletion of ADK, which is predominantly expressed in astrocytes of the adult brain (Studer et al., 2006). To exclude any Cre related experimental confounds, additional Nestin-$Cre^{+/-}$ mice without Adk-flox alleles were included in our *in vivo* studies. Nestin-$Cre^{+/-}$:$Adk^{fl/fl}$:$A_1R^{-/-}$ mice and Nestin-$Cre^{+/-}$:$Adk^{fl/fl}$:$A_{2A}R^{-/-}$ were generated by breeding Nestin-$Cre^{+/-}$:$Adk^{fl/fl}$ mice with global $A_1R^{-/-}$ (Johansson et al., 2001) or $A_{2A}R^{-/-}$ mice (Day et al., 2003), respectively. Offspring were then backcrossed until Nestin-$Cre^{+/-}$:$Adk^{fl/fl}$:$A_1R^{-/-}$ and Nestin-$Cre^{+/-}$:$Adk^{fl/fl}$:$A_{2A}R^{-/-}$ mice were generated. Breeding of the experimental animals followed either a Nestin-$Cre^{+/-}$:$Adk^{fl/fl}$ mice × Nestin-$Cre^{-/-}$:$Adk^{fl/fl}$, Nestin-$Cre^{+/-}$:$Adk^{fl/fl}$:$A_1R^{-/-}$ × Nestin-$Cre^{-/-}$:$Adk^{fl/fl}$:$A_1R^{-/-}$ or Nestin-$Cre^{+/-}$:$Adk^{fl/fl}$:$A_{2A}R^{-/-}$ × Nestin-$Cre^{-/-}$:$Adk^{fl/fl}$:$A_{2A}R^{-/-}$ mating protocol, which generated ADK-deficient and normal mice in a 1:1 ratio as littermates. All mice were generated and propagated on an identical C57BL/6 background and were genotyped at weaning by PCR. If not indicated otherwise, male subjects were used. All animals were social housed under standardized conditions of light, temperature, humidity, and environmental enrichment and had *ad libitum* access to food and water. Experimental animals used for this study were taken between 2 and 8 months of age. *In vivo* studies were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility in accordance with protocols approved by the Legacy Institutional Animal Care and Use Committee. *Ex vivo* assays were performed according to procedures approved by the Portuguese authorities (DL 113/2013) and European Community Guidelines for Animal Care (European Communities Council Directive 2010/63/EU).

PCR. Tissue from mice was obtained by tail clipping or from specific organs. Genomic DNA was prepared following standard procedures. For genotyping, Cre DNA sequence amplification with primers 5′-GGACATGTTCAGGGATCGCCAGGCG-3′ and 5′-GGACATGTTCAGGGATCGCCAGGCG-3′ was performed. For genomic Adk, the primer sequences were as follows: 5′-CCTCTATGAGTTGAGATCCTGTCTCC-3′ and 5′-ATTTATTAACTTTACATAGATTCAGACAG-3′. The Cre and Adk PCRs were paired with a genomic internal positive control using primers oIMR7338 (5′-CTAGGCCACAGAATTGAAAGATCT-3′) and oIMR7339 (5′-GTAGGTGGAAATTCTAGCATCATCC-3′) (The Jackson Laboratory). PCR products were loaded in a 2% agarose gel dyed with ethidium bromide for band visualization. For qRT-PCR, cortical samples were homogenization in QiAzol Lysis Reagent (Qiagen) and RNA was extracted, using the RNeasy Lipid Tissue Mini Kit (Qiagen). For first-strand cDNA synthesis, 1.5 μg of total RNA was applied in each reaction according to the manufacturer's protocol (SuperScript III Reverse Transcriptase; Invitrogen Life Technologies). For cDNA amplification, 2 μl of 1:10 diluted cDNA was added to 12.5 μl of 2× Power SYBR Green PCR Master Mix (Life Technologies) and 1 μl of each primer (5 μM) in a reaction volume of 25 μl. All reactions were performed in duplicate. The qRT-PCRs were performed on an RT-PCR Rotor Gene 6000 device (Corbett Life Science). Melting curves were analyzed to confirm primer specificity and the comparative C_t (threshold cycle) method was for quantification according to the following formula: $C_t = C_t(\text{target gene}) - C_t(\text{reference gene})$. The genes used to normalize the expression of the target sequences were PPIA peptidylprolyl isomerase A (cyclophilin A, CypA) and ribosomal protein L13A (RpL13A). Primers used were as follows: 5′-TATCTGCACTGCCAAGACTGAGTG-3′ and 5′-CTTCTTGCTGGTCTTGCCATTCC-3′ for CypA; 5′-GGATCCCTCCACCCTATGACA-3′ and 5′-CTGGTACTTCCACCCGACCTC-3′ for RpL13A; and 5′-TCGGCTGGCTACCACCCCTTG-3′ and 5′- CCAGCACCCAAGGTCACACCAAAGC-3′ for A_1R.

Analysis and quantification of induced seizures. Stress-induced seizures were evoked in $Adk^{\Delta brain}$ mice by placing animals into a novel environment and characterized by tonic–clonic seizure activity followed by rearing and falling. Seizures were scored for occurrence and duration. Please see Movie 1 for a representative stress-induced seizure. $Adk^{\Delta brain}$ mice used for the quantification of stress-induced seizures were naive to any additional treatments. During this period, the mice were exclusively handled by the same investigators. The probability of an evoked seizure was calculated for each mouse as the number of evoked seizures relative to the total number of trials within a month and then reported as the group average. Additional animals were equipped with a bipolar electrode surgically implanted into the hippocampus (AP: −1.94; ML: −1.25, DV: −1.5, relative to bregma) with a surface cortical monopolar screw electrode and cerebellum reference screw electrode while under general anesthesia (2% isofluo-

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Movie 1. Stress-induced tonic convulsive seizure in an Adk$^{\Delta brain}$ mouse. The movie is of a representative stress-induced seizure that is triggered by placing the mouse in a novel environment. The seizure is characterized by multiple bouts of tonic-clonic, rearing, and falling activity.

rane, 100% O_2). Video EEG recordings occurred at least 1 week after surgery. Mice were tethered for the EEG recordings. Electrical brain activity was amplified and digitized using a Nervus EEG recording system. Pharmacologically induced seizures were evoked by intraperitoneal injection of the A_1R antagonist DPCPX (up to 3.5 mg/kg; Sigma-Aldrich). For the DPCPX threshold test, mice received 0.5 mg/kg DPCPX intraperitoneally every 10 min until a convulsive tonic–clonic seizure was induced up to a maximum cumulative dose of 3.5 mg/kg DPCPX. For the high-dose DPCPX experiment, a single dose of 3.0 mg/kg intraperitoneal DPCPX was administered and the latency to tonic–clonic seizure and mortality were indexed. The $A_{2A}R$ antagonist SCH58261 or the TrkB antagonist Ana-12 (0.5 mg/kg, 5% DMSO, i.p.; Sigma-Aldrich) were administered either 30 min or 4 h before DPCPX, respectively.

Assessment of baseline EEG activity and spontaneous seizures. Spontaneous seizures were assessed by video EEG in 6-month-old Adk$^{\Delta brain}$ mice ($n = 6$) and nestin-Cre$^{+/-}$ mice ($n = 4$) for 7 d. Additional baseline EEG recordings were conducted in all three lines of control mice (wild-type, nestin-Cre$^{+/-}$, and Adk$^{fl/fl}$) plus Adk$^{\Delta brain}$ mice for 3 d ($n = 5$/genotype). Animals were equipped with EEG recording electrodes as described above. Electrical brain activity was monitored using a Nervus EEG recording system connected with a Nervus Magnus 32/8 Amplifier and filtered (high-pass filter 50 Hz cutoff, low-pass 1 Hz). The digital EEG signal was recorded, stored, and visualized using a NicoletOne-System (Viasys Healthcare). Videos were acquired with Lorex cameras and a DVR. EEG recordings were scored in their entirety for seizure activity. EEG seizure activity was defined as high-amplitude rhythmic discharges that clearly represented a new pattern of tracing lasting for >5 s. EEG seizures were confirmed to have a tonic–clonic behavioral correlated with the corresponding time-matched video.

Behavioral assessment of mice. Conditioned learning and contextual memory were conducted using a classic fear-conditioning paradigm (Wehner and Radcliffe, 2004) with a Med Associates Fear Conditioning chamber and Video Freeze software (RRID: SCR_014574). Testing consisted of a 2 d paradigm. Day 1 was conditioned learning, including a baseline activity trial (3 min in the arena) and then 4 trials of conditioning stimuli (CS, 90 dB white noise, 30 s) immediately followed by aversive foot shock (0.3 mA, 1 s) with an intertrial interval (ITI) of 3 min. Freezing was measured during the ITI. Day 2 was contextual memory testing, in which mice were returned to the same arena and freezing was measured during 8 consecutive 1 min bins. Data are represented as the group average for percentage time freezing. The fear-conditioning paradigm was performed in animals that did not respond to seizure induction or in animals that were in their refractory period 2 h after a preceding seizure. There were no significant differences between the two groups of animals (associative learning, $p = 0.58$; contextual memory, $p = 0.79$). Animals that had a stress-induced seizure during the fear-conditioning paradigm were excluded from the analysis.

Brain dissection for electrophysiology. All mice were quickly anesthetized with isofluorane before decapitation to minimize stress. No signs of seizures were detected in Adk$^{\Delta brain}$ mice at the time of euthanasia. The brain was quickly removed and hippocampi and cortices were dissected in ice-cold aCSF containing the following (in mM): NaCl 124, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 26, $MgSO_4$ 1, $CaCl_2$ 2, and glucose 10, pH 7.4. While the other brain areas were frozen at −80°C until further use, one of the hippocampi was sliced (400 μm) perpendicularly to its longitudinal axis using a McIlwain tissue chopper. Slices were then immediately transferred to a resting chamber filled with the same solution at room temperature and allowed to recover for at least 1 h before use in extracellular recordings. For each of the experimental paradigms described below, we used at least one slice per mouse; all *n* values stated are based on the group size of mice with their respective genotype.

Extracellular recordings. Slices were transferred to a recording chamber and submerged with oxygenated aCSF solution at 32°C, continuously superfused at a flow rate of 3 ml/min. When indicated, drugs were added to the superfused solution. Recordings were obtained with an Axoclamp 2B amplifier and digitized (Molecular Devices). Evoked field EPSPs (fEPSPs) were recorded extracellularly through a microelectrode filled with 4 M NaCl (2–6 MΩ resistance) placed in the stratum radiatum of the CA1 area. A concentric electrode was placed on the Schaffer collateral–commissural fibers in stratum radiatum near the CA3–CA1 border and used to deliver the stimulation (rectangular 0.1 ms pulses once every 15 s). Individual responses were monitored and averages of eight (basal synaptic transmission) or six (LTP induction and input–output curve) consecutive responses were continuously stored on a personal computer with the WinLTP software (RRID: SCR_008590).

Drugs used for electrophysiological studies. DPCPX (1,3-dipropyl-8-cyclopentylxanthine, A_1R antagonist) was obtained from Ascent Scientific (Bristol, UK). CGS21680 (2-[p-(2-carboxyethyl)phenethylamino]-5-*N*-ethyl-carboxamido adenosine, $A_{2A}R$ agonist) was purchased from Sigma (St Louis, MO). K252a (Tyrosine kinase inhibitor) and SCH58261 (2-(2-Furanyl)-7-(2-phenylethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine, $A_{2A}R$ antagonist were purchased from Tocris Bioscience Cookson (Ballwin, MO). DPCPX, SCH58261 and CGS21680 were prepared in a 5 mM and K252a in a 1 mM stock solution, all in DMSO. The percentage of DMSO in each experiment did not exceed 0.001%.

Basal synaptic transmission. The intensity of stimulus (80–200 μA) was initially adjusted to obtain a submaximal fEPSP slope (near 0.5 mV/ms) with a minimum population spike contamination. Alteration in synaptic transmission induced by drugs was evaluated as the percentage change in the average slope of the fEPSP in the presence of the drug in relation to the average slope of the fEPSP measured during the 10 min that preceded its addition.

Input–output curve. Once obtaining a stable baseline for at least 15 min, the stimulus delivered to the slice was decreased to 60 μA, with fEPSPs disappearance. The stimulus was delivered to the slice in successively increased steps of 20 μA until a supramaximal stimulation of 340 μA. For each stimulation condition, data from three consecutive average fEPSP were stored. The Input-Output curve was plotted as the relationship of fEPSP slope (ms) vs stimulus intensity (μA).

LTP induction. After obtaining a 14 min stable baseline of fEPSP slope by 0.5 ms, LTP was induced by a theta-burst protocol as described previously (Diógenes et al., 2011). This protocol consisted of three trains of three stimuli delivered at 100 Hz, each separated by 200 ms; the intensity of the stimulus was kept constant before, during, and after the induction protocol. LTP was quantified as the percentage change in the average slope of fEPSP taken from 52–60 min after LTP induction in relation to the average slope of the fEPSP measured during the baseline period (10 min before LTP induction). While assessing LTP in the presence of drugs, LTP was only induced no less than 30 min after starting the drug perfusion and only after fEPSP slope values had stabilized.

Western blot and saturation binding assays. Dissected brain samples were homogenized in chilled 0.32 M sucrose solution with 50 mM Tris, pH 7.6, plus protease inhibitors (Hoffmann LaRoche) and then centrifuged.

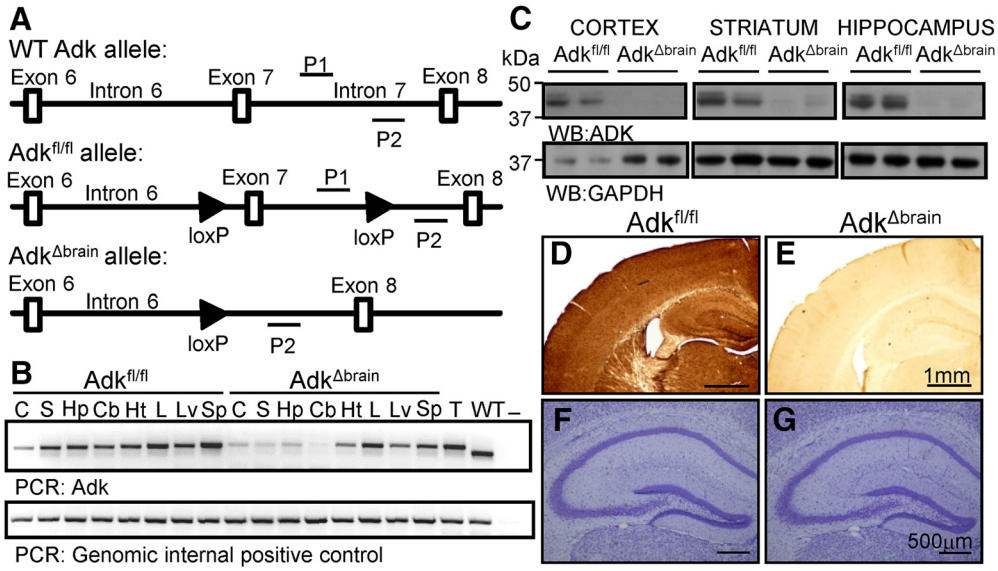

Figure 1. Brain-specific deletion of ADK in Adk $^{\Delta brain}$ mice. ***A***, Transgenic strategy: exon 7 of the *Adk* allele is flanked with loxP sites in Adk $^{fl/fl}$ mice. ***B***, *Adk* PCR on genomic DNA extracts from the cortex (C), striatum (S), hippocampus (Hp), cerebellum (Cb), heart (Ht), lung (L), liver (Lv), and spleen (Sp) from Adk $^{fl/fl}$ and Adk $^{\Delta brain}$ mice. Tail DNA from an Adk $^{fl/fl}$ (T) and wild-type mouse (WT) were included as positive controls. Water (−) was included as a no-template control. *Adk* forward and reverse primer (P1 and P2) sites are depicted in ***A***. ***C***, ADK (40 kDa) Western blots on cortical, striatal, and hippocampal protein extracts from Adk $^{fl/fl}$ and Adk $^{\Delta brain}$ mice; $n = 2$/genotype are used as representatives. ADK Western blots were reprobed with GAPDH (37 kDa) as a loading control. ***D***, ***E***, ADK immunohistochemistry of cortical brain tissue from Adk $^{fl/fl}$ (***D***) and Adk $^{\Delta brain}$ (***E***) mice. ***F***, ***G***, Nissl stain of hippocampal formation from Adk $^{fl/fl}$ (***F***) and Adk $^{\Delta brain}$ (***G***) mice.

Protein was quantified using a Bio-Rad Protein assay kit. Next, 25–200 μg of aqueous protein extracts from tissue were loaded and separated on 10% SDS-PAGE gels and transferred onto PVDF membranes (Millipore). The blots were probed overnight at 4°C with primary antibody anti-ADK (1:4000, RRID pending; Gouder et al., 2004) and anti-GAPDH (1:500, catalog #sc-47724 RRID:AB_627678; Santa Cruz Biotechnology) as internal standards. The specificity of the ADK antibody has been validated previously in knock-out samples (Fedele et al., 2004; Gouder et al., 2004). The membranes were incubated with secondary antibody anti-mouse (1:2000, catalog #A-10677 RRID:AB_2534060; Thermo Fisher Scientific) or anti-rabbit (1:10,000, catalog #G-21234 RRID: AB_2536530; Thermo Fisher Scientific) conjugated with horseradish peroxidase for 1 h at room temperature. Chemoluminescence detection was performed with an ECL-PLUS Western blot detection reagent (GE Healthcare) using X-ray films (Fujifilm). The saturation binding experiments were adapted from a previous protocol (Batalha et al., 2013). [^{3}H]DPCPX (radiolabeled A_1R antagonist, specific activity 120 Ci/mmol) was from GE Healthcare. [^{3}H]DPCPX (0–42 nM) (specific activity 120 Ci/mmol) was incubated for 2 h at room temperature with 39–77 μg of protein homogenate and 4 U/ml adenosine deaminase in a solution containing 50 mM Tris, 2 mM $MgCl_2 \cdot 6H_2O$, pH 7.4, with a final volume of 300 μl. Each reaction was performed in duplicate. Nonspecific binding was measured in the presence of 2 μM XAC and normalized for protein concentration. Binding reactions were stopped by vacuum filtration with a Skatron semiautomatic cell harvester using chilled incubation solution. Filtermats 1.5 μm (Molecular Devices) were used and placed in scintillation vials to which 3 ml of scintillation mixture (OptiPhase HiSafe 2; PerkinElmer) was added. Radioactivity bound to the filters was determined after 12 h with an efficiency of 55–60% for 2 min.

Immunohistochemistry. ADK and Nissl staining was performed on 4% paraformaldehyde-fixed coronal brain sections (40 μm) using standard protocols (Studer et al., 2006). High-resolution digital images were acquired using equivalent settings with a Leica DM1000 bright-field microscope equipped with a DCF295 camera and LAS AF Image Acquisition Software (RRID: SCR_013673; Leica Microsystems).

Statistical analysis. Analyses were conducted with Prism 7 software (RRID: SCR_002798; GraphPad) and statistical significance was assumed at $p < 0.05$. Repeated-measures two-way ANOVA followed by Tukey's, Dunnett's, or Sidak's multiple-comparison tests; two-way ANOVA followed by Tukey's multiple-comparison test; nonlinear regression fit test; or two-tailed unpaired *t* tests were used as appropriate. The two-tailed Mann–Whitney test or Kruskal–Wallis test followed by Dunn's multiple-comparisons test were used for nonparametric analysis where appropriate. Kaplan–Meier survival curves were analyzed by log–rank (Mantel–Cox) tests.

Results

Conditional deletion of the *Adk* gene causes brain-wide ADK deficiency

To provide mechanistic evidence that ADK deficiency in the brain could be a primary cause for neurological symptoms in ADK-deficient patients, we engineered a novel line of mice with brain-wide disruption of ADK. First, our *Adk* gene-targeting construct (Boison et al., 2002b) was modified to flank exon 7 of the *Adk* gene with loxP sites (Fig. 1*A*). This construct was used to generate *Adk* $^{fl/fl}$ mice, which were then crossed with *Nestin-Cre* mice (Burns et al., 2007) to generate mutant Adk $^{\Delta brain}$ mice and normal Adk $^{fl/fl}$ littermates. PCR analysis validated selective deletion of the *Adk* allele in representative brain regions of Adk $^{\Delta brain}$ mice, whereas the intact *Adk* gene was maintained in peripheral organs of Adk $^{\Delta brain}$ mice and in all tissues from Adk $^{fl/fl}$ mice (Fig. 1*B*). Consistent with intact *Adk* gene expression in the liver of Adk $^{\Delta brain}$ mice, the gross liver morphology was normal and did not show any signs of lipid accumulation with oil-red-O staining (data not shown). In addition, Adk $^{\Delta brain}$ mice did not show any appreciable differences in reproduction and life span. Western blot analysis (Fig. 1*C*) and ADK immunohistochemistry (Fig. 1*D*,*E*) corroborated a complete lack of ADK expression throughout the brains of the Adk $^{\Delta brain}$ mice without affecting gross morphology (Fig. 1*F*,*G*).

Brain-wide disruption of ADK results in increased susceptibility to seizure induction

Adk $^{\Delta brain}$ mice initially developed normally. However, from 2 months on, the animals developed increased susceptibility to stress-induced seizures that were reliably induced by placing animals into a novel environment (i.e., a clean observation

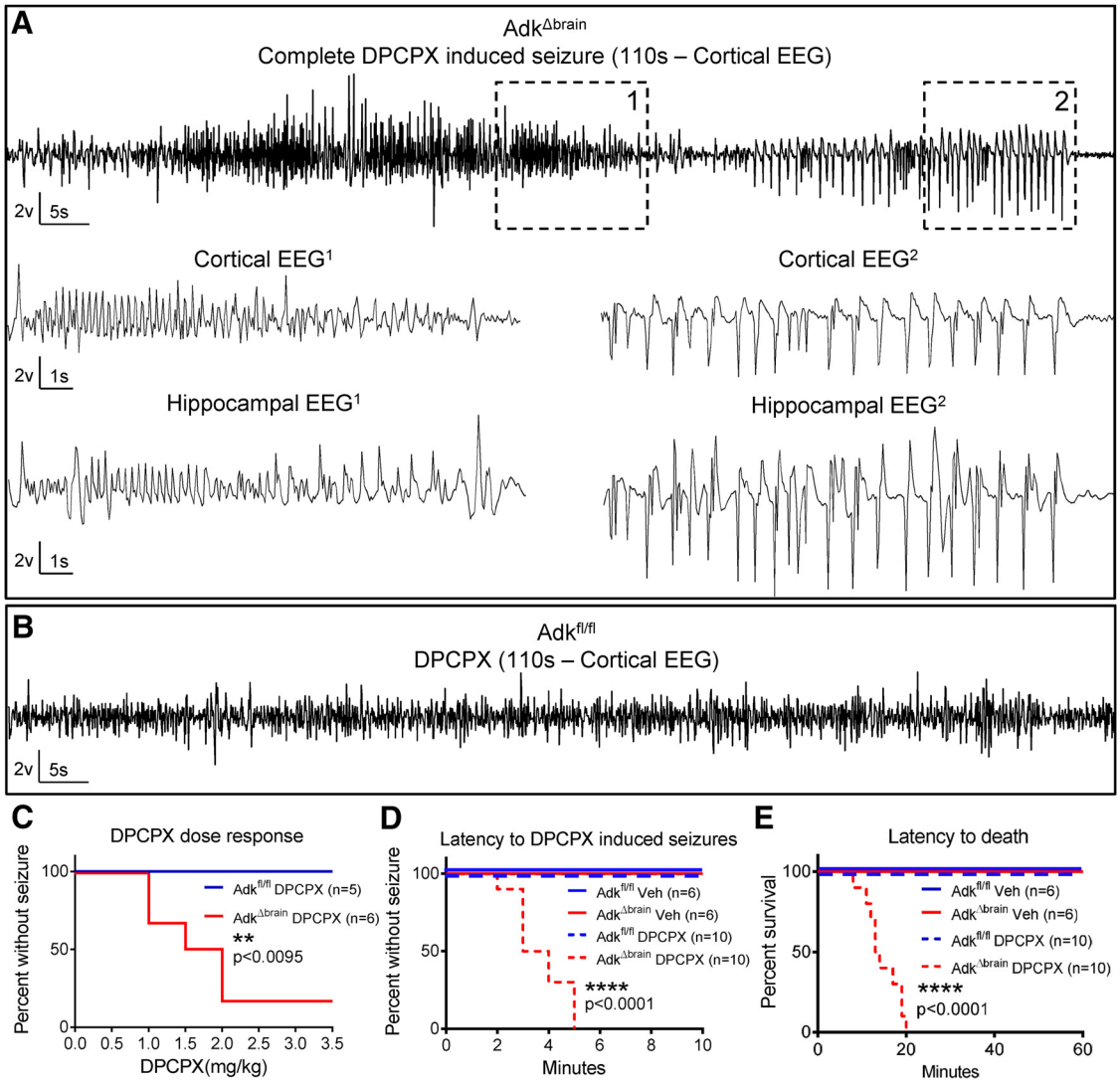

Figure 2. Loss of brain ADK results in increased susceptibility to seizure induction. ***A***, Representative cortical EEG trace that includes a complete DPCPX- induced seizure (110 s) in an Adk $^{\Delta brain}$ mouse that received a total cumulative dose of 2.0 mg/kg DPCPX intraperitoneally. High-resolution portions (15 s) of the DPCPX-induced seizure are depicted in cortical/hippocampal EEG ^{1} and EEG ^{2}. These EEG traces correspond to boxes demarcated with 1 and 2 in upper cortical trace. See Movie 2 for a matching behavioral seizure. ***B***, DPCPX does not trigger seizures in Adk $^{fl/fl}$ control mice. Shown is a representative section of a cortical EEG trace (110 s) recorded after a cumulative dose of 3.5 mg/kg DPCPX intraperitoneally. ***C***, DPCPX dose response (0.5 mg/kg, i.p., every 10 min) in Adk $^{\Delta brain}$ mice ($n = 6$) and Adk $^{fl/fl}$ mice ($n = 5$). ***D***, ***E***, DPCPX (3 mg/kg, i.p.) administered to Adk $^{\Delta brain}$ mice ($n = 10$) causes status epilepticus (***D***, $^{****}p < 0.0001$) and increased mortality (***E***, $^{****}p < 0.0001$) compared with DPCPX-treated Adk $^{fl/fl}$ mice ($n = 10$), vehicle-treated Adk $^{fl/fl}$ mice ($n = 6$), and vehicle-treated Adk $^{\Delta brain}$ mice ($n = 6$). Statistical analysis: log–rank (Mantel–Cox) test.

area) and characterized by tonic–clonic convulsions (see a representative seizure in Movie 1). These evoked seizures lasted an average of 2.48 ± 0.18 min ($n = 7$) and a marked progression of this seizure phenotype with age was found. By 3 months of age, 44% of all Adk $^{\Delta brain}$ mice reacted with a seizure upon being placed into a novel environment; by 4 months of age, the incidence of seizure response within the same Adk $^{\Delta brain}$ population was at 89% and continued to 100% by 6 months of age. Likewise, the probability of an evoked seizure response in the Adk $^{\Delta brain}$ mice rose from 7.4% at 2 months of age to 77% at 6 months. Because pharmacological blockade of ADK is a very effective therapeutic strategy to suppress seizures in clinically relevant animal models of epilepsy (Gouder et al., 2004; McGaraughty et al., 2005; Boison, 2016a, 2016b), the emergence of the stress-induced seizure phenotype in Adk $^{\Delta brain}$ mice was an unexpected finding. To identify the underlying mechanisms of seizure induction in Adk $^{\Delta brain}$ mice, we next sought to replicate seizure induction in Adk $^{\Delta brain}$ mice with well controlled pharmacological tools.

Because adenosine A_1Rs link to potent anticonvulsant mechanisms (Gouder et al., 2003; Fedele et al., 2006; Kochanek et al., 2006; Gomes et al., 2011; Chen et al., 2013), A_1R function in Adk $^{\Delta brain}$ mice was tested. Injection of the A_1R antagonist DPCPX (1.0 - 2.0 mg/kg, i.p.) at doses that do not trigger seizures in control mice (Masino et al., 2011; Fig. 2*B*) triggered a convulsive seizure in 5 of 6 Adk $^{\Delta brain}$ mice (Fig. 2*A*, *C*, Movie 2). Furthermore, Adk $^{fl/fl}$ control mice ($n = 5$) were unaffected by higher doses up 3.5 mg/kg (Fig. 2*B*, *C*). Therefore, pharmacological blockade of the A_1R could be used to induce seizures reliably in lieu of stress in Adk $^{\Delta brain}$ mice (Mantel–Cox test, $\chi^2_{(1)} = 6.73$, $^{**}p = 0.0095$; Fig. 2*C*). Next, it was determined that a single injection with a higher dose of the A_1R antagonist DPCPX (3 mg/kg i.p.) consistently triggered lethal status epilepticus in

Movie 2. DPCPX-induced tonic convulsive seizure in an Adk$^{\Delta brain}$ mouse. The movie is of a representative DPCPX-induced seizure that was triggered with a final cumulative dose of 2.0 mg/kg DPCPX. The seizure is the behavioral correlate of the cortical and hippocampal EEG traces shown in Figure 2*A*. The seizure is characterized by bouts of tonic-clonic, rearing, falling, running, and jumping activity.

Adk$^{\Delta brain}$ mice (10 of 10 mice), but not in Adk$^{fl/fl}$ mice (0 of 10 mice; Fig. 2*D*,*E*). Together, these data demonstrate that, despite increased susceptibility for inducible seizures in Adk$^{\Delta brain}$ mice, the A_1R maintains an inhibitory and protective function.

Brain-wide disruption of ADK results in spontaneous seizures

To rule out that the Nestin-Cre expression or the Adk flox mutation affects the EEG baseline, 72 h blocks of hippocampal EEGs obtained from C57BL/6 (WT), Adk$^{fl/fl}$, and Nestin-Cre$^{+/-}$ mice without floxed *Adk* alleles were compared with those from Adk$^{\Delta brain}$ mice ($n = 5$, each) All control lines had normal hippocampal baseline EEGs, similar to those recorded from Adk$^{\Delta brain}$ mice. Neither the Adk$^{\Delta brain}$ mutation nor the Nestin-Cre expression affected baseline EEG activity (Fig. 3*A*). During those initial recordings, spontaneous seizures were found only in the Adk$^{\Delta brain}$ mice, not in the controls. Therefore, an additional 7 d of vEEG recording blocks in a new set of Adk$^{\Delta brain}$ mice ($n = 6$) and Nestin-Cre$^{+/-}$ mice ($n = 4$; Fig. 3*B*) were performed. Five of six Adk$^{\Delta brain}$ mice had spontaneous seizures, whereas none of the controls was affected (χ^2 test of contingency, $\chi^2_{(1)} = 6.67$, $z = 2.58$, $^{**}p = 0.0098$). The spontaneous seizures were identified first on EEG and defined as high-amplitude rhythmic discharges that clearly represented a new pattern of tracing lasting for >5 s. The seizures identified by EEG always had a tonic–clonic behavioral correlate (Movie 3). The average spontaneous seizure rate assessed by vEEG was estimated to be 0.85 seizures per day with the average duration of each seizure being 43.6 ± 7.4 s.

Brain-wide disruption of ADK results in learning and memory impairment

Patients with ADK deficiency have severe cognitive impairments (Bjursell et al., 2011; Staufner et al., 2016); therefore, we next assessed learning and memory in our Adk$^{\Delta brain}$ mice. Adk$^{\Delta brain}$ and Adk$^{fl/fl}$ controls were subjected to a classic fear-conditioning paradigm in which a cue (CS) is paired with a mild electric foot shock. Adk$^{\Delta brain}$ mice had severe cognitive impairments reflected by deficits in both associative learning and contextual memory compared with Adk$^{fl/fl}$ mice (Fig. 4*A*,*B*). Associative learning was indexed by an increase in percentage time freezing during the CS, with CS1 being the first baseline tone response. Adk$^{\Delta brain}$ mice had a significant decrease in percentage time freezing during CS2 compared with Adk$^{fl/fl}$ mice (repeated-measures ANOVA, genotype effect $F_{(3,48)} = 6.62$; Adk$^{\Delta brain}$ vs Adk$^{fl/fl}$ $\#\#\#\#p < 0.0001$; Fig. 4*A*). Even though Adk$^{\Delta brain}$ mice had an associative learning deficit, they showed the general ability to learn, with a significant increase in freezing during CS3 and CS4, compared with CS1 (trial effect $F_{(3,144)} = 60.99$; CS3 vs CS1 $^{*}p = 0.0163$, CS4 vs CS1 $^{****}p < 0.0001$; Fig. 4*B*). Therefore, learning was considerably

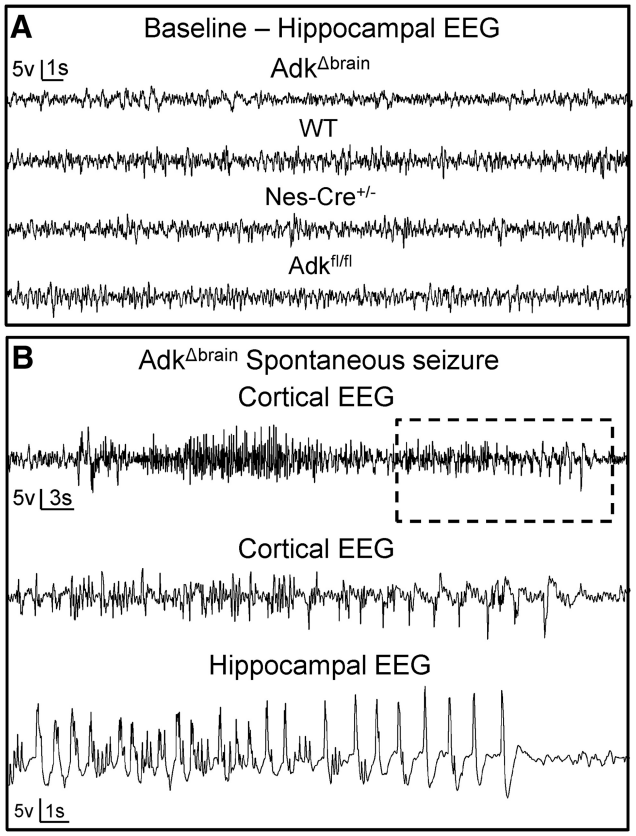

Figure 3. Loss of brain ADK results in spontaneous seizures. ***A***, Thirty seconds of representative hippocampal EEG traces from Adk$^{\Delta brain}$ mice and C57BL6 (WT), Nestin-Cre$^{+/-}$: Adk$^{+/+}$ (Nes-Cre$^{+/-}$), and Adk$^{fl/fl}$ control mice demonstrate comparable baseline seizure activity. ***B***, Representative cortical EEG recording of a complete spontaneous convulsive seizure (55 s) from an Adk$^{\Delta brain}$ mouse (top trace). Bottom traces are high-resolution cortical and hippocampal EEG recordings of a 20 s portion of seizure demarcated by the box in top trace. See Movie 3 for a matching behavioral seizure.

Movie 3. Spontaneous tonic convulsive seizure in an Adk$^{\Delta brain}$ mouse. The movie is of a representative spontaneous seizure characterized by tonic– clonic activity. The seizure is the behavioral correlate of the cortical and hippocampal EEG traces shown in Figure 3*B*.

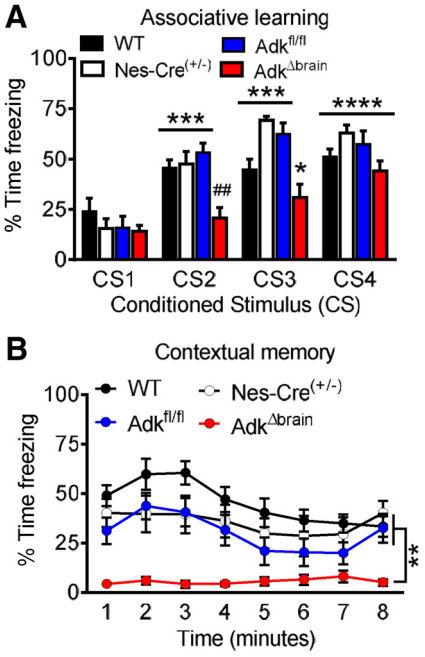

Figure 4. ADK deficiency in the brain results in cognitive impairment. ***A***, Associative learning in the conditioned freezing paradigm is significantly decreased during conditioned stimulus 2 (CS2) in Adk $^{\Delta brain}$ ($n = 13$) mice, compared with WT ($n = 15$), Nestin-Cre $^{+/-}$ ($n = 10$), and Adk $^{fl/fl}$ ($n = 14$) controls. Within Adk $^{\Delta brain}$ mice, the percentage time freezing during CS3 and CS4 is significantly increased compared with baseline CS1 freezing. ***B***, Contextual freezing is significantly impaired in Adk $^{\Delta brain}$ mice ($n = 13$), compared with WT ($n = 15$), Nestin-Cre $^{+/-}$ ($n = 10$), and Adk $^{fl/fl}$ ($n = 14$) controls. Data are represented as the mean ± SEM; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, $\#\#p < 0.01$.

delayed in Adk $^{\Delta brain}$ mice, compared with Adk $^{fl/fl}$ mice. Furthermore, Adk $^{\Delta brain}$ mice had a profound deficit in contextual memory as demonstrated by freezing rates comparable to baseline, which was significantly different from those observed in Adk $^{fl/fl}$ mice (repeated-measures ANOVA, genotype effect $F_{(3,48)} = 13.41$; Adk $^{\Delta brain}$ vs Adk $^{fl/fl}$ $**p = 0.0024$; Fig. 4*B*). To rule out genotype-related confounds, we conducted fear conditioning between three control lines (Adk $^{fl/fl}$, Nestin-Cre $^{+/-}$, and WT C57BL/6) and found that learning and memory deficits were not affected by either Cre or the floxed Adk allele (Fig. 4*A*, *B*). To rule out sensory-related confounds, we performed a basic behavioral screen and found no differences in the elevated plus maze, acoustic startle response, or spatial working memory tests in Adk $^{\Delta brain}$ versus Adk $^{fl/fl}$ mice (data not shown). Furthermore, the response to foot shock, indexed by average ($p = 0.51$) and maximum motion index ($p = 0.38$), was comparable between the Adk $^{\Delta brain}$ mice and all controls (data not shown). Together, these data demonstrate that brain ADK deficiency is associated with hippocampus-dependent cognitive impairment.

Adk $^{\Delta brain}$ mice have increased levels of synaptic adenosine

To provide a mechanistic basis for the Adk $^{\Delta brain}$ phenotype described above, we performed a detailed biochemical, pharmacological, and electrophysiological analysis of hippocampal brain slices derived from 4-month-old animals. Because ADK is the key metabolic route for adenosine clearance, we first tested for increased levels of brain adenosine. Due to the short half-life of adenosine and the need to gauge the magnitude of changes related to the synaptic pool of adenosine, we pursued a functional assay for synaptic adenosine by testing the influence of the A_1R antagonist DPCPX on fEPSPs. We used a hippocampal slice preparation with stimulating electrodes placed in the CA3/CA1 border and recording electrodes placed in CA1 to study excitatory glutamatergic inputs to CA1 pyramidal neurons. We used a supramaximal concentration of DPCPX (50 nM) corresponding to ~100× the K_i value for A_1Rs (Lohse et al., 1988; Sebastião et al., 1990). In hippocampal slices from epileptic Adk $^{\Delta brain}$ mice, DPCPX induced a significant increase in the fEPSP slope of 118 ± 20.1% compared with the predrug baseline (repeated-measures ANOVA, time effect $F_{(22,176)} = 23.23$; 10 min vs 0 min $\#p = 0.022$, 12–38 min vs 0 min $\#\#\#\#p < 0.0001$; Fig. 5*A*). This value contrasts with that obtained from age-matched Adk $^{fl/fl}$ mice, in which DPCPX increased the fEPSP slope by only 22 ± 7.8% (genotype effect $F_{(1,8)} = 16.25$; Adk $^{\Delta brain}$ vs Adk $^{fl/fl}$ $**p = 0.0042$, Fig. 5*A*; unpaired *t* test, $t_{(8)} = 4.43$, $**p = 0.0022$, Fig. 5*B*). The higher disinhibition of synaptic transmission caused by the A_1R antagonist in Adk $^{\Delta brain}$ mice suggests an enhanced A_1R-mediated inhibitory tonus. Using calculations described previously (Dunwiddie and Diao, 1994), we estimated the extracellular adenosine levels in the Adk $^{\Delta brain}$ brains to be in the 1 μM range, whereas control levels of extracellular adenosine in Adk $^{fl/fl}$ mice were maintained at <200 nM. To assess A_1R levels and function, we first quantified A_1R mRNA and protein from cortical lysates. qRT-PCR demonstrated significantly decreased A_1R mRNA in Adk $^{\Delta brain}$ mice compared with controls (Mann–Whitney test, U = 0, $**p = 0.0095$; Fig. 5*C*). A_1R protein was assessed by saturation binding experiments using [^{3}H]DPCPX, which allow quantification of the maximal number of binding sites (B_{max}) as well as the affinity (dissociation constant, K_d) of the receptor for the ligand. Both parameters were obtained by nonlinear regression analysis. The B_{max} value indicated a significant reduction of functional A_1Rs in Adk $^{\Delta brain}$ mice (unpaired *t* test, $t_{(6)} = 8.02$, $***p = 0.0002$; Fig. 5*D*). Based on the K_d values, there were no significant differences ($p > 0.05$) in the affinity of the receptor for its agonist between Adk $^{fl/fl}$ and Adk $^{\Delta brain}$ mice (K_d: 4.8 ± 0.9 vs 3.4 ± 1.1 nM). These data suggest decreased A_1R density, likely an adaptation to an increased synaptic adenosine tone in the Adk $^{\Delta brain}$ mice. Finally, to assess basal synaptic transmission in Adk $^{\Delta brain}$ mice, we performed input–output curve analysis by recording the fEPSP responses as a function of increased stimulation intensities delivered to hippocampal slices. The maximum fEPSP slope obtained from Adk $^{\Delta brain}$ slices was significantly higher than that from control slices (nonlinear regression fit; top values, $F_{(1,108)} = 5.79$; Adk $^{\Delta brain}$ vs Adk $^{fl/fl}$, $*p = 0.018$; Fig. 5*F*). Together, the data demonstrate increased facilitatory action of DPCPX (Fig. 5*A*) in the Adk $^{\Delta brain}$ mice, a finding that supports our *in vivo* data (Fig. 2).

Adk $^{\Delta brain}$ mice have increased A_{2A} receptor-mediated synaptic plasticity

Because synaptic levels of adenosine were found to be elevated in Adk $^{\Delta brain}$ samples (> 1 μM), we anticipated increased A_{2A}R activation, although adenosine has a slightly lower potency at A_{2A}Rs ($EC_{50} = 0.7$ μM) compared with A_1Rs ($EC_{50} = 0.3$ μM; Fredholm et al., 2001). To test whether baseline A_{2A}R signaling was affected in Adk $^{\Delta brain}$ mice, we assessed fEPSPs recorded from hippocampal slices in the presence of a selective A_{2A}R agonist or antagonist. Neither the A_{2A}R agonist CGS21680 nor the antagonist SCH58261 had any effects on fEPSPs recorded from hippocampal Adk $^{\Delta brain}$ or Adk $^{fl/fl}$ slices (repeated-measures ANOVA; Adk $^{\Delta brain}$ vs Adk $^{fl/fl}$ (*A*) $F_{(1,6)} = 2.58$, $p = 0.16$, Fig. 6*A*; $F_{(4,108)} = 0.029$, $p = 0.87$, Fig. 6*B*). Next, we tested whether the ADK deletion would affect the tonic influence of A_{2A}Rs on LTP. Consistent with previous reports using mild theta-burst LTP induction

(Costenla et al., 2011), $A_{2A}R$ blockade was virtually devoid of effect on LTP magnitude, the fEPSP slope 60 min after LTP induction being 122 ± 4.0% in control compared with 124 ± 4.5 in SCH58261 (repeated-measures ANOVA, treatment effect $F_{(1,8)} = 0.036$, control vs SCH58261, $p = 0.85$; Fig. 6*C*). When comparing LTP magnitude in the absence of any drug in Adk$^{fl/fl}$ versus Adk$^{\Delta brain}$ slices, a significant increase in LTP was detected (fEPSP: 122 ± 4.0% vs 152 ± 6.9%; two-way ANOVA, interaction $F_{(1,16)} = 14.36$, Adk$^{fl/fl}$ vs Adk$^{\Delta brain}$, $^{**}p = 0.0053$; Fig. 6*E*). Remarkably, the presence of SCH58261 in Adk$^{\Delta brain}$ slices reversed the LTP magnitude (fEPSP: 113 ± 5.6%) toward values close to the Adk$^{fl/fl}$ mice (repeated-measures ANOVA, treatment effect $F_{(1,8)} = 17.0$, control vs SCH58261, $^{**}p = 0.0033$, Fig. 6*E*; Adk$^{\Delta brain}$: control vs SCH58261, $^{***}p = 0.0005$; Fig. 6*F*), suggesting that Adk$^{\Delta brain}$ mice have enhanced tonic $A_{2A}R$ activation leading to increased synaptic plasticity.

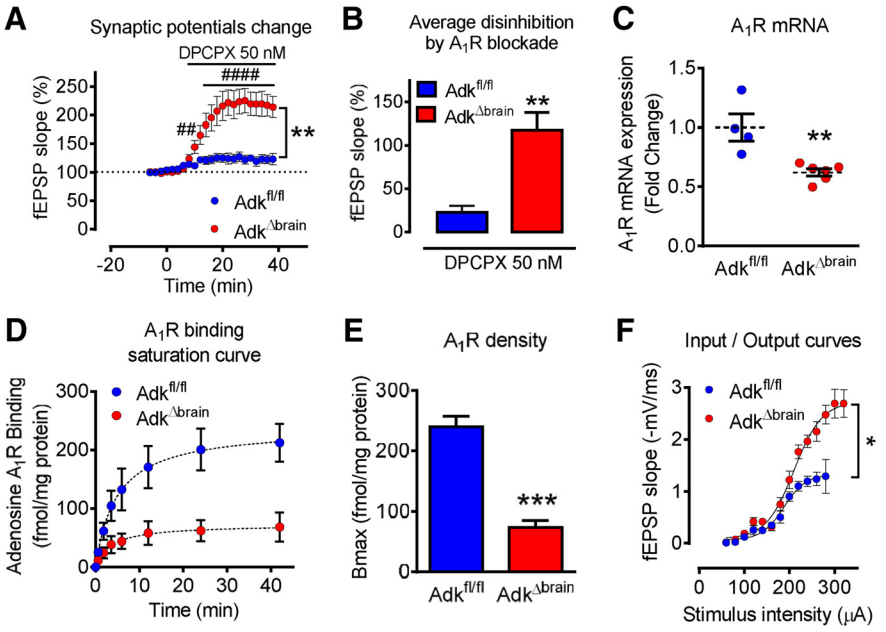

Figure 5. Adk$^{\Delta brain}$ mice have increased synaptic adenosine. ***A***, Disinhibition of synaptic transmission by the A_1R antagonist DPCPX (50 nM) is facilitated in Adk$^{\Delta brain}$ vs Adk$^{fl/fl}$ slices ($n = 5$ mice each). DPCPX increases the fEPSP slope in Adk$^{\Delta brain}$ slices versus baseline. ***B***, Percentage change in fEPSP slope during the last 10 min of DPCPX. ***C***, Decreased A_1R mRNA levels of Adk$^{\Delta brain}$ ($n = 6$) versus Adk$^{fl/fl}$ ($n = 4$ mice) cortex. ***D***, Binding curve for [^{3}H]DPCPX is reduced the Adk$^{\Delta brain}$ versus Adk$^{fl/fl}$ cortex ($n = 4$ mice each). ***E***, Average B_{max} values from the binding curves of Adk$^{\Delta brain}$ versus Adk$^{fl/fl}$ mice (B_{max}: 73 ± 6.5 vs 239 ± 13.4 fmol/mg protein). Data are presented as the mean ± SEM. 100% corresponds to the averaged fEPSP recorded 10 min before drug perfusion (***A***, ***B***, ***F***, ***G***). ***F***, Input–output curves were obtained from hippocampal slices to address changes in synaptic transmission level. The input–output curves correspond to responses generated by increasing stimulation intensities (60–340 mA) in Adk$^{fl/fl}$ ($n = 5$) and Adk$^{\Delta brain}$ ($n = 5$) mice. Results are shown as the mean ± SEM and statistical analysis was performed using an *F* test ($^*p < 0.05$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$.

BDNF signaling is increased in Adk$^{\Delta brain}$ mice

High-frequency stimulation triggers the release of BDNF and ATP, the major metabolic precursor of adenosine, therefore favoring the activation of $A_{2A}Rs$, which in turn gate TrkB receptor-mediated facilitatory actions of BDNF upon LTP (Fontinha et al., 2008; Dias et al., 2013). Taking into account the increased LTP magnitude in Adk$^{\Delta brain}$ animals and the prevention of this increase on $A_{2A}R$ blockade, we next assessed whether the LTP increase in Adk$^{\Delta brain}$ mice could result from enhanced BDNF signaling. To test this hypothesis, the tyrosine kinase inhibitor K252a (200 nM) was added to the perfusion solution and LTP was induced as before. K252a restored normal theta-burst-induced LTP in Adk$^{\Delta brain}$ slices (absence: 150 ± 6.7% vs presence: 101 ± 7.0%, $n = 5$ mice). Therefore, in Adk$^{\Delta brain}$ mice, the magnitude of LTP in slices in the presence of K252a was abolished (repeated-measures ANOVA, treatment effect, $F_{(1,4)} = 19.82$, control vs K252a, $^*p = 0.012$; Fig. 6*F*). K252a had virtually no effect on LTP magnitude in Adk$^{fl/fl}$ mice (data not shown), in agreement with data reported for wild-type rats (Fontinha et al., 2008). These results suggest a higher level of TrkB receptor activation accounting for a larger LTP in the Adk$^{\Delta brain}$ mice. Therefore, our results indicate that the enhanced LTP in Adk$^{\Delta brain}$ mice can be attributed to a higher influence of endogenous BDNF on LTP. Finally, we determined that Adk$^{\Delta brain}$ mice do not compensate for increased BDNF signaling with changes in TrkB receptor expression ($n =$ 8/genotype, unpaired *t* test, $t_{(14)} = 0.96$, $p = 0.36$; Fig. 6*H*). However, we observed a modest, but insignificant, decrease in the ratio of full-length TrkB receptor to truncated TrkB receptor (unpaired *t* test, $t_{(14)} = 1.68$, $p = 0.11$; Fig. 6*H*).

$A_{2A}R$ blockade ameliorates the inducible seizure phenotype and cognitive impairment in Adk$^{\Delta brain}$ mice

Increased $A_{2A}R$ activation, as identified here, may promote seizures in Adk$^{\Delta brain}$ mice through a BDNF-mediated mechanism. In this case, $A_{2A}R$ antagonists or TrkB antagonists might be of therapeutic value. Furthermore, the balance between inhibitory A_1Rs and stimulatory $A_{2A}Rs$ is one of the factors that determine the susceptibility to seizures (Sebastião and Ribeiro, 2009; Gomes et al., 2011; Chen et al., 2013). We therefore tested whether $A_{2A}Rs$ play a role in determining seizure thresholds in Adk$^{\Delta brain}$ mice by first administering a single injection of the $A_{2A}R$ agonist 5′-(N-cyclopropyl)carboxamidoadenosine (CPCA, 0.5 mg/kg). CPCA was ineffective at inducing a seizure (data not shown), which may be indicative of a ceiling effect for $A_{2A}R$ activation in Adk$^{\Delta brain}$ mice. To circumvent this, we next sought to focus on the effect of blocking $A_{2A}R$ activity. Pretreatment with antagonists to both $A_{2A}Rs$ (SCH58261) and TrkB (Ana-12) significantly extended the survival time after DPCPX-induced seizures in Adk$^{\Delta brain}$ mice, compared with vehicle-treated controls (Mantel–Cox test, $\chi^2_{(2)} = 12.51$, $^{**}p < 0.0019$; Fig. 7*A*). Adk$^{\Delta brain}$ mice that received vehicle injections in lieu of DPCPX and Adk$^{fl/fl}$ mice that received vehicle, SCH58261, or Ana-12 ± DPCPX did not have seizures (data not shown). Next, we generated Nestin-Cre:Adk$^{fl/fl}$:$A_1R^{-/-}$ (=Adk$^{\Delta brain}A_1R^{-/-}$) and Nestin-Cre:Adk$^{fl/fl}$:$A_{2A}R^{-/-}$ (=Adk$^{\Delta brain}A_{2A}R^{-/-}$) triple mutants. Importantly, Adk$^{\Delta brain}A_1R^{-/-}$ mice maintained their stress-induced seizure phenotype while exhibiting increased mortality, whereas Adk$^{\Delta brain}A_{2A}R^{-/-}$ mice showed a marked resistance to stress-induced seizures and normal life expectancy (χ^2 test of contingency, $\chi^2_{(2)} = 18.26$, $^{***}p = 0.0001$, Fig. 7*C*; Mantel–Cox test, $\chi^2_{(2)} = 26.51$, $^{****}p < 0.0001$, Fig. 7*B*). Together, these data suggest that Adk$^{\Delta brain}$ mice have an enhanced A_1R-mediated protective tonus and that additional factors such as stress, A_1R blockade, and increased $A_{2A}R$ activation are needed to overcome this hurdle and allow the emergence of induced seizures.

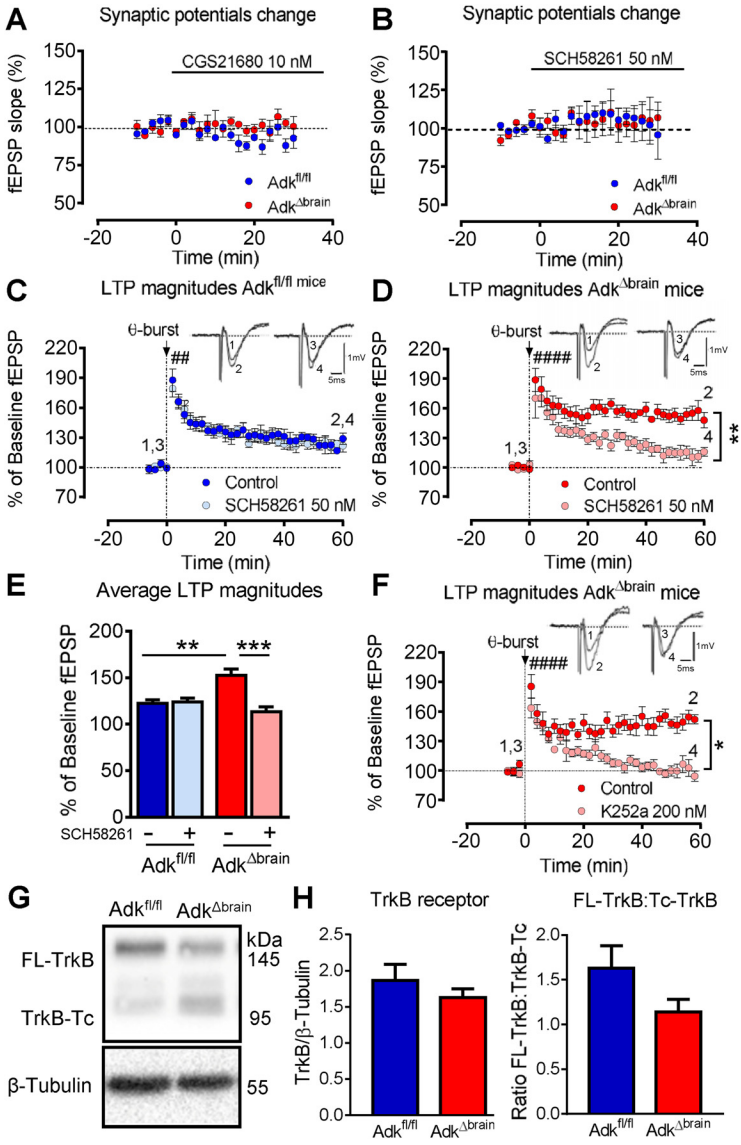

Figure 6. Adk $^{\Delta brain}$ mice show enhanced A_{2A} and TrkB receptor- dependent LTP. ***A***, ***B***, The A_{2A}R-selective agonist CGS21680 (10 nM) and antagonist SCH58261 (50 nM) does not affect the fEPSP slope in Adk $^{fl/fl}$ and Adk $^{\Delta brain}$ slices ($n = 4$ mice/genotype/drug). ***C***–***E***, SCH58261 restores normal theta-burst (3 × 3)-induced LTP in Adk $^{\Delta brain}$ mice. ***C***, ***D***, Average change in fEPSP slopes in the absence versus presence of SCH58261 in Adk $^{fl/fl}$ (***C***) and Adk $^{\Delta brain}$ (***D***) slices ($n = 5$ mice/genotype/drug). ***E***, Percentage of change in the fEPSP slopes recorded at 52–60 min after LTP induction. ***F***, The TrkB inhibitor K252a (200 nM) restores normal theta-burst (3 × 3)-induced LTP in Adk $^{\Delta brain}$ slices (absence: 150 ± 6.7% vs presence: 101 ± 7.0%, $n = 5$ mice each,). Insets in ***C***, ***D***, and ***F*** are representative traces of six consecutive responses composed of the stimulus artifact, presynaptic volley, and fEPSP obtained before (***1***, ***3***) and after (***2***, ***4***) the theta-burst stimuli in the absence (***1***, ***2***) and presence (***3***, ***4***) of drug. LTP was significant (####) after theta-burst stimuli versus baseline (***C***, ***D***, ***F***). ***G***, Representative blot of full-length TrkB receptor (FL-TrkB, 145 kDa), truncated TrkB receptor (TrkB-Tc, 95 kDa), and β-tubulin (55 kDa, loading control) is shown. ***H***, Averaged immunodensities of TrkB receptor normalized to β-tubulin and the ratio of FL-TrkB to TrkB-Tc ($n = 8$ mice/genotype). Results are presented as mean ± SEM. 100% corresponds to the averaged fEPSP slope recorded 10 min before LTP induction. *$p < 0.05$, **$p < 0.01$, ####$p < 0.0001$.

To establish whether the A_{2A}R is a target for the treatment of cognitive impairment in ADK deficiency, we tested Adk $^{\Delta brain}$:$A_{2A}R^{-/-}$ mice in the fear-conditioning paradigm. Remarkably, the deletion of A_{2A}Rs from Adk $^{\Delta brain}$ mice restored hippocampus-dependent associative learning (ANOVA, $F_{(2,35)} = 15.94$; Adk $^{\Delta brain}$ vs Adk $^{\Delta brain}$:$A_{2A}R^{-/-}$, ***$p = 0.0008$, Adk $^{fl/fl}$ vs Adk $^{\Delta brain}$:$A_{2A}R^{-/-}$, $p = 0.43$; Fig. 7*D*) and contextual memory (Kruskal–Wallis statistic = 27.54; Adk $^{\Delta brain}$ vs Adk $^{\Delta brain}$:$A_{2A}R^{-/-}$, ****$p < 0.0001$, Adk $^{fl/fl}$ vs Adk $^{\Delta brain}$:$A_{2A}R^{-/-}$, $p = 0.11$; Fig. 7*E*) to Adk $^{fl/fl}$ control levels. Together, our findings suggest that the A_{2A}R might be a therapeutic target for the treatment of the neurological phenotypes associated with ADK deficiency.

Discussion

We generated a novel mouse line, Adk $^{\Delta brain}$, to study specifically the neurological symptoms associated with global ADK deficiency such as those observed in human patients with mutations in the *Adk* gene (Bjursell et al., 2011; Staufner et al., 2016). We describe a novel mechanism by which ADK deficiency in the brain leads to neuronal adaptation processes that trigger enhanced A_{2A}R- and BDNF-dependent synaptic plasticity. Together, those adaptive processes cause a phenotype characterized by increased propensity to induced and spontaneous seizures and impairment in hippocampus-specific cognitive domains. Therefore, our findings suggest that global ADK deficiency has direct implications on neurological outcome parameters, which might be amenable to treatment. Importantly, we demonstrate that the genetic and pharmacological blockade of A_{2A}Rs ameliorates the seizure phenotype and cognitive impairment in Adk $^{\Delta brain}$ mice. Several aspects of our study warrant further discussion.

Loss of ADK function

Human patients with global ADK deficiency due to homozygosity of point mutations located in the coding sequence of the ADK gene are rare, with only 17 patients from 11 independent families identified to date (Bjursell et al., 2011; Staufner et al., 2016). Human ADK deficiency shares certain characteristics with a global deletion of ADK in the mouse (Boison et al., 2002b). Both conditions are characterized by disruption of the transmethylation pathway resulting in major physiological aberrations of liver metabolism. Liver is the organ in which 85% of all transmethylation reactions in the body take place and it is also the organ with the highest expression levels of ADK. In the mouse, homozygous deletion of the *Adk* gene leads to microvesicular hepatic steatosis, whereas the human condition is characterized by hepatic encephalopathy, developmental delay, cognitive impairment, and seizures (Boison et al., 2002b; Bjursell et al., 2011; Staufner et al., 2016). Because global ADK knock-out mice do not survive into adulthood, it was not possible to investigate direct consequences of ADK deficiency on brain physiology. Because *Adk* expression in the liver of Adk $^{\Delta brain}$ mice is normal and heterozygous *Adk* knock-out (*Adk* $^{+/-}$) have a normal liver despite a 50% reduction in liver ADK expression (Boison et al., 2002b), the increased brain adenosine levels in Adk $^{\Delta brain}$ mice are not likely to translate into increased peripheral levels of adenosine that affect liver physiology. Our present study with Adk $^{\Delta brain}$ mice, in

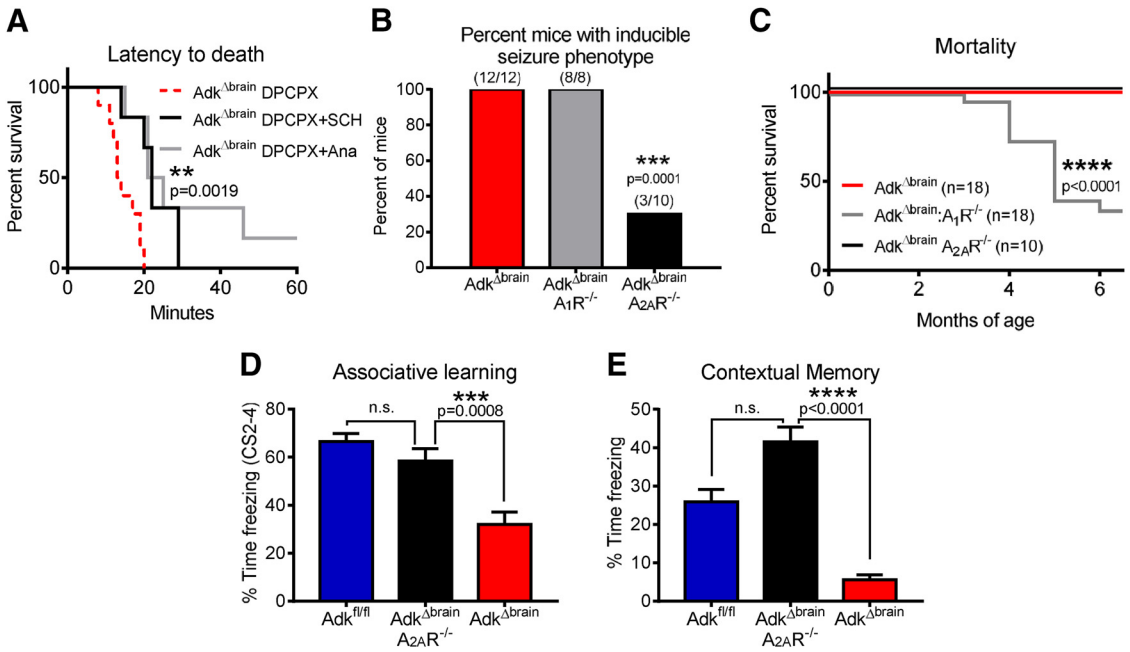

Figure 7. Blockade of $A_{2A}R$ activity ameliorates the inducible seizure phenotype and cognitive impairment in Adk $^{\Delta brain}$ mice. ***A***, Increased survival time of Adk $^{\Delta brain}$ mice treated with SCH 58261 (0.5 mg/kg, i.p., 30 min in advance, $n = 6$) or Ana-12 (0.5 mg/kg, i.p., 4 h in advance, $n = 6$) before the DPCPX (3 mg/kg, i.p.) challenge compared with DPCPX controls ($n = 10$). ***B***, Contingency analysis indicates a significant decrease in the percentage of Adk $^{\Delta brain}$:$A_{2A}R^{-/-}$ mice (3 of 10) that develop the inducible seizure phenotype compared with Adk $^{\Delta brain}$ (12 of 12) and Adk $^{\Delta brain}$:$A_1R^{-/-}$ (8 of 8) mice. ***C***, Kaplan–Meier survival curve indicating increased Adk $^{\Delta brain}$:$A_1R^{-/-}$ ($n = 18$) mortality compared with Adk $^{\Delta brain}$ ($n = 18$) and Adk $^{\Delta brain}$.$A_{2A}R^{-/-}$ ($n = 10$). ***D***, Associative learning indexed as total percentage time freezing during the CS 2–4 is restored to Adk $^{fl/fl}$ ($n = 13$) control levels in Adk $^{\Delta brain}$:$A_{2A}R^{-/-}$ ($n = 12$) mice and is significantly increased compared with Adk $^{\Delta brain}$ ($n = 13$) mice. ***E***, Contextual memory indexed as total percentage time freezing during the 8 min contextual freezing trial is significantly increased in Adk $^{\Delta brain}$:$A_{2A}R^{-/-}$ ($n = 12$) and Adk $^{fl/fl}$ ($n = 13$) mice, compared with Adk $^{\Delta brain}$ ($n = 13$) mice. Data are represented as the mean ± SEM; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

which development is not compromised by liver pathology, reveals a novel mechanism by which brain-wide ADK deficiency leads to neuronal adaptation processes resulting in increased $A_{2A}R$-dependent synaptic plasticity. We propose that similar mechanisms might play a role for seizure generation and the complex behavioral phenotype of human patients with inborn ADK deficiency. Given the enormous size of the ADK gene (546 kb in humans), it is surprising that only few patients with mutations this gene have been described so far. High evolutionary conservation of the *Adk* cDNA (>80% identical between man and rodents) suggests that mutations are rarely tolerated. Given the robust neurological phenotype described in homozygous human ADK deficiency (Bjursell et al., 2011; Staufner et al., 2016) and in mice with a homozygous deletion of the *Adk* gene in the brain, as described here, it remains to be determined whether heterozygosity of *Adk* gene mutations might affect the development of epilepsy or psychiatric disorders in the human population.

Gain vs loss of ADK function

Although no human gain-of-function mutations for the *Adk* gene have been described, ADK expression is subject to dynamic regulation during the course of epileptogenesis (Boison, 2008). Astrocytic ADK expression is significantly increased in the epileptogenic hippocampus of patients with epilepsy, as well as in rodent models of epilepsy (Boison, 2013), and is thought to be intrinsically linked to the development of epilepsy (Williams-Karnesky et al., 2013). Both transgenic overexpression of ADK (Li et al., 2008) and adeno-associated virus-mediated overexpression of ADK in astrocytes (Shen et al., 2014) are sufficient to induce spontaneous recurrent seizures in mice. This ADK gain-of-function-related seizure phenotype has been linked to adenosine deficiency and insufficient activation of adenosine A_1Rs (Gouder et al., 2004; Fedele et al., 2006). Seizure susceptibility is controlled, not only by the availability of adenosine (i.e., the synaptic adenosine pool), but also by the balance of inhibitory A_1R activation versus stimulatory $A_{2A}R$ activation, different distribution patterns of the receptors in the brain, and different potencies of adenosine on the two receptor types (Sebastião and Ribeiro, 2009; Gomes et al., 2011; Chen et al., 2013; Boison, 2016a). Effects mediated by the two receptors are discussed in more detail below.

A_1R-dependent effects

Evidence for an inverse relation between ADK expression and adenosine levels has been provided previously (Shen et al., 2011). The synaptic potential recordings reported here (Fig. 5) also revealed a higher synaptic adenosine tonus in epileptic Adk $^{\Delta brain}$ mice compared with aged-matched controls because the A_1R antagonist DPCPX caused a more pronounced disinhibition of synaptic transmission in Adk $^{\Delta brain}$ mice despite the lower expression levels of the A_1R and the lower inhibitory effect of A_1R agonists on synaptic transmission. Consistent with those findings, we show that a low dose of DPCPX can induce seizures in Adk $^{\Delta brain}$ mice that are remarkably similar to the stress-induced seizure phenotype (cf. Movies 1, 2).

$A_{2A}R$-dependent effects

Brain-specific deletion of ADK increased $A_{2A}R$ function. Interestingly, Adk $^{\Delta brain}$ mice expressed a significantly larger LTP magnitude, which was prevented in the presence of an $A_{2A}R$ antagonist, suggesting a higher tonic influence of this receptor in synaptic plasticity modulation. $A_{2A}R$ antagonists do not affect basal synaptic transmission in hippocampal slices (Sebastião and Ribeiro, 1992; Cunha et al., 1997), which suggests only a minor

role of the $A_{2A}R$ as a regulator of hippocampal synaptic transmission under low-frequency neuronal firing. However, the release of purines is more pronounced during physiologically relevant patterns of neuronal activity, namely those that induce hippocampal LTP (Wieraszko and Seyfried, 1989). Adenosine formed from released ATP activates $A_{2A}Rs$ preferentially (Cunha et al., 1996). All of these factors favor an influence of $A_{2A}R$ activation on synaptic plasticity. Our data show that these effects, which also result from increased $A_{2A}R$/BDNF signaling (see below), are markedly exacerbated in $Adk^{\Delta brain}$ mice.

Our findings of cognitive impairment in $Adk^{\Delta brain}$ mice are consistent with hippocampus-dependent memory deficits in transgenic rats with overexpression of the $A_{2A}R$ in the hippocampus and cortical brain structures (Giménez-Llort et al., 2007). Remarkably, enhanced LTP, together with enhanced $A_{2A}R$ and BDNF signaling, is associated with impaired learning and memory (Diógenes et al., 2011). Here, we show that the genetic ablation of $A_{2A}Rs$ restores cognitive function in $Adk^{\Delta brain}$ mice. These findings demonstrate directly that increased $A_{2A}R$ activity in $Adk^{\Delta brain}$ mice may precipitate the impaired learning and memory phenotype. Increased $A_{2A}R$ function is also consistent with previous findings in the kindling model of epilepsy, in which a long-term increased density of $A_{2A}Rs$ was observed (Rebola et al., 2003). Consistent with the anti-epileptic effects of an $A_{2A}R$ antagonist (Etherington and Frenguelli, 2004), $A_{2A}R$ knock-out mice are partially protected from convulsive activity in some experimental models of epilepsy (El Yacoubi et al., 2009). Therefore, enhanced $A_{2A}R$ signaling (Fig. 6) in addition to inhibitory A_1R activation (Fig. 5) resulting in an imbalance of $A_1R/A_{2A}R$ signaling is a plausible mechanism to permit a "breakthrough" of seizures in an otherwise protected brain environment in $Adk^{\Delta brain}$ mice.

BDNF-dependent effects

Our findings of enhanced BDNF-dependent plasticity in $Adk^{\Delta brain}$ mice are related to those from BDNF-overexpressing mice (BDNF-tg mice), which likewise develop stress-induced seizures triggered by placing the animals into a new cage. This seizure phenotype only develops later in life, at 5–6 months after birth (Papaleo et al., 2011). In our mechanistic study, we demonstrate that the enhanced $A_{2A}R$ activity in $Adk^{\Delta brain}$ mice leads to increased tonic activation of TrkB receptors by BDNF, thereby inducing enhanced synaptic plasticity. Adenosine, through $A_{2A}R$ activation, is an upstream regulator of BDNF-mediated synaptic plasticity (Fontinha et al., 2008; Sebastião and Ribeiro, 2015). Through activation of TrkB, BDNF has facilitatory actions on neuronal activity and synaptic plasticity by operating a cascade of events that lead to imbalanced excitatory transmission (Leal et al., 2015).

Therapeutic implications

Our findings strongly suggest that blocking the $A_{2A}R$ might be of therapeutic value for the treatment of neurological symptoms in ADK-deficient patients. We show here that the pharmacological and genetic ablation of $A_{2A}R$ function ameliorates the seizure phenotype of $Adk^{\Delta brain}$ mice. Importantly, the genetic deletion of the $A_{2A}R$ restores hippocampus-dependent learning and memory functions in $Adk^{\Delta brain}$ mice. This is a significant finding of translational significance because $A_{2A}R$ inhibitors are in clinical development for the treatment of motor symptoms in Parkinson's disease (Schwarzschild, 2007). We show that those agents might also have procognitive effects under conditions of enhanced adenosine levels in the brain.

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