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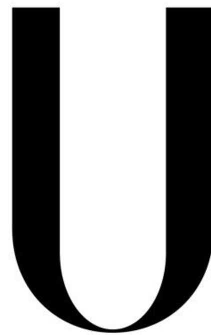

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Regulation of adenosine levels as a new therapeutic strategy for Rett Syndrome

Catarina Miranda Lourenço

Orientadora: Maria José de Oliveira Diógenes Nogueira

Tese especialmente elaborada para a obtenção de grau de Doutor em

Ciências Biomédicas, especialidade em Neurociências

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O trabalho experimental constante da presente tese foi realizado no Instituto de Farmacologia e Neurociências, Faculdade de Medicina de Lisboa e Unidade de Neurociências, Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina de Lisboa, sob a orientação da Professora Doutora Maria José Diógenes.

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Sísifo

Recomeça….

Se puderes

Sem angústia

E sem pressa.

E os passos que deres,

Nesse caminho duro

Do futuro

Dá-os em liberdade.

Enquanto não alcances

Não descanses.

De nenhum fruto queiras só metade.

E, nunca saciado,

Vai colhendo ilusões sucessivas no pomar.

Sempre a sonhar e vendo

O logro da aventura.

És homem, não te esqueças!

Só é tua a loucura

Onde, com lucidez, te reconheças…

Miguel Torga

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Com estas palavras dou início à “cerimónia de encerramento” deste percurso que é construir um doutoramento. São as últimas palavras que deixo impressas nesta tese tanto pelo seu simbolismo, mas também pelo seu peso. Aqui não há dados obtidos através de experiências repetidas múltiplas vezes, nem teorias que tantos outros foram validando ao longo dos anos. Sem heroísmos, sabemos que estes dados são um pequeno contributo para o avançar da ciência e do conhecimento, e se tudo correr bem, hão-de crescer com o contributo de outros para que seu o impacto seja cada vez mais real e positivo para a sociedade em que vivemos. As teorias, vão também elas mudando para algo que nos faça compreender cada vez melhor o mundo e o que fazer nele. É uma mudança positiva, só poderá ser.

O peso destas palavras é porque nelas não está só a racionalidade de fazer ciência. Está a emoção de fazer ciência. E estão todas as pessoas que são precisas para construir seja o que for. Neste caso, um projecto de doutoramento e uma parte importante da minha vida. Não são as palavras que imaginei escrever, mas isso também já não é o mais impactante. O receio está nestas palavras perderem o sentido com o tempo, porque ao contrário da positividade com que encaro as mutações na ciência, nem sempre há uma boa razão para haver mudanças nas pessoas que nos rodeiam. Acabam por ser, estas palavras, um acto de fé, e uma marca do presente, esperando que daqui a 5 ou 100 anos, continue rodeada de gente como esta. Gente que me faça crescer, atingir objectivos e concretizar sonhos, tanto a nível pessoal como profissional.

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Abbreviations list

2D - 2 dimensions

3D - 3 dimensions

A - Adenine

AR - Adenosine receptors

A_1R - Adenosine A_1 receptors

A_{2A}R - Adenosine A_{2A} receptors

A_{2B}R - Adenosine A_{2B} receptors

A_3R - Adenosine A_3 receptors

AAT - Adenosine augmentation therapy

aCSF - Artificial cerebrospinal fluid

ADA - Adenosine deaminase

ADK - Adenosine kinase

ADP - Adenosine diphosphate

AKT - Protein kinase B

AMP - Adenosine monophosphate

AR - Adenosine receptors

ASD - Anti-seizure drugs

ATP - Adenosine triphosphate

BBB - Blood-brain barrier

BDNF - Brain-derived neurotrophic factor

BS - Brainstem

BSA - Bovine serum albumin

C - Cytosine

CA1-3 - *Cornu Ammonis,* areas 1-3

cAMP - Cycle adenosine monophosphate

CB - Cerebellum

CD - Cluster of differentiation

CDKL5 - Cyclin dependent kinase like 5

CGS21860 - 2-p-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine

cN - cytosolic 5´-nucleotidase

CNS - Central nervous system

CNT - Sodium-dependent concentrative nucleoside transporters

CpG - -C-phosphate-G

CREB - cAMP response element-binding protein

Crh - Corticotropinreleasing hormone

CTD - C-terminal domain

CTEP - 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine

CTX - Cortex

D_2R - Dopamine receptor type 2

DAG - Diacylglycerol

DARPP-32 - dopamine- and cyclic AMP-regulated phosphoprotein 32

DLX5 - Distal-less homeobox 5

DNA - Deoxyribonucleic acid

D-NAC - Dendrimer NAC

DPCPX - 1,3-Dipropyl-8-cyclopentylxanthine

DTT - Dithiothreitol

DMSO - Dimethyl sulfoxide

EEG - Electroencephalogram

EDTA - Ethylenediaminetetraacetic acid

ENT - Sodium-independent equilibrative nucleoside transporters

ERK - Extracellular signal-regulated kinases

FDA - Food and drug administration

fEPSP - Field excitatory postsynaptic potential

FOXG1 - Forkhead box protein G1

FXYD1 - FXYD domain-containing transport regulator 1

G - Guanine

GA - Glatiramer acetate

GABA - γ-Aminobutyric Acid

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GMP - Guanosine monophosphate

GPCR - G-protein-coupled receptor

GPI - Glycosyl phosphatidyl inositol

HDACs - Histone deacetilases

HET - Heterozygous

HIP - Hippocampus

hIPSCs - Human induced pluripotent stem cells

HPLC - High performance liquid cromotography

ID - Inter domain

IDP - intrinsically disordered protein

IGF-1 - Insulin-like growth factor 1

IMP - Inosine monophosphate

i.p. - Intraperitoneal

IP3 - Inositol 1,4,5-triphosphate

IPSCs - Induced pluripotent stem cells

IRAK1 - Interleukin-1 receptor-associated kinase

ITU - 5-iodotubercidin

KD - Dissociation constant

K_m - Michaelis menton constant

KO - Knockout

LINE-1 - Long interspersed nuclear elements

LTP - Long-term potentiation

mBDNF - Mature protein

MAPK - Mitogen-activated protein kinases

MBD - Methyl binding domain

MeCP2 - Methyl CpG binding protein 2

MeCP2E1 - MeCP2_exon1

MeCP2E2 - MeCP2_exon2

mGluR - Metabotropic glutamate receptor

miRNA - Micro-RNA

MMP - Matrix-metalloproteinases

mRNA - Messenger RNA

mTOR - Mammalian target of rapamycin

NAC - N-acetyl cysteine

NCorR - Nuclear coreceptor co-repressor

NDP - Nucleoside diphosphate

NF-kB - Factor nuclear kappa B

NGF - Nerve growth factor

NLS - Nuclear localization signals

NMDA - N-methyl-D-aspartic acid

NP-40 - Nonidet P-40

NT-3(4/5) - Neurotrophin-3(4/5)

NTD - N-terminal domain

NTRK2 - Eurotrophic receptor tyrosine kinase 2

$p75^{NTR}$ - p75 neurotrophin receptor

PI3K - Phosphoinositide 3-kinase

PIP2 - Phosphatidylinositol-4,5-bisphosphate

PK - Protein kinase

PLC(γ) - Phospholipase C (gamma)

PLD - Phospholipase D

PNS - Peripheral nervous system

PolyA - Polyadenelation sites

Pre-pro-BDNF - Precursor protein form

Pro-BDNF - Immature precursor protein form

PT1B - Protein tyrosine phosphatase 1B

PVDF - Polyvinylidene fluoride

QT - QT interval from electrocardiogram

RCP/GCP - Red Opsin gene

RIPA - Radio-Immunoprecipitation Assay

RNA - Ribonucleic acid

RTT - Rett Syndrome

SAH - S-adenosylhomocysteine

SDS-(PAGE) - Sodium dodecyl sulphate-(polyacrylamide-gel electrophoresis)

SIN3A - Paired amphipathic helix protein Sin3a

ST - Striatum

T – Thymine

TBS-T - Tris-Buffered Saline and Tween 20

TRD - Transcription repression domain

Trk - Tropomyosin-related kinase

Trk(B)-FL - Tropomyosin-related kinase (B) full-length

TrkB-T1 - Tropomyosin-related kinase B T1 isoform

TrkB-T2 - Tropomyosin-related kinase B T2 isoform

Trk(B)-Tc - Tropomyosin-related kinase (B) truncated isoforms

TrkB-T-Shc - Tropomyosin-related kinase B isoform containing Src homologous

UTR - Untranslated region

WB - Western-Blot

Wg - Weeks of gestation

WT - Wild type

XCI - X-chromosome inactivation

Resumo

A Síndrome de Rett (RTT) é uma doença do neurodesenvolvimento rara e de causa genética que afeta cerca de 1:10.000-15.000 raparigas em todo o mundo. Caracteriza-se por um desenvolvimento aparentemente normal até aos 6 a 18 meses de idade, seguido de uma fase de regressão, na qual ocorre a perda de capacidades já adquiridas. Durante a progressão desta doença, que decorre ao longo de quatro etapas distintas, destacam-se: aparecimento de movimentos estereotipados e repetitivos das mãos com progressiva perda da sua funcionalidade, disfunção cognitiva e motora, e epilepsia. Atualmente, esta doença não tem cura existindo apenas algumas opções terapêuticas para controlo sintomático o que torna esta doença devastadora tanto para os seus portadores como para os respetivos cuidadores.

Estudos genéticos realizados nos últimos anos estabeleceram que esta síndrome se deve, maioritariamente, a mutações no gene *methyl-CpG-binding protein 2* (*MECP2)*, localizado no cromossoma X. Este gene codifica a proteína MeCP2, que desempenha múltiplas funções onde se destacam a sua atuação como modulador epigenético e regulador da estrutura da cromatina, controlando a expressão de vários outros genes. É, por isso, uma proteína primordial no desenvolvimento e maturação do sistema nervoso central (*central nervous system* – CNS). Uma das proteínas cuja expressão é controlada pela MeCP2 é o fator neurotrófico derivado do cérebro (*brain-derived neurotrophic factor* - BDNF), uma neurotrofina com funções essenciais na maturação e diferenciação celular, plasticidade sináptica e sobrevivência neuronal. Consequentemente, alterações na MeCP2 comprometem os níveis de expressão e função do BDNF, tendo esta evidência sido já demonstrada em múltiplos estudos em modelos animais de RTT

Simultaneamente, estudos nestes modelos demonstraram também que o aumento da expressão do *BDNF* consegue reverter algumas das disfunções e sintomas associados a esta síndrome. Contudo, a utilização terapêutica de BDNF não é ainda aplicável uma vez que a barreira hematoencefálica (*blood-brain barrier* - BBB) é impermeável a este fator neurotrófico, impedindo-o de chegar ao cérebro e desempenhar adequadamente as suas funções. Na tentativa de facilitar os efeitos do BDNF, têm-se desenvolvido novas estratégias envolvendo, por exemplo, a utilização de moléculas que atravessando a BBB potenciam a ação

neuroprotetora do BDNF. Uma das moléculas que tem merecido particular atenção é a adenosina. A adenosina é um neuromodulador do CNS que exerce as suas funções através da ativação de quatro recetores, A_1, A_{2A}, A_3 e A_{2B} (AR). Em particular, a ativação dos recetores A_{2A} é fulcral para a manutenção dos níveis de BDNF e do seu recetor, TrkB-FL (*tropomyosin-related kinase B full-length*), assim como para os seus efeitos sinápticos. É de realçar que, o sistema adenosinérgico, para além de ser crucial na sinalização mediada pelo BDNF, também tem um papel de destaque no controlo da excitabilidade sináptica através da ativação dos recetores inibitórios A_1R, reconhecidos como potenciais alvos terapêuticos no controlo da epilepsia. Estas ações da adenosina, assim como a presença de alguns sintomas nos doentes com RTT sobreponíveis a doenças com disfunção adenosinérgica já descrita, sugerem a possibilidade deste neuromodulador também estar afetado na RTT.

Assim, este projeto teve como objetivos: 1) caracterizar detalhadamente o sistema adenosinérgico e a sinalização mediada pelo BDNF, através da utilização de modelos com fenótipos distintos: 1.1) modelo animal com um fenótipo agressivo, ratinhos machos mutantes *Mecp2-null* ($Mecp2^{-/y}$); 1.2) modelo animal com um fenótipo intermédio, ratinhos fêmeas heterozigóticas para *Mecp2* ($Mecp2^{+/-}$); e 2) explorar o aumento dos níveis de adenosina como uma possível estratégia terapêutica.

Através de ensaios de *Western-Blot* (WB) foi possível detetar níveis proteicos de BDNF diminuídos em homogenatos de hipocampo, córtex, tronco cerebral e cerebelo obtidos de animais $Mecp2^{-/y}$ na fase sintomática. As alterações detetadas na fase pré-sintomática foram menos marcadas, tendo-se apenas se observado a diminuição dos níveis de BDNF no estriado de animais $Mecp2^{-/y}$ com 3 semanas de idade. Também nos animais $Mecp2^{+/-}$ foram detetadas diminuições nos níveis de BDNF tanto no córtex como no hipocampo na fase sintomática. Relativamente aos níveis proteicos dos recetores do BDNF, observaram-se diminuições de TrkB-FL na fase sintomática em homogenatos de córtex e de hipocampo, e na fase pré-sintomática no córtex e no estriado. Também no estriado, observou-se um aumento dos níveis de TrkB-FL na fase sintomática. Analisando os níveis proteicos das isoformas truncadas do recetor TrkB (TrkB-Tc), moduladores negativos da ação do BDNF, detetaram-se aumentos em homogenatos de tronco cerebral de animais $Mecp2^{-/y}$ com 1 semana e 6 semanas de idade.

Nos animais Mecp2$^{+/-}$ não foram detetadas alterações em nenhum dos recetores do BDNF estudados.

Registos eletrofisiológicos realizados na área CA1 do hipocampo permitiram o estudo da plasticidade sináptica, nomeadamente através do estudo da potenciação de longa duração (*long-term potentiation* - LTP). Nos animais Mecp2$^{-/y}$ observou-se uma diminuição da magnitude da LTP e uma ausência do efeito facilitador por parte do BDNF sobre a LTP. Nos animais Mecp2$^{+/-}$, apesar da magnitude basal da LTP não estar afetada, o BDNF perdeu também a sua capacidade potenciar este fenómeno.

O estudo do sistema adenosinérgico, através da quantificação por cromatografia líquida de alta performace (*high performance liquid cromotography* – HPLC), permitiu detetar uma diminuição dos níveis de adenosina e do seu percursor adenosina monofosfato (AMP), tanto em homogenatos de hipocampo como de córtex dos animais Mecp2$^{-/y}$ na fase sintomática do modelo animal. Simultaneamente, foram encontrados aumentos nos níveis proteicos dos A_1R no córtex e no hipocampo e uma diminuição dos $A_{2A}R$ no córtex. As mesmas alterações nos receptores de adenosina foram encontradas nos animais Mecp2$^{+/-}$, acrescendo também uma diminuição dos níveis de $A_{2A}R$ no hipocampo. Apesar da diminuição dos níveis de BDNF, em ambos os modelos, foi possível recuperar o efeito facilitador do BDNF na magnitude da LTP hipocampal através da ativação dos $A_{2A}R$, pelo agonista seletivo CGS21680.

Estes dados apoiam a hipótese de que uma terapêutica tendo como alvo o sistema adenosinérgico poderá ser benéfica. Assim, animais Mecp2$^{-/y}$ com 5-6 semanas de idade foram administrados intraperitonealmente com um fármaco inibidor do enzima adenosina cinase (*adenosine kinase* – ADK), 5-iodotubercidina (ITU). Este fármaco permite o aumento dos níveis de adenosina através da inibição da sua metabolização pelo enzima ADK. Para além da eficácia da inibição da ADK já demonstrada noutras doenças, como em modelos de epilepsia, nos animais Mecp2$^{-/y}$, por análise WB, foram detetados aumentos dos níveis proteicos da ADK em homogenatos de córtex durante a fase pré-sintomática, apoiando o estudo da ADK como alvo terapêutico.

Através do estudo da administração da ITU, realizado *in vivo*, foi possível observar uma recuperação do efeito do BDNF sobre a potenciação da LTP, em registos eletrofisiológicos

realizados em fatias de hipocampo, assim como uma recuperação dos níveis proteicos dos recetores TrkB-FL em homogenatos de hipocampos dos animais *Mecp2*$^{-/y}$ tratados com ITU.

Globalmente, os resultados apontam para uma disfunção na sinalização mediada quer pelo BDNF quer pelo sistema adenosinérgico, em dois fenótipos diferentes da doença, sugerindo um possível envolvimento de ambos na fisiopatologia da doença. A obtenção de dados positivos na reversão de alguns défices presentes nos modelos animais estudados, através da inibição farmacológica da ADK reforça esta importância, ao mesmo tempo que sugere o aumento dos níveis de adenosina como uma estratégia a explorar na RTT.

Palavras-Chave: Síndrome de Rett; Sistema adenosinérgico; Recetores A_1 e A_{2A}; BDNF; Terapias com aumento da adenosina

Abstract

Rett Syndrome (RTT) is a rare, genetically caused neurodevelopmental disorder that affects approximately 1:10000-15000 girls worldwide. It is characterized by an apparently normal development up to 6 to 18 months of age, followed by a regression phase, in which there is a loss of acquired abilities. During the progression of this disease, which takes place over four distinct stages, the following stand out: appearance of stereotyped and repetitive hand movements with progressive loss of functionality, cognitive and motor dysfunction and epilepsy. Currently, this disease has no cure and there are few therapeutic options for symptomatic control, which makes this disease devastating for both patients and caregivers.

Genetic studies carried out in recent years have established that this syndrome is mainly due to mutations in the methyl-CpG-binding protein 2 (*MECP2*) gene, located on the X chromosome. This gene encodes the MeCP2 protein, which performs multiple functions where its role stands out as an epigenetic modulator and regulator of the structure of chromatin, controlling the expression of several other genes, making it a key protein in the development and maturation of the central nervous system (CNS). One of the proteins whose expression is controlled by MeCP2 is the brain-derived neurotrophic factor (BDNF), a neurotrophin with essential functions in cell maturation and differentiation, synaptic plasticity and neuronal survival. Consequently, alterations in MeCP2 compromise BDNF expression levels and function, and this evidence has already been demonstrated in multiple studies in RTT animal models.

Those studies have also shown that the increase in BDNF expression can reverse some of the dysfunctions and symptoms present in RTT animal models. However, the therapeutic use of BDNF is not yet applicable since the blood-brain barrier (BBB) is impervious to this neurotrophic factor, preventing it from reaching the brain and performing its functions properly. In an attempt to facilitate the effects of BDNF, new strategies have been developed involving, for example, the use of molecules that cross the BBB and potentiate the neuroprotective action of BDNF. One of the molecules that has deserved particular attention is adenosine. Adenosine is a CNS neuromodulator that exerts its functions through the activation of four receptors, A_1, A_{2A}, A_3 and A_{2B} (AR). In particular, the activation of A_{2A}R is

crucial for the maintenance of BDNF and its receptor, TrkB-FL (full-length tropomyosin-related kinase B), levels as well as for its synaptic effects. It is noteworthy that the adenosinergic system, in addition to be crucial in BDNF-mediated signaling, also has a prominent role in the control of synaptic excitability through the activation of inhibitory A_1 receptors, recognized as potential therapeutic targets in the control of epilepsy. These actions of adenosine, as well as the presence of some symptoms in patients with RTT overlapping with diseases with adenosinergic dysfunction already described, suggest the possibility that this neuromodulator is also affected in RTT.

Thus, this project aimed to: 1) characterize in detail the adenosinergic system and BDNF-mediated signaling, through the use of models with distinct phenotypes: 1.1) animal model with an severe phenotype, Mecp2-null mutant male mice (Mecp2$^{-/y}$); 1.2) animal model with a moderate phenotype, female mice heterozygous for *Mecp2* (Mecp2$^{+/-}$); and 2) explore the augmentation of adenosine levels as a possible therapeutic strategy.

Through Western-Blot (WB) assays it was possible to detect decreased BDNF protein levels in hippocampus, cortex, brainstem, and cerebellum homogenates obtained from Mecp2$^{-/y}$ symptomatic animals. The changes detected in the pre-symptomatic stage were less marked, with only a decrease in BDNF levels observed in the striatum of 3-week-old Mecp2$^{-/y}$ animals. Also, in Mecp2$^{+/-}$ animals, decreases in BDNF levels were detected both in the cortex and in the hippocampus in the symptomatic stage. Regarding protein levels of BDNF receptors, decreases in TrkB-FL were observed in the symptomatic stage in cortical and hippocampal homogenates, and in the pre-symptomatic phase in the cortex and striatum. In this brain area, an increase in TrkB-FL levels was also observed in the symptomatic phase. Analyzing the protein levels of truncated isoforms of the TrkB receptor (TrkB-Tc), negative modulators of BDNF action, an increase were detected in brainstem homogenates from 1-week and 6-week-old animals. In Mecp2$^{+/-}$ animals, no alterations were detected in any of the studied BDNF receptors.

Electrophysiological recordings performed in the hippocampus allowed the study of synaptic plasticity, namely through the study of long-term potentiation (LTP). In Mecp2$^{-/y}$ animals there was a decrease in the magnitude of LTP and an absence of the facilitatory effect

of BDNF upon LTP. In Mecp2$^{+/-}$ animals, although the basal magnitude of LTP was not affected, BDNF also lost its ability to potentiate this phenomenon associated with synaptic plasticity.

The study of the adenosinergic system, analyzed by high performance liquid chromatography (HPLC), allowed to detect a decrease in the levels of adenosine and its precursor adenosine monophosphate (AMP), both in hippocampal and cortical homogenates of Mecp2$^{-/y}$ animals in the symptomatic stage. Simultaneously, increases in protein levels of A_1R in the cortex and hippocampus and a decrease in $A_{2A}R$ in the cortex were found. The same changes in adenosine receptors were found in Mecp2$^{+/-}$ animals, but also a decrease in $A_{2A}R$ levels in the hippocampus. Despite the decrease in BDNF levels, in both models, it was possible to recover the facilitatory effect of BDNF upon the magnitude of hippocampal LTP, through the activation of $A_{2A}R$ by the selective agonist CGS21680.

These data supported the hypothesis that a therapy targeting the adenosinergic system could be beneficial. Thus, 5-6 weeks old Mecp2$^{-/y}$ animals were administered intraperitoneally with an adenosine kinase (ADK) inhibitor drug, 5-iodotubercidin (ITU). This drug allows an increase in adenosine levels by inhibiting its metabolism. In addition to the efficacy of ADK inhibition already demonstrated in other diseases, such as epilepsy models, in Mecp2$^{-/y}$ animals, by WB analysis, increased ADK protein levels were detected in cortex homogenates during the pre-symptomatic stage, supporting the study of ADK as a potential therapeutic target in RTT.

Through the study of ITU administration, carried out *in vivo*, it was possible to observe a recovery of the effect of BDNF upon LTP potentiation, in electrophysiological recordings performed in hippocampal slices, as well as a recovery of protein levels of TrkB-FL receptors in hippocampal homogenates from ITU-treated Mecp2$^{-/y}$ animals.

Overall, the results point to a dysfunction either in signaling mediated by BDNF and in adenosinergic system, in two different phenotypes of the disease, suggesting a possible involvement of both in the pathophysiology of the disease. The positive data obtained regarding the reversal of some deficits present in the animal models studied, through the pharmacological inhibition of ADK, reinforces the importance of adenosinergic system involvement while suggesting the increase of adenosine levels as a strategy to be explored in RTT.

Keywords: Rett Syndrome; Adenosinergic system; Adenosine A_1 and A_{2A} receptors; BDNF; Adenosine augmentation therapy

Chapter 1

General introduction

Chapter 1 – General introduction

1.1 Rett Syndrome

In the 1960s, Dr. Andreas Rett was puzzled after observing two young girls with an identical presentation and clinical history: prominent hand stereotypies with a normal early development followed by a regression period, during which the loss of hand purposeful movements become evident. These findings motivated Dr. Rett to investigate other patients with similar clinical profile, and in 1966, with a total of 22 girl patients, he described these symptoms as a metabolic disorder named "cerebroatrophic hyperammonaemia" (Rett, 1966).

However, just 17 years later, when Dr. Bengt Hagberg and colleagues described 35 additional patients with identical clinical histories overlapping with the syndrome previously described by Dr. Rett, this syndrome was recognized by medical community. The clinical cases were published, as well as the diagnosis criteria for what is nowadays known as Rett Syndrome (RTT) (Hagberg et al., 1983).

RTT is recognized as a severe, progressive, and monogenic neurodevelopmental disorder. This syndrome primarily affects girls, and involves a wide range of clinical symptoms including neurological and behavioral alterations, autonomic dysfunctions, with significant respiratory abnormalities (Chahrour and Zoghbi, 2007; Banerjee et al., 2012). It is the second most common cause of intellectual disability in females after Down Syndrome, with no currently known cure available (Krajnc, 2015; Gomathi et al., 2020).

1.1.1 Clinical basis of Rett Syndrome

RTT is a neurodevelopmental disorder, which in most cases is caused by alterations in the methyl-CpG-binding protein 2 (*MECP2*) gene (Amir et al., 1999). However, mutations in other genes can also be implicated in some forms of RTT (see subchapter 1.1.2)(Percy et al., 2018). RTT presents an incidence of 1:10000-15000 live female births, corresponding to 350000 affected individuals worldwide. Considering the non-diagnosed cases, still in a significant number, it is expected that the incidence would increase in the next years (Katz et al., 2016; Banerjee et al., 2019).

Typically, RTT patients clinically present a normal development, reaching all the expected development milestones up to 6-18 months after birth, when some overt symptoms start to emerge (Chahrour and Zoghbi, 2007; Neul et al., 2011). However, there is evidence of molecular changes impacting development during gestation, mainly upon cell fate, early neurogenesis, cell signaling, synaptogenesis, and synaptic function (Feldman et al., 2016).

During syndrome progression, several symptoms may appear, such as stereotypic hand movements, loss of purposeful hand movements, altered motor coordination, epileptic seizures, language and learning deficits, and cognitive impairments ranging from mild to severe (Banerjee et al., 2012).

Indeed, four stages of disease progression have been described: 1) Developmental stagnation; 2) Rapid regression; 3) Stationary stage, and 4) Late motor deterioration (Figure 1.1). Although

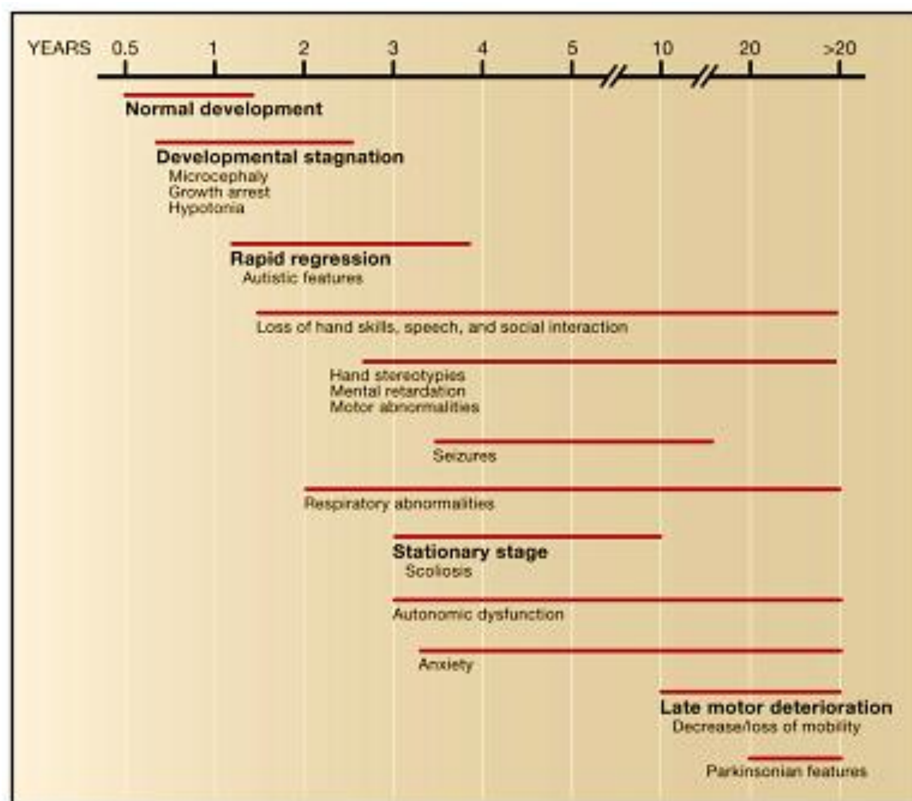

Figure 1.1| Progression of RTT and respective symptomatology. RTT patients develop normally during the first months of life. After this period, a phase of developmental stagnation begins followed by a rapid deterioration with loss of acquired speech, loss of purposeful hand use and midline stereotypies. Autistic features and epilepsy also emerge. Following this rapid regression, a stationary stage can be observed with autonomic dysfunction, anxiety, and scoliosis as prominent symptoms. In advanced disease stages, clinical condition deteriorates, with loss of motor skills (late motor deterioration). In addition, patients suffer from impactful autonomic abnormalities, *(adapted from Chahrour and Zoghbi, 2007).*

not all symptoms appear simultaneously, all of them severely impact the life of both patients and caregivers (Chahrour and Zoghbi, 2007; Kyle et al., 2018).

In detail, the first stage, “Development stagnation”, occurs from the 6-18 month of age. In this stage, one of the earliest signs of neurological involvement can be found: the deceleration of head growth, with consequent microcephaly, mainly caused by the suppressed proliferation of glial cells with *MECP2* dysfunction, and by abnormally small, densely packed neurons with reduced dendritic complexity and synapse density (Nagai et al., 2005; Leonard et al., 2016a; Kyle et al., 2018). Simultaneously, general growth retardation, weight loss, and muscle hypotonia resulting in weak posture, can also manifest (Chahrour and Zoghbi, 2007).

The second stage “Rapid regression”, spans from 1-4 years of age, and is the period where the most debilitating symptoms emerge. Patients acquire stereotypic hand movements such as wringing, washing, clapping, or tapping, as well as repeatedly moving the hands to the mouth which led to the loss of purposeful hand movements. The loss of language function, and social interaction withdrawal also become apparent at this stage. Behavior changes, such as irritability and self-aggressive actions, as well as autistic features, such as expressionless face, lack of eye-to-eye contact, hypersensitivity to sound, indifference to surrounding environment and unresponsiveness to social cues usually emerge in this stage (Nomura, 2005; Chahrour and Zoghbi, 2007; Quest et al., 2014). Both mental retardation and motor coordination deficits, like ataxia and gait apraxia, frequently appear simultaneously (Chahrour and Zoghbi, 2007). The first manifestations of autonomic dysfunction, characterized by hyperventilation during wakefulness, also appear during this stage (Chahrour and Zoghbi, 2007). Additionally, RTT patients also present multiple respiratory abnormalities, such as breath-holding, aerophagia, forced expulsion of air and saliva, and apnea (Ramirez et al., 2013). In the typical RTT form, even though electroencephalogram (EEG) records present pattern changes during the four syndrome stages, the occurrence of seizures only becomes apparent during the second stage, losing prominence at the fourth stage, and commonly is unlikely its onset after 20 years of age (Glaze et al., 2010; Operto et al., 2019). Nevertheless, the epilepsy could range from easily controlled to refractory, with seizures usually being partially complex and tonic-clonic (Jian et al., 2006; Operto et al., 2019).

The third stage, “Stationary stage”, ranges from 3-10 years old or, in some cases, until the end of life. In this stage an amelioration of the social deficits can be observed, as well as a stagnation

of the progression of cognitive function deficits. However, osteopenia, scoliosis and rigidity start to develop at this stage. At the same time, teeth grinding, night laughing or crying, screaming fits, but also low mood and anxiety episodes caused by distressful external events may appear. Additional autonomic disabilities, including severe constipation, oropharyngeal dysfunction, as well as cardiac abnormalities, such as prolonged QT interval, and tachycardia may occur (Nomura, 2005; Chahrour and Zoghbi, 2007; Kumar et al., 2017).

The last stage "Late motor deterioration", lasting from the age of 10 years to the end of life, is characterized by severe deterioration of motor function, with significant loss of mobility, leading most RTT affected girls become wheelchair dependent during the adolescence. Parkinsonian features usually appear at this stage (Chahrour and Zoghbi, 2007; Leuzzi et al., 2020).

RTT patients typically reach a plateau. Although completely dependent on a caregiver, mild cases, when properly accompanied, can live up to age 60-70, despite presenting a very sensitive clinical condition (Chahrour and Zoghbi, 2007).

In addition to this general view over the typical clinical RTT manifestations, it was necessary to stablish strict and recognized clinical criteria, so as to facilitate the exclusion of other neurological conditions whose symptoms overlap with those present in RTT patients. Accordingly, in 2011, the diagnosis criteria were revised, establishing a necessary period of regression followed by a recovery or stagnation, with a presence of four main criteria and two exclusion criteria for the diagnosis, as presented in table 1.1 (Neul et al., 2011).

Table 1.1| Revised diagnosis criteria for typical RTT, *(adapted from Neul et al., 2011)***.**

<table>
<tr><td colspan="2">Consider diagnosis when postnatal deceleration of head growth observed.</td></tr>
<tr><th>Main Criteria</th><th>Required for typical RTT</th></tr>
<tr><td>1.Partial or complete loss of acquired purposeful hand skills.
2.Partial or complete loss of acquired spoken language.
3.Gait abnormalities: Impaired (dyspraxic) or absence of ability.
4.Stereotypic hand movements such as hand wringing/squeezing, clapping/tapping, mouthing and washing/rubbing automatisms.</td><td rowspan="3">1.A period of regression followed by recovery or stabilization.
2.All main criteria and all exclusion criteria.
3.Supportive criteria are not required, although often present in typical RTT.</td></tr>
<tr><th>Exclusion Criteria</th></tr>
<tr><td>1.Brain injury secondary to trauma (peri- or postnatally), neurometabolic disease, or severe infection that causes neurological problems.
2.Grossly abnormal psychomotor development in first 6 months of life.</td></tr>
</table>

1.1.2 Molecular basis of Rett Syndrome

Molecular basis of RTT involves a high complexity and multiple influencing factors which manifest several forms of this disease (Matijevic et al., 2008).

The first identified RTT cases were in female individuals. This led the scientific community to hypothesize that RTT could be a X-linked syndrome, fatal to hemizygous males (Chahrour and Zoghbi, 2007).

The familial cases were crucial in deciphering the narrow location of the gene in Xq28 locus (Schanen et al., 1997; Sirianni et al., 1998). In 1999, Amir and colleagues (Amir et al., 1999) screened DNA from sporadic and familial RTT cases, analyzing more than 100 gene candidates present in that locus. This study allowed the identification of several types of mutation, such as missense, frameshift and nonsense mutations, in the coding region of the *MECP2* gene (Amir et al., 1999).

Nowadays, it is well known that mutations in *MECP2* gene are found in 95% of typical RTT cases, and in 73.2% of atypical RTT cases (Ehrhart et al., 2018). In 99% of RTT cases the mutations in *MECP2* occur *de novo*, and less than 1% are inherent from one parent (Wan et al., 1999; Operto et al., 2019). The *de novo* mutations usually emerge from the paternal germline, and usually involve a C to T transition in CpG dinucleotides (Girard et al., 2001; Trappe et al., 2001). The familial cases identified are often originated from inherited mutations from healthy to mildly affected mothers, from gonadal mosaicism, or favorable X-chromosome inactivation (XCI) (Matijevic et al., 2008).

More than 800 mutations in *MECP2* have been found to be associated to RTT, including different mutations types, such as point mutations, insertions, duplications, small or large deletions, which can be found in almost every location of the gene sequence (Ehrhart et al., 2018). However, 8 single-nucleotide polymorphism hotspots, such as missense and nonsense mutations, have been identified as the causative RTT mutation in approximately 70% of cases: T158M; R255X; R306C; R294X; R270X; R133C, R168X and R106W (presented by order of frequency) (Amir and Zoghbi, 2000; Percy et al., 2010) (Figure 1.2). It has also been described that C-terminal mutations represent 8% of cases, while large deletions represent 5% (Neul et al., 2008). This diversity in the genetic mechanisms underlying RTT may partially explain the wide range of phenotypic severity observed in different patients. Nonetheless, it is possible to find individuals

with the some mutation but different phenotypic severity (Kyle et al., 2018). Despite this lack of perfect correlation between mutation and phenotype, some studies have shown a correlation between genotype and the severity of clinical manifestations. Accordingly, early truncated mutations, such as R168X, R255X, and R270X, as well as large insertions and deletions, are associated to severe phenotypes (Neul et al., 2008). On the other hand, missense mutations, such as R133C and R306C, late truncating mutations, like R294X and others in the 3' with an intact MBD (methyl binding domain) and TRD (transcription repression domain) domain (see subchapter 1.1.2.1), are associated with the mildest phenotypes (Neul et al., 2008; Cuddapah et al., 2014). Additionally, mutation type tends to be located in specific MeCP2 domains, which will affect the respective protein function, thus impacting phenotype. Accordingly, missense mutations most commonly occur in the MBD domain, nonsense mutations generally occur downstream of the MBD domain, and frameshift mutations, resulting from small deletions, usually occur in the C-terminus (Lee et al., 2001; Neul et al., 2008).

Another important contributor for the phenotypic variability, besides the location and type of mutation, is the XCI. The majority of RTT patients are heterozygous for *MECP2*, carrying a normal (or wild-type - WT) and a mutant allele of the gene.

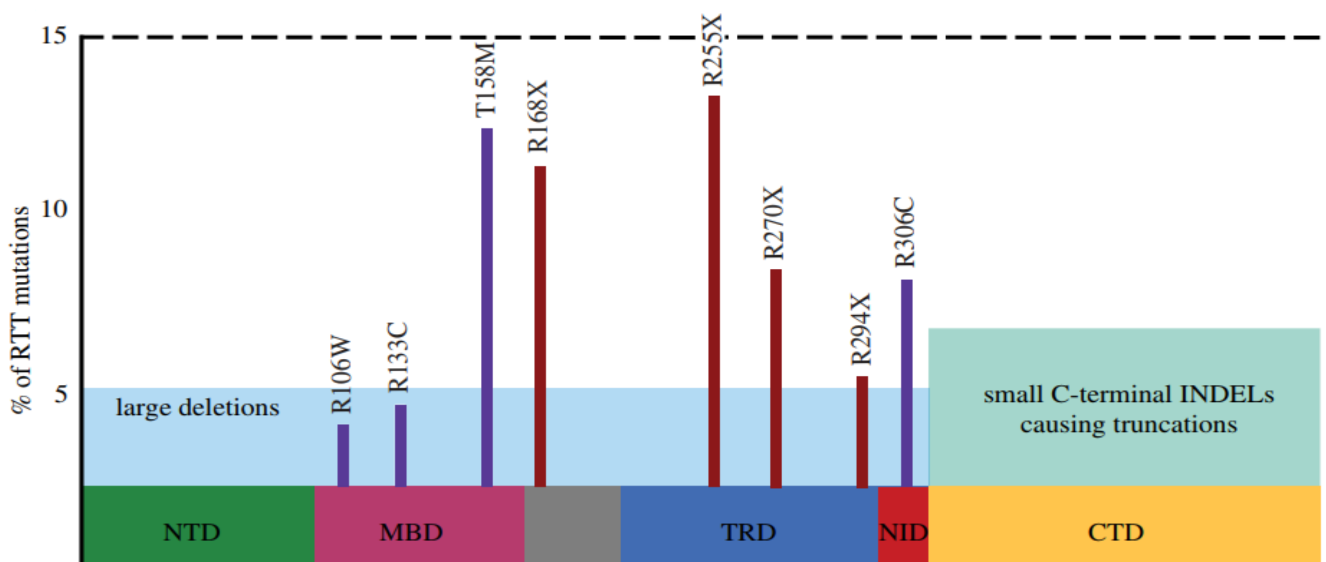

Figure 1.2| Most common mutations in RTT. T158M; R255X; R306C; R294X; R270X; R133C, R168X and R106W are the eight most common mutation present in RTT, located in methyl-binding domain (pink, MBD) and transcriptional repression domain (blue, TRD) of the Mecp2 protein. The representative percentage is shown in the bars, with missense mutations in purple and nonsense mutations in red. N-terminal domain (green, NTD); interaction domain (red, NID); C-terminal domain (yellow, CTD), (*adapted from Kyle et al., 2018*).

XCI allows the presence of a mosaicism in heterozygous females. Depending on the skewing of the mutant X chromosome, and of its consequent stochastic expression, the clinical severity changes: with symptom severity being inversely proportional to skewing (Ishii et al., 2001; Hoffbuhr et al., 2002). However, even with same mutations and equivalent XCI pattern in peripheral blood, clinical severity could differ between individuals. This has been explained by the presence of additional gene mutations that can either ameliorate or aggravate the clinical outcome (Zeev et al., 2009).

Despite RTT being a X-linked syndrome, males can also be affected. Indeed, some clinical cases had been reported, previous to the discovery of the gene involved in the disease. The first *MECP2* mutation was described in a male in 1999 (Wan et al., 1999). Hence, males with symptoms included in RTT clinical criteria, started to be screened for *MECP2* mutations and classified in four different groups: 1) severe neonatal encephalopathy and infantile death; 2) classical RTT; 3) less severe neuropsychiatric phenotypes and 4) *MECP2* duplication syndrome (Kyle et al., 2018) (Table 1.2).

Table 1.2| RTT classification found in males: genetic profile and respective clinical features, *(adapted from Oporto et al 2019).*

Classification	*Genetic profile*	*Clinical features*
Severe neonatal encephalopathy and infantile death	*MECP2* mutations inherited from mildly symptomatic or asymptomatic mothers.	When born, severe neonatal encephalopathy with respiratory arrest and seizures and death within 2 years of age.
Classical RTT	XXY karyotype or other somatic mosaicisms.	Like RTT clinical features in female patients.
Less severe neuropsychiatric symptoms	*MECP2* mutations less severe than those in female patients.	Intellectual disability and motor abnormalities (broad spectrum of symptoms and possible overlap with features of Angelman syndrome).
***MECP2* duplication syndrome**	Gain of *MECP2* dosage.	Hypotonia, severe intellectual disability, lung infections, seizures, absent or limited speech and walking, motor spasticity, and muscle stiffness.

Importantly, 3%-5% of RTT patients do not have *MECP2* mutations (Neul et al., 2008). There are mutations in other genes responsible for RTT variants, being the most common: the *FOXG1*, associated to the Rolando atypical RTT variant, and the *CDKL5*, associated to the Hanefeld atypical RTT variant (Weaving et al., 2004; Ariani et al., 2008).

FOXG1 is located in 14q12 chromosome and encodes a homonymous transcriptional repressor involved in neuronal differentiation. Its location, outside the X chromosome, has been associated to the congenital variant of RTT (Ariani et al., 2008). Patients carrying this type of mutation have a clinical history characterized by postnatal growth deficiency, microcephaly, developmental delay with absent speech, defective social reciprocity, poor sleep, stereotypies, dyskinesia, and severe early onset epilepsy (Brunetti-Pierri et al., 2011; Guerrini and Parrini, 2012).

Like *MECP2, CDKL5* is also an X chromosome gene, more precisely located on Xq22, encoding the CDKL5 protein, a ubiquitous protein with a key role in neuronal maturation. Mutations in this gene are associated with the early seizure variant of RTT (Mari et al., 2005; Rusconi et al., 2008; Guerrini and Parrini, 2012).

In recent years, the development of genetic and sequencing technology, such as the whole sequencing genome, allowed the identification of more genes associated with typical or atypical RTT. Around 69 new genes have recently been described, as causative of RTT, wherein 46 were described since 2017 (Vidal et al., 2019)(Figure. 1.3).

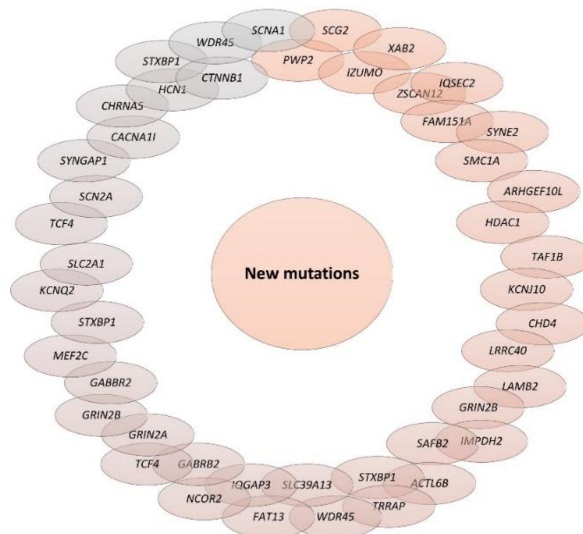

Figure 1.3| New genes associated with RTT. In this figure are represented the 46 new mutations, described since 2017, associated with RTT.

According to the RTT National History Study (NCT02738281), atypical RTT variants represent 14.9% of RTT patients in United States of America. Clinical criteria for these variants includes a period of regression, followed by recovery or stabilization, and at least two of the four main criteria and five of the eleven supportive criteria (Neul et al., 2011, 2014) (Table 1.3). Despite part of atypical RTT variants being associated with mutations in non-*MECP2* genes, the Zappella or preserved speech variant is primarily associated to *MECP2* gene mutations (Zappella et al., 2001; Townend et al., 2018). This variant is characterized by a regression stage from ages 1-3, followed by a prolonged plateau stage, a milder compromise of purposeful hand movements, as well as significant epilepsy and intellectual disability (Operto et al., 2019). Interestingly, in this variant, the patients can recover language functions, after their regression (Zappella, 1992; Zappella et al., 2001).

Table 1.3| Revised diagnosis criteria for atypical RTT, *(adapted from Neul et al., 2011)*.

Consider diagnosis when postnatal deceleration of head growth observed.	
Main Criteria	**Required for atypical RTT**
1.Partial or complete loss of acquired purposeful hand skills. 2.Partial or complete loss of acquired spoken language. 3.Gait abnormalities: Impaired (dyspraxic) or absence of ability. 4.Stereotypic hand movements such as hand wringing/squeezing, clapping/tapping, mouthing and washing/rubbing automatisms.	1.A period of regression followed by recovery or stabilization. 2.At least 2 out of the 4 main criteria. 3.5 out of 11 supportive criteria.
Supportive Criteria for atypical RTT	
1.Breathing disturbances when awake. 2.Bruxism when awake. 3.Impaired sleep pattern. 4.Abnormal muscle tone. 5.Peripheral vasomotor disturbances. 6.Scoliosis/kyphosis. 7.Growth retardation. 8.Small cold hands and feet. 9.Inappropriate laughing/screaming spells. 10.Diminished response to pain. 11.Intense eye communication - “eye pointing”,	

Undoubtedly, mutations in *MECP2* are the primary cause of RTT. Accordingly, in the next subchapters, *MECP2* gene and MeCP2 protein expression, as well as its structure, will be detailed.

1.1.2.1 From MECP2 gene to MeCP2 protein

MECP2, located on the long arm of the X chromosome (Xq28), maps between interleukin-1 receptor associated kinase gene (*IRAK1*) and red opsin (*RCP/GCP*) loci, spans around 76kb and, as mentioned before, undergoes XCI (Matijevic et al., 2008; Liyanage and Rastegar, 2014). This gene is constituted by four exons and three introns, encoding two isoforms of the MeCP2 protein, in both humans and rodents,: MeCP2_exon1 (MeCP2E1), in which the exon 2 is skipped, and MeCP2_exon2 (MeCP2E2), in which exon 1 is skipped, starting the translation at exon 2 (Mnatzakanian et al., 2004). MeCP2E1 has a ten times greater expression in the brain relative to MeCP2E2, in both humans and rodents, suggesting that MeCP2E1 isoform is the primary MeCP2 isoform in the brain. MeCP2E1 also shows an higher expression in the lung and thymus of mice but an equal ratio of both isoforms is described in the testis and liver of the same animals (Olson et al., 2014).

Exon 4 contains a long 3'-untranslated region (3'UTR), presenting three different polyadenylation sites (polyA), corresponding to four different transcripts that show quantitative changes in expression across tissues as well as across pre- and postnatal development. The 10.1-kb transcript shows an higher expression in the fetal brain, whereas in the adult brain it is the 5-kb transcript that becomes the most expressed transcript (Coy et al., 1999; Pelka et al., 2005). In this way, it is speculated that 3'-UTR could be a regulator of downstream *MECP2* transcription, controlling mRNA degradation and stability, nucleocytoplasmic transport, mRNA localization, and modulation of translation (McGowan and Pang, 2015) (Figure 1.4).

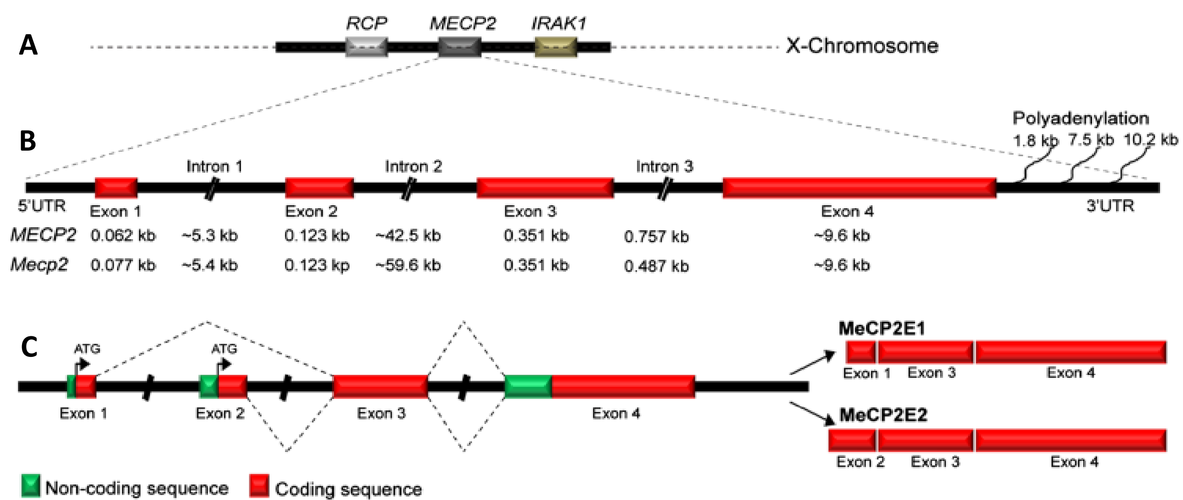

Figure 1.4| *MECP2* gene structure. In **A** *MECP2/Mecp2* gene location is represented: in X-chromosome (Xq28), surrounded by the *RCP* and *IRAK* genes. In **B** it is shown that the *MECP2/ Mecp2* gene is composed by four exons (exon 1–4) and three introns (intron 1–3). The gene has three polyadenylation sites at the 3'UTR. The sizes of each exonic and intronic region in human MECP2 and mouse Mecp2 genes are represented. The translation in two MeCP2 isoforms: MeCP2E1 and MeCP2E2 is represented in **C**. The translation start site (ATG) for each isoform is indicated by arrows. MeCP2E1 isoform is encoded by exons 1, 3 and 4. MeCP2E2 isoform is encoded by 2,3 and 4. *IRAK1*, interleukin-1 receptor associated kinase gene; MeCP2E1, MeCP2_exon1; MeCP2E2, MeCP2_exon2; *RCP*, red opsin, *(adapted from Liyanage and Rastegar, 2014).*

MeCP2 is a nuclear protein that is ubiquitously expressed in all human cells, but is particularly abundant in neurons (Zachariah et al., 2012; Liyanage and Rastegar, 2014). It is composed by five different domains: NTD (N-terminal domain), MBD, ID (Inter domain), TRD and CTD (C-terminal domain). From these, three are functional, with specific functions already uncover: 1) MBD is responsible for binding to 5-methyl cytosine with high affinity, and for transporting MeCP2 to the nucleus (Lyst et al., 2018); 2) TRD is responsible for interacting with histone deacetylase (HDAC), and transcriptional corepressor deacetylase complexes, SIN3A. It also contains the nuclear receptor co-repressor (NCoR)-ID; 3) CTD facilitates MeCP2 binding to the nucleosome core, allowing chromatin compaction, and consequent inaccessibility to transcriptional machinery (Matijevic et al., 2008; Hite et al., 2009). Although these well-defined domains, MeCP2 is classified as an intrinsically disordered protein (IDP), with around 60% of the protein being unstructured, just obtaining a tertiary structure when bounded to other proteins (Liyanage and Rastegar, 2014) (Figure 1.5). In this way, posttranslational modifications, such as acetylation, phosphorylation,

ubiquitination and SUMOylation, gain an important role in controlling MeCP2 binding to proper elements critical for its function (for review see (Kyle et al., 2018)).

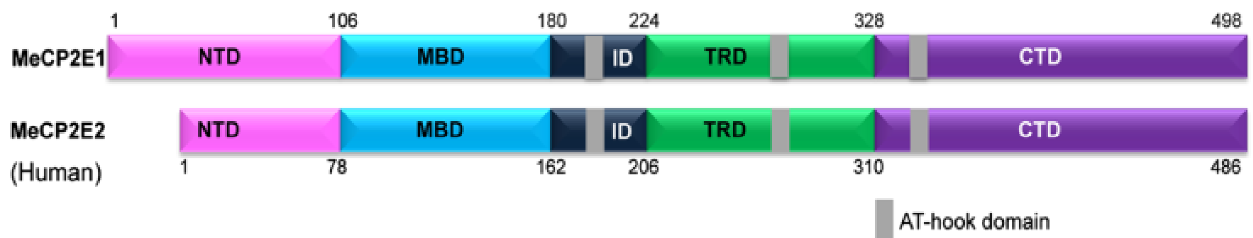

Figure 1.5| MeCP2 protein structure. Human MeCP2E1 and MeCP2E2 are 498 and 486 amino acids in size, respectively, while mouse MeCP2E1 and MeCP2E2 are 501 and 484 amino acids in size, respectively. CTD, C-terminal domain; ID, Inter domain; MBD, Methyl binding domain; MeCP2E1, MeCP2_exon1; MeCP2E2, MeCP2_exon2; TRD, Transription repression domain (*adapted from Liyanage and Rastegar, 2014).*

Initially, MeCP2 was thought to act as an epigenetic transcriptional repressor (Lewis et al., 1992). Multiple studies have been performed during the last years, to better unravel the functions of MeCP2, and its involvement in disorders, such as RTT. Its pleiotropic effects in multiple genes and signaling targets make a difficult task deciphering a linear correlation between MeCP2 functions and RTT pathophysiology. Work in this area, clarified that MeCP2 is an important regulator of transcription, acting as a transcriptional activator or repressor, depending on the context (Chahrour et al., 2008). Its ability to bind in DNA methylated cytosines was one of the first interactions associated to MeCP2, namely methylated cytosines followed by a guanine (Scarsdale et al., 2011). However, it was found recently that the MBD domain also binds methylated cytosines in the C-H context (where H = A/T/C), with similar affinity being observed both *in vitro* and *in vivo* (Lombardi et al., 2015). Insofar DNA methylation is an important epigenetic process, involved in the regulation of genomic imprinting, XCI, cellular differentiation and transposable silencing (Villicaña and Bell, 2021). MeCP2, a methyl binding protein, plays a key role in all of these processes (Lombardi et al., 2015). In addition, MeCP2 can interact with multiple proteins, such as histone SIN3A, NCOR and SMRT, already mentioned, which fine-tune the transcriptional regulation role of MeCP2 (Adachi and Monteggia, 2014).

The ability of MeCP2 to control the gene expression has been amply explored. Some of the most well-known genes under the influence of MeCP2, in neuronal cells, are: 1) *Brain-derived neurotrophic factor* (*BDNF*); 2) *Long interspersed nuclear elements* (*LINE-1*); 3) *Leukosialin*; 4) *Distal-less homeobox 5* (*DLX5*); 5) *Corticotropin-releasing hormone* (*Crh*); and 6) *FXYD domain-*

containing transport regulator 1 (FXYD1) (Matijevic et al., 2008). In astrocytes a set of other target genes has been described, all of which are important for adequate astrocytic function (Liyanage and Rastegar, 2014).

In addition, MeCP2 also plays a crucial role in chromatin remodeling, protein synthesis, regulation of alternative splicing, and microRNA (miRNA) processing, all of which are important for neuronal migration, differentiation, and dendritic morphogenesis (Leonard et al., 2016; Banerjee et al., 2019). miRNA, in turn, in parallel with activity-dependent phosphorylation, reciprocally regulates MeCP2 function (Leonard et al., 2016).

Despite the ubiquitous expression of MeCP2, the higher expression levels are detected in the brain, lungs and spleen and the lowest in the liver, heart, kidney and small intestines (Liyanage and Rastegar, 2014; Gulmez Karaca et al., 2019). Particularly, in the central nervous system (CNS), during the prenatal stage, MeCP2 expression is low, progressively increasing during neuronal maturation and synaptogenesis, with peaking in mature and post-migratory neurons (Figure 1.6) (Shahbazian et al., 2002; Kishi and Macklis, 2005; Bedogni et al., 2016). Across different brain regions, expression in whole cell extracts is higher in the cortex and cerebellum, comparing with the olfactory bulb, striatum, hippocampus, thalamus, and brainstem (Zachariah et al., 2012; Olson et al., 2014). Interestingly, MeCP2 expression is different in multiple brain regions implicated in the specific functional and behavioral impairments observed in RTT mouse models (Liyanage and Rastegar, 2014). At cellular level, MeCP2 expression is highest in neurons but it is also detected in astrocytes, microglia, and oligodendrocytes (Liyanage and Rastegar, 2014).

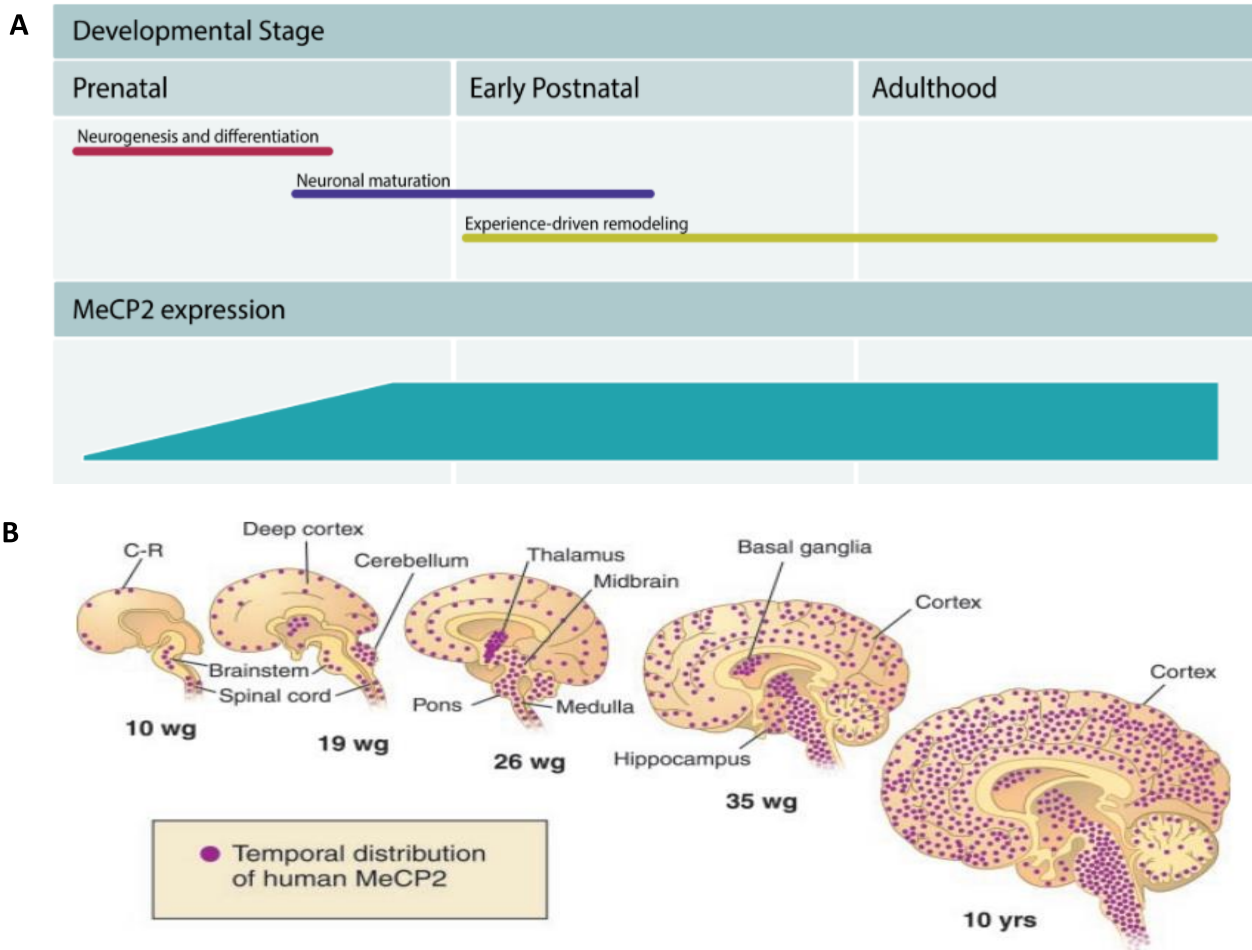

Figure 1.6| Mecp2 expression across the development. In **A** it is shown MeCP2 expression across ages: MeCP2 expression increases during the prenatal stage, remaining significant until adulthood. Initially, MeCP2 has an important role in neurogenesis and differentiation, later in neuronal maturation and postnatally, in driven remodeling. MeCP2 also regulates neuronal differentiation in early embryonic development, neuronal maturation, and circuit formation. **B** shows MeCP2 brain expression. Cajal-Retzius (C-R) neurons are the first cortical neurons to mature and express MeCP2, followed by midbrain, thalamus, cerebellum, and deep cortical neurons. Expression in basal ganglia, hypothalamus, hippocampus, and superficial cortical layers occurs later, and the number of MeCP2-positive neurons in the cerebral cortex continues to increase until ten years of age. Wg, weeks of gestation, (*adapted from Karaca et al., 2019 and Zoghbi 2003*).

1.1.2.2 Modelling Rett Syndrome

The finding that mutations in the *MECP2* gene are the main cause of RTT, as well as the advance of genetic and molecular techniques, allowed the development of different models of the disease. This development allowed a better understanding of RTT pathogenesis, and the exploration of possible therapeutic targets, and strategies.

Non-rodent animal models

The possibility of replicating a RTT-representative phenotype is limited to vertebrate genetic models, as *Mecp2* is not expressed in non-vertebrate species, thereby excluding fruit flies and nematodes as possible models (Hendrich and Tweedie, 2003). In fish, zebrafish *Mecp2*$^{Q63*/Q63*}$ was generated for the first time in 2013, leading to Mecp2 protein truncation (Pietri et al., 2013). This animal model presents minor motor abnormalities and shortened lifespan, but did not reproduce some of the disease features observed in humans, having normal viability and reproduction (Pietri et al., 2013). More recently, a *Mecp2*-mutant cynomolgus monkey was generated presenting some important features observed in RTT patients: impaired motor function, social withdrawal, increased stereotypical behavior and sleep abnormalities (Chen et al., 2017). This suggests simians as robust and consistent RTT models. However, the high costs associated with such models, limited their study to highly sophisticated analyses, or for specific assessment that are not possible to perform in other available models (Kyle et al., 2018).

Rodent animal models

Rodent models, in particular mouse models, are widely used, since mice are easy to genetically manipulate, require low maintenance costs, and can mimic the majority of RTT symptoms. Two years after MeCP2 loss-of-function was described as the cause of RTT (Amir et al., 1999), two null mouse models were generated, and remain as the most studied RTT models to this day: 1) *Mecp2*$^{tm1.1Bird/Y}$, lacking any Mecp2 protein product and 2) Mecp2$^{tm1.1Jae}$, expressing smaller *Mecp2* transcript and protein fragments. Both models show similar phenotypes with rapid regression, in which the symptomatology starts around 4 to 6 weeks of age and surviving being around 8 to 12 weeks of age. Microcephaly, reduced body weight, smaller somas, decreased dendritic branches, impaired synaptic plasticity, changes in neurotransmitters, motor and cognitive dysfunction, and seizures, are some of the typical features present in these mice models (Chen et al., 2001b; Guy et al., 2001). Despite these models had been develop in male mice, it is important to note that *Mecp2* heterozygous female mice present a more accurate genetic representation of the majority of RTT patients, due to XCI influence. On the other hand, Mecp2-null male mice are representative of more severe phenotypes, usually associated with truncated mutations in RTT patients (Neul et al., 2008). Due to XCI, *Mecp2* heterozygous female mice show

a more variable phenotype starting to develop the first symptoms, such as hypoactivity and hindlimb clasp, at around 3 months of age, much later than null male mice, or human patients (comparing development timepoints) (Leonard et al., 2016). However, female mice can remain asymptomatic until one year of age, with approximately 50% of the heterozygous females showing an almost overlapping phenotype with null male mice at around 9 months of age (Table 1.4) (Lombardi et al., 2015; Kyle et al., 2018).

Table 1.4| Differences between Mecp2-null male mice and heterozygous (Het) female mice. In red are highlighted the features discrepant between both genders. + feature present; - feature absent; +/- decreased; ND – not determined (adapted from *Lombardi et al*., 2015).

		Null mice	
	RTT patients	*Males*	*Females (Het)*
Motor	Limited mobility	+	+
	Ataxic gait	+	+
	Dystonia/rigidity	+	+
	Tremor	+	+
	Stereotypes	-	-
Cognitive and social abilities	Decreased cognition	+	+
	Speech loss	**ND**	**ND**
	Social avoidance	-	+
	Axiety	+/-	+/-
Morphological	Microcephaly	+	-
	Neuronal hypotrophy	+	+
Autonomic dysfunction	Breathing abnormalities	+	+
	Reduced lifespan	+	+
Other	Seizures	+	-

To study the phenotypic association with specific *MECP2* mutations and the impact of the mutated domain on MeCP2 function, some alleles have been constructed to replicate common *MECP2* mutations observed in RTT patients. Some of the already available allele mice models are: $Mecp2^{308}$ and R168X, mutations resulting in a truncated protein and Mecp2 T308A, Mecp2 R306C, and Mecp2 T158A that recapitulate mutations with partial loss of Mecp2 function (Goffin et al., 2011; Ebert et al., 2013; Lyst et al., 2013). These models usually present milder phenotypes, with

later onset of neurological symptoms, and tend to not recapitulate the complete disease phenotype (for review see (Liyanage and Rastegar, 2014; Kyle et al., 2018)).

In rat models where the expression of Mecp2 is reduced in the brain, such as the Mecp2 zinc-finger nuclease rat model, it is possible to observe transient abnormalities in behavior with decreased BDNF levels but without significant RTT phenotypes being present (Jin et al., 2008; Veeraragavan et al., 2016). In addition to this unfavorable characteristic, the high cost associated to their maintenance, and the lack of availability of genetic tools, support the use of mouse rather than rat models despite their advantages in physiologic and pre-clinical tests of the latter (Kyle et al., 2018) (Figure 1.7).

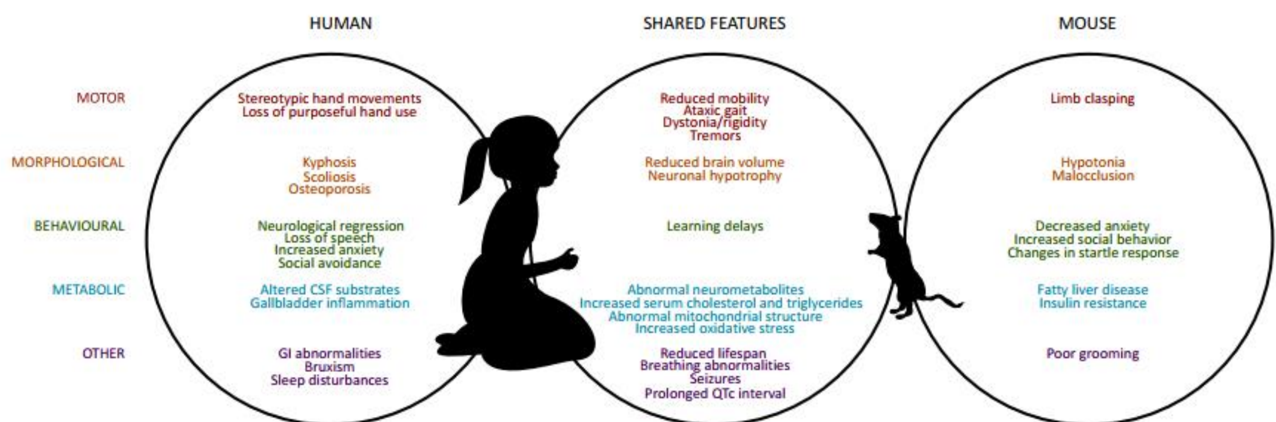

Figure 1.7| Shared features between RTT mouse models and Human RTT patients. In each circle is included: on left, phenotypic features present in human RTT patients; on right, in mouse models; in center, shared features between both, (*Vashi and Justice, 2019*).

Non-animal models

In addition to animal models, in the last two decades it became possible to model RTT using human patient cells. The development of induced pluripotent stem cell (iPSC) technology allowed the generation of different types of human neurons from somatic cells of RTT patients, thus creating an alternative strategy to understand specific disease mechanisms and allowing an alternative platform for drug-screening (Dajani et al., 2013). Despite *in vitro* studies allowing the answering of some questions, they inherently present some research caveats (for review see (Chailangkarn et al., 2012; Dajani et al., 2013). More recently, organoid technology allowed to model brain development and neurological disorders in a 3D platform, adding some

sophistication to usual 2D studies using iPSCs, where analysis of brain development was more limited (Lancaster et al., 2013). Studies carried out in these models have shown decreases in neurite growth, dendritic spines, axonal branches, neurite coalescence, soma size, and glutamatergic synapses, as well as impaired neurogenesis, neural progenitor migration deficits, and electrophysiological changes, such as decreased frequency of spontaneous postsynaptic currents (Chailangkarn et al., 2012; Mellios et al., 2018; Paiva Miranda et al., 2020; Xiang et al., 2020).

1.1.3 Drug development in RTT

Given that RTT is still a disease for which there is no cure, in the last 20 years, vast effort was made in the search for new drugs able to improve patients' life, mostly by controlling some symptoms. A recent review identified 70 investigated drugs in pre-clinical and clinical trials. Unfortunately, no clinical trial reached III/IV phase (Gomathi et al., 2020). This clearly show an urgent need of develop new therapeutic strategies to treat RTT.

Pre-clinical trials have been mostly conducted in *MECP2* transfected cell lines, *Mecp2* heterozygous female mice, or in *Mecp2*-null male mice, especially for *in vivo* gene therapy studies (Smeets et al., 2011; Gadalla et al., 2013; Garg et al., 2013). The neuropathology and the multiple systems affected in RTT make it so that different drug classes could be explored as possible strategies, targeting one or more specific RTT phenotypes (Ip et al., 2018; Gomathi et al., 2020). Given the already disclosed mechanisms affected in RTT, the most explored targets involve gene therapy or epigenetic modifiers, modulation of neurotransmitters and for their respective receptors, enhancement of growth factors, and metabolic and mitochondrial control (Vashi and Justice, 1234; Li and Pozzo-Miller, 2014; Kyle et al., 2018; Gomathi et al., 2020). The rationale beyond each of these components allows the study of new drugs, or compounds already approved for other diseases, which can be divided in 17 categories: 1) Genetic/epigenetic modifiers, (Nelson et al., 2006; Yu et al., 2011; Goldmann et al., 2012; Robinson et al., 2012; Pitcher et al., 2015); 2) Growth factor therapy (Tropea et al., 2009; Pitcher et al., 2013; Castro et al., 2014); 3) BDNF boosters or mimetics (explored in subchapter 1.2.4); 4) Protein tyrosine phosphatase 1B (PT1B) **i**nhibitors (Zhang and Zhang, 2007; Krishnan et al., 2015); 5) NMDA receptor antagonists, (Mierau et al., 2016; Patrizi et al., 2016); 6) HDAC inhibitors (Guo et al.,

2014); 7) Antidepressants (Roux et al., 2007; Zanella et al., 2008; Bittolo et al., 2016); 8) Serotonergic Drugs, (Toward et al., 2013; Valenti et al., 2017); 9) Dopaminergic drugs, (Szczesna et al., 2014); 10) Adrenergic drugs (Mellios et al., 2014); 11) Gabaergic drugs (Jin et al., 2013; El-Khoury et al., 2014; Chin et al., 2016); 12) Cholinergic drugs (Ricceri et al., 2011); 13) Glutamatergic Drugs (Gogliotti et al., 2016, 2017; Tao et al., 2016); 14) Mitochondrial effectors, (De Filippis et al., 2012; Panighini et al., 2013); 15) Metabolic boosters (Buchovecky et al., 2013; Park et al., 2014; Villani et al., 2016; Nance et al., 2017); 16) Anti-seizures drugs (ASD) and 17) Electrolyte Modifiers (De Filippis et al., 2013; Herrera et al., 2015).

In Figure 1.8 is detailed the multiple compounds belonging to each drug class studied in RTT stated above, with the exception for BDNF mimics and boosters (explored in subchapter 1.2.4).

All of these compounds demonstrated positive results at least in one of the following features

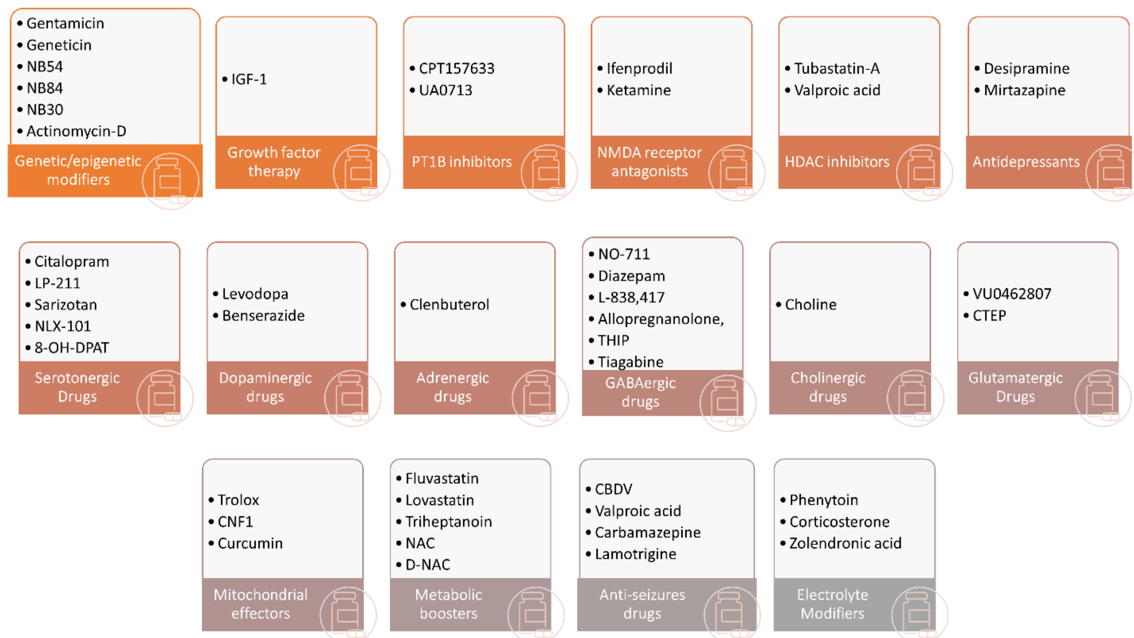

Figure 1.8| Drugs explored in non-clinical RTT studies. In each box is detailed the several compounds studied in RTT models, integrated in sixteen of the seventeen different drug categories.

present in RTT mouse models: behavioral deficits, cardiorespiratory abnormalities, defects in neuronal circuits, and lifespan (Vashi and Justice, 1234; Gomathi et al., 2020). Additionally, Gentamicine, IGF-1, HDAC inhibitors, and Choline have also been screened and studied in human-IPSCs, corroborating the potential beneficial observed in RTT mouse models (Marchetto et al., 2010; Chin et al., 2016; Delépine et al., 2016; Strebl et al., 2017; Landucci et al., 2018).

Of the above mentioned 17 classes explored in pre-clinical trials, only 13 progressed to clinical trials. Since 1994, 50 clinical trials have been conducted targeting drug efficacy endpoints, including: motor function, respiratory and gastrointestinal abnormalities, chewing, swallowing, constipation, epilepsy, muscle tone, and communication. The primary outcome measure in RTT trials is the quality of life (QoL), derived from clinical scores, communication assessments, EEG, plethysmography, breathing pattern recordings, and analysis and actigraphy studies to evaluate stereotypic hand movements (Gomathi et al., 2020). Table 1.5 summarizes the clinical trials conducted since 2017.

Table 1.5| Clinical trials conducted since 2017 in RTT patients, (*adapted from Gomathi et al., 2020).*

	Trial	Drug	Phase	Outcome
GROWTH FACTOR THERAPY	Pharmacological Treatment of Rett Syndrome by Stimulation of Synaptic Maturation With Recombinant Human IGF-1 (Mecasermin [rDNA] Injection)	rhIFG-1	II	Improvement in stereotypic behavior and social communication .
	A Phase II Randomized, Double-Blind, Placebo-Controlled, Parallel-Group, Dose-Escalation Study of NNZ-2566 in Rett Syndrome	NNZ-2566	II	Safety and tolerability with positive outcomes in communication and speech, behavior, breathing abnormalities, motor functions and seizures.
	A Randomized Double-blind, Placebocontrolled, Dose-ranging Study of the Safety and Pharmacokinetics of Oral NNZ-2566 in Pediatric Rett Syndrome	NNZ-2566	II	Achieved primary endpoint and safety outcomes with improved quality of life.
NMDA RECEPTOR ANTAGONISTS	A Study to Evaluate Ketamine for the Treatment of Rett Syndrome	Ketamine	II	Recruiting, no results reported yet.
	Placebo-Controlled Trial of Dextromethorphan in Rett Syndrome	Dextromethorphan	II	Open, no results reported yet.
ANTIDEPRESSANTS	Pilot Study of the Effects of the Desipramine on the Neurovegetative Parameters of the Child With Rett Syndrome	Desipramine	II	No efficacy in the number of apnea and hypopnea events.
SEROTONERGIC DRUGS	Open-label study of the effect of fluoxetine in patients aged 8–28 years with Rett Syndrome Typical	Fluoxetine and buspirone	II	Ongoing, no results reported yet.
	A Randomised, Double-Blind, PlaceboControlled 6-month Study to Evaluate the Efficacy, Safety, and Tolerability of Sarizotan in Patients With Rett Syndrome With Respiratory Symptoms	Sarizotan	II/III	Open, no results reported yet.
MITOCHONDRIAL EFFECTORS	A Phase 2A Randomized, PlaceboControlled Trial of EPI-743 in Children with Rett Syndrome	EPI-743	II	There was increase in head circumference, oxygenation and hand function. Not met the endpoint of RTT severity score.
	Treatment of Mitochondrial Dysfunction in Rett Syndrome With Triheptanoin: An Open-label, 10-subject Clinical Trial of UX007 (Triheptanoin) in the Treatment of Mitochondrial Dysfunction in Participants With Rett Syndrome, Dyskinesia, and Epilepsy	UX007	iiI	Open, no results reported yet
	Open Label Trial of Triheptanoin (UX007) in Treatment of Rett Syndrome	UX007	II	Recruiting, no results reported yet,
ANTI-SEIZURES	Effectiveness and tolerability of anti-seizures drugs in 104 girls with Rett syndrome	Valproic acid (VA), carbamazepine (CBZ) and lamotrigine (LTG)	NA	VPA is most effective anti-seizures drugs in younger girls, CBZ the most effective in the patients 15 years or older and LTG is the least tolerated drug.
ELECTROLYTE MODIFIERS	Annual Injection of Zoledronic Acid Improves Bone Status in Children with Cerebral Palsy and Rett Syndrome	Zolendronic acid	NA	Increased bone density and reduced incidence of fractures.

1.2 The neurotrophin BDNF

Neurotrophins belong to the neurotrophic factor family, and include nerve growth factor (NGF), BDNF, neurotrophin 3 (NT3) and neurotrophin 4 (also known as neurotrophin 5, NT4/5) (Bibel and Barde, 2000; Skaper, 2018). These proteins act both in CNS and in peripheral nervous system (PNS), and have relevant endogenous roles in neuronal proliferation, growth, differentiation, migration and survival (Skaper, 2018).

These molecules are first synthetized as immature forms, being later converted into their respective mature forms, which primarily act by binding to their high affinity receptors: tropomyosin-related kinase (Trk) receptors. However, they can also bind to a low affinity receptor, the p75 neurotrophin receptor ($p75^{NTR}$) (detailed in subchapter 1.2.2) (Pattarawarapan and Burgess, 2003).

BDNF is expressed in neuronal and non-neuronal cells, and exerts crucial functions in synaptic plasticity, neuronal maturation and differentiation, functions severely compromised in RTT patients. Moreover, BDNF is one of the most studied neurotrophins, given its involvement in several pathologies, and its potential use as a therapeutic agent, namely in RTT context (for review see (Miranda-Lourenço et al., 2020c)).

1.2.1 BDNF expression

BDNF was first purified in 1980s by Barde and colleagues (Barde et al., 1982). In the 1990s, the human *BDNF* gene was mapped, disclosing its location, on 11p14.1 loci, and its structure (Hanson et al., 1992; Kolbeck et al., 1999; Pruunsild et al., 2007). The *BDNF* gene is constituted by a total of 11 coding and noncoding exons, and it is regulated by 9 functional promoters. These promotors control, through alternative splicing, the expression of different transcripts of the same protein. Depending on the tissue, different transcripts are expressed with specific functions (Pruunsild et al., 2007).

BDNF is synthetized in the endoplasmic reticulum in its precursor protein form (pre-pro-BDNF), which is then cleaved to an immature precursor protein form (pro-BDNF) (Lessmann et al., 2003). Pro-BDNF is transported to the Golgi apparatus, where it is proteolytically transformed into the mature protein (mBDNF), by two distinct secretory pathways (Seidah et al., 1996; Mowla

et al., 1999). The constitutive pathway, occurs intracellularly, in the trans-Golgi, and under the action of the furin, a convertase responsible for pro-BDNF cleavage (Al-Qudah and Al-Dwairi, 2016; Miranda-Lourenço et al., 2020c). This pathway ends with mBDNF being packaged in small vesicles, that can be fuse with the cellular membrane and released in a calcium-independent way. On the other hand, the regulatory pathway occurs within secretory vesicles, where pro-BDNF is cleaved by convertases, with its transport and fusion with plasma membrane being calcium-dependent (Seidaha et al., 1996; Mowla et al., 1999; Lessmann et al., 2003). In addition, pro-BDNF processing could occur extracellularly, by the action of tissue plasminogen activator/plasmin system and by matrix-metalloproteinases (MMP) (Pang et al., 2004; Mizoguchi et al., 2011). Furthermore, pro-BDNF can be secreted without being cleaved in which case it will activate $p75^{NTR}$ (Miranda-Lourenço et al., 2020c) (Figure 1.9).

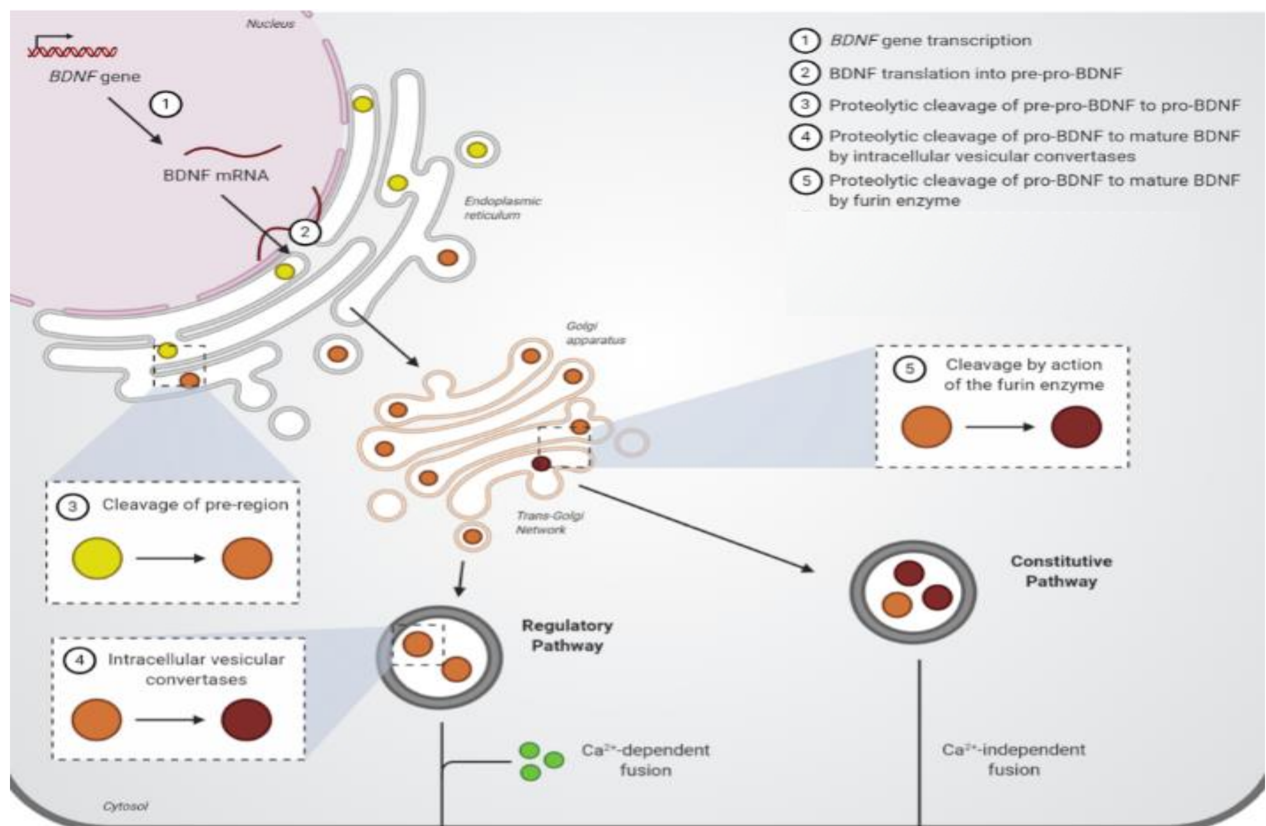

Figure 1.9| BDNF expression and synthesis. BDNF is synthesized as pre-pro-BDNF which is proteolytically cleaved to pro-BDNF. In the regulatory pathway, pro-BDNF is cleaved by proteases within secretory vesicles to form mature BDNF (mBDNF) to be extracellularly released through calcium-dependent mechanisms. In the constitutive pathway, pro-BDNF, localized to the Golgi apparatus, is cleaved by furin to form mBDNF, which is released by calcium-independent vesicular fusion with the plasma membrane. Alternatively, pro-BDNF is released as such and mBDNF maturation occurs extracellularly by tPA/plasmin system and matrix-metalloproteinases. pre-pro-BDNF, precursor protein form pro-BDNF; immature protein precursor; mBDNF, mature BDNF*;* tPA, tissue plasminogen activator *(*adapted from *Miranda-Lourenço et al. 2020b).*

1.2.2 BDNF signaling

BDNF has high affinity for the TrkB receptor, encoded by *neurotrophic receptor tyrosine kinase 2* (*NTRK2*) gene, and low affinity for $p75^{NTR}$ encoded by an homonymous gene (Bothwell, 1996; Reichardt, 2006; Bathina and Das, 2015). The TrkB full-length (FL) isoform is constituted by an intracellular domain composed by a tyrosine kinase domain flanked by a Shc binding site within the juxtamembrane region, and a C-terminal tail containing the phospholipase C gamma (PLCγ) binding site (Middlemas et al., 1991). Its extracellular domain is composed by a short transmembrane sequence, two immunoglobulin-like domains, two cysteine-rich domains, and a cluster of three leucine-rich motifs (Middlemas et al., 1991; Huang and Reichardt, 2003). In addition, *NTRK2* gene expression can also generate other TrkB isoforms, by alternative splicing, known as TrkB truncated isoforms (TrkB-Tc). These truncated forms lack the intracellular tyrosine kinase domain and include: isoform 1 (TrkB-T1), isoform 2 (TrkB-T2), and isoform containing Src homologous and collagen-like adaptor protein binding site (TrkB-T-Shc) (Klein et al., 1990; Middlemas et al., 1991).

After BDNF binds to TrkB-FL, the receptor dimerizes, and the autophosphorylation of tyrosine residues on the intracellular kinase domain occurs. This phosphorylation activates distinct intracellular signaling pathways: phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) pathways linked to cell survival and neuronal differentiation, and the PLC-γ pathway associated with synaptic activity and plasticity phenomena (Finkbeiner et al., 1997; Huang and Reichardt, 2003).

Importantly, both the expression and function of BDNF and TrkB receptor differ slightly according to cell type. In neurons, BDNF binds to TrkB-FL activating the previously mentioned intracellular signaling pathways and in addition it is also able to bind to TrkB-Tc receptors, which act as dominant negative modulators of TrkB-FL receptors (Eide et al., 1996; Dorsey et al., 2006). Otherwise, in astrocytes, the predominant TrkB isoform is the TrkB-T1, that respond to brief exposure to BDNF by triggering calcium release from intracellular stores (Holt et al., 2019). The BDNF/TrkB-T1 signaling is crucial for the maintenance of astrocytic morphology (Rose et al., 2003). Microglia expresses both TrkB-FL and TrkB-Tc receptors. The activation of these receptors, by BDNF, promotes survival and proliferation of microglia itself (Mizoguchi et al., 2011). In addition, upon microglial activation, BDNF secretion plays an important autocrine/paracrine role for microgliosis (Mizoguchi et al., 2011).

p75^{NTR} consists of an extracellular cysteine-rich domain, a single transmembrane domain, and a cytoplasmic death domain. The activation of this receptor by BDNF regulates transcriptional activity and cellular survival, through the recruitment of specific signaling pathway mediators, such as nuclear factor-κB (NF-κB), and Jun kinase (Bamji et al., 1998; Reichardt, 2006). On the other hand, p75^{NTR} signaling through pro-BDNF mediated action, promotes cJun N-terminal kinase signaling pathway, involved in cellular apoptosis (Bamji et al., 1998; Teng et al., 2005) (Figure 1.10).

Nevertheless, BDNF signaling can be influenced by other important players, where adenosine gain a important role.

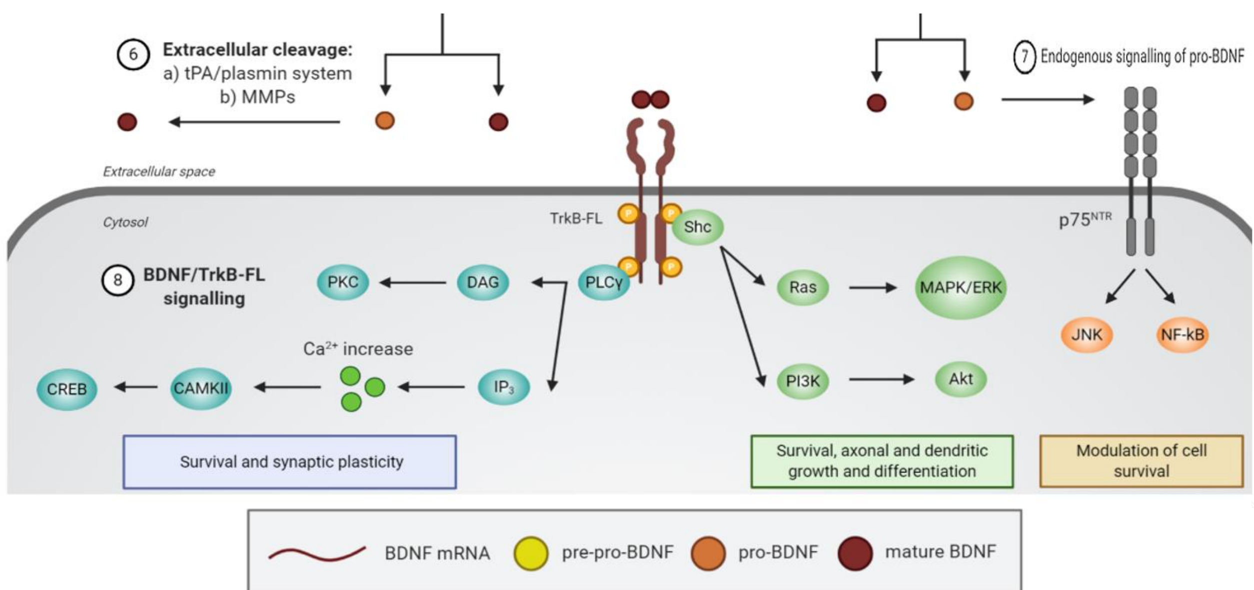

Figure 1.10| BDNF signaling pathways. mBDNF binds the high-affinity receptor, TrkB-FL, promoting receptor dimerization and further signal transduction. The signaling cascades serve many regulatory purposes pertaining survival, axonal and dendritic growth, differentiation, and synaptic plasticity. Pro-BDNF binds to p75^{NTR}, thereby activating apoptotic pathways and modulating synaptic plasticity. Akt, protein kinase B. BDNF, brain-derived neurotrophic factor; CAMKII, calcium/calmodulin-dependent kinase; CREB, cyclic adenosine monophosphate (cAMP) response element-binding protein; DAG, diacylglycerol; ERK, extracellular signal-regulated protein kinase; IP3, Inositol triphosphate; MAPK, mitogen-activated protein kinase; MMPs, matrix-metalloproteinases; p75^{NTR}, p75 neurotrophin receptor. PI3K, phophotydilinositol-3-kinase; PKC, Protein Kinase C. PLC-γ, phospholipase C; pre-pro-BDNF, precursor protein form; pro-BDNF, immature protein precursor; TrkB-FL, full-length tropomyosin-related kinase B, *(adapted from Miranda-Lourenço et al., 2020b).*

1.2.3 An important player for BDNF action: the adenosinergic system

The phosphorylation of TrkB-FL can also be achieved in the absence of BDNF in a mechanism known as transactivation, mainly mediated by ligands of the G-protein-coupled receptor (GPCR),

family of transmembrane receptors, such as adenosine receptors (Rajagopal et al., 2004). Indeed, it has been shown that adenosine and adenosine A_{2A} receptors ($A_{2A}R$) agonists (see section 3.2.2) can directly transactivate TrkB-FL receptors within specialized membrane domains (Lee and Chao, 2001) (Figure 1.11). Additionally, $A_{2A}R$ activation is also able to rapidly boost the effects of BDNF triggered TrkB activation, by promoting the translocation of receptors to lipid rafts (Assaife-Lopes et al., 2014). This crosstalk between TrkB-FL and $A_{2A}R$, facilitates the effects of BDNF on Hebbian forms of synaptic plasticity, modulates BDNF effects on GABA uptake by astrocytes and synaptosomes, and BDNF effects on GABA and glutamate release from nerve terminals (Fontinha et al., 2008; Vaz et al., 2008; Diógenes et al., 2011; Sebastião et al., 2011; Jerónimo-Santos et al., 2014; Rodrigues et al., 2014; Vaz et al., 2015). Moreover, the expression of *Bdnf* and *Nrtk2* genes also involves $A_{2A}R$ activation. Briefly, BDNF increased expression occurs through stimulation of ERK-CREB pathway, in a feed-forward mechanism, that allow a strong induction of BDNF expression that consequently implies a more efficient BDNF signaling (Tebano et al., 2008; Jeon et al., 2011). Since $A_{2A}R$ are G_s-coupled receptors, their activation results in an increased of cAMP followed by ERK-CREB phosphorylation (Huang et al., 2001), which contributes for the promotion of BDNF expression.

This evidence highlights the existence of significant crosstalk between the adenosinergic system and BDNF expression and signaling, that could be relevant in overcoming some BDNF signaling deficits.

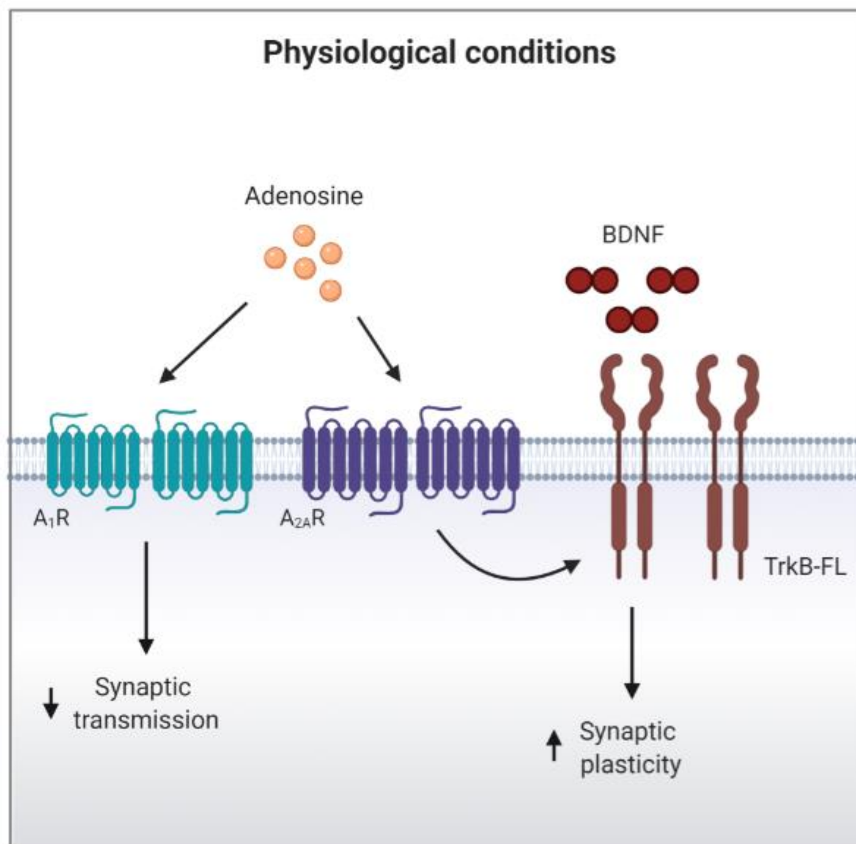

Figure 1.11| Crosstalk between $A_{2A}R$ and BDNF. Physiologically, adenosine acts through its two high affinity receptors, A_1R, and $A_{2A}R$ (see chapter 1.3.2). $A_{2A}R$ crosstalk with TrkB-FL receptors, promoting BDNF action, such as synaptic plasticity, highlighted in this figure, *(adapted from Miranda-Lourenço et al., 2020b)*

1.2.4 BDNF and RTT

Similarly to Mecp2, BDNF expression is low during prenatal development, but it dramatically increases during postnatal period, in the rodent brain (Kolbeck et al., 1999). The coincidence of the two expression patterns also becomes evident in data showing that BDNF levels remain unaffected in the cortex, cerebellum, brainstem and nuclear ganglia of Mecp2-null mice up to the onset of the first symptoms (Chang et al., 2006; Wang et al., 2006). In addition, postnatal conditional deletion of *Bdnf* in the forebrain of mice promotes a phenotype resembling that of Mecp2-null mice, while the overexpression of *Bdnf* in Mecp2-null mice reverses symptoms,

indicating a high relevance of BDNF in RTT pathophysiology (Chang et al., 2006). It is known that MeCP2 binds to *BDNF* at methylated CpG sites adjacent to A/T runs (Klose et al., 2005). But the mechanism behind the regulation of *BDNF* gene expression by MeCP2 is not fully understood. Two opposite models were proposed to explain the specific mechanism underlying this regulation: a repression model and an activation model (Li and Pozzo-Miller, 2014). Nowadays, a mixed model was constructed, integrating both views: the dual operation model, where BDNF control by MeCP2 changes dynamically between the activation or repression (Li and Pozzo-Miller, 2014). Here, epigenetic modifications of DNA and phosphorylation of Mecp2 are apparently relevant mechanisms for this process regulation (Li and Pozzo-Miller, 2014).

The proof-of-concept that the recovery of BDNF expression is able to blunt the phenotypic abnormalities of Mecp2-null mice opened multiple research lines focused on BDNF boosting strategies (Chang et al., 2006; Li and Pozzo-Miller, 2014).

Although the administration of recombinant BDNF could be considered the best way to restore BDNF levels in the brain, its low permeability to blood-brain barrier (BBB) constitutes a significant limitation to its application in humans (Poduslo and Curran, 1996). To overcome this limitation, other pharmacological approaches have been tested, in order to boost or mimic BDNF actions, such as AMPAkines, LMM22A-4, Fingolimod, 7,8-dihydroxyflavone (7,8-DHF) and Copaxone.

Briefly, AMPAkines are a family of nootropic agents that increase BDNF expression by slow desensitization of AMPA-type glutamate receptors, which strengthen excitatory input, consequently promoting the opening of voltage-sensitive calcium channels and NMDA receptors, known to be involved in the stimulation of BDNF gene expression (Ghosh et al., 1994; Lynch and Gall, 2006). These compounds showed good results in restoring breathing abnormalities of *Mecp2*-null mice (Ogier et al., 2007).

Copaxone or Glatiramer acetate (GA) is an anti-inflammatory drug, already approved for multiple sclerosis, and was shown to efficiently increase BDNF expression in Mecp2-mutant mice. This effect upon BDNF expression mainly occurs through GA-induced infiltrating T cells and bystander CNS resident cells (Aharoni et al., 2005; Ben-Zeev et al., 2011).

Fingolimod activates the Erk pathway, which promotes an increase of BDNF expression, with a consequent improvement of the RTT-like phenotype in *Mecp2*-null mice (Deogracias et al., 2012).

LM22A-4 mimics BDNF actions by selectively activating TrkB-FL receptors, leading to a significant amelioration of respiratory problems present in *Mecp2* heterozygous females (Schmid et al., 2012).

7,8-DHF is TrkB-FL agonist which demonstrates improvements on motor function, prevention of both weight loss and breathing pattern abnormalities which, in turn, result in increased lifespan in Mecp2-null mice (Johnson et al., 2012; Jang et al., 2014).

Cysteamine improves BDNF transport and secretion, leading to a decrease of motor deficits, and to increased lifespan in *Mecp2*-null mice (Borrell-Pagès et al., 2006; Roux et al., 2012) (Figure 1.12).

Of these drugs, only Copaxapone and Fingolimod entered in clinical trials. Copaxone was screened both in phase I and II, with reported improvements in gait velocity, memory, and breath holding, but with life-threatening effects caused by the daily injections (Nissenkorn et al., 2017). Simultaneously, a big discrepancy between human and mouse model results was obtained (Djukic et al., 2016). Fingolimod is now being tested in a phase I clinical trial (NCT02061137) to assess the safety and efficacy of its oral administration. Importantly, no adverse event has this far been reported in the RTT children enrolled in the study (Naegelin et al., 2021) (Figure 1.12).

Besides pharmacological approaches, it is known that physical exercise and cognitive stimulation promote an increase of BDNF levels (Baraldi et al., 2013; Hötting and Röder, 2013; Hannan, 2014; Hötting et al., 2016) (Figure 1.12). Indeed, *Mecp2* heterozygous female mice exposed to environmental enrichment during early postnatal stages show a slight increase in motor function (Kondo et al., 2008). Moreover, *Mecp2*-null mice subjected to environmental enrichment during the pre-weaning stage present increased motor coordination, improved spatial learning, reduced anxiety (Lonetti et al., 2010) and restored *ex vivo* LTP in cortical synapses (Kondo et al., 2008; Lonetti et al., 2010). Notably, the improvements in behavioral

phenotypes were correlated with increased levels of BDNF (Kondo et al., 2008; Lonetti et al., 2010).

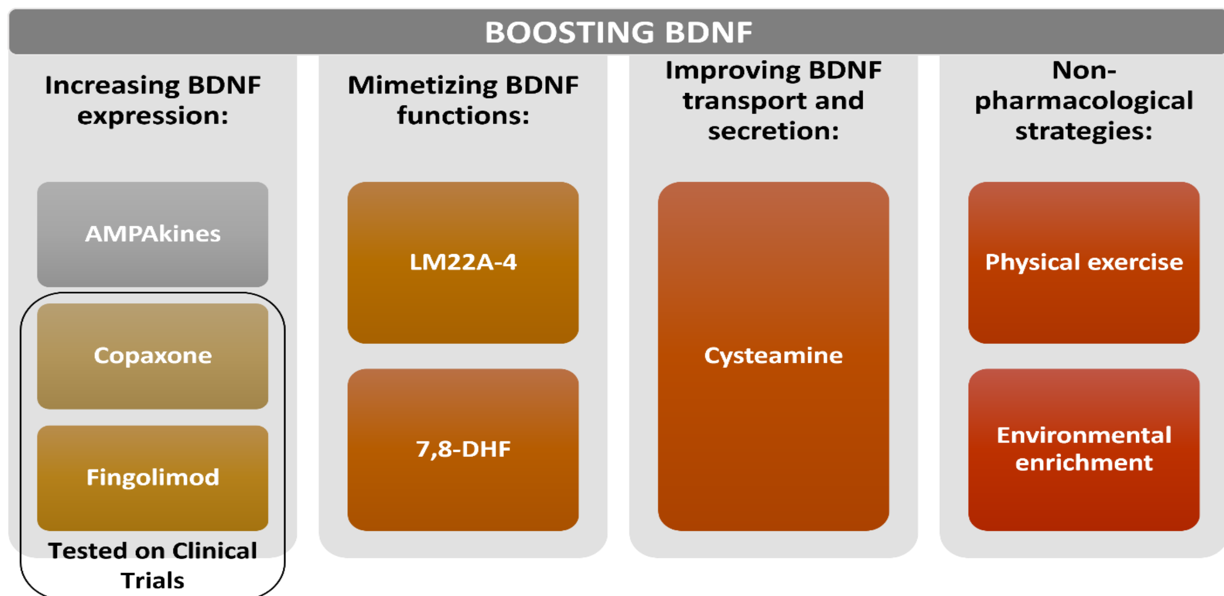

Figure 1.12| BDNF boosting strategies. Boosting BDNF actions could be achieve by pharmacological tools that enable to increase BDNF expression, mimics BDNF function (mainly through TrkB-FL agonists) and improve BDNF transport and secretion. For each mechanism are identified the associated drugs. Whitin these drugs just Copaxone and Fingolimod were tested in clinical trials. In non-pharmacological strategies, physical exercise, and environmental enrichment allow an increase of BDNF expression.

1.3 Adenosine

Adenosine has a key physiological role in several tissues and organs, most importantly in the CNS, where it acts as a neuromodulator (Sebastião and Ribeiro, 2009). Indeed, alterations in the adenosinergic system have been implicated in several pathological processes (for review see (Effendi et al., 2020)). As already mentioned, adenosine also exerts a crucial interaction with BDNF signalling (Sebastião and Ribeiro, 2009) (see subchapter 1.2.3).

Therefore, understanding metabolism and signalling of adenosine should be considered in pathologies with BDNF signalling altered. Its pertinence become more evident when those pathologies present symptomatology usually associated with adenosine dysfunction, such as RTT.

1.3.1 Adenosine metabolism

Adenosine is an endogenous purine nucleoside (Soliman et al., 2018) formed by a nucleobase adenine linked to a ribose by a glycosidic linkage.

Adenosine is produced either intra- or extracellularly (Klotz, 2000; Effendi et al., 2020). The main source of intracellular adenosine production is through dephosphorylation of the nucleotide 5´-adenosine monophosphate (AMP) by cytosolic 5´-nucleotidase (cN)-I. In turn, inosine monophosphate (IMP)/guanosine monophosphate (GMP)-selective cN-II is responsible for respectively converting IMP and GMP to inosine and guanosine. In addition, intracellular adenosine production also results from hydrolysis of S-adenosyl-homocysteine (SAH) by the enzyme SAH hydrolase (Broch and Ueland, 1980). Comparing the two intracellular mechanisms described, the adenosine production by adenosine monophosphate (AMP) dephosphorylation is faster than that relying on SAH hydrolysis. Since adenosine can be formed through endogenous purine synthesis, these alternative mechanisms are important to preserve adequate levels of adenosine triphosphate (ATP) in conditions of ischemia or hypoxia, where there is a mismatch between ATP formation and consumption, resulting in the accumulation of other purines besides adenosine (Kroll et al., 1993; Saito et al., 1999).

Adenosine is bidirectionally transported by sodium-dependent concentrative nucleoside transporters (CNT), in an active way, and by sodium-independent equilibrative nucleoside transporters (ENT), passively (Latini and Pedata, 2001; Gomes et al., 2021).

In physiologic conditions, extracellular adenosine concentration is usually low. Its levels are dependent on: 1) adenosine release from cells, 2) adenosine reuptake by bidirectional adenosine transport processes, and 3) adenosine conversion by ectonucleotidases located on the cell surface (Boison et al., 2010). Extracellular synthesis of adenosine results from the hydrolysis of intracellular nucleotides (AMP; ADP – adenosine diphosphate; ATP), released by exocytosis from vesicles, as well as membrane protein transport during stress, hypoxia, inflammation, or injury (Zimmermann, 1996). The ectonucleotidase cluster of differentiation (CD) 39 is main ectonucleotidase responsible for dephosphorylating ATP and ADP to AMP. This is a surface-located enzyme, mainly expressed in the endothelium, and its action can be reverted by adenylate kinase or extracellular diphosphate kinases, such as the nucleoside diphosphate (NDP) kinase (Kaczmarek et al., 1996; Chadwick and Frischauf, 1998; Bono et al., 2015). The final conversion of AMP to adenosine is mediated by CD73 ectonucleotidase, also known as glycosyl phosphatidyl inositol (GPI)-anchored enzyme, ecto-5'-nucleotidase (Zimmermann, 1992). The action of CD73 is only reversible by intracellular transport of adenosine, after which it can be reconverted to AMP by adenosine kinase (ADK). Alternatively, adenosine can also be metabolized to inosine by ADA (Blackburn and Kellems, 1996; Bono et al., 2015). ADK is the main enzyme responsible for the metabolic clearance of adenosine in physiological conditions, presenting a low-capacity and low-K_m, and rapidly changing its activity when adenosine concentration changes (Bontemps et al., 1983, 1993). ADK expression is highest in organs such as liver and placenta, where adenosine clearance demand is elevated (Fredholm et al., 2005; Boison et al., 2010). On the other hand, ADA, characterized by an high-capacity and - K_m, regulates adenosine levels when these become so elevated as to surpass the capacity of ADK (Boison et al., 2010) (Figure 1.13).

Additionally, extracellular adenosine is produced by inflammatory cells, such as mast cells, leucocytes, neutrophils and eosinophils, promoting healing process after injury and to regulate anabolic and catabolic hormones during stress (Marquardt et al., 1984; Mann et al., 1986; Madara et al., 1993; Effendi et al., 2020).

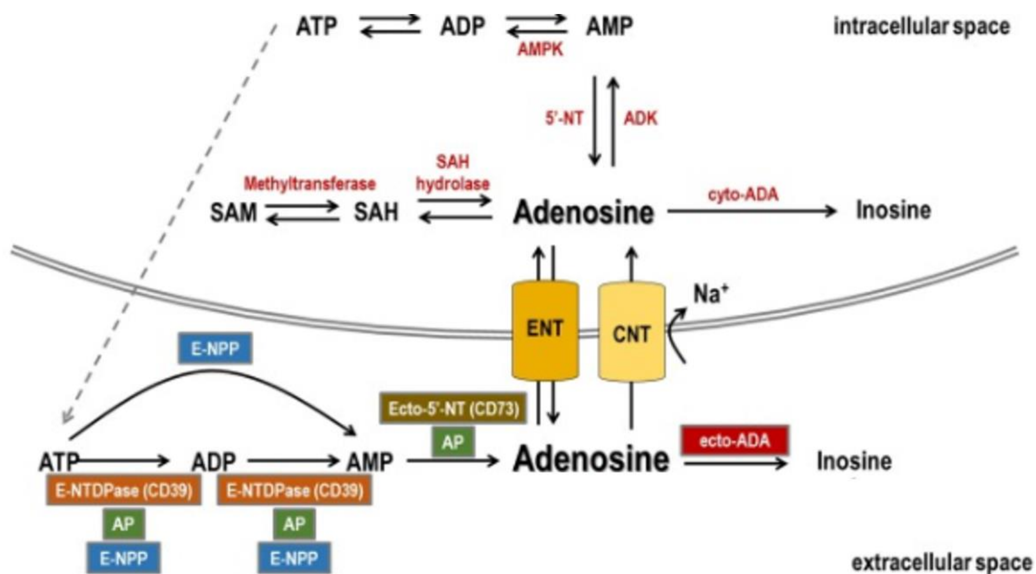

Figure 1.13| Adenosine metabolism. Intracellular adenosine is produced via dephosphorylation from the main source, AMP, via both cN I and cN-II and hydrolysis of S-adenosyl-homocysteine through the enzyme S-adenosyl-L-homocysteine hydrolase. The extracellular formation of adenosine is the result of enzymatic cascades consisting of ATP transport, hydrolysis of ATP and ADP by CD39 to form AMP, and dephosphorylation of AMP by CD73. ADA, adenosine deaminase; ADP, adenosine diphosphate, ADO, adenosine; AMP, adenosine monophosphate; AP, alkaline phosphatase; ATP, adenosine triphosphate; ADK, Adenosine kinase; CD39, ectonucleotidase cluster of differentiation 39; CD73, ectonucleotidase cluster of differentiation 73; ENT, equilibrative nucleoside transporters; ENTPase, Ecto-nucleoside triphosphate diphosphydrolase; GMP, guanosine monophosphate; IMP, inosine monophosphate; INO, inosine; SAH, S-adenosil-homocysteine; SAM, S-adenosil-methionine, *(adapted from Gomes et al. 2021).*

1.3.2 Adenosinergic signalling

The action of adenosine are accomplished through its binding to four different adenosine receptors (AR): A_1R, $A_{2A}R$, $A_{2B}R$ and A_3 (Fredholm et al., 2001). All of these receptors were isolated and purified from mammalian and nonmammalian species, including mice and humans, and are expressed in most cell types (Peleli et al., 2017). Regarding receptor affinity, A_1R and $A_{2A}R$ are the high affinity receptors, with KD ranging from 10-100 nM, whereas the KD for $A_{2B}R$ and A_3R range from 1-5 µM (Soliman et al., 2018).

Despite the fact that these receptors are all G-coupled receptors, they can be grouped into two sets, based on their sequence and its specific G protein binding: 1) A_1R and A_3R which share 49% of overlapping sequence, preferentially couple to $G_{\alpha i/o}$ and act as inhibitors of adenyl cyclase and 2) the second group is composed by $A_{2A}R$ and $A_{2B}R$, sharing 59% of its sequence and coupling to $G_{\alpha s}$, which induce the stimulation of adenyl cyclase (Piirainen et al., 2011) (Figure 1.14).

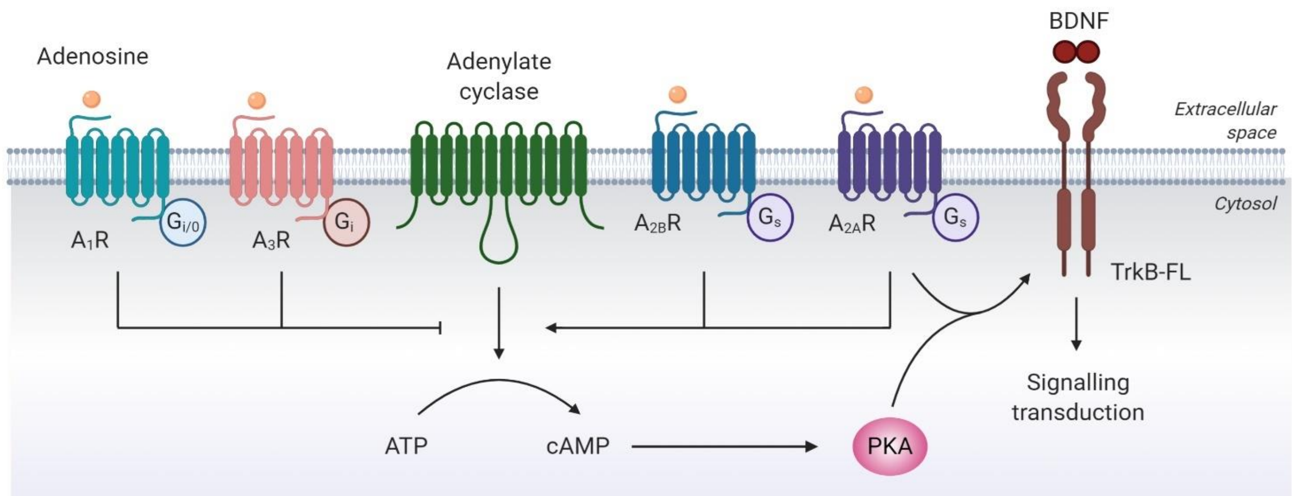

Figure 1.14| Adenosine receptors and respective signaling pathways. A_1R are $G_{i/o}$ protein coupled, while $A_{2A}R$ are G_s protein coupled, leading to opposite effects on cyclic-AMP (cAMP) levels. cAMP can act as a secondary messenger; among other effects, its levels regulate the activity of protein kinase A (PKA), which is necessary for the $A_{2A}R$-mediated facilitatory effect of BDNF fast synaptic actions. A_1R, adenosine 1A receptor. $A_{2A}R$, adenosine 2A receptor. $A_{2B}R$, adenosine 2B receptor. A_3R, adenosine 3 receptor. AKT, protein kinase B. ATP, adenosine triphosphate. BDNF, brain-derived neurotrophic factor. cAMP, cyclic adenosine monophosphate. PKA, Protein Kinase A. TrkB-FL, full-length tropomyosin-related kinase B, *(adapted from Miranda-Lourenço et al., 2020b).*

1.3.2.1 A_1 Receptor

A_1R couples to G_{i-1}, G_{i-2}, G_{i-3}, and G_o, but does not couple to G_s or G_z (Fredholm et al., 1994). As such, this receptor promotes several cellular responses that include an increase of potassium conductance, activation of PLC, inhibition of N-type calcium channels, and generation of calcium, protein kinase C (PKC) and PI3K/MAPK (Effendi et al., 2020).

These receptors are mainly expressed in CNS, testis, kidney and adipose tissue, presenting less expression in the lung and pancreas (Fredholm et al., 2001). In the CNS, they are abundantly expressed in neurons, but also in glial cells, such as astrocytes and microglia (Rebola et al., 2003; Cristóvão-Ferreira et al., 2013). Cortex, cerebellum, hippocampus and dorsal horn of spinal cord

are the CNS regions where A_1R are most expressed (Fredholm et al., 2011). A_1R located pre-synaptically decrease the release of several neurotransmitters, such as glutamate, acetylcholine, serotonin and dopamine. At postsynaptic level, A_1R inhibit NMDA mediated currents and voltage sensitive calcium channels, increase potassium conductance, and disinhibit inhibitory neurons. Therefore, A_1R work as important modulators of synaptic plasticity and synaptic transmission, allowing adenosine to act as a powerful endogenous antiepileptic agent (Boison, 2013a).

Importantly, adenosine action upon CNS is mainly conquered by the balance established between A_1R and $A_{2A}R$ (Fredholm et al., 2005).

1.3.2.2 A_{2A} Receptor

$A_{2A}R$ activation recruits G_s, activating adenylate cyclase which promotes cAMP-dependent protein kinase A (PKA) and PKC activation. The final result of this signaling pathway, is the phosphorylation of several important subtracts such as receptors, ion channels, enzymes and other proteins.

Regarding $A_{2A}R$ distribution, they can be found in the liver, heart, lungs, the immune system (spleen, thymus, leucocytes, and blood platelets) and CNS (Fredholm et al., 2007).

In the striatum, where these receptors are highly expressed, they interact with G_{olf} that presents a considerable expression in both dorsal and ventral striatum (Gessi et al., 2011). Additionally, $A_{2A}R$ are co-expressed with D_2 dopamine receptors in GABAergic striatopalliatal neurons (Fink et al., 1992; Chen et al., 2001a). On opposite to A_1R, when activated pre-synaptically, they increase the release of glutamate, acetylcholine, dopamine, serotonin and GABA (Gomes et al 2021). Postsynaptically, $A_{2A}R$ modulate the activation of NMDA receptors, cannabinoid type 1 receptor, mGluR5 and dopamine receptor type 2 receptors (D_2R) (Gomes et al 2021). Importantly, $A_{2A}R$ stablish an important crosstalk with TrkB-FL receptors, already detailed in 1.2.3 section (Sebastião and Ribeiro, 2009).

1.3.2.3 A_{2B} Receptor

Similarly to $A_{2A}R$, $A_{2B}R$ are G_s-coupled receptors, promoting PKA signaling and consequent stimulation of cAMP. Additionally, $A_{2B}R$ activation promotes G_{q11}-mediated activation of PLC

resulting in increased levels of 1,4,5-inositol triphosphate (IP3)/diacylglycerol (DAG), activation of PKC, and elevation of intracellular calcium levels (Kalla et al., 2009). Their affinity to adenosine can be increased by interaction with PKC, which enhances the affinity of these receptors, important for their participation in physiological responses, namely in myocardium ischemic conditions (Cohen et al., 2010).

$A_{2B}R$ have been found to be distributed in the bowel, bladder, heart, lungs, *vas deferens*, and mouse mast cells, but also in the CNS, where it was found to be expressed in both rat hypothalamus and in human hippocampus (Pierce et al., 1992; Rivkees and Reppert, 1992; Gessi et al., 2011).

1.3.2.4 A_3 Receptor

Likewise A_1R, A_3R are coupled to G_i protein, inhibiting adenylate cyclase, resulting in decreased cAMP stimulation as well as PKA activity. However, A_3R activation can also increase calcium levels and modulate PKC activity, through recruitment of G_q proteins that stimulate PLC (Jacobson et al., 2009; Effendi et al., 2020).

Contrary to what is seen in rats, where A_3R is primarily expressed in testis, in humans its expression is widespread, being most abundant in the lungs and liver (Zhou et al., 1992). In brain, A_3R are the AR with a lower expression, mainly distributed in hippocampus and cerebellum (Liu et al., 2019).

1.3.3 Adenosine and pathology

As the involvement of adenosine in several diseases has been studied, the importance of this physiological system in the maintenance of crucial biological functions has been observed. Moreover, understanding the involvement of adenosine in homeostasis has helped to uncover possible new therapeutic strategies for multiple disorders involving the adenosinergic system (Peleli et al., 2017). However, there is considerable complexity in applying adenosinergic system-targeting compounds in the treatment of any disease, insofar the highly integrated widespread influence of this system makes the prediction of its pharmacological modulation upon disease state and/or progression difficult (Chen et al., 2013). Despite this complexity, the role of AR in pathophysiological processes as well as the therapeutic ends have already been explored in

multiple systems, such as the cardiovascular, pulmonary, inflammatory, renal system, and, most importantly in CNS (Chen et al., 2013; Effendi et al., 2020).

RTT patients present many symptoms superimposed to those caused by the dysregulation of adenosinergic system (Figure 1.15). However, adenosine has never been explored in RTT so far.

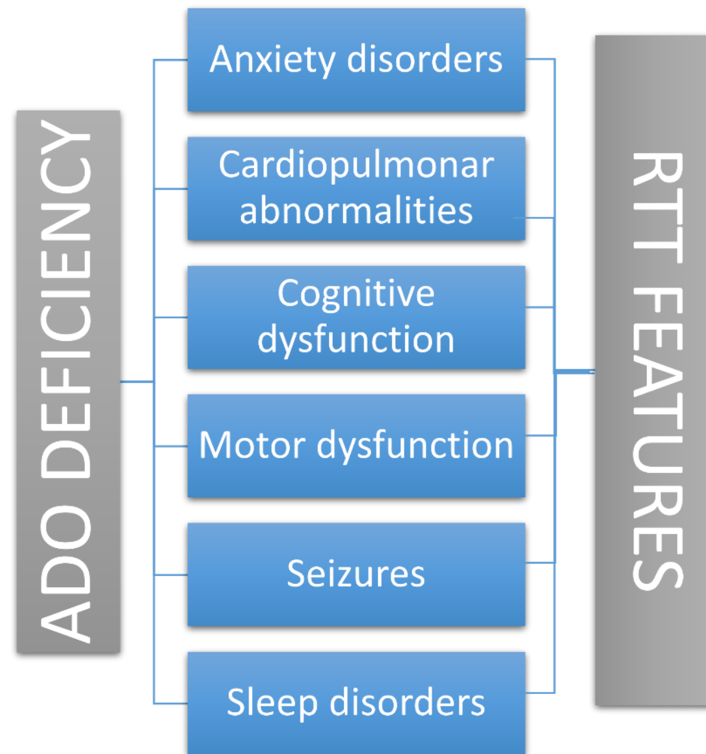

Figure 1.15 | Common features find in RTT and in adenosine deficiency associated pathologies.

Regarding cardiovascular system, A_1R and A_3R are important protective receptors in arrhythmia and ischemia context while $A_{2A}R$ gain relevance in vasodilatory action (Effendi et al., 2020). Concerning pulmonary disease it was suggested that A_1R could have a protective role in lung injury while in asthma the blockage of $A_{2B}R$ and A_3R or the activation of $A_{2A}R$ could be beneficial to contradict adenosine actions as an irritant and bronchoconstrictor (Polosa and Blackburn, 2009; Effendi et al., 2020).

In CNS, adenosine system dysregulation has been reported in several type of disorders: ischemic, neurodegenerative, neuropsychiatric, neurodevelopmental, and circadian rhythm dysfunction, some of them overlapping symptomatology with RTT. In dementia, A_1R antagonism showed potential benefits as a possible pharmacological strategy, since they promote an enhancement of cognitive function, while in pathological conditions A_1R agonism can control anxiety (Gomes et al., 2011; Vincenzi et al., 2017). In the specific case of Parkinson's Disease, the antagonists of $A_{2A}R$ appear as a possible solution, with an already approved drug, istradefylline,

by the Food and Drug Administration (FDA) (Chen and Cunha, 2020; Paton, 2020). The pre-clinical basis of this approach relates to co-expression and -localization of $A_{2A}R$ and D_2R in the striatum, and the formation of heterodimers by both receptors (Ribeiro et al., 2002; Diógenes et al., 2015). Given that striatopallidal pathway dopamine levels are reduced, $A_{2A}R$ antagonism allows to promote D_2R-mediated neurotransmitter release, early gene expression, and striatal D_2R binding, which contribute to the re-establishment of dopaminergic signaling (Ferré et al., 1997; Soliman et al., 2018).

Studies focusing on the action of caffeine, an inhibitor of AR, have helped to decipher adenosine role on sleep-wake cycle (Peleli et al., 2017). In sleep regulation, adenosine has been shown to be an important endogenous sleep promotor (Porkka-Heiskanen, 2009). The most relevant AR for sleep regulation are the A_1R and $A_{2A}R$. Accordingly, the activity of wakefulness-promoting neurons in the cholinergic basal forebrain is inhibited by A_1R activation. On the other hand, in the subarachnoid space below the rostral forebrain, $A_{2A}R$ have a role in the prostaglandin D_2R-mediated somnogenic effects of adenosine (Soliman et al., 2018).

In epilepsy, where a brain circuit imbalance between excitatory and inhibitory transmission is observed, adenosine has been a focus of interest. Its important action as an anti-epileptic agent has been demonstrated to be mediated by A_1R, widely distributed in excitatory synapses, when inhibiting glutamate release, decreasing glutamatergic responsiveness and hyperpolarising neurons (Boison, 2012). Additionally, it has been reported that adenosine levels increase during seizure, while in some forms of epilepsy a pathologic decrease of adenosine levels has been observed (During and Spencer, 1992; Etherington et al., 2009). Congruently, the activation of A_1R and/or the enhancement of adenosine levels have been shown to be promising strategies to combat epilepsy (Boison, 2009; Gomes et al., 2011). Since the outcomes from AR modulation might be different in the context of different pathologies, approaches targeting these receptors should be carefully studied. Adenosine augmentations therapies (AAT) are one possible strategy to be implemented, particularly when the activation of more than one AR has beneficial effects (Boison, 2009).

1.3.4 Adenosine augmentation therapies

The existing evidence showing adenosinergic system changes in multiple disorders encouraged the exploration of this system as a possible therapeutic target. Some strategies involve the use of specific receptor agonists or antagonists, according to the particular situation (Chen et al., 2013). However, the increase of adenosine levels, rather than specific receptor activation, has also been proven to be a beneficial strategy in select disorders, such as epilepsy (Boison, 2009), a condition significantly present in RTT patients.

As described in section 1.3.1, ADK is a key enzyme in adenosine metabolism (Boison, 2013b). Given the role of ADK in adenosine metabolism and, consequently, in adenosine homeostasis, several studies explored inhibition of ADK as possible way of increasing adenosine levels (Theofilas et al., 2012; Boison, 2013b). In epilepsy, this strategy was shown to be highly beneficial, since a link between increased ADK protein levels, and decreased adenosine levels, associated to neuronal dysregulation, were described (Li et al., 2008). Simultaneously, small increases in adenosine are sufficient to promote inhibitory A_1R-mediated effects, with importance in the control of epileptic states, suggesting that modulation of A_1R function is also useful in the context of epilepsy (Boison, 2013b). In fact, A_1R are the AR with the most significant involvement in seizures (Masino et al., 2014). In multiple types of experimental epilepsies and seizures it has been shown that both systemic and intracranial administration of A_1R agonists decrease seizures and convulsions (Gouder et al., 2003; Güttinger et al., 2005; Heidarianpour et al., 2006; Girardi et al., 2007, 2010). Conversely, systemic administration of A_1R antagonists aggravates seizures and convulsions (Fukuda et al., 2010). Interestingly, it has also been reported that A_1R agonists promote the efficacy of standard ASD while, A_1R antagonists have the opposite action (Borowicz et al., 2002; Łuszczki et al., 2005; Zuchora et al., 2005).

Despite its efficiency in the suppression of seizures, the systemic delivery of A_1R agonists or ADK inhibitors could be associated to side effects. ADK interferes with methionine metabolism in liver, leading to hypermethioninemia, inhibition of transmethylation, and severe liver pathology (Bjursell et al., 2011; Boison, 2013b). In addition, it and can increase the risk of brain hemorrhage (Jarvis, 2019). On the other hand A_1R agonism can lead to cardiac side effects (Tosh et al., 2012).

To overcome these problems alternative methods using focused delivery of these compounds have been tried, such as: synthetic slow-release polymers and pump systems, which

can be coupled to integrated seizure prediction systems (Boison, 2009). However, these systems demand repetitive device implantations, or specific procedures to refill the device over the lifespan of the patient. Long-term solutions are being tested, such as cell therapy, including stem cell transplantation, or gene therapy approaches to locally augment the adenosine levels (Boison, 2009).

The beneficial effects of adenosine upon seizure control have been demonstrated by the ability of adenosine to regulate epigenetic mechanisms, namely DNA methylation (Williams-Karnesky et al., 2013; Boison, 2016). It is known that DNA methylation inhibits epileptogenesis in multiple seizure models, which correlates with increased DNA methyltransferase activity, disruption of adenosine homeostasis, and spontaneous recurrent seizures when hypermethylation occurs (Williams-Karnesky et al., 2013). Given that, AAT can exert beneficial effects by different mechanisms.

Additionally, a link between ADK overexpression and cognitive impairment has been pointed in some pathological conditions, such as epilepsy, Alzheimer's Disease, Parkinsons's Disease and amyotrophic lateral sclerosis (Boison, 2007; Boison et al., 2010). All of these diseases have significant astrogliosis as an histopathological hallmark (Schiffer et al., 1996; Renkawek et al., 1999; Ala et al., 2000; Yamanaka et al., 2008), suggesting that astrogliosis-associated to ADK overexpression might be implicated in cognitive dysfunction present in different neurological conditions (Boison, 2013b).

Given the multiple evidence showing that increased adenosine tonus could be beneficial, strategies other than ADK inhibition could be leveraged to increase adenosine levels through decreased adenosine metabolism (Schmidt et al., 2009; Essawy and Elbaz, 2013). Allopurinol increases A_1R activity, and acts as an inhibitor of xanthine oxidase, increasing all the metabolites upstream of its action, from adenosine to acid uric pathway (Schmidt et al., 2009). Additionally, allopurinol is already used as an uricosuric drug, and is well tolerated by patients. Furthermore, it showed positive results in controlling seizures, in two double-blind and placebo-controlled studies (Coppola and Pascotto, 1996; Togha et al., 2007).

Non-pharmacological strategies, such as the ketogenic diet, also present some beneficial effects over seizure control, with the adenosinergic system participating in this action. The ketogenic diet increases ATP, involved in adenosine metabolism. Simultaneously, ketogenic

metabolism increases extracellular adenosine, leading to A_1R mediate anticonvulsant effect reliant on presynaptic inhibition of glutamatergic terminals and postsynaptic hyperpolarization by increasing potassium channel permeability (Masino et al., 2011; Ruskin et al., 2020).

Thereby, AAT, mainly through ADK inhibition, can target simultaneously different pathological mechanisms present in neurological disorders.

Chapter 2

Aims

Chapter 2 - Aims

RTT is an X-linked dominant disorder with severe intellectual disability and epilepsy among other symptoms. Abnormal expression and function of brain-derived neurotrophic factor (BDNF) have been highlighted as important contributor for neurological dysfunctions present in RTT patients. Therefore, restoring BDNF signaling would be an important achievement, but it has been hampered by the difficulty of BDNF to cross the blood-brain barrier and, consequently, to reach the brain when peripherally administered.

BDNF synaptic actions are facilitated by the activation of $A_{2A}R$, therefore agonists of $A_{2A}R$ would be a good strategy to potentiate BDNF actions. Simultaneously, it is known that A_1R act as an important anticonvulsant modulator which could be important in RTT context given the present imbalance of excitatory/inhibitory tonus. However, adenosinergic system has never been addressed in RTT.

Accordingly, the following specific objectives were pursued in this work:

AIM 1: Characterize adenosinergic system and BDNF signaling:

AIM 1.1: in a severe model of RTT (*Chapter 4.1*);

AIM 1.2: in a mild model of RTT (*Chapter 4.2*).

AIM 2: Explore adenosine augmentation therapy (AAT) as a possible strategy for RTT (*Chapter 4.3*).

Chapter 3

Methods

Chapter 3 - Methods

3.1 Animals

The experiments were performed in Mecp2tm1.1Bird/J (Mecp2 knockout/null (KO), deletion of exons 3 and 4 of the Mecp2 gene) mice (Guy et al., 2001), in particular in null male adult mice (Mecp2$^{-/y}$) in pre-symptomatic stage (1 and 3 weeks old animals) and in symptomatic stage (6 to 10 weeks old animals) and in heterozygous female adult mice (Mecp2$^{-/+}$) at symptomatic stage (26 to 30 weeks old).

Age- and sex-matched WT littermates were used as controls. The genotype of animals was determined by polymerase chain reaction, as previously described (Guy et al., 2007).

The animals were housed on a 12 hours light/dark cycle, with food and water provided *ad libitum*. Experiments involving animals were taken into careful consideration in order to reduce the number of animals sacrificed. All animals were handled according to the Portuguese law on Animal Care and European Union guidelines (86/609/EEC).

3.1.1 Adenosine Augmentation Therapy: 5-Iodotubercidin treatment

5-Iodotubercidin (ITU, Sigma-Aldrich, I100-5MG, Darmstadt, Germany) was properly reconstituted and stored in 10 mg/mL aliquots at -20ºC. ITU was dissolved in 20% DMSO at a final concentration of 0.16 mg/ml. During five consecutive days, at 36-41 days after birth (5 to 6 week of age), two intraperitoneal (i.p.) injections within 8 hours interval, in a dose of 1.6 mg/kg was injected in each test animal. Control injections of vehicle (20% DMSO in saline (v/v)) alone were performed (Sandau et al., 2019). Experimental design consisted of four groups defined by genotype (WT or Mecp2$^{-/y}$) and drug treatment (vehicle or ITU). Note that this set of experiments were just performed in Mecp2$^{-/y}$ symptomatic animals.

Animal weights were recorded before each i.p. injection. Animal well-fare were daily controlled, and any necessary support were given by the veterinarian of iMM Biotério (Rodent Facility).

3.2 *Ex vivo* electrophysiological recordings

3.2.1 Hippocampus isolation and slice preparation

The animals were first anesthetized with isoflurane (1,2-Propylenglycol 50% (v/v)) in an anaesthesia chamber. When animals showed the first signs of anaesthesia state, like reduction of respiratory rate and lack of reflexes, they were sacrificed by decapitation. In order to have access to the brain, the skull was exposed by cutting the skin at the top of the head and then the brain was carefully removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing: 124 mM NaCl; 3 mM KCl; 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1 mM $MgSO_4$, 2 mM $CaCl_2$ and 10 mM glucose previously gassed with 95% O_2 and 5% CO_2, pH 7.4. The two brain hemispheres were separated through the midline and the hippocampus was isolated. After isolated, the hippocampus was cut perpendicularly to the long axis into slices with 400 µm thickness with the Mcllwain tissue chopper. Slices were then placed in a resting chamber in Krebs' solution permanently oxygenated at room temperature for one hour in order to recover.

3.2.2 Basal synaptic transmission

After functional and energetic recovery, slices were transferred to the recording chamber for submerged slices, continuously superfused with bathing solution (aCSF) gassed with 95% O_2 and 5% CO_2 at 32˚C. The flux of bathing solution was established at 3ml/min and the drugs used were added to this superfusion solution.

Recordings were obtained with Axoclamp 2B amplifier and digitized (Axon Instruments, Foster City, CA). Individual responses were monitored and averages of eight consecutive responses were continuously store on personal computer with *LTP program* (Anderson and Collingridge, 2001). Field excitatory postsynaptic potentials (fEPSPs) were recorded through an extracellular microelectrode (2-8 MΩ resistance, Harvard apparatus LTD, Fircroft way, Edenbridge, Kent) placed in the *stratum radiatum* of the CA1 area (Figure 3.1 **A**). Stimulation (rectangular 0.1 ms pulses, once every 15 seconds) was delivered through a concentric electrode placed on the Schaffer collateral-commissural fibers, in the *stratum radiatum* near CA3-CA1 border. The stimulus intensity (80-200 µA) was initially adjusted to obtain a large fEPSP with a minimum population spike contamination (Figure 3.1 **B**).

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

These experiments were performed in Mecp2$^{-/+}$ symptomatic animals and aged matched WT animals.

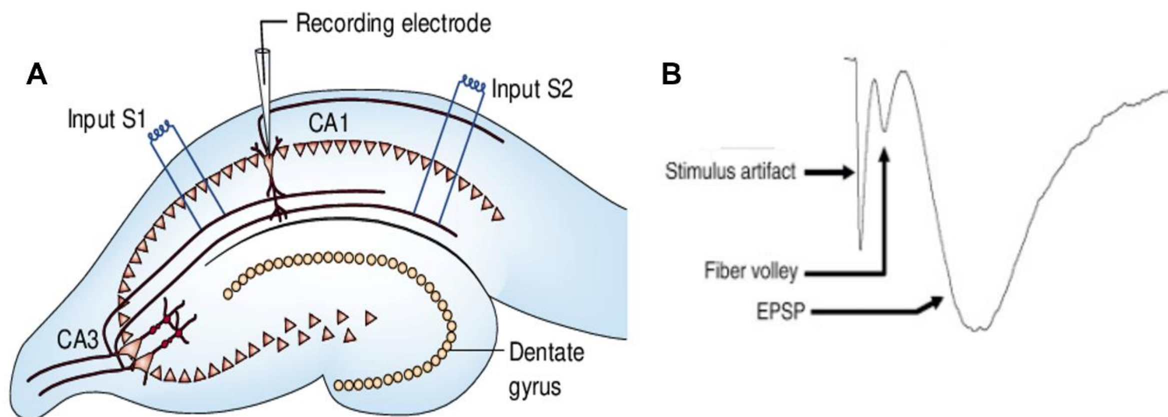

Figure 3.1| Extracellular recording from hippocampal slices. A Represents the stimulation of two sets of the Shaffer collateral-commissural pathway (S1, S2) in a hippocampal slice to record extracellular responses in the CA1 dendritic layer (*stratum radiatum*). **B** Representative tracing obtained, composed by stimulus artefact, followed by presynaptic/fiber volley and the field excitatory postsynaptic potentials (fEPSP), *(adapted from Martin and Kosik, 2002; Anderson et al., 2017).*

3.2.3 Long-term potentiation (LTP) induction and quantification

fEPSPs were elicited and recorded as outlined above. Stimulation was delivered alternatively to two independent pathways. LTP was induced by a θ-burst protocol consisting of three trains of 100 Hz, three stimuli, separated by 200 ms as previously described (Diógenes et al., 2011).

LTP was quantified as the % change in the average slope of the fEPSP taken from 50-60 min after LTP induction in relation to the average slope of the fEPSP measured during the 10 min that preceded the induction of LTP. In each individual experiment, the same LTP-inducing paradigm was delivered to each pathway. After 1 h of LTP induction in one of the pathways, BDNF (20 ng/ml) was added to the superfusion solution and LTP was induced in the second pathway, no less than 15-20 min after BDNF perfusion and only after stability of fEPSP slope values was observed for at least 10 min (Figure 3.1B). The effect of BDNF upon LTP was evaluated by comparing the magnitude of LTP in the first pathway in the absence of BDNF (control pathway), with the

magnitude of LTP in the second pathway in the presence of BDNF (test pathway); each pathway was used as control or test on alternate days.

These experiments were performed both in Mecp2$^{-/y}$ and Mecp2$^{-/+}$ symptomatic animals and aged matched WT animals.

3.2.4 Input–output curve (I/O)

After obtaining a stable baseline for at least 10 min, the stimulus delivered to the slice was decreased until no fEPSP was evoked and subsequently increased in 20 µA steps. For each stimulation intensity, data from three consecutive averages of 8 fEPSP were collected. Inputs delivered to slices typically ranged from 60 µA to a supramaximal stimulation of 320 µA. The I/O curve was plotted as the relationship of fEPSP slope vs stimulus intensity, which provides a measure of synaptic efficiency, as previously described (Diogenes et al., 2012).

These experiments were performed both in Mecp2$^{-/y}$ and Mecp2$^{-/+}$ symptomatic animals and aged matched WT animals.

3.2.5 Pharmacological tools

Table 3.1| Drugs used in electrophysiology experiments.

Abbreviation	Designation	Function	Used Concentration	Supplier
BDNF	Brain-derived neurotrophic factor	TrkB-FL endogenous ligand	20 ng/mL	Regeneron Pharmaceuticals
DPCPX	1,3-Dipropyl-8-cyclopentylxanthine	A_1R selective antagonist	50 nM	Tocris (Natick, MA, USA)
CGS-21680 (CGS)	2-p-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine	$A_{2A}R$ selective agonist	10 nM	Tocris (Natick, MA, USA)

Note: CGS was aliquoted as stock solutions of 5mM in DMSO (CPA and DPCPX) and BDNF in 1.0 mg/ml stock solution in 150 mM NaCl, 10 mM sodium phosphate buffer, and 0.004% Tween 20.

3.3 Molecular techniques

3.1.1 Western-Blot (WB)

3.1.1.1 Protein Extraction

The respective brain areas intended to study were first dissected in ice-cold aCSF solution: NaCl 124 mM; KCl 3 mM; NaH2PO4 1.25 mM; NaHCO3 26 mM; MgSO4 1 mM; CaCl2 2 mM; and glucose 10 mM, previously gassed with 95% O2 and 5% CO2, pH 7.4) solution, washed in PBS solution (NaCl 137mM, KCl 2.1mM, KH2PO4 1.8mM and Na2HPO4.2H2O 10mM, pH 7.4) and immediately snap-frozen and stored at -80ºC until homogenates preparation.

Snap-frozen brain samples were disrupted using a sonicator (Sonic & Materials Inc., CT, USA) in 250µl (hippocampus and striatum) or 500µl (cerebellum, cortex and brainstem) of Ristocetin Induced Platelet Agglutination (RIPA) lysis buffer (1 M Tris pH 8.0, 0.5 M EDTA pH 8.0, 5 M NaCl, 0.1% Sodium Dodecyl Sulfate (SDS), 10% Nonidet P-40 (NP-40), 50% Glycerol) supplemented with cOmpleteTM Mini protease inhibitor cocktail tablets (Mini-Complete EDTA-free; Roche Applied Science, Penzberg, Germany).

All lysates were then vortexed and sonicated (3 cycles of 15 seconds). The protein content in the supernatant was determined by Bio-Rad DC reagent, a commercial Bradford assay (Sigma®).

3.1.1.2 Protein Electrophoresis, Transfer and Detection/Quantification

Equal amounts of protein were loaded (70 µg except for A_{2A}R blot: 200 µg) and separated on 10-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare). To check protein transfer efficiency, membranes were stained with Ponceau S solution. After blocking with a 5% non-fat dry milk solution in TBS-T (20 mM Tris base, 137 mM NaCl and 0.1% Tween-20), membranes were washed three times with TBS-T, before incubation with the primary antibody, diluted in 3% BSA solution in TBS-T (overnight at 4˚C) and secondary antibodies diluted in blocking solution (1 hour at room temperature).

Immunoreactivity was visualized using ECL chemiluminescence detection system (Amersham-ECL Western Blotting Detection Reagents from GE Healthcare) and band intensities were quantified by digital densitometry (ImageJ 1.45 software). GAPDH was used as loading control.

3.1.1.3 Antibodies

Table 3.2| Western-Blot primary antibodies.

Protein	Primary Antibody	Dilution	Supplier
A_1R	Rabbit polyclonal, Anti-A_1R	1:1000	Santa Cruz Biotechnology (sc-28995)
$A_{2A}R$	Mouse monoclonal, Anti-$A_{2A}R$, clone 7F6-G5-A2	1:1500	Merk Millipore (05–717)
ADK	Rabbit polyclonal anti-ADK	1:1500	Bethyl Laboratories (A304-280A)
BDNF	Recombinant Anti-BDNF antibody [EPR1292]	1:1500	Abcam (ab108319)
GAPDH	Mouse monoclonal anti-GAPDH	1:5000	Ambion® by life technologies (AM4300)
TrkB-FL and TrkB-TC	Mouse monoclonal anti-TrkB	1:1500	BD Transduction Laboratories (610101)

Note: $A_{2A}R$ protein levels were assessed by WB using an antibody validated using $A_{2A}R$ KO samples (Figure 3.2).

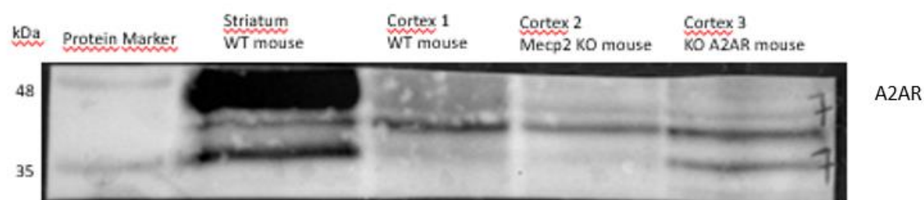

Figure 3.2| $A_{2A}R$ detection by WB. A represents $A_{2A}R$ density bands obtained in WT mouse striatum (positive control), WT and Mecp2 KO cortex (test samples from $Mecp2^{-/y}$) and in $A_{2A}R$ KO mouse cortex (negative control).

Table 3.3| Western-Blot secondary antibodies.

Secondary Antibody	Dilution	Supplier
Goat anti-mouse IgG-HRP	1:10000	Santa Cruz Biotechnology (sc-2005)
Goat anti-rabbit IgG-HRP	1:10000	Santa Cruz Biotechnology (sc-2004)

3.4 Liquid chromatography with diode array detection (LC/DAD)

The purines (ATP, AMP, adenosine and inosine) content of extracts from the cortex and hippocampus of WT and $Mecp2^{-/y}$ or $Mecp2^{-/+}$ mice was measured by liquid chromatography with diode array detection (LC/DAD). Snap-frozen cortex and hippocampus tissue samples were stored at -80ºC until use. For extraction, the samples were defrosted (250 ul) in round-bottom microcentrifuge tubes followed by thorough tissue homogenisation using a mixture of ice-cold acetonitrile : methanol : water (1:2:2) solution (Jackson et al., 2017) containing 2-chloro-adenosine (5 µM) as internal standard; the obtained mixture was centrifuged at 16000 g for 20 min at 4◦C. Tissue homogenization and centrifugation were repeated twice. The two recovered supernatant extracts (~250 µl each) were mixed together and, then, centrifuged again at 16000 g (for 20 min at 4◦C) using a 50-kDa cutoff filter (Amicon Ultra-0.5 50K Filter Device; Merck KGaA, Darmstadt, Germany). After filtration, supernatant extracts were divided in 15 µl aliquots and stored at -80ºC until analysis. Using this procedure, recovery of adenine nucleosides was higher than 95%, as determined by adding 2-chloro-adenosine (5 µM) as internal standard before extraction.

Extraction media containing purines were 1:10 diluted with water before LC/DAD analysis. Chromatographic separation of nucleosides was carried out using an elution gradient composed of ammonium acetate (5 mM, with a pH of 6 adjusted with acetic acid) and methanol (Silva et al., 2017, 2020; Vieira et al., 2017). Separation of adenine nucleotides was carried using a paired-ion chromatography reagent (PIC Reagent A; Waters Chromatography Europe BV, Etten-Leur, The Netherlands) containing tetrabutylamonium phosphate (1 mM) in KH2PO4 (100 mM, at pH=6) (Waters) and methanol. Separation of adenine nucleotides and nucleosides using both elution systems was achieved by reversed–phase liquid chromatography through a Hypersil GOLD C18 column (5 µM, 2.1 mm × 150 mm) equipped with a guard column (5 µm, 2.1 mm × 1 mm) and assayed using a Finigan Thermo Fisher Scientific System LC/DAD, equipped with an Accela Pump coupled to an Accela Autosample, a diode array detector and an Accela PDA running the X-Calibur software chromatography manager. Quantification of adenine nucleotides and nucleosides was carried out using calibration curves made of high-purity external standards, namely ATP, AMP, adenosine and inosine.

Experiments regarding this technique were performed in collaboration with Professor Paulo Correia de Sá in ICBAS, Porto.

3.4 Statistics

The data are expressed as mean ± SEM of the n number of independent experiments. The significance of differences between the means of 2 conditions was evaluated by paired or unpaired t-test; between the mean of 4 different groups by two-way ANOVA. Welch correction was used in unpaired t-test as appropriate. Nonlinear regressions were used to fit data pertaining I/O curves. Pearson correlation analyses were performed to analyze correlation between Mecp2 protein levels and BDNF. Values of $p < 0.05$ were considered to represent statistically significant differences. GraphPad Prism 5.00 was used to performed statistical analysis.

Chapter 4

Results

Chapter 4.1 – Alterations of BDNF signaling and adenosinergic system in a severe model of RTT

1. Rational

Previous studies have shown that BDNF levels are decreased in RTT mouse models. Thus, dysregulation of BDNF functions in RTT has been suggested as a disease mechanism for the deficits in synaptic transmission and plasticity, neuronal survival and development (Asaka et al., 2006; Moretti et al., 2006; Li et al., 2012; Robinson et al., 2012; Li and Pozzo-Miller, 2014). Accordingly, BDNF mRNA levels were shown to be decreased in *post-mortem* brain samples from human patients affected with this disease. However, no changes were detected in BDNF protein levels on cerebrospinal fluid and on blood serum samples collected from RTT patients (Vanhala et al., 1998; Riikonen, 2003; Abuhatzira et al., 2007; Deng et al., 2007). Experiments where BDNF expression levels were genetically manipulated, in RTT mouse models, have shown that BDNF overexpression led to symptomatic improvements (Chang et al., 2006; Guy et al., 2007; Li and Pozzo-Miller, 2014). However, the therapeutic designs involving BDNF delivery to the brain are still inefficient, because the BBB is impermeable to this neurotrophin (Poduslo and Curran, 1996; Li and Pozzo-Miller, 2014). In order to overcome this limitation, other strategies should be considered, including the use of other molecules able to boost BDNF actions.

It is now currently accepted that most of BDNF synaptic actions are dependent on the activation of a specific type of AR, the $A_{2A}R$ (Sebastião et al., 2011). On the other hand, another subtype of AR, the A_1R, have well-known inhibitory actions upon synaptic transmission with impact in epilepsy control (Rombo et al., 2016). Modulation of AR, mostly through A_1R and $A_{2A}R$, has long been considered as a useful strategy to treat several neurologic disorders, such as sleep disorders, epilepsy and neurodegenerative diseases (Jacobson and Gao, 2006; Sebastião and Ribeiro, 2009; Gomes et al., 2011), but it has never been put forward for RTT. Clinical trials using drugs that modulate AR have been developed already (Chen et al., 2007; Jacobson et al., 2019), but RTT had never been under focus. On the light of the knowledge on: i) the crosstalk between BDNF and AR, ii) the advantages of potentiating BDNF actions in RTT, and iii) the dysregulation of the adenosinergic system in several pathological conditions co-occurring with epilepsy; we hypothesized that AR or adenosine signalling could be valid therapeutic targets to explore in RTT.

To tackle that hypothesis, we used a well-recognized RTT mice model, *Mecp2*-null mice (*Mecp2*$^{-/y}$) to identify putative changes in the adenosinergic system and to assess if pharmacological modulation of AR could potentiate BDNF effects in this RTT model.

The data presented on this chapter have been partially published in the paper (Miranda-Lourenço et al., 2020a) (Attachment 1), exception for the characterization of the different BDNF and adenosinergic system proteins in multiple brain areas, excluding cortex and hippocampus during symptomatic stage (data in pre-symptomatic animals from subchapter 2.1, 2.2 and 2.3 – Figure 4.1.6 and 4.1.8). Some results here presented were integrated in Sofia Duarte PhD thesis (data from subchapter 2.3 - Figure 4.1.4 **C** and **B**; and subchapter 2.6) and in Cátia Palminha Master thesis (data from subchapter 2.3 – Figure 4.1.4 **F**). Adenosine measurements by HPLC (subchapter 2.2 – Figure 4.1.5) were performed in collaboration with Prof. Paulo Correia de Sá from ICBAS, Porto.

2. Results

2.1 BDNF protein levels evaluation

Previous data suggest that MeCP2 acts both as a repressor and as an activator of gene expression (Chahrour et al., 2008). Given that the *BDNF* gene is under MeCP2 transcriptional regulation (Chen et al., 2003) attention has been directed to BDNF protein changes on RTT. It was described that the profile of BDNF expression coincides with profile of MeCP2 expression: BDNF and Mecp2 present low expression levels during prenatal period, raising its level postnatally, in rodent brain (Kolbeck et al., 1999). Interestingly, it has been shown that BDNF levels remain unaffected during pre-symptomatic stage of Mecp2-null mice. However, its decrease in cortex, cerebellum, nodose ganglia and brainstem is observed with the onset of behavioral and neurological changes characteristic of RTT features (Chang et al., 2006; Wang et al., 2006). These descriptions were made during pre-symptomatic stage (2 weeks after birth) in all brain homogenates, not allowing a discrimination between brain regions (Chang et al., 2006). Or, when performed in different brain regions, the timepoints studied were limited to P0 (Wang et al.,

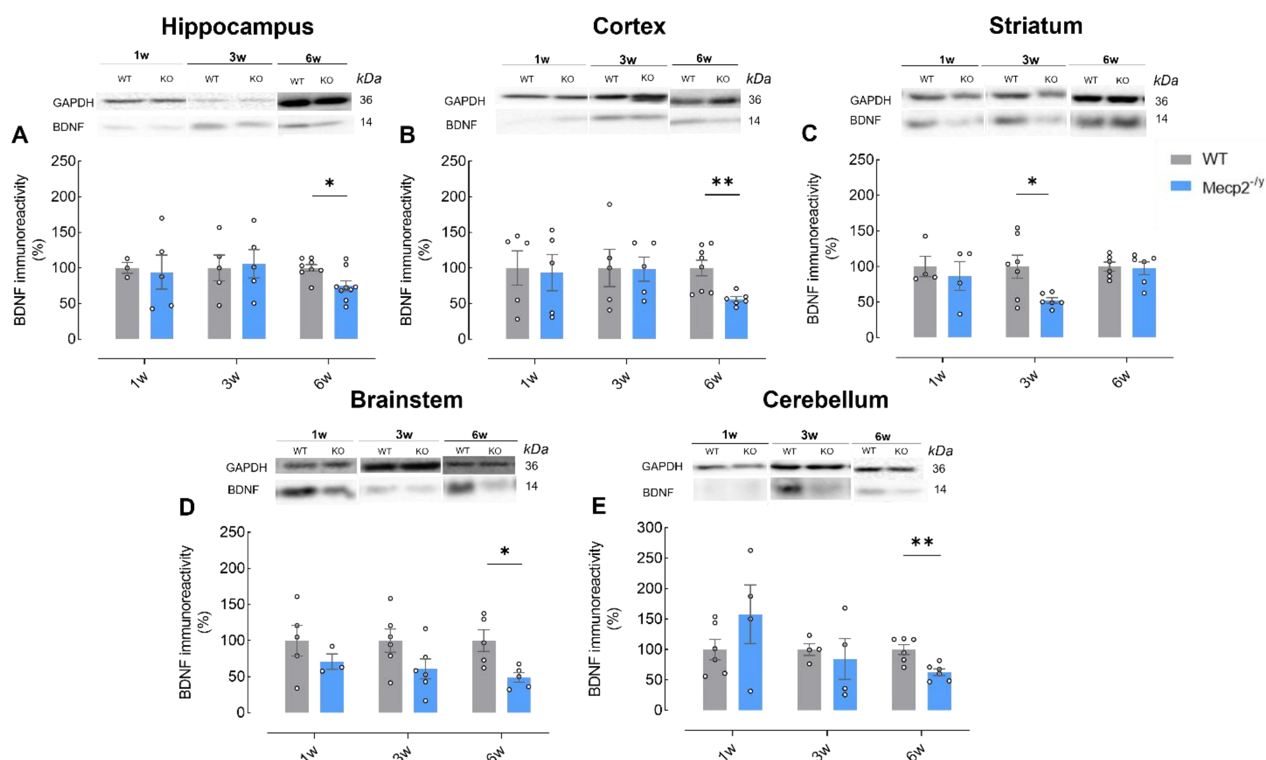

Figure 4.1.1| BDNF protein levels in pre-symptomatic and symptomatic stage in different brain areas. In panels **A-E** are shown the averaged BDNF density levels, respectively, in hippocampus, cortex, striatum, brainstem and cerebellum by Western Blot analysis of WT (grey bar, n=3-8) and Mecp2$^{-/y}$ (blue bars, n=3-6) animals with 1 (1w), 3 (3w) and 6-10 (6w) weeks of age. The results are represented in % of WT protein. Representative bands are shown for each brain area. All values are mean±standard error of mean (SEM). *p<0.05; **p<0.01 (Student's t-test).

2006). Given that, we characterized BDNF protein levels by WB in pre-symptomatic stage at different time points: 1 week (w) and 3w of age (week before the beginning of the symptoms); and also in symptomatic stage at 6w.

Regarding the results obtained in pre-symptomatic stage, we observed similar BDNF protein levels both at 1w and 3w in cortex (CTX) and hippocampus (HIP) of Mecp2$^{-/y}$ comparing with WT (Figure 4.1.1 **A;B**). Concerning brainstem (BS), BDNF protein levels in this brain region showed a tendency to lower levels at 1w and 3w despite not statistically different (Figure 4.1.1 **D**). In cerebellum (CB), we found a slight increase of BDNF protein levels at 1w, with protein levels similar to WT levels at 3w (Figure 4.1.1 **E**). From all brain regions studied, significant differences in BDNF protein levels were only detected in pre-symptomatic stage (3w) in striatum (ST), (1w: ST_{WT} = 100.0 ± 14.3%, n = 4 vs $ST_{Mecp2-/y}$ = 86.6 ± 20.2%, n = 4, p = 0.61; 3w: ST_{WT} = 100.0 ± 16.1%, n = 7 vs $HIP_{Mecp2-/y}$ = 52.5 ± 3.9%, n = 6, p = 0.02; unpaired t-test; Figure 4.1.1 **C**).

The results obtained in symptomatic stage were in line with the already published studies showing decreased BDNF protein levels in cortex, brainstem and cerebellum. Besides the changes in these brain areas, in the present work, a decrease of BDNF levels was also detected in hippocampus, in symptomatic stage (HIP_{WT} = 100.0 ± 4.8%, n = 8 vs $HIP_{Mecp2-/y}$ = 75.0 ± 7.2%, n = 9, p = 0.01; CTX_{WT} = 100.0 ± 10.9%, n = 8 vs $CTX_{Mecp2-/y}$ = 56.1 ± 3.7%, n = 6, p = 0.006; BS_{WT} = 100.00 ± 14.9%, n = 5 vs $BS_{Mecp2-/y}$ = 49.02 ± 6.6%, n=5, p = 0.01; CB_{WT} = 100.00 ± 8.2%, n = 6 vs $CB_{Mecp2-/y}$ = 62.8 ± 5.5%, n = 6, p = 0.004, unpaired t-test; Figure 4.1.1 **A;B;D;E**). In striatum was not observed statistically significant differences in symptomatic stage (Figure 4.1.1 **C**).

To facilitate all the analyses, a summarizing table (Table 4.1.1) is presented below.

Table 4.1.1| Resumed results for BDNF protein levels in Mecp2$^{-/y}$ mice. In red are shown the values significantly different, ↓ - significant decreased values, when compared to WT age-matched animals.

	1w		3w		6w	
	WT	**Mecp2$^{y/-}$**	**WT**	**Mecp2$^{y/-}$**	**WT**	**Mecp2$^{y/-}$**
Hippocampus	100.0 ± 7.6%,	94.1 ± 24.0%	100.0 ± 18.2%,	106.0± 19.9%	100.0 ± 4.8%	75.0 ± 7.2% ↓
Cortex	100.0 ± 24.3%	93.5 ± 25.5%,	100.0 ± 26.1%	98.4 ± 17.2%	100.0 ± 10.9%	56.1 ± 3.7% ↓
Striatum	100.0 ± 14.3%	86.6 ± 20.2%,	100.0 ± 16.1%	52.5 ± 3.9% ↓	100.00 ± 14.9%	97.7 ± 8.8%
Brainstem	100.0 ± 21.2%	71.0 ± 10.7%	100.0 ± 16.1%	60.8 ± 13.8%	100.00 ± 6.4%	49.02 ± 6.6% ↓
Cerebellum	100.0 ± 17.0%	158.2 ± 48.2%	100.0 ± 9.6%	84.5 ± 33.4%	100.00 ± 8.2%	62.8 ± 5.5 ↓

2.2 TrkB-FL Protein levels evaluation

Despite BDNF levels had been already explored in some studies (for review see (Li and Pozzo-Miller, 2014), the protein levels of TrkB-FL and of its truncated isoforms (TrkB-Tc), crucial players in BDNF-mediated synaptic actions, had not yet been fully addressed. Therefore, in this work the levels of TrkB receptors were evaluated in Mecp2$^{-/y}$ mice.

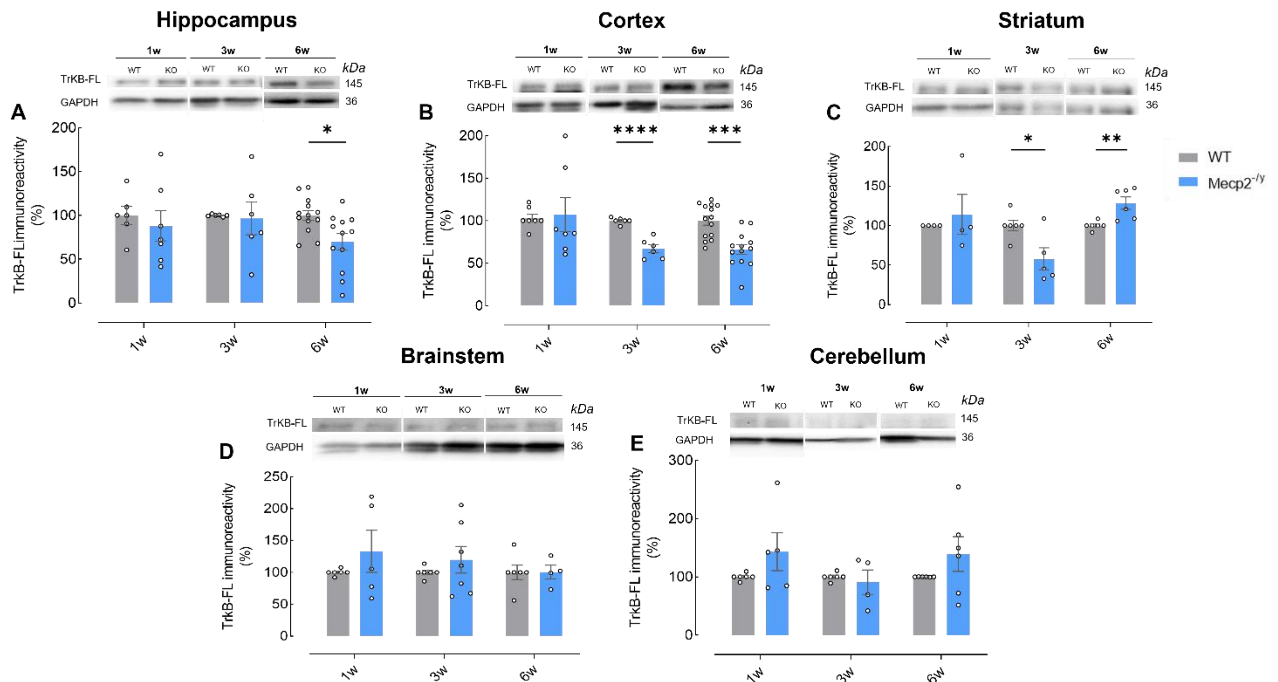

Figure 4.1.2| TrkB-FL protein levels during pre-symptomatic and symptomatic stage in different brain areas. In panels **A-E** are shown the averaged TrkB-FL density levels, respectively, in hippocampus, cortex, striatum, brainstem and cerebellum by Western Blot analysis of WT (grey bar, n=4-14) and Mecp2$^{-/y}$ (blue bars, n=4-13) animals with 1 (1w), 3 (3w) and 6-10 (6w) weeks of age. The results are represented in % of WT protein. Representative bands are shown for each brain area. All values are mean±standard error of mean (SEM). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (Student's t-test).

In pre-symptomatic stages, as shown in Figure 4.1.2, while in hippocampus no changes in TrkB-FL protein levels were found, in cortex it was possible to observe decreased TrkB-FL levels, particularly at 3w (CTX_{WT} = 100.0 ± 1.5%, n = 6 vs $CTX_{Mecp2-/y}$ = 67.1 ± 4.8%, n = 6, $p < 0.0001$, unpaired t-test; Figure 4.1.2 **A;B**). Additionally, in striatum a decrease in protein levels of TrkB-FL, at 3w, was detected (3w: ST_{WT} = 100.0 ± 6.7%, n = 6 vs $ST_{Mecp2-/y}$ = 58.1 ± 13.9%, n = 5, p = 0.02). Intriguingly at 6w, TrkB-FL protein levels were increased in striatum of Mecp2$^{-/y}$ mouse (6w: ST_{WT} = 100.0 ± 2.4%, n = 6 vs $ST_{Mecp2-/y}$ = 128.5 ± 7.7%, n = 6, p = 0.05; unpaired t-test; Figure 4.1.2 **C**). Regarding brainstem and cerebellum no changes were detected in TrkB-FL protein levels (Figure 4.1.2 **D;E**).

In symptomatic stage, a significant decrease of TrkB-FL protein levels was detected in cortex and hippocampus (HIP_{WT} = 100.0 ± 5.6%, n = 13 vs $HIP_{Mecp2-/y}$ = 70.0 ± 9.4%, n = 12, p = 0.01; CTX_{WT} = 100.0 ± 5.3%, n = 14 vs $CTX_{Mecp2-/y}$ = 66.2 ± 5.7%, n = 13, p = 0.0002, unpaired t-test; Figure 4.1.2 **A;B**). In Table 4.1.2 all obtained results are summarized.

Table 4.1.2| Resumed results for TrkB-FL protein levels in Mecp2$^{-/y}$ mice. In red are shown the values significantly decreased and in green, the results significantly increased; ↓ - significant decreased values. ↑ - significant increased values when compared to WT age-matched animals.

	1w		3w		6w	
	WT	**Mecp2$^{y/-}$**	**WT**	**Mecp2$^{y/-}$**	**WT**	**Mecp2$^{y/-}$**
Hippocampus	100.0 ± 10.5%	88.1 ± 17.4%	= 100.0 ± 0.6%	96.6 ± 18.8%	100.0 ± 5.6%	70.0 ± 9.4% ↓
Cortex	100.0 ± 3.5	105.4 ± 20.6%	100.0 ± 1.5%	67.1 ± 4.8% ↓	100.0 ± 5.3%	66.2 ± 5.7% ↓
Striatum	100.0 ± 0.0%	114.0 ± 25.4%	100.0 ± 6.7%	58.1 ± 13.9% ↓	100.0 ± 2.4%	128.5 ± 7.7% ↑
Brainstem	100.0 ± 2.0%	133.2 ± 33.0%	100.0 ± 3.5%	119.9 ± 21.0%	100.0 ± 11.3%	100.4 ± 10.9%
Cerebellum	100.0 ± 2.4%	143.2 ± 32.6%	100.0 ± 2.8%	90.8 ± 21.2%	100.0 ± 0.1%	139.3 ± 29.8%

In some neurological pathologies, changes in TrkB-FL protein levels have been associated to alterations in other TrkB isoforms, mainly in truncated isoforms (Wong et al., 2013; Fenner et al., 2014; Jerónimo-Santos et al., 2015). In order to understand if the same dysregulation could be found in RTT, TrkB-Tc levels were studied in the same brain regions and at the same disease progression stages that BDNF and TrkB-FL were.

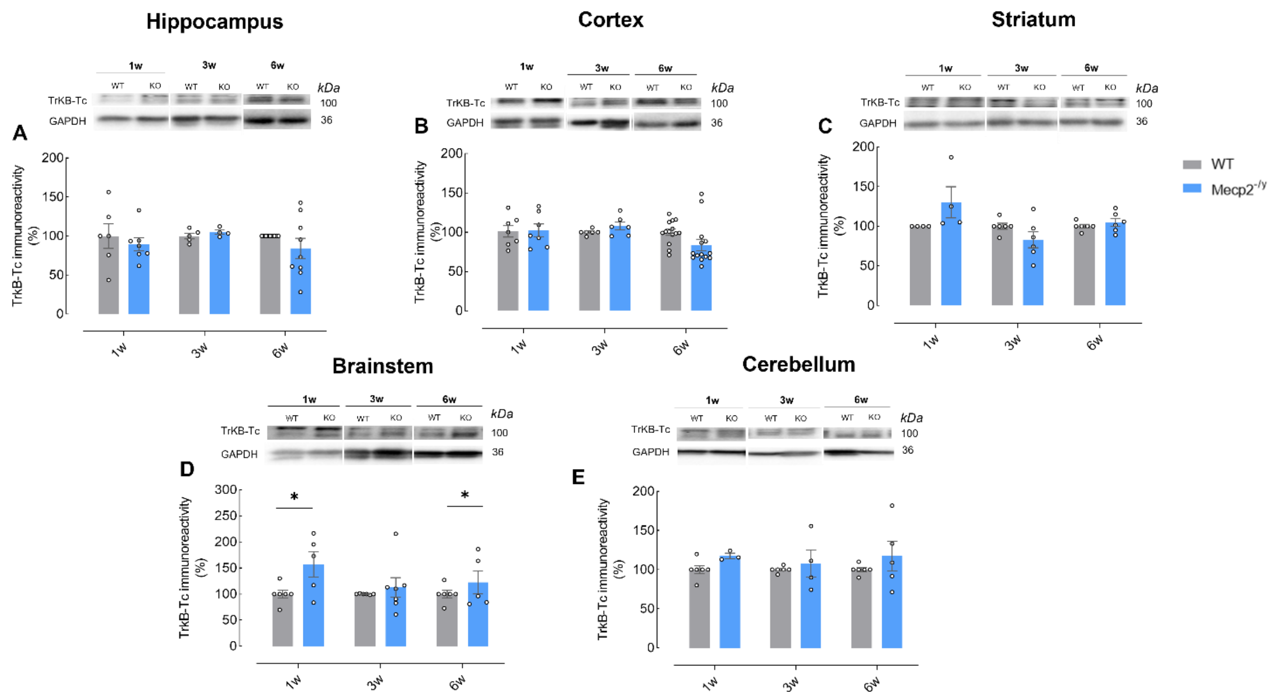

Figure 4.1.3| TrkB-Tc protein levels in pre-symptomatic and symptomatic stage in different brain areas. In panels **A-E** are shown the averaged TrkB-Tc levels density, respectively, in hippocampus, cortex, striatum, brainstem and cerebellum by Western Blot analysis of WT (grey bar, n=4-14) and Mecp2$^{-/y}$ (blue bars, n=4-14) animals with 1 (1w), 3 (3w) and 6-10 (6w) weeks old. The results are represented in % of WT protein. Representative bands are shown for each brain area. All values are mean±standard error of mean (SEM). *p<0.05 (Student's t-test).

As shown in figure 4.1.3, among the brain areas studied, the brainstem was the only in which alterations in TrkB-Tc levels were detected. Data show increased levels of TrkB-Tc protein in Mecp2$^{-/y}$ mouse at 1w and 6w when compared to WT aged-matched animals (1w: BS_{WT} = 100.0 ± 7.8%, n = 6 vs $BS_{Mecp2-/y}$ = 157.2 ± 24.7%, n = 5, p = 0.04; 6w: BS_{WT} = 100.0 ± 7.0%, n = 6 vs $BS_{Mecp2-/y}$ = 122.3 ± 22.0%, n = 5, p = 0.04; unpaired t-test; Figure 4.1.3 **D**). In table 4.1.3 all obtained results are summarized.

Table 4.1.3| Resumed results for TrkB-Tc protein levels in Mecp2$^{-/y}$ mice. In green, the results significantly increased are represented; ↑ - significant increased values, when compared to WT age-matched animals.

	1w		3w		6w	
	WT	**Mecp2$^{y/-}$**	**WT**	**Mecp2$^{y/-}$**	**WT**	**Mecp2$^{y/-}$**
Hippocampus	100.0 ± 15.9%	102.4 ± 8.1%	100.0 ± 3.0%	104.8 ± 2.8%	100.0 ± 6.1%	94.5 ± 11.8%
Cortex	100.0 ± 8.0%	102.4 ± 8.10%	100.0 ± 1.5%	107.9 ± 5.1%	100.0 ± 4.0%	83.7 ± 7.35%
Striatum	100.0 ± 0.0%	130.3 ± 19.6%	100.0 ± 3.8%	83.0 ± 10.1%	100.0 ± 2.3%	104.9 ± 5.1%
Brainstem	100.0 ± 7.8	157.2 ± 24.7% ↑	100.0 ± 0.3%	106.9 ± 16.4%	100.0 ± 7.0%	122.3 ± 22.0% ↑
Cerebellum	100.0 ± 5.1%	117.6 ± 3.4%	100.0 ± 1.6%	107.9 ± 17.7%	100.0 ± 2.6%	117.4 ± 19.0%

2.3 Evaluation of LTP magnitude and BDNF actions in hippocampal slices from symptomatic animals

LTP is generally regarded as the neurophysiological correlate for learning and memory and BDNF has a well-documented ability to increase its magnitude on hippocampal CA1 area through TrkB-FL receptors activation (Figurov et al., 1996; Minichiello et al., 1999). To assess the functional impact of the decrease in BDNF and TrkB-FL levels in RTT (see section 3.1 and 3.2), we evaluated the effect of exogenously applied BDNF (20 ng/ml) upon LTP.

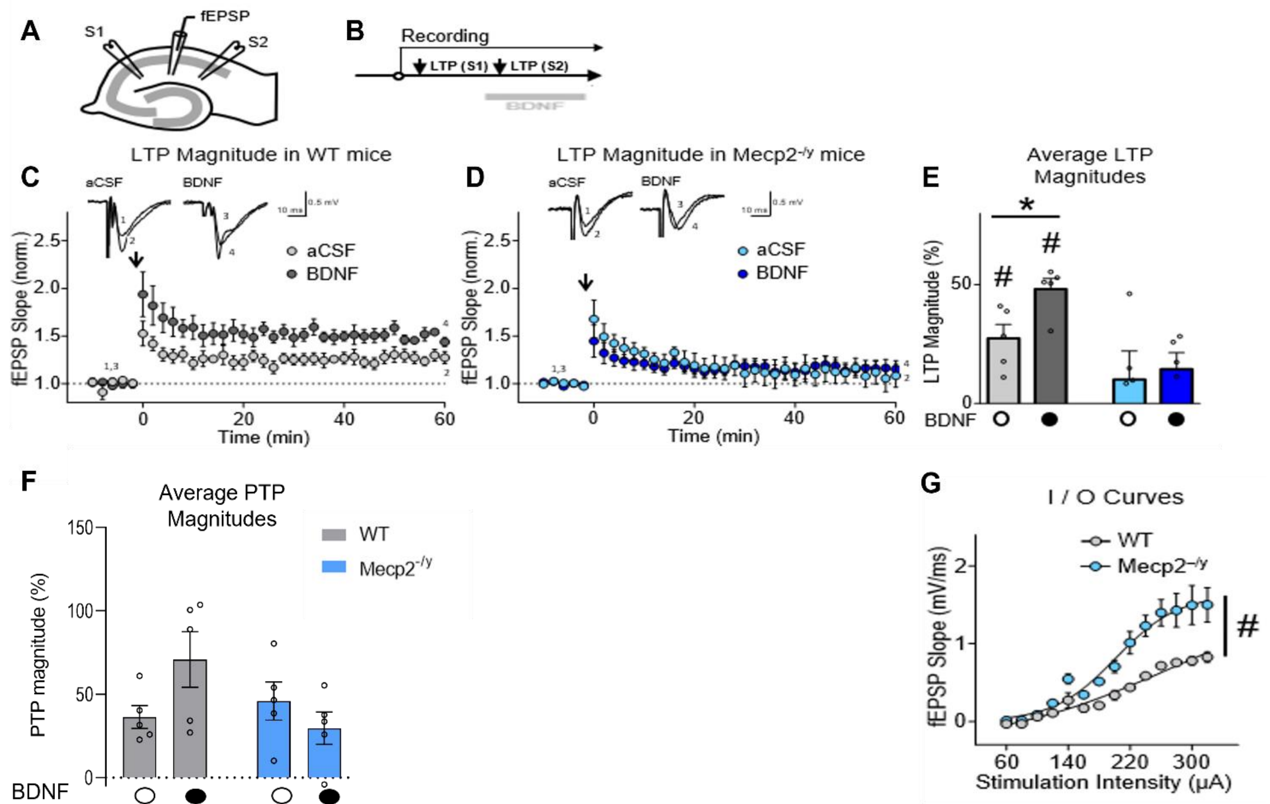

Figure 4.1.4| LTP defects in Mecp2$^{-/y}$ mice. A shows schematic representation of a transverse hippocampal slice and the recording configuration used, as described in methods section. **B** represents the protocol used to evaluate the effect of BDNF upon LTP (see methods). Panels **C** and **D** show time courses of averaged changes in fEPSP slope induced by the θ-burst stimulation in the absence (light circles) or in the presence (dark circles) of BDNF in hippocampal slices taken from WT (grey symbols,n=5) animals or from Mecp2$^{-/y}$ (blue symbols,n=5) animals. Traces from representative experiments are shown for WT and Mecp2$^{-/y}$ animals on the top of the panel (1,3 – baseline; 2.4 – LTP after 1 hour). The arrow marks LTP induction. Histogram **E** depicts the magnitude of LTP in the absence (light bars) and the presence of BDNF (20 ng/ml, dark bars) in hippocampal slices from WT (gray) or Mecp2$^{-/y}$ animals (blue). **F** shows the input/output (I/O) curves corresponding to responses generated by various stimulation intensities (60–340 μA) in WT slices (grey circles,n=4) and Mecp2$^{-/y}$ slices (blue circles,n=4). **G** represents the PTP magnitude in the absence (light bars) and the presence of BDNF (20 ng/ml, dark bars) in hippocampal slices from WT (gray) or Mecp2$^{-/y}$ animals (blue) *$p<0.05$ (paired Student's t-test) as compared with absence of BDNF in the same experiments; # $p<0.05$ (paired Student's t-test) as compared with respective baseline.

As expected (Fontinha et al., 2008; Diógenes et al., 2011), the θ-burst paradigm applied to hippocampal slices, taken from 6 weeks old WT animals induced a robust LTP in the presence of BDNF (48.2 ± 4.5%, n = 5; Figure 4.1.4 **A;E**), which was significantly higher than the obtained in the absence of BDNF (27.5 ± 5.8%, n = 5; p = 0.02, paired t-test; Figure 4.1.4 **A;E**). Importantly, in the absence of BDNF, θ-burst stimulation induced a small, yet significant LTP in WT mice (comparing with fESPs baseline (p = 0.009, paired t-test)), but not in $Mecp2^{-/y}$ mice (p = 0.45, paired t-test). Moreover, BDNF (20 ng/ml) did not further increase LTP magnitude in hippocampal slices taken from $Mecp2^{-/y}$ animals (LTP_{CTR} = 10.2 ± 11.9%, LTP_{BDNF} 14.5 ± 7.0%, n = 5; p = 0.72, paired t-test; Figure 4.1.4 **B;E**). To evaluate whether the impairment of LTP in $Mecp2^{-/y}$ animals could be due to changes in baseline synaptic efficiency, I/O curves were performed. Hippocampal slices taken from $Mecp2^{-/y}$ animals displayed higher Emax values when compared with WT animals (EmaxWT = 0.99 ± 0.04, n = 4; $EmaxMecp2^{-/y}$ = 1.78 ± 0.21, n = 4; p = 0.01, unpaired t-test; Figure 4.1.4 **G**). Regarding post-tetanic potential (PTP), representing the increase of neurotransmitters release after a high-frequency train (Powell and Castillo, 2007), such as LTP protocol, BDNF had no statistically significant effect on WT mice beside its tendency to an increased PTP magnitude (PTP_{CTR} = 36.3 ± 6.9%, PTP_{BDNF} 70.8 ± 16.7%, n = 5; p = 0.06, paired t-test; Figure 4.1.4 **F**). At the same time, in $Mecp2^{-/y}$ animals, BDNF also did not show an effect upon PTP magnitude (PTP_{CTR} = 45.9 ± 11.4%, PTP_{BDNF} = 29.6 ± 9.7%, n = 5; p = 0.09, paired t-test; Figure 4.1.4 **F**). Simultaneously, there was no significant differences between PTP control in both animal groups (p = 0.5; unpaired t-test).

All together, these data indicate higher neuronal excitability in the hippocampus of $Mecp2^{-/y}$ animals and that LTP impairment in $Mecp2^{-/y}$ animals is not due to a decrease in basal synaptic transmission efficiency.

2.4 Adenosine extracellular levels quantification

The lack of BDNF effect upon LTP could be explained by the decreased expression of TrkB-FL receptors in the hippocampus. However, since BDNF-induced facilitation of hippocampal LTP is tightly dependent on the activation of $A_{2A}R$ by endogenously generated adenosine (Fontinha et al., 2008), we next characterized the adenosinergic system in $Mecp2^{-/y}$ mice.

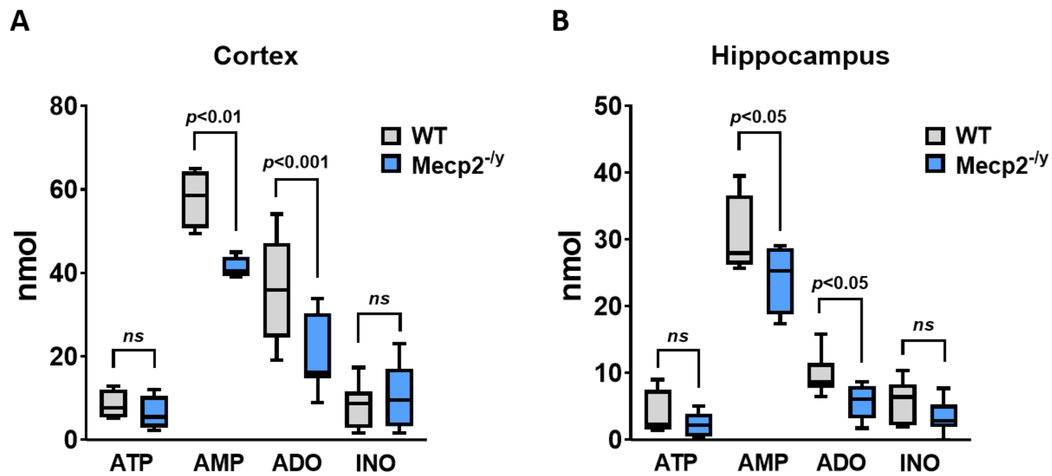

Figure 4.1.5| Adenosine levels measurement. In figure **A** and **B** the ordinates represent the amount of ATP, AMP, adenosine (ADO) and inosine (INO) in nmol extracted from the cortex and hippocampus, respectively, of WT (grey bars) and Mecp2$^{-/y}$ (blue bars) mice and detected by LC/DAD (for details, see Materials and Methods). Represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values; horizontal lines inside boxes indicate the corresponding medians. Each data point represents four to eight individuals (see text for details); duplicate measurements were performed for each individual experiment. $p<0.05$ (one-way ANOVA; uncorrected Fisher's LSD, with a single pooled variance) represent significant differences when compared to WT animals.

Brain extracts from Mecp2$^{-/y}$ mice exhibited lower adenosine amounts compared to WT animals in the cortex (WT = 35.49 ± 4.04 nmol, n = 8 vs Mecp2$^{-/y}$ = 20.60 ± 2.91 nmol; n = 9, p = 0.009, $p < 0.05$, Welch's t-test, Figure 4.1.5 **A**) and in the hippocampus (WT = 9.62 ± 0.99 nmol, n = 8 vs. Mecp2$^{-/y}$ = 5.55 ± 0.92 nmol; n = 8, p = 0.023, $p < 0.05$, Welch's t-test, Figure 4.1.5 **B**), without any measurable changes in the levels of inosine. These results suggest that adenosine deficiency in RTT animals might not be related to increases in adenosine ADA activity. This prompted us to measure the amount of adenosine precursors (e.g. ATP and AMP) in the same samples. Brain extracts exhibited very small amounts of ATP, which levels did not differ among WT and Mecp2$^{-/y}$ mice both in the cortex (WT = 8.36 ± 1.77 nmol vs. Mecp2$^{-/y}$ = 6.34 ± 2.07 nmol; n = 4; $p > 0.05$, Figure 4.1.5 **A**) and in the hippocampus (WT = 3.76 ± 1.77 nmol vs. Mecp2$^{-/y}$ = 2.19 ± 0.84 nmol; n = 4; $p > 0.05$, Figure 4.1.5 **B**). Contrariwise, higher AMP amounts compared to adenosine were extracted from the cortex (57.88 ± 3.57 nmol; n = 4) and hippocampus (30.28 ± 3.12 nmol; n = 4) of WT animals, but these levels also significantly ($p < 0.05$) decreased to 41.19 ± 1.30 nmol (n = 4) and 24.26 ± 2.64 nmol (n = 4) in the cortex and hippocampus of Mecp2$^{-/y}$ mice, respectively (Figure 4.1.5 **A;B**). The observed increase in the proportion of AMP *vis a vis* adenosine in cortical and hippocampal extracts of Mecp2$^{-/y}$ mice may indicate a decrease in 5'-nucleotidase

activity, the enzyme responsible for AMP dephosphorylation into adenosine, and/or a higher competence of intracellular ADK mediating phosphorylation of adenosine back to AMP.

To test this last hypothesis, we evaluated ADK protein levels by WB, in the hippocampus, cortex, striatum, brainstem and cerebellum in the three ages previously studied (1w, 3w and 6w). In the CNS, ADK is considered the main driving force to take up adenosine from the extracellular milieu by promoting the phosphorylation of adenosine with the consequent formation of AMP (Boison, 2013b). Overexpression of ADK has been detected in astrocytes of epileptic brains of mice, and humans (Aronica et al., 2011). This is consistent with the hypothesis that the pathophysiology of epilepsy involves lower levels of endogenous adenosine and, thus, a deficient inhibition of synaptic transmission (Boison, 2013).

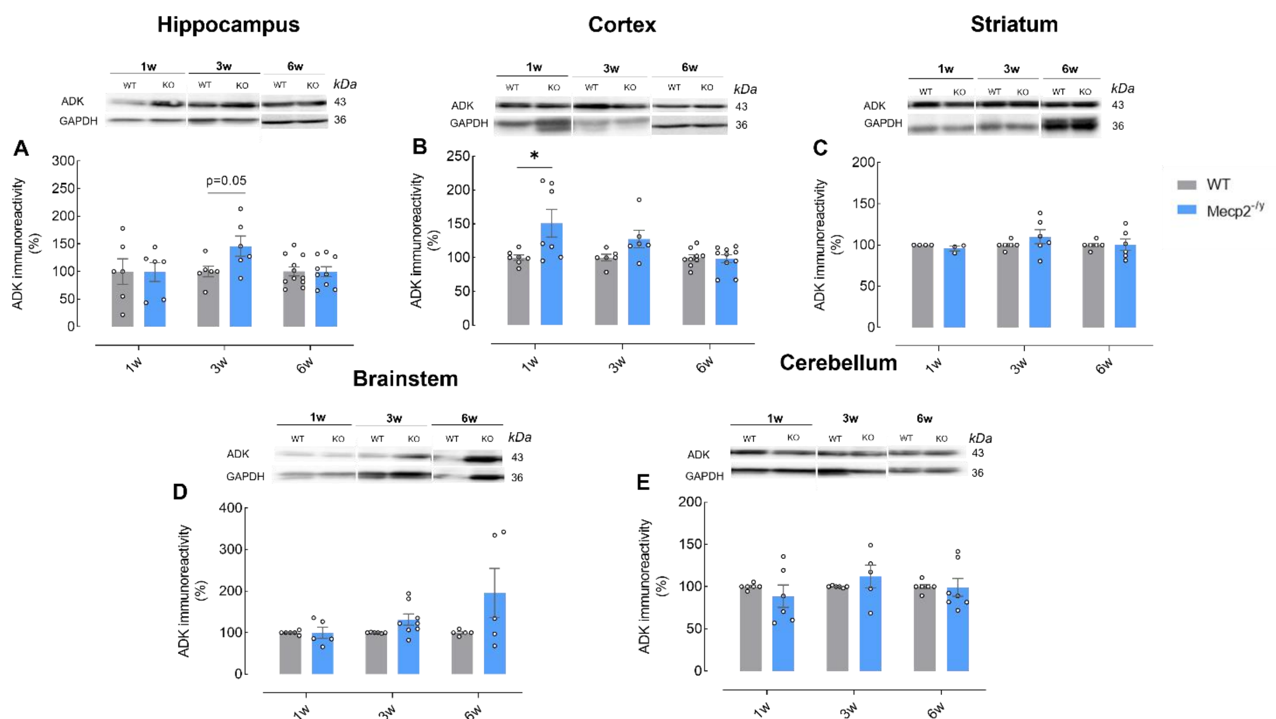

Figure 4.1.6| ADK protein levels in pre-symptomatic and symptomatic stage in different brain areas. In panels **A-E** are shown the averaged ADK levels density levels, respectively, in hippocampus, cortex, striatum, brainstem and cerebellum by Western Blot analysis of WT (grey bar, n=4-7) and Mecp2$^{-/y}$ (blue bars, n=3-8) animals with 1 (1w), 3 (3w) and 6-10 (6w) weeks old. The results are represented in % of WT protein. Representative bands are shown for each brain area. All values are mean±standard error of mean (SEM). *p<0.05 (Student's t-test).

In pre-symptomatic stage, we observed that in cortex, ADK protein levels are increased in Mecp2$^{-/y}$ animals at 1w, while in hippocampus, a tendency for increased levels are dected at 3w, (3w: HIP_{WT} = 100.0 ± 9.7%, n = 6 vs. $HIP_{Mecp2-/y}$ = 146.0 ± 18.4%, n = 6, p = 0.05; 1w: CTX_{WT} = 100.0 ± 3.8%, n = 7 vs. $CTX_{Mecp2-/y}$ = 150.8 ± 20.3%, n = 7, p = 0.03 Figure 4.1.6 **A-E**).

In symptomatic stage of Mecp2$^{-/y}$ animals, none of the studied brain regions show differences in ADK levels when compared to control animals (Figure 4.1.6 **A-E**). In table 4.1.4, these results are resumed.

Table 4.1.4| Resumed results for ADK protein levels in Mecp2$^{-/y}$ mice. In green are shown the values significantly decreased and in yellow the results with a tendency to increase but not significant; ↑ - significant increased values; when compared with age-matched WT animals.

	1w		3w		6w	
	WT	**Mecp2$^{y/-}$**	**WT**	**Mecp2$^{y/-}$**	**WT**	**Mecp2$^{y/-}$**
Hippocampus	100.0 ± 23.2%	99.8 ± 17.2%	100.0 ± 9.7%	146.0 ± 18.4% ↑	100.2 ± 8.1%	107.4 ± 11.2%
Cortex	100.0 ± 3.8%	150.8 ± 20.3% ↑	100.0 ± 5.0%	127.5 ± 12.8%	100.0 ± 4.3%	98.5 ± 5.8%
Striatum	100.0 ± 0.0%	95.9 ± 2.9%	100.0 ± 2.2%	110.1 ± 8.5%	100.0 ± 2.2%	100.4 ± 7.0%
Brainstem	100.0 ± 2.2%	99.9 ± 13.4%	100.0 ± 0.5%	131.8 ± 13.5%	100.0 ± 2.4%	195.5 ± 59.4%
Cerebellum	100.0 ± 1.2%	88.7 ± 13.3%	100.0 ± 0.4%	111.9 ± 13.6%	100.0 ± 2.3%	99.4 ± 10.5%

Therefore, these results show that adenosine levels as well as AMP are decreased in Mecp2$^{-/y}$ animalls and that ADK expression is altered in pre-symptomatic stage in some brain regions. It is, however, worth noting that WB and HPLC data reflects protein levels in whole tissue homogenates, not allowing the access to cell-specific protein contents at the synaptic level.

2.5 $A_{2A}R$ and A_1R protein levels evaluation

To further understand how the adenosinergic system is disturbed in Mecp2$^{-/y}$ mice, we evaluated the A_1R and $A_{2A}R$ protein levels.

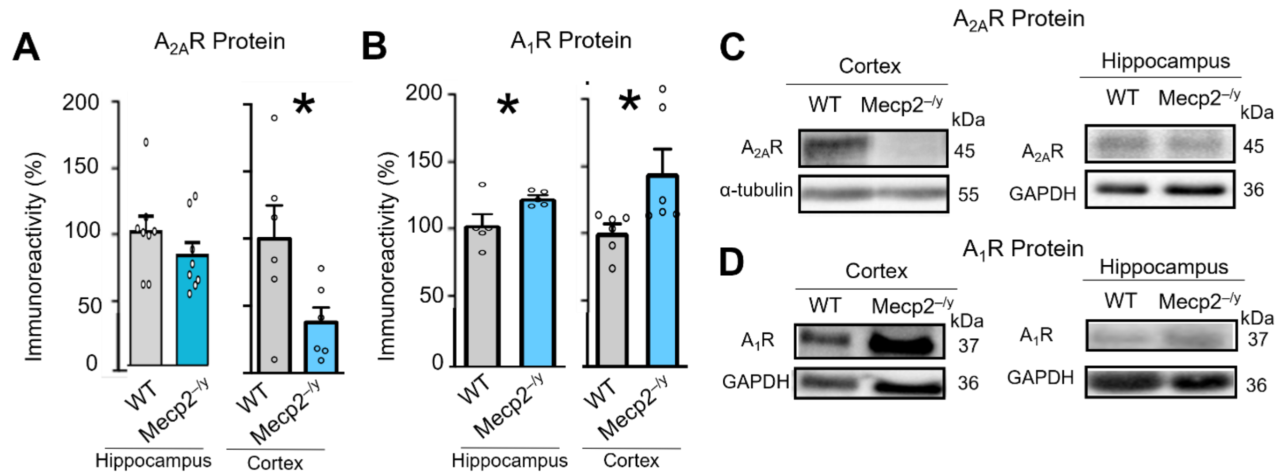

Figure 4.1.7| $A_{2A}R$ and A_1R protein levels in cortex and hippocampus during symptomatic stage. In **A** and **B** panels are shown the averaged A_{2A} and A_1 receptors density levels, respectively, all evaluated in hippocampal and cortical brain samples by Western Blot analysis of WT (grey bar, $n_{hipA2AR}$=8; $n_{ctxA2AR}$=6; and n_{hipA1R}=5; n_{ctxA1R}=6) and Mecp2$^{-/y}$ (blue bars, $n_{ctxA2AR}$=6; $n_{hipA2AR}$=8 and n_{ctxA1R}=6; n_{hipA1R}=5) animals 6-10 weeks old. The results are represented in % of WT protein. **C** and **D** representative bands. All values are mean±standard error of mean (SEM). *p<0.05 (Student's t-test).

WB analysis of cortical and hippocampal homogenates showed that $A_{2A}R$ protein levels were significantly decreased in Mecp2$^{-/y}$ mice cortex when compared to WT littermates and showed a discreet decreased of $A_{2A}R$ protein levels in hippocampus (HIP_{WT} = 100.00 ± 11.78%, n = 8 and $HIP_{Mecp2-/y}$ = 82.19 ± 9.86%, n = 8; p = 0.258; CTX_{WT} = 100.0 ± 24.8%, n = 6 vs $CTX_{Mecp2-/y}$ = 37.5 ± 11.3%, n = 6; p = 0.04; unpaired t-test; Figure 4.1.7 **A;C**).

Regarding A_1R protein levels, WB analysis showed increased levels in cortex and hippocampus of Mecp2$^{-/y}$ animals comparing with WT animals (HIP_{WT}= 100.00 ± 7.88%, n = 5 vs. $HIP_{Mecp2-/y}$ = 119 ± 5.17, n = 5; p = 0.049; CTX_{WT} = 100.0 ± 6.34%, n = 6 vs $CTX_{Mecp2-/y}$ = 144 ± 17.81%, n = 6; p = 0.041, unpaired t-test; Figure 4.1.7 **B;D**).

Overall, data show that A_1R protein levels are significantly increased in cortex and hippocampus while $A_{2A}R$ protein levels are decreased in cortex of Mecp2$^{-/y}$.

Given the higher levels of $A_{2A}R$ expression in the striatum comparing to the other brain regions, we evaluated the levels of these receptors in this specific brain region.

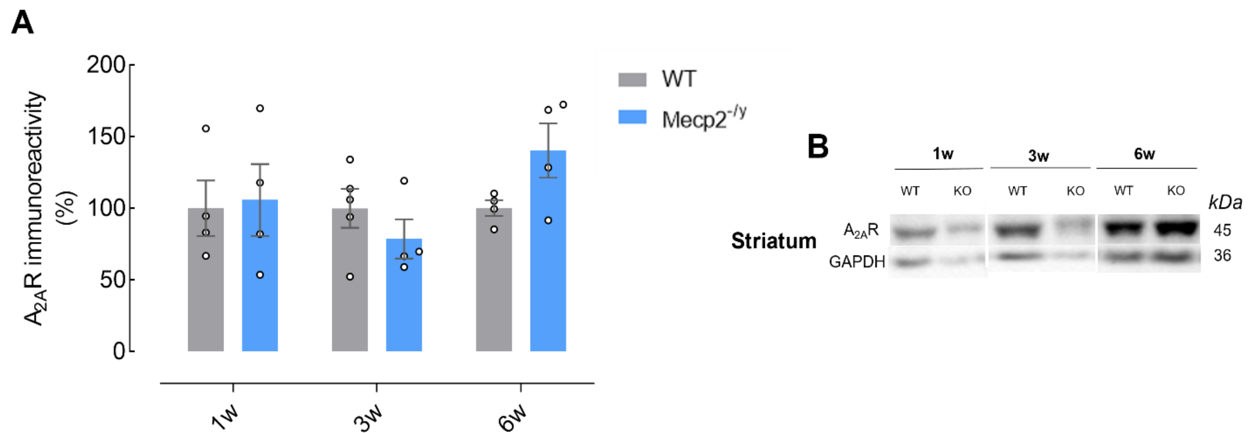

Figure 4.1.8| $A_{2A}R$ protein levels in pre-symptomatic and symptomatic stage in striatum. In **A** panel is shown the averaged $A_{2A}R$ density levels, respectively, in striatum brain samples by Western Blot analysis of WT (grey bar, n=4-5) and Mecp2$^{-/y}$ (blue bars, n=4) animals with 1 (1w), 3 (3w) and 6-10 (6w) weeks old. The results are represented in % of WT protein. **B** shows representative bands. All values are mean±standard error of mean (SEM). *p<0.05 (Student's t-test).

The obtained results showed no significant alterations in $A_{2A}R$ protein levels at different ages explored, despite some tendencies could be explored at 6w (1w: ST_{WT} = 100.00 ± 19.4%, n = 4 and $ST_{Mecp2-/y}$ = 105.8 ± 25.0%, n = 4; p = 0.9; 3w: ST_{WT} = 100.0 ± 13.6%, n = 5 vs $ST_{Mecp2-/y}$ = 78.6 ± 13.7%, n = 4; p = 0.3; 6w: ST_{WT} = 100.0 ± 5.4%, n = 4 vs $ST_{Mecp2-/y}$ = 140.2 ± 19.0%, n = 4; p = 0.08; unpaired t-test; Figure 4.1.8 **A;B**).

2.6 Activation of $A_{2A}R$ to restore BDNF actions upon LTP magnitude

The results reported above indicate that endogenous levels of adenosine and, thus, the adenosine-mediated control of synaptic transmission are deficient in the hippocampus of Mecp2$^{-/y}$ mice. Therefore, we hypothesized that reduced adenosine tonus could also contribute to impairment of BDNF-mediated actions in RTT. Indeed, a strong body of evidence demonstrates that several BDNF actions rely on the activation of $A_{2A}R$ (Sebastião et al., 2011). Furthermore, this crosstalk was shown to be dynamic, for instance, during aging where the reduction of TrkB-FL receptor levels is accompanied by increases in $A_{2A}R$ levels (Diogenes et al., 2007). This prompted us to investigate whether activation of $A_{2A}R$ could be a feasible strategy to overcome BDNF signaling deficits in RTT patients, along with the reduced levels of this neurotrophin and to the partial loss of its TrkB-FL receptors.

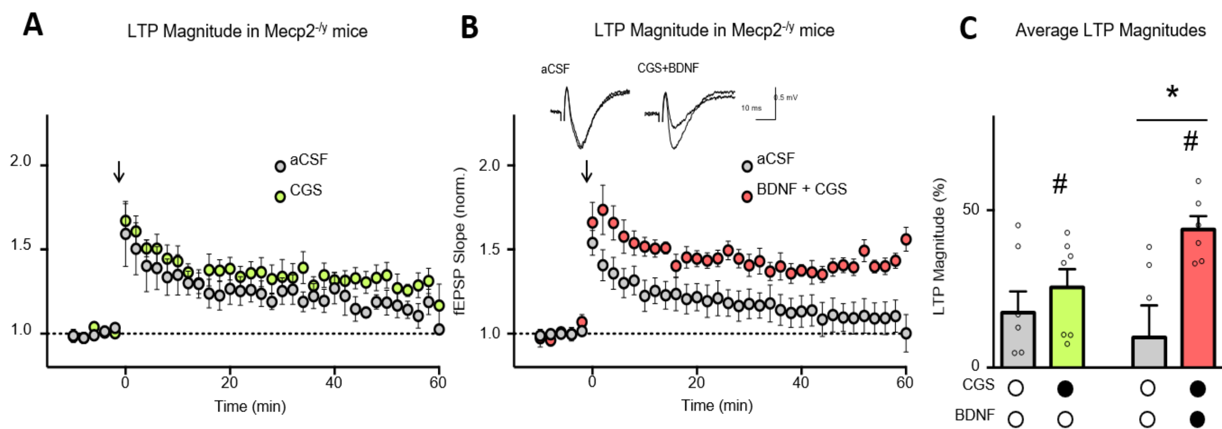

Figure 4.1.9| CGS effect upon LTP. A shows averaged time courses changes in field excitatory postsynaptic potential (fEPSP) slope induced by the θ-burst stimulation in the absence (grey circles) or in the presence (green circles) of the selective A_{2A} receptor agonist, CGS21680 (10nM) in hippocampal slices taken from Mecp2$^{-/y}$ mice (n=7). CGS21680 (10nM) was applied 60 min after the induction of LTP in the first pathway (grey circles) and at least 20 min before induction of LTP in the second pathway (green circles). **B** shows time courses changes of averaged fEPSP slopes in response to θ-burst stimulation in the absence (grey circles) or presence (red circles) of both CGS21680 (10nM) and BDNF (20ng/ml) in hippocampal slices taken from Mecp2$^{-/y}$ animals (n=6). Representative traces from representative experiments are shown on the top of the panel (1,3 – baseline; 2.4 – LTP after 1 hour). The arrow marks LTP induction. Panel **C** depicts corresponding LTP magnitudes. All values are mean±standard error of mean (SEM). *$p<0.05$ (Student's t-test) #$p<0.05$ (Student's t-test).

The selective $A_{2A}R$ agonist, CGS21680 (10 nM) (Jarvis et al., 1989) had no effect on the magnitude of LTP when it was applied alone to hippocampal slices of Mecp2$^{-/y}$ mice 1 h after LTP induction in the first pathway and at least 30 min before LTP induction in the second pathway;

the magnitude of LTP in the absence (LTP_{CTR} = 17.51 ± 6.70, n = 7) and presence (LTP_{CGS} = 25.52 ± 5.79, n = 7) of CGS21680 did not significantly differ (p = 0.25, paired t-test; Figure 4.1.9 A;C). However, in the presence of CGS21680, the LTP magnitude (LTP_{CGS} = 25.52 ± 5.79, n = 7) was significantly different from the baseline (values obtained before the θ-burst; n = 7; p = 0.01, paired t-test, Figure 4.1.9 **A;C**), in contrast with what occurred in the absence of CGS21680. Next, we set to test whether activation of $A_{2A}R$ with CGS21680 could rehabilitate the facilitatory effect of BDNF on LTP when the neurotrophin was applied in a concentration (20 ng/ml) that was devoid of effect in hippocampal slices of $Mecp2^{-/y}$ mice (see section 3.3). In these experiments, LTP was induced in the first pathway in the absence of test drugs, then CGS21680 (10 nM) was applied 1 h after induction of the first LTP and BDNF (20 ng/ml) was applied 20 min after starting CGS21680 perfusion. The second pathway LTP was induced at least 30 min after starting BDNF application together with CGS21680. Co-application of BDNF (20 ng/ml) plus CGS21680 (10 nM) increased the magnitude of LTP in the hippocampus of $Mecp2^{-/y}$ mice (LTP_{CTR} = 9.6 ± 10.2% vs. $LTP_{CGS+BDNF}$ = 43.9 ± 4.3%, n = 6; p = 0.048, paired t-test; Figure 4.1.9 **B;C**); the LTP magnitude attained under these conditions was fairly comparable to that obtained with BDNF alone in WT animals (see section 3.3).

Data suggest that exogenous activation of $A_{2A}R$ with CGS21680 may rehabilitate BDNF signaling deficits observed in hippocampal LTP in $Mecp2^{-/y}$ mice, strengthening our theory that inadequacy of endogenous adenosine plays a major role in synaptic transmission deficits in RTT patients.

3.Discussion

In this part of the work, we addressed an innovative strategy to potentiate BDNF effects in RTT through pharmacological modulation of AR. While evaluating potential causes for the inability of exogenous BDNF to facilitate synaptic plasticity, we demonstrated that TrkB-FL receptors are decreased in the hippocampus at symptomatic stage and in cerebral cortex both at pre- and symptomatic stage in $Mecp2^{-/y}$ mice. Simultaneously, the adenosinergic system is fully dysregulated, both at the receptor expression and basal adenosine levels. Nevertheless, pharmacological activation of $A_{2A}R$ was shown to recover the facilitatory action of BDNF upon synaptic plasticity.

BDNF is widely accepted as a neuroprotective molecule and, thus, BDNF-based therapies have been thoroughly explored to prevent neurodegeneration (Lu et al., 2013). Although RTT is not considered a neurodegenerative disorder, this interest has been extended to RTT were BDNF levels are known to be altered mainly in cortex, cerebellum and brainstem (Chang et al., 2006; Wang et al., 2006; Abuhatzira et al., 2007; Li et al., 2012). A decrease of mRNA BDNF levels in *post-mortem* human brain samples of RTT patients has also been reported (Abuhatzira et al., 2007), though differences in BDNF protein levels in blood serum and cerebrospinal fluid from RTT human patients have been more difficult to show (Vanhala et al., 1998; Riikonen, 2003). Here we could observe that during symptomatic stage, BDNF protein levels are decreased in cortex, cerebellum and brainstem (as previously reported, (Chang et al., 2006; Wang et al., 2006; Abuhatzira et al., 2007; Li et al., 2012)) but also in hippocampus. Regarding pre-symptomatic stage, only in striatum it is possible to find changes, with decreased BDNF protein levels at 3w. These decrease of BDNF levels in striatum could be a reflex of already impaired BDNF protein levels in cortex, since it is known that BDNF transport through cortico-striatal projections is impaired in RTT neuronal cultures (Roux et al., 2012). However, it would be interesting to explore why striatum is the only brain area with affected BDNF levels during pre-symptomatic stage.

Much less was known about BDNF receptor signaling in RTT, and the existing data are conflicting. One study showed no differences in TrkB-FL mRNA levels between human embryonic stem cell derived from RTT neurons and controls (Li et al., 2013). Another study (Abuhatzira et al., 2007) showed that TrkB-FL mRNA expression levels are increased in a mouse model and in RTT human cortical brain samples, a change interpreted as a compensatory mechanism for the reduced BDNF protein levels detected in whole brain homogenates collected from newborn

asymptomatic female mutant mice (Abuhatzira et al., 2007). By analysing the hippocampus and cortex of symptomatic male Mecp2$^{-/y}$ mice separately, we detected a significant decrease of BDNF protein levels while mRNA expression studied in other project showed a tendency for an increase in mRNA levels of TrkB-FL and Trk-B-Tc in both brain areas (Miranda-Lourenço et al., 2020a). A tendency for an increase in TrkB-FL mRNA in a *post-mortem* temporal cortex sample from a RTT patient was also observed (Miranda-Lourenço et al., 2020a). Importantly, none of the previous studies analysed, in detail, the protein levels of the different isoforms of the TrkB receptor. In particular, if putative alterations in the complete signaling form of the TrkB receptor, the TrkB-FL, were accompanied by changes in the truncated form of the receptor, the TrkB-Tc form, known to counteract TrkB signaling. Our data clearly shows that TrkB-FL protein levels are decreased in the hippocampus and in the cortex of symptomatic Mecp2$^{-/y}$ mice, without appreciable alterations in the other brain areas. Coincident with BDNF decreased levels at pre-symptomatic stage, more precisely at 3w, in striatum we can also observe decreased TrkB-FL levels. However, in cortex it is also observed diminished TrkB-FL levels at 3w besides no changes in BDNF levels at this age. This may alter the way we should think about treatment, since nor only it is important to increase BDNF levels, but it is also crucial to assure the presence of functional TrkB-FL receptors. Interestingly, no significant changes were found in TrkB-Tc protein levels, with exception for brainstem presenting increased levels both in pre- (1w) and symptomatic stage. The presence of increased TrkB-Tc levels is important to explore in this brain region given its role as negative modulator of TrkB-FL signaling (Skaper, 2018).

The idea that BDNF impairment could contribute to RTT pathophysiology was greatly reinforced by the finding that BDNF overexpression could partially ameliorate some of RTT symptoms in Mecp2$^{-/y}$ mice, including locomotor function, lifespan and the deficits on cortical electrophysiological activity (Chang et al., 2006). Overexpression of BDNF is, however, of very limited therapeutic use, if feasible at all. Other strategies are thus necessary to rescue BDNF function and to assure proper levels of TrkB-FL receptor. We aimed to potentiate BDNF actions through the major neuromodulator in the brain, adenosine. The reason to try this strategy is the previous evidence that, at least in healthy conditions, the synaptic actions of BDNF can be potentiated by activation of an adenosine receptor, the $A_{2A}R$ (Diógenes et al., 2004). $A_{2A}R$ activation induces transactivation of TrkB-FL (Lee and Chao, 2001), promotes translocation of TrkB receptors to lipid rafts (Sebastião et al., 2011; Assaife-Lopes et al., 2014) and regulates BDNF

(Tebano et al., 2008) and TrkB-FL (Jerónimo-Santos et al., 2015) levels. Moreover, the effect of exogenous BDNF upon hippocampal synaptic transmission and LTP is dependent on a fully functional adenosinergic tonus via activation of $A_{2A}R$ (Fontinha et al., 2008; Diógenes et al., 2011; Diogenes et al., 2014). However, not all BDNF functions are adenosine dependent, which explain why some BDNF actions can be revealed even in the presence of an adenosinergic dysfunction, as shown in some previous papers (Chang et al., 2006). Dysfunction of adenosine signaling has been highlighted in several pathologies, such as sleep/arousal dysfunction, neurodegeneration, epilepsy, pain, neuronal maturation and central control of breathing (Ribeiro et al., 2002; Gomes et al., 2011), but has never been considered for RTT. This is surprising on the light of the putative dual role of adenosine in this pathology: on one hand, and as mentioned above, adenosine through $A_{2A}R$ influences BDNF actions; on the other hand, adenosine, mostly through A_1R, can control seizures in epileptic syndromes such it is RTT (Boison, 2007; Sandau et al., 2016). Adenosine is an endogenous homeostatic regulator of network activity (Dunwiddie and Masino, 2001; Theofilas et al., 2012; Diogenes et al., 2014) and adenosine deficiency has been identified as a pathologic hallmark of the epileptic brain (Aronica et al., 2013). The work developed in this thesis and the work developed in other related projects revealed that $Mecp2^{-/y}$ mice displayed lower tonic inhibitory adenosinergic signaling in the hippocampus, as indicated by: 1) the lower disinhibition of excitatory synaptic transmission while blocking inhibitory A_1R (Miranda-Lourenço et al., 2020a); 2) the lower inhibition of synaptic transmission caused by an adenosine-releasing drug, as an ADK inhibitor (Miranda-Lourenço et al., 2020a); 3) lower levels of adenosine and AMP. This lower tonic adenosinergic inhibition is mostly likely due to the decreased levels of adenosine which might be related to lower expression and/or activity of 5'-nucleotidase resulting in the increased AMP/adenosine concentration ratio detected by us in hippocampus and cortex. On contrary, lower tonic adenosinergic inhibition cannot be accounted by lower ADK levels. Although an increase in ADK levels had been detected in hippocampus, at 1w of age, the $Mecp2^{-/y}$ mice have maintained ADK protein levels in the evaluated symptomatic stage. Adenosine levels are also dependent on ATP levels (Boison, 2016), therefore, we cannot exclude the potential role of deregulated bioenergetics due to dysfunctional mitochondria in RTT. Although lower levels of ATP were already reported in the brain of $Mecp2^{-/y}$ mice (Saywell et al., 2006; Toloe et al., 2014), in the present work, we did not detected significant differences on the content of ATP when comparing hippocampus and cortex from WT and $Mecp2^{-/y}$ mice. Interestingly, studies performed in organotypic hippocampal cultures have shown a downregulation on mitochondrial gene

expression (Großer et al., 2012). Moreover, a quantitative decrease in electron transport chain units and reduced efficiency of glucose metabolism were found in RTT mouse models (Kriaucionis et al., 2006; Saywell et al., 2006; De Filippis et al., 2015). Also lower A_1R receptor number or lower responsiveness of the A_1R did not explain lower tonic adenosinergic inhibition because $Mecp2^{-/y}$ mice had enhanced A_1R protein levels (as revealed by binding assays and WB technique), and the response to an added A_1R agonist was even higher in slices of $Mecp2^{-/y}$ mice (Miranda-Lourenço et al., 2020a). The mechanism underlying the overall increased levels of A_1R remains unknown. However, we can speculate that this could be a compensatory mechanism to decrease levels of adenosine and to the overexcitability associated to this pathology. Indeed, in the brain of $Mecp2^{-/y}$ mice there is a tendency for hyperexcitability, associated to changes in basal inhibitory rhythms and pre- and postsynaptic defects in GABAergic synapses (Calfa et al., 2011). Low intracellular adenosine levels may favour DNA methylation and hence epileptogenesis (Williams-Karnesky et al., 2013). Consequently, adenosine augmentation therapies constitute an effective strategy to reduce seizures, even in cases refractory to conventional antiepileptic drugs (Boison, 2009, 2013b).

In contrast with A_1Rs, the protein levels of $A_{2A}R$ were decreased in cortex of $Mecp2^{-/y}$ mice. The available receptors in hippocampus were although slight decreased enough to respond to exogenous activation with a selective agonist, which was able to rescue the action of BDNF on synaptic plasticity (LTP). This further reinforces the hypothesis that AAT may be a particularly useful strategy in a disease that simultaneously involves low BDNF signaling and low capability of endogenous adenosine to act as an endogenous anticonvulsant. As it occurred with the mRNA of the A_1R, the mRNA expression level of $A_{2A}R$ was not significantly affected in $Mecp2^{-/y}$ mice (Miranda-Lourenço et al., 2020a). The discrepancy between mRNA and protein expression levels could be attributed to several posttranscriptional mechanisms such as dysregulation of AKT/mTOR in RTT, an important signaling pathway for protein synthesis and which it is under the regulatory influence of MeCP2 (Li et al., 2013). Intriguingly, it was possible to detect changes in the mRNA levels of both A_1R and $A_{2A}R$ in one *post-mortem* human brain sample from an RTT patient, and the changes detected closely mimicked the changes detected at the protein level in $Mecp2^{-/y}$ mice (Miranda-Lourenço et al., 2020a).

Taken together, the results show a decrease on endogenous adenosine levels and a consequent impairment on adenosinergic signaling in RTT. This dysregulation could hamper BDNF

signaling and may account for epileptogenesis and decrease seizure control opening a new avenue for RTT therapy: AAT. Augmentation adenosine strategies have been already explored in other pathologies, such as epilepsy (Boison, 2009). In fact, RTT presents some disturbances related with adenosinergic system changes as already mentioned in this chapter. Our finding that adenosine changes are also present in RTT reinforces a possible contribution of adenosinergic system in RTT pathophysiology. AATs could have a higher benefit comparing with $A_{2A}R$ activation alone (using a specific agonist, p.e.), since we demonstrate in this paper that adenosine levels are decreased. Augmentation of adenosine levels could act on both dominant adenosine receptor and trigger their activation, promoting: 1) the rescue of BDNF effects, through $A_{2A}R$; 2) a control of hyperexcitability through A_1R. In addition, it was already shown that adenosine also plays an important role in epileptogenesis through an epigenetic action: adenosine contributes to DNA methylation which inhibits epileptogenesis (Williams-Karnesky et al., 2013). The adenosine-enhancing strategies could be performed by genetic approaches and cell-therapy strategies, still under investigation, or pharmacologically, taking into account possible side effects (as discussed in Boison, 2009). However, it is crucial to understand if the adenosinergic system changes are focal or systemic in order to achieve a better strategy for RTT patients.

Subsequent work should elucidate whether MeCP2 directly affects the expression of enzymes that regulate adenosine and its receptors. Nevertheless, it is possible that changes in receptor levels result of homeostatic mechanisms (Sandau et al., 2016), attempting to preserve inhibitory adenosine synaptic actions by increasing inhibitory A_1R and decreasing excitatory $A_{2A}R$ expression levels. However, in the case of RTT where BDNF expression and signaling is impaired, the decrease of $A_{2A}R$ further aggravates the disease. Importantly, as we herein show, in spite of the reduction of $A_{2A}R$ expression and the decreased levels of endogenous adenosine, the pharmacological activation of $A_{2A}R$ could efficiently rescue BDNF effects upon synaptic plasticity.

Chapter 4.2 – BDNF signaling and adenosinergic system changes in a mild model of RTT

1. Rational

The previous results presented and discussed in the Chapter 4.1 of this dissertation corroborated changes in BDNF protein levels and clarify that TrkB-FL receptors are also decreased in symptomatic stage of the mouse model studied. The molecular changes found in multiple stages of the disease progression, but mainly in symptomatic stage, and in different brain regions, highlight how BDNF signaling is widely dysregulated in Mecp2$^{-/y}$ animals. This might contribute to better understand how BDNF signaling dysregulation could impact RTT pathophysiology. Additionally, for the first time, adenosinergic system was added to this equation, with results pointing out to decreased levels of adenosine and changes in its A_1R and $A_{2A}R$ protein levels. Reported these new findings, showing a dysregulation of adenosinergic system in RTT, a new possible target emerged: adenosinergic system. Indeed, the activation of $A_{2A}R$ in hippocampal slices taken from male Mecp2$^{-/y}$ mice was able to restore BDNF action upon LTP.

Although male Mecp2-null mice are the most well studied RTT model, by better recapitulate the severe forms of the disorder, RTT is a disease that affects mainly girls and it is characterized by a broad spectrum of clinical manifestations with a variable severity (Kyle et al., 2018). These considerations prompted the investigators to also conduct studies in heterozygous females (Mecp2$^{+/-}$). These animals manifest a more variable phenotype due to XCI, which represents a closer scenario of what occurs in human patients (Leonard et al., 2016; Shah and Bird, 2017). In fact, the use of different animal models in RTT field is nowadays highly recommended (Katz et al., 2012). In this way, in this chapter, similarly to the previous chapter, we evaluated BDNF and adenosinergic systems in a milder RTT animal model phenotype, Mecp2$^{+/-}$.

Note that A_1R protein levels obtained by WB analysis present in these chapter (subchapter 2.4, Figure 4.2.6**A;C**) were obtained in collaboration with Nadia Rei from ASebastião lab.

2. Results

2.1 BDNF protein levels evaluation

Given the role of BDNF in CNS, the known regulation of *BDNF* gene by Mecp2 is already assumed as an important factor for RTT pathophysiology (Chang et al., 2006). Despite several descriptions showing BDNF decreased levels in RTT mouse models, most of the studies were conducted in Mecp2-null male animals neglecting heterozygous female mice, representative of a milder phenotype. Given that, we evaluated the BDNF protein levels in Mecp2$^{+/-}$ mice, in symptomatic stage. Cortex and hippocampus were the two brain regions studied, since these were the two brain regions where more alterations were detected in the Mecp2$^{-/y}$ animals.

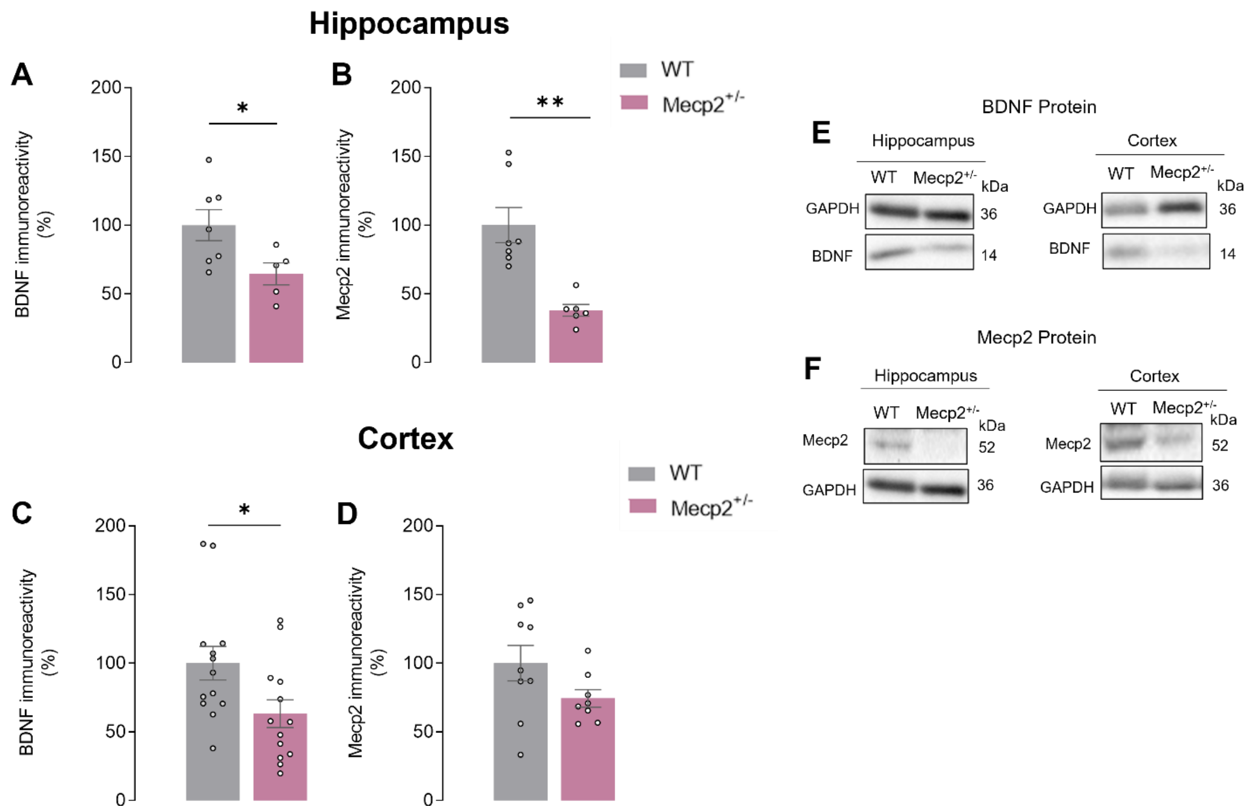

Figure 4.2.1| BDNF and Mecp2 protein levels in Mecp2$^{+/-}$ animals. In **A** and **C** are shown the averaged of BDNF density levels (WT_{hip}, n=7;WT_{ctx}, n=13;Mecp2$^{+/-}_{hip}$, n=5;Mecp2$^{-/y}_{ctx}$, n=13) while **B** and **D** show Mecp2 (WT_{hip}, n=7;WT_{ctx}, n=9;Mecp2$^{+/-}_{hip}$, n=6;Mecp2$^{+/-}_{ctx}$, n=8) density levels evaluated by Western-Blot analysis in hippocampal and cortical homogenates from WT (grey) and Mecp2$^{+/-}$ (pink) animals with 26-30 weeks of age. **E** and **F** show representative bands obtained. All values are mean±standard error of mean (SEM). *p<0.05; **p<0.01 (Student's t-test).

BDNF protein levels, in line to what had been previously described for Mecp2$^{-/y}$, were decreased both in hippocampus and cortex of Mecp2$^{+/-}$ when compared with aged-matched WT animals (HIP_{WT} = 100.0 ± 11.31%, n = 7 and $HIP_{Mecp2+/-}$ = 64.6 ± 8.02, n = 5; p = 0.03; CTX_{WT} = 100.0 ± 12.26%, n = 13 and $CTX_{Mecp2+/-}$ = 63.2 ± 10.09, n = 13; p = 0.04; unpaired t-test; Figure 4.2.1 **A;C;E**). Due to the heterozygosity present, Mecp2 expression may diverge among the Mecp2$^{+/-}$ animals. To clarify this, Mecp2 levels were analyzed. Data show that Mecp2 protein levels were significantly decreased in hippocampal brain homogenates of Mecp2$^{+/-}$ animals (HIP_{WT} = 100.0 ± 12.80%, n = 7 and $HIP_{Mecp2+/-}$ = 38.0 ± 4.31, n = 6; p = 0.001; CTX_{WT} = 100.0 ± 12.98%, n = 9 and $CTX_{Mecp2+/-}$ = 74.3 ± 6.40, n = 8; p = 0.11; unpaired t-test; Figure 4.2.1 **B;D;E**).

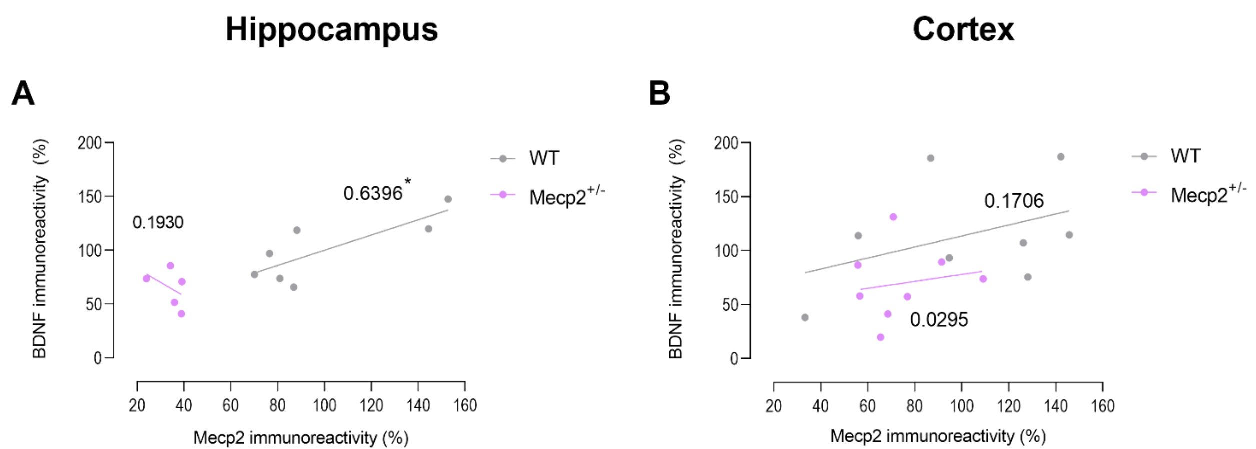

Figure 4.2.2| Correlation between BDNF and Mecp2 protein levels in Mecp2$^{+/-}$ animals. In **A** and **B** are shown the correlations of BDNF and Mecp2 in hippocampus (WThip, n=7;Mecp2$^{+/-}$ hip, n=5) and in cortex (WTctx, n=8;Mecp2$^{+/-}$ ctx, n=8), respectevely, analysed by Pearson method.

Although a similar profile of BDNF and Mecp2 proteins were detected, both decreased in Mecp2$^{+/-}$ animals, the Pearson correlation analysis, only revealed a statistically significant correlation between BDNF and Mecp2 protein levels in the hippocampus of WT animals (HIP_{WT} = 0.6396, p = 0.03; n = 7; Figure 4.2.2).

The results show that in hippocampus and cortex of Mecp2$^{+/-}$ animals, BDNF levels were significantly decreased in the evaluated symptomatic stage (26w), clarifying that in a milder phenotype BDNF levels are also affected. Simultaneously, it was possible to stablish a correlation between Mecp2 and BDNF levels in the hippocampus of WT animals and a complete dissociation between both proteins in Mecp2$^{+/-}$ animals, both in hippocampus and cortex. It is important to

state that these analyses only included the samples where it was possible to quantify both proteins by WB (in the same gel).

2.2 TrkB receptors protein levels evaluation

Enlightened the similarities in BDNF changes, both in Mecp2$^{-/y}$ and Mecp2$^{+/-}$ animals, it was considered to study possible changes in TrkB protein levels in Mecp2$^{+/-}$ animals. The knowledge about possible alterations in protein levels of TrkB receptors is fundamental to elucidate the severity of BDNF dysfunction.

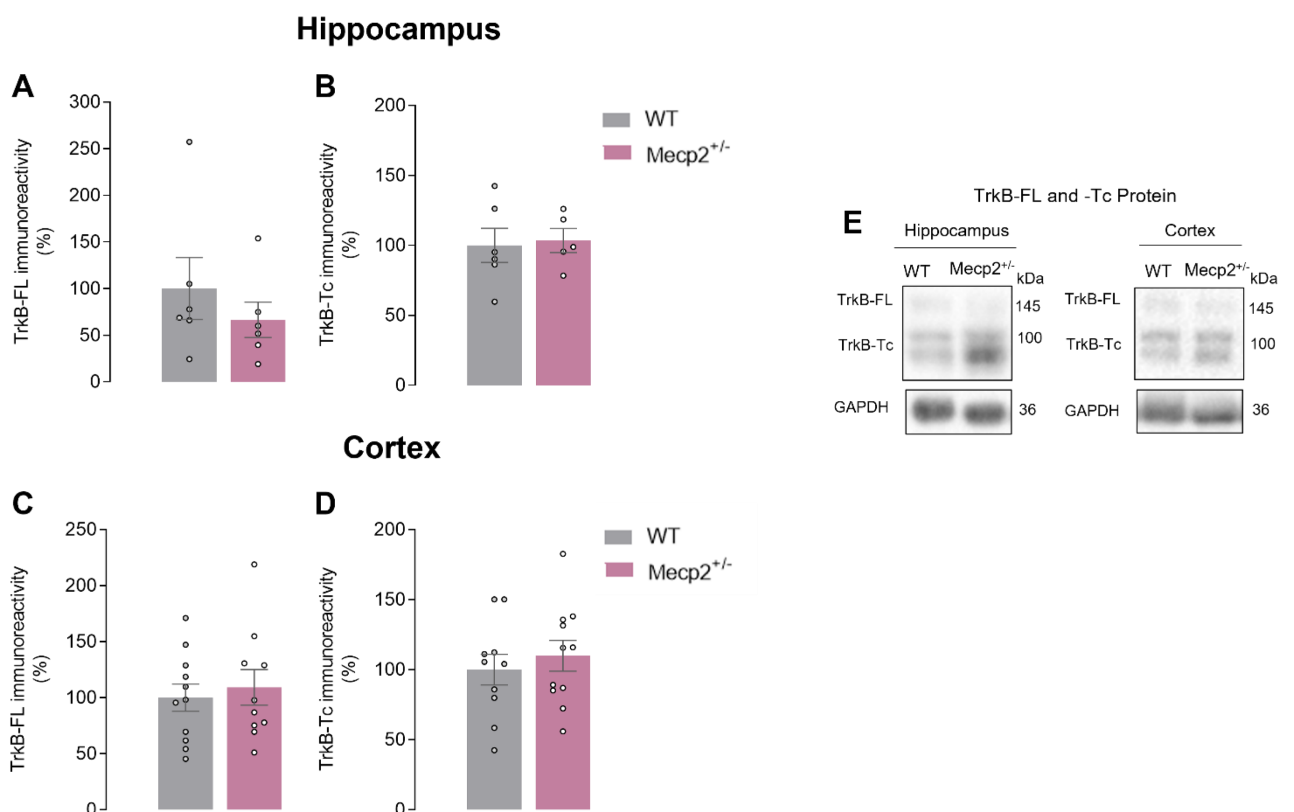

Figure 4.2.3| TrkB-FL and TrkB-Tc protein levels in Mecp2$^{+/-}$ animals. In **A** and **C** are shown the averaged of TrkB-FL levels density (WThip, n=;6;WTctx, n=11;Mecp2$^{+/-}$hip, n=6;Mecp2$^{+/-}$ctx, n=11) while **B** and **D** show TrkB-Tc (WThip, n=6;WTctx, n=10;Mecp2$^{+/-}$hip, n=5;Mecp2$^{+/-}$ctx, n=11) density evaluated by Western-Blot analysis in hippocampal and cortical homogenates from WT (grey) and Mecp2$^{+/-}$ (pink) animals with 26-30 weeks of age. **E** shows representative bands obtained.

Contrary to what was observe in Mecp2$^{-/y}$ animals, no statistically significant changes were detected in TrkB-FL levels (HIP_{WT} = 100.0 ± 33.21%, n = 6 and $HIP_{Mecp2+/-}$ = 66.6 ± 19.07, n = 6; p = 0.4; CTX_{WT} = 100.0 ± 12.08%, n = 11 and $CTX_{Mecp2+/-}$ = 109.2 ± 15.88, n = 11; p = 0.65; unpaired t-

test; (Figure 4.2.3 **A;C;E**). In addition, in accordance to what was already described in $Mecp2^{-/y}$ animals, no changes in TrkB-Tc protein levels were found in $Mecp2^{+/-}$ animals at 26 weeks (HIP_{WT} = 100.0 ± 12.14%, n = 6 and $HIP_{Mecp2+/-}$ = 103.5 ± 8.51, n = 5; p = 0.8; CTX_{WT} = 100.0 ± 11.06%, n = 10 and $CTX_{Mecp2+/-}$ = 109.9 ± 10.95, n = 11; p = 0.16; unpaired t-test; Figure 4.2.3 **B;D;E**).

In this way, in a milder phenotype model of RTT syndrome, TrkB-FL levels present normal protein levels which could reflect a BDNF signaling less affected comparing to the severe phenotype studied.

2.3 Evaluation of LTP magnitude and BDNF actions in hippocampal slices from symptomatic animals

Given the changes in BDNF levels depicted in RTT syndrome and the importance of this neurotrophin through synaptic plasticity, the magnitude of LTP was evaluated as well as the effect of exogenous BDNF upon LTP in hippocampal slices from Mecp2$^{+/-}$ animals.

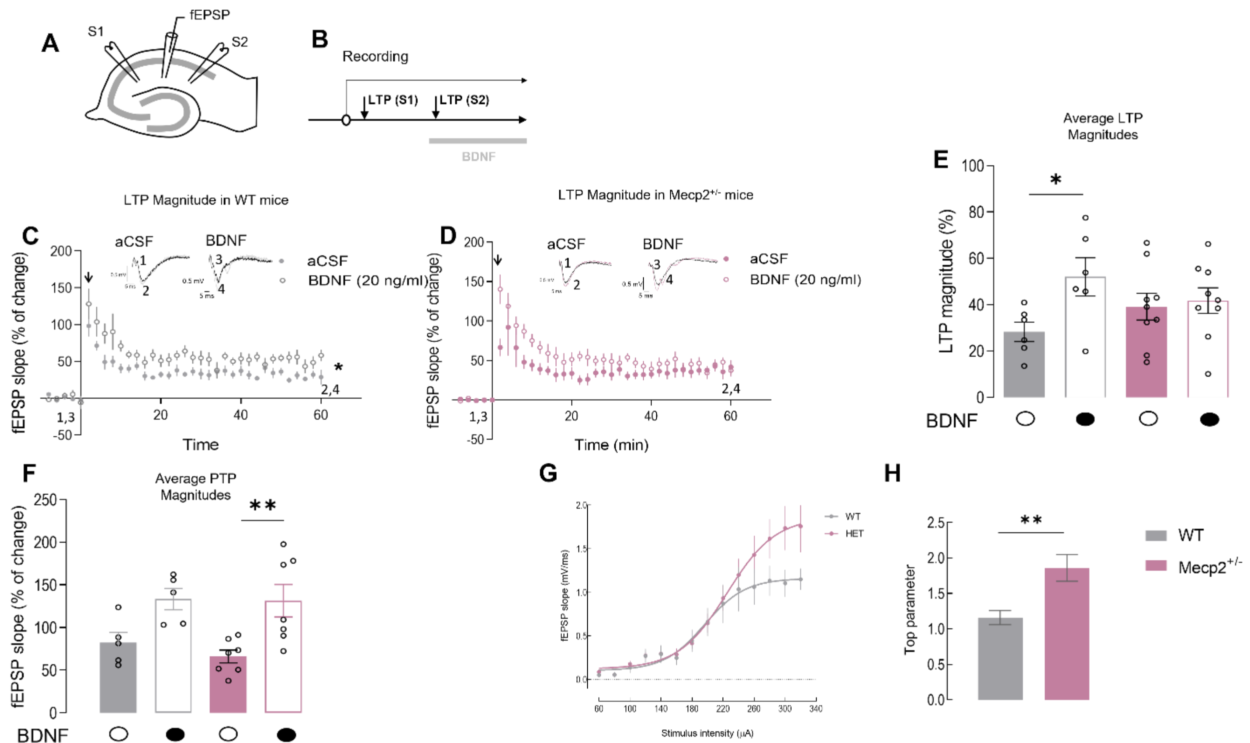

Figure 4.2.4| BDNF loses the facilitatory effect upon LTP in Mecp2$^{+/-}$ animals. A shows schematic representation of a transverse hippocampal slice and the recording configuration used, as described in methods section. **B** represents the protocol used to evaluate the effect of BDNF upon LTP (see methods). Panels **C** and **D** show time courses of averaged changes in fEPSP slope induced by the θ-burst stimulation in the absence (dark circles) or in the presence (light circles) of BDNF in hippocampal slices taken from WT (grey symbols, n=6) animals or from Mecp2$^{+/-}$ (pink symbols, n=9) animals. Traces from representative experiments are shown for WT and Mecp2$^{+/-}$ animals, on the top of the panel (1,3 – baseline; 2.4 – LTP after 1 hour). The arrow marks LTP induction. Histogram **E** depicts the magnitude of LTP in the absence (dark bars) and the presence of BDNF (20 ng/ml, dark bars) in hippocampal slices from WT (gray) or Mecp2$^{+/-}$ animals (pink) while **F** the PTP magnitude. **H** shows the input/output (I/O) curves corresponding to responses generated by various stimulation intensities (60–340 μA) in WT slices (grey circles, n=5) and Mecp2$^{+/-}$ slices (pink circles, n=7) and **G** the respective means of top parameter. *p<0.05;**p<0.01 (paired Student's t-test) as compared with absence of BDNF in the same experiments.

It was possible to observe that LTP magnitude was not changed in hippocampal slices taken form Mecp2$^{+/-}$ when compared to hippocampal slices taken from aged-matched WT animals (WT-LTP_{CTR} = 28.3 ± 4.2%, n = 6 and Mecp2$^{+/-}$-LTP_{CTR} = 39.1 ± 6.5%, n = 8; p = 0.2, unpaired t-test; Figure

4.2.4 **C;D;E**). However, in $Mecp2^{+/-}$ animals, it was possible to observe the loss of BDNF functions upon LTP: exogenous BDNF was unable to further potentiate LTP as previously observed in hippocampal slices from $Mecp2^{-/y}$ animals (WT-LTP_{BDNF} = 52.0 ± 8.2%, n = 6, paired t-test; p = 0.02; Figure 4.2.4 **C;E**; $Mecp2^{+/-}$-LTP_{BDNF} = 48.2 ± 6.3%, n = 9; p = 0.5, paired t-test; Figure 4.2.4 **D;E**). On the other side, despite BDNF presented a tendency to increase PTP in WT animals, in $Mecp2^{+/-}$ animals was possible to observe a significant effect of BDNF upon PTP, not reflected in LTP, as previously mentioned (WT: PTP_{CTR} = 86.3 ± 12.0% and PTP_{BDNF} = 133.3 ± 12.5%, n = 5; p = 0.06 and $Mecp2^{+/-}$: PTP_{CTR} = 66.0 ± 7.6% and PTP_{BDNF} = 131.3 ± 19.0%, n = 7; p = 0.009; Figure 4.2.4 **C;D;F**).

I/O curves were performed to evaluate synaptic efficiency (Figure 4.2.4 **G;H**) as mentioned in chapter 4.1. Higher levels of Emax parameter were obtained in hippocampal sliced from $Mecp2^{+/-}$ animals (EmaxWT = 1.12 ± 0.09, n = 8; $EmaxMecp2^{+/-}$ = 1.86 ± 0.21, n = 8; p = 0.005, unpaired t-test; Figure 4.2.4 **G;H**), demonstrating a higher excitability in $Mecp2^{+/-}$ animals.

2.4 A_1R and $A_{2A}R$ protein levels evaluation

Similarity to what had been evaluated in brain samples from Mecp2$^{-/y}$, the levels of both A_1R and $A_{2A}R$ were also evaluated.

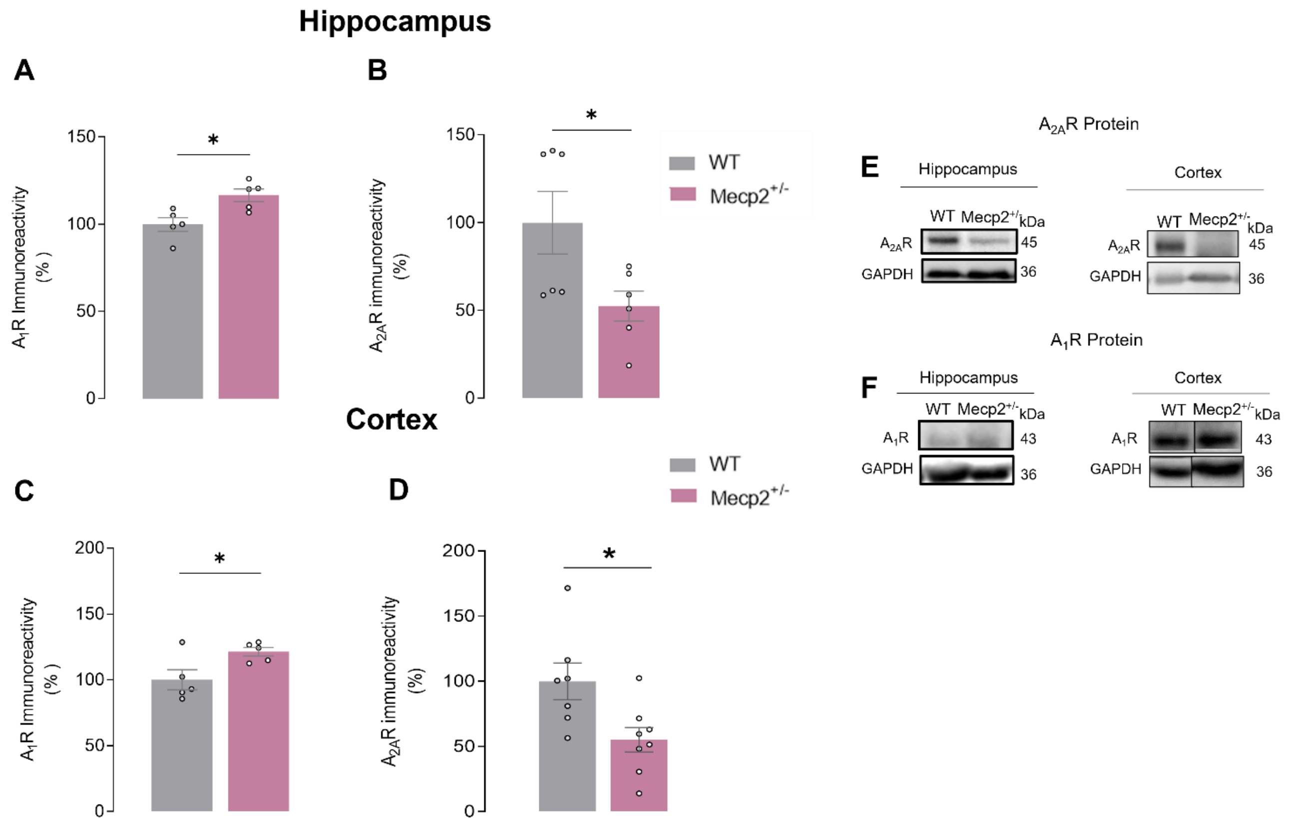

Figure 4.2.5| A_1R and $A_{2A}R$ protein levels in Mecp2$^{+/-}$ animals. In **A** and **C** are shown the averaged of A_1R density levels (WThip, n=5;WTctx, n=5;Mecp2$^{+/-}$hip, n=5;Mecp2$^{-/y}$ctx, n=5) while **B** and **D** shows $A_{2A}R$ (WThip, n=7 ;WTctx, n=9;Mecp2$^{+/-}$hip, n=6;Mecp2+/-ctx, n=8) density levels evaluated by Western-Blot analysis in hippocampal and cortical homogenates from WT (grey) and Mecp2$^{+/-}$ (pink) animals with 26-30 weeks of age. **E** and **F** show representative bands obtained. All values are mean±standard error of mean (SEM). *p<0.05 (Student's t-test).

Similar changes to what were found in A_1R and $A_{2A}R$ protein levels of Mecp2$^{-/y}$ animals were detected in hippocampal and cortical homogenates of Mecp2$^{+/-}$ animals when compared to respective controls: 1) significant increase of A_1R protein levels in the hippocampus (~16%) and cortex (~21%) of Mecp2$^{+/-}$ animals (HIP_{WT} = 100.00 ± 3.89%, n = 5 and $HIP_{Mecp2+/-}$ = 116.60 ± 3.62%, n = 5, p = 0.01, unpaired t-test; Figure 4.2.5 **A;F** and CTX_{WT} = 100.0% ± 7.6%, n = 5 and $CTX_{Mecp2+/-}$ = 121.3 ± 3.2%, n = 5, p = 0.03, unpaired t-test, 4.2.5 **C;F**); 2) significant decreased of $A_{2A}R$ protein levels in the hippocampus (~47 %) and in the cortex (~45%) of Mecp2$^{+/-}$ animals (HIP_{WT} = 100.00 ± 17.78%, n = 6 and $HIP_{Mecp2+/-}$ = 52.47 ± 8.54%, n = 6, p = 0.04, unpaired t-test; Figure 4.2.5 **B;E**

and CTX_{WT} = 100.0 ± 14.16%, n = 7 and $CTX_{Mecp2+/-}$ = 55.12 ± 9.40%, n = 8, p = 0.02, unpaired t-test; Figure 4.2.5 **D;E**).

Taken together, and in line to what was observed in BDNF signaling, these data show that that adenosinergic system is altered in several types of RTT severity.

2.5 Activation of $A_{2A}R$ to restore BDNF actions on LTP magnitude

Although no significant changes were found in the levels of hippocampal TrkB-FL protein in $Mecp2^{+/-}$ animals, the obtained results have shown that BDNF levels and its effects upon synaptic plasticity are impaired. Simultaneously, $A_{2A}R$ were found to be decreased in this brain region. With these two systems impaired and taking into account the well-known crosstalk between them (Ribeiro et al., 2002), we next investigated if the selective $A_{2A}R$ agonist, CGS21680 (10 nM) (Jarvis et al., 1989), could recover BDNF action on LTP magnitude as it did in a severe phenotype study (chapter 4.1, Miranda-Lourenço et al, 2020a).

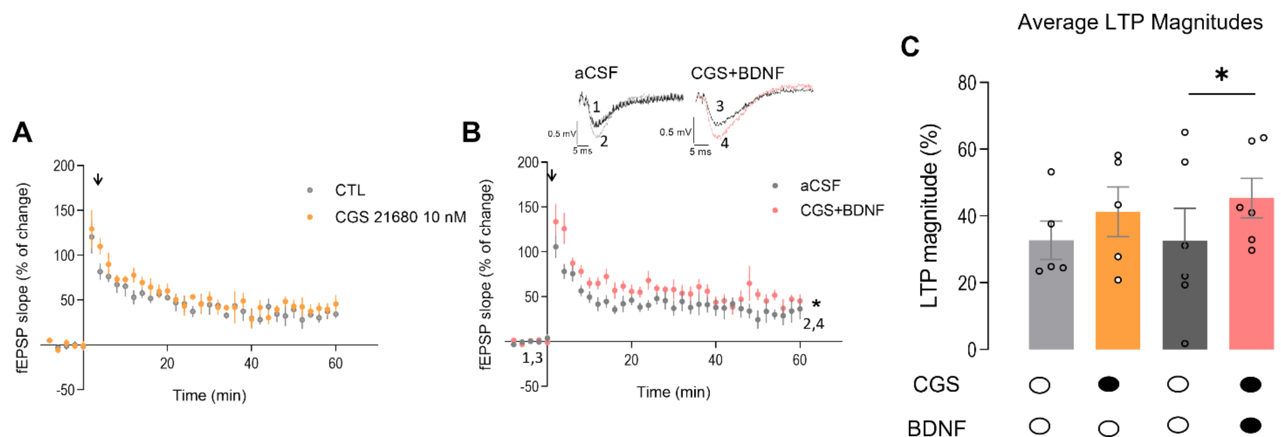

Figure 4.2.6| Acute $A_{2A}R$ activation restores BDNF effect upon LTP. A shows averaged time courses changes in field excitatory postsynaptic potential (fEPSP) slope induced by the θ-burst stimulation in the absence (grey circles) or in the presence (orange circles) of the selective A_{2A} receptor agonist, CGS21680 (10nM) in hippocampal slices taken from $Mecp2^{+/-}$ mice (n=5). CGS21680 (10nM) was applied 60 min after the induction of LTP in the first pathway (grey circles) and at least 15 min before induction of LTP in the second pathway (orange circles). **B** shows time courses changes of averaged fEPSP slopes in response to θ-burst stimulation in the absence (grey circles) or presence (pink circles) of both CGS21680 (10nM) and BDNF (20ng/ml) in hippocampal slices taken from $Mecp2^{+/-}$ animals (n=6). Representative traces from representative experiments are shown, on the top of the panel (1,3 – baseline; 2.4 – LTP after 1 hour). The arrow marks LTP induction. Panel **C** depicts corresponding LTP magnitudes. All values are mean±standard error of mean (SEM). *p<0.05 (paired t-test).

CGS21680, when applied alone to hippocampal slices 1h after LTP induction in first pathway and at least 15 min alone before LTP induction in the second pathway, caused no statistically significant effect on LTP magnitude (LTP_{CTR} = 32,74 ± 5.739, n = 5 and LTP_{CGS} = 46.37 ± 7.00, n = 4; p = 0.46, paired t-test; Figure 4.2.6 **A;C**). However, when applied concomitantly with BDNF (20ng/ml), the LTP magnitude significantly increased (LTP_{CTR} = 32.60 ± 9.7, n = 6 and $LTP_{CGS+BDNF}$ = 45.36 ± 5.90, n = 6; p = 0.04, paired t-test; Figure 4.2.6 **B;C**), suggesting that exogenous activation of $A_{2A}R$ with CGS21680 recovers BDNF signaling deficits.

Taken together, the results obtain in a RTT mouse model representative of a milder phenotype, present in the majority of RTT patients, corroborates the initial hypothesis that adenosinergic system dysregulation contributes for a worsen efficiency of BDNF signaling.

Importantly, in both phenotypes, the recovery of BDNF action upon synaptic plasticity is possible to achieve by the activation of $A_{2A}R$.

3. Discussion

Data obtained in this chapter showed decreased levels of BDNF protein levels both in cortex and hippocampus of symptomatic *Mecp2* heterozygous females, Mecp2$^{+/-}$, and a loss of BDNF effect upon hippocampal LTP facilitation. Although a dysregulation of adenosinergic system had also been detected at molecular level, the pharmacological activation of $A_{2A}R$ allowed the recovery of BDNF effect upon LTP. These findings are in line with the results previously described in Mecp2$^{-/y}$ mice (Chapter 4.1), suggesting that the dual dysregulation of BDNF signaling and adenosinergic system is inherent to RTT pathophysiology, presenting small variation concordant with the disease severity.

The work presented in Chapter 4.1 of this dissertation together with work published recently by the group (Miranda-Lourenço et al., 2020a) clearly showed that BDNF signaling and adenosinergic system are molecularly and functionally dysregulated in Mecp2$^{-/y}$ symptomatic mice. The well known crosstalk between $A_{2A}R$ and TrkB-FL receptors, as well as the dependency on $A_{2A}R$ activation to maintain proper BDNF and TrkB-FL levels (Tebano et al., 2008; Jerónimo-Santos et al., 2015) and to translocate TrkB-FL to lipid rafts (Assaife-Lopes et al., 2010), should be further addressed to better understand the mechanisms contributing for BDNF impairment in RTT. Indeed, the pharmacological activation of $A_{2A}R$ was shown to recover the facilitatory action of BDNF upon synaptic plasticity, supporting the need of a proper adenosinergic system for the efficient function of BDNF in RTT (Chapter 4.1 of this thesis and Miranda-Lourenço et al., 2020a).

Considering the genetic characteristics of RTT, comparing with Mecp2-null male mice, the heterozygous female mice are more representative of the genetics' phenomena that occurs in human patients (Leonard et al., 2016). Undoubtably, XCI is an important event present in RTT heterozygous patient (the majority), explaining why patients with the same mutation show different phenotypes (Ishii et al., 2001; Hoffbuhr et al., 2002). However, heterozygous female mice are still considered as a supportive/backup model for RTT studies since the advantages associated with studies with Mecp2-null mice are many, such as high reproducibility, good model characterization, and rapid establishment of symptomatology that mimics much of the symptoms present in patients (Calfa et al., 2011b; Lombardi et al., 2015).

Although the regulation of *BDNF* gene by MeCP2 has already been stated, the exact mechanism beyond this regulation is not yet completely clarified (Li and Pozzo-Miller, 2014). Most

of the studies described BDNF decreased protein levels in Mecp2-null male mice, detectable within the beginning of the first behavioral impairments, and with a progression from the caudal brain regions to rostral brain regions, with all brain showing BDNF decreased levels around 7 weeks of age (Wang et al., 2006; Lonetti et al., 2010). In heterozygous female, less studies are available, with only one study describing decreased BDNF levels in medulla, pons and nodose ganglia during symptomatic stage have been published so far (Schmid et al., 2012). In this thesis, in a different heterozygous female model from the mentioned study (Schmid et al., 2012), decreased BDNF levels were also detected in two brain regions, cortex and hippocampus. In addition, it was possible to correlate BDNF and Mecp2 levels in hippocampus of WT animals but not in Mecp2$^{+/-}$ animals, indicating that BDNF dysregulation could be beyond Mecp2 dysfunction, with other factors contributing to BDNF impairment. However, in cortex it was not possible to observe a correlation between the two protein levels. Similarly to BDNF, Mecp2 expression progress from posterior to more rostral brain structures, being cerebral cortex the last brain region where Mecp2 is expressed. This expression is dependent on diverse epigenetic mechanisms (Balmer et al., 2003) and indeed, these epigenetic phenomena could explain why different patterns of Mecp2 expression could be found in different brain regions, influencing differently other proteins under Mecp2 regulation in each brain region, like it was observed in hippocampus and cortex. Simultaneously, the dimension of cerebral cortex and its functional heterogeneity could also contribute for the differences found between Mecp2 and BDNF levels correlation in both brain areas.

Contrary to what was found in *Mecp2*-null males animals (Chapter 4.1) (Miranda-Lourenço et al., 2020a), no significant changes were found in TrkB-FL receptor protein levels, in the hippocampus and cortex of Mecp2$^{+/-}$ animals. Nevertheless, BDNF decreased protein levels were also found in the milder phenotype model, indicating BDNF signaling dysregulation as a possible hallmark of RTT.

In opposite to what was observe in Mecp2$^{-/y}$ animals (Asaka et al., 2006; Miranda-Lourenço et al., 2020a), hippocampal slices from Mecp2$^{+/-}$ animals did not present changes in LTP magnitude. One possible explanation is the non-detectable alterations in TrkB-FL levels, since TrkB-FL receptors are one of the multiple factors contributing to fine-tuning LTP fenomena. However, other study using a different heterozygous female model (and a different LTP protocol) had shown a decreased LTP in Mecp2 heterozygous females (Li et al., 2017). Indeed, these results

suggests that further studies should be done to better understand which mechanism is causing such changes in hippocampal LTP in different RTT models. Additionally, these differences found between models could be explained by the high variability associated to heterozygous female mice, demanding more studies in the future.

Exogenous BDNF had no effect upon LTP in a similar way of what was observed in hippocampal slices from *Mecp2*-null male mice. These results could indicate that BDNF signaling is impaired despite TrkB-FL protein levels are not altered.

On opposite to what was observed in Mecp2$^{-/y}$ animals PTP was potentiated by exogenous BDNF in hippocampal slices from Mecp2$^{+/-}$ animals. Indeed, it was previously shown that BDNF increases PTP in hippocampal neurons, via MAPK-dependent phosphorylation of synapsin I (Valente et al., 2012). Although we did not observe a statistically significant effect of BDNF upon PTP in hippocampal slices from WT animals, the p-value revealed a strong tendency for an increase ($p=0.06$). One could speculate on whether the BDNF loss of function on PTP, only detected in Mecp2$^{-/y}$ animals, could be related to a dysregulation on MAPK-dependent phosphorylation of synapsin I together with the decreased levels of TrkB-FL.

Regarding adenosinergic system, and concomitantly to what was observed in Mecp2$^{-/y}$ animals, A_1R protein levels are increased and $A_{2A}R$ protein levels are decreased, both in cortex and hippocampus of heterozygous females. As already discussed chapter 4.1 and in (Miranda-Lourenço et al., 2020a), the mechanism underlying the overall increased levels of A_1R and decreased levels of $A_{2A}R$ remains unknown. However, the overexcitability associated to this pathology could be a trigger for these changes in order to compensate the excitatory-inhibitory imbalance described in Mecp2-null animals (Boggio, 2010). Despite the lower levels of $A_{2A}R$, the available receptors were enough to respond to exogenous activation with the selective agonist, CGS21680, which was able to rescue the action of BDNF on synaptic plasticity (LTP).

The demonstrated efficiency of $A_{2A}R$ activation in restoring BDNF actions upon LTP, in two different phenotypes of the same disease, gives strength to explore AAT as a possible therapeutic strategy for RTT, in future.

Besides these findings suggesting that adenosinergic changes and BDNF signaling dysregulation are transversal to different RTT phenotypes, it is necessary to elucidate if these changes are focal or systemic, and if these changes could be prevented rather than reverted. This

would allow to better understand which type of AAT should be considered as well as the therapeutic schedule in future experimental work.

Table 4.2.1 summarizes the results obtained in both phenotypes regarding BDNF signaling and adenosinergic system. It is clear an overlap between the two studied models.

Table 4.2.1| Molecular and functional differences found between Mecp2$^{-/y}$ (Males) and Mecp2$^{+/-}$ (Females) mice. 🡅 - increased; 🡇 - decreased; ≈ without differences.

	MALES	FEMALES
BDNF CTX	🡇	🡇
BDNF HIP	🡇	🡇
TrkB-FL CTX	🡇	≈
TrkB-FL HIP	🡇	≈
$A_{2A}R$ CTX	🡇	🡇
$A_{2A}R$ HIP	≈	🡇
A_1R CTX	🡅	🡅
A_1R HIP	🡅	🡅
LTP	🡇(NO BDNF EFFECT)	≈ (NO BDNF EFFECT)
I/O	🡅	🡅

Chapter 4.3 – Adenosine augmentation therapy

1. Rational

Our work placed adenosinergic system as a new player in RTT, revealing new possible therapeutic targets for this pathology. In fact, the lower levels of adenosine detected in Mecp2$^{-/y}$ animals together with the ability of exogenous activation of $A_{2A}R$ recovers BDNF action upon LTP support AAT as a possible strategy to be explored in RTT. Actually, in other pathologies such as epilepsy and pain, AAT has been explored as possible therapeutic strategy (Theofilas et al., 2012; Boison and Jarvis, 2021), mainly through ADK inhibition .

Different approaches could be used in order to promote an augmentation of adenosine levels: i) polymer-based brain implants loaded with adenosine; ii) brain implants composed by cells engineered to release adenosine and embedded in a cell-encapsulation device; iii) direct transplantation of stem cells engineered to release adenosine; iv) focal adenosine augmentation by genetic disruption of ADK using viral tools and v) pharmacological blockage of ADK (Boison, 2009).

ADK due to its high affinity and activity is the most important enzyme involved in the clearance of adenosine under physiological levels (Boison, 2013b). Its important function allied with its potential role in pathophysiological mechanisms become this enzyme a potential target to promote the increase of adenosine levels (Li et al., 2008). Some evidences showed that promoting ADK inhibition using an ADK inhibitor, like 5-iodotubercidine (ITU), could suppress seizures in epileptic models (Gouder et al., 2004; Sandau et al., 2019).

Considering that augmentation of adenosine levels in RTT could be useful to: 1) promote the inhibition of synaptic transmission throughout A_1R activation; 2) boost BDNF actions through the crosstalk between $A_{2A}R$ and TrkB-FL and 3) promote epigenetic mechanisms involved in prevention of epileptogenesis, ITU was systemically administered to Mecp2$^{-/y}$ animals to evaluate if BDNF signaling and its actions upon LTP could be re-established in symptomatic stage.

2. Results

2.1 Adenosine augmentation therapy in $Mecp2^{-/y}$ symptomatic animals: efficacy of ITU administration

As shown in Chapter 4.1, adenosine and AMP levels present in cortical and hippocampal extracts from $Mecp2^{-/y}$ animals are lower when compared with samples from WT animals. The lower adenosine levels present in the brain of $Mecp2^{-/y}$ animals could contribute for functional repercussions in synaptic transmission, as hypothesized in Chapter 4.1 and in (Miranda-Lourenço et al., 2020a). To explore if the increase of adenosine levels could revert some dysfunctions found in $Mecp2^{-/y}$ animals, ITU was intraperitoneally administrated (Sandau et al., 2019) to symptomatic $Mecp2^{-/y}$ animals (see protocol in Methods 3.1.1).

First, to set out if the implemented AAT was efficient, a supramaximal concentration of DPCPX (50 nM; Ki value of 0.5 nM in the hippocampus (Sebastião et al., 1990)) was used to evaluate the effect of A_1R blockage on basal synaptic transmission in hippocampal slices from $Mecp2^{-/y}$ animals and WT littermates. Previously we showed that the magnitude of the decrease of fEPSP slope in the presence of DPCPX (50 nM) is significantly higher in hippocampal slices from WT when compared to those obtained from $Mecp2^{-/y}$ mice. This difference reflects a reduction in adenosine inhibitory tonus in $Mecp2^{-/y}$ mice (Miranda-Lourenço et al., 2020a). Given this, the re-establishment of adenosine levels promoted by ITU administration should produce an higher

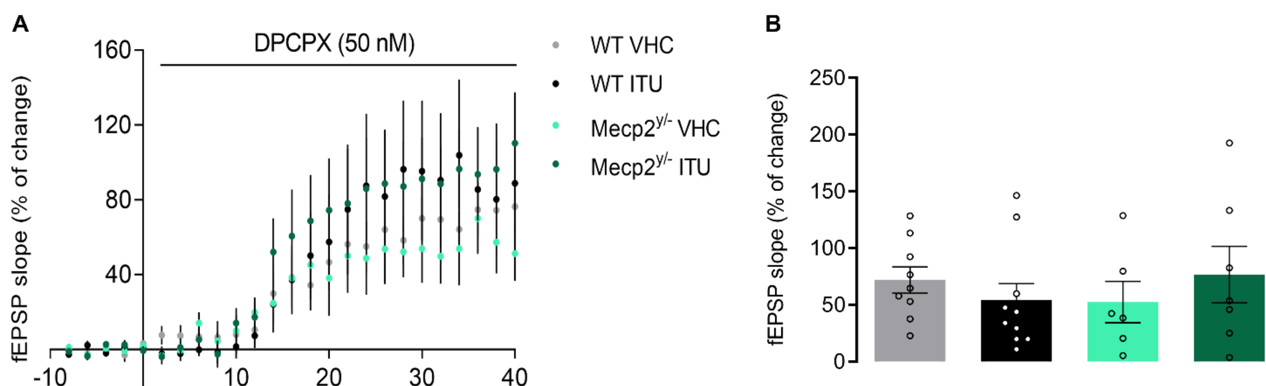

Figure 4.3.1| Synaptic transmission evaluation upon DPCPX effect. The histogram **A** represents the averaged normalized fEPSPs slope in each animal group in response to DPCPX (50 nM). The right panel **B** shows the averaged time courses of changes in fEPSP slope induced by application of DPCPX in slices taken from WT-VHC (grey, n=9), WT-ITU (black, n=10) and $Mecp2^{-/y}$-VHC (light green, n=6), $Mecp2^{-/y}$-ITU (dark green, n=7) animals.

disinhibition of synaptic transmission in presence of DPCPX, similarly to the effect produced in WT non-treated animals, described before (Miranda-Lourenço et al., 2020a).

ITU administration has no significant effect in the disinhibition of synaptic transmission promoted by DPCPX (Figure 4.3.1). In fact, not even the expected differences between the controlled groups, already described (Miranda-Lourenço et al., 2020a), were found (WT-VHC vs Mecp2$^{-/y}$-VHC). Analyzing more carefully, although there are no significant differences between the groups it is possible to observe that Mecp2$^{-/y}$ animals treated with ITU presented a response to DPCPX more similar to the response observed in WT-VHC animals (WT-VHC = 71.84 ± 11.52, n = 9; WT-ITU = 53.96 ± 14.63, n = 10; Mecp2$^{-/y}$-VHC = 52.50 ± 18.32, n = 6; Mecp2$^{-/y}$-ITU = 76.66 ± 24.95, n = 7; two-way ANOVA; Figure 4.3.1 **A;B**).

2.2 Evaluation of LTP magnitude and BDNF actions in hippocampal slices from symptomatic treated animals

The dysregulation found both in BDNF signaling and in adenosinergic system apparent to be an important feature present in RTT, as described in the previous chapters. One of the observed impacts, at functional level, associated with the dysfunction of both systems is the loss of BDNF effect upon LTP. It was shown that the activation of $A_{2A}R$, with a selective agonist, is able to recover BDNF effect upon LTP. In this way, the augmentation of adenosine levels should trigger a higher efficiency of AR activation, including $A_{2A}R$. The efficient activation of these receptors would promote the re-establishment of their functions, including the crosstalk between $A_{2A}R$ and TrkB-FL receptors, boosting BDNF actions indirectly. To prompt this hypothesis, LTP magnitude were evaluated in the four studied group of animals.

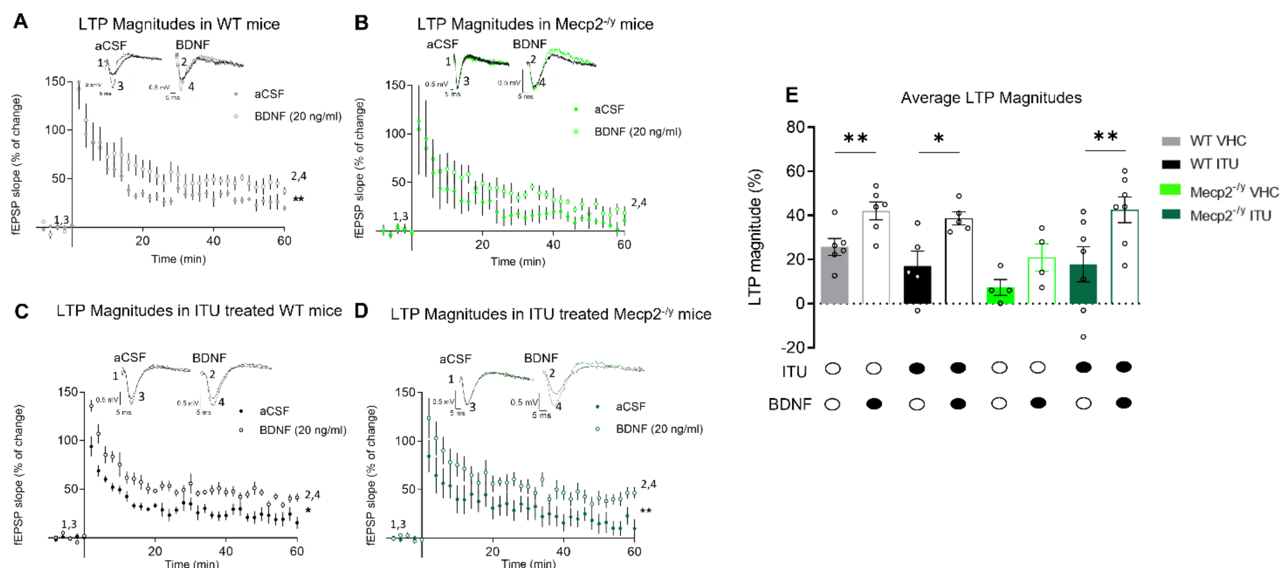

Figure 4.3.2| BDNF facilitatory effect upon LTP in ITU treated animals. Panels **A-D** show time courses of averaged changes in fEPSP slope induced by the θ-burst stimulation in the absence (dark circles) or in the presence (light circles) of BDNF in hippocampal slices taken from WT-VHC (grey, n=6), WT-ITU (black, n=5) and $Mecp2^{-/y}$-VHC (light green, n=4), $Mecp2^{-/y}$-ITU (dark green, n=7) animals. Traces from representative experiments are shown on the top of the panel (1,3 – baseline; 2.4 – LTP after 1 hour). The arrow marks LTP induction. Panel **E** depicts the magnitude of LTP in the absence (dark bars) and the presence of BDNF (20 ng/ml, dark bars) in hippocampal slices from each group of animals. *p<0.05, **p<0.01 (paired Student's t-test).

As observed in Figure 4.3.2, as expected, BDNF shows an effect upon LTP potentiation in both WT animal groups ($WT_{VHC-CTL}$ = 25.75 ± 3.81%, n = 6 and $WT_{VHC-BDNF}$ = 42.06 ± 4.07%, n = 6, p = 0.006, paired t-test; Figure 4.3.2 **A;E**; $WT_{ITU-CTL}$ = 17.12 ± 6.67%, n = 5 and $WT_{ITU-BDNF}$ = 38.70 ± 2.89%, n = 5, p = 0.02, paired t-test; Figure 4.3.2 **C,E**). Additionally, and as observed previously, in $Mecp2^{-/y}$ VHC group, BDNF lost its effect upon LTP ($Mecp2^{-/y}{}_{VHC-CTL}$ = 7.4 ± 3.59%, n = 4 and $Mecp2^{-/y}{}_{VHC-BDNF}$ = 20.96 ± 6.19%, n = 4, p = 0.25, paired t-test; Figure 4.3.2 **B;E**). Remarkably, in $Mecp2^{-/y}$ ITU treated animals, BDNF effect upon LTP was restored, congruous to $A_{2A}R$ selective agonist (CGS21680) results showed in chapter 4.1 ($Mecp2^{-/y}{}_{ITU-CTL}$ = 17.84 ± 7.97%, n = 7 and $Mecp2^{-/y}{}_{ITU-BDNF}$ = 42.55 ± 5.81%, n = 7, p = 0.005 0.25, paired t-test; Figure 4.3.2 **D;E**).

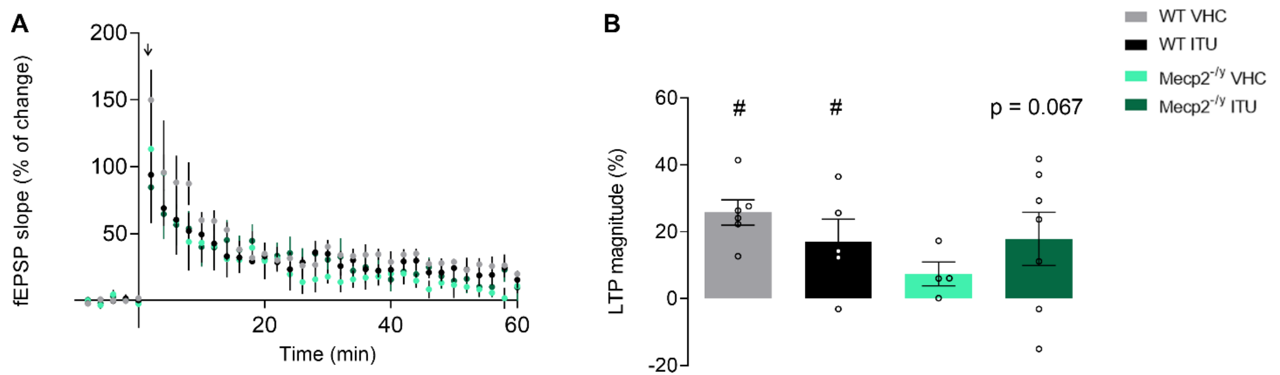

Figure 4.3.3| Comparison of basal LTP. Histogram **A** depicts the magnitude of LTP in the absence of BDNF (20 ng/ml, dark bars) in hippocampal slices from each group of animals: WT-VHC (grey, n=6), WT-ITU (black, n=5) and Mecp2$^{-/y}$-VHC (light green, n=4), Mecp2$^{-/y}$-ITU (dark green, n=7). The arrow marks LTP induction. Panel **B** shows time courses of averaged changes in fEPSP slope induced by the θ-burst stimulation in the absence (dark circles) of BDNF in hippocampal slices taken from animals. # $p<0.05$, (paired Student's t-test).

However, when evaluating LTP magnitude in the control pathway (the first LTP induced in each group of animals, in the absence of BDNF), it was observed, once again, that θ-burst stimulation induced a small, yet significant LTP in WT animals ($WT_{VHC-CTL}$: $p = 0.0024$, paired t-test; $WT_{ITU-CTL}$: $p = 0.023$, paired t-test; Figure 4.3.3 **A;B**), but not in Mecp2$^{-/y}$ animals ($Mecp2^{-/y}_{VHC-CTL}$: $p = 0.14$, paired t-test; $Mecp2^{-/y}_{ITU-CTL}$: $p = 0.067$, paired t-test; Figure 4.3.3 **A;B**). Despite the statistical significance was not achieved in the θ-burst stimulation induced in both groups of Mecp2$^{-/y}$ animals, Mecp2$^{-/y}$-ITU treated animals were closer to the statistical significance, with a LTP magnitude similar to WT-ITU group.

Given these results, it is possible to affirm that *in vivo* ITU administration is able to restore BDNF effect upon LTP in Mecp2$^{-/y}$ animals. Moreover, ITU treatment increases LTP magnitude, in the absence of exogenous BDNF, to levels close to those observed in WT animals.

To investigate if any changes in baseline synaptic efficiency were induced by ITU treatment, I/O curves were performed, as in previous chapters.

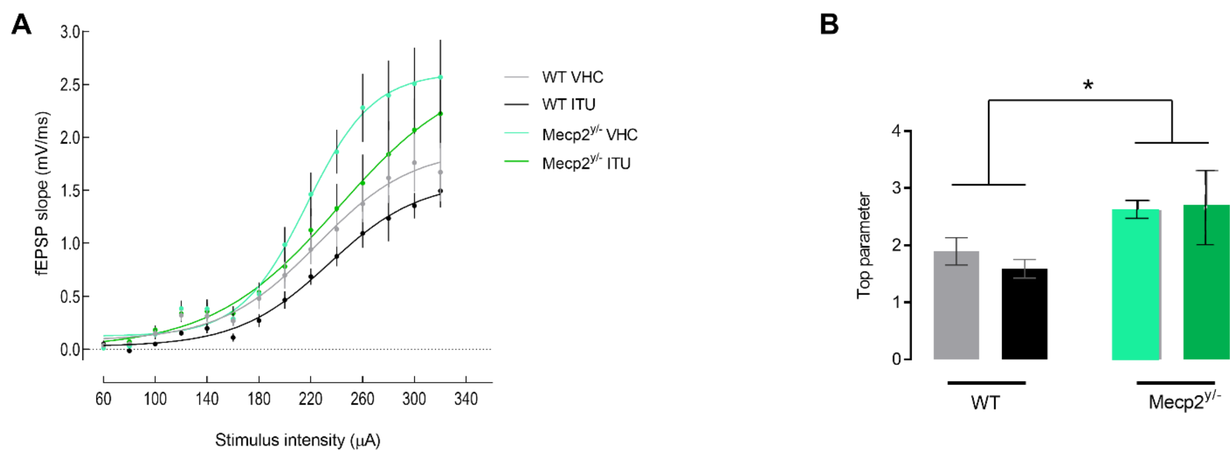

Figure 4.3.4| ITU treatment effect on input-output curves recordings. A shows the input/output (I/O) curves corresponding to responses generated by various stimulation intensities (60–340 µA) in WT-VHC (grey, n=11), WT-ITU (black, n=5) and Mecp2$^{-/y}$-VHC (light green,n=7), Mecp2$^{-/y}$-ITU (dark green, n=8) and **B** the respective means of top parameter. *p<0.05 (two-way ANOVA).

It was observed that the increased synaptic efficiency detected in hippocampal slices from Mecp2$^{-/y}$ animals was not affected by ITU administration. Accordingly, the two-way ANOVA analyzes revealed that the differences found in Emax are only influenced by the genotype but not by the treatment ($Emax_{WT\text{-}VHC}$ = 1.89 ± 0.24, n = 11; $Emax_{WT\text{-}ITU}$ = 1.59 ± 0.16, n = 5; $Emax_{Mecp\text{-}/y\text{-}VHC}$ = 2.61 ± 0.11, n = 7; $Emax_{Mecp\text{-}/y\text{-}ITU}$ = 2.67 ± 0.11, n = 8; two-way ANOVA multiple comparisons; Figure 4.3.4 **A;B**).

2.3 BDNF and TrkB-FL protein levels evaluation upon ITU treatment

Given the promising results obtained regarding LTP recovery in Mecp2$^{-/y}$ treated animals, it was explored if this regain of function could be related to molecular changes in BDNF signaling promoted by ITU treatment. Taking into account that an efficient $A_{2A}R$ activation is required for maintenance and regulation of both BDNF and TrkB expression (Tebano et al., 2008; Jerónimo-Santos et al., 2015), and knowing that BDNF and TrkB-FL levels are decreased in Mecp2$^{-/y}$ animals

(as described in chapter 4.1), in this chapter , the levels of BDNF and its receptors were evaluated in ITU-treated animals.

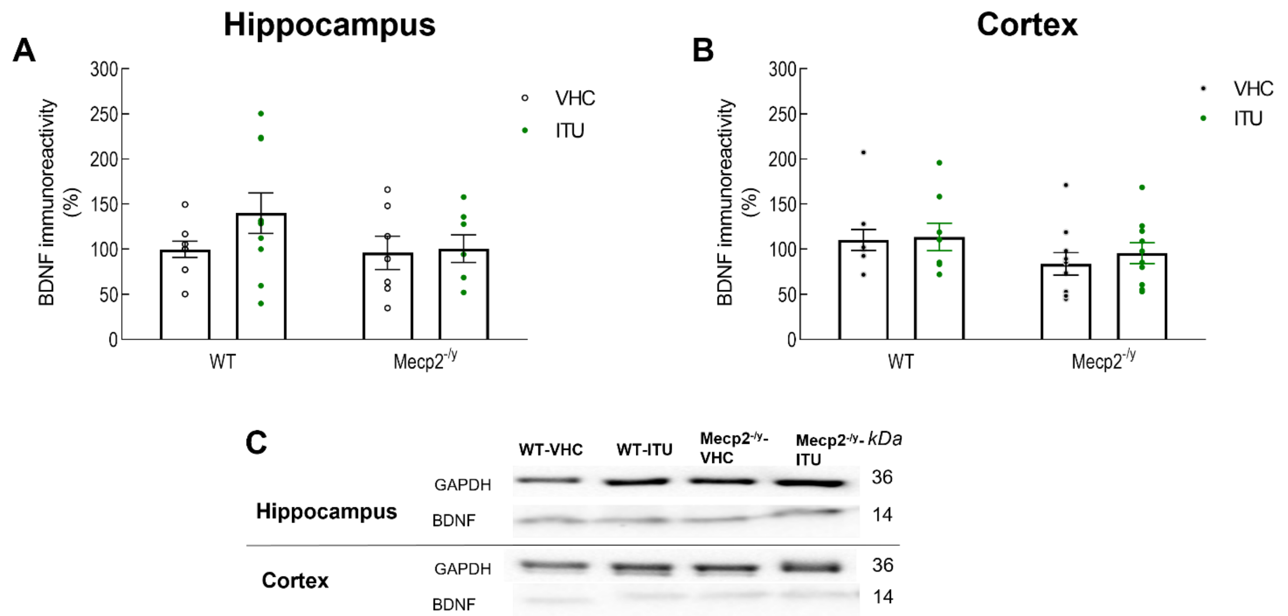

Figure 4.3.5| BDNF protein levels after ITU treatment. In **A** and **B** are shown the averaged of BDNF density levels evaluated by Western-Blot analysis in hippocampal (WT-VHC, n=9; WT-ITU, n=10; Mecp2$^{-/y}$-VHC, n=7; Mecp2$^{-/y}$-ITU, n=7) and cortical (WT-VHC, n=10; WT-ITU, n=8; Mecp2$^{-/y}$-VHC, n=10; Mecp2$^{-/y}$-ITU, n=10) homogenates, respectively. ITU treated animals are represented in green circles while control animals in open circles. **C** shows representative bands obtained. All values are mean±standard error of mean (SEM).

As observed in figure 4.3.5, ITU treatment did not promote any significant changes in BDNF protein levels in both brain regions studied (cortex and hippocampus). At the same time, the significant changes described in chapter 4.1, regarding decreased BDNF protein levels in Mecp2$^{-/y}$ animals, were not detected in this set of experiments ($HIP_{WT\text{-}VHC}$ = 100.0 ± 8.95%, n = 9 vs $HIP_{WT\text{-}ITU}$ = 139.9 ± 22.41%, n = 10 vs $HIP_{Mecp2-/y\text{-}VHC}$ = 96.2 ± 18.49%, n = 7, $HIP_{Mecp2-/y\text{-}ITU}$ = 100.7 ± 15.23%, n = 7; $CTX_{WT\text{-}VHC}$ = 100.0 ± 7.10%, n = 10 vs $CTX_{WT\text{-}ITU}$ = 113.6 ± 15.29%, n = 8 vs $CTX_{Mecp2-/y\text{-}VHC}$ = 83.7 ± 12.40%, n = 10 vs $CTX_{Mecp2-/y\text{-}ITU}$ = 95.6 ± 11.58%, n = 10, two-way ANOVA; Figure 4.3.5 **A;B;C**).

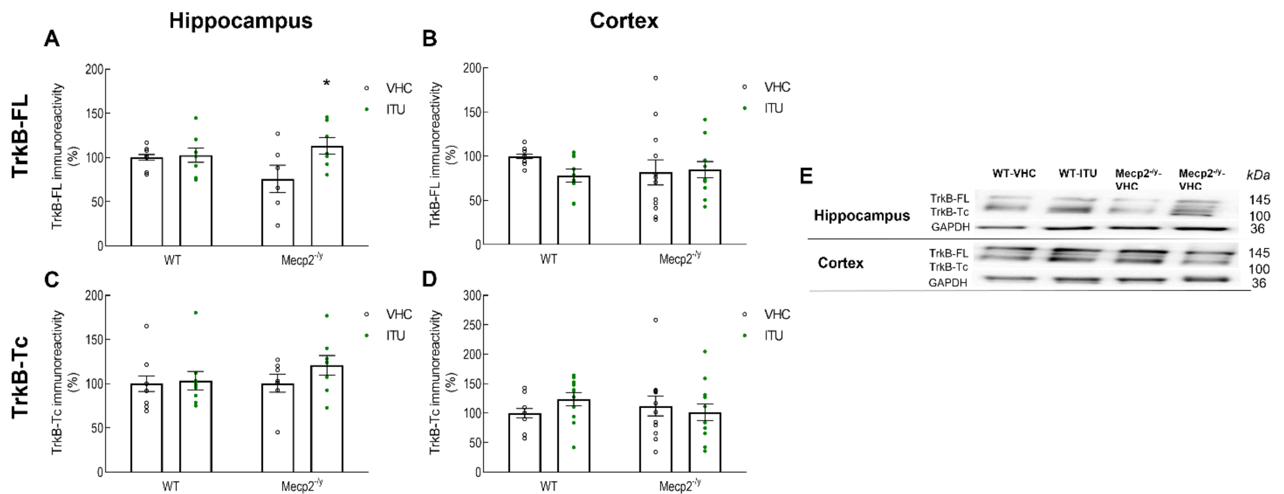

Figure 4.3.6| TrkB-FL and -Tc levels after ITU treatment. In **A** and **B** are shown the averaged TrkB-FL density levels evaluated by Western-Blot analysis in hippocampal (WT-VHC, n=10; WT-ITU, n=10; Mecp2$^{-/y}$-VHC, n=6; Mecp2$^{-/y}$-ITU, n=7) and cortical (WT-VHC, n=11; WT-ITU, n=9; Mecp2$^{-/y}$-VHC, n=12; Mecp2$^{-/y}$-ITU, n=12) homogenates, respectively. In **C** and **D** are shown the averaged TrkB-Tc density levels evaluated by Western-Blot analysis in hippocampal (WT-VHC, n=10; WT-ITU, n=9; Mecp2$^{-/y}$-VHC, n=7; Mecp2$^{-/y}$-ITU, n=8) and cortical (WT-VHC, n=11; WT-ITU, n=11; Mecp2$^{-/y}$-VHC, n=12; Mecp2$^{-/y}$-ITU, n=12) homogenates, respectively. ITU treated animals are represented in green circles while control animals in open circles. **E** show representative bands obtained. All values are mean±standard error of mean (SEM). *p<0.05 (two-way ANOVA).

However, when TrkB-FL protein levels were analyzed, it was possible to detect changes in hippocampus of Mecp2$^{-/y}$ treated animals. In these animals, TrkB-FL protein levels recovered its levels. ($HIP_{WT\text{-}VHC}$ = 100.0 ± 3.48%, n = 10 vs $HIP_{WT\text{-}ITU}$ = 102.7 ± 8.14%, n = 10 vs $HIP_{Mecp2-/y\text{-}VHC}$ = 76.0 ± 15.40%, n = 6, $HIP_{Mecp2-/y\text{-}ITU}$ = 113.0 ± 9.42%, n = 7; $CTX_{WT\text{-}VHC}$ = 100.0 ± 2.56%, n = 11 vs $CTX_{WT\text{-}ITU}$ = 78.28 ± 7.33%, n = 9 vs $CTX_{Mecp2-/y\text{-}VHC}$ = 81.7 ± 14.26%, n = 12 vs $CTX_{Mecp2-/y\text{-}ITU}$ = 84.8 ± 9.15%, n = 12, two-way ANOVA; Figure 4.3.6 **A-C**). This recovery of hippocampal TrkB-FL protein levels could contribute for the recovery of BDNF effect upon LTP observed in Mecp2$^{-/y}$ animals.

Once again, no changes in TrkB-Tc levels were detected between genotype or treatment groups ($HIP_{WT\text{-}VHC}$ = 100.0 ± 8.75%, n = 10 vs $HIP_{WT\text{-}ITU}$ = 103.6 ± 10.41%, n = 9 vs $HIP_{Mecp2-/y\text{-}VHC}$ = 100.7 ± 10.32%, n = 7, $HIP_{Mecp2-/y\text{-}ITU}$ = 120.8 ± 11.10%, n = 8; $CTX_{WT\text{-}VHC}$ = 100.0 ± 7.72%, n = 11 vs $CTX_{WT\text{-}ITU}$ = 123.8 ± 11.39%, n = 11 vs $CTX_{Mecp2-/y\text{-}VHC}$ = 112.2 ± 16.78%, n = 12 vs $CTX_{Mecp2-/y\text{-}ITU}$ = 101.70 ± 14.19%, n = 12, two-way ANOVA; Figure 4.3.6 **A-C**).

In this way, ITU treatment showed an effect in hippocampus, promoting a recovery of TrkB-FL protein levels, crucial for synaptic plasticity also recovered in Mecp2$^{-/y}$ treated animals.

3. Discussion

In this chapter, ADK blockage was studied as an AAT. ITU intraperitoneal administration showed to have a positive impact in the recovery of BDNF effect upon LTP animals. Simultaneously, ITU treatment also restored TrkB-FL protein levels in hippocampus of Mecp2$^{-/y}$ animals. These results set a promising new strategy to be explored in RTT, stablishing adenosinergic system dysregulation as an important contributor for RTT pathophysiology.

The first two chapters of results of this dissertation revealed that BDNF signaling is impaired in two RTT mouse models with different phenotypic severity (Mecp2$^{-/y}$ and Mecp2$^{+/-}$ animals). In both, significant changes in BDNF signaling were observed, but more prominently in Mecp2$^{-/y}$ animals (Miranda-Lourenço et al., 2020a). Importantly, it was shown that adenosinergic system is dysregulated. The decreased levels of adenosine found in the hippocampus and cortex of symptomatic Mecp2$^{-/y}$ add new information about RTT pathophysiology and how other systems might contribute for BDNF signaling impairment and for excitatory-inhibitory imbalance widely discussed in this field (Boggio, 2010; Li and Pozzo-Miller, 2014). Moreover, it was shown that a proper activation of $A_{2A}R$ can recover BDNF effect upon LTP lost in both models studied. This opened a new avenue to be explored in RTT: the modulation of adenosinergic system, namely through AAT. The re-establishment of adenosine levels would promote the activation of both $A_{2A}R$ and A_1R, acting in two signaling pathways potentially beneficial in RTT: while the activation of $A_{2A}R$ would potentiate BDNF actions, A_1R activation could be useful to control hyperexcitability present in RTT.

AAT have been discussed as possible alternative to be applied in several pathologies, such us epilepsy, chronic pain, trauma and inflammation (Kowaluk and Jarvis, 2000; Boison, 2009; Jarvis, 2019). Although several strategies could be used to increase adenosine levels, the blockage of ADK is one of the strategies that have been already tested in epilepsy. ITU is one of the drugs used for this purpose, already studied in epilepsy models with a good outcome as an ASD, reducing seizures burden and activity (Sandau et al., 2019). In fact, in epilepsy, ADK have been considered as an important player in epileptogenesis, presenting increased expression levels in epileptic models (Li et al., 2008; Theofilas et al., 2012). The data obtained in the present thesis (chapter 4.1), revealed increased ADK protein levels in cortex and a tendency for increased ADK protein levels in hippocampus of pre-symptomatic Mecp2$^{-/y}$ animals. These alterations, congruent to what is described in epilepsy, may suggest that the alterations in ADK protein levels might have

repercussions in the decreased adenosine levels found in these animals. Indeed, these results further support the implementation of ADK blockage as a strategy to increase adenosine levels also in RTT.

Despite promising results, the blockage of ADK by ITU has some hindrances related to liver toxicity, cardiovascular side effects, and neurovascular toxic effects. These side effects are transposable to the majority of ADK inhibitors (Boison et al., 2002; McGaraughty et al., 2005; Jarvis, 2019) and could limit long term administration of ADK inhibitors. One way to overcome this problem would be the short-time administration of the ADK inhibitor. However it would require a precise determination of the critical timepoints where it should be administered (Sandau et al., 2019). Other option would be the use of more selective ADK inhibitors, as already screened in the last years and explored for renal injury treatment (Köse et al., 2016; Zhu et al., 2016). The selectivity increase of these compounds could be achieved by developing ADK inhibitors selective for ADK-nuclear isoform instead for ADK-cytoplasmatic isoform, as the majority of ADK inhibitors are (Toti et al., 2016; Jarvis, 2019). ADK exists in two isoforms: 1) a cytoplasmatic isoform, with a shorter length; 2) a nuclear isoform, with longer length (Cui et al., 2011). The side effects associated to systemic administrations of ADK inhibitors, despite not completely deciphered, could be related with a mediated-AR toxicity instead of adenosine increased concentrations *per se*. In this way, developing ADK inhibitors selective to nuclear-ADK isoform could overcome this unwanted effects (Toti et al., 2016; Jarvis, 2019). Targeting selectively nuclear-ADK, with a prominent effect upon epigenetic modulation, would possibly allow to reach satisfying safety and toxicity outcomes once it would allow to obtain therapeutic effects using a lower dose or a shorter administration time when compared with non-selective ADK inhibitors. The epigenetic effects associated to adenosine, which in higher levels promotes an increase of SAH and a consequent inhibition of DNA methyltransferases would allow to reduce the duration of drug administration, avoiding toxicity associated to chronic therapy and high dose administration (Williams-Karnesky et al., 2013; Boison, 2016; Toti et al., 2016).

Regarding dose optimization, it was shown in pain studies that to reach therapeutic effects, in particular analgesia, it would be necessary three to ten-folder lower doses than those needed to cause psychomotor and cardiovascular side effects (Jarvis, 2019). In this way, impactful side effects consequent of ADK administration usually happen for higher doses and/or long duration administrations. However, in specific context of RTT where adenosine levels are decreased, and

AR are altered, these noxious effects would not be probably observed once the adenosine augmentation strategy will re-establish adenosine levels rather than increase it above the physiological levels not causing an overactivation of AR.

Specifically considering ITU, hepatic side effects are described but those are observed at an half maximal inhibitory concentration (IC_{50}, usually used as a measure of an antagonist drug potency in pharmacological studies), about 1000-fold higher than the IC_{50} for ADK (26 nM) (Boison, 2013b; Sandau et al., 2019). In addition, an advantage of ITU is its ability to block adenosine transport at a similar IC_{50} (Foga et al., 1996). This allows to trap adenosine inside the cell and consequently enable adenosine receptor-independent epigenetic activity to play a key role in epilepsy prevention, where it was shown that adenosine inhibits DNA methylation important for epileptogenesis process (Williams-Karnesky et al., 2013).

Interestingly, it was possible to observed that ITU administration: 1) recovered BDNF effect upon LTP; 2) increased basal LTP; and 3) restored hippocampal TrkB-FL protein levels in Mecp2$^{-/y}$ animals. This site- and event-specific effects were already described in other studies that showed that systemically administration of non-selective ADK inhibitors induces endogenous stress, enhancing adenosine actions in cells undergoing accelerated adenosine release (Newby et al., 1983; Pak et al., 1994; Britton et al., 1999). This means that ADK inhibition effect is more pronounced in tissues where pathological changes resulted in adenosine release, already observed in hippocampus, spinal cord (*in vitro*) and in striatum after peripheral inflammation and excitotoxic insults in rats (*in vivo*) (Pak et al., 1994; Golembiowska et al., 1996; Liu et al., 2000). Besides the noted decreased adenosine levels, no information regarding adenosine release was obtain in Mecp2$^{-/y}$ animals. ADK effectiveness in cells undergoing pathological adenosine release point to explore if the brain regions where we obtained positive results under ITU administration could be related with this feature.

However, it should be explored why in cortex no specific changes were promoted after ITU treatment. Some aspects like the administration timepoints, the administered dose or the cell-specific effect (given the high cortical heterogeneity) should be address and properly explored to better interpret the results. Besides the differences in BDNF and Mecp2 across different brain regions that could explain this different outcome in cortex, it has been explored how $A_{2A}R$ activation exerts its effect upon TrkB transactivation over different brain regions. Indeed, it has been suggested that A_{2A} receptor KO mice shows reduced BDNF levels in hippocampus and

striatum, but not cerebral cortex (Tebano et al., 2008). This suggests that $A_{2A}R$ may not be involved in BDNF expression in cerebral cortex (Jeon et al., 2011). Additionally, different regulatory mechanisms have been proposed for $A_{2A}R$ subtype distribution across all brain, which could influence $A_{2A}R$ action in each brain region (Weaver, 1993; Latini et al., 1996; Rebola et al., 2005).

Despite the positive outcome obtained in this first exploratory study, present in this chapter, more studies should be performed. It should be explored adenosine augmentation effects upon other impaired functions associated to BDNF/adenosine impairment in RTT, but should be also clarified possible toxicity and safety issues that could arise.

Given the endogenous anticonvulsant effect of adenosine, it should be expected a decreased excitability in I/O performed in ITU treated animals. However, no significant effect was obtained, even considering the increased A_1R expression in Mecp2$^{-/y}$, with no changes in these receptors affinity or in its function (Miranda-Lourenço et al., 2020a). More specific techniques should be performed to characterize hyperexcitability in Mecp2$^{-/y}$ after adenosine augmentation, to ensure that this particular RTT feature could also be re-established. Simultaneously, the previous mentioned caveats should be addressed to understand what optimizations should be done in this therapeutic strategy to also revert the hyperexcitability in RTT.

In fact, it is necessary to confirm with more precision if the AAT applied increased adenosine levels in an effective way (e.g. using HPLC). The study of DPCPX effect upon synaptic transmition did not show any differences between the four groups studied, when was expected, at least, differences between WT-CTL and Mecp2$^{-/y}$-CTL, replicating the results obtained in (Miranda-Lourenço et al., 2020a). In this way, it is not possible to understand if ITU administration augmented adenosine levels as desirable. Indeed, these group of experiments should be performed again to clarify the real effect of DPCPX upon synaptic transmission and if it is possible to extrapolate more information regarding ITU administration efficiency in increasing adenosine levels. Simultaneously, BDNF decreased protein levels were not found in Mecp2$^{-/y}$ animals as previously described, adding necessity to clarify the results obtained in this set of experiments.

In fact, one of the main advantages of using ADK inhibitors is that its action allows to target multiple AR by increasing adenosine levels. This strategy was shown to be useful in specific pathologies, such as chronic pain and in inflammation, where multiple AR are beneficial to target

(Williams and Jarvis, 2000; McGaraughty et al., 2005). The results present in this dissertation show that $A_{2A}R$ targeting brings positive outcomes, allowing a recover of BDNF functions. It is now crucial to understand if this strategy is also efficient in promoting inhibitory tonus through A_1R activation.

All together, these results point to a promising action of ADK inhibitors throughout hippocampal BDNF signaling recovery. However further investigation is needed in order to understand how AAT could be applied as a therapy for RTT.

General Conclusions and Future Perspectives

General Conclusions and Future Perspectives

For the first time, adenosinergic system was considered as a possible disrupted system influencing RTT pathophysiology.

Overall, the results here obtain showed that, as initially hypothesized, adenosinergic system is significantly altered, with both adenosine and/or its receptor levels changed, in two different RTT mouse models. These changes accompany BDNF signaling alterations. Besides to corroborate studies already published showing decreased BDNF levels in RTT, our study added new information about BDNF dysregulation in RTT: 1) TrkB-FL receptor protein levels are decreased in cortex and hippocampus of symptomatic Mecp2-null mice; 2) changes in BDNF signaling occur across disease progression in *Mecp2*-null animals, and 3) BDNF loss its facilitatory action upon synaptic plasticity in both studied models. Moreover, an *in vivo* AAT strategy was tested, reaching positive results by recovering BDNF facilitatory effect upon hippocampal LTP and re-establishing hippocampal TrkB-FL protein levels, in Mecp2$^{-/y}$ animals.

It will be important to address new questions to better explore these new findings. Understanding why adenosine levels are decreased in *Mecp2*-null male mice may allowed to find a more direct target that could revert or prevent changes in adenosine levels. A deep analysis of adenosine metabolic regulators, such as ADA, endonucleotidases and ADK should be considered. Both its expression levels and enzymatic activities could give us important data to better understand why adenosine levels are affected. Moreover, a timeline of these changes, but also of AR alterations, will allow to observe when adenosinergic disruption starts, which will also facilitate to optimize a better target. Unfortunately, in results present in this dissertation, it was only possible to perform this timeline for ADK protein levels, in *Mecp2*-null animals.

Remarkably, this study brings to discussion an important topic regarding RTT, which reinforces its strength: the high variability of RTT phenotypes caused by its highly complex genetic background. Studying, in parallel, two different RTT phenotypes through *Mecp2*-null male and -heterozygous female mice depicted that BDNF and adenosinergic changes could be transversal to different disease severities. However, some details are missing regarding heterozygous female characterization. Adenosine levels quantification is primordial to understand if the less severity observed in this model

is also supported by minor impacts detected in adenosinergic system. Moreover, no information regarding BDNF and adenosinergic changes were obtained during the pre-symptomatic stage of this model. Considering the high difficulty of this study, since the symptoms establishment do not start simultaneously in all heterozygous females, correlation studies between behavior, molecular changes, and functional responses might be a good strategy to address this question and decipher how symptoms and molecular changes in adenosine and BDNF signaling could be related.

Regarding the study using an *in vivo* ATT strategy, the data obtain reinforces adenosinergic system as a promising pharmacological target in RTT. However, this study was mainly exploratory. First, it is important to clarify why systemic ADK blockage only revert TrkB-FL protein levels in hippocampus, functionally translated in the recovery of BDNF potentiation over hippocampal LTP. Additionally, to understand if this treatment has an important repercussion at behavioral level, in particular in cognitive functions, it is crucial to validate the pertinence of this strategy by performing behavioral tests. Furthermore, given that choosing an AAT as a beneficial strategy for RTT is related to its ability to simultaneously target A_1R and $A_{2A}R$, it is also important to study ADK blockage in inhibitory/excitatory imbalance.

It is also important to notice that ADK blockage through ITU administration worked as a proof-of-concept. In future work, a careful follow-up study should be performed in order to detect possible side effects promoted by ADK blockage, and if necessary to explore other alternatives to increase adenosine levels. Simultaneously, different timepoints of AAT administration should be tested to understand if this treatment could also give the possibility to prevent the progression of this syndrome rather than revert symptomatology. Importantly, if reasonable, all these questions should be address to other RTT models before clinical studies, to guaranteed that AAT is beneficial for different RTT phenotypes.

Overall, this project added valuable information to RTT pathophysiology understanding, bringing questions to be explored and a new possible therapeutic strategy to be studied in RTT.

Publications

The scientific content of the present thesis is included in the published manuscripts:

1. **Miranda-Lourenço C**, Duarte ST, Palminha C, Gaspar C, Rodrigues TM, Magalhães-Cardoso T, Rei N, Colino-Oliveira M, Gomes R, Ferreira S, Rosa J, Xapelli S, Armstrong J, García-Cazorla À, Correia-de-Sá P, Sebastião AM, Diógenes MJ (2020). Impairment of adenosinergic system in Rett syndrome: Novel therapeutic target to boost BDNF signalling; Neurobiol Dis doi: 10.1016/j.nbd.2020.105043 (Attachment 1);
2. **Miranda-Lourenço C**, Ribeiro-Rodrigues L, Fonseca-Gomes J, Tanqueiro SR, Belo RF, Ferreira CB, Rei N, Ferreira-Manso M, de Almeida-Borlido C, Costa-Coelho T, Freitas CF, Zavalko S, Mouro FM, Sebastião AM, Xapelli S, Rodrigues TM, Diógenes MJ. (2020) Challenges of BDNF-based therapies: from common to rare diseases; Pharmacol Res doi: 10.1016/j.phrs.2020.105281;
3. **Miranda-Lourenço C**, Rosa J, Rei N, Belo RF, Magalhães-Cardoso T, Correia-de-Sá P, Sebastião AM, Diógenes MJ (in preparation) Adenosinergic system changes as a transversal featured of RTT: characterization of Mecp2 heterozygous female phenotype.

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

1. Sa de Almeida J, Vargas M, Fonseca-Gomes J, Tanqueiro SR, Belo RF, **Miranda-Lourenço C**, Sebastião AM, Diógenes MJ, Pais TF (2020) Microglial Sirtuin 2 Shapes Long-Term Potentiation in Hippocampal Slices; Front Neurosci doi: 10.3389/fnins.2020.00614;
2. Ruffolo G, Cifelli P, **Miranda-Lourenço C**, De Felice E, Limatola C, Sebastião AM, Diógenes MJ, Aronica E, Palma E. Rare Diseases of Neurodevelopment: Maintain the Mystery or Use a Dazzling Tool for Investigation? The Case of Rett Syndrome. (2020) Neuroscience doi: 10.1016/j.neuroscience.2019.06.015;
3. Mouro FM, **Miranda-Lourenço C**, Sebastião AM, Diógenes MJ (2019) From Cannabinoids and Neurosteroids to Statins and the Ketogenic Diet: New Therapeutic Avenues in Rett Syndrome? Front Neurosci doi: 10.3389/fnins.2019.00680;

Attachments

Attachment 1: Impairment of adenosinergic system in Rett syndrome: Novel therapeutic target to boost BDNF signalling (Miranda-Lourenço et al., 2020a)

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Impairment of adenosinergic system in Rett syndrome: Novel therapeutic target to boost BDNF signalling

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ABSTRACT

Rett syndrome (RTT; OMIM#312750) is mainly caused by mutations in the X-linked *MECP2* gene (methyl-CpG-binding protein 2 gene; OMIM*300005), which leads to impairments in the brain-derived neurotrophic factor (BDNF) signalling. The boost of BDNF mediated effects would be a significant breakthrough but it has been hampered by the difficulty to administer BDNF to the central nervous system. Adenosine, an endogenous neuromodulator, may accomplish that role since through $A_{2A}R$ it potentiates BDNF synaptic actions in healthy animals. We thus characterized several hallmarks of the adenosinergic and BDNF signalling in RTT and explored whether $A_{2A}R$ activation could boost BDNF actions.

For this study, the RTT animal model, the *Mecp2 knockout* (*Mecp2*$^{-/y}$) (B6.129P2 (C)-Mecp2tm1.1Bird/J) mouse was used. Whenever possible, parallel data was also obtained from *post-mortem* brain samples from one RTT patient. *Ex vivo* extracellular recordings of field excitatory post-synaptic potentials in CA1 hippocampal area were performed to evaluate synaptic transmission and long-term potentiation (LTP). RT-PCR was used to assess mRNA levels and Western Blot or radioligand binding assays were performed to evaluate protein levels. Changes in cortical and hippocampal adenosine content were assessed by liquid chromatography with diode array detection (LC/DAD).

Hippocampal *ex vivo* experiments revealed that the facilitatory actions of BDNF upon LTP is absent in *Mecp2*$^{-/y}$ mice and that TrkB full-length (TrkB-FL) receptor levels are significantly decreased. Extracts of the hippocampus and cortex of *Mecp2*$^{-/y}$ mice revealed less adenosine amount as well as less $A_{2A}R$ protein levels when compared to WT littermates, which may partially explain the deficits in adenosinergic tonus in these

Abbreviations: [^{3}H]DPCPX, 1,3-[3H]-dipropyl-8-cyclopentylxanthine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; 5-ITU, 5-iodotubercidin; A_1R, A_1 receptors; $A_{2A}R$, A_{2A} receptors; BDNF, brain-derived neurotrophic factor; aCSF, cold artificial cerebrospinal fluid; fEPSPs, Field excitatory postsynaptic potentials; I/O, Input–output curve; LTP, long-term potentiation; Mecp2$^{-/y}$, MECP2 knockout; MECP2, methyl-CpG binding protein 2; CPA, N6-Cyclopentyladenosine; PVDF, polyvinylidene fluoride; qPCR, quantitative PCR; RIPA, Radio-Immunoprecipitation Assay; RTT, Rett Syndrome; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TrkB-FL, Tropomyosin receptor kinase B full-length (TrkB-FL); WT, wild type

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animals. Remarkably, the lack of BDNF effect on hippocampal LTP in *Mecp2*$^{-/y}$ mice was overcome by selective activation of $A_{2A}R$ with CGS21680.

Overall, in *Mecp2*$^{-/y}$ mice there is an impairment on adenosinergic system and BDNF signalling. These findings set the stage for adenosine-based pharmacological therapeutic strategies for RTT, highlighting $A_{2A}R$ as a therapeutic target in this devastating pathology.

1. Introduction

Rett Syndrome (RTT) is a progressive genetic neurodevelopmental disorder characterized by cognitive and motor impairments, development of stereotypic hand movements and epilepsy. A developmental regression with loss of skills acquired during an apparently normal period after birth (*ca.* 6 months) is typical (Neul et al., 2014). RTT affects 1:10.000 female live births worldwide and it is the main genetic cause of intellectual disability in girls (Chahrour and Zoghbi, 2007). About 90% of RTT cases are caused by mutations in methyl-CpG binding protein 2 gene (*MECP2*), located on the X chromosome, responsible for encoding the MeCP2 protein (Amir et al., 1999; Liyanage and Rastegar, 2014). This protein is an essential epigenetic factor that regulates the expression of several genes (Bedogni et al., 2014; Lewis et al., 1992; Liyanage and Rastegar, 2014). One of the genes under the jurisdiction of MeCP2 is the brain-derived neurotrophic factor (BDNF) gene, which encodes the neurotrophin BDNF (Li and Pozzo-Miller, 2014). BDNF plays essential functions during neuronal development and maturation, including axonal targeting, dendritic growth, synaptic maturation and it is also a modulator of synaptic plasticity (Binder and Scharfman, 2004). Its actions are mediated by the high-affinity Tropomyosin receptor kinase B full-length (TrkB-FL) receptors. BDNF binding to TrkB-FL receptors induces receptor dimerization and kinase domain transactivation, subsequently leading to activation of specific signalling transduction pathways (Huang and Reichardt, 2003). BDNF also has affinity to TrkB truncated isoforms (TrkB-Tc), which act as negative effectors of full-length receptors through their own pathways (Eide, 1996; Rose et al., 2003).

Previous studies have shown that BDNF levels are decreased in RTT mouse models; thus, deregulation of BDNF functions in RTT has been suggested as a disease mechanism for the deficits in synaptic transmission and plasticity, neuronal survival and development (Asaka et al., 2006; Deng et al., 2007; Li et al., 2012; Li and Pozzo-Miller, 2014; Moretti et al., 2006; Robinson et al., 2012). Accordingly, BDNF mRNA levels were shown to be decreased in *post-mortem* brain samples from human patients (Abuhatzira et al., 2007). However, no changes were detected in BDNF protein levels on cerebrospinal fluid and blood serum samples (Riikonen, 2003; Vanhala et al., 1998). Experiments where BDNF expression levels were genetically manipulated in RTT mouse models have shown that BDNF overexpression led to symptomatic improvements (Chen et al., 2007; Guy et al., 2007, for review see Li and Pozzo-Miller, 2014). However, therapeutic designs involving BDNF delivery to the brain are still inefficient, given BDNF's inability to cross the blood-brain barrier (Poduslo and Curran, 1996). In order to overcome this limitation, novel strategies should be considered, including the use of small molecules able to boost BDNF actions.

It is now currently accepted that most BDNF actions are dependent on the activation of a specific type of adenosine receptors, the A_{2A} receptors ($A_{2A}R$) (Sebastião et al., 2011). On the other hand, another subtype of adenosine receptor, the A_1R, has well known inhibitory actions upon synaptic transmission with impact in epilepsy control (Rombo et al., 2014). Modulation of adenosine receptors, mostly A_1 and $A_{2A}R$ has long been considered as a useful strategy to treat several neurologic disorders, such as sleep disorders, epilepsy and neurodegenerative diseases (Sebastião and Ribeiro, 2009), but has never been tested in RTT. Modulators of adenosine receptors were already tested in clinical trials for treatment of other diseases (Chen et al., 2007). In light of the knowledge on: i) the cross-talk between BDNF and adenosine receptors, ii) the advantages of potentiating BDNF actions in RTT, and iii) the deregulation of the adenosinergic system in several pathological conditions co-occurring with epilepsy, we hypothesized that adenosine receptors could be therapeutic targets in RTT. To tackle this hypothesis, we used a well-recognized RTT mice model, the *Mecp2 knockout* (*Mecp2*$^{-/y}$), to identify putative changes in the adenosinergic system and to assess whether pharmacological modulation of adenosine receptors could potentiate BDNF effects in this RTT model.

2. Materials and methods

2.1. Animals

Experiments were performed in a mouse model of RTT (B6.129P2)C)-Mecp2$^{tm1.1Bird}$/J [*MECP2* knockout; *Mecp2*$^{-/y}$], JAX #003890; (Guy et al., 2001) during the symptomatic stage (males between 6 and 10 weeks old; females between 26 and 28 weeks); wild type (WT) littermates were used as control. The genotype of animals was determined by PCR analysis, as previously described (Guy et al., 2007). The animals were housed on a 12 h light/dark cycle, with food and water provided *ad libitum*. Throughout the experimental work, care was taken to minimize the number of animals sacrificed. All animals were handled according to the Portuguese law on Animal Care and European Community guidelines (86/609/EEC). Mice were sacrificed by decapitation under deep isoflurane anaesthesia.

2.2. Post-mortem human brain sample

The brain tissue of one 11 year-old girl with RTT (MeCP2 mutation - R255X) who died after a severe pneumonia was dissected and different anatomic regions were immediately frozen at −80 °C. Age- and sex-matched cortical tissue was kindly provided by the "Biobank de Hospital Infantil Sant Joan de Déu (HSJD) per la Investigació" integrated in the "Spanish Biobank Network of ISCIII for the sample and the data procurement", to whom we are indebted.

2.3. Ex vivo electrophysiological recordings

After decapitation, the mouse brain was quickly removed, hemisected and both hippocampi were dissected free within ice-cold artificial cerebrospinal fluid (aCSF) solution: NaCl 124 mM; KCl 3 mM; NaH_2PO_4 1.25 mM; $NaHCO_3$ 26 mM; $MgSO_4$ 1 mM; $CaCl_2$ 2 mM; and glucose 10 mM, previously gassed with 95% O_2 and 5% CO_2, pH 7.4. Slices (400 µm thick) were cut perpendicularly to the long axis of the hippocampus with a McIlwain tissue chopper and allowed to recover functionally and energetically for at least 1 h in a resting chamber, filled with aCSF, at room temperature. Slices were then transferred to a submerging chamber (1 ml) and continuously superfused at 3 ml/min with gassed aCSF at 32 °C; drugs were added to this superfusion solution. Field excitatory postsynaptic potentials (fEPSPs) were recorded extracellularly through a microelectrode (2–8 MΩ resistance, Harvard apparatus Ltd., Cambridge, MA, USA) placed in *stratum radiatum* of hippocampal CA1 area. One or two pathways (as indicated) of the Schaffer collateral/commissural fibers were stimulated (rectangular 0.1 ms pulses), every 15 s (for input-output [I/O] curves and basal synaptic transmission) or every 20 s (for long-term potentiation [LTP] recordings) through a bipolar concentric electrode placed on the Schaffer fibers, in *stratum radiatum* near CA3–CA1 border. The intensity

of stimulus (80–200 μA) was initially adjusted to obtain a large fEPSP slope with a minimum population spike contamination. The averages of 8 (for I/O curves and basal synaptic transmission) or 6 (for LTP recordings) consecutive responses were obtained and the slope of the initial phase of the potential was quantified. Recordings were obtained with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA), digitized and continuously stored on a personal computer with the LTP software (Anderson and Collingridge, 2001).

2.3.1. Basal synaptic transmission

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

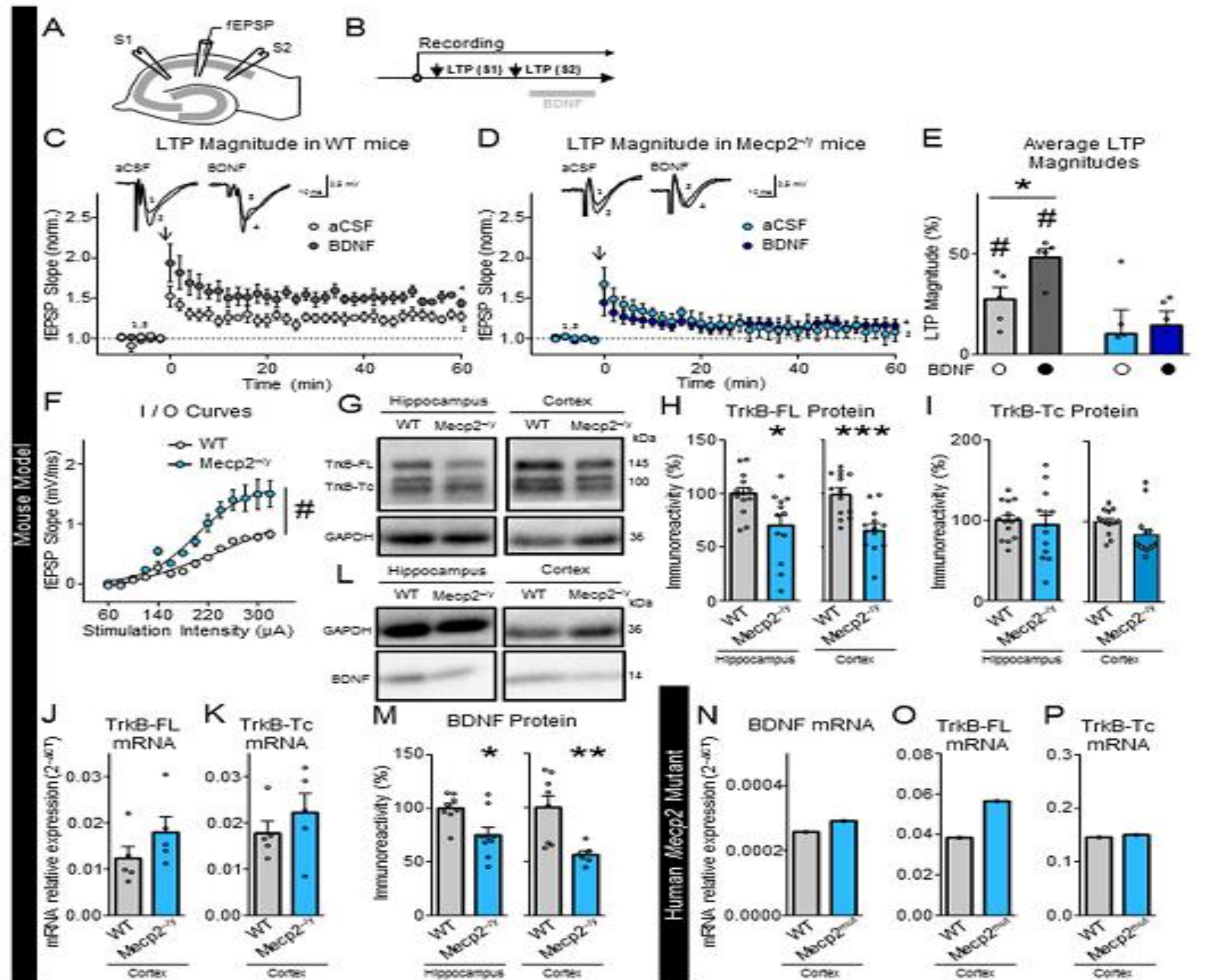

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Fig. 1. BDNF loses the facilitatory effect upon LTP in Mecp2$^{-/Y}$ animals: A shows schematic representation of a transverse hippocampal slice and the recording configuration used, as described in methods section. B represents the protocol used to evaluate the effect of BDNF upon LTP (see methods). Panels C and D show time courses of averaged changes in fEPSP slope induced by the θ-burst stimulation in the absence (light circles) or in the presence (dark circles) of BDNF in hippocampal slices taken from WT (grey symbols,n = 5) animals or from Mecp2$^{-/Y}$ (blue symbols,n = 5) animals. Traces from representative experiments are shown for WT and Mecp2$^{-/Y}$ animals. Histogram E depicts the magnitude of LTP in the absence (light bars) and the presence of BDNF (20 ng/ml, dark bars) in hippocampal slices from WT (grey) or Mecp2$^{-/Y}$ animals (blue). F shows the input/output (I/O) curves corresponding to responses generated by various stimulation intensities (60–340 μA) in WT slices (grey circles,*n* = 4) and Mecp2$^{-/Y}$ slices (blue circles,n = 4). **p* < 0.05 (paired Student's *t*-test) as compared with absence of BDNF in the same experiments.
In H,I and M are shown the averaged of TrkB-FL(WT_{hipp},n = 13;WT_{ctx},n = 14;$Mecp2^{-/Y}_{hip}$,n = 12;$Mecp2^{-/Y}_{ctx}$,n = 13), TrkB-Tc (WT_{hipp}, n = 14;WT_{ctx},n = 14;$Mecp2^{-/Y}_{hip}$,n = 13;$Mecp2^{-/Y}_{ctx}$,n = 14) and BDNF(WT_{hip},n = 8;WT_{ctx},n = 8;$Mecp2^{-/Y}_{hip}$,n = 9;$Mecp2^{-/Y}_{ctx}$,n = 6) density evaluated by Western-Blot analysis in hippocampal and cortical homogenates from WT(grey) and Mecp2$^{-/Y}$(blue) animals with 6–10 weeks of age. G and L show representative bands obtained by Western Blot for each studied protein. All values presented are mean ± SEM and are represented in % of WT protein. J,K and N–P depicted data obtained by qPCR showing mRNA levels of TrkB-FL(J,O), TrkB-Tc(K,P) and BDNF(N) in cortical samples from WT(light bar,n = 5) and Mecp2$^{-/Y}$(dark bar,n = 5) animals (J and K) and from a human temporal cortex sample (N,O and P) taken from a control patient (white bar,n = 1) and a RTT patient (black bar,n = 1). All values are mean ± standard error of mean (SEM). *p < 0.05; **p < 0.01; ***p < 0.001 (Student's t-test); #p < 0.05 (F test).

2.3.2. LTP induction and quantification

fEPSPs were elicited and recorded as outlined above. Stimulation was delivered alternatively to two independent pathways (Fig. 1A). LTP was induced by a θ-burst protocol consisting of three trains of 100 Hz, three stimuli, separated by 200 ms as previously described (Diógenes et al., 2011). LTP was quantified as the % change in the average slope of the fEPSP taken from 50 to 60 min after LTP induction in relation to the average slope of the fEPSP measured during the 10 min that preceded the induction of LTP. In each individual experiment, the same LTP-inducing paradigm was delivered to each pathway. At 1 h after LTP induction in one of the pathways, BDNF (20 ng/ml) was added to the superfusion solution and LTP was induced in the second pathway, no less than 20 min after BDNF perfusion and only after stability of fEPSP slope values was observed for at least 10 min (Fig. 1B). The effect of BDNF upon LTP was evaluated by comparing the magnitude of LTP in the first pathway in the absence of BDNF (control pathway), with the magnitude of LTP in the second pathway in the presence of BDNF (test pathway); each pathway was used as control or test on alternate days. The same experimental protocol was used to test the effect of CGS21680, an $A_{2A}R$ agonist, on LTP magnitude. In order to test the modulatory effect of $A_{2A}R$ activation on BDNF effect upon LTP, CGS21680 was added 60 min after LTP induction in the first pathway and at least 20 min before BDNF were added to the superfusion bath. Thus, in these experiments, LTP was induced in the second pathway in the presence of both CGS21680 and BDNF. These drugs remained in bath until the end of the experiment.

2.3.3. Input–output curve (I/O)

After obtaining a stable baseline for at least 10 min, the stimulus delivered to the slice was decreased until no fEPSP was evoked and subsequently increased in 20 μA steps. For each stimulation intensity, data from three consecutive averages of 8 fEPSP were collected. Inputs delivered to slices typically ranged from 60 μA to a supramaximal stimulation of 320 μA. The input–output curve was plotted as the relationship of fEPSP slope *vs* stimulus intensity, which provides a measure of synaptic efficiency as previously described (Diógenes et al., 2012).

2.4. Western Blot

Snap-frozen cortex and hippocampus samples from mice of each genotype (WT and *Mecp2*$^{-/Y}$) were first disrupted with a Teflon pestle in Radio-Immunoprecipitation Assay (RIPA) buffer containing: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM ethyl-enediamine tetra-acetic acid (EDTA), 0.1% SDS and 1%Triton X-100 and protease inhibitors cocktail (Mini-Complete EDTA-free; Roche Applied Science, Penzberg, Germany). All lysates were then vortexed and sonicated (3 cycles of 15 s), and clarified by centrifugation (13,000 g, 10 min). The protein content in the supernatant was determined by Bio-Rad DC reagent, a commercial Bradford assay (Sigma-Aldrich, St. Louis, MO, USA). Equal amount of protein was loaded (70 μg, except for $A_{2A}R$ blot: 200 μg) and separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, UK). To check protein transfer efficiency, membranes were stained with Ponceau S solution. After blocking with a 5% non-fat dry milk solution in TBS-Tween (20 mM Tris base, 137 mM NaCl and 0.1% Tween-20), membranes were incubated with the primary antibody, diluted in 3% BSA solution in TBS-Tween (overnight at 4 °C) and then with secondary antibodies HRP-conjugated (1:10000, Santa Cruz Biotechnology, Dallas, TX, USA) diluted in blocking solution (1 h at room temperature). Immunoreactivity was visualized using ECL chemiluminescence detection system (Amersham-ECL Western Blotting Detection Reagents from GE Healthcare) and band intensities were quantified by digital densitometry (ImageJ 1.45 software). α-tubulin (1:3000, #ab4074, Abcam, Cambridge, MA, USA) or GAPDH (1:5000, #AM4300, Ambion by life technologies, Carlsbad, CA, USA) bands were used as loading control. The primary antibodies used were Anti-$A_{2A}R$ from Merk Millipore (1:1500, 05–717, Darmstadt, Germany) (Rei et al., 2020), Anti-A_1R from Santa Cruz Biotecnology (1:1000, sc-28,995, Dallas, TX, USA), Anti-TrkB from BD Transduction Laboratories (1:1500, 610,101, Franklin Lakes, NJ, USA) and Anti-ADK from Bethyl Laboratories (1:1500, A304-280A, Montgomery, TX, USA).

2.5. Radioligand binding experiments

1,3-[3H]-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) binding studies were performed in cortical brain homogenates (40–80 μg protein per assay) of *Mecp2*$^{-/Y}$ mice and WT littermates. [^{3}H]DPCPX binding was performed in an incubation solution (50 mM Tris-HCl buffer and 10 mM $MgCl_2$ (pH 7.4) and 4 U/ml ADA) for 120 min at room temperature, in a final volume of 200 μl. Specific binding was calculated by subtraction of the nonspecific binding, defined in the presence of 2 μM XAC. The reaction was stopped by addition of cold incubation buffer and vacuum filtration through glass fiber filters (FilterMAT for receptor binding, Skatron Instruments, Mountain View, CA, USA) using a semiautomatic cell harvester from Skatron Instruments. The samples were then transferred to scintillation vials and radioactivity was measured by a liquid scintillation analyser (Tri Carb 2900TR, Perkin-Elmer, IL). Membrane protein content was measured using the Bio-Rad DC reagent, a commercial Bradford assay (Berkeley, CA, USA).

2.6. RNA extraction and qPCR

Total RNA was extracted from cerebral cortex samples of *Mecp2*$^{-/Y}$ mice, WT littermates and human samples using RNeasy Lipid Tissue Mini Kit (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions. RNA concentration and purity were then evaluated by spectrophotometry based on optical density (OD) measurements at 260

and 280 mm (Model: NanoDrop-100; Thermo Scientific, Rockford, IL, USA). cDNA synthesis was performed in a 20 µl reaction mixture. 2 µg of total RNA were mixed with 1 µl random primer hexamers (Amersham Life Sciences, Buckinghamshire, UK) and 1 µl (each) of dATP, dTTP, dCTP and dGTP (10 mM). This mix was incubated 5 min at 65 °C and after cooling for 2 min on ice, the remaining reagents were added (4 µl of 25 mM $MgCl_2$, 2 µl of 10× RT buffer, 2 µl of 0.1 M DTT, 0.5 µl Superscript II Reverse Transcriptase (200 U); all reagents from Invitrogen, Life Technologies, Waltham, MA, USA). Parallel reactions for each RNA sample were run without Superscript II to evaluate the degree of genomic DNA contamination. Gene expression level was addressed by quantitative PCR (qPCR) using Power SYBR® Green Master Mix (Life Technologies, Waltham, MA, USA), and cDNA was used as template for the real-time PCR run in Rotor Gene 6000 (Corbett Life Science, Uithoom, Netherlands). Negative control PCR samples were run with no template. Relative mRNA expression was calculated using $2^{-\Delta Ct}$ method and primers were previously optimized in order to establish annealing temperature and primer efficiency (e = 2 ± 0.2), calculated after serial primer dilutions and construction of respective standard curve (slope and R^2 respectively around −3.3 and 0.99; described in Schmittgen and Livak, 2008). Data were normalized to the expression of PPIA peptidylprolyl isomerase A (cyclophilin A) (CypA) and ribosomal protein L13A (Rpl13A) (Batalha et al., 2016). The 5′-3′ primer sequences used for Cyp A (125 bp) were TAT CTG CAC TGC CAA GAC TGA GTG (forward) and CTT CTT GCT GGT CTT GCC ATT CC (reverse); for Rpl13A (130 bp) were GGA TCC CTC CAC CCT ATG ACA (forward) and CTG GTA CTT CCA CCC GAC CTC (reverse); for $A_{2A}R$ (113 bp) were ATT CCA CTC CGG TAC AAT GG (forward) and AGT TGT TCC AGC CCA GCA T (reverse) (Batalha et al., 2016); for A_1R (155 bp) were TCG GCT GGC TAC CAC CCC TTG (forward) and CCA GCA CCC AAG GTC ACA CCA AAG C (reverse); for TrkB-FL were GAG CTG CTG ACC AAC CTC CA (forward) and GTC CCC GTG CTT CAT GTA CTC A (reverse); for TrkB-T1 were TAA GAT CCC ACT GGA TGG GTA G (forward) and AAG CAG CAC TTC CTG GGA TA (reverse) (Karpova et al., 2014). Data acquisition was performed with Rotor-Gene Series Software 1.7 (Corbett Life Science) and data analysis was performed with Microsoft Excel, Office 2016, Redmond, WA, USA. For human brain cortex samples, the real time-PCR was run in 7500 Fast (Applied Biosystems, Foster City, CA, USA) and the normalization was done to GAPDH expression. Data acquisition was performed with 7500 software v2.0.6 (Applied Biosystems, Foster City, CA, USA) and data analysis was performed in Microsoft Excel, Office 2016, Redmond, WA, USA. The 5′-3′ primer sequences used for GAPDH were GGA GCT AAC GGA TTT GGT CG (forward) and GAC AAG CTT CCC GTT CTA G; for $A_{2A}R$ were AAC CTG CAG AAC GTC AC (forward) and GTC ACC AAG CCA TTG TAC CG (reverse) (Batalha et al., 2016); for A_1R were GCC ACA GAC CTA CTT CCA CA (forward) and CCT TCT CGA ACT CGC ACT TG (reverse); for TrkB-FL were GGC CCA GAT GCT GTC ATT AT (forward) and TTC TGC TCA GGA CAG AGG TT (reverse) (Nicolini et al., 2015); for TrkB-T1 were TCT ATG CTG TGG TGG TGA TTG (forward) and GAG TCC AGC TTA CAT GGC AG (reverse) (Luberg et al., 2010).

2.7. *Extraction and analysis of purines by liquid chromatography with diode array detection (LC/DAD)*

The purines (ATP, AMP, adenosine and inosine) content of extracts from the cortex and hippocampus of WT and *Mecp2*$^{-/y}$ mice was measured by liquid chromatography with diode array detection (LC/DAD). Snap-frozen cortex and hippocampal tissue samples were stored at −80 °C until use. For extraction, the samples were defrosted (250 µl) in round-bottom microcentrifuge tubes followed by thorough tissue homogenization using a mixture of ice-cold acetonitrile: methanol: water (1:2:2) solution (Jackson et al., 2017) containing 2-chloro-adenosine (5 µM) as internal standard; the obtained mixture was centrifuged at 16000 g for 20 min at 4 °C. Tissue homogenization and centrifugation were repeated twice. The two recovered supernatant extracts (~250 µl each) were mixed together and, then, centrifuged again at 16000 g (for 20 min at 4 °C) using a 50-kDa cutoff filter (Amicon Ultra-0.5 50 K Filter Device; Merck KGaA, Darmstadt, Germany). After filtration, supernatant extracts were divided in 15-µl aliquots and stored at −80 °C until analysis. Using this procedure, recovery of adenine nucleosides was higher than 95%, as determined by adding 2-chloro-adenosine (5 µM) as internal standard before extraction.

Extraction media containing purines were 1/10 diluted with water before LC/DAD analysis. Chromatographic separation of nucleosides was carried out using an elution gradient composed of ammonium acetate (5 mM, with a pH of 6 adjusted with acetic acid) and methanol (Silva et al., 2020, 2017; Vieira et al., 2017). Separation of adenine nucleotides was carried using a paired-ion chromatography reagent (PIC Reagent A; Waters Chromatography Europe BV, Etten-Leur, The Netherlands) containing tetrabutylamonium phosphate (1 mM) in KH_2PO_4 (100 mM, at pH = 6) (Waters) and methanol. Separation of adenine nucleotides and nucleosides using both elution systems was achieved by reversed-phase liquid chromatography through a Hypersil GOLD C18 column (5 µM, 2.1 mm × 150 mm) equipped with a guard column (5 µm, 2.1 mm × 1 mm) and assayed using a Finigan Thermo Fisher Scientific System LC/DAD, equipped with an Accela Pump coupled to an Accela Autosample, a diode array detector and an Accela PDA running the X-Calibur software chromatography manager. Quantification of adenine nucleotides and nucleosides was carried out using calibration curves made of high-purity external standards, namely ATP, AMP, adenosine and inosine.

2.8. *Drugs*

BDNF was generously provided by Regeneron Pharmaceuticals; 2-[p-(2-carboxyethyl)phenethylamino]-50-N-ethylcarboxamido adenosine (CGS21680) was purchased from *Sigma*; N6-Cyclopentyladenosine (CPA) and 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX) were purchased from Tocris Bioscience (Ballwin, MO, USA); 1[^{3}H]DPCPX was purchased from American Radiolabeled Chemicals, Inc.; ATP, AMP, adenosine, inosine, 2-chloro-adenosine and 5-iodotubercidin (ITU) were purchased from *Sigma* (St. Louis, MO, USA). BDNF was supplied in a 1.0 mg/ml stock solution in 150 mM NaCl, 10 mM sodium phosphate buffer, and 0.004% Tween 20. CGS21680, CPA and DPCPX were made up in 5 mM and ITU in 50 mM stock solutions in DMSO. [^{3}H] DPCPX was purchased as a 36.5 µM solution in ethanol. The maximum DMSO concentration (0.02%) applied to the preparations was devoid of action on fEPSPs (Tsvyetlynska et al., n.d.). Aliquots of the stock solutions were kept frozen at −20 °C until use.

2.9. *Data analysis*

The data are expressed as mean ± SEM of the n number of independent experiments. The significance of differences between the means of 2 conditions was evaluated by paired or unpaired *t*-tests; Welch correction was used in unpaired t-test as appropriate. Nonlinear regressions were used to fit data pertaining I/O curves and radioligand binding experiments. Values of $p < 0.05$ were considered to represent statistically significant differences. GraphPad Prism 5.00 was used to performed statistical analysis.

3. Results

3.1. *BDNF does not increase the magnitude of LTP in the hippocampus of Mecp2*$^{-/y}$ *mice*

LTP is generally regarded as the neurophysiological correlate for learning and memory and BDNF has a well-documented ability to increase its magnitude on hippocampal CA1 area through TrkB-FL receptors activation (Figurov et al., 1996; Minichiello et al., 1999). To

assess the functional impact of the decrease in BDNF levels in RTT (Li and Pozzo-Miller, 2014), we evaluated the effect of exogenously applied BDNF (20 ng/ml) upon LTP. As expected (Diógenes et al., 2011; Fontinha et al., 2008), the θ-burst paradigm applied to hippocampal slices, taken from wild type (WT) animals induced a robust LTP in the presence of BDNF (48.2 ± 4.5%, $n = 5$; Fig. 1C;E), which was significantly higher than the obtained in the absence of BDNF (27.5 ± 5.8%, n = 5; $p = 0.02$, paired t-test; Fig. 1C;E). Importantly, in the absence of BDNF, θ-burst stimulation induced a small, yet significant LTP in WT mice ($p = 0.009$, paired t-test), but not in $Mecp2^{-/y}$ mice ($p = 0.45$, paired t-test). Moreover, BDNF (20 ng/ml) did not further increase LTP magnitude in hippocampal slices taken from

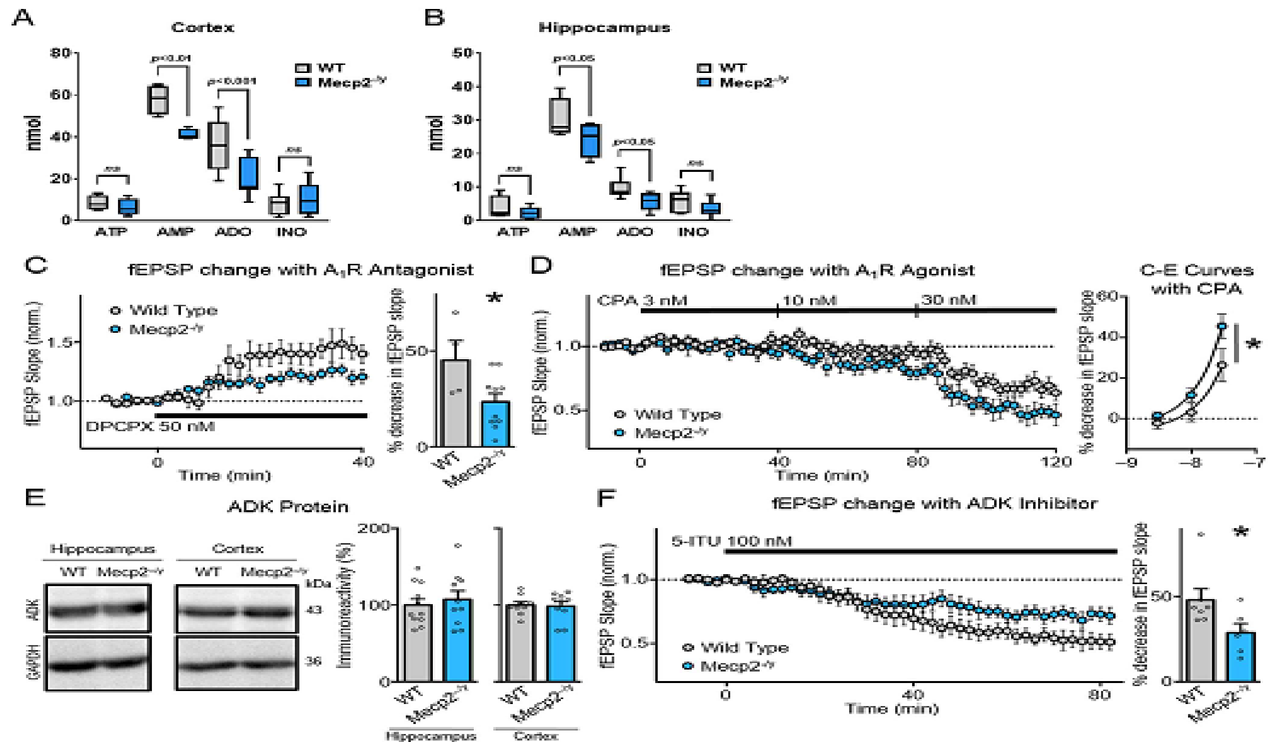

Fig. 2. Extracellular levels of adenosine are decreased in $Mecp2^{-/y}$ mice. In fig. A and B the ordinates represent the amount of ATP, AMP, adenosine (ADO) and inosine (INO) in nmol extracted from the cortex and hippocampus, respectively, of WT (grey bars) and $Mecp2^{-/y}$ (blue bars) mice and detected by LC/DAD (for details, see Materials and Methods). Represented are box-and-whiskers plots, with whiskers ranging from minimum to maximum values; horizontal lines inside boxes indicate the corresponding medians. Each data point represents four to eight individuals (see text for details); duplicate measurements were performed for each individual experiment. $p < 0.05$ (one-way ANOVA; uncorrected Fisher's LSD, with a single pooled variance) represent significant differences when compared to WT animals. The left panel of C shows the averaged time courses of changes in fEPSP slope induced by application of DPCPX (50 nM) in slices taken from WT (grey circles, $n = 4$) and $Mecp2^{-/y}$ (blue circles, $n = 10$) animals. The histogram on the right represent the averaged normalized decrease in fEPSPs slope in each animal group in response to DPCPX. D left panel shows the averaged time courses of changes in fEPSP slope induced by application of three different concentrations of CPA (3 nM, 10 nM, 30 nM) in slices taken from WT (grey circles, $n = 7$) and $Mecp2^{-/y}$ (blue circles, $n = 7$) animals. The right panel shows the comparison of the averaged effects of different concentrations of CPA in the different genotypes. Left panel of F shows the averaged time course of changes in fEPSP slope induced by ITU (100 nM) applied for 84 min. Hippocampal slices were taken from WT (grey circles, $n = 7$) and $Mecp2^{-/y}$ mice (blue circles, $n = 6$). The right panel shows the comparison of the averaged effects of ITU in the different genotypes. Left panel in E shows representative bands obtained by Western Blot of cortical and hippocampal tissue homogenates from $Mecp2^{-/y}$ and WT mice with 6–10 weeks of age. On the left panel, the histograms represent average band intensity for ADK obtained in samples from WT (grey bars, $n_{hip} = 11$; $n_{ctx} = 10$) and $Mecp2^{-/y}$ (blue bars, $n_{hip} = 9$; $n_{ctx} = 10$) hippocampal and cortical homogenates. All values presented are mean ± SEM and are represented in % of WT protein. *$p < 0.05$ (Student's t-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Mecp2$^{-/y}$ animals (LTP_{CTR} = 10.2 ± 11.9%, LTP_{BDNF} 14.5 ± 7.0%, n = 5; p = 0.72, paired t-test; Fig. 1D;E).

To evaluate whether the impairment of LTP in *Mecp2*$^{-/y}$ animals could be due to changes in baseline synaptic efficiency, I/O curves were performed. Hippocampal slices taken from *Mecp2*$^{-/y}$ animals displayed higher E_{max} values when compared with WT animals ($Emax_{WT}$ = 0.99 ± 0.04, n = 4; $Emax_{Mecp2-/y}$ = 1.78 ± 0.21, n = 4; p = 0.01, unpaired t-test; Fig. 1F). These data indicate higher neuronal excitability in the hippocampus of *Mecp2*$^{-/y}$ animals and that LTP impairment in *Mecp2*$^{-/y}$ animals is not due to a decrease in basal synaptic transmission efficiency.

3.2. BDNF and TrkB-FL receptor proteins are decreased in Mecp2$^{-/y}$ *mice*

Previous data suggest that MeCP2 acts both as a repressor and as an activator of gene expression (Chahrour et al., 2008). Given that the BDNF gene is under MeCP2 transcriptional regulation (Chen et al., 2003) attention has been directed to BDNF protein changes on RTT. Our results corroborate the previously described decrease in BDNF protein levels (for review see Li and Pozzo-Miller, 2014), both in the cerebral cortex and hippocampus (HIP_{WT} = 100.0 ± 4.8%, n = 8 *vs* $HIP_{Mecp2-/y}$ = 75.0 ± 7.2%, n = 9, p = 0.01, unpaired t-test; CTX_{WT} = 100.0 ± 10.9%, n = 8 *vs* $CTX_{Mecp2-/y}$ = 56.1 ± 3.7%, n = 6, p = 0.006, unpaired t-test; Fig. 1M;L).

However, the expression levels of TrkB-FL and its truncated isoform (TrkB-Tc), crucial players in BDNF mediated synaptic plasticity, had not yet been fully addressed. Therefore, we evaluated TrkB receptors levels in *Mecp2*$^{-/y}$ mice. As shown in Fig. 1G;H, TrkB-FL protein expression levels are decreased in both the cerebral cortex and the hippocampus of *Mecp2*$^{-/y}$ mice when compared to WT littermates (HIP_{WT} = 100 ± 5.6%, n = 13 *vs* $HIP_{Mecp2-/y}$ = 70.0 ± 9.4%, n = 12; p = 0.01, unpaired t-test; CTX_{WT} = 100.0 ± 5.3%, n = 14 *vs* $CTX_{Mecp2-/y}$ = 66.2 ± 5.7%, n = 13, p = 0.0002, unpaired t-test; Fig. 1H). Regarding TrkB-Tc protein expression levels, no significant changes were detected (HIP_{WT} = 100.0 ± 6.1%, n = 14 *vs* $HIP_{Mecp2-/y}$ = 94.5 ± 11.8%, n = 13, p = 0.67, unpaired t-test; CTX_{WT} = 100.0 ± 4.0%, n = 14 *vs* $CTX_{Mecp2-/y}$ = 83.7 ± 7.345%, n = 14, p = 0.06, unpaired t-test Fig. 1I).

In addition, mRNA relative expression of both *TrkB-FL* and the most expressed TrkB-Tc (Luberg et al., 2010), *TrkB-T1*, were analysed by qPCR. As shown in Fig. 1J;K, no significant differences between WT and *Mecp2*$^{-/y}$ mice were detected ($TrkB\text{-}FL_{WT}$ = 0.12 ± 0.03, n = 5 *vs* $TrkB\text{-}FL_{Mecp2-/y}$ = 0.18 ± 0.03, n = 5, p = 0.22, unpaired t-test; $TrkB\text{-}T1_{WT}$ = 0.18 ± 0.03, n = 5 *vs* $TrkB\text{-}T1_{Mecp2-/y}$ = 0.2 ± 0.04, n = 5, p = 0.39, unpaired t-test). Moreover, one *post-mortem* temporal cortex sample from a RTT patient was evaluated together with an aged-matched control. *TrkB-FL* mRNA expression in the temporal cortex of RTT patient showed a small increase (32.14%) when compared with the control (Fig. 1O). No apparent changes were observed regarding the mRNA expression levels of BDNF (Fig. 1N) and TrkB-Tc (Fig. 1P).

3.3. The adenosinergic modulation of synaptic transmission is compromised in Mecp2$^{-/y}$ *mice*

The lack of BDNF effect upon LTP could be explained by the decreased expression of TrkB-FL receptors in the hippocampus. However, since BDNF-induced facilitation of hippocampal LTP is tightly dependent on the activation of $A_{2A}R$ by endogenously generated adenosine (Fontinha et al., 2008), we next characterized the adenosinergic system in *Mecp2*$^{-/y}$ mice.

Brain extracts from *Mecp2*$^{-/y}$ mice exhibit lower ($p < 0.05$) adenosine amounts compared to WT animals in the cortex (WT = 35.49 ± 4.04 nmol, n = 8 *vs*. *Mecp2*$^{-/y}$ = 20.60 ± 2.91 nmol; n = 9, p-value = 0.009, Welch's t-test, Fig. 2A) and in the hippocampus (WT = 9.62 ± 0.99 nmol, n = 8 *vs*. *Mecp2*$^{-/y}$ = 5.55 ± 0.92 nmol; n = 8, p-value = 0.023, Welch's t-test, Fig. 2B), without any measurable changes in the levels of inosine. These results suggest that adenosine deficiency in RTT animals might not be related to increases in adenosine deaminase (ADA) activity. This prompted us to measure the amount of adenosine precursors (*e.g.* ATP and AMP) in the same samples. Brain extracts exhibit very small amounts of ATP, which levels did not differ among WT and *Mecp2*$^{-/y}$ mice both in the cortex (WT = 8.36 ± 1.77 nmol *vs*. *Mecp2*$^{-/y}$ = 6.34 ± 2.07 nmol; n = 4; $p > 0.05$, Fig. 2A) and in the hippocampus (WT = 3.76 ± 1.77 nmol *vs*. *Mecp2*$^{-/y}$ = 2.19 ± 0.84 nmol; n = 4; $p > 0.05$, Fig. 2B). Contrariwise, higher AMP amounts compared to adenosine were extracted from the cortex (57.88 ± 3.57 nmol; n = 4) and hippocampus (30.28 ± 3.12 nmol; n = 4) of WT animals, but these levels also significantly ($p < 0.05$) decreased to 41.19 ± 1.30 nmol (n = 4) and 24.26 ± 2.64 nmol (n = 4) in the cortex and hippocampus of *Mecp2*$^{-/y}$ mice, respectively (Fig. 2A;B). The observed increase in the proportion of AMP *vis a vis* adenosine in cortical and hippocampal extracts of *Mecp2*$^{-/y}$ mice may indicate a decrease in 5′-nucleotidase activity, the enzyme responsible for AMP dephosphorylation into adenosine, and/or a higher competence of intracellular adenosine kinase (ADK) mediating phosphorylation of adenosine back to AMP. Next, we set to test the functional repercussions of having lower adenosine amounts in the hippocampus of *Mecp2*$^{-/y}$ mice compared to WT littermates. If our prediction is correct, facilitation (disinhibition) of hippocampal synaptic transmission produced by the selective A_1R antagonist, DPCPX (Diogenes et al., 2014) should be less prominent in *Mecp2*$^{-/y}$ mice compared to WT animals. A supramaximal concentration of DPCPX (50 nM; K_i value of 0.5 nM in the hippocampus (Sebastião et al., 1990)) was used to evaluate the effect of A_1R blockage on basal synaptic transmission in hippocampal slices from *Mecp2*$^{-/y}$ mice and WT littermates. The magnitude of fEPSP slope in the presence of DPCPX (50 nM) was significantly higher in hippocampal slices from WT compared to those obtained from *Mecp2*$^{-/y}$ mice (WT = 45.3 ± 10.1%, n = 4, *vs*. *Mecp2*$^{-/y}$ = 23.4 ± 4.4%, n = 10; p = 0.0374, unpaired t-test; Fig. 2C).

Despite the reduction of the adenosine inhibitory tonus in hippocampal synaptic transmission in *Mecp2*$^{-/y}$ mice is in keeping with the observed low endogenous levels of the nucleoside in brain extracts (see above), A_1R hypoactivity could also explain the results obtained. To sort this out, we tested the effect of the selective A_1R agonist, CPA, at increasing concentrations (3, 10 and 30 nM). Exogenously added CPA concentration-dependently decreased fEPSP slope to a higher extent in *Mecp2*$^{-/y}$ mice compared to WT animals; this difference turn out to be significant ($p < 0.05$) at the highest concentration tested (30 nM) (WT = 31.4 ± 5.9%, n = 7 *vs*. *Mecp2*$^{-/y}$ = 52.4 ± 6.530%, n = 7; p-value = 0.0345, unpaired t-test; Fig. 2D). These results (1) exclude the hypothesis that A_1R are hypoactive in *Mecp2*$^{-/y}$ mice, (2) predict high expression rates of inhibitory A_1R in the hippocampus of these animals, and (3) strengthens the theory that reduction of the inhibitory control of adenosine on hippocampal synaptic transmission in *Mecp2*$^{-/y}$ mice is mainly due to insufficient availability of the nucleoside at A_1R sites. In the CNS, phosphorylation of adenosine leading to AMP formation by ADK is paramount to keep low intracellular levels of the nucleoside and, thus, it is considered the main driving force to take up adenosine from the extracellular milieu (Boison, 2013). Overexpression of ADK has been observed in astrocytes of epileptic brains of mice and humans (Aronica et al., 2011), which is consistent with the hypothesis that the pathophysiology of epilepsy involves lower endogenous adenosine levels and, thus, a deficient inhibition of synaptic transmission (Boison, 2013). Fig. 2E shows that the ADK protein level is not different in *Mecp2*$^{-/y}$ and WT mice, both in the hippocampus (HIP_{WT} = 100.2 ± 8.1%, n = 11 *vs*. $HIP_{Mecp2-/y}$ = 107.4 ± 11.24%, n = 10, p = 0.60, unpaired t-test) and in the cerebral cortex (CTX_{WT} = 100.0 ± 4.3%, n = 9 *vs*. $CTX_{Mecp2-/y}$ = 98.5 ± 5.8%, n = 10, p = 0.84, unpaired t-test; Fig. 2E). It is, however, worth noting that Western Blot data reflects protein levels in whole tissue homogenates, not allowing the access to cell-specific protein contents at the synaptic level. Inhibition of

ADK is often used to reverse the nucleoside gradient across the plasma membrane leading to adenosine outflow *via* equilibrative nucleoside transporters (ENT) and its, subsequent, extracellular accumulation (Lee and Chao, 2001; Li et al., 2012). Here, we tested the effect of the ADK inhibitor, 5-iodotubercidin (5-ITU) (Pak et al., 1994), on basal hippocampal synaptic transmission. As shown in Fig. 2F, 5-ITU (100 nM) decreased fEPSP slope in hippocampal slices of both *Mecp2*$^{-/y}$ mice and WT littermates, but the effect of the ADK inhibitor had a higher ($P = 0.02$, unpaired *t*-test) inhibitory magnitude in WT (48.0 ± 6.6%, $n = 7$) compared to *Mecp2*$^{-/y}$ (25.2 ± 5.3%, $n = 6$) animals. Data show that inhibition of ADK could not fully overcome adenosine deficiency at the A_1R level in the hippocampus of *Mecp2*$^{-/y}$ mice, thus indicating lower levels of endogenous adenosine.

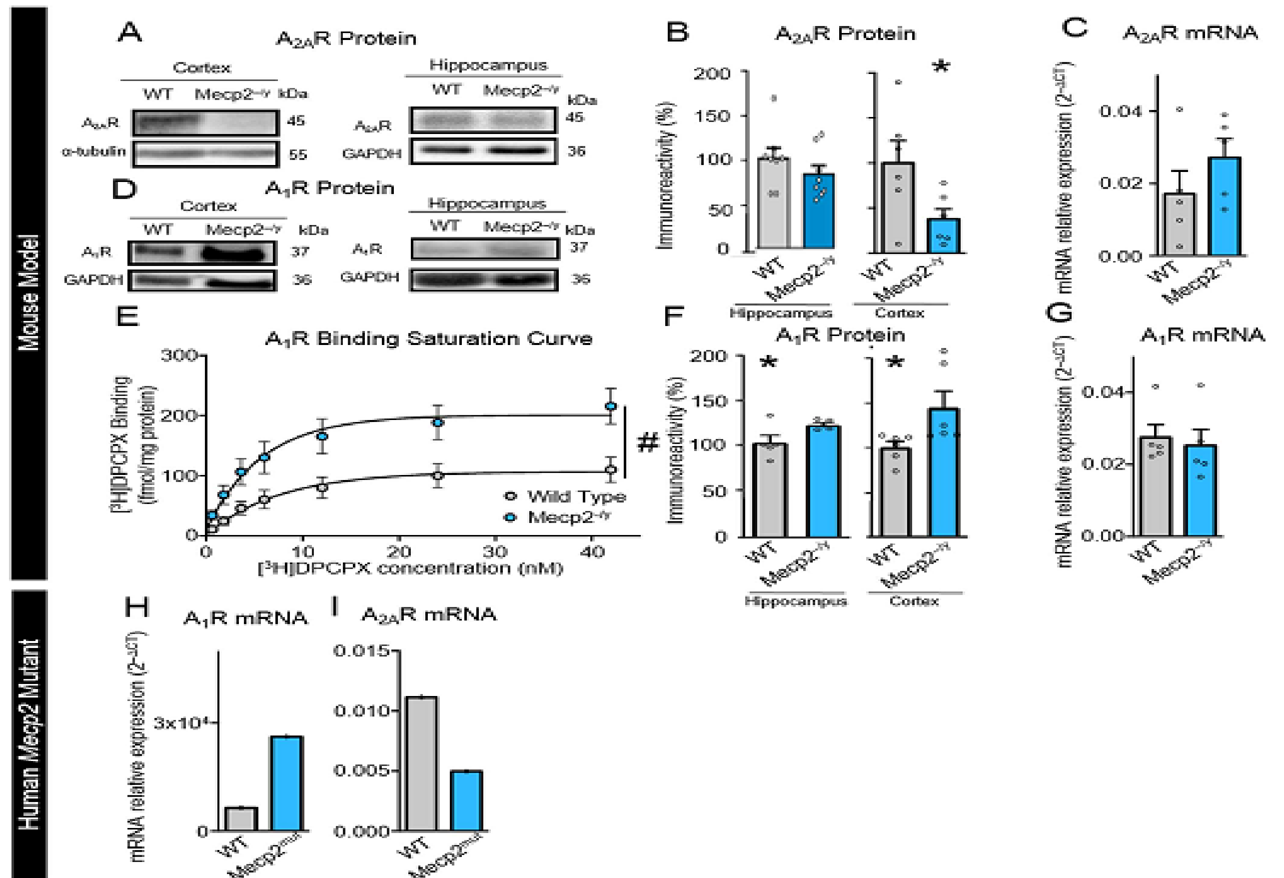

Fig. 3. $A_{2A}R$ protein expression is decreased and A_1R is increased in Mecp2$^{-/y}$ mice: In A and B panels are shown the averaged $A_{2A}R$ receptors density while in D and F panels are shown the averaged A_1R receptors density, all evaluated in hippocampal and cortical brain samples, respectively, by Western Blot analysis of WT (grey bar, $n_{hipA2AR} = 8$; $n_{ctxA2AR} = 6$; and $n_{hipA1R} = 5$; $n_{ctxA1R} = 6$) and Mecp2$^{-/y}$ (blue bars, $n_{ctxA2AR} = 6$; $n_{hipA2AR} = 8$ and $n_{ctxA1R} = 6$; $n_{hipA1R} = 5$) animals 6–10 weeks old. The results are represented in % of WT protein. In E are shown saturation isotherms for the binding of the selective A_1R receptor antagonist [^{3}H]DPCPX to the cortical homogenates of WT (grey circles, $n = 5$) and Mecp2$^{-/y}$ (blue circles, $n = 6$) animals. In C and G are shown histograms representing the relative qPCR data showing mRNA levels of $A_{2A}R$ and A_1R, respectively, from cortical samples of WT (grey bar, $n = 5$) and Mecp2$^{-/y}$ mice (blue bar, $n = 5$). H and I histograms represent relative qPCR data showing mRNA levels of A_1R and $A_{2A}R$, respectively, present in temporal cortex from a healthy control (grey bar, $n = 1$) and from a human RTT patient (blue bar, $n = 1$). All values are mean ± standard error of mean (SEM). *$p < 0.05$ (Student's t-test); #$p < 0.05$ (F test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. $A_{2A}R$ expression is decreased and A_1R expression is increased in $Mecp2^{-/y}$ mice

To further understand how the adenosinergic system is disturbed in $Mecp2^{-/y}$ mice, we evaluated the A_1R and $A_{2A}R$ expression at mRNA and protein levels. $A_{2A}R$ protein levels were assessed by Western Blot using an antibody validated against $A_{2A}R$ KO samples (supplementary Fig. 5A). Western Blot analysis of cortical and hippocampal homogenates show that $A_{2A}R$ protein amounts are significantly decreased in $Mecp2^{-/y}$ mice cortex compared to WT littermates and show a tendency for decreased $A_{2A}R$ protein levels in the hippocampus (CTX_{WT} = 100.0% ± 24.8, $n = 6$ vs $CTX_{Mecp2-/y}$ = 37.5% ± 11.3, $n = 6$; $p = 0.04$, unpaired t-test; and HIP_{WT} = 100.00 ± 11.78%, $n = 8$ and $HIP_{Mecp2-/y}$ = 82.19 ± 9.86%, $n = 8$; $p = 0.258$, unpaired t-test; Fig. 3A;B). Despite this, no significant differences were detected upon evaluating the $A_{2A}R$ mRNA relative expression in the two genotypes (WT = 0.017 ± 0.006, $n = 5$ vs. $Mecp2^{-/y}$ = 0.027 ± 0.005, $n = 5$, $p = 0.26$, unpaired t-test; Fig. 3C).

The existence of commercially available high affinity/high selectivity A_1R ligands allowed us to perform receptor binding assays, which are frequently preferred (over Western Blot) for quantitative analysis of receptor expression whenever a considerable amount of tissue is available. Therefore, A_1R were firstly evaluated by binding assays in cortical homogenates given the higher levels of protein content in this particular brain area. In Fig. 3E the saturation isotherms for the specific binding of the A_1R antagonist, [^{3}H]DPCPX, show that this ligand binds more extensively to cortical brain homogenates of $Mecp2^{-/y}$ mice than to their WT littermates. The B_{max} value obtained by nonlinear regression analysis, a measure of A_1R expression levels, was significantly higher for $Mecp2^{-/y}$ mice (WT = 127.9 ± 17.1 fmol mg/protein, $n = 5$ vs. $Mecp2^{-/y}$ = 229.5 ± 22.1 fmol mg/protein, $n = 6$, $p = 0.007$, unpaired t-test). No significant differences in the K_d values, a measure of receptor binding affinity, were found among the two genotypes (WT = 6.8 ± 2.7 nM, n = 5 vs. d $Mecp2^{-/y}$ = 4.3 ± 1.4 nM, n = 6, $p = 0.42$, unpaired t-test). By Western Blot technique similar data were obtained in cortex and hippocampus (HIP_{WT} = 100.00 ± 7.88%, n = 5 vs. $HIP_{Mecp2-/y}$ = 119 ± 5.17, n = 5; $p = 0.049$, unpaired t-test; Fig. 3D;F and CTX_{WT} = 100.0 ± 6.34, n = 6 vs $CTX_{Mecp2-/y}$ = 144 ± 17.81, n = 6; $p = 0.041$, unpaired t-test; Fig. 3D;F). Overall, data suggest that A_1R expression levels are significantly increased in the cortex and hippocampus of $Mecp2^{-/y}$ animals compared to WT littermates. This difference may be owe to post-translational protein modifications, given to the fact that qPCR assays revealed no significant changes in cortical mRNA A_1R gene transcripts (WT = 0.027 ± 0.004, n = 5 vs. $Mecp2^{-/y}$ = 0.025 ± 0.004, n = 5, $p = 0.7$, unpaired t-test; Fig. 3G). Interestingly, analyses of the temporal cortex from a RTT patient revealed a mRNA expression profile that is more consistent with the results obtained at the protein level in the mouse cortex, revealing an increase in A_1R and a decrease in $A_{2A}R$ mRNA expression levels (Fig. 3H,I). The hippocampus of symptomatic heterozygous females (*Het*), where the phenotype is less severe, were also analysed (supplementary Fig. 5B). Similar changes in A_1R and $A_{2A}R$ expression were detected in *Het* when compared to controls:1) significant decreased of $A_{2A}R$ protein levels in *Het* (WT = 100.00 ± 17.78%, n = 6 vs. *Het* = 52.47 ± 8.54%, n = 6, p = 0.04, unpaired t-test); 2) significant increase of A_1R protein levels *Het* (WT = 100.00 ± 3.89%, n = 5 vs. *Het* = 116.60 ± 3.62%, n = 5, $p = 0.01$, unpaired t-test) supporting that adenosinergic system can be altered in several degrees of RTT severity.

3.5. Exogenous activation of $A_{2A}R$ restores BDNF-induced LTP facilitation in $Mecp2^{-/y}$ mice

The results reported above indicate that endogenous levels of adenosine and, thus, the nucleoside-mediated control of synaptic transmission are both deficient in the hippocampus of $Mecp2^{-/y}$ mice, along with a concomitant reduction of $A_{2A}R$ protein expression in this brain region. Therefore, we hypothesized that reduced adenosine $A_{2A}R$ tonus could also contribute to impairment of BDNF-mediated actions in RTT patients. Indeed, a strong body of evidence demonstrates that several BDNF actions rely on the activation of $A_{2A}R$ (Sebastião et al., 2011). Furthermore, this cross-talk was shown to be dynamic, for instance, during aging where the reduction of TrkB-FL receptor levels is accompanied by increases in $A_{2A}R$ levels (Diogenes et al., 2007). This prompted us to investigate whether activation of $A_{2A}R$ could be a feasible strategy to overcome BDNF signalling deficits in RTT patients, along with the reduced levels of this neurotrophin and to the partial loss of its TrkB-FL receptors. The selective $A_{2A}R$ agonist, CGS21680 (10 nM) (Jarvis et al., 1989) had no effect on the magnitude of LTP when it was applied alone to hippocampal slices of $Mecp2^{-/y}$ mice 1 h after LTP induction in the first pathway and at least 30 min before LTP induction in the second pathway; the magnitude of LTP in the absence (LTP_{CTR} = 17,51 ± 6.70, $n = 7$) and presence

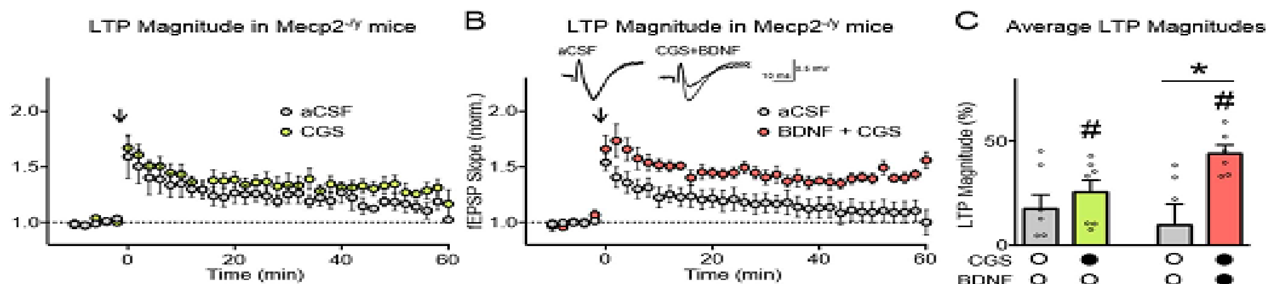

Fig. 4. Acute $A_{2A}R$ Activation restores BDNF effect upon LTP: A shows averaged time courses changes in field excitatory postsynaptic potential (fEPSP) slope induced by the θ-burst stimulation in the absence (grey circles) or in the presence (green circles) of the selective A_{2A} receptor agonist, CGS21680 (10 nM) in hippocampal slices taken from $Mecp2^{-/y}$ mice ($n = 7$). CGS21680 (10 nM) was applied 60 min after the induction of LTP in the first pathway (grey circles) and at least 20 min before induction of LTP in the second pathway (green circles). B shows time courses changes of averaged fEPSP slopes in response to θ-burst stimulation in the absence (grey circles) or presence (red circles) of both CGS21680 (10 nM) and BDNF (20 ng/ml) in hippocampal slices taken from $Mecp2^{-/y}$ animals ($n = 6$). Representative traces from representative experiments are shown. Panel C depicts corresponding LTP magnitudes. All values are mean ± standard error of mean (SEM). *p < 0.05 (Student's t-test) #p < 0.05 (F test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(LTP_{CGS} = 25.52 ± 5,79, n = 7) of CGS21680 did not significantly differ (p = 0.25, paired t-test; Fig. 4A;C). However, in the presence of CGS21680, the LTP magnitude (LTP_{CGS} = 25.52 ± 5,79, n = 7) is significantly different from the baseline (values obtained before the θ-burst; n = 7; p = 0.01, paired t-test, Fig. 4C), in contrast with what occurred in the absence of CGS21680. Next, we set to test whether activation of $A_{2A}R$ with CGS21680 could rehabilitate the facilitatory effect of BDNF on LTP when the neurotrophin was applied in a concentration (20 ng/ml) that was devoid of effect in hippocampal slices of *Mecp2*$^{-/y}$ mice (see Fig. 1D;E). In these experiments, LTP was induced in the first pathway in the absence of test drugs, then CGS21680 (10 nM) was applied 1 h after induction of the first LTP and BDNF (20 ng/ml) was applied 20 min after starting CGS21680 perfusion. The second pathway LTP was induced at least 30 min after starting BDNF application together with CGS21680. Co-application of BDNF (20 ng/ml) plus CGS21680 (10 nM) increased the magnitude of LTP in the hippocampus of *Mecp2*$^{-/y}$ mice (LTP_{CTR} = 9.6 ± 10.2% *vs.* $LTP_{CGS+BDNF}$ = 43.9 ± 4.3%, n = 6; p = 0.048, paired t-test; Fig. 4B;C); the LTP magnitude attained under these conditions was fairly comparable to that obtained with BDNF alone in WT animals (see Fig. 1C;E). Data suggest that exogenous activation of $A_{2A}R$ with CGS21680 may rehabilitate BDNF signalling deficits observed in hippocampal LTP in *Mecp2*$^{-/y}$ mice, strengthening our theory that inadequacy of endogenous adenosine production plays a major role in synaptic transmission deficits in RTT patients.

4. Discussion

In the present work, we addressed an innovative strategy to potentiate BDNF effects in RTT through pharmacological modulation of adenosine receptors. While evaluating potential causes for the inability of exogenous BDNF to facilitate synaptic plasticity, we demonstrated that TrkB-FL receptors are decreased in the hippocampus and cerebral cortex of symptomatic *Mecp2*$^{-/y}$ mice and that the adenosinergic system is fully dysregulated, both at the receptor expression and functional levels. Pharmacological activation of $A_{2A}R$ was shown to recover the facilitatory action of BDNF upon synaptic plasticity.

BDNF is widely accepted as a neuroprotective molecule and, thus, BDNF-based therapies have been thoroughly explored to prevent neurodegeneration (Lu et al., 2013). Although RTT is not considered a neurodegenerative disorder, this interest has been extended to RTT were BDNF levels are known to be altered mainly in cortex, cerebellum and hippocampus (Abuhatzira et al., 2007; Chang et al., 2006; Li et al., 2012; Wang et al., 2006). A decrease of mRNA BDNF levels in *post-mortem* human brain samples of RTT patients has also been reported (Abuhatzira et al., 2007), though differences in BDNF protein levels in blood serum and cerebrospinal fluid from RTT human patients have been more difficult to show (Riikonen, 2003; Vanhala et al., 1998).

Much less was known about BDNF receptor signalling in RTT, and the existing data were conflicting. One study showed no differences in TrkB-FL mRNA levels between human embryonic stem cell derived from RTT neurons and controls (Li et al., 2013). Another study (Abuhatzira et al., 2007) showed that TrkB-FL mRNA expression levels are increased in a mouse model and in RTT human cortical brain samples, a change interpreted as a compensatory mechanism for the reduced BDNF protein levels detected in whole brain homogenates collected from newborn asymptomatic female mutant mice (Abuhatzira et al., 2007). By analysing the hippocampus and cortex of symptomatic male *Mecp2*$^{-/y}$ mice separately, we detected a significant decrease of BDNF protein levels and a tendency for an increase in mRNA levels of TrkB-FL and TrkB-Tc in both brain areas. A tendency for an increase in TrkB-FL mRNA in a *post-mortem* temporal cortex sample from a RTT patient was also observed. Importantly, none of the previous studies analysed in detail the protein levels of the different isoforms of the TrkB receptor, in particular whether putative alterations in the signalling form of the TrkB receptor, the TrkB–FL, were accompanied by changes in the truncated form of the receptor, the TrkB-Tc form, known to counteract TrkB signalling. Our data clearly shows that TrkB-FL protein levels are decreased in the hippocampus as well as in the cortex of symptomatic *Mecp2*$^{-/y}$ mice, without appreciable alterations in the truncated receptor protein levels. This may alter the way we should think about treatment, it is not only important to increase BDNF levels but it is also crucial to assure the presence of functional TrkB-FL receptors.

The idea that BDNF impairment could contribute to RTT pathophysiology was greatly reinforced by the finding that BDNF overexpression could partially ameliorate some of RTT symptoms in *Mecp2*$^{-/y}$ mice, including locomotor function, lifespan and the deficits on cortical electrophysiological activity (Chang et al., 2006). Overexpression of BDNF is, however, of very limited therapeutic use, if feasible at all. Other strategies are thus necessary to rescue BDNF function and to assure proper levels of TrkB-FL receptor. We aimed to potentiate BDNF actions through the major neuromodulator in the brain, adenosine. The reason to try this strategy is the previous evidence that, at least in healthy conditions, the synaptic actions of BDNF can be potentiated by activation of an adenosine receptor, the $A_{2A}R$ (Diógenes et al., 2004). $A_{2A}R$ activation induces transactivation of TrkB-FL (Lee and Chao, 2001), promotes translocation of TrkB receptors to lipid rafts (Assaife-Lopes et al., 2014; Sebastião et al., 2011) and regulates BDNF (Tebano et al., 2008) and TrkB-FL (Jerónimo-Santos et al., 2014) levels. Moreover, the effect of exogenous BDNF upon hippocampal synaptic transmission and LTP is dependent on a fully functional adenosinergic tonus *via* activation of $A_{2A}R$ (Diógenes et al., 2011, 2004; Fontinha et al., 2008). However, not all BDNF functions are adenosine dependent, which explain why some BDNF actions can be revealed even in the presence of and adenosinergic dysfunction, as shown in some previous papers (Chang et al., 2006). Dysfunction of adenosine signalling has been highlighted in several pathologies, such as sleep/arousal dysfunction, neurodegeneration, epilepsy, pain, neuronal maturation and central control of breathing (Gomes et al., 2011; Ribeiro et al., 2002), but has never been considered for RTT. This is surprising in light of the putative dual role of adenosine in this pathology: on the one hand, and as mentioned above, adenosine through $A_{2A}R$ influences BDNF actions; on the other hand, adenosine, mostly through A_1R, can control seizures in epileptic syndromes such as RTT (Boison, 2007; Sandau et al., 2016). Adenosine is an endogenous homeostatic regulator of network activity (Boison, 2012; Diogenes et al., 2014; Dunwiddie and Masino, 2001) and adenosine deficiency has been identified as a pathologic hallmark of the epileptic brain (Aronica et al., 2013). We found that *Mecp2*$^{-/y}$ mice displayed lower tonic inhibitory adenosinergic signalling in the hippocampus, as indicated by: 1) the lower disinhibition of excitatory synaptic transmission while blocking inhibitory A_1R; 2) the lower inhibition of synaptic transmission caused by an adenosine-releasing drug, as an ADK inhibitor; 3) lower levels of adenosine and AMP. This lower tonic adenosinergic inhibition is mostly likely due to the decreased levels of adenosine which might be related to lower expression and/or activity of 5′-nucleotidase resulting in the increased AMP/adenosine concentration ratio detected by us in hippocampus and cortex. On contrary, lower tonic adenosinergic inhibition cannot be accounted by lower ADK levels since *Mecp2*$^{-/y}$ mice had maintained ADK protein levels. The results obtained with the ADK inhibitor, which caused a lower inhibition of synaptic transmission in *Mecp2*$^{-/y}$ mice, are compatible with a lower adenosine gradient across the membrane, thus with decreased intracellular adenosine levels in *Mecp2*$^{-/y}$ mice, highly suggestive of dysregulated adenosine homeostasis. Adenosine levels are also dependent on ATP levels (Boison, 2015), therefore, we cannot exclude the potential role of dysregulated bioenergetics due to dysfunctional mitochondria in RTT. Although lower levels of ATP were already reported in the brain of *Mecp2*$^{-/y}$ mice (Saywell et al., 2006; Toloe et al., 2014), in the present work, we did not detected significant differences on the content of ATP when comparing hippocampus and cortex from WT and *Mecp2*$^{-/y}$ mice. Interestingly, studies performed in

organotypic hippocampal cultures have shown a downregulation on mitochondrial gene expression (Großer et al., 2012). Moreover, a quantitative decrease in electron transport chain units and reduced efficiency of glucose metabolism were found in RTT mouse models (De Filippis et al., 2015; Kriaucionis et al., 2006; Saywell et al., 2006). Also lower A_1R receptor number or lower responsiveness of the A_1R did not explain lower tonic adenosinergic inhibition because *Mecp2*$^{-/y}$ mice had enhanced A_1R protein levels (as revealed by binding assays and western blot technique), and the response to an added A_1R agonist was even higher in slices of *Mecp2*$^{-/y}$ mice. The mechanism underlying the overall increased levels of A_1R remains unknown. However, we can speculate that this could be a compensatory mechanism to decreased levels of adenosine and to the overexcitability associated to this pathology. Indeed, in the brain of *Mecp2*$^{-/y}$ mice there is a tendency for hyperexcitability, associated to changes in basal inhibitory rhythms and pre- and postsynaptic defects in GABAergic synapses (Calfa et al., 2011). Low intracellular adenosine levels may favour DNA methylation and hence epileptogenesis (Williams-Karnesky et al., 2013). Consequently, adenosine augmentation therapies constitute an effective strategy to reduce seizures, even in cases refractory to conventional antiepileptic drugs (Boison, 2013, 2009).

In contrast with A_1Rs, the protein levels of $A_{2A}R$ were decreased in cortex of *Mecp2*$^{-/y}$ mice. The available receptors were nevertheless enough to respond to exogenous activation with a selective agonist, which was able to rescue the action of BDNF on synaptic plasticity (LTP). This further reinforces the hypothesis that adenosine augmentation therapies may be a particularly useful strategy in a disease that simultaneously involves low BDNF signalling and low capability of endogenous adenosine to act as an endogenous anticonvulsant. As it occurred with the mRNA of the A_1R, the mRNA expression level of $A_{2A}R$ was not significantly affected in *Mecp2*$^{-/y}$ mice. The discrepancy between mRNA and protein expression levels could be attributed to several posttranscriptional mechanisms such as deregulation of AKT/mTOR in RTT, an important signalling pathway for protein synthesis and which it is under the regulatory influence of MeCP2 (Li et al., 2013). Intriguingly, it was possible to detect changes in the mRNA levels of both A_1R and $A_{2A}R$ in one *post-mortem* human brain sample from an RTT patient, and the changes detected closely mimicked the changes detected at the protein level in *Mecp2*$^{-/y}$ mice. The advanced disease stage of the donor child and the inherent differences between mice models and humans may explain the discrepancy regarding mRNA expression levels. Nevertheless, we should acknowledge the limitations of this analysis: samples from only one mRNA RTT patient were available, and it was thus not possible to access variability in different subjects. Considering the high influence of the heterozygosity in this syndrome, we conducted some preliminary experiences to know if the adenosinergic system is also changed in a milder phenotype such as the heterozygotic mice female. The results showed similar changes found in the hippocampus from *Mecp2*$^{-/y}$: decreased protein levels of $A_{2A}R$ and increased protein levels in A_1R. Moreover, the alterations detected in mRNA expression levels of $A_{2A}R$ and A_1R support a similar deregulation in adenosinergic system in the sample from a RTT patient with a different genotype (*MeCP2* mutation - R255X). These results strongly support the hypothesis that the adenosinergic system is changed in multiple phenotypes of RTT. Taken together, the results show a decrease on endogenous adenosine levels and a consequent impairment on adenosinergic signalling in RTT. This deregulation could hamper BDNF signalling and may account for epileptogenesis and decrease seizure control, therefore opening new avenues for RTT therapy: adenosine augmentation therapies (AAT). AAT have been already explored in other pathologies, such as in epilepsy (Boison, 2009). Interestingly, RTT patients present some disturbances related with deregulation on adenosinergic system such dysfunction in sleep/arousal, neuronal maturation, central control of breathing and epilepsy, (Gomes et al., 2011; Ribeiro et al., 2002). Our findings showing alterations in adenosine system in RTT strongly point towards a possible contribution of adenosine system in RTT pathophysiology. To potentiate BDNF signalling it could be enough to target only $A_{2A}R$. However, our results shed light into the relevance of using AATs instead of direct $A_{2A}R$ activation. IndeeTs would target the two high affinity adenosine receptors and trigger their activation to promote: 1) the rescue of BDNF effects, through $A_{2A}R$; 2) the control of hyperexcitability through A_1R. In addition, it was already shown that adenosine plays an important role in epileptogenesis through an epigenetic action by DNA methylation, which inhibits epileptogenesis (Williams-Karnesky et al., 2013). As previous discussed, AAT could be achieved by genetic approaches and cell-therapy strategies (as discussed in Boison, 2009).

Subsequent work should elucidate whether MeCP2 directly affects the expression of enzymes that regulate adenosine and adenosine receptor expression. It is possible that changes in receptor levels result of homeostatic mechanisms (Sandau et al., 2016) attempting to preserve inhibitory ADO synaptic actions by increasing inhibitory A_1R and decreasing excitatory $A_{2A}R$ expression levels. However, in the case of RTT where BDNF expression and signalling is impaired, the decrease of $A_{2A}R$ further aggravates the disease. Importantly, as we herein show, in spite of the reduction of $A_{2A}R$ expression and the decreased levels of endogenous adenosine, the pharmacological activation of $A_{2A}R$ could efficiently rescue BDNF effects upon synaptic plasticity.

5. Conclusion

In conclusion, we found that BDNF actions upon hippocampal LTP are impaired in RTT. This dysfunction can be explained by the changes in TrkB receptor levels and in adenosinergic neuromodulation. Importantly, by understanding that the adenosinergic system is compromised in RTT, we can now advance that deregulation of BDNF signalling can also be due to impairment of endogenous $A_{2A}R$ activation. This study thus sets the stage for new adenosine-based pharmacological therapeutic strategies for RTT, for which disease modifying drugs are still lacking.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2020.105043.

Ethics approval

Studies in *post-mortem* brain of *MECP2* patients were included in the project "Therapeutic approaches in Rett syndrome" funded by "Mi Princesa Rett", a Spanish Patient Association. This study was approved by the Ethics Committee of HSJD and parents provided informed written consent.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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References

Abuhatzira, L., Makedonski, K., Kaufman, Y., Razin, A., Shemer, R., 2007. MeCP2 deficiency in the brain decreases BDNF levels by REST/CoREST-mediated repression and increases TRKB production. Epigenetics 2, 214–222. https://doi.org/10.4161/epi.2.4.5212.

Amir, R.E., Van den Veyver, I.B., Wan, M., Tran, C.Q., Francke, U., Zoghbi, H.Y., 1999. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat. Genet. 23, 185–188. https://doi.org/10.1038/13810.

Anderson, W.W., Collingridge, G.L., 2001. The LTP Program: A data acquisition program for on-line analysis of long-term potentiation and other synaptic events. J. Neurosci. Methods 108, 71–83. https://doi.org/10.1016/S0165-0270(01)00374-0.

Aronica, E., Zurolo, E., Iyer, A., De Groot, M. De, Anink, J., 2011. Upregulation of adenosine kinase in astrocytes in experimental and human temporal lobe epilepsy. Epilepsia 52, 1645–1655. https://doi.org/10.1111/j.1528-1167.2011.03115.x.Upregulation.

Aronica, E., Sandau, U.S., Iyer, A., Boison, D., 2013. Glial adenosine kinase - A neuropathological marker of the epileptic brain. Neurochem. Int. 63, 688–695. https://doi.org/10.1016/j.neuint.2013.01.028.

Asaka, Y., Jugloff, D.G.M., Zhang, L., Eubanks, J.H., Fitzsimonds, R.M., 2006. Hippocampal synaptic plasticity is impaired in the Mecp2-null mouse model of Rett syndrome. Neurobiol. Dis. 21, 217–227. https://doi.org/10.1016/j.nbd.2005.07.005.

Assaife-Lopes, N., Sousa, V.C., Pereira, D.B., Ribeiro, J.A., Sebastião, A.M., 2014. Regulation of TrkB receptor translocation to lipid rafts by adenosine A(2A) receptors and its functional implications for BDNF-induced regulation of synaptic plasticity. Purinergic Signal 10, 251–267. https://doi.org/10.1007/s11302-013-9389-9.

Batalha, V.L., Ferreira, D.G., Coelho, J.E., Valadas, J.S., Gomes, R., Temido-Ferreira, M., Shmidt, T., Baqi, Y., Buée, L., Müller, C.E., Hamdane, M., Outeiro, T.F., Bader, M., Meijsing, S.H., Sadri-Vakili, G., Blum, D., Lopes, L.V., 2016. The caffeine-binding adenosine A2A receptor induces age-like HPA-axis dysfunction by targeting glucocorticoid receptor function. Sci. Rep. 6, 31493. https://doi.org/10.1038/srep31493.

Bedogni, F., Rossi, R.L., Galli, F., Cobolli Gigli, C., Gandaglia, A., Kilstrup-Nielsen, C., Landsberger, N., 2014. Rett syndrome and the urge of novel approaches to study MeCP2 functions and mechanisms of action. Neurosci. Biobehav. Rev. 46, 187–201. https://doi.org/10.1016/j.neubiorev.2014.01.011.

Binder, D.K., Scharfman, H.E., 2004. Brain-derived neurotrophic factor. Growth Factors 22, 123–131. https://doi.org/10.1016/j.bbi.2008.05.010.

Boison, D., 2007. Adenosine as a modulator of brain activity. Drug News Perspect. 20, 607. https://doi.org/10.1358/dnp.2007.20.10.1181353.

Boison, D., 2009. Adenosine augmentation therapies (AATs) for epilepsy: Prospect of cell and gene therapies. Epilepsy Res. 85, 131–141. https://doi.org/10.1016/j.eplepsyres.2009.03.019.

Boison, D., 2012. Adenosine dysfunction in epilepsy. Glia 29, 997–1003. https://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted.

Boison, D., 2013. Adenosine kinase: exploitation for therapeutic gain. Pharmacol. Rev. 65, 906–943. https://doi.org/10.1124/pr.112.006361.

Boison, D., 2015. Adenosinergic signaling in epilepsy. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2015.08.046.

Calfa, G., Hablitz, J.J., Pozzo-Miller, L., 2011. Network hyperexcitability in hippocampal slices from Mecp2 mutant mice revealed by voltage-sensitive dye imaging. J. Neurophysiol. 105, 1768–1784. https://doi.org/10.1152/jn.00800.2010.

Chahrour, M., Zoghbi, H.Y., 2007. The story of Rett syndrome: From clinic to neurobiology. Neuron 56, 422–437. https://doi.org/10.1016/j.neuron.2007.10.001.

Chahrour, M., Jung, S.Y., Shaw, C., Zhou, X., Wong, S.T.C., Qin, J., Zoghbi, H.Y., 2008. MeCP2, a key contributor to neurological disease, activates and represses transcription. Science 320, 1224–1229. https://doi.org/10.1126/science.1153252.

Chang, Q., Khare, G., Dani, V., Nelson, S., Jaenisch, R., 2006. The disease progression of Mecp2 mutant mice is affected by the level of BDNF expression. Neuron 49, 341–348. https://doi.org/10.1016/j.neuron.2005.12.027.

Chen, W.G., Chang, Q., Lin, Y., Meissner, A., West, A.E., Griffith, E.C., Jaenisch, R., Greenberg, M.E., 2003. Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. Science 302, 885–889. https://doi.org/10.1126/science.1086446.

Chen, J.F., Sonsalla, P.K., Pedata, F., Melani, A., Domenici, M.R., Popoli, P., Geiger, J., Lopes, L.V., de Mendonça, A., 2007. Adenosine A2A receptors and brain injury: Broad spectrum of neuroprotection, multifaceted actions and "fine tuning" modulation. Prog. Neurobiol. 83, 310–331. https://doi.org/10.1016/j.pneurobio.2007.09.002.

De Filippis, B., Valenti, D., De Bari, L., De Rasmo, D., Musto, M., Fabbri, A., Ricceri, L., Fiorentini, C., Laviola, G., Vacca, R.A., 2015. Mitochondrial free radical overproduction due to respiratory chain impairment in the brain of a mouse model of Rett syndrome: Protective effect of CNF1. Free Radic. Biol. Med. 83, 167–177. https://doi.org/10.1016/j.freeradbiomed.2015.02.014.

Deng, V., Matagne, V., Banine, F., Frerking, M., Ohliger, P., Budden, S., Pevsner, J., Dissen, G.a., Sherman, L.S., Ojeda, S.R., 2007. FXYD1 is an MeCP2 target gene overexpressed in the brains of Rett syndrome patients and Mecp2-null mice. Hum. Mol. Genet. 16, 640–650. https://doi.org/10.1093/hmg/ddm007.

Diógenes, M.J., Fernandes, C.C., Sebastião, A.M., Ribeiro, J.A., 2004. Activation of adenosine A2A receptor facilitates brain-derived neurotrophic factor modulation of synaptic transmission in hippocampal slices. J. Neurosci. 24, 2905–2913. https://doi.org/10.1523/JNEUROSCI.4454-03.2004.

Diogenes, M. j, Assaife-Lopes, N., Pinto-Duarte, A., Ribeiro, J.A., Sebastião, A.M., 2007. Influence of age on bdnf modulation of hippocampal synaptic transmission: Interplay with adenosine a2a receptors. Hippocampus 000, 1–3. https://doi.org/10.1002/hipo.

Diógenes, M.J., Costenla, A.R., Lopes, L.V., Jerónimo-Santos, A., Sousa, V.C., Fontinha, B.M., Ribeiro, J. a, Sebastião, A.M., 2011. Enhancement of LTP in aged rats is dependent on endogenous BDNF. Neuropsychopharmacology 36, 1823–1836. https://doi.org/10.1038/npp.2011.64.

Diogenes, M.J., Dias, R.B., Rombo, D.M., Vicente Miranda, H., Maiolino, F., Guerreiro, P., Naastrom, T., Franquelim, H.G., Oliveira, L.M.a., Castanho, M.a.R.B., Lannfelt, L., Bergstrom, J., Ingelsson, M., Quintas, a., Sebastiao, a.M., Lopes, L.V., Outeiro, T.F., 2012. Extracellular alpha-synuclein oligomers modulate synaptic transmission and impair LTP via NMDA-receptor activation. J. Neurosci. 32, 11750–11762. https://doi.org/10.1523/JNEUROSCI.0234-12.2012.

Diogenes, M.J., Neves-Tome, R., Fucile, S., Martinello, K., Scianni, M., Theofilas, P., Lopatar, J., Ribeiro, J.A., Maggi, L., Frenguelli, B.G., Limatola, C., Boison, D., Sebastiao, A.M., 2014. Homeostatic control of synaptic activity by endogenous adenosine is mediated by adenosine kinase. Cereb. Cortex 24, 67–80. https://doi.org/10.1093/cercor/bhs284.

Dunwiddie, T.V., Masino, S.A., 2001. The role and regulation of adenosine in the central nervous system. Annu. Rev. Neurosci. 24, 31–55. https://doi.org/10.1146/annurev.neuro.24.1.31.

Eide, F.F., 1996. Naturally occuring truncated trkb receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. J. Neurosci. 16, 3123–3129. https://doi.org/10.1055/s-0029-1237430.Imprinting.

Figurov, A., Pozzo-Miller, L.D., Olafsson, P., Wang, T., Lu, B., 1996. Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381, 706–709. https://doi.org/10.1038/381706a0.

Fontinha, B.M., Diógenes, M.J., Ribeiro, J.a., Sebastião, a.M., 2008. Enhancement of long-term potentiation by brain-derived neurotrophic factor requires adenosine A2A receptor activation by endogenous adenosine. Neuropharmacology 54, 924–933. https://doi.org/10.1016/j.neuropharm.2008.01.011.

Gomes, C.V., Kaster, M.P., Tomé, A.R., Agostinho, P.M., Cunha, R. a, 2011. Adenosine receptors and brain diseases: Neuroprotection and neurodegeneration. Biochim. Biophys. Acta 1808, 1380–1399. https://doi.org/10.1016/j.bbamem.2010.12.001.

Großer, E., Hirt, U., Janc, O.A., Menzfeld, C., Fischer, M., Kempkes, B., Vogelgesang, S., Manzke, T.U., Opitz, L., Salinas-Riester, G., Müller, M., 2012. Oxidative burden and mitochondrial dysfunction in a mouse model of Rett syndrome. Neurobiol. Dis. 48, 102–114. https://doi.org/10.1016/j.nbd.2012.06.007.

Guy, J., Hendrich, B., Holmes, M., Martin, J.E., Bird, a, 2001. A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nat. Genet. 27, 322–326. https://doi.org/10.1038/85899.

Guy, J., Gan, J., Selfridge, J., Cobb, S., Bird, A., 2007. Reversal of neurological defects in a mouse model of Rett syndrome. Science 315, 1143–1147. https://doi.org/10.1126/science.1138389.

Huang, E.J., Reichardt, L.F., 2003. Trk receptors: Roles in neuronal signal transduction. Annu. Rev. Biochem. 72, 609–642. https://doi.org/10.1146/annurev.biochem.72.121801.161629.

Jackson, E.K., Kotermanski, S.E., Menshikova, E.V., Dubey, R.K., Jackson, T.C., Kochanek, P.M., 2017. Adenosine production by brain cells. J. Neurochem. 141, 676–693. https://doi.org/10.1111/jnc.14018.

Jarvis, M.F., Schulz, R., Hutchison, A.J., Do, U.H., Sills, M.A., Williams, M., 1989. [3H]CGS 21680, a selective A2 adenosine receptor agonist directly labels A2 receptors in rat brain. J. Pharmacol. Exp. Ther. 251, 888–893.

Jerónimo-Santos, A., Batalha, V.L., Müller, C.E., Baqi, Y., Sebastião, A.M., Lopes, L.V., Diógenes, M.J., 2014. Impact of in vivo chronic blockade of adenosine A2A receptors on the BDNF-mediated facilitation of LTP. Neuropharmacology 83, 99–106. https://doi.org/10.1016/j.neuropharm.2014.04.006.

Karpova, N.N., Lindholm, J.S.O., Kulesskaya, N., Onishchenko, N., Vahter, M., Popova, D., Ceccatelli, S., Castrén, E., 2014. TrkB overexpression in mice buffers against memory deficits and depression-like behavior but not all anxiety- and stress-related symptoms induced by developmental exposure to methylmercury. Front. Behav. Neurosci. 8, 315. https://doi.org/10.3389/fnbeh.2014.00315.

Kriaucionis, S., Paterson, A., Curtis, J., Guy, J., MacLeod, N., Bird, A., 2006. Gene expression analysis exposes mitochondrial abnormalities in a mouse model of Rett syndrome. Mol. Cell. Biol. 26, 5033–5042. https://doi.org/10.1128/MCB.01665-05.

Lee, F.S., Chao, M.V., 2001. Activation of Trk neurotrophin receptors in the absence of neurotrophins. Proc. Natl. Acad. Sci. U. S. A. 98, 3555–3560. https://doi.org/10.1073/pnas.061020198.

Lewis, J.D., Meehan, R.R., Henzel, W.J., Maurer-Fogy, I., Jeppesen, P., Klein, F., Bird, a, 1992. Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. Cell 69, 905–914. https://doi.org/10.1016/0092-8674(92)90610-O.

Li, W., Pozzo-Miller, L., 2014. BDNF deregulation in Rett syndrome. Neuropharmacology 76, 737–746. https://doi.org/10.1016/j.neuropharm.2013.03.024.

Li, W., Calfa, G., Larimore, J., Pozzo-Miller, L., 2012. Activity-dependent BDNF release and TRPC signaling is impaired in hippocampal neurons of Mecp2 mutant mice. Proc. Natl. Acad. Sci. 109, 17087–17092. https://doi.org/10.1073/pnas.1205271109.

Li, Y., Wang, H., Muffat, J., Cheng, a W., Orlando, D. a, Loven, J., Kwok, S.M., Feldman, D. a, Bateup, H.S., Gao, Q., Hockemeyer, D., Mitalipova, M., Lewis, C. a, Vander Heiden, M.G., Sur, M., Young, R. a, Jaenisch, R., 2013. Global transcriptional and translational repression in human-embryonic-stem-cell-derived Rett syndrome neurons. Cell Stem Cell 13, 446–458. https://doi.org/10.1016/j.stem.2013.09.001.
Liyanage, V.R.B., Rastegar, M., 2014. Rett syndrome and MeCP2. NeuroMolecular Med. 16, 231–264. https://doi.org/10.1007/s12017-014-8295-9.
Lu, B., Nagappan, G., Guan, X., Nathan, P.J., Wren, P., 2013. BDNF-based synaptic repair as a disease-modifying strategy for neurodegenerative diseases. Nat. Rev. Neurosci. 14, 401–416. https://doi.org/10.1038/nrn3505.
Luberg, K., Wong, J., Weickert, C.S., Timmusk, T., 2010. Human TrkB gene: Novel alternative transcripts, protein isoforms and expression pattern in the prefrontal cerebral cortex during postnatal development. J. Neurochem. 113, 952–964. https://doi.org/10.1111/j.1471-4159.2010.06662.x.
Minichiello, L., Korte, M., Wolfer, D., Kühn, R., Unsicker, K., Cestari, V., Rossi-Arnaud, C., Lipp, H.P., Bonhoeffer, T., Klein, R., 1999. Essential role for TrkB receptors in hippocampus-mediated learning. Neuron 24, 401–414.
Moretti, P., Levenson, J.M., Battaglia, F., Atkinson, R., Teague, R., Antalffy, B., Armstrong, D., Arancio, O., Sweatt, J.D., Zoghbi, H.Y., 2006. Learning and memory and synaptic plasticity are impaired in a mouse model of Rett syndrome. J. Neurosci. 26, 319–327. https://doi.org/10.1523/JNEUROSCI.2623-05.2006.
Neul, J.L., Lane, J.B., Lee, H.-S., Geerts, S., Barrish, J.O., Annese, F., Baggett, L.M., Barnes, K., Skinner, S. a, Motil, K.J., Glaze, D.G., Kaufmann, W.E., Percy, A.K., 2014. Developmental delay in Rett syndrome: Data from the natural history study. J. Neurodev. Disord. 6, 20. https://doi.org/10.1186/1866-1955-6-20.
Nicolini, C., Ahn, Y., Michalski, B., Rho, J.M., Fahnestock, M., 2015. Decreased mTOR signaling pathway in human idiopathic autism and in rats exposed to valproic acid. Acta Neuropathol. Commun. 3, 3. https://doi.org/10.1186/s40478-015-0184-4.
Pak, M.A., Haas, H.L., Decking, U.K., Schrader, J., 1994. Inhibition of adenosine kinase increases endogenous adenosine and depresses neuronal activity in hippocampal slices. Neuropharmacology 33, 1049–1053.
Poduslo, J.F., Curran, G.L., 1996. Permeability at the blood-brain and blood-nerve barriers of the neurotrophic factors: NGF, CNTF, NT-3, BDNF. Mol. Brain Res. 36, 280–286. https://doi.org/10.1016/0169-328X(95)00250-V.
Rei, N., Rombo, D.M., Ferreira, M.F., Baqi, Y., Müller, C.E., Ribeiro, J.A., Sebastião, A.M., Vaz, S.H., 2020. Hippocampal synaptic dysfunction in the SOD1G93A mouse model of amyotrophic lateral sclerosis: Reversal by adenosine A2AR blockade. Neuropharmacology 171. https://doi.org/10.1016/j.neuropharm.2020.108106.
Ribeiro, J.A., Sebastião, A.M., de Mendonça, A., 2002. Adenosine receptors in the nervous system: Pathophysiological implications. Prog. Neurobiol. 68, 377–392. https://doi.org/10.1016/S0301-0082(02)00155-7.
Riikonen, R., 2003. Neurotrophic factors in the pathogenesis of Rett syndrome. J. Child Neurol. 18, 693–697. https://doi.org/10.1177/08830738030180101101.
Robinson, L., Guy, J., McKay, L., Brockett, E., Spike, R.C., Selfridge, J., De Sousa, D., Merusi, C., Riedel, G., Bird, A., Cobb, S.R., 2012. Morphological and functional reversal of phenotypes in a mouse model of Rett syndrome. Brain 135, 2699–2710. https://doi.org/10.1093/brain/aws096.
Rombo, D.M., Dias, R.B., Duarte, S.T., Ribeiro, J. a, Lamsa, K.P., Sebastião, A.M., 2014. Adenosine A1 receptor suppresses tonic GABAA receptor currents in hippocampal pyramidal cells and in a defined subpopulation of interneurons. Cereb. Cortex 1–15. https://doi.org/10.1093/cercor/bhu288.
Rose, C.R., Blum, R., Pichler, B., Lepier, A., Kafitz, K.W., Konnerth, A., 2003. Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. Nature 426, 74–78. https://doi.org/10.1038/nature01983.
Sandau, U.S., Colino-Oliveira, M., Jones, A., Saleumvong, B., Coffman, S.Q., Liu, L., Miranda-Lourenço, C., Palminha, C., Batalha, V.L., Xu, Y., Huo, Y., Diógenes, M.J., Sebastião, A.M., Boison, D., 2016. Adenosine kinase deficiency in the brain results in maladaptive synaptic plasticity. J. Neurosci. 36.
Saywell, V., Viola, A., Confort-Gouny, S., Le Fur, Y., Villard, L., Cozzone, P.J., 2006. Brain magnetic resonance study of Mecp2 deletion effects on anatomy and metabolism. Biochem. Biophys. Res. Commun. 340, 776–783. https://doi.org/10.1016/j.bbrc.2005.12.080.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3, 1101–1108. https://doi.org/10.1038/nprot.2008.73.
Sebastião, a M., Ribeiro, J. a, 2009. Tuning and fine-tuning of synapses with adenosine. Curr. Neuropharmacol. 7, 180–194. https://doi.org/10.2174/157015909789152128.
Sebastião, A.M., Stone, T.W., Ribeiro, J.A., 1990. The inhibitory adenosine receptor at the neuromuscular junction and hippocampus of the rat: Antagonism by 1,3,8-substituted xanthines. Br. J. Pharmacol. 101, 453–459.
Sebastião, A.M., Assaife-Lopes, N., Diógenes, M.J., Vaz, S.H., Ribeiro, J. a, 2011. Modulation of brain-derived neurotrophic factor (BDNF) actions in the nervous system by adenosine A(2A) receptors and the role of lipid rafts. Biochim. Biophys. Acta 1808, 1340–1349. https://doi.org/10.1016/j.bbamem.2010.06.028.
Silva, I., Costa, A.F., Moreira, S., Ferreirinha, F., Magalhães-Cardoso, M.T., Calejo, I., Silva-Ramos, M., Correia-de-Sá, P., 2017. Inhibition of cholinergic neurotransmission by β_3 -adrenoceptors depends on adenosine release and A_1 -receptor activation in human and rat urinary bladders. Am. J. Physiol. Physiol. 313, F388–F403. https://doi.org/10.1152/ajprenal.00392.2016.
Silva, I., Magalhães-Cardoso, M.T., Ferreirinha, F., Moreira, S., Costa, A.F., Silva, D., Vieira, C., Silva-Ramos, M., Correia-de-Sá, P., 2020. β_3 Adrenoceptor-induced cholinergic inhibition in human and rat urinary bladders involves the exchange protein directly activated by cyclic AMP 1 favoring adenosine release. Br. J. Pharmacol. 14921. https://doi.org/10.1111/bph.14921.
Tebano, M.T., Martire, a., Potenza, R.L., Grò, C., Pepponi, R., Armida, M., Domenici, M.R., Schwarzschild, M.a., Chen, J.F., Popoli, P., 2008. Adenosine A2A receptors are required for normal BDNF levels and BDNF-induced potentiation of synaptic transmission in the mouse hippocampus. J. Neurochem. 104, 279–286. https://doi.org/10.1111/j.1471-4159.2007.05046.x.
Toloe, J., Mollajew, R., Kügler, S., Mironov, S.L., 2014. Metabolic differences in hippocampal "Rett" neurons revealed by ATP imaging. Mol. Cell. Neurosci. 59, 47–56. https://doi.org/10.1016/j.mcn.2013.12.008.
Tsvyetlynska, N.A., Hill, R.H., Grillner, S., n.d. Role of AMPA Receptor Desensitization and the Side Effects of a DMSO Vehicle on Reticulospinal EPSPs and Locomotor Activity. https://doi.org/10.1152/jn.00201.2005.
Vanhala, R., Korhonen, L., Mikelsaar, M., Lindholm, D., Riikonen, R., 1998. Neurotrophic factors in cerebrospinal fluid and serum of patients with Rett syndrome. J. Child Neurol. 13, 429–433. https://doi.org/10.1177/088307389801300903.
Vieira, C., Ferreirinha, F., Magalhães-Cardoso, M.T., Silva, I., Marques, P., Correia-de-Sá, P., 2017. Post-inflammatory ileitis induces non-neuronal purinergic signaling adjustments of cholinergic neurotransmission in the myenteric plexus. Front. Pharmacol. 8, 811. https://doi.org/10.3389/fphar.2017.00811.
Wang, H., Chan, S., Ogier, M., Hellard, D., Wang, Q., Smith, C., Katz, D.M., 2006. Dysregulation of brain-derived neurotrophic factor expression and neurosecretory function in Mecp2 null mice. J. Neurosci. 26, 10911–10915. https://doi.org/10.1523/JNEUROSCI.1810-06.2006.
Williams-Karnesky, R.L., Sandau, U.S., Lusardi, T.A., Lytle, N.K., Farrell, J.M., Pritchard, E.M., Kaplan, D.L., Boison, D., 2013. Epigenetic changes induced by adenosine augmentation therapy prevent epileptogenesis. J. Clin. Invest. 123, 3552–3563. https://doi.org/10.1172/JCI65636.

Bibliographic references

Abuhatzira L, Makedonski K, Kaufman Y, Razin A, Shemer R (2007) MeCP2 deficiency in the brain decreases BDNF levels by REST/CoREST-mediated repression and increases TRKB production. Epigenetics 2:214–222.

Adachi M, Monteggia LM (2014) Decoding transcriptional repressor complexes in the adult central nervous system. Neuropharmacology 0:45.

Aharoni R, Eilam R, Domev H, Labunskay G, Sela M, Arnon R (2005) The immunomodulator glatiramer acetate augments the expression of neurotrophic factors in brains of experimental autoimmune encephalomyelitis mice. Proc Natl Acad Sci U S A 102:19045–19050.

Ala TA, Beh GO, Frey WH (2000) Pure hippocampal sclerosis: a rare cause of dementia mimicking Alzheimer's disease. Neurology 54:843–848.

Al-Qudah MA, Al-Dwairi A (2016) Mechanisms and regulation of neurotrophin synthesis and secretion. Neurosciences 21:306.

Amir RE, Van Den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY (1999) Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl- CpG-binding protein 2. Nature Genetics.

Amir RE, Zoghbi HY (2000) Rett syndrome: Methyl-CpG-binding protein 2 mutations and phenotype-genotype correlations. American Journal of Medical Genetics - Seminars in Medical Genetics 97:147–152.

Anderson WW, Collingridge GL (2001) The LTP Program: A data acquisition program for on-line analysis of long-term potentiation and other synaptic events. Journal of Neuroscience Methods 108:71–83.

Ariani F, Hayek G, Rondinella D, Artuso R, Mencarelli MA, Spanhol-Rosseto A, Pollazzon M, Buoni S, Spiga O, Ricciardi S, Meloni I, Longo I, Mari F, Broccoli V, Zappella M, Renieri A (2008) FOXG1 Is Responsible for the Congenital Variant of Rett Syndrome. American Journal of Human Genetics 83:89–93.

Aronica E, Sandau US, Iyer A, Boison D (2013) Glial adenosine kinase - A neuropathological marker of the epileptic brain. Neurochemistry International 63:688–695.

Asaka Y, Jugloff DGM, Zhang L, Eubanks JH, Fitzsimonds RM (2006) Hippocampal synaptic plasticity is impaired in the Mecp2-null mouse model of Rett syndrome. Neurobiology of disease 21:217–227.

Assaife-Lopes N, Sousa VC, Pereira DB, Ribeiro J a, Chao M V, Sebastião AM (2010) Activation of adenosine A2A receptors induces TrkB translocation and increases BDNF-mediated phospho-TrkB localization in lipid rafts: implications for neuromodulation. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:8468–8480.

Assaife-Lopes N, Sousa VC, Pereira DB, Ribeiro JA, Sebastião AM (2014) Regulation of TrkB receptor translocation to lipid rafts by adenosine A(2A) receptors and its functional implications for BDNF-induced regulation of synaptic plasticity. Purinergic signalling 10:251–267.

Balmer D, Goldstine J, Rao YM, LaSalle JM (2003) Elevated methyl-CpG-binding protein 2 expression is acquired during postnatal human brain development and is correlated with alternative polyadenylation. Journal of Molecular Medicine 81:61–68.

Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG, Miller FD (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. Journal of Cell Biology 140:911–923.

Banerjee A, Castro J, Sur M (2012) Rett syndrome: Genes, synapses, circuits, and therapeutics. Frontiers in Psychiatry 3:1–13.

Banerjee A, Miller MT, Li K, Sur M, Kaufmann WE (2019) Towards a better diagnosis and treatment of Rett syndrome: A model synaptic disorder. Brain 142:239–248.

Baraldi T, Schöwe NM, Balthazar J, Monteiro-Silva KC, Albuquerque MS, Buck HS, Viel TA (2013) Cognitive stimulation during lifetime and in the aged phase improved spatial memory, and altered neuroplasticity and cholinergic markers of mice. Experimental Gerontology 48:831–838.

Barde Y a, Edgar D, Thoenen H (1982) Purification of a new neurotrophic factor from mammalian brain. The EMBO journal 1:549–553.

Bathina S, Das UN (2015) Brain-derived neurotrophic factor and its clinical Implications. Archives of Medical Science 11:1164–1178.

Bedogni F, Cobolli Gigli C, Pozzi D, Rossi RL, Scaramuzza L, Rossetti G, Pagani M, Kilstrup-Nielsen C, Matteoli M, Landsberger N (2016) Defects During Mecp2 Null Embryonic Cortex Development Precede the Onset of Overt Neurological Symptoms. Cereb Cortex 26:2517–2529.

Ben-Zeev B, Aharoni R, Nissenkorn A, Arnon R (2011) Glatiramer acetate (GA, Copolymer-1) an hypothetical treatment option for Rett syndrome. Med Hypotheses 76:190–193.

Bibel M, Barde YA (2000) Neurotrophins: Key regulators of cell fate and cell shape in the vertebrate nervous system. Genes and Development 14:2919–2937.

Bittolo T, Raminelli CA, Deiana C, Baj G, Vaghi V, Ferrazzo S, Bernareggi A, Tongiorgi E (2016) Pharmacological treatment with mirtazapine rescues cortical atrophy and respiratory deficits in MeCP2 null mice. Scientific Reports 2016 6:1 6:1–14.

Bjursell MK, Blom HJ, Cayuela JA, Engvall ML, Lesko N, Balasubramaniam S, Brandberg G, Halldin M, Falkenberg M, Jakobs C, Smith D, Struys E, von Döbeln U, Gustafsson CM, Lundeberg J, Wedell A (2011) Adenosine kinase deficiency disrupts the methionine cycle and causes hypermethioninemia, encephalopathy, and abnormal liver function. Am J Hum Genet 89:507–515.

Blackburn MR, Kellems RE (1996) Regulation and function of adenosine deaminase in mice. Progress in nucleic acid research and molecular biology 55:195–226.

Boggio (2010) Synaptic determinants of Rett syndrome. Frontiers in Synaptic Neuroscience.

Boison D (2007) Adenosine as a modulator of brain activity. Drug News & Perspectives 20:607.

Boison D (2009) Adenosine augmentation therapies (AATs) for epilepsy: Prospect of cell and gene therapies. Epilepsy Research 85:131–141.

Boison D (2012) Adenosine Dysfunction in epilepsy. Glia 29:997–1003.

Boison D (2013a) Adenosine and seizure termination: Endogenous mechanisms. Epilepsy Currents 13:35–37.

Boison D (2013b) Adenosine kinase: exploitation for therapeutic gain. Pharmacological reviews 65:906–943.

Boison D (2016) Adenosinergic signaling in epilepsy. Neuropharmacology 104:131–139.

Boison D, Chen JF, Fredholm BB (2010) Adenosine Signalling and Function in Glial Cells Detlev. Cell Death Differ 17:1071–1082.

Boison D, Scheurer L, Zumsteg V, Rülicke T, Litynski P, Fowler B, Brandner S, Mohler H (2002) Neonatal hepatic steatosis by disruption of the adenosine kinase gene. Proceedings of the National Academy of Sciences of the United States of America 99:6985–6990.

Boison D, Jarvis MF (2021) Adenosine kinase: A key regulator of purinergic physiology. Biochemical pharmacology 187.

Bono MR, Fernández D, Flores-Santibáñez F, Rosemblatt M, Sauma D (2015) CD73 and CD39 ectonucleotidases in T cell differentiation: Beyond immunosuppression. FEBS Letters 589:3454–3460.

Bontemps F, Van den Berghe G, Hers HG (1983) Evidence for a substrate cycle between AMP and adenosine in isolated hepatocytes. Proceedings of the National Academy of Sciences of the United States of America 80:2829–2833.

Bontemps F, Vincent MF, Van den Berghe G (1993) Mechanisms of elevation of adenosine levels in anoxic hepatocytes. The Biochemical journal 290 (Pt 3:671–677.

Borowicz KK, Łuszczki J, Czuczwar SJ (2002) 2-Chloroadenosine, a preferential agonist of adenosine A1 receptors, enhances the anticonvulsant activity of carbamazepine and clonazepam in mice. Eur Neuropsychopharmacol 12:173–179.

Borrell-Pagès M, Canals JM, Cordelières FP, Parker JA, Pineda JR, Grange G, Bryson EA, Guillermier M, Hirsch E, Hantraye P, Cheetham ME, Néri C, Alberch J, Brouillet E, Saudou F, Humbert S (2006) Cystamine and cysteamine increase brain levels of BDNF in Huntington disease via HSJ1b and transglutaminase. J Clin Invest 116:1410–1424.

Bothwell M (1996) p75NTR: A Receptor After All. Science 272:506–507.

Britton DR, Mikusa J, Lee CH, Jarvis MF, Williams M, Kowaluk EA (1999) Site and event specific increase of striatal adenosine release by adenosine kinase inhibition in rats. Neuroscience Letters 266:93–96.

Broch OJ, Ueland PM (1980) Regional and subcellular distribution of S-adenosylhomocysteine hydrolase in the adult rat brain. Journal of neurochemistry 35:484–488.

Brunetti-Pierri N et al. (2011) Duplications of FOXG1 in 14q12 are associated with developmental epilepsy, mental retardation, and severe speech impairment. European Journal of Human Genetics 19:102–107.

Buchovecky CM, Turley SD, Brown HM, Kyle SM, McDonald JG, Liu B, Pieper AA, Huang W, Katz DM, Russell DW, Shendure J, Justice MJ (2013) A suppressor screen in Mecp2 mutant mice implicates cholesterol metabolism in Rett syndrome. Nat Genet 45:1013–1020.

Calfa G, Hablitz JJ, Pozzo-Miller L (2011) Network hyperexcitability in hippocampal slices from Mecp2 mutant mice revealed by voltage-sensitive dye imaging. Journal of neurophysiology 105:1768–1784.

Castro J, Garcia RI, Kwok S, Banerjee A, Petravicz J, Woodson J, Mellios N, Tropea D, Sur M (2014) Functional recovery with recombinant human IGF1 treatment in a mouse model of Rett Syndrome. Proc Natl Acad Sci U S A 111:9941–9946.

Chadwick BP, Frischauf AM (1998) The CD39-like gene family: Identification of three new human members (CD39L2, CD39L3, and CD39L4), their murine homologues, and a member of the gene family from Drosophila melanogaster. Genomics 50:357–367.

Chahrour M, Jung SY, Shaw C, Zhou X, Wong STC, Qin J, Zoghbi HY (2008) MeCP2, a key contributor to neurological disease, activates and represses transcription. Science (New York, NY) 320:1224–1229.

Chahrour M, Zoghbi HY (2007) The Story of Rett Syndrome: From Clinic to Neurobiology. Neuron 56:422–437.

Chailangkarn T, Acab a, Renato Muotri a (2012) Modeling neurodevelopmental disorders using human neurons. Current opinion in neurobiology 22:785–790.

Chang Q, Khare G, Dani V, Nelson S, Jaenisch R (2006) The disease progression of Mecp2 mutant mice is affected by the level of BDNF expression. Neuron 49:341–348.

Chen JF, Cunha R (2020) The belated US FDA approval of the adenosine A 2A receptor antagonist istradefylline for treatment of Parkinson's disease - PubMed. Purinergic Signalling 16(2):167–174.

Chen JF, Eltzschig HK, Fredholm BB (2013) Adenosine receptors as drug targets-what are the challenges? Nature Reviews Drug Discovery 12:265–286.

Chen JF, Moratalla R, Impagnatiello F, Grandy DK, Cuellar B, Rubinstein M, Beilstein MA, Hackett E, Fink JS, Low MJ, Ongini E, Schwarzschild MA (2001a) The role of the D2 dopamine receptor (D2R) in A2A adenosine receptor (A2AR)-mediated behavioral and cellular responses as revealed by A2A and D2 receptor knockout mice. Proceedings of the National Academy of Sciences 98:1970–1975.

Chen JF, Sonsalla PK, Pedata F, Melani A, Domenici MR, Popoli P, Geiger J, Lopes L V., de Mendonça A (2007) Adenosine A2A receptors and brain injury: Broad spectrum of neuroprotection, multifaceted actions and "fine tuning" modulation. Progress in Neurobiology 83:310–331.

Chen RZ, Akbarian S, Tudor M, Jaenisch R (2001b) Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. Nature genetics 27:327–331.

Chen WG, Chang Q, Lin Y, Meissner A, West AE, Griffith EC, Jaenisch R, Greenberg ME (2003) Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. Science (New York, NY) 302:885–889.

Chen Y et al. (2017) Modeling Rett Syndrome Using TALEN-Edited MECP2 Mutant Cynomolgus Monkeys. Cell 169:945-955.e10.

Chin EWM, Marcy G, Yoon S-I, Ma D, Rosales FJ, Augustine GJ, Goh ELK (2016) Choline Ameliorates Disease Phenotypes in Human iPSC Models of Rett Syndrome. Neuromolecular Med 18:364–377.

Cohen M V., Yang X, Downey JM (2010) A 2b adenosine receptors can change their spots. British Journal of Pharmacology 159:1595–1597.

Coppola G, Pascotto A (1996) Double-blind, placebo-controlled, cross-over trial of allopurinol as add-on therapy in childhood refractory epilepsy. Brain Dev 18:50–52.

Coy JF, Sedlacek Z, Bächner D, Delius H, Poustka A (1999) A complex pattern of evolutionary conservation and alternative polyadenylation within the long 3'-untranslated region of the methyl-CpG-binding protein 2 gene (MeCP2) suggests a regulatory role in gene expression.

Cristóvão-Ferreira S, Navarro G, Brugarolas M, Pérez-Capote K, Vaz V, Fattorina G, Conti F, Lluis C, Ribeiro JA, McCormick PJ, Casadó V, Franco R, Sebastião AM (2013) A1R-A2AR heteromers coupled to Gs and G i/0 proteins modulate GABA transport into astrocytes. Purinergic signalling 9:433–449.

Cuddapah VA, Pillai RB, Shekar K V., Lane JB, Motil KJ, Skinner SA, Tarquinio DC, Glaze DG, McGwin G, Kaufmann WE, Percy AK, Neul JL, Olsen ML (2014) Methyl-CpG-binding protein 2 (MECP2) mutation type is associated with disease severity in rett syndrome. Journal of Medical Genetics 51:152–158.

Cui XA, Agarwal T, Singh B, Gupta RS (2011) Molecular characterization of Chinese hamster cells mutants affected in adenosine kinase and showing novel genetic and biochemical characteristics. BMC Biochem 12:22.

Dajani R, Koo S-E, Sullivan GJ, Park I-H (2013) Investigation of Rett syndrome using pluripotent stem cells. Journal of cellular biochemistry 114:2446–2453.

De Filippis B, Fabbri A, Simone D, Canese R, Ricceri L, Malchiodi-Albedi F, Laviola G, Fiorentini C (2012) Modulation of RhoGTPases improves the behavioral phenotype and reverses astrocytic deficits in a mouse model of Rett syndrome. Neuropsychopharmacology 37:1152–1163.

De Filippis B, Ricceri L, Fuso A, Laviola G (2013) Neonatal exposure to low dose corticosterone persistently modulates hippocampal mineralocorticoid receptor expression and improves locomotor/exploratory behaviour in a mouse model of Rett syndrome. Neuropharmacology 68:174–183.

De Filippis B, Valenti D, De Bari L, De Rasmo D, Musto M, Fabbri A, Ricceri L, Fiorentini C, Laviola G, Vacca RA (2015) Mitochondrial free radical overproduction due to respiratory chain impairment in the brain of a mouse model of Rett syndrome: Protective effect of CNF1. Free Radical Biology and Medicine 83:167–177.

Delépine C, Meziane H, Nectoux J, Opitz M, Smith AB, Ballatore C, Saillour Y, Bennaceur-Griscelli A, Chang Q, Williams EC, Dahan M, Duboin A, Billuart P, Herault Y, Bienvenu T (2016) Altered microtubule dynamics and vesicular transport in mouse and human MeCP2-deficient astrocytes. Human molecular genetics 25:146–157.

Deng V, Matagne V, Banine F, Frerking M, Ohliger P, Budden S, Pevsner J, Dissen G a., Sherman LS, Ojeda SR (2007) FXYD1 is an MeCP2 target gene overexpressed in the brains of Rett syndrome patients and Mecp2-null mice. Human Molecular Genetics 16:640–650.

Deogracias R, Yazdani M, Dekkers MPJ, Guy J, Ionescu MCS, Vogt KE, Barde Y -a. (2012) Fingolimod, a sphingosine-1 phosphate receptor modulator, increases BDNF levels and improves symptoms of a mouse model of Rett syndrome. Proceedings of the National Academy of Sciences 109:14230–14235.

Diógenes M j, Assaife-Lopes N, Pinto-Duarte A, Ribeiro JA, Sebastião AM (2007) Influence of age on bdnf modulation of hippocampal synaptic transmission:interplay with adenosine a2a receptors. Hippocampus 000:1–3.

Diógenes MJ, Costenla AR, Lopes L V, Jerónimo-Santos A, Sousa VC, Fontinha BM, Ribeiro J a, Sebastião AM (2011) Enhancement of LTP in aged rats is dependent on endogenous BDNF. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 36:1823–1836.

Diógenes MJ, Dias RB, Rombo DM, Vicente Miranda H, Maiolino F, Guerreiro P, Nasstrom T, Franquelim HG, Oliveira LM a., Castanho M a. RB, Lannfelt L, Bergstrom J, Ingelsson M, Quintas a., Sebastiao a. M, Lopes L V., Outeiro TF (2012) Extracellular Alpha-Synuclein Oligomers Modulate Synaptic Transmission and Impair LTP Via NMDA-Receptor Activation. Journal of Neuroscience 32:11750–11762.

Diógenes MJ, Fernandes CC, Sebastião AM, Ribeiro JA (2004) Activation of adenosine A2A receptor facilitates brain-derived neurotrophic factor modulation of synaptic transmission in hippocampal slices. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:2905–2913.

Diógenes MJ, Neves-Tome R, Fucile S, Martinello K, Scianni M, Theofilas P, Lopatar J, Ribeiro JA, Maggi L, Frenguelli BG, Limatola C, Boison D, Sebastiao AM (2014) Homeostatic Control of Synaptic Activity by Endogenous Adenosine is Mediated by Adenosine Kinase. Cerebral Cortex 24:67–80.

Diógenes MJ, Ribeiro JA, Sebastião AM (2015) Adenosine A2A Receptors and Neurotrophic Factors: Relevance for Parkinson's Disease. In: The Adenosinergic System SE - 4 (Morelli M, Simola N, Wardas J, eds), pp 57–79 Current Topics in Neurotoxicity. Springer International Publishing.

Djukic A, Holtzer R, Shinnar S, Muzumdar H, Rose SA, Mowrey W, Galanopoulou AS, Shinnar R, Jankowski JJ, Feldman JF, Pillai S, Moshé SL (2016) Pharmacologic Treatment of Rett Syndrome With Glatiramer Acetate. Pediatr Neurol 61:51–57.

Dorsey SG, Renn CL, Carim-Todd L, Barrick CA, Bambrick L, Krueger BK, Ward CW, Tessarollo L (2006) In Vivo Restoration of Physiological Levels of Truncated TrkB.T1 Receptor Rescues Neuronal Cell Death in a Trisomic Mouse Model. Neuron 51:21–28.

Dunwiddie T V, Masino SA (2001) The role and regulation of adenosine in the central nervous system. Annual review of neuroscience 24:31–55.

During MJ, Spencer DD (1992) Adenosine: a potential mediator of seizure arrest and postictal refractoriness. Ann Neurol 32:618–624.

Ebert DH, Gabel HW, Robinson ND, Kastan NR, Hu LS, Cohen S, Navarro AJ, Lyst MJ, Ekiert R, Bird AP, Greenberg ME (2013) Activity-dependent phosphorylation of MeCP2 threonine 308 regulates interaction with NCoR. Nature 2013 499:7458 499:341–345.

Effendi WI, Nagano T, Kobayashi K, Nishimura Y (2020) Focusing on Adenosine Receptors as a Potential Targeted Therapy in Human Diseases. Cells 9:785.

Ehrhart F, Sangani NB, Curfs LMG (2018) Current developments in the genetics of Rett and Rett-like syndrome. Current Opinion in Psychiatry 31:103–108.

Eide FF, Vining ER, Eide BL, Zang K, Wang XY, Reichardt LF (1996) Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. Journal of Neuroscience 16:3123–3129.

El-Khoury R, Panayotis N, Matagne V, Ghata A, Villard L, Roux J-C (2014) GABA and glutamate pathways are spatially and developmentally affected in the brain of Mecp2-deficient mice. PLoS One 9:e92169.

Essawy SS, Elbaz AA (2013) Role of adenosine receptors in the anti-nociceptive effects of allopurinol in mice. Eur Rev Med Pharmacol Sci 17:1857–1863.

Etherington L-AV, Patterson GE, Meechan L, Boison D, Irving AJ, Dale N, Frenguelli BG (2009) Astrocytic adenosine kinase regulates basal synaptic adenosine levels and seizure activity but not activity-dependent adenosine release in the hippocampus. Neuropharmacology 56:429–437.

Feldman D, Banerjee A, Sur M (2016) Developmental Dynamics of Rett Syndrome. Neural Plasticity 2016.

Fenner ME, Achim CL, Fenner BM (2014) Expression of full-length and truncated trkB in human striatum and substantia nigra neurons: Implications for Parkinson's disease. Journal of Molecular Histology 45:349–361.

Ferré S, Fredholm BB, Morelli M, Popoli P, Fuxe K (1997) Adenosine-dopamine receptor-receptor interactions as an integrative mechanism in the basal ganglia. Trends in neurosciences 20:482–487.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B (1996) Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381:706–709.

Fink JS, Weaver DR, Rivkees SA, Peterfreund RA, Pollack AE, Adler EM, Reppert SM (1992) Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D2 dopamine receptors in rat striatum. Brain Res Mol Brain Res 14:186–195.

Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME (1997) CREB: A major mediator of neuronal neurotrophin responses. Neuron 19:1031–1047.

Foga IO, Geiger JD, Parkinson FE (1996) Nucleoside transporter-mediated uptake and release of [3H]L-adenosine in DDT1 MF-2 smooth muscle cells. European Journal of Pharmacology 318:455–460.

Fontinha BM, Diógenes MJ, Ribeiro J a., Sebastião a. M (2008) Enhancement of long-term potentiation by brain-derived neurotrophic factor requires adenosine A2A receptor activation by endogenous adenosine. Neuropharmacology 54:924–933.

Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, Williams M (1994) VI. Nomenclature and classification of purinoceptors. Pharmacological Reviews 46:143–156

Fredholm BB, Chen J-F, Cunha RA, Svenningsson P, Vaugeois J-M (2005) Adenosine and brain function. International review of neurobiology 63:191–270.

Fredholm BB, Chern Y, Franco R, Sitkovsky M (2007) Aspects of the general biology of adenosine A2A signaling. Progress in Neurobiology 83:263–276.

Fredholm BB, IJzerman a P, Jacobson K a, Klotz KN, Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacological reviews 53:527–552.

Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Müller CE (2011) International union of basic and clinical pharmacology. LXXXI. Nomenclature and classification of adenosine receptors - An update. Pharmacological Reviews 63:1–34.

Fukuda M, Suzuki Y, Hino H, Kuzume K, Morimoto T, Ishii E (2010) Adenosine A1 receptor blockage mediates theophylline-associated seizures. Epilepsia 51:483–487.

Gadalla KKE, Bailey MES, Spike RC, Ross PD, Woodard KT, Kalburgi SN, Bachaboina L, Deng J V, West AE, Samulski RJ, Gray SJ, Cobb SR (2013) Improved survival and reduced phenotypic severity following AAV9/MECP2 gene transfer to neonatal and juvenile male Mecp2 knockout mice. Molecular therapy : the journal of the American Society of Gene Therapy 21.

Garg SK, Lioy DT, Cheval H, McGann JC, Bissonnette JM, Murtha MJ, Foust KD, Kaspar BK, Bird A, Mandel G (2013) Systemic delivery of MeCP2 rescues behavioral and cellular deficits in female mouse models of Rett syndrome. The Journal of neuroscience : the official journal of the Society for Neuroscience 33:13612–13620.

Gessi S, Merighi S, Fazzi D, Stefanelli A, Varani K, Borea PA (2011) Adenosine receptor targeting in health and disease. Expert Opinion on Investigational Drugs 20:1591–1609.

Ghosh A, Carnahan J, Greenberg ME (1994) Requirement for BDNF in activity-dependent survival of cortical neurons. Science 263:1618–1623.

Girard M, Couvert P, Carrié a, Tardieu M, Chelly J, Beldjord C, Bienvenu T (2001) Parental origin of de novo MECP2 mutations in Rett syndrome. European journal of human genetics : EJHG 9:231–236.

Girardi E, Auzmendi J, Charó N, Gori MB, Castro M (2010) 3-mercaptopropionic acid-induced seizures decrease NR2B expression in Purkinje cells: cyclopentyladenosine effect. Cell Mol Neurobiol 30:985–990.

Girardi ES, Canitrot J, Antonelli M, González NN, Coirini H (2007) Differential expression of cerebellar metabotropic glutamate receptors mGLUR2/3 and mGLUR4a after the administration of a convulsant drug and the adenosine analogue cyclopentyladenosine. Neurochem Res 32:1120–1128.

Glaze DG, Percy AK, Skinner S, Motil KJ, Neul JL, Barrish JO, Lane JB, Geerts SP, Annese F, Graham J, McNair L, Lee H-S (2010) Epilepsy and the natural history of Rett syndrome. Neurology 74:909.

Goffin D, Allen M, Zhang L, Amorim M, Wang I-TJ, Reyes A-RS, Mercado-Berton A, Ong C, Cohen S, Hu L, Blendy JA, Carlson GC, Siegel SJ, Greenberg ME, Zhou Z (2011) Rett syndrome mutation MeCP2 T158A disrupts DNA binding, protein stability and ERP responses. Nat Neurosci 15:274–283.

Gogliotti RG, Senter RK, Fisher NM, Adams J, Zamorano R, Walker AG, Blobaum AL, Engers DW, Hopkins CR, Daniels JS, Jones CK, Lindsley CW, Xiang Z, Conn PJ, Niswender CM (2017) mGlu7 potentiation rescues cognitive, social, and respiratory phenotypes in a mouse model of Rett syndrome. Science Translational Medicine 9.

Gogliotti RG, Senter RK, Rook JM, Ghoshal A, Zamorano R, Malosh C, Stauffer SR, Bridges TM, Bartolome JM, Daniels JS, Jones CK, Lindsley CW, Conn PJ, Niswender CM (2016) mGlu5 positive allosteric modulation normalizes synaptic plasticity defects and motor phenotypes in a mouse model of Rett syndrome. Hum Mol Genet 25:1990–2004.

Goldmann T, Overlack N, Möller F, Belakhov V, Wyk M van, Baasov T, Wolfrum U, Nagel-Wolfrum K (2012) A comparative evaluation of NB30, NB54 and PTC124 in translational read-through efficacy for treatment of an USH1C nonsense mutation. EMBO Molecular Medicine 4:1186.

Golembiowska K, White TD, Sawynok J (1996) Adenosine kinase inhibitors augment release of adenosine from spinal cord slices. European Journal of Pharmacology 307:157–162.

Gomathi M, Padmapriya S, Balachandar V (2020) Drug Studies on Rett Syndrome: From Bench to Bedside. Journal of Autism and Developmental Disorders 2020 50:8 50:2740–2764.

Gomes JI, Farinha-Ferreira M, Rei N, Gonçalves-Ribeiro J, Ribeiro JA, Sebastião AM, Vaz SH (2021) Of adenosine and the blues: The adenosinergic system in the pathophysiology and treatment of major depressive disorder. Pharmacological Research 163:1043–6618.

Gomes C V, Kaster MP, Tomé AR, Agostinho PM, Cunha R a (2011) Adenosine receptors and brain diseases: neuroprotection and neurodegeneration. Biochimica et biophysica acta 1808:1380–1399.

Gouder N, Fritschy J-M, Boison D (2003) Seizure suppression by adenosine A1 receptor activation in a mouse model of pharmacoresistant epilepsy. Epilepsia 44:877–885.

Gouder N, Scheurer L, Fritschy J-M, Boison D (2004) Overexpression of adenosine kinase in epileptic hippocampus contributes to epileptogenesis. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:692–701.

Großer E, Hirt U, Janc OA, Menzfeld C, Fischer M, Kempkes B, Vogelgesang S, Manzke TU, Opitz L, Salinas-Riester G, Müller M (2012) Oxidative burden and mitochondrial dysfunction in a mouse model of Rett syndrome. Neurobiology of Disease 48:102–114.

Guerrini R, Parrini E (2012) Epilepsy in Rett syndrome, and CDKL5- and FOXG1-gene-related encephalopathies. Epilepsia 53:2067–2078.

Gulmez Karaca K, Brito DVC, Oliveira AMM (2019) MeCP2: A Critical Regulator of Chromatin in Neurodevelopment and Adult Brain Function. Int J Mol Sci 20:4577.

Guo W, Tsujimura K, Otsuka I M, Irie K, Igarashi K, Nakashima K, Zhao X (2014) VPA alleviates neurological deficits and restores gene expression in a mouse model of Rett syndrome. PLoS One 9:e100215.

Güttinger M, Padrun V, Pralong WF, Boison D (2005) Seizure suppression and lack of adenosine A1 receptor desensitization after focal long-term delivery of adenosine by encapsulated myoblasts. Exp Neurol 193:53–64.

Guy J, Gan J, Selfridge J, Cobb S, Bird A (2007) Reversal of neurological defects in a mouse model of Rett syndrome. Science (New York, NY) 315:1143–1147.

Guy J, Hendrich B, Holmes M, Martin JE, Bird a (2001) A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nature genetics 27:322–326.

Hagberg B, Aicardi J, Dias K, Ramos O (1983) A progressive syndrome of autism, dementia, ataxia, and loss of purposeful hand use in girls: Rett's syndrome: report of 35 cases. Annals of neurology 14:471–479.

Hannan AJ (2014) Review: Environmental enrichment and brain repair: harnessing the therapeutic effects of cognitive stimulation and physical activity to enhance experience-dependent plasticity. Neuropathology and Applied Neurobiology 40:13–25.

Hanson IM, Seawright A, van Heyningen V (1992) The human BDNF gene maps between FSHB and HVBS1 at the boundary of 11p13-p14. Genomics 13:1331–1333.

Heidarianpour A, Sadeghian E, Mirnajafi-Zadeh J, Fathollahi Y, Mohammad-Zadeh M (2006) Anticonvulsant effects of N^6-cyclohexyladenosine microinjected into the CA1 region of the hippocampus on entorhinal cortex-kindled seizures in rats. Epileptic Disorders 8:259–266.

Hendrich B, Tweedie S (2003) The methyl-CpG binding domain and the evolving role of DNA methylation in animals. Trends in Genetics 19:269–277.

Herrera JA, Ward CS, Pitcher MR, Percy AK, Skinner S, Kaufmann WE, Glaze DG, Wehrens XHT, Neul JL (2015) Treatment of cardiac arrhythmias in a mouse model of Rett syndrome with Na+-channel-blocking antiepileptic drugs. Dis Model Mech 8:363–371.

Hite KC, Adams VH, Hansen JC (2009) Recent advances in MeCP2 structure and function. Biochemistry and cell biology = Biochimie et biologie cellulaire 87:219.

Hoffbuhr KC, Moses LM, Jerdonek MA, Naidu S, Hoffman EP (2002) Associations between MeCP2 mutations, X-chromosome inactivation, and phenotype. Mental Retardation and Developmental Disabilities Research Reviews 8:99–105.

Holt LM, Hernandez RD, Pacheco NL, Torres Ceja B, Hossain M, Olsen ML (2019) Astrocyte morphogenesis is dependent on BDNF signaling via astrocytic TrkB.T1. Elife 8:e44667.

Hötting K, Röder B (2013) Beneficial effects of physical exercise on neuroplasticity and cognition. Neuroscience and Biobehavioral Reviews 37:2243–2257.

Hötting K, Schickert N, Kaiser J, Röder B, Schmidt-Kassow M (2016) The effects of acute physical exercise on memory, peripheral BDNF, and cortisol in young adults. Neural Plasticity 2016.

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annual review of biochemistry 72:609–642.

Huang NK, Lin YW, Huang CL, Messing RO, Chern Y (2001) Activation of protein kinase A and atypical protein kinase C by A(2A) adenosine receptors antagonizes apoptosis due to serum deprivation in PC12 cells. J Biol Chem 276:13838–13846.

Ip JPK, Mellios N, Sur M (2018) Rett syndrome: Insights into genetic, molecular and circuit mechanisms. Nature Reviews Neuroscience.

Ishii T, Makita Y, Ogawa A, Amamiya S, Yamamoto M, Miyamoto A, Oki J (2001) The role of different X-inactivation pattern on the variable clinical phenotype with Rett syndrome. In: Brain and Development. Brain Dev.

Jackson EK, Kotermanski SE, Menshikova E V., Dubey RK, Jackson TC, Kochanek PM (2017) Adenosine production by brain cells. Journal of Neurochemistry 141:676–693.

Jacobson K a., Gao Z-G (2006) Adenosine receptors as therapeutic targets. Nature review Drug discovery 5:247–264.

Jacobson KA, Klutz AM, Tosh DK, Ivanov AA, Preti D, Baraldi PG (2009) Medicinal chemistry of the A3 adenosine receptor: Agonists, antagonists, and receptor engineering. Handbook of Experimental Pharmacology 193:123–159.

Jacobson KA, Tosh DK, Jain S, Gao Z-G (2019) Historical and Current Adenosine Receptor Agonists in Preclinical and Clinical Development. Frontiers in Cellular Neuroscience 13:124.

Jang J, Quan Z, Yum YJ, Song HS, Paek S, Kang HC (2014) Induced pluripotent stem cells for modeling of pediatric neurological disorders. Biotechnology Journal 9:871–891.

Jarvis MF (2019) Therapeutic potential of adenosine kinase inhibition—Revisited. Pharmacol Res Perspect 00:506.

Jarvis MF, Schulz R, Hutchison AJ, Do UH, Sills MA, Williams M (1989) [3H]CGS 21680, a selective A2 adenosine receptor agonist directly labels A2 receptors in rat brain. The Journal of pharmacology and experimental therapeutics 251:888–893.

Jeon SJ, Rhee SY, Ryu JH, Cheong JH, Kwon K, Yang S-I, Park SH, Lee J, Kim HY, Han S-H, Ko KH, Shin CY (2011) Activation of adenosine A2A receptor up-regulates BDNF expression in rat primary cortical neurons. Neurochem Res 36:2259–2269.

Jerónimo-Santos A, Batalha VL, Müller CE, Baqi Y, Sebastião AM, Lopes L V., Diógenes MJ (2014) Impact of in vivo chronic blockade of adenosine A2A receptors on the BDNF-mediated facilitation of LTP. Neuropharmacology 83:99–106.

Jerónimo-Santos A, Vaz SH, Parreira S, Rapaz-Lérias S, Caetano AP, Scherrer VB, Castrén E, Valente CA, Blum D, Sebastião AM, Diógenes MJ (2015) Dysregulation of TrkB Receptors and BDNF function by amyloid-β peptide is mediated by calpain. Cerebral Cortex 25:3107–3121.

Jian L, Nagarajan L, de Klerk N, Ravine D, Bower C, Anderson A, Williamson S, Christodoulou J, Leonard H (2006) Predictors of seizure onset in Rett syndrome. Journal of Pediatrics 149:542-547.e3.

Jin J, Bao X, Wang H, Pan H, Zhang Y, Wu X (2008) RNAi-induced down-regulation of Mecp2 expression in the rat brain. International Journal of Developmental Neuroscience 26:457–465.

Jin X, Zhong W, Jiang C (2013) Time-dependent modulation of GABA(A)-ergic synaptic transmission by allopregnanolone in locus coeruleus neurons of Mecp2-null mice. Am J Physiol Cell Physiol 305:C1151-1160.

Johnson RA, Lam M, Punzo AM, Li H, Lin BR, Ye K, Mitchell GS, Chang Q (2012) 7,8-dihydroxyflavone exhibits therapeutic efficacy in a mouse model of Rett syndrome. Journal of applied physiology (Bethesda, Md : 1985) 112:704–710.

Kaczmarek E, Koziak K, Sévigny J, Siegel JB, Anrather J, Beaudoin AR, Bach FH, Robson SC (1996) Identification and characterization of CD39/vascular ATP diphosphohydrolase. Journal of Biological Chemistry 271:33116–33122.

Kalla R V., Zablocki J, Tabrizi MA, Baraldi PG (2009) Recent developments in A2B adenosine receptor ligands. Handbook of Experimental Pharmacology 193:99–122.

Katz DM et al. (2012) Preclinical research in Rett syndrome: setting the foundation for translational success. Disease Models & Mechanisms 5:733–745.

Katz DM, Bird A, Coenraads M, Gray SJ, Menon DU, Philpot BD, Tarquinio DC (2016) Rett Syndrome: Crossing the Threshold to Clinical Translation. Trends in Neurosciences 39:100–113.

Kishi N, Macklis JD (2005) Dissecting MECP2 function in the central nervous system. J Child Neurol 20:753–759.

Klein R, Conway D, Parada LF, Barbacid M (1990) The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell 61:647–656.

Klose RJ, Sarraf S a., Schmiedeberg L, McDermott SM, Stancheva I, Bird AP (2005) DNA binding selectivity of MeCP2 due to a requirement for A/T sequences adjacent to methyl-CpG. Molecular Cell 19:667–678.

Klotz KN (2000) Adenosine receptors and their ligands. Naunyn-Schmiedeberg's Archives of Pharmacology 362:382–391.

Kolbeck R, Bartke I, Eberle W, Barde Y a (1999) Brain-derived neurotrophic factor levels in the nervous system of wild-type and neurotrophin gene mutant mice. Journal of neurochemistry 72:1930–1938.

Kondo M, Gray LJ, Pelka GJ, Christodoulou J, Tam PPL, Hannan AJ (2008) Environmental enrichment ameliorates a motor coordination deficit in a mouse model of Rett syndrome--Mecp2 gene dosage effects and BDNF expression. Eur J Neurosci 27:3342–3350.

Köse M, Schiedel AC, Bauer AA, Poschenrieder H, Burbiel JC, Akkinepally RR, Stachel HD, Müller CE (2016) Focused screening to identify new adenosine kinase inhibitors. Bioorganic and Medicinal Chemistry 24:5127–5133.

Kowaluk EA, Jarvis MF (2000) Therapeutic potential of adenosine kinase inhibitors. Expert opinion on investigational drugs 9:551–564.

Krajnc N (2015) Management of epilepsy in patients with Rett syndrome: perspectives and considerations. Therapeutics and Clinical Risk Management 11:925.

Kriaucionis S, Paterson A, Curtis J, Guy J, MacLeod N, Bird A (2006) Gene Expression Analysis Exposes Mitochondrial Abnormalities in a Mouse Model of Rett Syndrome. Molecular and Cellular Biology 26:5033–5042.

Krishnan N, K K, Connors C, Choy M, Page R, Peti W, Aelst L, Shea S, Tonks N (2015) PTP1B: a new therapeutic target for Rett syndrome. Journal of Clinical Investigation 125:2931–2934.

Kroll K, Decking UKM, Dreikorn K, Schrader J (1993) Rapid turnover of the AMP-adenosine metabolic cycle in the guinea pig heart. Circulation Research 73:846–856.

Kumar A, Jaryal A, Gulati S, Chakrabarty B, Singh A, Deepak KK, Pandey RM, Gupta N, Sapra S, Kabra M, Khajuria R (2017) Cardiovascular Autonomic Dysfunction in Children and Adolescents With Rett Syndrome. Pediatric Neurology 70:61–66.

Kyle SM, Vashi N, Justice MJ (2018) Rett syndrome: A neurological disorder with metabolic components. Open Biology 8.

Lancaster MA, Renner M, Martin CA, Wenzel D, Bicknell LS, Hurles ME, Homfray T, Penninger JM, Jackson AP, Knoblich JA (2013) Cerebral organoids model human brain development and microcephaly. Nature 501:373–379.

Landucci E et al. (2018) iPSC-derived neurons profiling reveals GABAergic circuit disruption and acetylated α-tubulin defect which improves after iHDAC6 treatment in Rett syndrome. Exp Cell Res 368:225–235.

Latini S, Pazzagli M, Pepeu G, Pedata F (1996) A2 adenosine receptors: their presence and neuromodulatory role in the central nervous system. Gen Pharmacol 27:925–933.

Latini S, Pedata F (2001) Adenosine in the central nervous system: Release mechanisms and extracellular concentrations. Journal of Neurochemistry 79:463–484.

Lee FS, Chao M V (2001) Activation of Trk neurotrophin receptors in the absence of neurotrophins. Proceedings of the National Academy of Sciences of the United States of America 98:3555–3560.

Lee SS, Wan M, Francke U (2001) Spectrum of MECP2 mutations in Rett syndrome. Brain Dev 23 Suppl 1:S138-143.

Leonard H, Cobb S, Downs J (2016) Clinical and biological progress over 50 years in Rett syndrome. Nature Reviews Neurology 13:37–51.

Lessmann V, Gottmann K, Malcangio M (2003) Neurotrophin secretion: current facts and future prospects. Prog Neurobiol 69:341–374.

Leuzzi V, Nardecchia F, Pons R, Galosi S (2020) Parkinsonism in children: Clinical classification and etiological spectrum. Parkinsonism and Related Disorders 82.

Lewis JD, Meehan RR, Henzel WJ, Maurer-Fogy I, Jeppesen P, Klein F, Bird a (1992) Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. Cell 69:905–914.

Li T, Ren G, Lusardi T, Wilz A, Lan JQ, Iwasato T, Itohara S, Simon RP, Boison D (2008) Adenosine kinase is a target for the prediction and prevention of epileptogenesis in mice. The Journal of clinical investigation 118:571–582.

Li W, Bellot-Saez A, Phillips ML, Yang T, Longo FM, Pozzo-Miller L (2017) A small-molecule TrkB ligand restores hippocampal synaptic plasticity and object location memory in Rett syndrome mice. Disease Models & Mechanisms 10:837–845.

Li W, Calfa G, Larimore J, Pozzo-Miller L (2012) Activity-dependent BDNF release and TRPC signaling is impaired in hippocampal neurons of Mecp2 mutant mice. Proceedings of the National Academy of Sciences 109:17087–17092.

Li W, Pozzo-Miller L (2014) BDNF deregulation in Rett syndrome. Neuropharmacology 76:737–746.

Li Y, Wang H, Muffat J, Cheng a W, Orlando D a, Loven J, Kwok SM, Feldman D a, Bateup HS, Gao Q, Hockemeyer D, Mitalipova M, Lewis C a, Vander Heiden MG, Sur M, Young R a, Jaenisch R (2013) Global transcriptional

and translational repression in human-embryonic-stem-cell-derived Rett syndrome neurons. Cell Stem Cell 13:446–458.

Liu XJ, White TD, Sawynok J (2000) Potentiation of formalin-evoked adenosine release by an adenosine kinase inhibitor and an adenosine deaminase inhibitor in the rat hind paw: A microdialysis study. European Journal of Pharmacology 408:143–152.

Liu YJ, Chen J, Li | Xun, Zhou X, Hu Y-M, Chu S-F, Peng Y, Chen N-H (2019) Research progress on adenosine in central nervous system diseases.

Liyanage VRB, Rastegar M (2014) Rett syndrome and MeCP2. NeuroMolecular Medicine 16:231–264.

Lombardi LM, Baker SA, Zoghbi HY (2015) MECP2 disorders: From the clinic to mice and back. Journal of Clinical Investigation 125:2914–2923.

Lonetti G, Angelucci A, Morando L, Boggio EM, Giustetto M, Pizzorusso T (2010) Early environmental enrichment moderates the behavioral and synaptic phenotype of MeCP2 null mice. Biol Psychiatry 67:657–665.

Lu B, Nagappan G, Guan X, Nathan PJ, Wren P (2013) BDNF-based synaptic repair as a disease-modifying strategy for neurodegenerative diseases. Nature Reviews Neuroscience 14:401–416.

Łuszczki JJ, Kozicka M, Swiader MJ, Czuczwar SJ (2005) 2-Chloro-N6-cyclopentyladenosine enhances the anticonvulsant action of carbamazepine in the mouse maximal electroshock-induced seizure model. Pharmacol Rep 57:787–794.

Lynch G, Gall CM (2006) Ampakines and the threefold path to cognitive enhancement. Trends Neurosci 29:554–562.

Lyst MJ, Ekiert R, Ebert DH, Merusi C, Nowak J, Selfridge J, Guy J, Kastan NR, Robinson ND, de Lima Alves F, Rappsilber J, Greenberg ME, Bird A (2013) Rett syndrome mutations abolish the interaction of MeCP2 with the NCoR/SMRT co-repressor. Nat Neurosci 16:898–902.

Lyst MJ, Ekiert R, Guy J, Merusi C, De Sousa D, Bird A, Selfridge J, Koerner M V (2018) Affinity for DNA Contributes to NLS Independent Nuclear Localization of MeCP2 Graphical Abstract Highlights d Nuclear localization of MeCP2 does not require its NLS d DNA binding by MeCP2 contributes to its NLS-independent nuclear localization d MeCP2 NLS mutation does not affect pathology in a mouse model of Rett syndrome Cell Reports Affinity for DNA Contributes to NLS Independent Nuclear Localization of MeCP2.

Madara JL, Patapoff TW, Gillece-Castro B, Colgan SP, Parkos CA, Delp C, Mrsny RJ (1993) 5′-adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. Journal of Clinical Investigation 91:2320–2325.

Mann JS, Renwick AG, Holgate ST (1986) Release of adenosine and its metabolites from activated human leucocytes. Clinical Science 70:461–468.

Marchetto MC, Carromeu C, Acab A, Yu D, Yeo Y, Mu Y, Chen G, Gage FH, Muotri AR (2010) A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. Cell 143:527–539.

Mari F et al. (2005) CDKL5 belongs to the same molecular pathway of MeCP2 and it is responsible for the early-onset seizure variant of Rett syndrome. Human Molecular Genetics 14:1935–1946.

Marquardt DL, Gruber HE, Wasserman SI (1984) Adenosine release from stimulated mast cells. Proceedings of the National Academy of Sciences of the United States of America 81:6192–6196.

Masino SA, Kawamura M, Jr, Ruskin DN (2014) Adenosine Receptors and Epilepsy: Current Evidence and Future Potential. International review of neurobiology 119:233.

Masino SA, Li T, Theofilas P, Sandau US, Ruskin DN, Fredholm BB, Geiger JD, Aronica E, Boison D (2011) A ketogenic diet suppresses seizures in mice through adenosine A_1 receptors. The Journal of clinical investigation 121:2679–2683.

Matijevic T, Knezevic J, Slavica M, Pavelic J (2008) Rett syndrome: From the gene to the disease. European Neurology 61:3–10.

McGaraughty S, Cowart M, Jarvis M, Berman R (2005) Anticonvulsant and Antinociceptive Actions of Novel Adenosine Kinase Inhibitors. Current Topics in Medicinal Chemistry 5:43–58.

McGowan H, Pang ZP (2015) Regulatory functions and pathological relevance of the MECP2 3'UTR in the central nervous system. Cell Regeneration 4:4:9.

Mellios N, Feldman DA, Sheridan SD, Ip JPK, Kwok S, Amoah SK, Rosen B, Rodriguez BA, Crawford B, Swaminathan R, Chou S, Li Y, Ziats M, Ernst C, Jaenisch R, Haggarty SJ, Sur M (2018) MeCP2-regulated miRNAs control early human neurogenesis through differential effects on ERK and AKT signaling. Molecular Psychiatry 23:1051–1065.

Mellios N, Woodson J, Garcia RI, Crawford B, Sharma J, Sheridan SD, Haggarty SJ, Sur M (2014) β2-Adrenergic receptor agonist ameliorates phenotypes and corrects microRNA-mediated IGF1 deficits in a mouse model of Rett syndrome. Proc Natl Acad Sci U S A 111:9947–9952.

Middlemas DS, Lindberg RA, Hunter T (1991) trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Molecular and Cellular Biology 11:143–153.

Mierau SB, Patrizi A, Hensch TK, Fagiolini M (2016) Cell-Specific Regulation of N-Methyl-D-Aspartate Receptor Maturation by Mecp2 in Cortical Circuits. Biological psychiatry 79:746–754.

Minichiello L, Korte M, Wolfer D, Kühn R, Unsicker K, Cestari V, Rossi-Arnaud C, Lipp HP, Bonhoeffer T, Klein R (1999) Essential role for TrkB receptors in hippocampus-mediated learning. Neuron 24:401–414.

Miranda-Lourenço C, Duarte ST, Palminha C, Gaspar C, Rodrigues TM, Magalhães-Cardosof T, Rei N, Colino-Oliveira M, Gomes R, Ferreira S, Rosa J, Xapelli S, Armstrong J, García-Cazorla À, Correia-de-Sá P, Sebastião AM, Diógenes MJ (2020a) Impairment of adenosinergic system in Rett syndrome: Novel therapeutic target to boost BDNF signalling. Neurobiology of Disease 145

Miranda-Lourenço C, Ribeiro-Rodrigues L, Fonseca-Gomes J, Tanqueiro SR, Belo RF, Ferreira CB, Rei N, Ferreira-Manso M, de Almeida-Borlido C, Costa-Coelho T, Freitas CF, Zavalko S, Mouro FM, Sebastião AM, Xapelli S, Rodrigues TM, Diógenes MJ (2020b) Challenges of BDNF-based therapies: From common to rare diseases. Pharmacological Research 162:105281.

Mizoguchi Y, Monji A, A. Kato T, Horikawa H, Seki Y, Kasai M, Kanba S, Yamada S (2011) Possible Role of BDNF-Induced Microglial Intracellular Ca^{2+} Elevation in the Pathophysiology of Neuropsychiatric Disorders. Mini-Reviews in Medicinal Chemistry 11:575–581.

Mnatzakanian GN, Lohi H, Munteanu I, Alfred SE, Yamada T, MacLeod PJ, Jones JR, Scherer SW, Carolyn Schanen N, Friez MJ, Vincent JB, Minassian BA (2004) B R I E F C O M M U N I C A T I O N S A previously unidentified MECP2 open reading frame defines a new protein isoform relevant to Rett syndrome. NATURE GENETICS 36:339.

Moretti P, Levenson JM, Battaglia F, Atkinson R, Teague R, Antalffy B, Armstrong D, Arancio O, Sweatt JD, Zoghbi HY (2006) Learning and memory and synaptic plasticity are impaired in a mouse model of Rett syndrome. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:319–327.

Mowla SJ, Pareek S, Farhadi HF, Petrecca K, Fawcett JP, Seidah NG, Morris SJ, Sossin WS, Murphy RA (1999) Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. J Neurosci 19:2069–2080.

Naegelin Y, Kuhle J, Schädelin S, Datta AN, Magon S, Amann M, Barro C, Ramelli GP, Heesom K, Barde Y-A, Weber P, Kappos L (2021) Fingolimod in children with Rett syndrome: the FINGORETT study. Orphanet Journal of Rare Diseases 16.

Nagai K, Miyake K, Kubota T (2005) A transcriptional repressor MeCP2 causing Rett syndrome is expressed in embryonic non-neuronal cells and controls their growth. Developmental Brain Research 157:103–106.

Nance E, Kambhampati SP, Smith ES, Zhang Z, Zhang F, Singh S, Johnston MV, Kannan RM, Blue ME, Kannan S (2017) Dendrimer-mediated delivery of N-acetyl cysteine to microglia in a mouse model of Rett syndrome. J Neuroinflammation 14:252.

Nelson ED, Kavalali ET, Monteggia LM (2006) MeCP2-Dependent Transcriptional Repression Regulates Excitatory Neurotransmission. Current Biology 16:710–716.

Neul JL, Fang P, Barrish J, Lane J, Caeg EB, Smith EO, Zoghbi H, Percy A, Glaze DG (2008) Specific mutations in Methyl-CpG-Binding Protein 2 confer different severity in Rett syndrome. Neurology 70:1313–1321.

Neul JL, Kaufmann WE, Glaze DG, Clarke AJ, Leonard H, Bailey MES, Carolyn N, Zappella M, Renieri A (2011) Rett syndrome: revised diagnostic criteria and nomenclature. 68:944–950.

Neul JL, Lane JB, Lee H-S, Geerts S, Barrish JO, Annese F, Baggett LM, Barnes K, Skinner S a, Motil KJ, Glaze DG, Kaufmann WE, Percy AK (2014) Developmental delay in Rett syndrome: data from the natural history study. Journal of neurodevelopmental disorders 6:20.

Newby AC, Holmquist CA, Illingworth J, Pearson JD (1983) The control of adenosine concentration in polymorphonuclear leucocytes, cultured heart cells and isolated perfused heart from the rat. Biochemical Journal 214:317–323.

Nissenkorn A, Kidon M, Ben-Zeev B (2017) A Potential Life-Threatening Reaction to Glatiramer Acetate in Rett Syndrome. Pediatr Neurol 68:40–43.

Nomura Y (2005) Early behavior characteristics and sleep disturbance in Rett syndrome. In: Brain and Development, pp S35–S42.

Ogier M, Wang H, Hong E, Wang Q, Greenberg ME, Katz DM (2007) Brain-derived neurotrophic factor expression and respiratory function improve after ampakine treatment in a mouse model of Rett syndrome. J Neurosci 27:10912–10917.

Olson CO, Zachariah RM, Ezeonwuka CD, Liyanage VRB, Rastegar M (2014) Brain region-specific expression of MeCP2 isoforms correlates with DNA methylation within Mecp2 regulatory elements. PLoS ONE 9:90645.

Operto FF, Mazza R, Pastorino GMG, Verrotti A, Coppola G (2019) Epilepsy and genetic in Rett syndrome: A review. Brain and Behavior 9.

Paiva Miranda J, Lindholm D, Margarida Diogo M, Rita Gomes A, Fernandes TG, Vaz SH, Silva TP, Bekman EP, Xapelli S, Duarte S, Ghazvini M, Gribnau J, Muotri AR, Trujillo CA, Sebastião AM, S Cabral JM (2020) Modeling Rett Syndrome With Human Patient-Specific Forebrain Organoids.

Pak MA, Haas HL, Decking UK, Schrader J (1994) Inhibition of adenosine kinase increases endogenous adenosine and depresses neuronal activity in hippocampal slices. Neuropharmacology 33:1049–1053.

Pang PT, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung W-H, Hempstead BL, Lu B (2004) Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science (New York, NY) 306:487–491.

Panighini A, Duranti E, Santini F, Maffei M, Pizzorusso T, Funel N, Taddei S, Bernardini N, Ippolito C, Virdis A, Costa M (2013) Vascular Dysfunction in a Mouse Model of Rett Syndrome and Effects of Curcumin Treatment. PLOS ONE 8:e64863.

Park MJ, Aja S, Li Q, Degano AL, Penati J, Zhuo J, Roe CR, Ronnett GV (2014) Anaplerotic triheptanoin diet enhances mitochondrial substrate use to remodel the metabolome and improve lifespan, motor function, and sociability in MeCP2-null mice. PLoS One 9:e109527.

Paton DM (2020) Istradefylline: Adenosine A2A receptor antagonist to reduce “OFF” time in Parkinson’s disease. Drugs of Today 56:125–134.

Patrizi A, Picard N, Simon AJ, Gunner G, Centofante E, Andrews NA, Fagiolini M (2016) Chronic Administration of the N-Methyl-D-Aspartate Receptor Antagonist Ketamine Improves Rett Syndrome Phenotype. Biological psychiatry 79:755–764.

Pattarawarapan M, Burgess K (2003) Molecular Basis of Neurotrophin-Receptor Interactions. Journal of Medicinal Chemistry 46:5277–5291.

Peleli M, Fredholm BB, Sobrevia L, Carlström M (2017) Pharmacological targeting of adenosine receptor signaling. Molecular Aspects of Medicine 55:4–8.

Pelka GJ, Watson CM, Christodoulou J, Tam PPL (2005) Distinct expression profiles of Mecp2 transcripts with different lengths of 3’UTR in the brain and visceral organs during mouse development. Genomics 85:441–452.

Percy AK, Lane J, Annese F, Warren H, Skinner SA, Neul JL (2018) When Rett syndrome is due to genes other than MECP2. Translational Science of Rare Diseases 3:49–53.

Percy AK, Neul JL, Glaze DG, Motil KJ, Skinner SA, Khwaja O, Lee HS, Lane JB, Barrish JO, Annese F, McNair L, Graham J, Barnes K (2010) Rett syndrome diagnostic criteria: Lessons from the Natural History Study. Annals of Neurology 68:951–955.

Pierce KD, Furlong TJ, Selbie LA, Shine J (1992) Molecular cloning and expression of an adenosine A2b receptor from human brain. Biochemical and Biophysical Research Communications 187:86–93.

Pietri T, Roman AC, Guyon N, Romano SA, Washbourne P, Moens CB, de Polavieja GG, Sumbre G (2013) The first mecp2-null zebrafish model shows altered motor behaviors. Frontiers in Neural Circuits 7.

Piirainen H, Ashok Y, Nanekar RT, Jaakola VP (2011) Structural features of adenosine receptors: From crystal to function. Biochimica et Biophysica Acta - Biomembranes 1808:1233–1244.

Pitcher MR, Herrera JA, Buffington SA, Kochukov MY, Merritt JK, Fisher AR, Schanen NC, Costa-Mattioli M, Neul JL (2015) Rett syndrome like phenotypes in the R255X Mecp2 mutant mouse are rescued by MECP2 transgene. Human Molecular Genetics 24:2662.

Pitcher MR, Ward CS, Arvide EM, Chapleau CA, Pozzo-Miller L, Hoeflich A, Sivaramakrishnan M, Saenger S, Metzger F, Neul JL (2013) Insulinotropic treatments exacerbate metabolic syndrome in mice lacking MeCP2 function. Human Molecular Genetics 22:2626.

Poduslo JF, Curran GL (1996) Permeability at the blood-brain and blood-nerve barriers of the neurotrophic factors: NGF, CNTF, NT-3, BDNF. Molecular Brain Research 36:280–286.

Polosa R, Blackburn MR (2009) Adenosine receptors as targets for therapeutic intervention in asthma and chronic obstructive pulmonary disease. Trends in Pharmacological Sciences 30:528–535.

Porkka-Heiskanen T (2009) Adenosine in sleep and wakefulness.

Powell CM, Castillo PE (2007) Presynaptic mechanisms in plasticity and memory. In: Learning and Memory: A Comprehensive Reference, pp 741–769. Elsevier.

Pruunsild P, Kazantseval A, Aid T, Palm K, Timmusk T (2007) Dissecting the human BDNF locus: Bidirectional transcription, complex splicing, and multiple promoters. Genomics 90:397–406.

Quest KM, Byiers BJ, Payen A, Symons FJ (2014) Rett syndrome: A preliminary analysis of stereotypy, stress, and negative affect. Research in Developmental Disabilities 35:1191–1197.

Rajagopal R, Chen Z-Y, Lee FS, Chao MV (2004) Transactivation of Trk Neurotrophin Receptors by G-Protein-Coupled Receptor Ligands Occurs on Intracellular Membranes. The Journal of Neuroscience 24:6650.

Ramirez JM, Ward CS, Neul JL (2013) Breathing challenges in Rett Syndrome: Lessons learned from humans and animal models. Respiratory Physiology and Neurobiology 189:280–287.

Rebola N, Pinheiro PC, Oliveira CR, Malva JO, Cunha RA (2003) Subcellular localization of adenosine A(1) receptors in nerve terminals and synapses of the rat hippocampus. Brain Res 987:49–58.

Rebola N, Rodrigues RJ, Oliveira CR, Cunha RA (2005) Different roles of adenosine A1, A2A and A3 receptors in controlling kainate-induced toxicity in cortical cultured neurons. Neurochem Int 47:317–325.

Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philosophical Transactions of the Royal Society B: Biological Sciences 361:1545–1564.

Renkawek K, Stege GJ, Bosman GJ (1999) Dementia, gliosis and expression of the small heat shock proteins hsp27 and alpha B-crystallin in Parkinson’s disease. Neuroreport 10:2273–2276.

Rett A (1966) On a remarkable syndrome of cerebral atrophy associated with hyperammonaemia in childhood. Wiener Medizinische Wochenschrift 166:322–324.

Ribeiro JA, Sebastião AM, de Mendonça A (2002a) Adenosine receptors in the nervous system: pathophysiological implications. Progress in Neurobiology 68:377–392.

Ricceri L, De Filippis BD, Fuso A, Laviola G (2011) Cholinergic hypofunction in MeCP2-308 mice: beneficial neurobehavioural effects of neonatal choline supplementation. Behavioural brain research 221:623–629.

Riikonen R (2003) Neurotrophic factors in the pathogenesis of Rett syndrome. Journal of child neurology 18:693–697.

Rivkees SA, Reppert SM (1992) RFL9 encodes an A2b-adenosine receptor. Molecular Endocrinology 6:1598–1604.

Robinson L, Guy J, McKay L, Brockett E, Spike RC, Selfridge J, De Sousa D, Merusi C, Riedel G, Bird A, Cobb SR (2012) Morphological and functional reversal of phenotypes in a mouse model of Rett syndrome. Brain 135:2699–2710.

Rodrigues TM, Jerónimo-Santos A, Sebastião AM, Diógenes MJ (2014) Adenosine A2A Receptors as novel upstream regulators of BDNF-mediated attenuation of hippocampal Long-Term Depression (LTD). Neuropharmacology 79:389–398.

Rombo DM, Dias RB, Duarte ST, Ribeiro JA, Lamsa KP, Sebastião AM (2016) Adenosine A1 Receptor Suppresses Tonic GABAA Receptor Currents in Hippocampal Pyramidal Cells and in a Defined Subpopulation of Interneurons. Cereb Cortex 26:1081–1095.

Rose CR, Blum R, Pichler B, Lepier A, Kafitz KW, Konnerth A (2003) Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. Nature 426:74–78.

Roux J-C, Dura E, Moncla A, Mancini J, Villard L (2007) Treatment with desipramine improves breathing and survival in a mouse model for Rett syndrome. Eur J Neurosci 25:1915–1922.

Roux J-C, Zala D, Panayotis N, Borges-Correia A, Saudou F, Villard L (2012) Modification of Mecp2 dosage alters axonal transport through the Huntingtin/Hap1 pathway. Neurobiology of disease 45:786–795.

Rusconi L, Salvatoni L, Giudici L, Bertani I, Kilstrup-Nielsen C, Broccoli V, Landsberger N (2008) CDKL5 expression is modulated during neuronal development and its subcellular distribution is tightly regulated by the C-terminal tail. Journal of Biological Chemistry 283:30101–30111.

Ruskin DN, Jr MK, Masino SA (2020) Adenosine and Ketogenic Treatments. https://home.liebertpub.com/caff 10:104–109.

Saito H, Nishimura M, Shinano H, Makita H, Tsujino I, Shibuya E, Sato F, Miyamoto K, Kawakami Y (1999) Plasma concentration of adenosine during normoxia and moderate hypoxia in humans. American Journal of Respiratory and Critical Care Medicine 159:1014–1018.

Sala-Newby GB, Freeman N V, Skladanowski AC, Newby AC (2000) Distinct roles for recombinant cytosolic 5'-nucleotidase-I and -II in AMP and IMP catabolism in COS-7 and H9c2 rat myoblast cell lines. The Journal of biological chemistry 275:11666–11671.

Sandau US, Colino-Oliveira M, Jones A, Saleumvong B, Coffman SQ, Liu L, Miranda-Lourenço C, Palminha C, Batalha VL, Xu Y, Huo Y, Diógenes MJ, Sebastião AM, Boison D (2016) Adenosine Kinase Deficiency in the Brain Results in Maladaptive Synaptic Plasticity. Journal of Neuroscience 36.

Sandau US, Yahya M, Bigej R, Friedman JL, Saleumvong B, Boison D (2019) Transient use of a systemic adenosine kinase inhibitor attenuates epilepsy development in mice. Epilepsia 60:615–625.

Saywell V, Viola A, Confort-Gouny S, Le Fur Y, Villard L, Cozzone PJ (2006) Brain magnetic resonance study of Mecp2 deletion effects on anatomy and metabolism. Biochemical and Biophysical Research Communications 340:776–783.

Scarsdale JN, Webb HD, Ginder GD, Williams DC (2011) Solution structure and dynamic analysis of chicken MBD2 methyl binding domain bound to a target-methylated DNA sequence. Nucleic Acids Research 39:6741–6752.

Schanen NC, Dahle EJR, Capozzoli F, Holm VA, Zoghbi HY, Francke U (1997) A new Rett syndrome family consistent with X-linked inheritance expands the X chromosome exclusion map. American Journal of Human Genetics 61:634–641.

Schiffer D, Cordera S, Cavalla P, Migheli A (1996) Reactive astrogliosis of the spinal cord in amyotrophic lateral sclerosis. J Neurol Sci 139 Suppl:27–33.

Schmid DA, Yang T, Ogier M, Adams I, Mirakhur Y, Wang Q, Massa SM, Longo FM, Katz DM (2012) A TrkB Small Molecule Partial Agonist Rescues TrkB Phosphorylation Deficits and Improves Respiratory Function in a Mouse Model of Rett Syndrome. Journal of Neuroscience 32:1803–1810.

Schmidt AP, Böhmer AE, Antunes C, Schallenberger C, Porciúncula LO, Elisabetsky E, Lara DR, Souza DO (2009) Anti-nociceptive properties of the xanthine oxidase inhibitor allopurinol in mice: role of A1 adenosine receptors. Br J Pharmacol 156:163–172.

Sebastião AM, Ribeiro JA (2009) Tuning and fine-tuning of synapses with adenosine. Current neuropharmacology 7:180–194.

Sebastião AM, Assaife-Lopes N, Diógenes MJ, Vaz SH, Ribeiro J a (2011) Modulation of brain-derived neurotrophic factor (BDNF) actions in the nervous system by adenosine A(2A) receptors and the role of lipid rafts. Biochimica et biophysica acta 1808:1340–1349.

Sebastião AM, Stone TW, Ribeiro JA (1990) The inhibitory adenosine receptor at the neuromuscular junction and hippocampus of the rat: antagonism by 1,3,8-substituted xanthines. British journal of pharmacology 101:453–459.

Seidah NG, Benjannet S, Pareek S, Chrétien M, Murphy RA (1996) Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. FEBS Lett 379:247–250.

Shah RR, Bird AP (2017) MeCP2 mutations: progress towards understanding and treating Rett syndrome. Genome Med 9:17.

Shahbazian MD, Antalffy B, Armstrong DL, Zoghbi HY (2002) Insight into Rett syndrome: MeCP2 levels display tissue- and cell-specific differences and correlate with neuronal maturation. Hum Mol Genet 11:115–124.

Silva I, Costa AF, Moreira S, Ferreirinha F, Magalhães-Cardoso MT, Calejo I, Silva-Ramos M, Correia-de-Sá P (2017) Inhibition of cholinergic neurotransmission by β_3 -adrenoceptors depends on adenosine release and A_1 -receptor activation in human and rat urinary bladders. American Journal of Physiology-Renal Physiology 313:F388–F403.

Silva I, Magalhães-Cardoso MT, Ferreirinha F, Moreira S, Costa AF, Silva D, Vieira C, Silva-Ramos M, Correia-de-Sá P (2020) β_3 Adrenoceptor-induced cholinergic inhibition in human and rat urinary bladders involves the exchange protein directly activated by cyclic AMP 1 favoring adenosine release. British Journal of Pharmacology:bph.14921.

Sirianni N, Naidu S, Pereira J, Pillotto RF, Hoffman EP (1998) Rett syndrome: Confirmation of X-linked dominant inheritance, and localization of the gene to Xq28 [3]. American Journal of Human Genetics 63:1552–1557.

Skaper SD (2018) Neurotrophic factors: An overview. In: Methods in Molecular Biology, pp 1–17. Humana Press Inc.

Smeets EEJ, Pelc K, Dan B (2011) Rett Syndrome. Molecular Syndromology 2:113–127.

Soliman AM, Fathalla AM, Moustafa AA (2018a) Adenosine role in brain functions: Pathophysiological influence on Parkinson's disease and other brain disorders. Pharmacological Reports 70:661–667.

Soliman AM, Fathalla AM, Moustafa AA (2018b) Adenosine role in brain functions: Pathophysiological influence on Parkinson's disease and other brain disorders. Pharmacological Reports 70:661–667 Available at: https://pubmed.ncbi.nlm.nih.gov/29909246/.

Strebl MG, Campbell AJ, Zhao W-N, Schroeder FA, Riley MM, Chindavong PS, Morin TM, Haggarty SJ, Wagner FF, Ritter T, Hooker JM (2017) HDAC6 Brain Mapping with [18F]Bavarostat Enabled by a Ru-Mediated Deoxyfluorination. ACS Central Science 3:1006–1014.

Szczesna K, Caridad O de la, Petazzi P, Soler M, Roa L, Saez MA, Fourcade S, Pujol A, Artuch-Iriberri R, Molero-Luis M, Vidal A, Huertas D, Esteller M (2014) Improvement of the Rett Syndrome Phenotype in a Mecp2 Mouse Model Upon Treatment with Levodopa and a Dopa-Decarboxylase Inhibitor. Neuropsychopharmacology 39:2846.

Tao J, Wu H, Coronado AA, de Laittre E, Osterweil EK, Zhang Y, Bear MF (2016) Negative Allosteric Modulation of mGluR5 Partially Corrects Pathophysiology in a Mouse Model of Rett Syndrome. J Neurosci 36:11946–11958.

Tebano MT, Martire A, Potenza RL, Grò C, Pepponi P, Armida M, Domenici MR, Schwarzschild MA, Chen JF, Popoli P (2008) Adenosine A(2A) receptors are required for normal BDNF levels and BDNF-induced potentiation of synaptic transmission in the mouse hippocampus. Journal of neurochemistry 104:279–286.

Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R, Chen Z-Y, Lee FS, Kraemer RT, Nykjaer A, Hempstead BL (2005) ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J Neurosci 25:5455–5463.

Theofilas P, Brar S, Stewart K, Shen H, Ursula S, Poulsen D, Boison D (2012) Adenosine kinase as a target for therapeutic antisense stratagies in epilepsy. Epilepsia 52:589–601.

Togha M, Akhondzadeh S, Motamedi M, Ahmadi B, Razeghi S (2007) Allopurinol as adjunctive therapy in intractable epilepsy: a double-blind and placebo-controlled trial. Arch Med Res 38:313–316.

Toloe J, Mollajew R, Kügler S, Mironov SL (2014) Metabolic differences in hippocampal “Rett” neurons revealed by ATP imaging. Molecular and cellular neurosciences 59:47–56.

Tosh DK, Paoletta S, Deflorian F, Phan K, Moss SM, Gao Z-G, Jiang X, Jacobson KA (2012) Structural Sweet Spot for A1 Adenosine Receptor Activation by Truncated (N)- Methanocarba Nucleosides: Receptor Docking and Potent Anticonvulsant Activity. Journal of medicinal chemistry 55:8075.

Toti KS, Osborne D, Ciancetta A, Boison D, Jacobson KA (2016) South (S)- and north (N)-methanocarba-7-deazaadenosine analogues as inhibitors of human adenosine kinase. Journal of Medicinal Chemistry 59:6860–6877.

Toward MA, Abdala AP, Knopp SJ, Paton JFR, Bissonnette JM (2013) Increasing brain serotonin corrects CO2 chemosensitivity in methyl-CpG-binding protein 2 (Mecp2)-deficient mice. Exp Physiol 98:842–849.

Townend GS, Ehrhart F, van Kranen HJ, Wilkinson M, Jacobsen A, Roos M, Willighagen EL, van Enckevort D, Evelo CT, Curfs LMG (2018) MECP2 variation in Rett syndrome—An overview of current coverage of genetic and phenotype data within existing databases. Hum Mutat 39:914–924.

Trappe R, Laccone F, Cobilanschi J, Meins M, Huppke P, Hanefeld F, Engel W (2001) MECP2 mutations in sporadic cases of Rett syndrome are almost exclusively of paternal origin. American journal of human genetics 68:1093–1101.

Tropea D, Giacometti E, Wilson NR, Beard C, McCurry C, Dong DF, Flannery R, Jaenisch R, Sur M (2009) Partial reversal of Rett Syndrome-like symptoms in MeCP2 mutant mice. Proceedings of the National Academy of Sciences of the United States of America 106:2029–2034.

Valente P, Casagrande S, Nieus T, Verstegen AMJ, Valtorta F, Benfenati F, Baldelli P (2012) Site-Specific Synapsin I Phosphorylation Participates in the Expression of Post-Tetanic Potentiation and Its Enhancement by BDNF. Journal of Neuroscience 32:5868–5879.

Valenti D, de Bari L, Vigli D, Lacivita E, Leopoldo M, Laviola G, Vacca RA, De Filippis B (2017) Stimulation of the brain serotonin receptor 7 rescues mitochondrial dysfunction in female mice from two models of Rett syndrome. Neuropharmacology 121:79–88.

Vanhala R, Korhonen L, Mikelsaar M, Lindholm D, Riikonen R (1998) Neurotrophic Factors in Cerebrospinal Fluid and Serum of Patients With Rett Syndrome. Journal of Child Neurology 13:429–433.

Vashi N, Justice MJ (1234) Treating Rett syndrome: from mouse models to human therapies Understanding and developing therapies for rare disorders. Mammalian Genome 30:90–110.

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

Vaz SH, Lérias SR, Parreira S, Diógenes MJ, Sebastião AM (2015) Adenosine A2A receptor activation is determinant for BDNF actions upon GABA and glutamate release from rat hippocampal synaptosomes. Purinergic Signalling 11:607–612.

Veeraragavan S, Wan Y-W, Connolly DR, Hamilton SM, Ward CS, Soriano S, Pitcher MR, McGraw CM, Huang SG, Green JR, Yuva LA, Liang AJ, Neul JL, Yasui DH, LaSalle JM, Liu Z, Paylor R, Samaco RC (2016) Loss of MeCP2 in the rat models regression, impaired sociability and transcriptional deficits of Rett syndrome. Human Molecular Genetics 25:3284.

Vidal S, Xiol C, Pascual-alonso A, O'callaghan M, Pineda M, Armstrong J (2019) Genetic landscape of rett syndrome spectrum: Improvements and challenges. International Journal of Molecular Sciences 20.

Vieira C, Ferreirinha F, Magalhães-Cardoso MT, Silva I, Marques P, Correia-de-Sá P (2017) Post-inflammatory Ileitis Induces Non-neuronal Purinergic Signaling Adjustments of Cholinergic Neurotransmission in the Myenteric Plexus. Frontiers in Pharmacology 8:811.

Villani C, Sacchetti G, Bagnati R, Passoni A, Fusco F, Carli M, Invernizzi RW (2016) Lovastatin fails to improve motor performance and survival in methyl-CpG-binding protein2-null mice. eLife 5.

Villicaña S, Bell JT (2021) Genetic impacts on DNA methylation: research findings and future perspectives. Genome Biol 22:127.

Vincenzi F, Borea PA, Varani K (2017) Anxiolytic properties of A1 adenosine receptor PAMs. Oncotarget 8:7216–7217.

Wan M, Lee SS, Zhang X, Houwink-Manville I, Song HR, Amir RE, Budden S, Naidu S, Pereira JL, Lo IF, Zoghbi HY, Schanen NC, Francke U (1999) Rett syndrome and beyond: recurrent spontaneous and familial MECP2 mutations at CpG hotspots. American journal of human genetics 65:1520–1529.

Wang H, Chan S, Ogier M, Hellard D, Wang Q, Smith C, Katz DM (2006) Dysregulation of brain-derived neurotrophic factor expression and neurosecretory function in Mecp2 null mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:10911–10915.

Weaver DR (1993) A2a adenosine receptor gene expression in developing rat brain. Brain Res Mol Brain Res 20:313–327.

Weaving LS, Christodoulou J, Williamson SL, Friend KL, McKenzie OLD, Archer H, Evans J, Clarke A, Pelka GJ, Tam PPL, Watson C, Lahooti H, Ellaway CJ, Bennetts B, Leonard H, Gécz J (2004) Mutations of CDKL5 cause a

severe neurodevelopmental disorder with infantile spasms and mental retardation. American Journal of Human Genetics 75:1079–1093.

Williams M, Jarvis MF (2000) Purinergic and pyrimidinergic receptors as potential drug targets. Biochemical Pharmacology 59:1173–1185.

Williams-Karnesky RL, Sandau US, Lusardi TA, Lytle NK, Farrell JM, Pritchard EM, Kaplan DL, Boison D (2013) Epigenetic changes induced by adenosine augmentation therapy prevent epileptogenesis. The Journal of clinical investigation 123:3552–3563.

Wong J, Rothmond DA, Webster MJ, Weickert CS (2013) Increases in two truncated TrkB isoforms in the prefrontal cortex of people with schizophrenia. Schizophrenia Bulletin 39:130–140.

Xiang Y, Tanaka Y, Patterson B, Hwang SM, Hysolli E, Cakir B, Kim KY, Wang W, Kang YJ, Clement EM, Zhong M, Lee SH, Cho YS, Patra P, Sullivan GJ, Weissman SM, Park IH (2020) Dysregulation of BRD4 Function Underlies the Functional Abnormalities of MeCP2 Mutant Neurons. Molecular Cell 79:84-98.e9.

Yamanaka K, Chun SJ, Boillee S, Fujimori-Tonou N, Yamashita H, Gutmann DH, Takahashi R, Misawa H, Cleveland DW (2008) Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. Nat Neurosci 11:251–253.

Yu D, Sakurai F, Corey DR (2011) Clonal Rett-syndrome cell lines to test compounds for activation of wild-type MeCP2 expression. Bioorganic & medicinal chemistry letters 21:5202.

Zachariah RM, Olson CO, Ezeonwuka C, Rastegar M (2012) Novel MeCP2 isoform-specific antibody reveals the endogenous MeCP2E1 expression in murine brain, primary neurons and astrocytes. PLoS One 7:e49763.

Zanella S, Mebarek S, Lajard A-M, Picard N, Dutschmann M, Hilaire G (2008) Oral treatment with desipramine improves breathing and life span in Rett syndrome mouse model. Respir Physiol Neurobiol 160:116–121.

Zappella M (1992) The rett girls with preserved speech. Brain and Development 14:98–101.

Zappella M, Meloni I, Longo I, Hayek G, Renieri A (2001) Preserved speech variants of the Rett syndrome: molecular and clinical analysis. Am J Med Genet 104:14–22.

Zeev B Ben, Bebbington A, Ho G, Leonard H, De Klerk N, Gak E, Vecksler M, Christodoulou J (2009) The common BDNF polymorphism may be a modifier of disease severity in Rett syndrome. Neurology 72:1242–1247.

Zhang S, Zhang Z-Y (2007) PTP1B as a drug target: recent developments in PTP1B inhibitor discovery. Drug Discov Today 12:373–381.

Zhou QY, Chuanyu LI, Olah ME, Johnson RA, Stiles GL, Civelli O (1992) Molecular cloning and characterization of an adenosine receptor: The A3 adenosine receptor. Proceedings of the National Academy of Sciences of the United States of America 89:7432–7436.

Zhu CZ, Gopalakrishnan S, Doyle K, Nikkel AL, Olson L, Abraham VC, Leys L, Widomski D, Salte K, Putman B, Pratt S, Ma J, Su Z, Gopalakrishnan M, Lee CH, McGaraughty SP (2016) A-306989, an inhibitor of adenosine kinase, is renoprotective in rodent models of podocyte, basement membrane, and obstructive injury. European Journal of Pharmacology 788:1–11.

Zimmermann H (1992) 5’-Nucleotidase: Molecular structure and functional aspects. Biochemical Journal 285:345–365.

Zimmermann H (1996) Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. Progress in Neurobiology 49:589–618.

Zuchora B, Wielosz M, Urbańska EM (2005) Adenosine A1 receptors and the anticonvulsant potential of drugs effective in the model of 3-nitropropionic acid-induced seizures in mice. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology 15:85–93.