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Tese especialmente elaborada para otenção do grau de Doutor em Medicina, especialidade de Neurologia

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Abstract

The synapse is the functional unit for neuronal communication. Mutations in genes that encode relevant proteins for synaptic functions are being increasingly identified in neuropediatric disorders. The present work is focused on synaptic dysfunction in Rett Syndrome, which is caused by mutation in the *MECP2* gene. This encodes for methyl-CpG-binding protein 2 (MeCP2), an essential epigenetic regulator in mammalian brain development. Rett syndrome is characterized by a period of cognitive decline, hand stereotypies, autistic traits and seizures, following an apparently normal early infancy. This disease can be classified as a synaptopathy, since it comprises simultaneously impairments in synaptogenesis, synaptic maturation and synaptic plasticity. There is evidence for the possibility of phenotypical rescue in *Mecp2* deficient mice models, but current treatments for Rett Syndrome are primarily symptomatic therapies for epilepsy or behavioral disturbances, and not for correction of the underlying brain abnormalities.

The molecule γ-amino butyric acid (GABA) is the main inhibitory (hyperpolarizing) neurotransmitter in the adult brain, but has excitatory (depolarizing) action in the developing brain, when it binds to GABA A receptors. This functional shift is dependent of neuronal maturational changes that include differences in the expression of cation chloride cotransporters, which regulate intracellular chloride concentration. One of the key molecular changes for this process is a perinatal neuronal membrane upregulation of potassium chloride cotransporter 2 (KCC2), an ion cotransporter that extrudes chloride form the cell, together with sodium potassium chloride cotransporter 1 (NKCC1), which transports choride into the cell. Deregulation of cation chloride cotransporters' expression or function has been associated with neurodevelopmental disorders like neonatal seizures, fragile X, tuberous sclerosis, Down syndrome, underpinning the relevance of this balance for an adequate central nervous system postnatal development.

In the present thesis, I describe a disturbed GABAergic maturational process in Rett Syndrome, regarding intracellular chloride regulation. Starting from the clinic, we searched for a method to detect synaptic proteins in the cerebrospinal fluid of pediatric patients. The earlier postnatal period is

characterized by intense synaptogenesis and synaptic pruning, allowing the detection of these proteins in the cerebrospinal fluid, using immunoblot analysis. Since we were interested in GABAergic function maturation, and this process was dependent on the expression of NKCC1 and KCC2, abnormalities in the cerebrospinal fluid levels of these proteins were searched in Rett Syndrome patients and a decrease in KCC2 was observed.

In order to obtain a model to understand the impact of this reduction of KCC2 in neuronal function, human iPSCs were reprogramed from Rett patients' fibroblasts. Human skin biopsies were collected in accordance with European and National ethical regulation and induced pluripotent stem cells were generated from fibroblasts upon infection with a retroviral vector expressing the four canonical transcription factors (Oct4, Sox2, Klf4, and Myc). Neural commitment of patient specific induced pluripotent stem cells was induced under defined conditions. Neuronal cortical populations were then derived in a monolayer culture system using a protocol that mimicks human cortical development in vitro. Perforated patch recordings were performed in these neurons, and GABA-evoked postsynaptic currents were measured to evaluate GABA A receptor equilibrium potencial. Our preliminary data indicates that recordings from *MECP2* mutant cells exhibit a GABA A receptor equilibrium potential that is more positive than in recordings from control cells, suggesting pathologic changes in chloride gradient, characteristic of an immature state.

These results were complemented with experiments in the extensively characterized *Mecp2* knock out mouse model (*Mecp2*tm1.1Bird/J). The level of KCC2 protein expression is lower in *Mecp2*-KO mice, when compared to control littermates, as addressed by western blot analysis of 6 week old hippocampi. Moreover, hippocampal electrophysiological recordings show reduction of membrane resting potential and threshold potential in the *Mecp2* knock-out model, where synaptic transmission evaluated by Input/Output curves reveals an increased excitatory synaptic transmission.

Brain derived neurotrophic factor (BDNF) is a neurotrophin relevant for synaptic function, neuronal maturation and neuronal survival. Several groups have reproduced experiments that consistently support a regulatory role of MeCP2 upon BDNF expression. There is also evidence supporting the

interference of BDNF upon KCC2 expression. Since BDNF is highly relevant in Rett Syndrome pathology, several pre-clinical and clinical strategies are being designed and tested to improve Rett Syndrome, restoring BDNF levels and physiological actions. Based on the knowledge about a facilitatory effect of adenosine on BDNF actions, we hypothesized that the modulation of BDNF with adenosine receptor agonists would have a positive effect in synaptic function in the Rett Syndrome mouse model. Long-term potentiation (LTP) is accepted as a neurophysiological paradigm to test synaptic plasticity, the basic process underlying learning and memory. Adenosine is a neuromodulator that acts mainly through A1 and A2A receptors. The activation of A2A receptors (A2ARs) potentiates BDNF synaptic actions in healthy animals. Therefore, we explored whether the activation of A2ARs in the Rett Syndrome animal model facilitates BDNF action upon LTP. We found that BDNF facilitatory actions upon LTP are absent in the Rett Syndrome animal model, suggesting that, in addition to BDNF reduction, there is also impairment in its actions, even when it is administered exogenously. This dysfunction could be explained by a reduction in the levels of the main BDNF receptor (the full length tropomyosin-related kinase B), which we describe for the first time in the present study. When BDNF was combined with the selective A2AR agonist, CGS2168, the BDNF effect upon LTP was restored, similar to what was observed in hippocampal slices from wild type animals with BDNF alone. Together these data highlight A2ARs as new possible therapeutic targets to increase BDNF actions in Rett Syndrome.

In conclusion, this work contributes to elucidate two significant downstream effects of MeCP2 impairment. The first is the abnormality of GABA postsynaptic actions upon GABAA receptors, suggested by a cerebrospinal fluid proteomic change and corroborated by findings in the *Mecp2* knock out animal model and in human neurons, derived from induced pluripotent stem cells of Rett Syndrome patients. This system is now available for pharmacological screening of compounds that target the detected disturbances with direct evaluation of phenotypical rescue, at a cellular level. KCC2 reduction or impaired function appears to have also impact on synaptic structure and plasticity, and is a pathophysiological mechanism that

contributes to several neurodevelopmental disorders. The second is the reduction of the main BDNF receptors in the hippocampi of the Rett Syndrome animal model. We have also shown that, using an adenosine A2ARs agonist, it is possible to restore BDNF actions upon LTP, a paradigm for synaptic plasticity. Adenosine, through A2ARs, positively modulates the intracellular signaling cascades activated by BDNF, bypassing the BDNF-tropomyosin-related kinase B receptor impairment that occurs in Rett Syndrome.

Key words: Rett Syndrome; Epilepsy; Synapse; GABA; BDNF; KCC2; Bumetanide; Induced Pluripotent Stem Cells; Autism Spectrum Disorders; Cerebrospinal Fluid; TrkB Receptor; Adenosine A2A receptor; Neural commitment; defined culture conditions; dual-SMAD inhibition; neurodevelopment modeling; matrigel; vitronectin;

RESUMO

A sinapse é a unidade funcional da comunicação neuronal. Mutações em genes que codificam proteínas relevantes para a função sináptica são descritas frequentemente como causa de doenças neuropediátricas. Esta tese tem o seu foco na disfunção sináptica na Síndrome de Rett, predominantemente causada por mutações ou deleções no gene *MECP2*. Este gene codifica a proteína "*methyl-CpG-binding protein* 2" (MeCP2), um regulador epigenético essencial para o desenvolvimento e função do cérebro dos mamíferos. A Síndrome de Rett é caracterizada por um período de desenvolvimento pós natal normal, seguido de perda das aquisições prévias, estereotipias manuais, traços autistas e convulsões. Já foram descritas alterações na sinaptogenese, na maturação sináptica e na plasticidade sináptica na Síndrome de Rett, que pode ser considerada uma sinaptopatia.

Existem estudos que demonstram a possibilidade de reversão do fenótipo em modelos animais com alteração no gene Mecp2, mas as terapêuticas atualmente utilizadas na clínica são apenas sintomáticas, dirigidas à epilepsia ou a perturbações do comportamento e não à correção da disfunção cerebral subjacente.

O ácido γ-amino butirico (GABA, γ-amino butyric acid) é o principal neurotransmissor inibitório (hiperpolarizante) no cérebro adulto, mas tem ação excitatória (despolarizante) no cérebro em desenvolvimento, quando se liga aos receptores GABA A. Esta modificação funcional depende de alterações moleculares nos neurónios em maturação que incluem diferenças na expressão dos cotransportadores de iões cloreto por catiões (*Cation Chloride Cotransporters*), reguladores da concentração intracelular de iões cloreto. Uma das alterações moleculares evolutivas relevantes para este processo é o aumento da expressão do cotransportador de cloreto e potássio do tipo 2 (KCC2), um cotransportador iónico transmembranar, especifico dos neurónios, que transporta cloreto para fora da célula. Por outro lado, o cotransportador de cloreto, potássio e sódio do tipo 1 (NKCC1) leva os iões cloreto para dentro da célula e verifica-se uma redução da sua expressão ao longo deste processo. A desregulação da expressão ou da função dos

cotransportadores de iões cloreto (sobretudo o KCC2 e o NKCC1), tem sido associada a doenças do desenvolvimento cerebral, tais como convulsões neonatais, Síndrome do X frágil, esclerose tuberosa, Síndrome de Down, sustentando a relevância deste equilíbrio para um desenvolvimento adequado do sistema nervoso central pós natal.

Nesta tese, eu descrevo uma perturbação no processo de maturação GABAérgico na Síndrome de Rett, nomeadamente na regulação intracelular de iões cloreto que por sua vez determina a resposta à estimulação GABAérgica, ao nível dos receptores GABA A.

Partindo da clínica, foi desenvolvido um método para detetar proteínas sinápticas no líquido cefalorraquidiano dos pacientes pediátricos. Nesta população verificam-se períodos de desenvolvimento caracterizados por sinaptogenese marcada e intensa remodelação sináptica o que permite a deteção destas proteínas no liquido cefalorraquidiano (LCR) através de análise por western *blot*.

Uma vez que a maturação da resposta GABAérgica depende de alterações evolutivas na expressão das proteínas NKCC1 e KCC2, foram determinados os níveis destas proteínas no LCR de doentes com Síndrome de Rett e de controlos saudáveis. Neste estudo foi detetada uma redução de KCC2 no LCR destas doentes.

Com o objetivo de obter um modelo celular humano para compreender o impacto desta redução de KCC2 na função dos neurónios, foram reprogramadas células humanas pluripotentes induzidas a partir de fibroblastos de doentes com Síndrome de Rett. Foram recolhidas biópsias de pele humana de doentes e controlos saudáveis, respeitando as recomendações éticas Europeias e Nacionais. A partir dos fibroblastos humanos em cultura, foram geradas células pluripotentes induzidas, através da infeção por retrovírus com um vetor que induz a expressão simultânea dos quatro fatores de transcrição (Oct4, Sox2, Klf4 e Myc) que permitem a reprogramação.

A partir das células pluripotentes induzidas humanas, foram então derivados neurónios corticais num sistema de cultura em monocamada, utilizando um protocolo que mimetiza, in vitro, o desenvolvimento cortical humano. Este

protocolo foi optimizado para a obtenção de progenitores neuronais em condições definidas.

Para estudar a resposta neuronal à estimulação com GABA nos neurónios humanos dos doentes e controlos, foram realizados registros neurofisiológicos de *perforated patch-clamp* nestes neurónios e foi medida a corrente pós sináptica evocada de GABA para avaliar o potencial de equilíbrio do receptor GABAA.

Os nossos resultados preliminares indicam que os registros de células com mutações no gene MECP2 exibem um potencial de equilíbrio do receptor GABAA que é mais positivo do que o potencial obtido nos registros de células controlo, sugerindo uma alteração patológica da resposta GABAérgica, característica de um estado imaturo.

Estes resultados foram complementados com experiências num modelo animal da Síndrome de Rett com ablação do gene *Mecp2* (*Mecp2* KO, *Mecp2*-knock out) (Mecp2tm1.1Bird/J), extensamente caracterizado na literatura. O nível de expressão da proteína KCC2 está significativamente reduzido nos ratos *Mecp2*-KO, quando comparado com animais controlo, tal como demonstramos através da análise de hipocampos dissecados do cérebro destes animais. Por outro lado, os registros eletrofisiológicos no hipocampo do mesmo modelo revelam um aumento da excitabilidade intrínseca destas células.

O factor neurotrófico derivado do cérebro (BNDF, *Brain Derived Neurotrophic Factor*) é uma neurotrofina determinante para a função sináptica, maturação neuronal e sobrevivência neuronal. Vários grupos têm reproduzido experiências que suportam de uma forma consistente um papel de regulação do MeCP2 na expressão do BDNF. Existe também evidência que suporta a interferência de BDNF na expressão do gene que codifica o KCC2, *SLC12A5*. Considera-se que a desregulação das ações desta neurotrofina está na base das muitas das alterações encontradas nos modelos de Síndrome de Rett, pelo que várias estratégias pré clinicas e clinicas estão a ser desenhadas e testadas repor os níveis de BDNF e as suas acções fisiológicas nestes doentes. Baseados no conhecimento do efeito facilitador da adenosina nas

acções do BDNF, colocámos a hipótese de que a modulação do BDNF com agonistas dos receptores de adenosina terá um efeito positivo na função sináptica dos animais sem expressão de Mecp2. Utilizámos um paradigma neurofisiológico, a Potenciação de Longa Duração (LTP, Long Term Potentiation), desenhado para testar a plasticidade sináptica, o processo básico subjacente à aprendizagem e à memória.

A adenosina é um neuromodulador que atua sobretudo através dos receptores A1 e A2A. A activação dos receptores A2A potencia as acções do BDNF sobre a plasticidade sináptica em animais saudáveis. Foi testada esta reconhecida ação potenciadora da adenosina no efeito do BDNF sobre a plasticidade sináptica.

Os nossos resultados indicam que a ação facilitadora do BDNF na LTP está ausente no modelo animal de RTT, sugerindo que, para além da redução do BDNF, existe também perda das suas acções, mesmo quando administrado exogenamente. Esta disfunção pode ser explicada por uma redução dos níveis do principal receptor de BDNF (TrkB, tropomyosin-related kinase B), que é descrita pela primeira vez neste estudo. Quando o BDNF foi associado ao agonista selectivo do receptor A2A, CGS2168, o efeito BDNF na LTP foi recuperado, de forma semelhante ao que foi observado em fatias de hipocampo de animais controlo, apenas com o BDNF. Estes dados apontam para os receptores A2A como um novo alvo terapêutico para recuperar a acção do BDNF na Síndrome de Rett.

Em conclusão, este trabalho contribui para elucidar dois efeitos significativos do défice de MeCP2:

O primeiro é a alteração das ações pós sinápticas do GABA nos receptores GABAA, sugerida por uma alteração na proteómica no LCR destas doentes e corroborada pelas descobertas no modelo animal de *Mecp2*-KO e em neurónios humanos, derivados de células pluripotentes induzidas de pacientes com Síndrome de Rett. Este sistema, baseado no modelo celular humano, está agora disponível para *screening* farmacológico de compostos que podem atenuar esta alteração. Com este modelo podemos avaliar directamente o efeito de um potencial fármaco no fenótipo celular. A redução

e/ou disfunção do KCC2 parece ser um mecanismo comum a várias doenças do desenvolvimento cerebral, com consequências na função GABAérgica, mas também na estrutura e plasticidade de sinapses glutamatérgicas, uma vez que este cotransportador exerce funções diretas nesses mecanismos.

O segundo é a redução dos recetores TrkB do BDNF no hipocampo do modelo animal da Síndrome de Rett. Demonstramos também que, utilizando um agonista do receptor A2A, é possível recuperar as acções do BDNF na LTP, um paradigma neurofisiológico para a plasticidade sináptica. Esta modulação da acção do BDNF pela adenosina é possível pela ativação intracelular de vias de sinalização do BDNF pelo receptor A2A, reduzindo as consequências do défice do recetor TrkB.

Abbreviations list

[cl-]- Intracellular Chloride Concentration
A1R- A1 Receptor
A2AR- A2A Receptor
aCSF- artificial CSF
ADA- Adenosine Deaminase
ADK- Adenosine Kinase
AED- anti epileptic drug
AFP- Alpha-fetoprotein
ALS- Amyotrophic Lateral Sclerosis
AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP- Action Potential
ASD- Autism Spectrum disorder
BDNF- Brain-Derived Neurotrophic Factor
BF- bright field
bFGF- Basic Fibroblast Growth Factor
BMP- Bone Morphogenetic Protein
BSA- Bovine Serum Albumin
CAMK II- Calcium calmodulin-dependent kinase II
CFP- Cyan Fluorescent Protein
CHLN- Centro Hospitalar de Lisboa Norte
CNS- Central Nervous System
CSF- Cerebrospinal Fluid
cTNT- cardiac troponin T
D2 R- D2 Receptor
DAPI- 4',6-diamidino-2-phenylindol
DAT- dopamine transporter
DNA- Deoxyribonucleic acid
E GABAA- GABA A receptor equilibrium potential
EB- Embrioid Bodies
Ecl- chloride equilibrium potential
ECL- enhanced chemiluminescence detection method

EE- Epileptic Encephalopathies

EEG- electroencephalogram

EIEE- Early Infantile Epileptic Encephalopathy

EME- Early Mioclonic Encephalopathy

ESCs- Embryonic stem cells

ESG1- Embryonal stem cell-specific gene 1

EU- European Union

FDA- US Food and Drug Administration

fEPSP- Field excitatory post-synaptic potential

FGF4- Fibroblast growth factor 4

FOXG1- Forkhead Box G1

FRET- Fluorescence resonance energy transfer

GABA VT- GABA Vesicular Transporter

GABA- γ amino aminobutyric acid

GABAAR- GABA A receptor

GABA-PSCs- GABA-evoked postsynaptic currents

GAPDH- Glyceraldehyde-3-phosphate-dehydrogenase

GATA2- GATA binding protein 2

GDF3- Growth differentiation factor 3

Gly R- Glicine receptors

hESC- Human Embryonic Stem Cell

HSJD- Hospital San Joan de Deu

I/O- Input/Output

IMM- Instituto de Medicina Molecular

iPSCs- Induced Pluripotent Stem Cells

IPSPs- Inhibitory Postsynaptic potentials

IST- Instituto Superior Técnico

KCC2- K+– Cl− cotransporter 2

KLF4- Kruppel-like factor 4

KO- Knock Out

KOSR- Knockout Serum Replacement

LDN- LDN-193189

LEV- Levetiracetam

LP- Lumbar Puncture
LTD- Long Term Depression
LTP- Long Term Potentiation
MAGI2- Membrane-associated guanylate kinase inverted-2
MAP2- Microtubule associated protein 2
mCG- methylated cytosine with guanine
MeCP2- Methyl-CpG binding protein 2
mRNA- Messenger RNA
mTOR- mammalian target of rapamycin
MRS- Magnetic Ressonance Spectroscopy
NANOG- Nanog homeobox
NKCC1- Na+-K+-2Cl- cotransporter 1
NLGN- Neurologin
NMDA- N-Methyl-D-aspartic acid or N-Methyl-D-aspartate
NPs- Neural precursors
NRXN- Neurexin
NTs- Neurotransmitters
OCT4- POU class 5 homeobox 1
OD- Optic Density
OS- Ohtahara Syndrome
PAX 6- Paired box 6
PB- Phenobarbital
PFA- Paraformaldehyde
PI3K- phosphatidylinositol 3- kinase
PSCs- Pluripotent Stem Cells
PSD- Postsynaptic Density
PVDF- polyvinylidene difluoride
REX1- Reduced expression protein 1
RMP- Resting Membrane Potential
RNA- Ribonucleic acid
RTT- Rett Syndrome
SB- SB-431542
SDS- Sodium dodecyl sulfate

SFFV- Spleen Focus Forming Virus

SHANK3- SH3 And Multiple Ankyrin Repeat Domains 3

Shc (Src homology 2/α-collagen-related protein)

SM- “Sec1/Munc18-like”

SMA- Smooth Muscle Actin

SNARE-“Soluble NSF attachment protein receptor”

SOX 1- SRY (Sex Determining Region Y)-Box 1

SOX2- SRY (Sex Determining Region Y)-Box 2

SR- Serum replacement

SSEA-4- Stage-specific embryonic antigen-4

STXBP1- Syntaxin Binding Protein 1

SV2- Synaptic vesicle proteins 2

SV-Synaptic Vesicle

T-Tesla

TGFβ- transforming growth factor beta

Trk- tropomyosin-related kinase

TrkB-FL- TrkB- Full Length

Tuj1- βIII-tubilin

VAMP2- Vesicle associated membrane protein 2

VGLUT- Vesicular glutamate transporter

VMAT2- Vesicular Monoamine Transporter 2

VTN- Vitronectin

WS- West Syndrome

WT- Wild Type

XIST- X inactive specific transcript

YFP- Yellow Fluorescent Protein

ZO-1- Tight junction protein ZO-1

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

Analysis of synaptic proteins in the cerebrospinal fluid as a new tool in the study of inborn errors of neurotransmission.
Duarte ST, Ortez C, Pérez A, Artuch R, García-Cazorla A. J Inherit Metab Dis. 2011 34(2): 523-8.

Abnormal expression of cerebrospinal fluid cation chloride cotransporters in patients with Rett syndrome.
Duarte ST, Armstrong J, Roche A, Ortez C, Pérez A, O'Callaghan M del M, Pereira A, Sanmartí F, Ormazábal A, Artuch R, Pineda M, García-Cazorla A. PLoS One. 2013 Jul 19;8(7):e68851.

Neural Commitment of Human Pluripotent Stem Cells under Defined Conditions Recapitulates Neural Development and Generates Patient-Specific Neural Cells
Tiago G. Fernandes[*], Sofia T. Duarte[*], Mehrnaz Ghazvini, Cláudia Gaspar, Diana C. Santos, Ana R. Porteira, Gonçalo M. C. Rodrigues, Simone Haupt, Diogo M. Rombo, Judith Armstrong, Ana M. Sebastião, Joost Gribnau, Àngels Garcia-Cazorla, Oliver Brüstle, Domingos Henrique, Joaquim M. S. Cabral and Maria M. Diogo
Biotechnology Journal, 2015, in press
(*) Both authors contributed equally to this work

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

CHAPTER 4: Adenosine Receptor as new pharmacological targets for Rett Syndrome

CHAPTER 6: GABAergic dysfunction in neurons derived from induced pluripotent stem cells of patients with Rett Syndrome

Other published articles where the author of this thesis participated during her doctoral studies:

Undetectable levels of CSF amyloid-ß peptide in a patient with 17ß-hydroxysteroid dehydrogenase deficiency.
Ortez C, Villar C, Fons C, Duarte ST, Pérez A, García-Villoria J, Ribes A, Ormazábal A, Casado M, Campistol J, Vilaseca MA, García-Cazorla A.. J Alzheimers Dis. 2011 27(2): 253-7

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Neuronal communication and synaptic metabolism in childhood epilepsy.
García-Cazorla À, Cortès-Saladelafont E, Duarte S. Rev Neurol. 2015 Mar 1;60(5):219-28.

Chapter 1 Introduction

1.1 Child neurology through synaptic communication

We are facing a fascinating era of exponential growth of knowledge in neuroscience and in biomedical technology. The application of this knowledge and the translation to clinical practice raises serious challenges for both clinicians and scientists. Projects focused in bringing these two fields together are certainly needed and relevant for the progress of our understanding about normal brain physiology and disease, leading to an improvement in treatment strategies and in disease prevention. In accordance, previous experience in the medical field suggests that therapeutic strategies and prognostic decision-making are usually greatly facilitated as classifications move closer to the underlying pathophysiology (Craddock and Owen, 2010).

The gap between clinical doctors and scientists results in an increased time lag between basic research discoveries and their application to patients, and also in the loss of precious information about normal physiology that arises from patients and their disorders. In particular, brain pathology has always been an excellent school to understand brain function, as long as studies focused on both aims, clinical and basic research ones, are done in coordination. Moreover, the study of the brain and brain diseases is amongst the most complex in human biology. Most current research addresses neurological disease in adults and the elderly, whereas the study of the child's brain is proportionately underreported in the literature. Childhood represents a specific phase in the development of the synaptic network in the brain, characterized by overall remarkable plasticity. Although plasticity is present in the adult brain, it does not appear to involve the same set of mechanisms that are present in the developing brain (Ehninger et al., 2008). Studies on mechanisms that underlie activation and regulation of developmental synaptic plasticity in the central nervous system (CNS) are seminal in neuroscience, since reactivation of such mechanisms may potentially facilitate neural circuit plasticity during times when the adult brain is less plastic, like periods of rewiring after a stroke or other CNS insult. Additionally, research in this field may help to develop rational pharmacological approaches in order to correct alterations in the brain of children and adults with neurodevelopmental

disorders involving altered synapse formation and/or plasticity (Chattopadhyaya, 2011; Ehninger et al., 2008).

The synapse is the basic unit for neuronal communication. Neuronal networking and neuron-to-neuron communication occurs at these specialized junctions, and one of the relevant fields expanding in neuroscience is the unraveling of the mechanisms of synaptic communication. Neuronal connection complexity, specifically dendrite branching and morphology, facilitates and allows individual neurons to carry out specialized brain functions, such as social networking, learning, and memory (Kulkarni and Firestein, 2012). The establishment and maintenance of synaptic contacts, as well as synaptic plasticity, are crucial factors for normal brain function and the functional properties of a synapse are largely dependent on the molecular setup of synaptic proteins (Grabrucker, 2014). The mechanism of action of these pre and postsynaptic proteins is currently being unraveled but information about the impact of their dysfunction is also arising from several useful models, with mutations or deletions of genes that code synaptic proteins or their regulatory factors. These models exhibit impaired neuronal circuitry by disrupted formation, plasticity and/or maturation of synapses. If we bridge this complex field of scientific knowledge with the clinical practice in child neurology, we can find that an extensive number of diseases, with different origins and heterogeneous symptoms, have a common pathophysiological element of disrupted synaptic function. Accordingly, the vast majority of child neurology disorders that exhibit manifestations like epilepsy, neuropsychiatric features, intellectual disability or movement disturbances, display primary or secondary synaptic dysfunctions (Garcia-Cazorla et al., 2015). Actually, the concept of synaptopathy is increasingly being used to describe disorders in which synaptic dysfunction underlies the pathogenesis process.

This work aims to contribute to the understanding of neurodevelopment disorders, particularly Rett syndrome (RTT) and other early encephalopathies with epilepsy and autistic traits. Using different approaches and different disease models, we have searched for molecular dysfunctions at the synaptic level that might underlie the signs and symptoms of these disorders.

1.2 The Synapse

The synapse is the functional unit of neuronal communication, corresponding to a specialized intracellular area, characterized at the pre synaptic level by a secretory machinery that allows fast, sub millisecond neurotransmitter release, through synaptic vesicle exocytosis when an action potential invades the nerve terminal (Katz, 1969) (Sudhof, 2004). At the postsynaptic level, the synaptic junction is enriched with clusters of neurotransmitter receptors, connected with intracellular signaling transduction proteins. Pre and postsynaptic compartments are linked by transsynaptic cell-adhesion molecules, which in turn are coupled to the presynaptic release machinery or to postsynaptic receptors, like neurexins and neuroligins, enriched at pre and postsynaptic membranes, respectively. Specific interactions between neurexins and neuroligins regulate various aspects of synaptogenesis and synaptic development and function, regulating the excitatory or inhibitory currents and their balance.

1.2.1 Pre synaptic release machinery

Presynaptic nerve terminals release neurotransmitters by synaptic vesicle (SV) exocytosis. SVs are the repositories of neurotransmitters (NTs). They are locally recycled at nerve terminals following exocytosis (Poudel and Bai, 2014). These vesicles undergo Ca2+-dependent fusion with plasma membrane and, consequently, NTs are released to propagate chemical signaling between neurons. SVs exhibit an extremely high protein/lipid ratio (Takamori et al., 2006). Proteins account for more than 60% of the total mass of vesicles, suggesting that the vesicle surface is fully covered by proteins (Figure 1.1). SVs fuse with active zones, where they contact (“dock”) with plasma membrane and fusion leads to NT release. NT-filled vesicles can be observed in clusters in the vicinity of the active zone. These vesicles are subsequently primed for release. The rise in cytosolic Ca2+ that occurs during an action potential triggers the opening of a fusion pore between some of the primed, docked vesicles and the plasma membrane. A complex machinery that includes the SNARE (for “soluble NSF-attachment protein receptor”) complex and SM (for “Sec1/Munc18-like”) proteins brings the vesicle and

plasma membranes into close proximity before vesicle fusion and exocytosis (Sudhof and Rizo, 2011). Synaptobrevin, syntaxin and SNAP-25 are core proteins of the SNARE complex. Several of the genes that code synaptic vesicle proteins and presynaptic membrane proteins have been implicated in epilepsy and other neurological disorders (Waites and Garner, 2011).

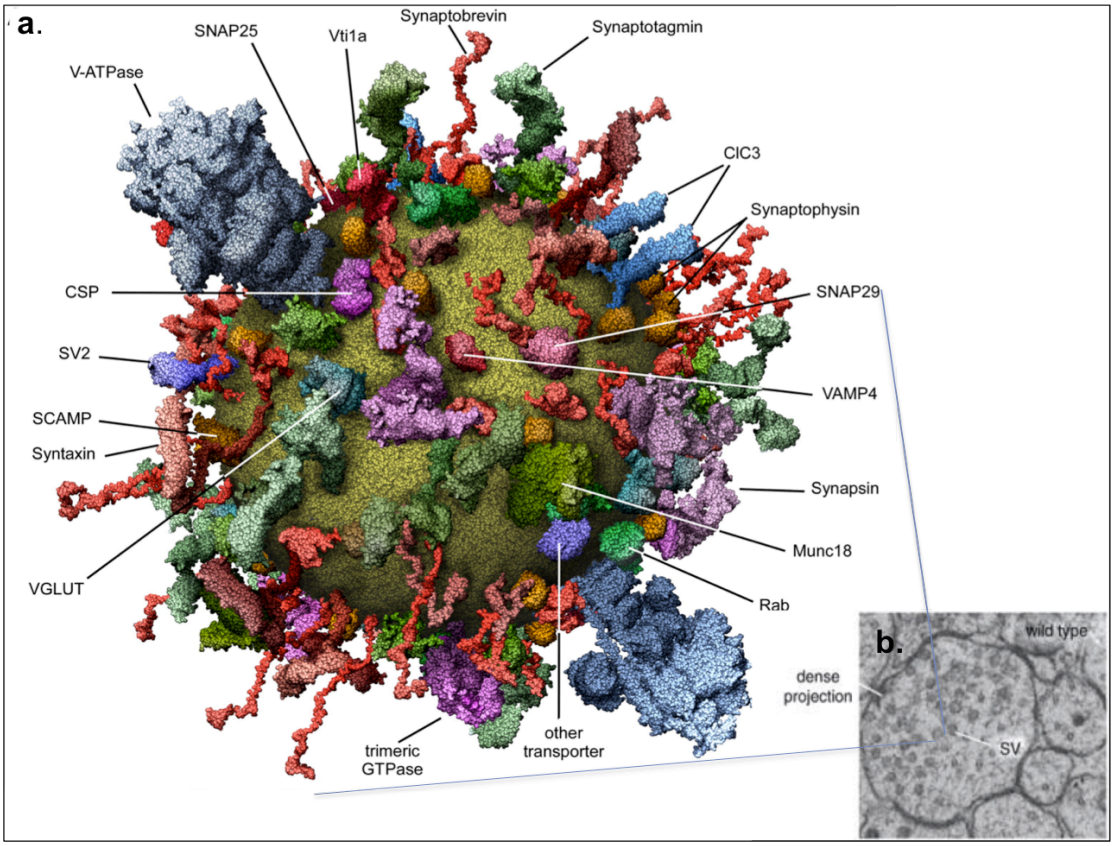


Figure 1.1 The glutamatergic synaptic vesicle

a. Outside view of a SV. The model is based on space-filling models of all macromolecules at near atomic resolution. b. Electron Microscopy view.

Adapted from *Takamori S et al, Molecular anatomy of a trafficking organelle. Cell. 2006;127(4):831-46* (Takamori et al., 2006).

1.2.2 Postsynaptic density

The cardinal functional components of the postsynaptic specialization of excitatory and inhibitory synapses are the neurotransmitter receptors for glutamate and γ-aminobutyric acid (GABA), respectively. The postsynaptic side of excitatory synapses differs from inhibitory synapses not only in their content of NT receptors but also in their location, morphology, molecular composition and organization. The functional specialization at the postsynaptic membrane of the synapse is called the postsynaptic density (PSD). Receptor channels are concentrated at the postsynaptic membrane and interact with a dense and rich protein network comprised of anchoring and scaffolding molecules, signaling enzymes, cytoskeletal components, as well as other membrane proteins. During the development of nervous systems, immature neurons usually form synapses with a large number of target cells; many of these early connections are eliminated as the CNS matures, while the remaining ones are strengthened. The selective elimination (pruning) and strengthening of immature synapses, termed synaptic refinement, is essential for the development of neural circuits and behaviors. These events are based in changes in conformation or concentration of PSD proteins, which can lead to long-term changes in synapses and neuronal circuits. Not only composition but also structure of PSDs change during the maturation of synapses (Sheng and Kim, 2011). Very relevant proteins like PSD-95, calcium calmodulin-dependent kinase II (CaMKII) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits increase during early postnatal development, when synaptogenesis and functional maturation of the synapses is taking place (Petralia et al., 2005). N-Methyl-D-aspartic acid or N-Methyl-D-aspartate (NMDA) receptors can also be found on neurons in early stages prior to synaptogenesis, where they may be involved in migration and differentiation (Petralia, 2012). Given the functional relationship of PSD proteins to synapse development, structure, and function, it is not surprising that mutations in many PSD proteins are associated with human neurologic and psychiatric disease (Bayes et al., 2011) and particularly with neurodevelopmental disorders.

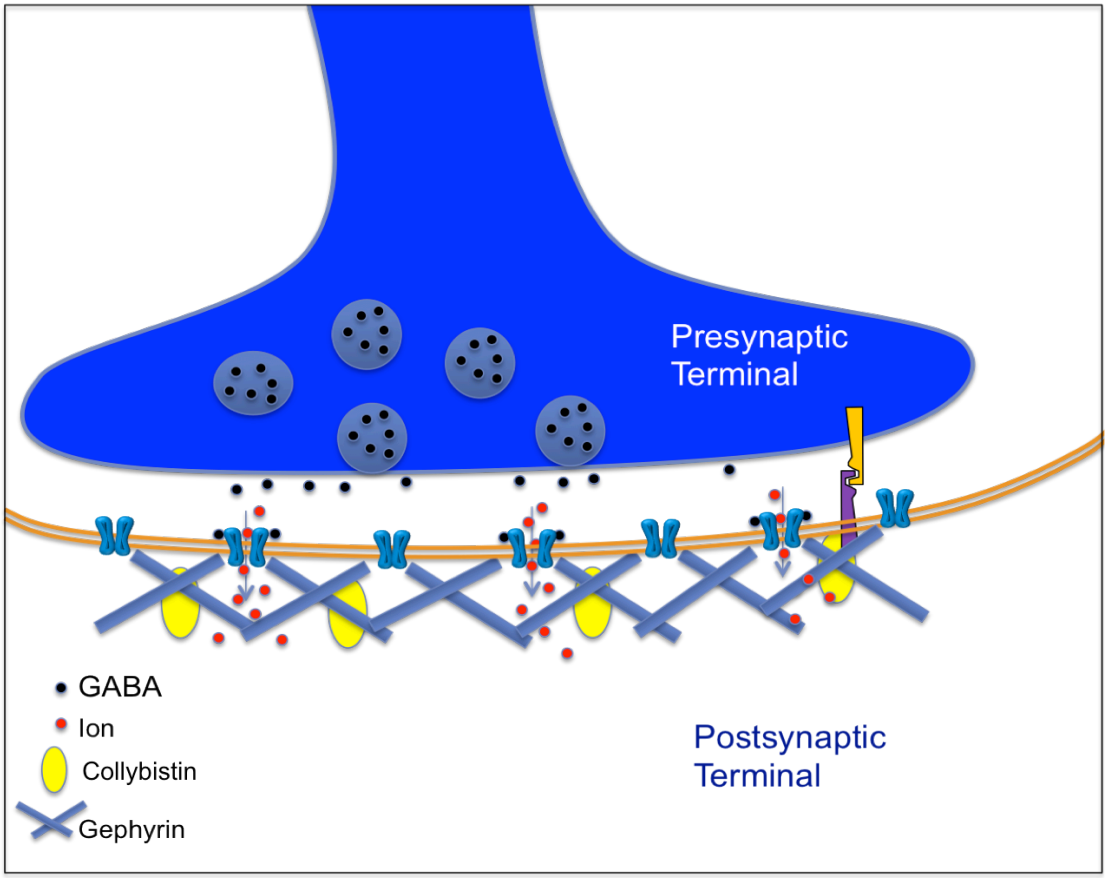


Figure 1.2 GABAergic synapse

Schematic representation of the role of collybistin and gephyrin at the post synaptic site of the GABAergic synapse. Gephyrin recessive mutations result in molybdenium cofactor deficiency, a severe encephalopathy with intractable seizures. Collybistin (*AHRGEF9* gene) result in severe epileptic encephalopathy.

The postsynaptic region of GABAergic and glycinergic synapses is organized around a core scaffolding protein, gephyrin (GPHN), which forms multimeric complexes by auto-aggregation, and by interacting with other postsynaptic proteins, including GABAA receptors (GABAAR), glycine receptors (GlyR), neuroligins, and collybistin (Fritschy et al., 2008). Gephyrin was named from the Greek term for “bridge”, in line with its proposed function of connecting NT receptors with the postsynaptic microtubule cytoskeleton (Lionel et al., 2013), while collybistin is a brain-specific guanine nucleotide exchange factor, crucial for inhibitory synapse development. Gephyrin is also essential for postsynaptic stabilization of inhibitory synapses, and abnormal *GPHN* expression has been described in temporal lobe epilepsy, both in patients and

in animal models (Fang et al., 2011). As at excitatory synapses, synaptic adhesion molecules may guide the differentiation and maturation of inhibitory synapses by coupling axodendritic adhesion events with the recruitment of specific membrane and signaling proteins.

1.3 Excitation and inhibition in the CNS

Excitation and inhibition in the mature CNS are mediated mainly by the NTs glutamate and GABA, respectively. Processing of neural information is thought to occur by integration of excitatory and inhibitory synaptic inputs. As such, precise control mechanisms are needed to maintain an appropriate balance between each synapse type. Remarkably, neurons outfit each synapse type with characteristic structural and neurochemical features (Kim and Sheng, 2004; Luscher and Keller, 2004). For example, most excitatory synapses are formed through contact between glutamate-releasing axonal terminals and postsynaptic dendritic spines containing glutamate receptors (Levinson and El-Husseini, 2005). One of the basic hypotheses of the present thesis is based on this concept of unbalance between excitation and inhibition in neurodevelopmental disorders, particularly those that include epilepsy and autistic traits in the symptoms' repertoire. Excitatory/inhibitory unbalance should be addressed from a neurodevelopmental perspective to study these disorders, considering the evolving changes of the CNS. In this work, focus was directed to the maturation of the GABAergic system, that can have opposite effects, depending on the developmental stage, and evolves from a relevant excitatory role to become the principal inhibitory system in the adult brain.

1.3.1 Epilepsy and the Synapse

Epilepsy, a disorder that affects 1% of the population worldwide (www.who.int/mental_health/neurology/epilepsy/euro_report.pdf) is classically thought to arise from an unbalance between excitation and inhibition either in a localized region, multiple brain areas or the whole brain. However, this is clearly a simplistic view, since several mechanisms related with synaptic release, ionic channels physiology, energetic metabolism and others, have

been arising from molecular and neurophysiological studies in epilepsy models.

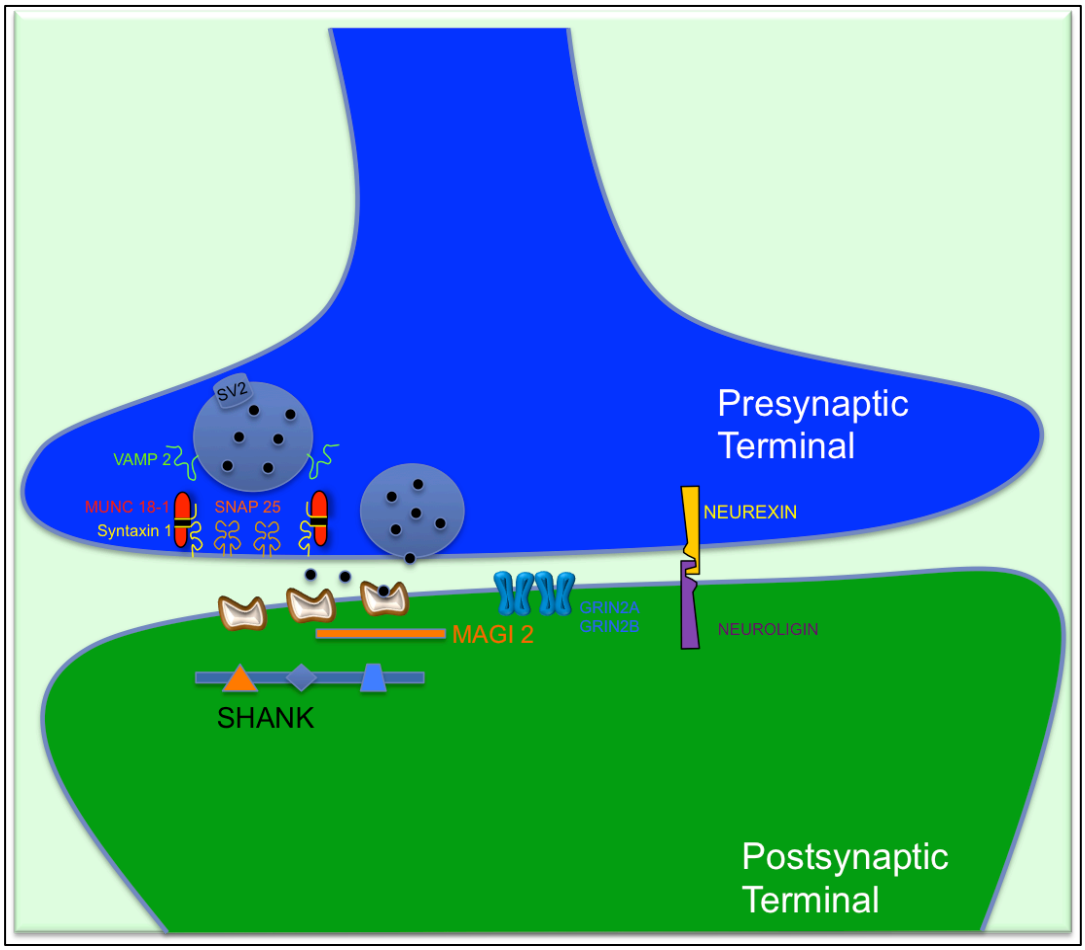


Figure 1.3 The Glutamatergic Synapse and Synaptic Proteins implicated in Epilepsy

Synaptic vesicle proteins 2 (SV2) are a small family of integral transmembrane glycoproteins that are localized to synaptic vesicles and appear to function as modulators of Ca2+-dependent exocytosis. Both clinical and experimental data suggest that SV2, and particularly SV2A, are involved in epilepsy (Crevecoeur et al., 2014). The anticonvulsant activity of levetiracetam (LEV), a powerful antiepileptic drug (AED), has been linked to its ability to bind to SV2A (Klitgaard and Verdru, 2007). More recently developed LEV analogues, such as brivaracetam and seletracetam, also bind to SV2A (Matagne et al., 2008).

The gene encoding syntaxin binding protein 1 (STXBP1, also known as MUNC18-1), an essential protein for synaptic vesicle release, has been associated with different epileptic syndromes: Ohtahara Syndrome (Saitsu et al., 2008; Saitsu et al., 2010), Infantile Spasms (Otsuka et al., 2010), Dravet Syndrome (Carvill et al., 2014) and

also other forms of epilepsy (Hamdan et al., 2011) or intellectual disability without epilepsy (Hamdan et al., 2011). Moreover, genetic variants of vesicle-associated membrane protein 2 (VAMP 2) and Synaptogamin XI have been associated with idiopathic generalized epilepsy (Yilmaz et al., 2014). At the post synaptic level, several neurotransmitter receptors, ionic channels and cotransporters, scaffolding molecules and signaling pathways have been implicated in epilepsy and epileptogenesis, and also as targets for anti epileptic drugs. Mutations in GRIN2A and GRIN2B encoding regulatory subunits of NMDA receptors cause mental retardation and early onset epileptic encephalopathy (Endele et al., 2010). Membrane-associated guanylate kinase inverted-2 (*MAGI2*) maps to 7q11.23-q21.1, and encodes for a scaffolding enzyme interacting with different presynaptic and postsynaptic receptors. A deletion including *MAGI2* has been associated with Infantile Spasms (Marshall et al., 2008), although Röthlisberger and colaborators found the same deletion without including *MAGI2* in patients with infantile spasms (Rothlisberger et al., 2010). SHANK proteins are elements of the postsynaptic density, linking synaptic transmission with the cytoskeleton. Deletions in *SHANK2* and *SHANK3* are known genetic risk factors for a broad range of neurodevelopmental disorders. *SHANK3* is located on the terminal end of chromosome 22 and is the main responsible gene for the phenotype of the 22q terminal deletion syndrome, also called Phelan-McDermid Syndrome. This neurodevelopmental syndrome presents with intellectual disability, autism, and a prominent severe speech delay. Seizures are common in patients with *SHANK 3* genetic defects (Soorya et al., 2013). Adapted from *[Neuronal communication and synaptic metabolism in childhood epilepsy]. García-Cazorla À, Cortès-Saladelafont E, Duarte S. Rev Neurol. 2015 Mar 1;60(5):219-28.* (Garcia-Cazorla et al., 2015)

Long-term changes in presynaptic morphology and synaptic vesicle recycling have been described in animal models (Zhou and Roper, 2010). These changes include alterations in the structure of mossy fiber synaptic buttons, including increased size, number of release sites, and number of vesicles in the reserve pool and in the readily releasable pool (Casillas-Espinosa et al., 2012). Moreover, mutations in genes that code for elements of the NT release machinery, and the efficacy of anti-epileptic drugs that act at the synaptic vesicle level bring the attention for the relevance of the presynaptic compartment for epilepsy. At the postsynaptic level, NT receptors, scaffolding

molecules and ion channels have also been implicated, as depicted in Figure 1.3.

From a child neurology perspective, it is particularly relevant to consider the normal patterns of brain maturation, regarding the GABAergic and glutamatergic systems. The immature brain is skewed towards excitation and different molecular mechanisms contribute for this status: the depolarizing effect of GABA and the high expression of AMPA and NMDA receptors with a composition that enhances excitability of neuronal networks.

1.4 Synaptic Plasticity

Chemical synapses are remarkable for their evolutionary specialization, serving as highly specialized cell junctions in the body, and retain considerable plasticity throughout their lifetime. Synaptic plasticity can be defined as enduring activity-driven changes in the efficacy of synaptic transmission (Chapleau et al., 2009). The strength of synaptic transmission can be modulated by either presynaptic or postsynaptic mechanisms and changes in this strength can last from a few hundred milliseconds to weeks or even longer. Short-term plasticity mechanisms are predominantly mediated through local modifications of proteins, while long-term plasticity leads to structural changes in synaptic contacts and is dependent on de novo protein synthesis (Lynch, 2004). Synaptic plasticity is considered as the basis for all key functions of the brain, including the processes of learning and memory (Grabrucker, 2014). The “hot spot” of this structural plasticity is the dendritic spine. Dendritic spines are small protrusions extending from dendrites that are the main postsynaptic site of excitatory glutamatergic synapses in the brain. The dynamic and plastic nature of spines observed *in vitro* (Fischer et al., 1998) and *in vivo* (Mizrahi et al., 2004) supports the hypothesis that changes in the shape and number of spines contribute to the mechanisms of memory formation and storage (Chapleau et al., 2009; Ethell and Pasquale, 2005; Nimchinsky et al., 2002; Yuste and Bonhoeffer, 2001)

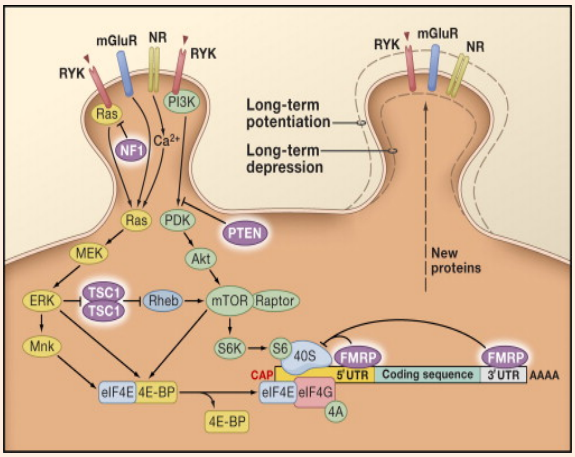
The most well established models for activity-dependent synaptic strengthening and weakening are long-term potentiation (LTP) and long-term depression (LTD), respectively. Structural changes linked to synaptic strength can be addressed experimentally with stimulation patterns that induce long-

term potentiation (LTP). LTP induces changes in spine number and morphology in regions important in learning and memory, such as the hippocampus and cortex. Induction of LTP followed by electron microscopy analysis revealed increases in spine size and number in the hippocampus and spine number in the neocortex (Harms and Dunaevsky, 2007). Unraveling the molecular mechanisms governing long-term synaptic plasticity is a key to understanding how the brain stores information in neural circuits and adapts to a changing environment. Mutations in genes that code proteins which are key players in synaptic plasticity, are considered the cause of several genetic neurodevelopmental disorders like RTT, Angelman Syndrome, Tuberous Sclerosis, Neurofibromatosis type 1 and Fragile X.

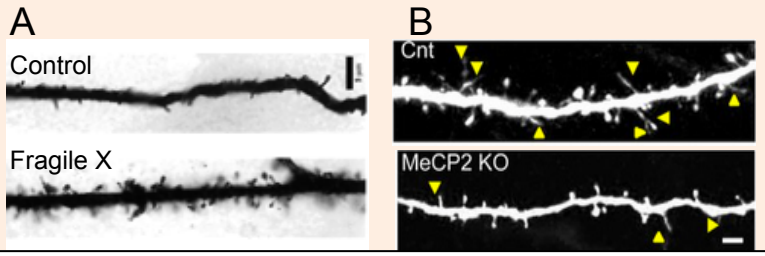
Box 1. Glutamatergic Synapse Plasticity and Genetic Neurodevelopmental disorders

Evidence indicates that many of the genes that are mutated in neurodevelopmental disorders with epilepsy, autistic traits and cognitive impairment are crucial components of the activity-dependent signaling networks that regulate synapse development and plasticity. Deregulation of activity-dependent signaling pathways in neurons may therefore have a key role in these disorders. The Ras/ERK and PI3K/mTOR signaling pathways couple synaptic activity to the translational machinery and play essential roles in protein synthesis-dependent LTP and LTD. The PI3K/mTOR signaling cascade is a central regulator of cell growth, proliferation, survival, and cap-dependent protein translation. In brain, components of the mTOR pathway are present at synapses, where they regulate dendritic spine morphology, and are essential to synaptogenesis. Inactivating mutations in several negative regulators of the ERK and mTOR pathways, including *NF1*, *PTEN*, and *TSC1/2*, are responsible for genetic disorders with a high prevalence of cognitive impairment and autism. *FMRP* represses translation of specific target mRNAs, and loss of *FMRP* expression in fragile X syndrome also leads to cognitive impairment and autism. These signaling pathways are recruited downstream of the activation of NMDA receptors, metabotropic glutamate receptors, and neurotrophin Trk receptors.

Interestingly, the Akt-mTOR (but not the ERK) signaling pathway is also affected in this RTT mouse model, although in this case the Akt-mTOR proteins (Akt, mTOR, p70S6K and S6) are hypophosphorylated, resulting in reduced protein synthesis. Additionally, BDNF levels reduction described in RTT can contribute to the pathway downregulation, since BDNF/TrKB signaling is a relevant upstream trigger . It has been postulated that the performance of neuronal networks mediating cognition is a function of the level of synaptic protein synthesis.



Increases or decreases in the levels of plasticity-related proteins available to active synapses in autistic neurons may cause corresponding changes in synaptic connectivity, compromising network performance and producing cognitive impairment. In several single-gene disorders associated with autism, such as Fragile X syndrome, levels of synaptic protein availability and connectivity are increased, but in Rett's syndrome these levels are decreased. In spite of an upregulation or a downregulation of protein synthesis synaptic pathways, with an increase (A) or decrease (B) in synaptic density, these disorders have in common clinical features like epilepsy, autistic traits and cognitive impairment and also aberrant spine morphology an impaired LTP in their animal models.



Adapted from Kelleher and Bear, 2008, Irwin et al, 2000, Landi et al, 2011

1.5 GABA

GABA is the major inhibitory neurotransmitter in the adult brain. Glutamatergic neurons correspond to 80 to 90% of the neuronal population in the cortical

circuits whereas GABAergic neurons constitute the remaining 20 to 10%. However, in spite of this underrepresentation, GABA plays a crucial role in controlling the activity of neurons in the entire brain (Hu et al., 2014). GABA-producing inhibitory interneurons are essential for regulating cortical excitability and coordinating appropriate behaviors. GABAergic interneurons control several aspects of neuronal circuit function from neuronal excitability (Swadlow, 2003) and integration (Pouille and Scanziani, 2001), to the generation of temporal synchrony and oscillation among networks of excitatory neurons (Somogyi and Klausberger, 2005) thought to support distinct brain states and high cognitive functions (Klausberger and Somogyi, 2008). Interneuron dysfunction has been implicated in many disorders like epilepsy (Freund and Katona, 2007), autism (Yip et al., 2008), schizophrenia (Nakazawa et al., 2012), several neurodevelopmental disorders like fragile X (Cea-Del Rio and Huntsman, 2014) and RTT (Tomassy et al., 2014) and can contribute to impairments in learning and memory or social behaviors.

GABA receptors are the major inhibitory receptors in human synapses. They are of two types. GABA A receptors (GABAAR) are fast-acting ligand gated chloride ion channels that mediate membrane depolarization and thus inhibit NT release (Michels and Moss, 2007). GABA B receptors are slow acting metabotropic G protein coupled receptors that act via the inhibitory action of their G alpha/Go subunits on adenylate cyclase to reduce cAMP levels and decrease Protein Kinase A activity. In addition, their G beta/gamma subunits interact directly with N and P/Q Ca2+ channels to decrease the release of Ca2+. GABA B receptors also interact with Kir3 K+ channels and increase the influx of K+, leading to cell membrane hyperpolarization and inhibition of channels such as NMDA receptors (Pinard et al., 2010). Antagonists of GABA were recognized as epileptic agents leading to the classical concept of the excitatory/inhibitory balance needed to avoid seizures and hyperactivity. This was further reinforced by the discovery of the antiepileptic actions of pro-GABA drugs like the benzodiazepines and phenobarbital (de Los Heros et al.). However, GABAergic systems fulfill a plethora of roles (Ben-Ari, 2014) that evolve during neural development and, as a consequence, pharmacological actions of GABA receptors targeted drugs translate these

developmental changes. Finding viable therapeutic treatments to modulate GABAergic transmission in neurodevelopmental disorders is thus both of great relevance and a big challenge. Addressing this issue implies the study of the fine modulation of GABAergic transmission during physiological conditions as a starting point to tackle dysfunctional transmission in neurodevelopmental disorders. The overall goal of such a challenging approach is to find a way to safely modulate GABAergic signaling without questioning the delicate equilibrium of the neuronal-network activity in a developing brain (Deidda et al., 2014).

1.5.1 GABA during development

The immature brain is highly susceptible to seizures. This heightened susceptibility is related, at least in part, to developmental changes that skew the balance between excitatory and inhibitory neurotransmitter systems in the brain, in favor of the former (Brooks-Kayal, 2005). During early development, GABA is depolarizing and mostly excitatory due to high intracellular chloride concentration (see section 1.5.1.1) and GABAergic interneurons regulate key developmental steps, from cell migration and differentiation to experience-dependent refinement of neuronal connections (Ben-Ari, 2002). In recent years, many studies have started to elucidate the development and function of cortical GABAergic circuits (Chattopadhyaya, 2011). BDNF also plays a critical role in migration and maturation of GABAergic interneurons. Once neurons have finished their migration and differentiation, they start shifting from a largely genetically oriented sequence of development to one in which the environment plays a progressively determinant role via synaptic currents. One of the first problems that the developing neuron must solve is how to establish and preserve equilibrium between excitation and inhibition. In adult neurons, inhibition is principally mediated by GABAergic synapses that generate a hyperpolarization via chloride permeable channels (Gozlan and Ben-Ari, 2003). However, during the first stages of development GABA actions can skew the excitatory/inhibitory actions towards excitation.

1.5.1.1 Cation Chloride Cotransporters

GABA exerts its fast hyperpolarizing effect through activation of anion-permeable GABAA receptors, predominantly Cl^- (Kaila, 1994). The inhibitory postsynaptic potentials (IPSPs) generated in mature neurons by GABAA and glycine receptors activation are dependent of a low intracellular chloride concentration (Kaila et al., 2014a). Intracellular chloride concentration is reduced in mature neurons due to a developmental upregulation and functional activation of the neuron-specific K^+– Cl^- cotransporter 2 (KCC2) that extrudes chloride from the cell (Rivera et al., 1999; Stein et al., 2004) (Zhu et al., 2005). However, during early development, GABAA-receptor-mediated responses are often depolarizing and this property might be relevant for several developmental phenomena like neuronal proliferation, migration and consolidation of synaptic connections. The magnitude and direction of the ionic current through GABAARs depends on its driving force, defined as the difference between the electrochemical equilibrium potential of Cl- anions (reversal potential, Ecl) and the resting membrane potential of the neuron. If this difference is positive or negative, there will be a net flux of Cl- anions through the plasma membrane following GABAAR opening, and this will result in a change in the membrane potential of the neuron. In particular, the net flux of Cl- anions through GABAAR (i.e., toward inside or outside the cell) is defined by its intracellular concentration ([Cl-]i). (Deidda et al., 2014). The higher intracellular chloride concentration in young neurons depends on high expression of NKCC1 (Dzhala et al., 2005), and results in depolarizing GABAAR transmission. As development proceeds, KCC2 becomes expressed at higher levels than NKCC1 (Dzhala et al., 2005; Rivera et al., 1999), and hyperpolarizing GABAergic transmission takes over (Ben-Ari, 2002) (Cancedda et al., 2007) (Sernagor et al., 2010). Balanced activity of NKCC1 and KCC2 is therefore determinant for neuronal response to GABA (Deidda et al., 2014).

The mammalian KCC2 gene generates two N-terminal splice isoforms, KCC2a and KCC2b, but only the of expression of KCC2b is strongly up-regulated during development, and accounts for most (> 90%) of the total KCC2 protein in the adult murine cortex (Uvarov et al., 2009). GABAergic

responses remain depolarizing in KCC2b knockout cortical cultures (Zhu et al., 2005), indicating that this isoform is responsible for the establishment of hyperpolarizing GABAAR-mediated transmission (Blaesse et al., 2009). Homozygous KCC2b knockout mice exhibit generalized seizures and die during the second postnatal week, whereas KCC2b heterozygotes, which express ~50% of the wild-type mouse KCC2 (mKCC2) protein level (Huber et al.), show increased susceptibility to induced seizures (Woo et al., 2002). Aditionally, genetic ablation of *KCC2* prevents spinogenesis and excitatory synapse formation in immature neurons, through a mechanism independent of KCC2 transporter function that requires instead its interaction with actin binding (Chamma et al., 2013). Indeed, KCC2 could act as a synchronizing factor in the functional development of glutamatergic and GABAergic synapses in cortical neurons and networks(Li et al., 2007).

In immature neurons, BDNF–TrkB signalling promotes KCC2 expression (Aguado et al., 2003; Carmona et al., 2006; Ludwig et al., 2011). In contrast, in mature neurons the BDNF/TrkB signaling mediates activity-dependent decrease in KCC2 expression, and this decrease can contribute to the pathogenesis of epilepsy after an insult like ischemia or trauma, bringing the cells to a hyperexcitable state (Overman and Carmichael, 2014) (Kaila et al., 2014b). BDNF is secreted after an insult and the KCC2 decrease seems to be an adaptative mechanism that has the secondary effect of increased excitability and epilepsy. For instance, the frequently reported downregulation of KCC2 following neuronal trauma may be part of a general adaptive cellular response that facilitates neuronal survival by reducing the energetic costs, needed to preserve low intracellular chloride concentration and also by facilitating functional recovery through the removal of GABAergic inhibitory constraints on neuroplasticity (Kaila et al., 2014a).

The process of development of hyperpolarization after GABAAR activation seems to include an upregulation of KCC2 expression, but also post transcriptional mechanisms like phosphorylation and fast membrane trafficking of the cotransporter. Bumetanide sensitive NKCC1 (Na^+-K^+-$2Cl^-$ cotransporter, which allows chloride to accumulate inside the cell) is also involved, since higher intracellular chloride concentration in young neurons

depends on high expression of NKCC1 (Dzhala et al., 2005), as depicted in Figure 1.4.

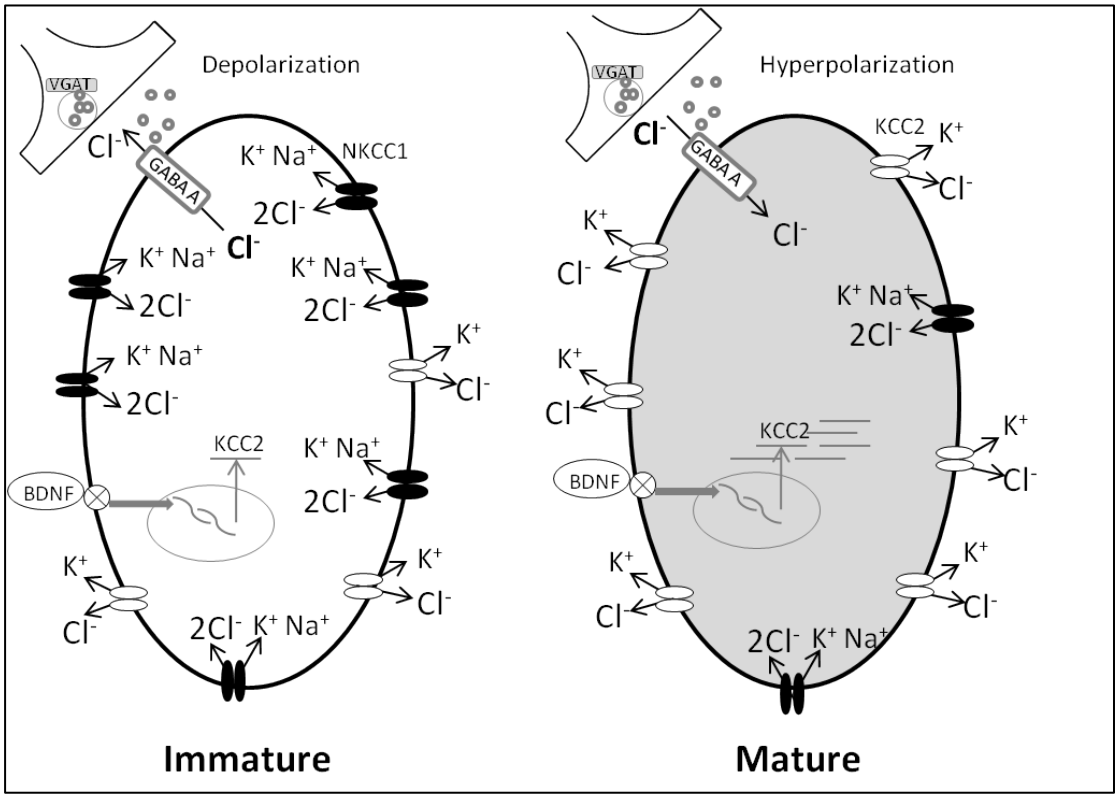


Figure 1.4 Schematic representation of the developmental switch in chloride cotransporters expression

KCC2 transports Cl− out of the cell; NKCC1, transports Cl− into the cell. The developmental increase in KCC2 and the decrease in NKCC1 triggers the switch from depolarizing to hyperpolarizing GABAAergic signaling (Brooks-Kayal, 2005; Galanopoulou, 2008a). Adapted from *[Neuronal communication and synaptic metabolism in childhood epilepsy]. García-Cazorla À, Cortès-Saladelafont E, Duarte S. Rev Neurol. 2015 Mar 1;60(5):219-28.* (Garcia-Cazorla et al., 2015)

Since the limitations of experimentation in humans have been an obstacle in obtaining direct evidence for the age-related changes in GABAAR physiology (Galanopoulou, 2008a), we need to find strategies to understand GABAAR physiology *in vivo*, in humans. This understanding will allow the delineation of improved treatments for early epilepsies, where this process might be in course and not completely developed (Puskarjov et al., 2014).

1.5.2 Rett Syndrome and the Synapse

Rett syndrome (RTT; MIM 312750) was first identified by Andreas Rett in 1966, after the observation of 22 patients with similar unique symptoms (Rett, 1966). The constellation of features that represent RTT became more widely recognized as a specific neurodevelopmental disorder after subsequent larger studies by Hagberg et al (Hagberg et al., 1983). This rare neurodevelopmental disorder is caused primarily by mutations in the X-linked gene *MECP2* (Methyl-CpG binding Protein 2). RTT is considered the leading cause of severe intellectual disability in females with a prevalence estimated at 1/9,000 in girls under the age of 12, whereas prevalence in the general population is estimated at approximately 1/30,000 (source: Orphanet).

1.5.3 Clinical features

Patients with RTT appear to develop normally up to 6–18 months of age. They typically achieve normal neurodevelopmental milestones, from gross and fine motor functions to social communication skills. The head circumference of RTT patients is normal at birth but head growth begins to decelerate and leads to microcephaly by the second or third year of life. Distinctive aspects contributing to the diagnosis include developmental regression, with accompanying loss of hand skills, mobility skills, and speech and stereotypic hand movements. As the syndrome progresses, social withdrawal and loss of language become apparent with features reminiscent of autistic traits. The onset of cognitive deterioration is accompanied by loss of motor coordination and the development of ataxia and gait apraxia. Associated features such as respiratory/autonomic abnormalities (Julu et al., 2001), seizures, scoliosis, growth deficits and early hypotonia are very prevalent. Neurophysiologic evaluations show cortical hyperexcitability on the electroencephalogram (Willemsen et al.) and the occurrence of rhythmic slow activity, primarily in the frontal-central regions. Epilepsy is frequent in RTT, has an age-related pattern of occurrence and is associated with greater clinical severity. Other associated abnormalities during the postregression phase include teeth grinding, night laughing or crying, screaming fits and anxiety episodes elicited by distressing external events (Mount et al., 2001).

Most girls with RTT lose mobility and are often wheelchair-bound with severe scoliosis and dystonia during the teenage years. Impairment of the autonomic nervous system in RTT is suggested by vasomotor disturbances like hypotrophic cold blue feet, life threatening cardiac abnormalities (tachycardia, prolonged corrected QT interval, and sinus bradycardia) that can contribute to the higher incidence rate of sudden unexpected death in RTT patients, severe constipation, oropharyngeal dysfunction and abnormal breathing patterns. Even with high risk of sudden death because of respiratory and cardiac dysfunctions, several patients survive till the 6th or 7th decade of life with limited mobility (Chahrour and Zoghbi, 2007; Glaze et al., 2010; Hagberg, 2005; Neul et al., 2010; Zoghbi and Bear, 2012).

1.5.4 MeCP2

During development, the expression pattern of different groups of genes are precisely regulated in a spatiotemporal manner, with intervention of multiple gene regulatory mechanisms (Ding, 2015). Time and space are the key metrics to all cortical operations, and developmental events such as regional patterning, neurogenesis, differentiation, migration, axogenesis, and synaptogenesis have different developmental timings that may vary throughout the CNS, increasing the complexity of this process. As in other cell types, precise control of neuronal gene expression is accomplished in part through the regulation of chromatin. Early studies found that MeCP2 interacts specifically with methylated cytosine with guanine (mCG) (Jones et al., 1998; Lewis et al., 1992; Nan et al., 1996). Recent in vivo studies in the adult mouse brain confirm the preference of MeCP2 for mCG over nonmethylated CG, although MeCP2 clearly binds widely across the entire genome (Skene et al., 2010). Using mice in which the MeCP2 protein is tagged with a fluorescent green protein, Chen and coworkers (Chen et al., 2015) determined genome-wide MeCP2 binding profiles in the adult animal brain. In addition to the expected finding of MeCP2 binding to methylated cytosine with guanine (mCG) with high affinity, they also found that MeCP2 binds to cytosine when it is followed by either adenine, cytosine or thymine instead of guanine (non-CG methylation or mCH). Whole-genome analysis using base-pair DNA

methylation and MeCP2 binding profiles indicate that MeCP2 shows the highest binding affinity for mCG, followed by mCH, and then significantly lower affinity for nonmethylated DNA elements. Chen and coworkers propose a model to explain why RTT patients have an initial period of postnatal life without symptoms: (i) During postnatal brain development the levels of MeCP2 and mCH increase as neurons mature, whereas mCG remains relatively stable. (ii) When DNA methylation patterns are fully developed together with higher levels of MeCP2 in the adult brain, differential binding affinity of MeCP2 for mCG, mCH and nonmethylated DNA becomes apparent. (iii) In this context, MeCP2 can now bind available mCH sites to influence transcription. Thus, changes to MeCP2 levels or function, which lead to disease related phenotypes, only become apparent after a long period of brain maturation.

MeCP2 functions at multiple levels, from transcriptional control to post-transcriptional regulation, and it is recognized as a key regulator in various cell types including excitatory neurons, inhibitory neurons, and glia. Furthermore, MeCP2 protein undergoes multiple posttranslational modifications such as phosphorylation, SUMOylation and acetylation, which impact its functions.

Neuronal activity induces the phosphorylation of MeCP2 at Ser421 (Zhou et al., 2006) raising the possibility that activity-dependent phosphorylation of MECP2 mediates a genome-wide chromatin response to neuronal activity (Cohen et al., 2011). This fact can also contribute for the first manifestations of RTT in the first year of life — a time when experience is shaping synapse development. MeCP2 is a chromatin regulator that is modified in response to neuronal activity, which suggests that RTT may be due to a defect in the process of activity-dependent MecP2 mediated chromatin regulation or gene transcription that controls synaptic development, plasticity and behavior. The formation and adaptation of functional circuits within the brain is modulated by experience, like what happens with the acquisition of language during a child's development: the sounds of words spoken and heard lead to signaling between neurons within the brain and trigger changes in gene expression that promote connectivity. This type of experience-dependent gene activation

appears to rely on MeCP2 phosphorylation (Cohen et al., 2011; Tao et al., 2009; Zhou et al., 2006). MeCP2's role in translating extracellular cues into the changes in gene expression is determinant for brain development and function (Cohen and Greenberg, 2010) and provides some insights to understand synaptic impairment in RTT. Accordingly, MECP2 has recently been shown to be required for experience-dependent synaptic remodelling of the retinogeniculate synapse. In Mecp2 knockout mice, the initial development and refinement of the retinogeniculate circuit proceeds without impairment, during a phase of development that does not depend on visual experience. However, during a late stage of development when visual experience is required for modulation and maintenance of the retinogeniculate circuit, the synapses that form on the neurons within this circuit are abnormal in Mecp2 knockout mice, resulting in impaired synaptic plasticity on visual deprivation. This supports the possibility that during the late phase of development of the retinogeniculate circuit, MECP2 may regulate an experience-dependent gene expression program that controls synaptic remodelling, a program that could potentially be controlled by activity-dependent phosphorylation of MECP2 (Noutel et al., 2011).

RTT is mainly caused by MeCP2 deficiency, but duplications of MECP2-containing loci are also detrimental to neural development and proper brain functions. Patients carrying MECP2 duplications usually manifest autistic features, mild RTT phenotypes, and mental retardation (Ramocki et al., 2009). All these results underscore the importance of the homeostatic modulation of MeCP2 expression, indicating that the dosage of MeCP2 protein is critical for the development of the CNS (Cheng and Qiu, 2014).

There is also evidence that loss of MeCP2 impacts on the balance between inhibition and excitation in the brain, altering the neuronal response to sensory experience, with interference in glutamatergic system (Dani et al., 2005) and also in GABAergic system (Chao et al., 2010). Regional quantifications of GABA and glutamate suggest that this unbalance varies through different brain areas, and seems to impact mainly GABAergic system and, to a less extent, the glutamatergic one (El-Khoury et al., 2014).

Together, these studies suggest that MeCP2 is a key regulator of chromatin architecture and gene expression that modulates synaptic development, function and plasticity, and that RTT can be considered a synaptopathy (Boggio et al., 2010) (Weng et al., 2011).

1.5.4.1 Rett Syndrome, BDNF and adenosine

Since its discovery almost three decades ago, the secreted neurotrophin BDNF has been firmly implicated in the differentiation and survival of neurons in the CNS. More recently, BDNF has also emerged as an important regulator of synaptogenesis and synaptic plasticity mechanisms, during development and adult life. BDNF actions are exerted through p75 neurotrophin receptors and also Trk receptors (Figure 1.5). Among neurotrophins, BDNF, and its major receptor TrkB, has the most abundant and widespread expression in the developing and adult mammalian brain (Murer et al., 2001). BDNF is a critical player in activity-dependent neuronal development, not only for its role in normal physiology but also for the multiple links to neurodevelopmental disorders associated with mental retardation and autism spectrum disorders. Indeed, deregulation of any step in BDNF synthesis and release (i.e. transcription, translation, vesicular packaging, processing and trafficking, Ca^{2+}-dependent regulated release, and signaling) may result in improper axonal, dendritic and synaptic development, as well as impaired activity-dependent refinement of synaptic connections during brain development. BDNF binding to the TrkB receptor has been shown to promote the induction and maintenance of LTP, and BDNF or TrkB deficient mice exhibit a marked reduction in LTP (Cao et al., 2013). Through TrkB signaling, BDNF exerts a determinant action in synaptic plasticity orquestrating a process that involves *de novo* synthesis of synaptic proteins, along with protein trafficking and degradation. In response to synaptic inputs, the time, place, and amount of cellular protein synthesis needs to be fine-tuned. Disruptions in this process are at the basis of several neurodevelopmental disorders and BDNF interacts with several protein products of genes already implicated in these disorders like *TSC 1* and *2*, *PTEN* or *FMRP* (Panja and Bramham, 2014).

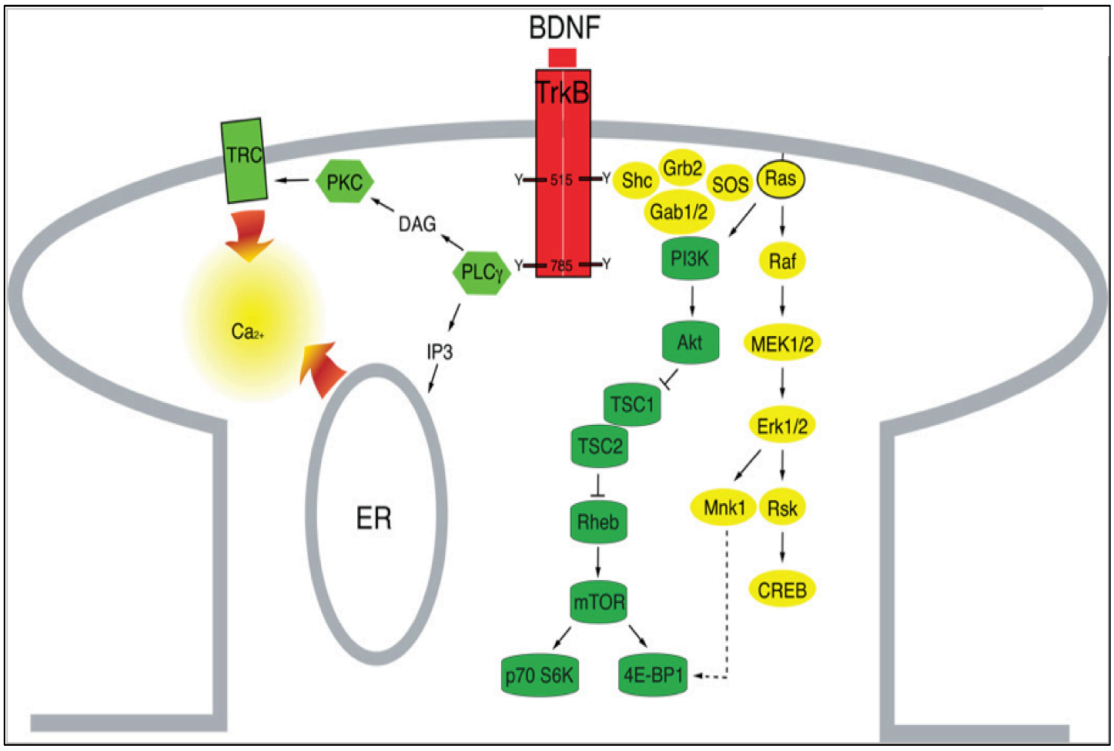


Figure 1.5 BDNF-TrkB signaling Regulates Multiple Events through PLCγ, PI3K and MAPK pathways.

PI3K pathway regulates trafficking and PSD-95 to a synapse and cAMP regulates formation of synaptic PSD-95-TrkB complex. BDNF-TrkB signaling activates MAPK/Erk, increasese cAMP and activate CREB-regulated gene transcription. BDNF-TrkB signaling regulates protein translation through both MAPK/Erk and PI3K-Akt-mTOR pathways. *Adapted from Yoshii A, Constantine-Paton M. Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease.* (Yoshii and Constantine-Paton, 2010)

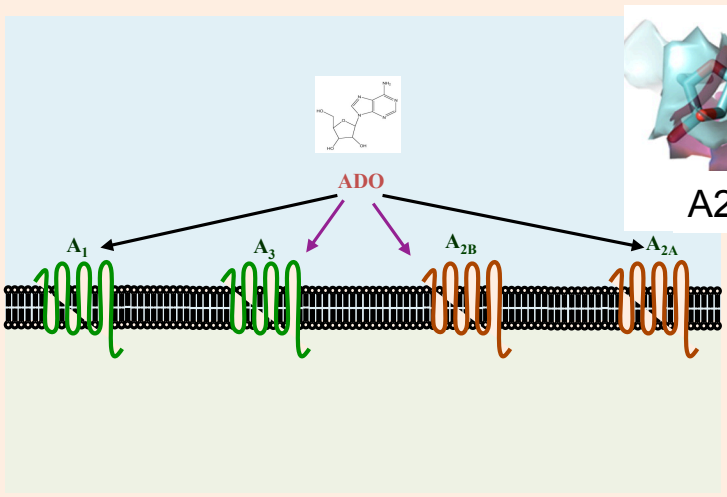
While a physiological amount of BDNF in the normal brain has been demonstrated to have positive effects on learning and memory, both increased and decreased levels of BDNF may disrupt the equilibrium between inhibitory and excitatory neurotransmission in the brain (Singh et al., 2006), leading to a loss of synaptic refinement and consequently impairing LTP, learning and memory (Johnson et al., 2007).

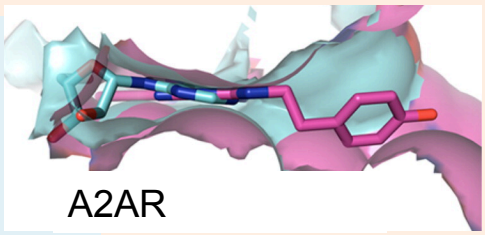
An increasing body of evidence points to a deregulation of BDNF signaling in RTT. MeCP2 transcriptionally regulates BDNF. Lower *Bdnf* mRNA and BDNF protein levels, as well as impaired BDNF trafficking and activity-dependent release, have been highlighted as pathophysiological mechanisms of RTT

disease progression (Xu et al., 2014). Indeed, conditional *Bdnf* mutant mice show a similar phenotype to that observed in *Mecp2* deficient mice. The similar phenotypic traits are: hindlimb clasping that mimics the stereotypic hand-wringing behavior characteristic of RTT patients, small brain weight and small neuronal size (Chang et al., 2006). On the other hand, several strategies to treat RTT through the manipulation of BDNF signaling have been tried with promising results (Chapleau et al., 2009). In spite of these results, the clinical use of BDNF has been hampered by critical limitations like the inability of BDNF to penetrate the blood brain barrier (BBB) and its short *in vivo* bioavailability (Zuccato and Cattaneo, 2009). Recently, a new line of investigation was initiated to identify small molecules that could potentiate the actions of endogenous neurotrophic factors present in the nervous system. One of the drugs attracting much attention is adenosine, due to its ability to modulate the actions of several molecules, including BDNF, in the nervous system mostly through the activation of adenosine receptors of the A_{2A} (A2AR) and A_1 (A1R) subtypes. Modulation of adenosine receptors has long thought to be a useful strategy in the treatment of multiple pathological states in the nervous system, such as sleep disorders, epilepsy and neurodegenerative diseases (Sebastiao and Ribeiro, 2009a). Actually, clinical trials using drugs that modulate adenosine receptors were already developed (Chen et al., 2007). Moreover, adenosine kinase (ADK) manipulation could also be other way to modulate adenosine levels. ADK, by phosphorylating intracellular adenosine to AMP, maintains an inward adenosine gradient, driving adenosine influx into the cell (Boison, 2009). When ADK levels are decreased, the resulting increase in adenosine levels leads to resistance to epileptic seizures and neuronal cell loss (Li et al., 2008). Interestingly, it was recently demonstrated that in transgenic mice that underexpress ADK in the forebrain, and therefore where adenosine levels are increased, BDNF effects upon synaptic transmission are strongly favoured due to the activation of A2ARs by endogenous adenosine (Diogenes et al., 2012).

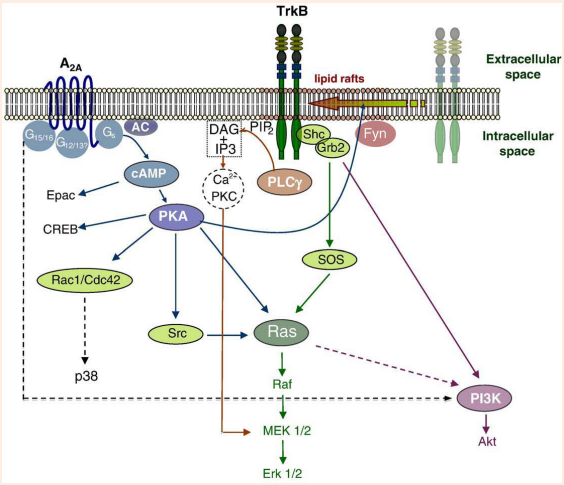
Box 2. Adenosine, Adenosine receptors and modulation of BDNF signaling by A2AR

ATP and adenosine (ado) are essential components of all living cells, where they mediate energy conversion and nucleic acid synthesis. In addition to these functions, both molecules play a pivotal role in extracellular signaling. Whereas ATP may function as a neurotransmitter, co-transmitter, gliotransmitter, or even a neuromodulator at specific synapses, adenosine always assumes neuromodulatory actions. Adenosine clearance is mostly mediated by astrocytic adenosine kinase, which phosphorylates intracellular adenosine to AMP and critically controls homeostatic adenosine actions. Adenosine activates P1 receptors, composed of high-affinity (A1 and A2A) and low-affinity (A2B) receptor subtypes; A3 receptor affinity for adenosine is species-dependent, and is high in humans and low in rodents.
Basal extracellular adenosine levels are likely to only activate high-affinity A1R and A2ARs, the main receptors activated with basal adenosinergic brain tonus.





The adenosine A2AR is a G-protein-coupled receptor that has been extensively studied during the past few decades because it offers numerous possibilities for therapeutic applications.



Signaling cascades activated by adenosine A 2A and TrkB receptors: Adenosine A2AR are mainly implicated in the activation of G proteins and the cAMP/PKA signaling pathway. TrkB receptors predominantly signal through the activation of PLCγ, Akt and MAPK pathways. As depicted in the figure, the presence of common signaling intermediates downstream A2A and TrkB receptors might account for the facilitatory action of adenosine A2ARs activation upon TrkB-mediated effects. Activation of A2ARs also induces translocation of TrkB receptors to lipid rafts. TrkB localization in these membrane microdomains is important for BDNF actions and may be involved in the modulation of TrkB-mediated signaling cascades by A2ARs.

Adapted from De Lera Ruiz et al, 2014; Dias et al, 2013; Sebastião et al, 2011

In summary, A1R are typically inhibitory receptors and its activation has been suggested to be important in the control of epilepsy (Boison, 2009). A2ARs are excitatory receptors which activation is known to potentiate synaptic actions of BDNF (Sebastiao et al., 2011). Therefore, controlling epilepsy through the activation of A1Rs, and potentiating BDNF effects by the

activation of A2ARs, adenosine-based strategies emerges as a great possibility for RTT pharmacological treatment. Although systemic application of adenosine is precluded by peripheral side effects, several delivery approaches have been designed to overcome this problem (Boison, 2009). Focal adenosine achieved by devices such as synthetic slow-release polymers, pump systems, cellular implants or cell therapy or gene therapy approaches to locally augment the adenosine system are options already explored for epilepsy treatment and therefore reasonable to be considered for RTT patients. Another possibility is the use of specific receptor agonists, in order to modulate adenosinergic system.

1.6 Epileptic encephalopathies and the Synapse

Early seizures impact the neural development of thousands of human newborns, annually. Understanding the deleterious effects of seizures in early brain development and the molecular mechanisms underlying their origin is fundamental for age and mechanism specific treatment. Epileptic Encephalopathies (EEs; MIM 308350) are conditions in which epileptic activity dominates the clinical picture (Dulac, 2001), associated with cognitive impairment and autism.

Classically, the term epileptic encephalopathy presupposes that epilepsy itself contributes to encephalopathy. However it is often difficult to weigh the role of epilepsy in newborns or infants against pre-existing severe neurological dysfunction, given that neither seizures nor electroencephalographic abnormalities substantially modify a poor outcome that is mainly explained by the underlying etiology, and its interference with normal brain development (Basel-Vanagaite et al., 2013). EEs are age-dependent, and the corresponding unique developmental window of onset seems to confer disease susceptibility. Ohtahara Syndrome (OS), Early Mioclonic Epilepsy (EME), and West Syndrome (WS) are associated with severe developmental outcomes. The etiology in many patients remains undetermined.

Early infantile epileptic encephalopathy with suppression-burst (EIEE) is the earliest form of infantile epileptic syndrome and includes OS and EME. The transition from EIEE to WS occurs in 75% of individuals with EIEE, suggesting

a common pathological mechanism between these two syndromes (Saitsu et al., 2010).

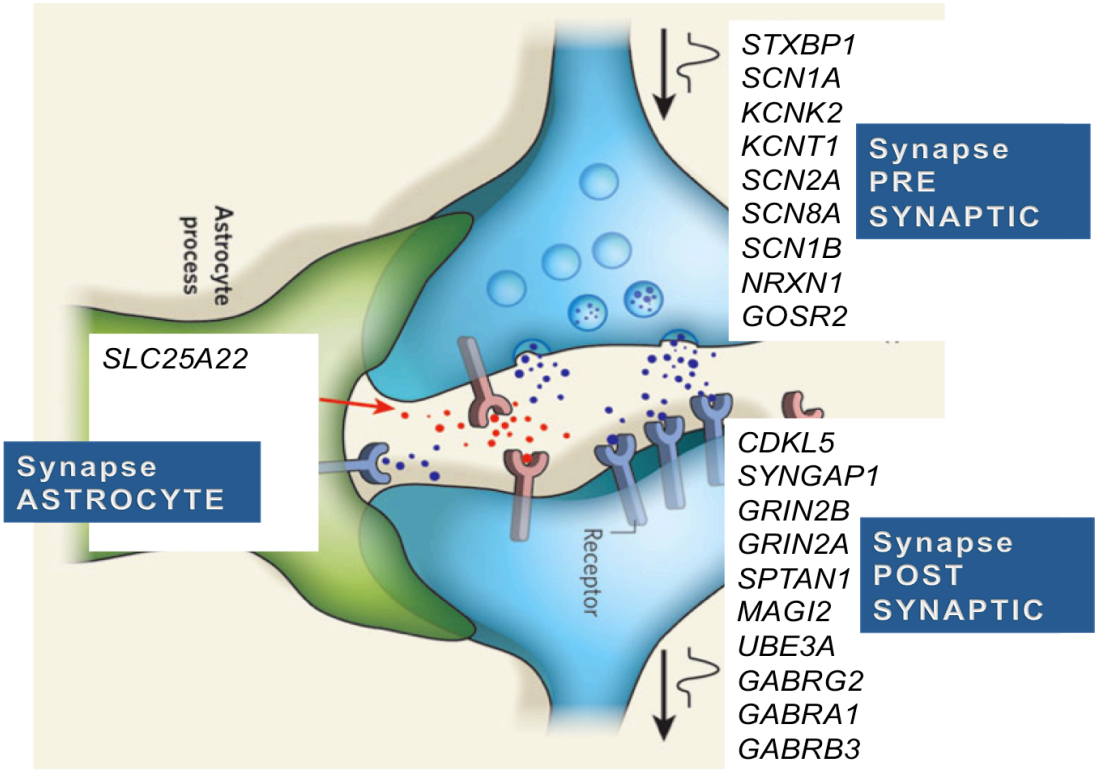


Figure 1.6 Genes implicated in early epileptic encephalopathies and the synapse

A significant group of proteins coded by genes mutated in EIEEs have relevant roles at the synaptic level, mainly at the pre and postsynaptic densities, but also in astrocytes, that reduce the excess of glutamate in the synaptic cleft. *Adapted from Allen NJ, Barres BA. Neuroscience: Glia - more than just brain glue. Nature. 2009; 457(7230):675-7* (Allen and Barres, 2009)

Recent genetic studies have identified a variety of genes implicated in EE (Backx et al., 2009; Kato et al., 2010; Kurian et al., 2010; Otsuka et al., 2010; Paciorkowski et al., 2011a; Paciorkowski et al., 2011b; Saitsu et al., 2012). These genes fall broadly into two categories: either linked to the development of the GABA forebrain system, or to synaptic function. GABA is considered to have a role not only in ictogenesis but also in the pathogenesis of the associated cognitive and behaviour impairments. In particular, genetic predisposition to EE may create an unbalance between excitation and inhibition through a preferential impair of GABAergic inhibition. Identifying new

genetic players in patients with both cognitive dysfunction and epilepsy may define key cellular pathways involved. The molecular diagnosis workup is challenging since similar phenotypes are associated with mutations in different genes and since mutations in one given gene can be associated with very different phenotypes (Milh et al., 2013). The immaturity of GABA inhibitory system and its dysregulation induced by seizures has been postulated as a pathophysiology mechanism.

1.7 Autism Spectrum Disorders and the Synapse

Autism spectrum disorders (ASD) affect about 0.7-1% of children and are characterized by deficits in social communication, absence or delay in language, and stereotyped and repetitive behaviors. In Portugal, the prevalence estimation of ASD for mainland Portugal and the Azores is close to 10 per 10 000 children (Oliveira et al., 2007). ASD are defined by 1) deficits in social communication and social interaction and 2) restricted repetitive behaviors, interests, and activities.

Several studies have identified genes implicated in synaptic dysfunction in patients with ASD. Moreover, mutations in genes associated with abnormal cellular or synaptic growth rate like *TSC1/TSC2*, *NF1* or *PTEN* are identified in patients with syndromic ASD with tuberous sclerosis, neurofibromatosis or macrocephaly.

Mutations in *NLGN3/4*, *SHANK3*, or *NRXN1* alter synaptic function and lead to mental retardation, typical autism, or Asperger syndrome. The mTOR/ PI3K pathway is associated with abnormal cellular/synaptic growth rate, whereas the NRXN–NLGN–SHANK pathway is associated with synaptogenesis and imbalance between excitatory and inhibitory currents.

The unbalance between excitation and inhibition can also be related with an abnormal maturation of GABAergic function in autism (Zimmerman and Connors, 2014).

In summary, several synaptic role players have been reported as relevant for ASD and the study of these mechanisms is opening new therapeutic perspectives.

1.8 From rare to common disorders

Rare disorders, also called orphan disorders are individually rare but collectively have a significant prevalence. In the European Union (EU), rare disorders, defined as prevalence of less of 5 in 10 000, affect 6-8% of EU citizens, since there are 5000-8000 disorders in this category.

Their burden is amplified by the fact that clinical trials are difficult to perform in small numbers of patients, and commercial and political interest is reduced since the potential use for a particular drug is limited to a small group of patients.

However, mendelian monogenic disorders provide an opportunity for research in disease and eventually normal physiology. When a mutation is identified in a particular gene, one can then understand how that genetic defect impairs the function of a particular protein, and can establish a relationshiop between signs or symptoms and a molecular underlying disturbance. This approach is not that simple since, from genotype to phenotype, there is a serious variability imposed by the genetic background of the patient, epigenetic modifications and also environmental influence. In the brain, the plasticity and continuous adaptation to external stimulus increase the complexity of this interpretation, particularly during periods of higher synaptogenesis and circuit remodeling like infancy and childhood. That being said, when evidence converges, one can establish a link between a molecular defect and a certain sign or symptom, or a constellation of both. Like experience has been teaching us, in a group of patients with similar clinical features, mutations have been identified in different genes but that are related with each other at the protein functional level: they are part of the same pathway, they interfere with each other's regulation or are similar or redundant in their functions. One paradigmatic example is the one of "RASopathies", a class of developmental disorders that is caused by germline mutations in genes that encode protein components of the Ras/MAPK pathway. The vast majority of these mutations result in increased signal transduction down the Ras/MAPK pathway, but usually to a lesser extent than somatic mutations associated with oncogenesis. Each syndrome exhibits unique phenotypic features, however,

since they all cause dysregulation of the Ras/MAPK pathway, there are numerous overlapping phenotypic features between the syndromes, including characteristic facial features, cardiac defects, cutaneous abnormalities, neurocognitive delay and a predisposition to malignancies (Rauen, 2013).
The same has happened with inborn errors of metabolism. They cause genetically determined alterations in biochemical pathways and generate a repertoire of neurological signs that can be associated with biological markers. They can produce symptoms such as severe epilepsy due to cerebral glucose deficiency, acute psychosis/encephalopathy due to cerebral ammonia intoxication, cognitive impairment and autistic traits due to cerebral creatine transport deficiency or parkinsonism due to neurotransmitter primary metabolic disorders (Garcia-Cazorla and Duarte, 2014). The study of neuropaediatric rare mendelian or syndromic disorders can also bring interesting inputs to common complex neurological disorders that afflict the pediatric and adult population.

1.9 Animal Models for neurodevelopmental disorders: Strengths and Limitations

In spite of the enormous contribution to our understanding of pathophysiology, research based exclusively on animal models of neurodevelopmental disorders is severely hampered by several problems:

i) The complexity of the phenotype, with physiological developmental events taking place simultaneously with the disease manifestations, leading to a specific time dependent progression, and ii) the fact that neocortical development is highly divergent in mouse and mammalian species (Florio and Huttner, 2014) (Rakic, 2009), making the recapitulation of human disease phenotypes in rodents improbable, in particular for higher cortical functions. iii) The fact that most animal models are produced through knockout approaches in which gene function is totally abolished can be misleading in the sense that human mutations are far less disruptive and can originate completely different phenotypes, even at the molecular level.
Questions have also arisen concerning the value even of genetic mouse models constructed with highly penetrant alleles that cause human disease,

because some effective therapeutic approaches in mouse models have failed in human trials (Hyman, 2014; Seok et al., 2013).

Although some research is performed purely for the sake of studying the physiology and pathophysiology of animals, the goal of the majority of animal studies is to gain knowledge and insights that are useful for understanding human biology, the response of humans to treatments or other interventions, or both. However, care should be taken to consider the effects of the genetic backgrounds of the mouse lines being studied and introduction of bias in the analysis of phenotypes, particularly for more complex behavior paradigms (Ioannidis, 2012). Successful cross species translation requires the synthesis of data from different models and the acknowledgement that there are many biological steps between genes and behavioral outputs; moreover, the social and environmental contexts that shaped human behavior compared with that of rodents have been highly divergent over the course of evolutionary time. The primate cortex, and particularly the human cerebral cortex, differs in several respects from that of the rodent (Abuhatzira et al., 2007; Finlay and Darlington, 1995). In addition to a marked increase in the size of the cerebral cortex relative to the rest of the nervous system, these include the size, complexity and the nature of its developing stem cell populations, quantitative and qualitative differences in the gene composition and expression between the neocortex of rodents and primates (Rakic, 2009; Shi et al., 2012b), an increase in the diversity of upper layers, late-born neuronal cell types, and the presence of primate-specific neuron types in deep layers (Hill and Walsh, 2005; Shi et al., 2012b). Additionally, the plasticity and the dynamic nature of the brain, specially in early developmental stages, further complicates the interpretation of data, because most of the observed molecular changes can be the result of an adaptation to the imposed genetic defect and contribute to an attempt of reducing their impact and not originally part of the pathological process.

In summary, when interpreting the data arising from rodent animal models regarding human disorders, we have to consider **the complex relationship of genotypes and phenotypes,** considering the influence of environment and epigenetic factors, and also the differences arising from species specific

brain evolution with impact in the development of neocortex and also in mechanisms of adaptation to genetic defects. For studies of the nervous system in animal models, it is therefore required that these focus on neural phenotypes that involve cells, synapses, and circuits that are plausibly conserved in evolution, and that are thus potentially relevant to the human condition.

1.10 Induced Pluripotent Stem Cells to model neurodevelopmental disorders: Strengths and Limitations

Pluripotent stem cells (PSCs) are cells capable to differentiate and give rise to every tissue/cell in the human body (Mitalipov and Wolf, 2009). Until recently, these cells could only be isolated from the inner cell mass of the blastocyst, being designated embryonic stem cells (ESCs) (Keller and Snodgrass, 1999; Thomson et al., 1998). However, in 2007, Shinya Yamanaka and co-workers were able to reprogram human somatic cells into the pluripotent stem cell state using transfection of four transcription factors (Oct4, Sox2, Klf4, c-myc). These human induced pluripotent stem cells (iPSCs) brought many expectations into the biomedical field due to their potential applications in disease modeling, drug and toxicity screening, patient-tailored therapies and engineered tissues (Takahashi et al., 2007), potentially preventing immunosuppression and graft rejection, and opening new perspectives for the next generation of personalized medicine (Yamanaka, 2009).

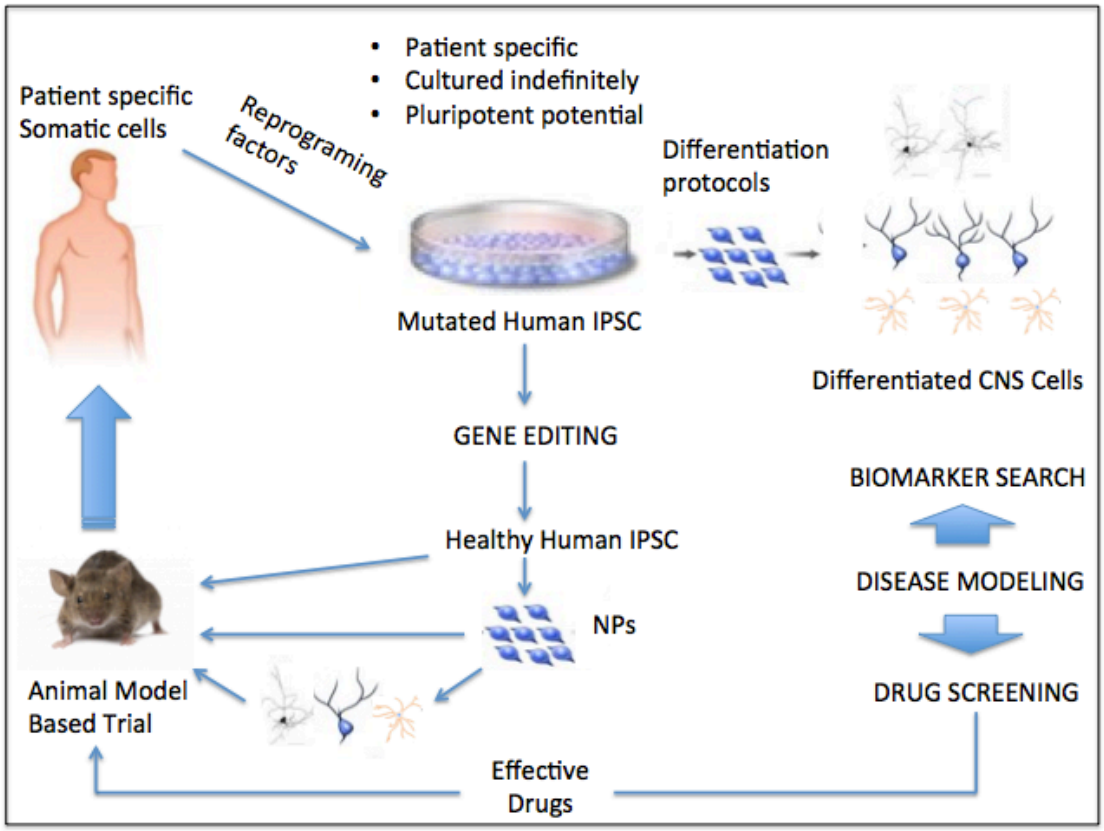


Figure 1.7 Potential applications of iPSCs to the study of genetic neurological disorders

The reprograming of human iPSCs from neurological somatic cells opens several possibilities for disease understanding and treatment. These cells can be differentiated into mature CNS cells for disease modeling and drug screening. They can also be genetically corrected through different genome editing approaches and be used for therapeutic trials using undifferentiated cells or neural precursors (NPs) or even fully differentiated cells. Adapted from http://science.psu.edu/news-and-events/2010-news/Chen11-2010.

In recent years, patient-derived iPSCs have been used to recapitulate the phenotypes of neurological diseases and broaden our understanding of the pathogenesis of many neurological diseases (Ito et al., 2012). The ultimate goal would be to develop cell based therapeutics using cells derived from the recipient, without the risks of immune rejection, but we still have some serious challenges to overcome:

First, the generation, characterization and differentiation of human iPSCs is a time consuming and costly procedure. In rapidly progressive diseases this process configures a serious limitation. The methods are still not

optimized for this kind of application but published strategies like direct trans differentiation from fibroblasts to neurons can reduce the time issue (Xue et al., 2013).

Second, another serious obstacle for iPSC based therapies is teratoma formation. Even a small number of undifferentiated cells can result in the formation of teratomas (germ cell tumors comprising several cell types, which confirm the pluripotency of stem cells). A key goal is to induce differentiation of human ESCs (hESCs) or iPSC into the required cell type while leaving few undifferentiated cells behind. Aberrant reprogramming may result in an impaired ability to differentiate and may increase the risk of immature teratoma formation after directed differentiation into the required cell type.

Third, most iPSCs are generated by transduction of somatic cells with retroviruses or lentiviruses carrying transgenes, which are integrated into the host cell genome. Transgenes are largely silenced in iPSCs, but the reactivation of such transgenes (especially the transgene encoding c-Myc) could lead to tumorigenesis (Okita et al., 2007). When patients with X-linked severe combined immunodeficiency were treated with gene therapy using retroviruses, activation of the proto-oncogene *LMO2* resulted in leukemia (Hacein-Bey-Abina et al., 2003). Leaky expression of these transgenes may also inhibit complete iPSC differentiation and maturation, leading to a greater risk of immature teratoma formation.

The high risk of tumorigenesis and mutagenesis (Han et al., 2011) led to the development of strategies to eliminate or reduce genome integration that include: development of excision systems, such as the cre-lox (Chang et al., 2009) and PiggyBac transposon systems (Kaji et al., 2009), use of non-integrating delivery approaches, such as episomal vectors (Yu et al., 2009), Sendai virus (Nakanishi and Otsu, 2012), plasmid DNA (Okita et al., 2008), mRNA (Schott et al., 2011) or direct protein delivery (Zhou et al., 2009). Further research has focused on improving the method of reprogramming with the goal of efficiently, rapidly and inexpensively generating non-tumorigenic iPSCs without use of oncogenes, such as c-Myc, or animal-derived products (Kramer et al., 2013; Lee-Kubli and Lu, 2015). Another way to avoid viral integration is to generate iPSCs using chemicals or small molecules.

Transplantation of iPSC-derived cells into animal models of sickle cell anemia, hemophilia, Parkinson's disease, spinal cord injury, and diabetes has shown therapeutic potential (Alipio et al., 2010; Hanna et al., 2007; Tsuji et al., 2010; Wernig et al., 2008; Xu et al., 2009). More recently, transplantation of Pax7-induced iPSC-derived myogenic progenitors into dystrophic mice resulted in extensive engraftment and an improvement in the contractile properties of treated muscles (Darabi et al., 2011). Thus, these types of approaches hold out much hope for future trial. In January 2009, the US Food and Drug Administration (FDA) approved the first clinical trial for using human ES cells to treat patients with spinal cord injury (Yamanaka, 2009). From that moment, several therapeutic strategies have been developed and some results have been published (Thomsen et al., 2014), focusing mainly in safety issues, which are very relevant as first steps in such a long journey.

Neuronal transplants have serious connectivity and functional issues (Emsley et al., 2004), since transplanted neurons would have to anatomically integrate into the host brain, become localized to the diseased portion of the brain, receive afferent inputs, express appropriate neurotransmitters and receptors, and form precise axonal projections. However, the successful wiring of the cortical populations is normally achieved with isolated genetic programs (without environmental interference) initially and posteriorly results from the combination of genetic and environmental cues that newly integrating neurons missed during previous development and /or mature brain function. These limitations can be reduced if neuronal progenitors are used, as these could mature *in place* or simply provide neurotrophic factors or other relevant cues that potentially facilitate the recovery or improve the function of the original neuronal population. Accordingly, several stem cell transplantation studies involving animal models of CNS disease have attributed observed beneficial effects to grafted cells that were not part of the established circuits, but were genetically engineered to produce trophic factors, immunomodulatory molecules or other neuroprotective factors (Gowing and Svendsen, 2011). Knowing the important functions carried out by astrocytes in the CNS, astrocyte precursors or stem cell-derived astrocytes could promote axonal growth, support mechanisms and cells involved in myelination, modulate the

host immune response, deliver neurotrophic factors and provide protective molecules against oxidative or excitotoxic insults (Nicaise et al., 2015), amongst many possible benefits, including direct strategies to treat white matter disorders.

Although many proof-of-principle reports support the potential of using tissues produced from iPSC for therapeutic purposes, the actual feasibility and safety of these modalities for human clinical applications remains controversial, given very limited relevant preclinical data (Hong et al., 2014). Notwithstanding, the use of iPSCs as models of neurological disease is an approach already validated in the literature. There are also several limitations that should be considered: first, in many neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS) or Parkinson's Disease, it takes several decades for symptoms to develop. We need to find ways to facilitate disease pathogenesis in patient-specific iPSCs and to mimic epigenetic changes caused by aging and the environment. Stimulation like oxidative stress or UV irradiation may be required to attain this aim. When studying monogenic neurodevelopmental disorders, an advantage is that molecular and functional defects are expected at earlier stages, and protocols that reproduce embryonic neuronal development are promising for the recapitulation of the first consequences of a mutation in neuronal and synaptic function.

Secondly, another important issue is that many diseases may be cell non-autonomous. For example, motor neurons derived from ALS patient-specific iPSCs may not be able to reconstitute alone full disease pathogenesis, as they may need the interaction with mutated glial cells (Di Giorgio et al., 2007). Thus, multiple cell types may need to be generated from patient-specific iPSCs. In fact, regarding RTT, astrocytes have been implicated in disease pathology and effects of mutated astrocytes in wild type neurons have been identified (Ballas et al., 2009; Lioy et al., 2011). Protocols that start from iPSCs to derive cortical populations of neurons and astrocytes, may be a useful strategy to overcome this difficulty.

1.11 Aims

Clinical practice in child neurology has allowed me to be in contact with very severe disorders that affect the child brain, and this perspective was the starting point for the questions that motivated this work.

Two main aims were always present:

- To search for a synaptic phenotype in early encephalopathies: improve diagnosis and classification of child neurology disorders, searching for biomarkers that pointed to the molecular pathophysiologic signature, particularly focusing on the synapse.
- Direct research to molecular mechanisms that could be targeted pharmacologically, improving the treatment strategies in these disorders with a rational approach.

The specificities of the developing brain are frequently forgotten when we treat early encephalopathies, since there is usually a dose adjustment but not clearly an adjustment to the developing state of the central nervous system, which is different in neonates, infants, toddlers, pre-school, school age, adolescents and adults. Another usually forgotten aspect when therapeutic strategies are delineated is the individual variability, which should be increasingly tackled when we move towards a “personalized medicine” approach.

An increasing body of evidence points to the disrupted maturation of GABAergic system as implicated in several neurodevelopmental disorders, including RTT, Fragile X, epileptic encephalopathies and ASDs. The GABAergic system physiology was shown to exhibit several dramatic changes during postnatal development in rodents, but these changes are not yet sufficiently characterized in humans. Moreover, in the clinics, treatment of neonatal seizures has been crystalized during decades, with drugs that target the GABAergic system as if the maturation process was complete. The difficulties of performing clinical trials in neonates and the lack of translational research in the field have hampered the expected improvement of treatments. I focused on the control of intracellular chloride concentration, one of the

mechanisms with documented changes during CNS development, with a determinant impact in GABAergic actions (see 1.5.1.1) section in the introduction).

First, I searched for a strategy to understand the individual status of the GABAergic system in a particular patient. A first possibility would be to try to obtain some information from individual biological samples. Cerebrospinal fluid (CSF) analysis is used to diagnose several child neurology conditions and CSF accumulates products from different CNS cellular populations, reflecting cellular physiology in a "global perspective". The first experiments described in this thesis were performed with the objective of detecting synaptic proteins in the CSF, searching for differences in these proteins between patients and controls, in order to guide treatment and/or further research.

The results from CSF analysis contributed to the delineation of new experiments, particularly in RTT, a disorder caused by mutations in the gene encoding MeCP2, a fascinating protein with multiple roles in development and neuronal function. The multiplicity of signs and symptoms in RTT patients document the relevance and extension of MecP2 actions in the CNS.

I aimed to study a disease model that reflected the individual patient specificities and also allowed functional studies. These objectives led me to the resolution of generating iPSCs from patients with different *MECP2* mutations, and also different phenotypical severity. With iPSCs, I could obtain patient-specific neurons to study and possibly optimize treatment strategies.

Reprogramming of human iPSCs is mainly applied for disease modeling, but there are also many studies oriented for cell-based treatment. Transplantation of iPSCs or their progeny into an immunocompetent recipient in a clinical setting requires feeder-free, and ideally, completely xenogeneic-free defined cell culture conditions. We aimed to develop an approach that reproduces neural commitment from human iPSCs using dual-SMAD inhibition under defined conditions in a vitronectin-based monolayer system, in order to obtain neuronal precursors in xeno-free conditions.

One of the limitations of disease modeling with human iPSCs is that it reflects individual cell pathological changes, but neuronal circuit formation is highly artificial in a culture plate. This limitation was the reason to perform additional experiments in a rodent model of RTT, in order to combine results from different disease models and increase the relevance of the results.

BDNF is also a crucial role player in the CNS, during development, as well as for normal brain physiology in health and in response to insults. The *BDNF* gene has been consistently reported to be under the jurisdiction of MeCP2, regarding transcriptional control. Adenosine is a crucial modulator of BDNF and GABAergic actions, but the adenosinergic system was not previously studied in RTT, particularly as a modulator of BDNF actions. Importantly, adenosine A2AR activation gates the synaptic actions of BDNF. Accordingly, A2AR activation is required for the facilitatory action of BDNF on hippocampal synaptic transmission on LTP.
One of the aims of the present work was to characterize BDNF-TrkB receptor signaling in RTT disease models. If we confirmed the expected impairment in this system, a second step would be to try to modulate BDNF-TrkB signaling, through A2AR activation.

In summary, the main objectives of this work were:
1. To search for biomarkers of early encephalopathies with epilepsy and autistic traits, particularly synaptic proteins in the cerebrospinal fluid. These biomarkers could contribute to the delineation of a synaptic phenotype as an additional molecular signature of disease.
2. To obtain patient specific neuronal populations for functional studies.
3. To obtain neuronal progenitors in defined conditions.
4. To study GABAergic developmental changes in early encephalopathies.
5. To contribute for treatment strategies of RTT, including the pharmacological modulation of two significant synaptic dysfunctions in RTT: GABAergic and BDNF-TrkB signaling impairments.

Chapter 2 Analysis of synaptic proteins in the cerebrospinal fluid as a new tool in the study of inborn errors of neurotransmission

Sofia T. Duarte, Carlos Ortez, Ana Pérez, Rafael Artuch, Angels García-Cazorla

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In this work Sofia Duarte performed part of the western blot experiments, analysed the data and wrote the first draft of the manuscript.

Abstract

In a few rare diseases, specialised studies in the CSF are required to identify the underlying metabolic disorder. We aimed to explore the possibility of detecting key synaptic proteins in the CSF, in particular dopaminergic and GABAergic, as new procedures that could be useful for both pathophysiological and diagnostic purposes in investigation of inherited disorders of neurotransmission.

Dopamine receptor type 2 (D2R), dopamine transporter (DAT) and vesicular monoamine transporter type 2 (VMAT2), were analysed in CSF samples from 30 healthy controls (11 days to 17 years) by western blot analysis. Because VMAT2 was the only protein with intracellular localization, and in order to compare results, GABA vesicular transporter, which is another intracellular protein, was also studied. Spearman's correlation and Student-t tests were applied to compare optical density signals between different proteins.
All these synaptic proteins could be easily detected and quantified in the CSF. DAT, D2R and GABA VT expression decrease with age, particularly in the first months of life, reflecting the expected intense synaptic activity and neuronal circuitry formation. A statistically significant relation was found between D2R and DAT expression, reinforcing the previous evidence of DAT regulation by D2R. To our knowledge, there are no previous studies in human CSF reporting a reliable analysis of these proteins. These kinds of studies could help elucidate new causes of disturbed dopaminergic and gabaergic transmission as well as understanding of different responses to L-dopa in inherited disorders affecting dopamine metabolism. Moreover, this approach to synaptic activity in vivo can be extended to different groups of proteins and diseases.
Synopsis: The detection and quantification of key synaptic proteins in the cerebrospinal fluid, in particular dopaminergic and gabaergic, is reported as a new procedure that could be useful for both pathophysiological and diagnostic purposes in the investigation of inherited disorders of neurotransmission.

2.1 Introduction

Specialised investigations in cerebrospinal fluid can be crucial to identify some inborn errors of metabolism. Defects of biogenic amines (dopamine and serotonine), GABA and glycine make up the group of diseases that are currently considered to be inborn errors of neurotransmitters. In these disorders, quantification of some particular CSF metabolites (biogenic amines, GABA or glycine) is necessary in the diagnostic process. However, although growing evidence suggests that genetic or pathologic alterations of proteins involved in synaptic transmission may underlie a number of neurological and psychiatric disorders (Sudhof, 2008; Sudhof and Malenka, 2008) (Corradini et al., 2009; Kauer and Malenka, 2007; Witzmann et al., 2005), the study of these proteins is not normally included in the array of investigations carried out in disorders of this kind. *In vivo* measurement of synaptic activity is difficult to carry out in humans. CSF samples are easily accessible by standard lumbar puncture techniques. Additionally, biochemical changes in CSF may reflect pathological alteration of CNS physiology, as previously demonstrated (Ormazabal et al., 2005). Accordingly, CSF receives a wide variety of molecules released by different CNS cell populations (Thouvenot et al., 2008). Several authors, including some from our group, have shown that there is a strong variability of CSF biogenic amines with age in children not suffering neurologic disease (Ormazabal et al., 2005), which suggests that the same variability may be expected when analysing synaptic proteins related with classical neurotransmitter function.

Synaptic transmission depends on neurotransmitter pools stored within vesicles that undergo regulated exocytosis. In the case of dopaminergic transmission, VMAT2 is responsible for the loading of dopamine and other monoamines into synaptic vesicles (Cartier et al.). DAT carries dopamine across the plasmalemmal membrane from the synaptic cleft into the cytoplasm (Volz et al., 2009). The central actions of dopamine are mediated by five distinct receptors that belong to the G-protein receptor family. They are classified as D1-like (D1 and D5) and D2-like (D2, D3 and D4), and their interaction with dopamine translates into activation/inhibition of specific

neurons and circuitries (De Mei et al., 2009). Those suffering *DRD2* (D2 R gene) mutations exhibit postural abnormalities, bradykinesia with delayed initiation of movements, impaired coordination and prolonged periods of immobility (Glickstein and Schmauss, 2001). These symptoms are also characteristic of patients suffering dopaminergic neurotransmission disorders.
In spite of the critical role of these proteins in dopaminergic transmission (Figure 2.1), information about procedures for studying them in CSF is very scarce, to our knowledge. However, such knowledge would be useful to increase understanding of the mechanisms of disturbed dopaminergic transmission in primary (inherited disorders of dopamine) and secondary deficiencies.

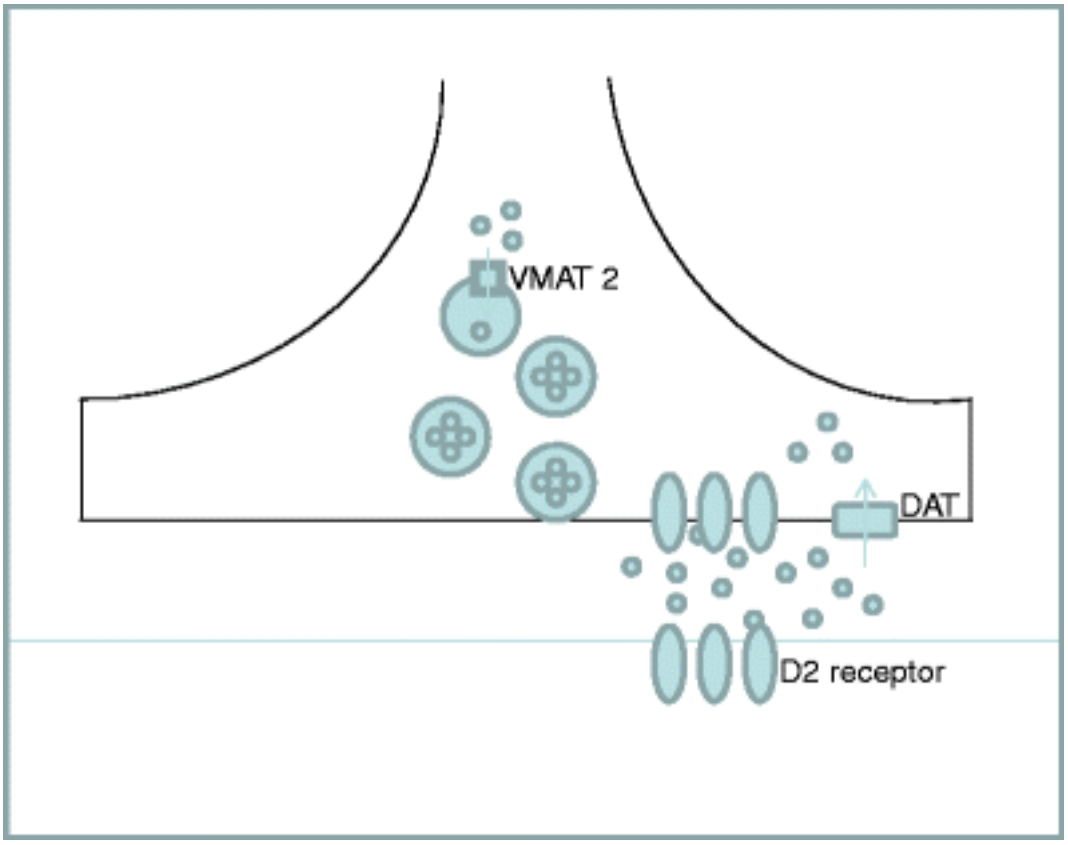

Figure 2.1 Schematic representation of the dopaminergic synapse.

The figure illustrates the link between VMAT2 (vesicular monoamine transporter 2), DAT (dopamine transporter) and D2 R (D2 receptor).

We aimed to analyse, in the CSF of a control population, the synaptic proteins VMAT2, DAT and D2 R. Because VMAT2 was the only protein with intracellular localization, GABA VT, which is another intracellular protein, was also included in the study. We evaluated the correlation of these proteins with each other and with the age of controls. Additionally, because total CSF protein concentration is known to decrease with age, we introduced this variable in the age correlation studies.

2.2 Methods

2.2.1 CSF Samples

The study was performed in 30 subjects (age range: 11 days - 17 years; average: 1.8 years) whose CSF samples were submitted to Hospital San Joan de Déu laboratory for analysis under suspicion of viral or bacterial meningitis or encephalitis. Lumbar puncture was performed in the emergency room, and the first ten drops were used for routine cytochemical/microbiological studies. Inflammatory markers (C-reactive protein and procalcitonin) were also analysed. Exclusion criteria were diagnosis of viral or bacterial meningitis, a chronic neurological condition, and hematic or xanthochromic CSF (blood contamination). Samples from patients were obtained in accordance with the Helsinki Declaration of 1964, as revised in 2000. The ethical committee of the Hospital Sant Joan de Déu (HSJD) approved the study. After collection, samples were stored at -80ºC up to the moment of the analysis.

2.2.2 Western blot

Western blot analysis was performed for each protein (DAT, D2 R, VMAT2, GABA VT). Twenty µL of CSF sample was loaded into the gel and proteins were separated on 10% sodium dodecyl sulphate-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham™ Hybond™ –ECL, GE Healthcare). The membranes were incubated in TBST buffer (0.02M Tris-base, pH7.6, 0.8% NaCl, 0.1% Tween 20) supplemented with 5% dried skimmed milk for 60 min to block non-specific binding. Anti-DAT extracellular loop (1:1000; Sigma®), anti-D2 R (1:1000; Millipore®), anti-VMAT

2 (1:1000; Santa Cruz Biotechnology®), and anti-GABA VT (1/500; Millipore®) antibodies were added, and the preparations were incubated at 4 ºC overnight. The membranes were washed three times with TBST buffer and then incubated with appropriate anti-rabbit (1:3000, Promega®) or anti-mouse (1:5000, Promega®) IgG secondary antibodies at room temperature for 1 h. The blot was then washed six times with TBST and prepared with ECL (Pierce® ECL Western Blotting Substract, Thermo Scientific) for developing. Relative levels of each protein were quantified by measuring optical densities (OD) of the corresponding bands with Quantity One ® V 4.3.1. software.

2.2.3 CSF total protein concentration, procalcitonin and C-reactive protein determination

CSF total protein and C-Reactive Protein concentrations were measured by standard automated procedures in an Architect ci8200 analyzer (Abbott, USA). Procalcitonin was measured by fluorimetric detection, with Brahams-Kryptor-Atom® analyser.

2.2.4 Statistical analysis

Statistical analysis was performed with the SPSS 17.0 program. Spearman's correlation test was applied to search for associations among the OD signals of the different proteins and with the age of controls. Multivariate linear regression analysis was used to adjust the results by total CSF protein concentration. Student-t test was applied to compare OD signals of the different proteins according to gender. Statistical significance was considered as $p < 0.05$.

2.3 Results

DAT, D2R, VMAT2 and GABA VT were clearly detectable at different ages using conventional western blot analysis in all the CSF samples studied (Figure 2.2).

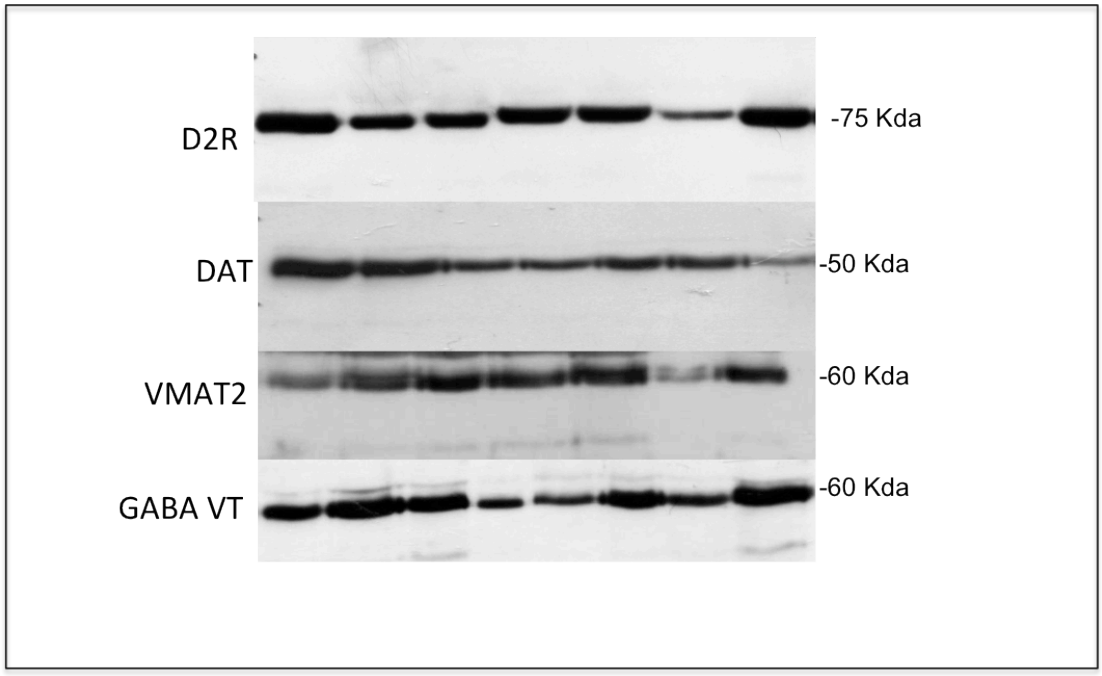

Figure 2.2 D2, DAT, VMAT and GABAVT detection in the CSF by western blot in different control samples.

VMAT2 expression measured as OD showed a statistically significant negative correlation with age (Table 2.1). Correlation between DAT expression and age showed a strong tendency to significance (p value=0.05). D2R expression and GABA vesicular transporter expression did not show statistical association with age when all the CSF samples were included in the statistical study. However, since our sample population was predominantly younger than 200 days (for age distribution see Figure 2.3.A), we searched for a correlation between D2R and GABA VT expression with age in this group. A significant negative correlation was found for D2R and GABA VT. In order to rule out interference of the expected decrease in total CSF protein concentration with age, we adjusted these results by total CSF protein concentration (range, 7–77 g/l; mean, 34.7; median, 31; standard deviation, 17.2. All values were within normal limits according to different age ranges)

(Biou et al., 2000). Except for VMAT2, the relation between age and expression of these synaptic proteins remained statistically significant (Table 2.1).

Table 2.1

	D2 R	DAT	VMAT2	GABA VT
Mean (OD measure)	580755.5	825805.8	654848.24	945490.3
Standard deviation (OD measure)	302541.9	598210.0	385135.9	356870.5
Upper limit (OD measure)	1126336	2115196	50255	1582609
Lower limit (OD measure)	99224.4	87053.87	1574680	395287.83
Spearman's Rho correlation test between age and OD measure	r=-0.591; p=0.01 (if age under 200 days)	r =-0.329; p=0.05 r =-0.482 p=0.015 (if age under 200 days)	r=-0.532 p=0.002	r=-0.681 p=0.002 (if age under 200 days)
Coefficients of a multivariate linear regression model adjusted by total protein concentration	β=-6139.738 p=0.007 (if age under 200 days)	β=16.294 p=0.834 β=-8119,042 p=0,016 (if age under 200 days)	β=-28.736 p=0.358	β=-5886.624 p=0.001 (if age under 200 days)

Table 1. Statistical data of the results for the synaptic proteins studied. Optic densities are expressed in arbitrary units. D2R: D2 receptor, DAT: dopamine transporter, VMAT2: vesicular monoamine transporter; GABA VT: GABA vesicular transporter. OD: Optic density.

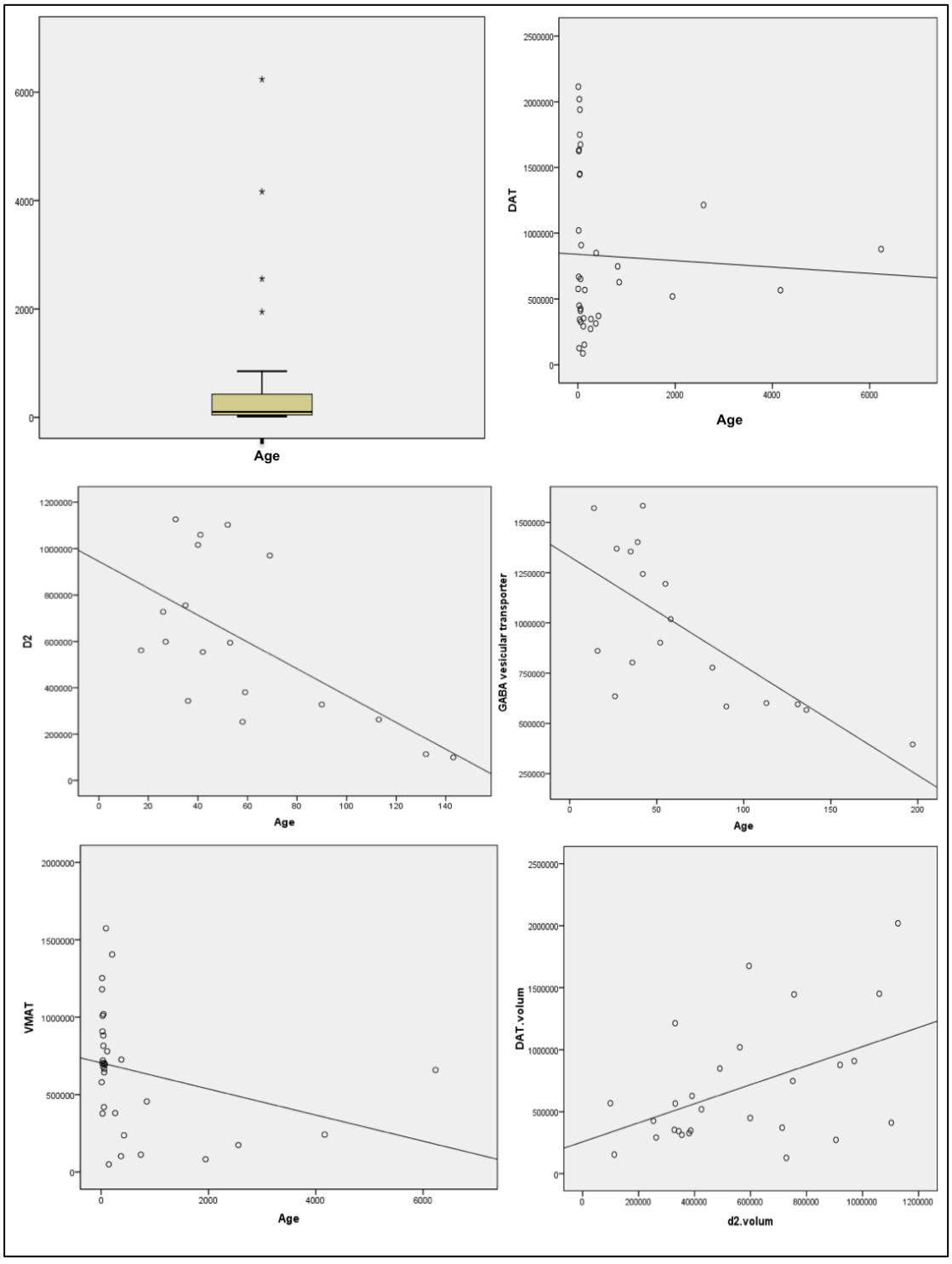

Figure 2.3 Upper left: Population age distribution. Upper right, middle right, middle left, lower right: Correlation between age and synaptic proteins. Lower left: Correlation between DAT and D2.

No differences were observed when we compared DAT, D2, VMAT2 and GABA VT protein expression according to gender.

Concerning association studies among the different proteins, OD signal of D2R, and DAT showed a statistically significant positive correlation (Spearman's rho correlation test r=0.016; p=0.443), while no correlation was found between D2 R and VMAT, or between DAT and VMAT.

Because VMAT2 is also involved in adrenaline and noradrenaline transport, and its release can be triggered by acute stress situations, we also tried to correlate VMAT2 expression with acute phase reactants (C-reactive protein and procalcitonin), in order to rule out the possible influence of fever or acute disease in the expression of this protein, since our control population was selected from a group of children under suspicion of an active infectious process. C-reactive protein and procalcitonin levels were not related with total CSF protein concentration, nor with VMAT 2 expression.

2.4 Discussion

We aimed to explore the possibility of detecting key dopaminergic and GABAergic synaptic proteins in a control population of children. To our knowledge, this is the first report of detection of these proteins in CSF in a paediatric population.

In spite of their low abundance compared to global CSF proteome (Thouvenot et al., 2008), the intensity and definition of the different bands obtained with the present procedure (Figure 2.1) lends strong support to the applicability of this analysis in neurochemical research.

The presence of transmembrane synaptic proteins in CSF can be explained in several ways. It has been suggested that membrane protein detection is the consequence of extensive protease actions that cleave the fragments from proteins that are embedded in membranes, and that these fragments enter the CSF (Egana et al., 2009) (Zougman et al., 2008). However, our study demonstrates that these proteins are detected at the expected molecular weight, indicating that there is no fragmentation, or at least, that they are intact in part. Harrington and co-workers (Harrington et al., 2009) identified the presence of CSF membranous nanostructures, thought to play a physiologically active role, and not merely the result of blebbing, apocrine secretion, or apoptosis events, or cellular debris. These structures can

provide an appropriate environment for transmembrane proteins, which are hydrophobic in nature. Their morphology is similar to that of synaptic vesicles and exosomes; their structure resembles that of nanotubules, cell-to-cell interactions that facilitate the selective transfer of membrane vesicles and organelles but which seem to impede the flow of small molecules (Rustom et al., 2004). In the first months of life, a period of intense synaptogenesis and neuronal circuitry formation, cell-to-cell exchange of synaptic machinery is thought to be carried out by small dense core vesicles (~80nm) (Sorra et al., 2006). However, extracellular localization of these structures is not well documented.

Based on our previous experience with the quantification of biogenic amines in a population of children without neurological disease (Ormazabal et al., 2005) who exhibited a decrease from the first days of life until reaching adult age, we aimed to learn whether the same tendency was observed with the synaptic proteins included in the study. For DAT, D2R and GABA VT, it was possible to establish a statistically significant relation, particularly in the group of children under 6 months of age from whom most of our sample was drawn. Age-related changes in DAT functionally active protein have been described in rats (Volz et al., 2009), showing a decrease between young adolescent rat levels and adult levels. Interestingly, in the same report, no difference between VMAT 2 immunoreactivity in the two groups was found, although conflicting data had previously been published (Volz et al., 2006). Furthermore, because VMAT2 is involved in fast neurotransmission, its regulation could appear to be related to rapid communication needs and not to age. Another result that deserves discussion is the relation discovered between D2 and DAT expression. Numerous studies have supported the notion that DAT is subjected to dynamic regulation in the plasma membrane, and this regulation has been extensively reviewed by Eriksen and co-workers (Eriksen et al., 2010). There is strong evidence that D2 R causes an increase in dopamine uptake through an increase in DAT surface expression. Additionally, loss of D2R/DAT co-immunoprecipitation has been described in schizophrenia, suggesting a role for the loss of the interaction in the disease process (Bolan et al., 2007).

Currently, several CSF investigations in disorders of suspected neurometabolic origin are performed; these include examination of cytochemistry (cells, glucose, proteins), lactate, amino acids, biogenic amine metabolites, pterins and 5-methyltetrahydrofolate. However, only a few disorders can be detected by means of these analyses (Garcia-Cazorla, 2010). Furthermore, the diagnosis of monogenic defects of dopaminergic neurotransmission is almost exclusively based on the quantitative determination of their metabolites in CSF (Marin-Valencia et al., 2008). Secondary deficiencies of dopamine are found in about 10% of patients who undergo a lumbar puncture in the diagnostic work-up of a suspected neurometabolic disorder (Garcia-Cazorla et al., 2007) (Van Der Heyden et al., 2003). Disorders such as mitochondrial diseases (Garcia-Cazorla et al., 2008) and Lesch-Nyhan disease (Serrano et al., 2008) are amongst the main known causes of secondary deficiencies. The analysis of these and other synaptic proteins is expected to be useful to both increased understanding of the mechanisms of disturbed neurotransmission and identification of new causes of dopaminergic defects. Concerning primary deficiencies such as tyrosine hydroxylase deficiency, this approach could be useful to improve understanding of the variable response that patients have to L-dopa therapy (from normalisation to almost absent effect) (Willemsen et al., 2010), but we need further data to document this hypothesis.

To conclude, we report the detection of transmembrane synaptic proteins in the CSF of a population of children without neurological disorders. This approach was made as a means of monitoring neurotransmitter genetic disease outcome and response to treatment, but its applications can be extended to the investigation of a growing group of neurological and psychiatric disorders related to neurotransmission. The proteins included in this study were chosen because of their relevance for dopaminergic and GABAergic transmission but there are many other proteins of interest that can be selected for analysis with this methodology.

The findings reported here deserve further investigation, especially in the field of CSF-neuron and inter-cellular interactions, in order to better understanding of their physiological implications in the developing brain and their

pathophysiological role in disease states. Significant correlation with age was found with D2R, DAT and GABA VT expression, as expected from the previously reported decrease of biogenic amines in a paediatric population. Coordinated regulation of DAT by stimulation of D2 R is further reinforced by our results.

2.5 Acknowledgements

We greatly appreciate the technical assistance of Nuria Valmanzo and Belén Ramos (Mental Health laboratory, Fundació Sant Joan de Déu, Barcelona). Statistical studies were done with the collaboration of Raquel Iniesta (Fundació Sant Joan de Déu, Barcelona). CIBERER is an initiative of the ISCIII (MICINN, Spain). This study was funded by the grant FIS PS09/01132. CO is the recipient of a grant from Caja Navarra.

In the next chapter, I will describe the results of the study of cation chloride cotransporters in the cerebrospinal fluid of RTT patients. Before performing this study, we studied a control pediatric population, searching for changes in CSF during normal postnatal development. Jansen and co-workers (Jansen et al., 2010) demonstrated in frozen brains of normal controls that the ratio of NKCC1 to KCC2 expression is very high in the youngest infants, decreases rapidly until around 2 years of age, and then remains low into adulthood.

We have determined KCC2 and NKCC1 expression in the CSF of a pediatric population without neurological disorders and this work gave us the possibility of having a control population for the work described in Chapter 2. Our findings suggest that this maturation is very variable, but in the CSF, KCC2 shows some predominance in patients after 7 months of life. The discrepancy between the age of functional switch suggested by the neuropathology study (2 years of life) and the present findings (global switch around 7 months of life) could be explained by the fact that this physiological pattern develops in a caudal-rostral progression. The CSF levels are expected to reflect a global functional state of all GABAergic neurons, while the cortical tissue quantification of these proteins reflects the encephalic changes that occur later in life. The variability is also supported by our experience in the neonatal intensive care unit, where different newborns have different and sometimes paradoxical responses to pharmacological treatment with GABAAR agonists that can increase epileptic activity at this neurodevelopmental stage.

The major advantage of doing this study *in vivo*, in children with severe neurologic disorders like epileptic encephalopathies of the first year of life or RTT, is the possibility of searching for disturbances in the normal developmental pattern that can underlie the pathogenesis of these disorders, and also the possible orientation of a rationale treatment with anti-epileptic agents.

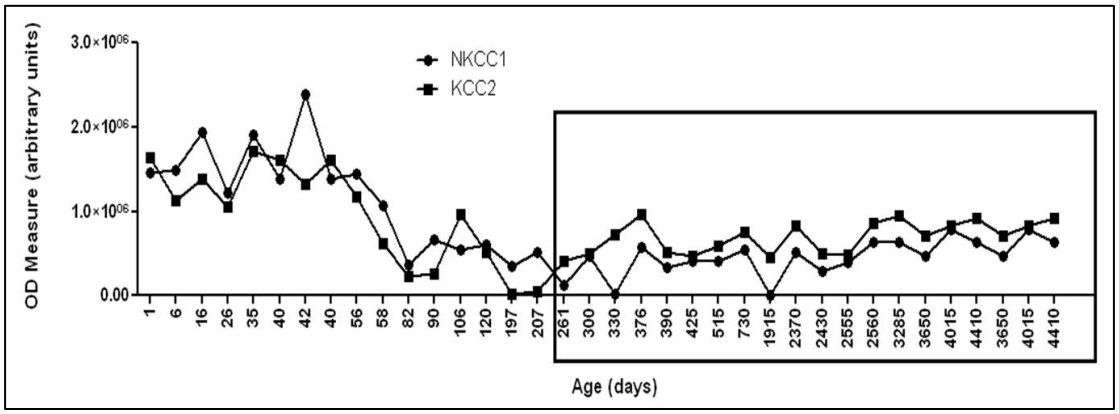

Figure 2.4 Evolution of the relative expression of NKCC1 and KCC2, with age.

Before 7 months of extra-uterine life, NKCC1 and KCC2 are both expressed without a clear predominance. After this period, the expression of KCC2 shows a tendency to be higher, contributing to a mature pattern of neuronal chloride physiology, related with the hyperpolarizing effect of GABAAR activation. Optic densities are expressed in arbitrary units.

Starting from the results in this control population, we searched for differences in RTT patients, as described in Chapter 3.

Chapter 3 Abnormal Expression of Cerebrospinal Fluid Cation Chloride Cotransporters in Patients with Rett Syndrome

Sofia Temudo Duarte, Judith Armstrong, Ana Roche, Carlos Ortez, Ana Perez, Maria del Mar O'Callaghan, Antonina Pereira, Francesc Sanmartí, Aida Ormazabal, Rafael Artuch, Mercedes Pineda, Angels García-Cazorla

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In this work Sofia Duarte helped to conceive and design the experiments, collected part of the biological samples, performed the main part of experiments, contributed to data analysis and wrote the first draft of the manuscript.

Abstract

Objective: RTT is a neurodevelopmental disorder caused mainly by mutations in the gene encoding MeCP 2. The relevance of MeCP2 for GABAergic function was previously documented in animal models. In these models, animals show deficits in BDNF, which is thought to contribute to the pathogenesis of this disease. Neuronal Cation Chloride Cotransporters play a key role in GABAergic neuronal maturation, and BDNF is implicated in the regulation of Cation Chloride Cotransporters expression during development. Our aim was to analyse the expression of two relevant Cation Chloride Cotransporters, NKCC1 and KCC2, in the CSF of RTT patients and compare it with a normal control group.

Methods: The presence of bumetanide sensitive NKCC1 and KCC2 was analysed in CSF samples from a control pediatric population (1 day to 14 years of life) and from RTT patients (2 to 19 years of life), by immunoblot analysis.

Results: Both proteins were detected in the CSF and their levels are higher in the early postnatal period. However, RTT patients showed significantly reduced levels of KCC2 and KCC2/NKCC1 ratio when compared to the control group.

Conclusions: Reduced KCC2/NKCC1 ratio in the CSF of RTT patients suggests a disturbed process of GABAergic neuronal maturation and open up a new therapeutic perspective.

3.1 Introduction

RTT is an X-linked neurodevelopmental disorder with an incidence of 1:10000 live female births and is one of the leading causes of mental retardation and autistic behavior in females (Hagberg et al., 1983). Loss-of-function mutations in the gene encoding MeCP2 cause most cases of RTT. Individuals affected with RTT experience normal development up to the age of 6–18 months, at which time they fail to acquire new skills and enter a period of motor regression (Monteggia and Kavalali, 2009). Autistic features are a hallmark of this disorder and epilepsy is frequent (Nissenkorn et al., 2010). RTT patient brain does not show obvious signs of neurodegeneration, atrophy, gliosis, demyelination, or neuronal migration defects (Jellinger et al., 1988) (Reiss et al., 1993), suggesting that neurological symptoms may primarily stem from subtle defects of subcellular compartments such as dendrites, axons, or synaptic structures (Boggio et al., 2010). MeCP2 is a transcriptional regulatory protein, and in its absence, a large number of genes exhibit abnormal expression with implications in the balance between synaptic excitation and inhibition (Chao et al., 2010; Kron et al., 2012). MeCP2 might be particularly important for GABAergic function and there is evidence that the expression of MeCP2 is approximatelly 50% higher in GABAergic neurons when compared to non GABAergic neurons. Mice with conditional deletion of *Mecp2* in GABAergic neurons initially show normal behavior but in the course of development start displaying forepaw stereotyped movements, compulsive grooming, impaired motor coordination, learning/ memory deficits, abnormal EEG hyperexcitability, severe respiratory dysrhythmias and premature lethality (Chao et al., 2010).

GABA is the main inhibitory neurotransmitter in the adult brain. During early development, activation of the chloride- permeable, postsynaptic, GABAAR can induce depolarization and the basal intracellular chloride concentration is determinant for the action of GABA in the developing neurons (Blaesse et al., 2009). Two major contributors to intracellular chloride concentration are NKCC1 (Na^+, K^+, $2Cl^-$ cotransporter, that accumulates chloride in the cell), and KCC2 (K^+, Cl^- cotransporter, that extrudes chloride). Several lines of

research correlate epileptogenesis with altered function of NKCC1 and KCC2 (Munoz et al., 2007; Woo et al., 2002). *In vitro*, experiments suggest that bumetanide, a potent NKCC1 inhibitor, can increase GABAergic inhibition, in combination with phenobarbital (Dzhala et al., 2005). Bumetanide has also been reported useful in a neonatal patient with seizures (Kahle et al., 2009) and in autistic children (Lemonnier et al., 2012).

Moreover, the brain of MeCP2 deficient animal models shows deficits in BDNF (Wang et al., 2006), which is thought to contribute to the pathogenesis of RTT. BDNF can also promote the functional maturation of GABAAR mediated responses by inducing upregulation of KCC2 (Aguado et al., 2003; Carmona et al., 2006; Plotkin et al., 1997).

Human age related changes in GABAAR physiology remain controversial, although neuropathological studies have already identified postnatal developmental changes of NKCC1 and KCC2 cortical expression (Jansen et al., 2010). The detection of synaptic proteins in the CSF gives us the possibility to indirectly access synaptic composition and alterations, using the CSF of patients with disorders related to neurotransmission, with the advantage of performing these studies *in vivo* (Duarte et al., 2011).

We hypothesize that changes in BDNF expression levels or the direct effect of the underlying *MECP2* genetic mutation can interfere with the normal expression of NKCC1 and KCC2 leading to a reduction in the KCC2/NKCC1 ratio, characteristic of the immature GABAergic system. A comparison of NKCC1 and KCC2 protein levels in the CSF of patients affected with RTT and a control population was made in order to address this question.

3.2 Methods

3.2.1 Patients and controls

Sixteen patients with RTT were recruited to this study, aged between 2 to 19 years at the moment of CSF collection. Patients' clinical characteristics are summarized in table 1. Patients without a documented mutation fulfilled clinical criteria for RTT according to the last updated revision (Neul et al., 2010). The control study was performed in 67 subjects (age range: 1 day - 14 years; mean: 740 days; female: 27; male: 40) whose CSF samples were

submitted to HSJD laboratory under suspicion of viral or bacterial meningitis or encephalitis. Exclusion criteria were: diagnosis of viral or bacterial meningitis, neurologic disease, and hematic or xantocromic CSF (blood contamination).

3.2.2 CSF Samples

CSF samples were collected by lumbar puncture as previously described (Ormazabal et al., 2005). They were obtained after parent's written informed consent and in accordance with the Helsinki Declaration of 1964, as revised in 2000. The ethical committee of HSJD approved the study. After lumbar puncture, the first ten drops were used for routine cytochemical/microbiological studies and then CSF was immediately stored in 4 aliquots at -80ºC until the moment of analysis. Biogenic amines metabolites and synaptic proteins were studied using the following 20 drops.

3.2.3 Western Blot analysis

NKCC1 and KCC2 expression levels were analyzed by western blot. Twenty µL of CSF were loaded on gel and proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (AmershamTM HybondTM –ECL, GE Healthcare). Membranes were blocked in TBST buffer (0.02M Tris-base, pH7.6, 0.8% NaCl, 0.1% Tween 20) with 5% dry skimmed milk for 60 min at room temperature. Anti-NKCC1 (1:500; Santa Cruz Biotechnology®) and anti-KCC2 (1:500; Millipore®) antibodies were added and incubated at 4 ºC overnight. Membranes were washed three times with TBST buffer followed by incubation with appropriate anti-rabbit (1:3000, Promega®) IgG secondary antibody at room temperature for 1 h. The blot was then washed six times with TBST and signal was revealed with ECL (Pierce® ECL Western Blotting Substract, Thermo Scientific). Relative levels of each protein were quantified by measuring ODs of the corresponding bands with Quantity One® V 4.3.1.software.

3.2.4 Statistical analysis

Statistical analysis was performed using IBM Statistical Package for the Social Sciences (IBM SPSS Statistics Version 19.0, SPSS Inc: Chicago, IL). A significance level of .05 was used in all analyses. Outlier analysis was done taking into account the primary variable in the study – healthy/RTT. Outliers (defined as values 1.5 times lower than the 1st quartile or 1.5 times higher than the 3rd quartile (Maroco, 2007 #631) were found in 8 cases (1 RTT patient, 7 healthy controls) for KCC2/ NKCC1 ratio, 4 cases for NKCC1 (1 RTT patient, 3 healthy controls) and in 1 case (healthy control) for KCC2. Outliers were excluded from the respective analyses.

Non-parametric tests were applied when possible for age, NKCC1 and KCC2, since the assumption of normal distribution was not fulfilled for these variables.

3.3 Results

Total CSF protein concentration values (*M* = 33.27, *SD* = 19.04, range: 7 – 73 g/l) were within normal limits according to different age ranges (Biou et al., 2000). Clinical and genetic features of RTT patients are described in table 1. NKCC1 and KCC2 western blot analysis were performed on the CSF of controls and RTT patients. Cation Chloride Cotransporters were detected in the CSF of this population, at the expected molecular weight (Figure 3.1.A). Considering the reported sexually dimorphic expression of KCC2 and GABA function in the substantia nigra (Galanopoulou, 2008b) it was decided to control for gender in all reported analysis.

Furthermore, the homogenous distribution of the demographical variable age was verified with Mann-Whitney Test (*U*). Controls (*M* = 746.00 days, *SD* = 1075.89) were significantly (*U* = 932.00, $p < .001$) younger than RTT patients (*M* = 3444.69 days, *SD* = 2173.59) therefore we have controlled for this variable in all reported analysis (table 3.1).

	Rett Patients (n=16)	**Controls** (n=65)	**Statistics**	***p-value***
Age	3444.69 (2173.59)	746.00 (1075.89)	*U*=932.00	*< .001*

Table 3.1 Socio-demographic variable: mean values (and standard deviations) of age. Significant differences among the groups were assessed with Mann-Whitney Test (*U*).

	Age	Genetic studies	Mutations type /Domain altered	Epilepsy	Medication (when LP was performed)	Respiratory anomalies	KCC1/KCC2 (optical densities)
1	2	*MECP2/CDKL5*/ *FOXG1* screened, no alteration found	-	No	NO AED	No	310023 / 36795
2	2	*MECP2* p.Y141X	Nonsense/MBD	Refractory epilepsy Generalized seizures	NO AED	No	242968 / 0
3	2	*MECP2* p.R270X	Nonsense/NLS-TRD	Refractory epilepsy	No AED	Severe hyperventilation bursts and apneas	323889 / 0
4	4	*MECP2* p.P302H	Missense/TRD	Generalized seizures from 2 years of life	No AED	Hyperventilation bursts	228307 / 167071
5	5	*MECP2* p.R306C	Missense/TRD	Epileptic status Generalized seizures from 4 years of life	VPA	Hyperventilation bursts and apneas	169395 / 0
6	6	*MECP2*,CDKL5 screened, no alteration found	-	Generalized seizures since 8 years of life	CBZ	Hyperventilation bursts	257110 / 177102
7	7	*MECP2/CDKL5/ FOXG1* screened, no alteration found	-	Absences; Atonic seizures;	No AED	No	296735 / 28646
8	8	*MECP2* p.R255X	Nonsense/NLS-TRD	Generalized seizures from 2 years of life	VPA	Hyperventilation bursts and apneas	342592 / 15584
9	9	*MECP2/CDKL5/* *FOXG1* screened, no alteration found	-	Refractory epilepsy	VPA TPM	Severe hyperventilation and apneas	54010/ 0
10	10	*MECP2* p.R306C	Missense/TRD	Absences and partial seizures from 8 years	CBZ	Hyperventilation bursts	621440 / 155391
11	11	*CDKL5* deletion Exons.1-2 (g.c.1-100.234_65-289del103.669ins CCCGGTTTTATGCC)	Big deletion/no protein produced	Generalized seizures from 8 years	No AED	Hyperventilation	527745 / 91959
12	16	*MECP2/CDKL5* screened, no alteration found	-	Generalized seizures from 11 years of life	No AED (VPA was withdrawn 2 years before LP)	Hyperventilation	445649 / 156883
13	16	*MECP2/CDKL5* screened, no alteration found.	-	Generalized and absence seizures from 14 years of life	VPA CBZ	Hyperventilation bursts	259927 / 164531
14	16	*MECP2* p.R294X	Nonsense/TRD	Generalized seizures from 6 years of life	CBZ LEV	Hyperventilation bursts	281395 / 219795
15	18	*MECP2/CDKL5/* *FOXG1* screened, no alteration found		Partial seizures	CBZ	Hyperventilation	535292 / 7000
16	19	*MECP2* p.R294X	Nonsense/TRD	Partial, secondarily generalized and absences	CBZ	Hyperventilation bursts and apneas	28867 / 604155

Table 3.2. Clinical and laboratory features of RTT patients included in the study. Age is expressed in years at CSF collection. DEL: Deletion. LP: Lumbar Puncture. AED: Anti epileptic drugs. VPA: Valproic Acid. CBZ: Carbamazepine. LEV: Levetiracetam. TPM: Topiramate

3.3.1 KCC2 expression is decreased in the CSF of RTT patients

As patients grew older their OD signal of KCC2 in the CSF decreased. Partial correlation analysis was performed to clarify the relationship between age and the OD signal of the different synaptic proteins (NKCC1, KCC2) in each group (RTT, Controls), controlling for the effect of gender. A negative correlation between age and the OD signal of KCC2 (r (74) = -.292, n = 80, p < .05) has been clearly identified in our data, i.e. as the participants grew older their OD signal of KCC2 decreased, even when controlling for the effect of gender. Meaning that, KCC2 levels decrease in CSF throughout aging. However, concerning the OD signal of NKCC1 this correlation did not reach statistical significance (r (74) = -.207, p > .05). Interestingly, if the effect of gender was not taken into consideration, a strong, negative correlation between age and the OD signal of KCC2 (r_s (80) = -.509, p < .0001) and NKCC1 (r_s (78)= -.472, p < .0001) would have been identified in our data, i.e. as the participants grew older their OD signals of KCC2 and NKCC1 decrease.

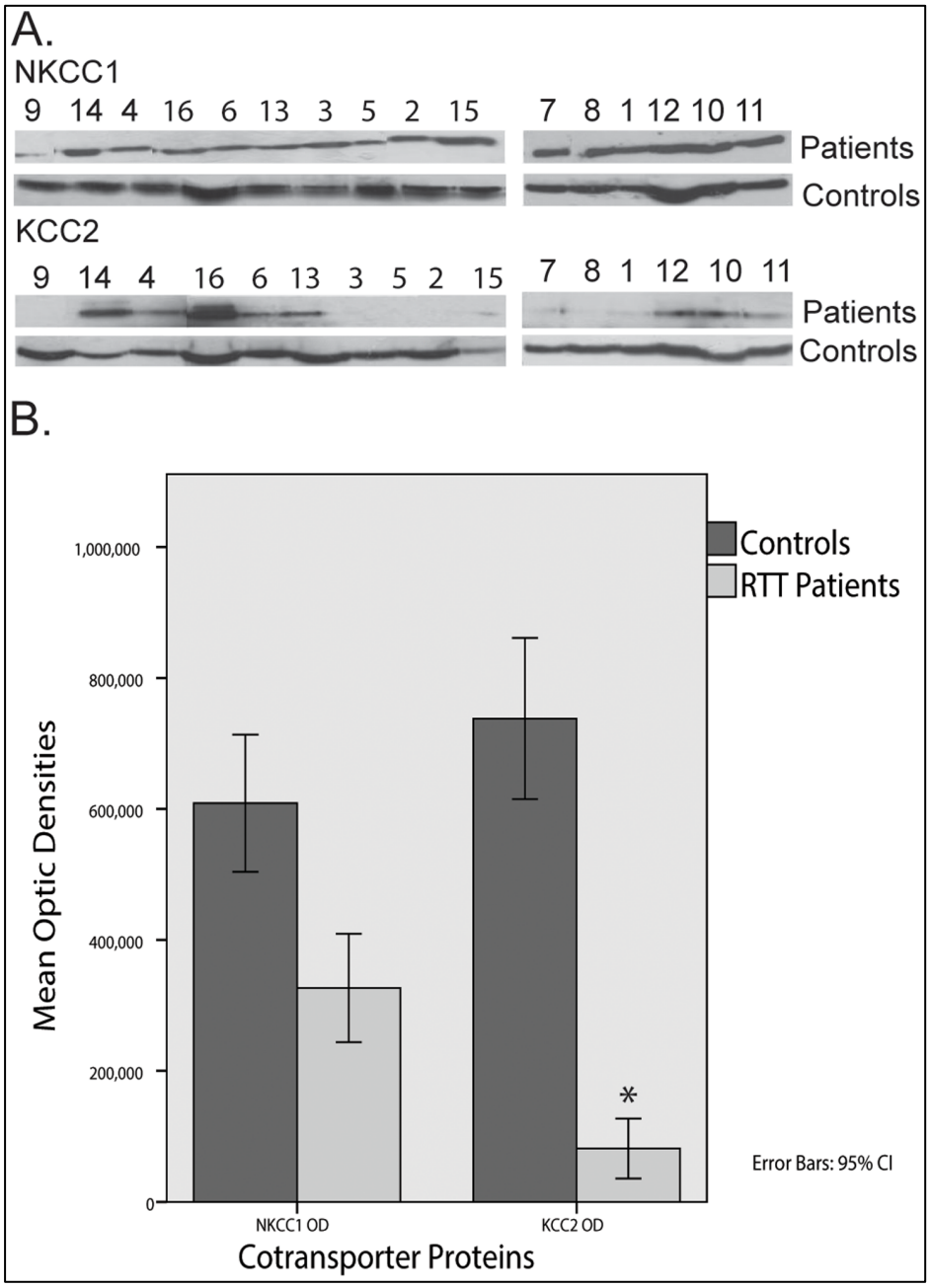


Figure 3.1 NKCC1 and KCC2 Cotransporters in the CSF of RTT Patients and Controls.

(A) Immunoblot results in RTT patients and controls. Numbers refer to the patient ID numbers of Table 1. Comparison of patients and age matched controls. (B) Mean Optic Densities of NKCC1 and KCC2 Cotransporter Proteins for RTT Patients and Controls suggesting discrepant cotransporter levels between Patients and Controls supported by the respective MANCOVA (F (1, 73) = 6.99, p<.01, η_p^2 = .087). Error bars represent 95% Confidence Interval. *p<.01.

RTT patients present a significantly lower OD signal of KCC2 than Healthy controls (Figure 3.1.B): To determine whether there were statistically significant differences in the OD signal of the different synaptic proteins (NKCC1, KCC2) between healthy controls and RTT patients, a between-subjects MANCOVA was performed, controlling for the effects of age and gender. This analysis revealed a statistically significant difference between healthy controls and RTT patients, F (1, 73) = 6.99, p < .01, η_p^2 = .087 even when controlling for the possible confounding effects of age and gender. The subsequent follow-up Univariate ANCOVAs run to specify the characteristics of this finding revealed that RTT patients presented a significantly lower OD signal of KCC2 (M= 81383.80, SD= 82370.99; F (1, 76) = 12.28, p < .001, η_p^2 = .139) than healthy controls (M= 692663.52, SD= 425753.91). Notwithstanding, NKCC1 expression does not differ significantly between patients (M= 307834.25, SD= 162592.59) and healthy controls, (M= 608703.27, SD= 416157.33; F (1, 74) = 1.87, p > .05).

3.3.2 KCC2/NKCC1 ratio is decreased in the CSF of RTT patients

To determine whether there were significant differences in the ratio of the two synaptic proteins (KCC2/NKCC1) on the CSF of the healthy population and RTT patients a between-subjects ANCOVA was performed, controlling for the effect of age and gender. This analysis revealed a statistically significant difference between Healthy controls and RTT patients, F (1, 70) = 26.56, p < .001, η_p^2 = .28 even when controlling for the possible confounding effect of age and gender (Figure 3.2.B and Figure 3.2.C). RTT patients presented a significantly lower KCC2/ NKCC1 ratio (M= .26, SD= .30) than Healthy controls (M= 1.08, SD= .56). Even if the effects of age and gender would not have been controlled for in the analysis the decrease in KCC2/NKCC1 ratio was still significant (F (1, 70) = 30.08, p = .001, η_p^2 = .29).

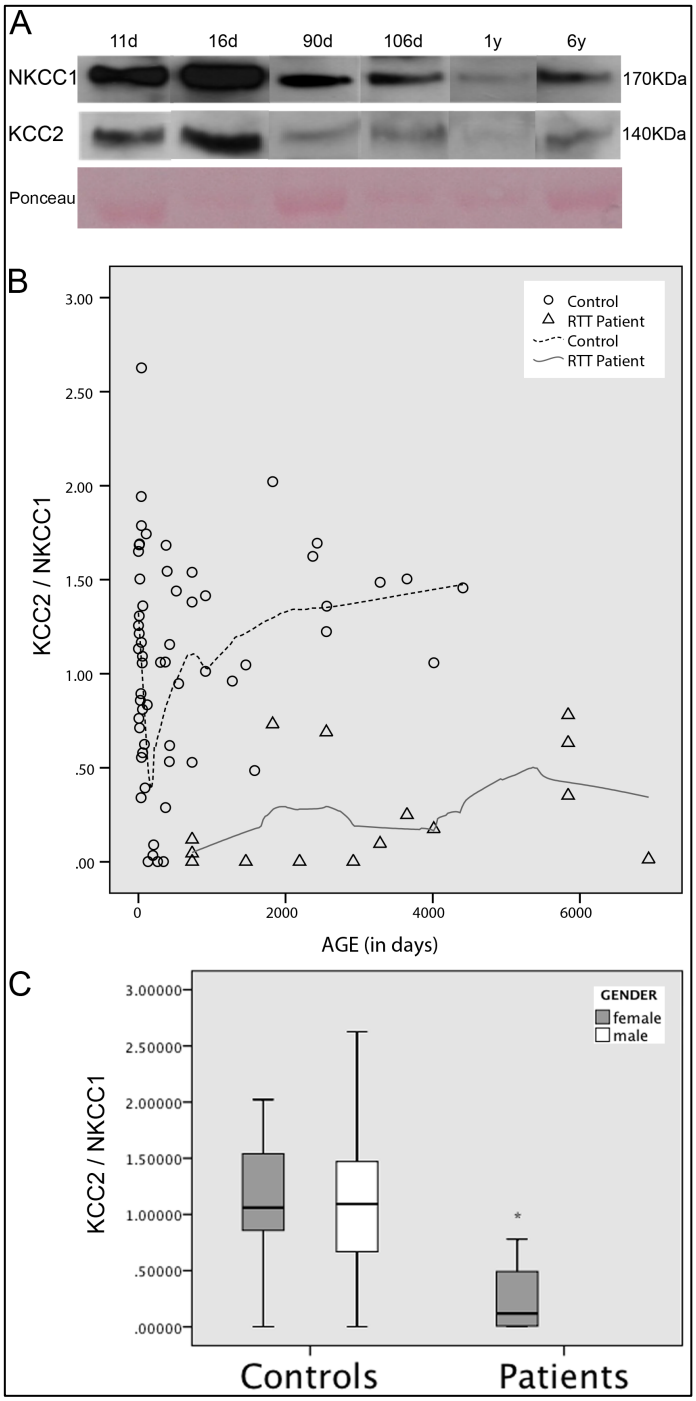


Figure 3.2 Cation Chloride Cotransporters ratio.

(A) Immunoblot analysis of NKCC1 and KCC2. (B) Scatterplot of the relationship between Cotrasporters ratio (KCC2/NKCC1) and age for RTT Patients and Controls. Lines show a LOWESS smooth (locally-weighted polynomial regression - nonparametric smooth) suggesting a discrepant cotransporter ratio between Patients and Controls supported by the respective ANCOVA (F (1, 70) = 30.08, p = .001, η_p^2 = .29). (C) Boxplot of Cotransporters ratio (KCC2/NKCC1) for RTT Patients and Controls (males and females). *p = 0.001.

3.4 Discussion

In this study, we demonstrate reduced KCC2 levels and KCC2/NKCC1 ratio in the CSF of RTT patients. These findings suggest that altered inhibitory GABA function can underlie the pathophysiology of RTT and also play a role in the epileptogenesis of this neurodevelopmental disorder, in which epilepsy is present in around 70% of patients (Nissenkorn et al., 2010). Detection of transmembrane synaptic proteins in the CSF is a useful tool in the study of neurotransmission disorders, as recently reported by our group (Duarte et al., 2011). Despite their low abundance compared to the global CSF proteome (Thouvenot et al., 2008), the proteins here studied (NKCC1 and KCC2) were readily detected and at the expected molecular weight. Harrington and co-workers (Harrington et al., 2009) identified the presence of CSF membranous nanostructures that can provide an appropriate environment for transmembrane proteins, which are hydrophobic in nature. Their morphology is similar to that of synaptic vesicles and exosomes; their structure resembles that of nanotubules, cell-to-cell interacting structures that facilitate the selective transfer of membrane vesicles and organelles but which seem to impede the flow of small molecules (Rustom et al., 2004). The intensity and resolution of the different bands obtained with the immunoblot procedure (Figure 3.1.A and Figure 3.2.A) strongly supports the applicability of this analysis in neurochemical research. To our knowledge, this is the first report of detection of cation chloride cotransporters in CSF.

CSF turnover ratio and the extent of central nervous system cell death and synaptic pruning can influence circulating protein levels but these factors are likely to affect both proteins, NKCC1 and KCC2, equally. During the first months of postnatal life there is a period of intense synaptogenesis that subsequentially decreases. This is probably the cause for the reduction of protein levels in CSF observed during the first year of life. In fact, regarding transmembrane proteins like NKCC1 and KCC2, we have observed the same tendency that also was detected with other synaptic proteins (Duarte et al., 2011). The same phenomena could explain the fact that, in the CSF, both cation chloride cotransporters exhibit a reduction and KCC2 does not increase

in the CSF, as was expected from previous studies in brain tissue (Deng et al., 2007; Galanopoulou, 2008a). Experimental limitations in humans have been an obstacle in obtaining direct evidence of age-related changes in GABAA-R physiology (Galanopoulou, 2008a).

In mice, loss of MeCP2 leads to reduced expression of BDNF after birth (Wang et al., 2006) and in humans evidence of BDNF reduction in RTT has also been detected (Deng et al., 2007). The effects of BDNF on neurotransmission in developing and mature neurons have been partly associated with the regulation of GABAergic transmission. Apart from its effects on GABAergic innervation (Danglot et al., 2006), BDNF can also promote the functional maturation of GABAA-R mediated responses by inducing upregulation of KCC2 (Aguado et al., 2003; Carmona et al., 2006). The imbalance between excitatory and inhibitory functions in RTT has been associated with reduced BDNF (Kline et al., 2010) and GABA levels, decreased expression of GABA receptor subunits (Medrihan et al., 2008), reduced expression of the enzymes glutamic acid decarboxylase 67 and glutamic acid decarboxylase 65 (Chao et al., 2010), reduced number of glutamatergic synapses (Chao et al., 2007) and reduced strength of basal inhibitory rhythms (Zhang et al., 2008). Moreover, exogenous BDNF has been shown to rescue synaptic dysfunction in *Mecp-2* null mice. However, the mechanism by which reduced levels of BDNF contribute to disease and also to the phenotypical rescue is not completely understood (Kline et al., 2010).

Our results suggest an immature pattern of GABAergic neurotransmission in RTT patients, by revealing a dysregulation on the KCC2/NKCC1 ratio (the two major contributors to intracellular chloride concentration) and this evidence in humans is in accordance with the relevance of MeCP2 for GABAergic function described in animal models (Chao et al., 2010). An imbalance between excitatory and inhibitory synaptic events, in the brain of children with neurodevelopmental disorders that have epilepsy and autism as key features, is a postulated general mechanism. Moreover, KCC2/NKCC1 ratio dysregulation is a particularly interesting specific molecular change, already described for diseases like tuberous sclerosis (Talos et al., 2012).

3.5 Conclusions

We describe a significant decrease of KCC2 in the cerebrospinal fluid of RTT patients. A major advantage of doing these *in vivo* studies in children with severe neurologic disorders like RTT, is that it allows to search for disturbances in the normal developmental pattern. Therefore, our findings might have implications for the understanding of RTT pathophysiology, considering that KCC2 is a neuronal specific protein with a key role for neuronal electrical function and structure, properties that are known to be altered in *Mecp2* mutated neurons. Moreover, these results could bring light to new therapeutic approaches, particularly through the pharmacological manipulation of the cation chloride cotransporters. Further studies in the *Mecp2* knockout model and other models to study the disease process are needed to explore these possibilities.

Chapter 4 Adenosine Receptors As New Pharmacological Targets For Rett Syndrome Treatment

Sofia Duarte performed performed LTP experiments in collaboration with Maria José Diógenes who supervised the work described in this chapter.
The following also contributed to the work in this chapter:

Cátia Palminha that performed part of input/output curves, under the grounds of her Master thesis supervised by Maria José Diógenes.

Catarina Lourenço that performed RT-PCR in human samples uder the grouds of her Master thesis co-supervised by of Sofia Duarte and Maria José Diógenes, together with Cláudia Gaspar.

Abstract

RTT is the main cause of intellectual disability in females and is caused mainly by mutations in the X-linked *MECP2* gene. In RTT, BDNF signaling is impaired. BDNF regulates neuronal survival, differentiation and synaptic plasticity such as LTP, an accepted neurophysiological paradigm for learning and memory. The increase of BDNF signaling in these patients' brain would be a significant breakthrough, but has been hampered by the difficulty to administer BDNF to the central nervous system. Adenosine is a neuromodulator that acts mainly through A1 and A2AR. The activation of A2AR potentiates BDNF synaptic actions in healthy animals. Therefore, we explored whether the activation of A2AR facilitates BDNF action upon LTP in a RTT animal model. *Mecp*2 KO (B6.129P2 (C)-*Mecp*2tm1.1Bird/J) animals were used for neurophysiology and molecular assays.

BDNF facilitatory actions upon LTP are absent in the RTT animal model. A reduction in TrkB-FL receptor levels, described for the first time in the present study, can contribute to this impairment. Additionally, mRNA quantification of A1Rs and A2ARs, performed in human brain tissue from a RTT patient and compared with an aged matched control, suggests an increase in A1Rs and a decrease in A2ARs in RTT.

When BDNF was combined with the selective A2ARs agonist CGS2168, before the LTP experiment, BDNF effect upon LTP was restored, similar to what was observed in hippocampal slices from WT animals with BDNF alone.

Together our data highlight A2ARs as new possible therapeutic targets to overcome the BDNF-TrkB signaling impairment in RTT.

4.1 Introduction

RTT (MIM312750) is a rare neurodevelopmental disorder caused primarily by mutations in the X-linked gene *MECP2*. RTT is considered the leading cause of severe intellectual disability in females with a prevalence estimated at 1/9,000 in girls under the age of 12, whereas prevalence in the general population is estimated at approximately 1/30,000 (source: Orphanet). Developmental stagnation usually becomes apparent between 6 to 18 months of life and head growth typically decelerates, leading to microcephaly, by the second or third year of life. Loss of purposeful hand skills combined with the development of stereotypic hand movements is a hallmark of the disease. With disease evolution cognitive deficit and motor disturbances like ataxia, apraxia and dystonia are observed together with autonomic features like vasomotor disturbances and abnormal breathing patterns. Epilepsy is common in RTT, has an age-related pattern of occurrence and younger age of onset is associated with greater epilepsy severity. Other signs and symptoms like anxiety, sleep disturbances, scoliosis, life threatening cardiac abnormalities (tachycardia, prolonged corrected QT interval, and sinus bradycardia) are frequently described (Chahrour and Zoghbi, 2007; Glaze et al., 2010; Hagberg, 2005; Neul et al., 2010; Nissenkorn et al., 2015; Zoghbi and Bear, 2012). MeCP2 is a multifunctional protein involved not only in transcriptional silencing but also in transcriptional activation, chromatin remodeling, RNA splicing and microRNA processing control, with key regulatory activity during neurodevelopment (Cheng et al., 2014; Johnson et al., 2012). In accordance with the wide spectrum of clinical and particularly neurological features, MeCP2 is widely expressed in the CNS and not limited to specific cell types, indicating that the pathogenesis of RTT is caused by multiple impairments of the CNS development and mature function (Guy et al., 2007; Robinson et al., 2012). RTT is mainly caused by MeCP2 deficiency, but duplications of *MECP2*-containing loci are also detrimental to neural development and proper brain functions (Schmid et al., 2012). These observations underpin the relevance of a fine regulation of MeCP2 expression, since the levels of MeCP2 protein are critical for the development of the central nervous system. Although no specific treatments for RTT are

currently available, several studies have demonstrated that the *Mecp2*-related condition in mice is, at least, partially reversible, as phenotypic rescue is possible (Guy et al., 2007; Pitcher et al., 2015; Robinson et al., 2012). These studies suggest that therapeutic strategies aiming to restore MeCP2 dependent functions and circuits, even in symptomatic patients, might be beneficial. However, since the slightest perturbation in MeCP2 levels is deleterious for brain functioning, MeCP2-encoding gene therapy does not seem to be a valid approach in the near future. Thus, great advances in the RTT research field will be made if useful therapeutic approaches are developed, targeting MeCP2-associated dysfunctions.

MeCP2 functions are still being unraveled, despite of extensive research in the field. The recent consensual view is that it acts as a fine-tuning regulator of gene transcription. Along with the transcriptional control, MeCP2 interferes with various posttranscriptional regulatory mechanisms (Cheng et al., 2014). One of the most prominent genes under the jurisdiction of MeCP2 is the BDNF gene (Chen et al., 2003). BDNF is a neurotrophin that acts through a dual receptor system, to modulate diverse and sometimes opposing biological actions, that consists of a specific high affinity receptor, the full lenght tyrosine kinase receptor B (TrkB-FL), and a common low affinity receptor, p75 neurotrophin receptor (Encinas et al., 1999; Tsai, 2015).
Through TrkB-FL receptor activation, BDNF regulates neuronal survival, differentiation and synaptic plasticity (Huang and Reichardt, 2003) such as LTP, the neurophysiological basis for learning and memory (Bliss and Collingridge, 1993). Reduced BDNF signaling through TrkB-FL leads to impaired spatial memory (Kemppainen et al., 2012; Minichiello, 2009) while overexpression of TrkB-FL enhances memory (Kemppainen et al., 2012; Koponen et al., 2004). The TrkB gene (*NTRK2*) also encodes for truncated isoforms (Klein et al., 1990), which may act as negative modulators of TrkB-FL signaling (Dorsey et al., 2006; Eide et al., 1996) and decreases in the ratio between FL and truncated (Tc) receptors have been detected in several neurological disorders (Jeronimo-Santos et al., 2014). In symptomatic mice models of RTT, which recapitulate the most prominent symptoms presented by RTT patients, BDNF levels are decreased in the whole brain lysate (Chang

et al., 2006), suggesting that MeCP2 acts as an activator of *BDNF* expression. However, the regulation of *BDNF* expression is highly complex and this complexity is necessary for appropriate response to different neuronal functional states and region specific demands (Li and Pozzo-Miller, 2014).

Inspired in the rational that impairment of BDNF signaling could be strongly involved in the symptoms presented by RTT patients, work has been developed in order to study the impact of potentiating BDNF effects in animal models. Experiments designed to increase BDNF signaling mediated by TrkB-FL phosphorylation had positive results regarding breathing function and locomotor activity (Johnson et al., 2012; Ogier et al., 2007; Schmid et al., 2012) and the overexpression of BDNF demonstrated to extend the lifespan and reverse some electrophysiological deficits observed in these animals (Chang et al., 2006). However, until now there is no commercially available pharmacological treatment directed towards boosting BDNF effects. This might be explained by the high complexity of BDNF signaling deregulation in RTT, that it is not yet fully understood. Additionally, BDNF short bioavailability and low blood–brain barrier penetration have also been a constraint. Therefore, in order to overcome this limitation, other strategies should be considered, such as the use of other molecules able to boost BDNF actions. It is now currently accepted that most of BDNF actions are dependent on the activation of a particular type of adenosine receptors, the A2ARs (Sebastiao et al., 2011).

Adenosine is an ubiquitous homeostatic substance present in all cells including neurons and glia (see (Ribeiro et al., 2003)). This neuromodulator is neither stored nor released as a classical neurotransmitter since it does not accumulate in synaptic vesicles, but is instead released from the cytoplasm into extracellular space through a nucleoside transporter (see e.g. (Ribeiro et al., 2003)). Adenosine receptors have been intensively studied, and to the present date, four different receptors have been cloned and designated as A_1, A_{2A}, A_{2B} and A_3 receptors. Modulation of adenosine receptors, mostly A1R and A2AR is potentially a useful strategy in the treatment of multiple pathological states in the nervous system, such as sleep disorders, epilepsy and neurodegenerative diseases (Sebastiao and Ribeiro, 2009b). Actually,

clinical trials using drugs that modulate adenosine receptors were already developed (Chen et al., 2007).

In spite of the previous knowledge about: i) the crosstalk between BDNF and adenosine receptors, ii) the advantages of potentiating BDNF actions in RTT, and iii) the deregulation of adenosinergic system in several pathological conditions occurring with epilepsy, no research has been developed so far, considering adenosine receptors as possible therapeutic targets in RTT. Accordingly, in the present work, we used the *Mecp2* knockout (KO) (B6.129P2 (C)-Mecp2tm1.1Bird/J) RTT mice model, to study strategies to potentiate BDNF effects through pharmacological modulation of adenosine receptors. In order to better characterize adenosine receptors expression in RTT, we also quantified their mRNA expression in tissue dissected from a RTT human brain, compared with an age-matched control.

4.2 Materials and Methods:

4.2.1 Animals

The molecular characterization of BDNF and TrkB receptors was done in hippocampi taken from 1, 3, and 6 week old wild type (Huber et al.) and *Mecp2* Knockout (KO) (B6.129P2 (C)-*Mecp*2tm1.1Bird/J) animals. These animals exhibit an initial period free of symptoms and, at around 3 to 8 weeks of life, develop a stiff, uncoordinated gait and reduced spontaneous movement. Most animals subsequently developed hindlimb clasping and irregular breathing. Variable progression of symptoms lead ultimately to rapid weight loss and death at approximately 54 days. (Guy et al., 2001) Several studies have shown that this model mimics a panoply of human RTT symptoms, including repetitive movements, microcephaly, abnormal breathing, seizures, cognitive and motor impairment (Katz et al., 2012).

Electrophysiological recordings were performed in hippocampi and cortex from 6 week old animals (WT and KO). All the animals were handled according to the Portuguese law on Animal Care and European Union guidelines (86/609/EEC). Animals were housed in the local Animal House on a 12hours light/dark cycle and were provided food and water *ad libitum*. Care

was taken so to reduce the number of used animals to the absolutely necessary. The genotype of the mice was determined by polymerase chain reaction (Guy et al., 2007).

4.2.2 Human Brain Tissue Samples

The brain tissue of a 11 year-old girl affected with RTT (*MECP2* mutation - R255X) who died after a severe pneumonia, was dissected in different anatomic regions and were immediately frozen at -80ºC. Parent informed consent and ethical approval from HSJD were obtained. Control tissue was kindly provided by the biobank of HSJD.

4.2.3 Western Blot Analysis

Protein extracts for western blot analysis were prepared from snap-frozen hippocampi or cortex. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Zeng et al.) was used to evaluate the levels of BDNF and TrkB receptors. Brain tissue was disrupted in sucrose-Tris solution with a Teflon pestle and the protein content in the supernatants was determined using a commercial Bradford assay (Sigma, MO, United States of America). Total proteins (70 µg) were separated on 10% SDS-polyacrylamide electrophoresis gels and blotted onto a polyvinylidene fluoride membrane according to the standard procedures. The blots were probed overnight at 4ºC either with the polyclonal rabbit anti-BDNF or monoclonal mouse anti-TrkB in 3% BSA (Bovine Serum Albumin). After washing (3 x 5 min in TBST [10 mM Tris, 150 mM NaCl, 0,05% Tween 20 in H_2O]), blots were then incubated with secondary antibodies conjugated with horseradish peroxidase and bands were visualized with a commercial enhanced chemiluminescence detection method (ECL) kit (PerkinElmer Life Sciences, MA, United States of America). Values were normalized to the β-actin or α-Tubulin or GAPDH loading control and the relative intensities were normalized to WT (set as 1). Densitometry of the bands was performed using the Image J processing software (NIH, MD, United States of America).

4.2.4 Ex vivo electrophysiological recordings

The hippocampus was dissected free within ice-cold aCSF solution composed of: 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 hippocampus with a McIlwain tissue chopper and allowed to recover functionally and energetically for 1 h in a resting chamber, filled with same solution, at room temperature. Slices were transferred to a recording chamber for submerged slices and continuously superfused at 3 ml/min with gassed bathing solution at 32ºC; drugs were added to this superfusion solution in an open circuit. Recordings were obtained with an Axoclamp 2B amplifier and digitized (Axon Instruments, Foster City, CA). Individual responses were monitored, and averages of six consecutive responses continuously stored on a personal computer with the LTP software (Anderson and Collingridge, 2001).

Basal synaptic transmission: Field excitatory post-synaptic potentials (fEPSPs) were recorded through an extracellular microelectrode (4M NaCl, 2–6 MΩ resistance) placed in *stratum radiatum* of CA1 area. Stimulation (rectangular 0.1 ms pulses, once every 15 s) was delivered through a concentric electrode placed on the Schaffer collateral–commissural 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. 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 as described previously (Diogenes et al., 2004).

LTP induction and quantification: fEPSPs (Figure 4.1.A2) were recorded through extracellular microelectrode (4M NaCl, 2–6MΩ resistance) placed in stratum radiatum of CA1 area as represented in Figure 4.1.A1 In LTP experiments, stimulation (rectangular 0.1 ms pulses, once every 10 s) was delivered alternatively to two independent pathways through bipolar concentric electrodes placed on Shaffer collateral/commissural fibers in

stratum radiatum (Figure 4.1.A1). LTP was induced by θ-burst protocol consisting of three trains of 100 Hz, three stimuli, separated by 200 ms as previously described (Diogenes et al., 2011) and shown in Figure 4.1.A3.

The intensity of the stimulus was maintained during the induction protocol. LTP was quantified as the % change in the average slope of the fEPSP taken from 46 to 60 min after LTP induction in relation to the average slope of the fEPSP measured during the 14 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. The effect of BDNF upon LTP was evaluated by comparing the magnitude of LTP in the first pathway in the absence of BDNF (control pathway), with the magnitude of LTP in the second pathway in the presence of BDNF (test pathway); each pathway was used as control or test on alternate days. To test the modification of the effect of BDNF upon LTP, the modulatory drugs were added to superfusion bath at least 20 min before induction of LTP in the first pathway and remained in bath up to the end of the experiment. BDNF was added, as usual, 60 min after the induction of LTP in the first pathway. Thus, modulatory drugs were present during both LTP-inducing periods, whereas BDNF was only present during the second induction of LTP. This protocol allows the comparison between the effects of BDNF upon LTP under different experimental conditions, keeping the magnitude of LTP under the same drug condition, without BDNF in the same slice as an internal control. When testing the effect of a drug upon LTP (rather than the modulation of the BDNF effect on LTP), the drug was added to bath 20 min before induction of LTP in the second pathway and the magnitude of the resulting LTP was compared with that previously obtained (first pathway) in the absence of the drug (see, (Diogenes et al., 2011)). In control experiments performed with hippocampal slices taken from any age group, we confirmed that when the θ-burst paradigm was sequentially applied to each of the two pathways in the absence of any drug, the synaptic potentiation obtained in the first pathway was similar to that achieved in the second pathway.

Input–output curve: Input–output (I/O) curves in slices from the two genotypes were performed to ensure that modifications in LTP magnitude were not due to changes in basal synaptic transmission. After obtaining a stable baseline for at least 15 min, the stimulus delivered to the slice was decreased until disappearance of the fEPSPs. The stimuli delivered to the slice were successively increased by steps of 20 µA. For each stimulation condition, data from three consecutive averaged fEPSP (each averaged fEPSP is the computerized mean of six individual fEPSP) were stored. The range of all inputs delivered to the slice was typically from 60 µA to supra-maximum stimulation amplitude of 340 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 (Diogenes et al., 2012).

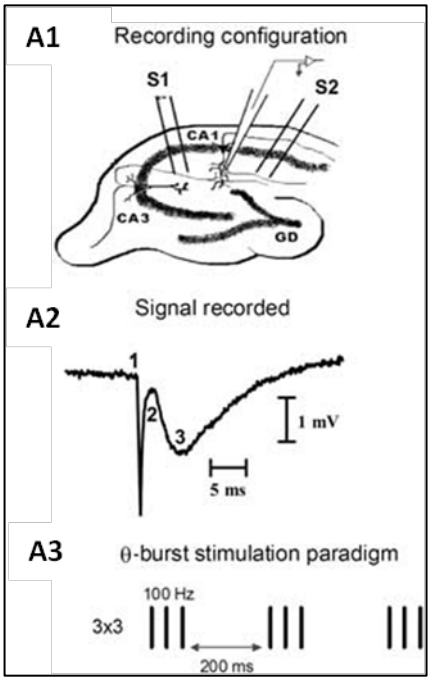


Figure 4.1 Extracellular recordings in hippocampal slices

Schematic representation of a hippocampal transverse slice preparation showing the recording configuration used to obtain extracellular responses in the CA1 dendritic layer (*stratum radiatum*) evoked by stimulation of two (**A1**) separate sets of the Schaffer pathway (S1 and S2). (**A2**) Example of a representative trace obtained after stimulation composed by the stimulus artifact (1), followed by the pre-synaptic volley (2) and the field excitatory post-synaptic potentials (fEPSP) (3). A schematic representation of the stimulation paradigms used in plasticity experiments is represented in (**A3**). Adapted from Diógenes et al., 2011 (Diogenes et al., 2011).

4.2.5 RNA extraction and cDNA synthesis

Total RNA was extracted from brain cortex using RNeasy Lipid Tissue Mini Kit (Qiagen) from cortex tissue, according to the manufacturer's instructions. RNA concentration and purity were evaluated by spectrometry on the basis of optical density (OD) measurements at 260 and 280 nm.

cDNA synthesis was performed in a 20 µl reaction mixture. A 4.3 µg total RNA was mixed with 1 µl random primer hexamer (Amersham) and 1 µl each of dATP, dTTP, dCTP, dGTP (each 10 mM) and incubated for 5 min at 65ºC. After cooling for 2 min at 4ºC, the solution was mixed with 4 µl of 25 mM $MgCl_2$, 2 µl of 10X RT Buffer, 2 µl of 0.1M DTT, 0.5 µl SuperScript III Reverse Transcriptase (200 U; Invitrogen Life Technologies). The reaction was performed for 50 min at 42ºC and terminated by 15 min incubation at 70ºC. Parallel reactions for each RNA sample were run in the absence of SuperScript III to assess the degree of any genomic DNA contamination. Completed RT reactions were stored at −20 ◦C until use.

Quantative PCR

qPCR for mRNA was done using Power SYBR® Green PCR Master Mix (Life Technologies), the pre amplified cDNA was used as the template for the real-time PCR run Rotor Gene 6000 (Corbett Life Science), according to the manufacturer´s recommendations. Negative Control PCR samples were run with no template. Fold-changes were calculated using Cq method and normalized to the expression of GAPDH with Rotor-Gene Series Software 1.7 (Corbett Life Science). The primers used are depicted in table 4.1.

Gene	Primers (5'-->3')
TrkB-FL	F: 5′-GGCCCAGATGCTGTCATT AT-3′ R: 5′-TGCCTTTTGGTAATGCTGTTT-3′
TrkB-Tc	F:5'-TCTATGCTGTGGTGGTGATTG-3' R:5'- GAGTCCAGCTTACATGGCAG-3'
A_1R	F: 5'- GCCACAGACCTACTTCCACA-3' R: 5'- CCTTCTCGAACTCGCACTTG-3'
$A_{2A}R$	F: 5'- AACCTGCAGAACGTCAC-3' R:5'- GTCACCAAGCCATTGTACCG-3'
BDNF	F:5'- TAACGGCGGCAGACAAAAAGA-3' R: 5'-GAAGTATTGCTTCAGTTGGCCT
GAPDH	F:5'-GGAGTCAACGGATTTGGTCG-3' R:5'-GACAAGCTTCCCGTTCTAG-3'

Table 4.1. Primers used for qPCR.

Data was normalized according to the following formula: Cq = Cq (target gene) – Cq (reference gene).

4.2.6 Drugs

BDNF was generously provided by Regeneron Pharmaceuticals, 2-[p-(2-carboxyethyl)phenethylamino]-50-N-ethylcarboxamido adenosine (CGS-21680) was from Sigma; Aliquots of the stock solutions were kept frozen at -20ºC until use.

4.2.7 Antibodies

Anti-TrkB antibody was from BD Biosciences, anti-β-actin antibody, anti-BDNF and anti-α-Tubulin were obtained from Abcam, anti-GAPDH was purchased from Life Technologies and secondary antibodies were from Biorad Laboratories.

4.2.8 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 Student's t-test. Values of $P<0.05$ were considered to represent statistically significant differences.

4.3 Results

4.3.1 BDNF and TrkB-FL receptor are severely decreased in symptomatic MeCp2 KO animals

Evidence supports MeCP2 actions as a repressor and also as an activator of gene expression (Chahrour and Zoghbi, 2007). Given that the *BDNF* gene is under MeCP2 regulation (Chen et al., 2003) attention has been directed to BDNF alterations on RTT.

Bdnf expression levels are low in the rodent brain during prenatal development, and rise dramatically during the postnatal period, which coincides with the pattern of *Mecp2* expression (Li and Pozzo-Miller, 2014). It was already described that *Bdnf* expression is impaired in RTT mice model exhibiting neuropathological and behavioral phenotypes (Chang et al., 2006). Less attention has been directed to TrkB receptors, crucial players in BDNF mediated neuroprotection (Huang and Reichardt, 2003). Accordingly, together with the evaluation of BDNF levels, TrkB receptors levels were also evaluated in both presymptomatic and symptomatic stages.

During the presymptomatic stage, hippocampi from 1 and 3 week old animals were studied. In the symptomatic period samples from 6-8 week old animals were evaluated. Interestingly, we observed a decrease of BDNF levels by approximately 20 % in the hippocampi taken from presymptomatic 3 week old KO animals when compared with the age matched WT animals ($p<0.05$, n=4, Figure 4.2.B1,2). Our preliminary data from 1 week old animals (n=2, Figure 4.2.A1,2), suggest increased levels of BDNF in this period of life.

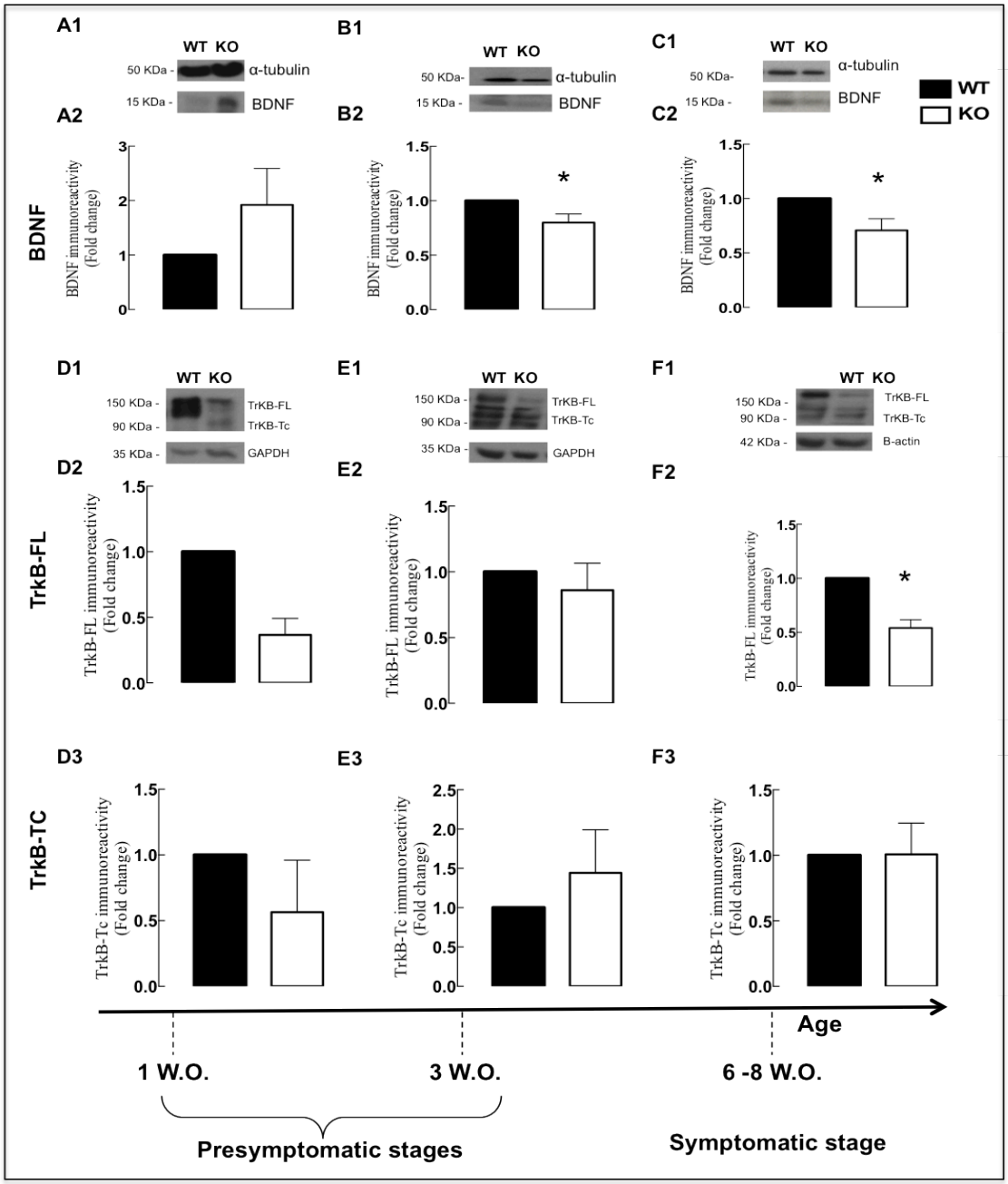


Figure 4.2 BDNF and TrkB receptor are decreased in symptomatic *MECP*2 KO animals

In **A2, B2** and **C2** are shown the averaged of BDNF density evaluated by western blot analysis in hippocampal homogenates prepared from hippocampi taken from WT (black bars) and KO (white bars) in different stages: presymptomatic stage, 1 week of age (**A2**) and 3 weeks of age (**B2**) and symptomatic stage, 6 weeks of age (**C2**). **A1, B1** and **C1** show representative western blots for each age of the animals using an antibody which recognizes BDNF (~15 KDa). A-Tubulin (~50 KDa) was used as loading control. In **D2**, **E2** and **F2** are shown the averaged OD of TrkB-FL receptors evaluated by western blot analysis in hippocampal homogenates prepared from

hippocampi taken from WT (black bars) and KO (white bars) in different stages: presymptomatic stage, 1 week of age (**D2**) and 3 weeks of age (**E2**) and symptomatic stage, 6 weeks of age (**F2**). In **D3**, **E3** and **F3** are shown the averaged of TrkB-TC receptors density evaluated by western blot analysis in hippocampal homogenates prepared from hippocampi taken from WT (black bars) and KO (white bars) in different stages: presymptomatic stage, 1 week of age (**D3**) and 3 weeks of age (**E3**) and symptomatic stage, 6 weeks of age (**F3**). **D1, E1** and **F1** show representative western blots for each age of the animals using an antibody that recognizes TrkB-FL receptors (~145 KDa) and TrkB-Tc receptors (~95 KDa). GAPDH (~35 KDa) or β-Actin (~42 KDa) were used as loading controls. All values are mean ± standard error of mean. *P<0.05 (Student's t-test). The hippocampal homogenates used in the analysis were taken from the same animals used in electrophysiological experiments. The values obtained for WT samples were considered 1.

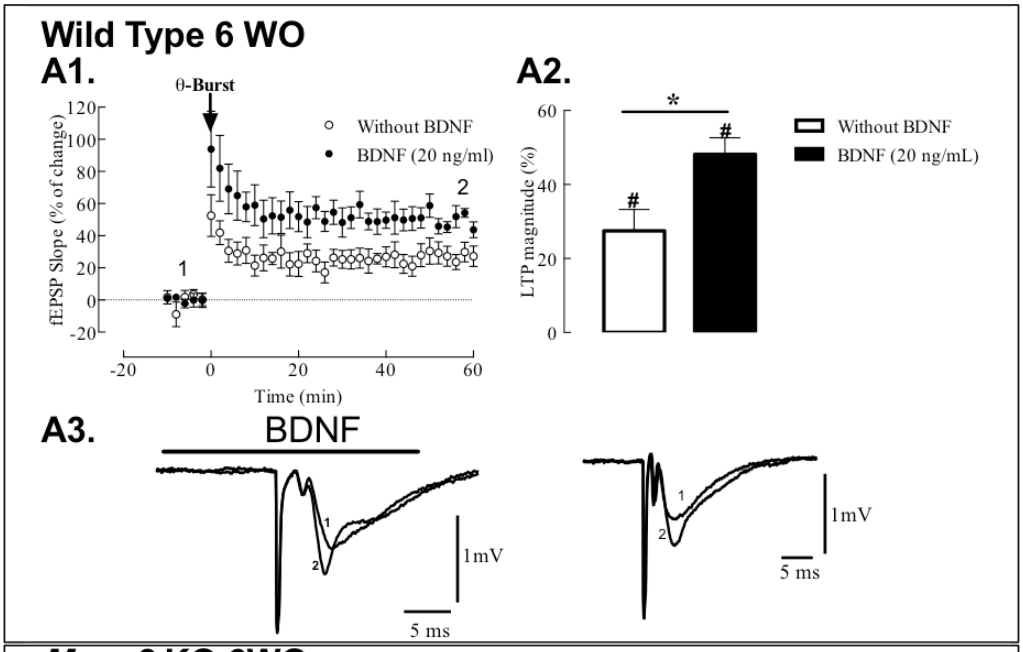
As expected, in the symptomatic KO animals (Chang et al., 2006) the hippocampal levels of BDNF are decreased by approximately 30% ($p<0.05$, n=5, Figure 4.2.C1,2). Regarding TrkB receptor levels, there is a significant reduction on the full length (FL) protein by approximately 46% ($p<0.05$, n=8-10, Figure 4.2.F1,2) in symptomatic 6-8 week old KO animals comparing to the age matched controls. The data obtained in two hippocampal samples from 1 week old animals, suggest the presence of decreased levels of TrkB-FL receptors in this period of life (n=2, Figure 4.2.D1,2). No significant changes were detected on the protein levels of TrkB truncated forms throughout ages studied (Figure 4.2.D1,3; E1,3; F1,3).

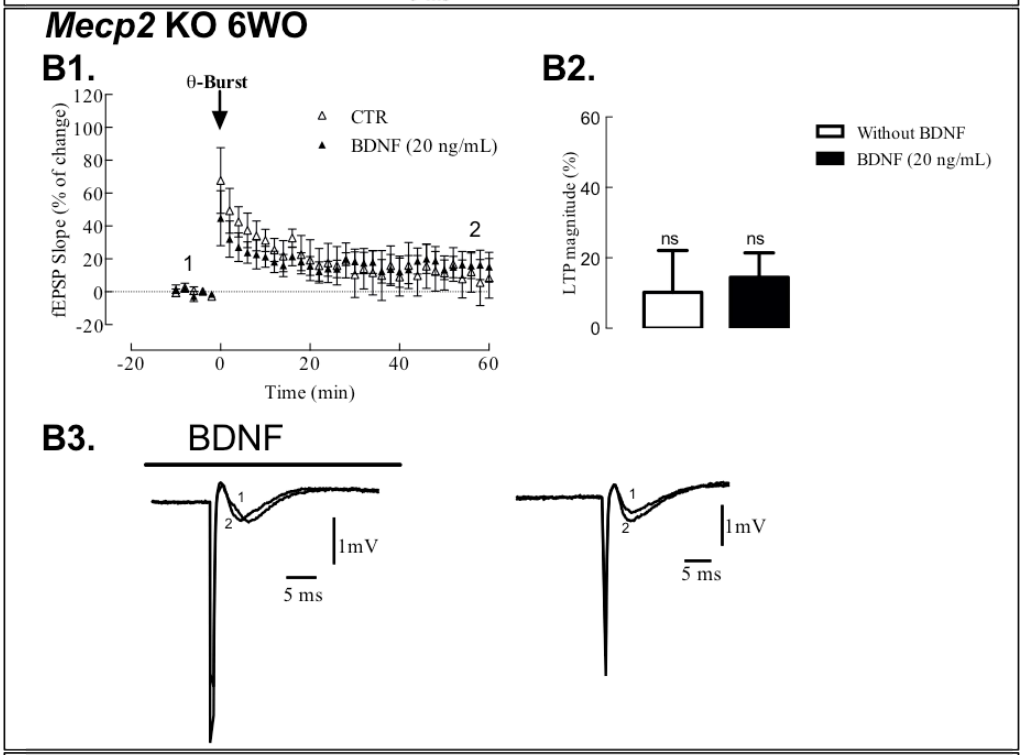
4.3.2 BDNF loses the facilitatory effect upon LTP in Mecp2 KO animals

BDNF has a well-documented ability to increase LTP, an accepted neurophysiological paradigm for learning and memory, on hippocampal CA1 area through TrkB-FL receptors activation (Figurov et al., 1996; Korte et al., 1996). In order to evaluate the functional impact of the molecular changes detected in symptomatic KO animals, the effect of exogenous BDNF upon LTP was evaluated. Therefore, as described in the methods section, electrophysiological recordings were performed using hippocampal slices taken from 6-8 week old WT and KO animals (symptomatic stage). As

expected (Diogenes et al., 2011; Fontinha et al., 2008), the θ-burst stimulus applied to hippocampal slices, taken from WT animals, in the presence of BDNF (20 ng/mL) induced a robust LTP ($LTP_{WT-BDNF}$: 46.9 ± 5.6% increase in fEPSP slope, n=4, Figure 4.2.A), which was significantly higher ($P<0.05$) than the obtained in the absence of the neurotrophin (LTP_{WT-CTR}: 24.1 ± 6.1% increase in fEPSP slope; n = 4, Figure 4.2.A1,2). The θ-burst stimulation induced a small LTP, in the absence of BDNF (control pathway), but statistically significant when compared to the baseline ($P<0.05$, n=4). On the contrary, in hippocampal slices taken from KO animals the same θ-burst protocol did not induced a significant increase on the slope of fEPSP compared to the baseline. Moreover, BDNF (20 ng/ml) did not further increased LTP magnitude (LTP_{KO-CTR}: 10.1 ± 11.9% and $LTP_{KO-BDNF}$: 14.5 ± 7.0%, n=5, Figure 4.2.B) in hippocampal slices taken from KO animals.

To evaluate whether the changes observed in LTP magnitude could be due to changes in baseline synaptic efficiency, I/O curves were performed and are shown in Figure 4.3. Hippocampal slices taken from KO animals displayed higher Emax values when compared with WT animals ($Emax_{WT=}$ 0.9901 ± 0.04626, n= 4; $Emax_{KO=}$ 1.781 ± 0.2129, n=4, $p<0.05$, F test;). Therefore suggesting, that the absence of a robust LTP in KO animals is not due to a decrease in basal synaptic transmission.


Wild Type 6 WO
A1.
θ-Burst
Without BDNF
BDNF (20 ng/ml)
fEPSP Slope (% of change)
Time (min)
A2.
LTP magnitude (%)
Without BDNF
BDNF (20 ng/mL)
A3.
BDNF
1mV
5 ms


Mecp2 KO 6WO
B1.
θ-Burst
CTR
BDNF (20 ng/mL)
fEPSP Slope (% of change)
Time (min)
B2.
LTP magnitude (%)
Without BDNF
BDNF (20 ng/mL)
ns
B3.
BDNF
1mV
5 ms

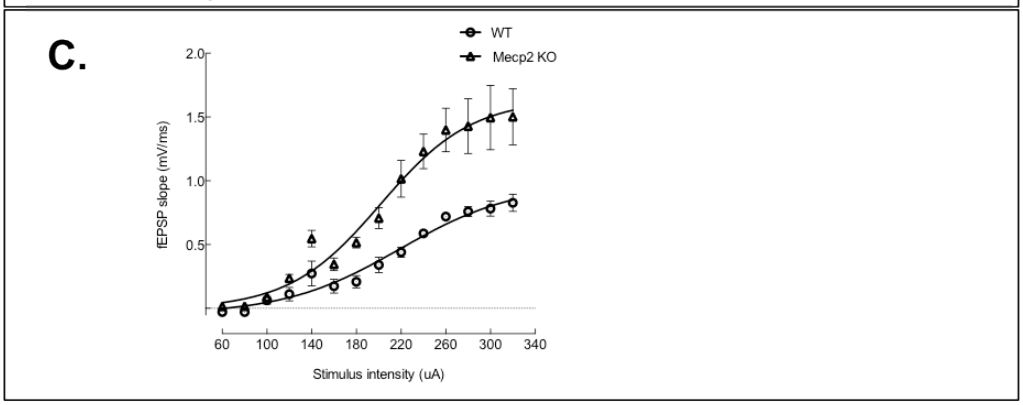

C.
WT
Mecp2 KO
fEPSP slope (mV/ms)
Stimulus intensity (uA)

Figure 4.3 BDNF loses the facilitatory effect upon LTP in *MECP2* KO animals

Panels **A1** and **B1** show averaged time courses changes in field excitatory post-synaptic potential (fEPSP) slope induced by the θ-burst stimulation in the absence (white symbols) or in the presence (black symbols) of BDNF (20 ng/ml) in hippocampal slices taken from wild type animals (A) or from *Mecp2* KO animals (B). BDNF (20 ng/ml) was applied 60 min after the induction of LTP in the first pathway (white symbols) and at least 20 min before induction of LTP in the second pathway (black symbols). The ordinates represent normalized fEPSP slopes, where 0% corresponds to the averaged slopes recorded for 14 min before θ-burst stimulation and the abscissa represents the time of every recording. Panels (**A2** and **B2**) depict the magnitude of LTP (change in the fEPSP slope at 46–60 min) induced by θ-burst stimulation in relation to pre-θ-burst values (0%) in the absence of (white bars), or in the presence of BDNF (black bars) alone in hippocampal slices from WT animals (**A2**) or KO animals (**B2**). In **A3** and **B3** are shown traces obtained in representative experiments in **A1** and **B1,** respectively; Panel **C** shows the input/output (O/I) curves corresponding to responses generated by various stimulation intensities (60–340 μA) in WT slices (filled circles) and KO slices (filled triangles).

All values are mean ± standard error of mean. *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 baseline in the same experiments. n.s. not significant;

Part of the I/O curve experiments were performed by Cátia Palminha and are included in her Master thesis.

4.3.3 The activation of A2AR restores LTP magnitude and the effect of BDNF upon LTP

A considerable number of data have been published demonstrating that there is a cross talk between BDNF and a particular type of adenosine receptors the A2ARs (Sebastiao et al., 2011). Accordingly, many BDNF effects are dependent and or facilitated by activation of A2ARs. This cross talk is present even when there is a strong reduction on TrkB-FL receptor levels, as in aging (Diogenes et al., 2007), where the activation of A2ARs is able to promote synaptic actions of BDNF (Diogenes et al., 2007). Therefore, the activation of A2ARs has been seen as a way to potentiate synaptic effects of BDNF. Indeed, in the presence of the selective A2ARs agonist, CGS 21680 (10 nM)

(Jarvis et al., 1989) added 60 minutes after the LTP induction in the first pathway and at least 20 minutes before BDNF (20 ng/ml) superfusion, the neurotrophin significantly increased LTP magnitude (LTPKO-CTR: 9.1 ± 10.0% and LTPKO-CGS+BDNF: 43.9 ± 4.3%, n=6, $p<0.05$; Figure 4.4.B1,2,3). Alone CGS 21680 did not affect LTP magnitude when compared to the LTP induced in the first pathway (control-CTR) (Figure 4.4.A1,2,3). Interestingly, the θ-burst stimulation in the presence of CGS 21680 induced an increase on fEPSP slope ($P<0.05$, Figure 4.4.A2) as compared with baseline.

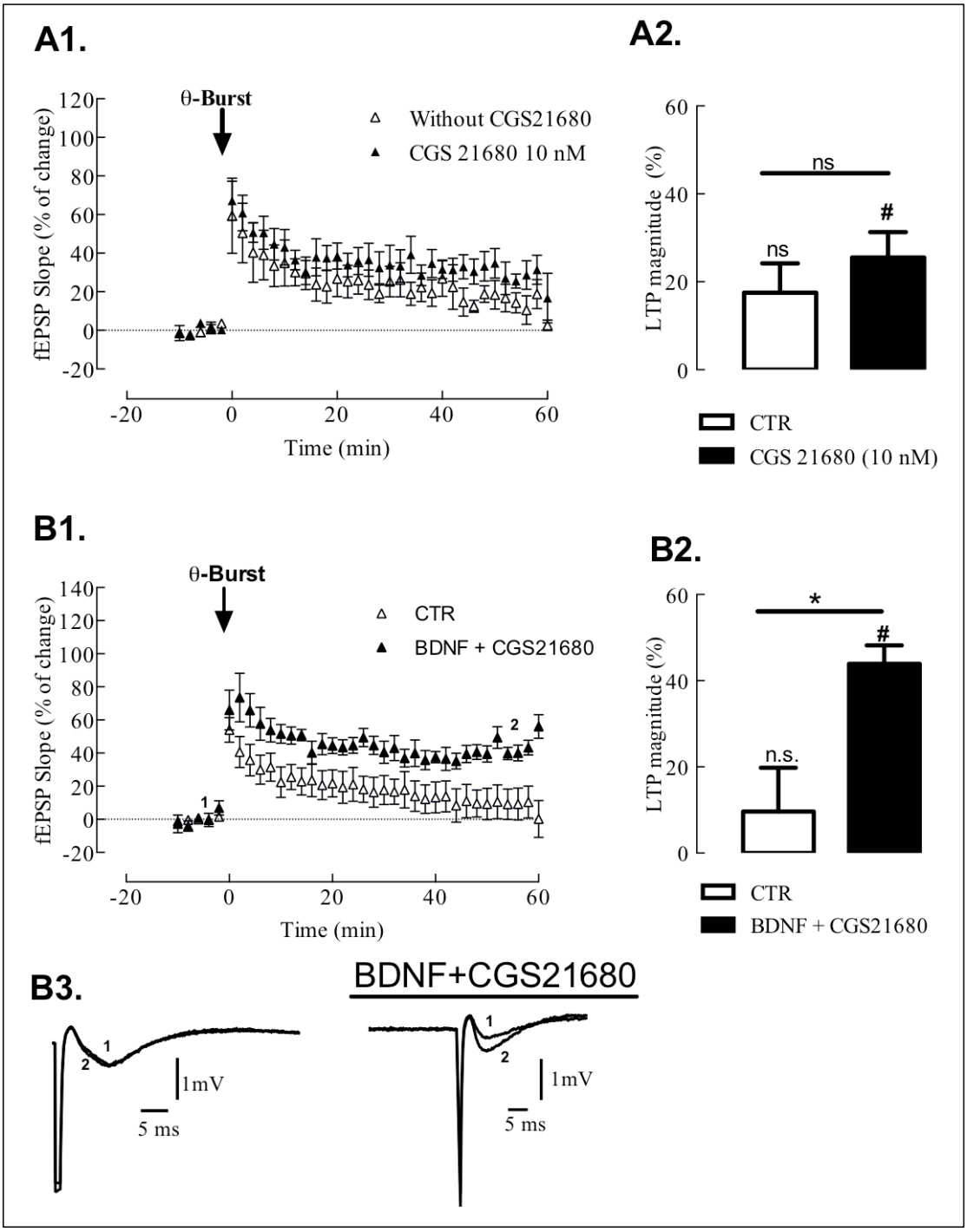


Figure 4.4 Activation of A2ARs restores the effect of BDNF upon LTP

Panel **A1** shows averaged time courses changes in field excitatory post-synaptic potential (fEPSP) slope induced by the θ-burst stimulation in the absence (open triangles) or in the presence (filled triangles) of CGS21680 (10nM) and BDNF (20ng/ml) in hippocampal slices taken from *MECP2* knockout (KO) animals. BDNF was applied at least 20 min after CGS21680 (10nM) applications which was applied 60 min after the induction of LTP in the first pathway (open triangles). Panel **A2** shows averaged time courses changes in fEPSP slope induced by the θ-burst

stimulation in the absence (open triangles) or in the presence (filled triangles) of the selective A2A agonist, CGS21680 (10nM) in hippocampal slices taken from *Mecp2* KO animals. CGS21680 (10nM) was applied 60 min after the induction of LTP in the first pathway (open triangles) and at least 20 min before induction of LTP in the second pathway (filled triangles). In **A1** and **A2** the ordinates represent normalized fEPSP slopes, where 0% corresponds to the averaged slopes recorded for 14 min before θ-burst stimulation and the abscissa represents the time of every recording. Panels (**B1** and **B2**) depict the magnitude of LTP (change in the fEPSP slope at 46–60 min) induced by θ-burst stimulation in relation to pre-θ-burst values (0%) in the absence of any drug (open bars), in the presence of BDNF + CGS21680 (**B1**) (filled bars) or in the presence of CGS 21680 (**B2**) in hippocampal slices from KO animals. In **B3** traces from representative experiments are shown. All values are mean ± standard error of mean. *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 baseline in the same experiments. n.s.- not significant;

4.3.4 A1R and A2AR expression is reduced in one human brain sample

Given the results obtained regarding BDNF, TrkB-FL levels and also unpublished results from Maria Jose Diogenes lab that suggested increased A1R and decreased A2AR expression changes in the animal RTT model, we performed mRNA quantification experiments in brain tissue from an 11 years old girl with RTT and compared the results with an age matched control mRNA sample extracted from the same cortical region. Adenosine A2AR mRNA level is reduced (Figure 4.5.A) (0.44 when control value is considered 1). Adenosine A1R mRNA level is increased (Figure 4.5.A) (4 fold increase, when control value is considered 1). Fold changes between mRNA quantification of TrkB-FL (1.4), TrkB-TC (1.1) and BDNF (1.2) were not remarkable (Figure 4.5.B).

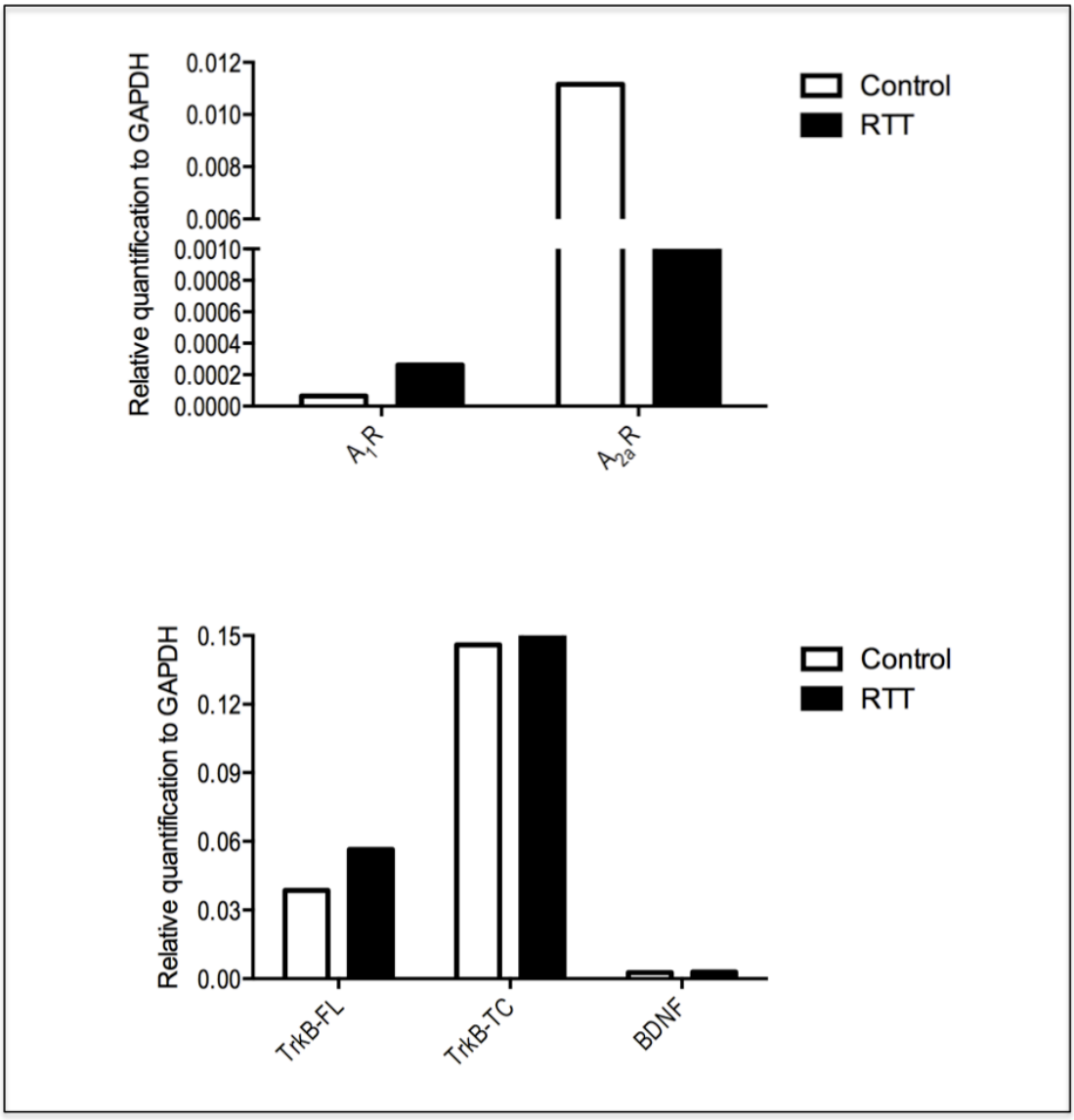


Figure 4.5 mRNA quantification studies of adenosine A2AR, A1R, TrkB-FL and TrkB-TC in RTT human cortical tissue.

A. Adenosine A2AR mRNA level in the RTT patient cortical tissue was reduced (44 % of control). Adenosine A1R mRNA level was increased (4 fold increase, black bars), when compared with an age matched control (white bars). B. Differences between mRNA levels of TrkB- FL (1.4), TrkB-TC (1.1) and BDNF (1.2) of control (white bars) and patient (black bar) were irrelevant.

4.4 Discussion

In the present work, in addition to the previously documented decreased levels of endogenous hippocampal BDNF in *Mecp2* KO symptomatic animals, a severe decrease on the TrkB-FL receptors levels was found. The detailed evaluation of BDNF and TrkB receptor levels throughout postnatal

development shows increased levels of BDNF during the first week of life, coupled with a down regulation of TrkB-FL receptors. In symptomatic stages, both BDNF levels and TrkB-FL receptors are decreased in the hippocampus. Hippocampal BDNF signaling has been pointed as crucial for memory formation. Therefore, these changes can be involved in the intectual disabilities described in RTT.

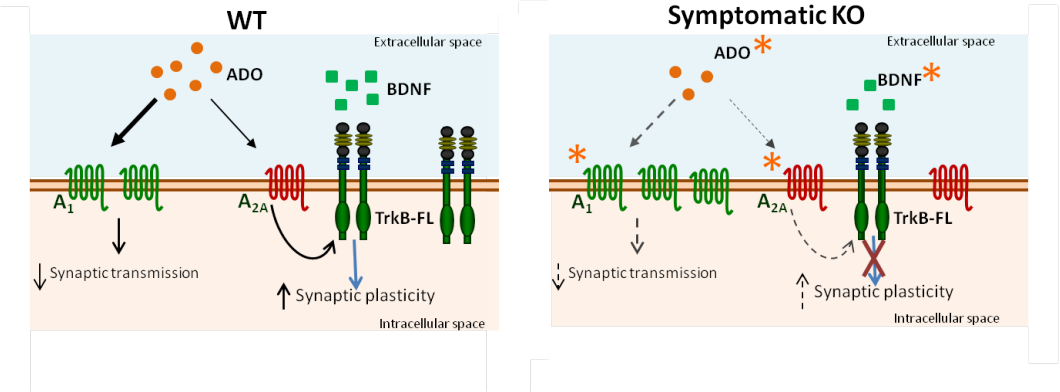


Figure 4.6 In *MECP2* KO animals the deregulation of endogenous adenosine tone can aggravate the impairments on BDNF signaling.

Hippocampal extracellular tone of adenosine, exerts upstream control over 2 major adenosine-dependent pathways: (1) A2AR-dependent promotion of BDNF-signaling, and (2) A1R-dependent inhibition of synaptic transmission. In WT animals (left panel), normal levels of adenosine facilitate BDNF actions by TrkB-FL receptors activation upon synaptic plasticity through the activation of A2A and inhibit synaptic transmission through A1R. In *Mecp2* KO animals (right panel), decreased levels of BDNF and TrkB-FL receptors were observed and also a possible decrease on adenosine levels, which in turn could induce an upregulation on A1R. The reduced activation of adenosine receptors (dashed arrows) in particular A2AR, that appears to be downregulated, implies an aggravation on the BDNF signaling impairment. These findings reinforce BDNF signaling pathways (orange asterisks) as possible pharmacological targets but importantly add adenosine and its receptors as new promising pharmacological targets (orange asterisks).

Increased BDNF levels detected during the early post natal life could contribute to a possible downregulation of TrkB-FL receptors (Sommerfeld et al., 2000). However, a direct effect of Mecp2 in TrkB-FL receptor levels should also be considered. RTT is a dynamic disorder with evolving clinical stages (Hagberg, 2002) that reflect the effect of MeCP2 impairment upon the developing brain, normal brain function and also secondary adaptative

changes to the original defect. The follow up of molecular and functional changes throughout development seems like a valuable approach to understand how neurodevelopmental disorders progress and also to delineate earlier therapeutic strategies that can prevent adaptative responses, which further aggravate the disease phenotype.

The increase on BDNF levels in RTT patients has been pointed as a promising therapeutic strategy. In that way, multiple strategies have been developed in order to increase BDNF actions (Li and Pozzo-Miller, 2014) on animal models. Notwithstanding, less attention has been devoted to BDNF receptors. In the present work, for the first time, we demonstrated that there is a strong decrease on TrkB-FL receptors in *Mecp*2 KO animals without any significant changes in the truncated forms. Both decreases in TrkB-FL receptors and BDNF levels have been detected in several neurologic disorders associated with cognitive deficits and are also now reported in this thesis in the RTT animal model.

BDNF is a neurotrophin that promotes neuronal survival, differentiation, and synaptic plasticity such as LTP through activation of TrkB-FL. Accordingly, LTP is deeply impaired in BDNF homozygous and heterozygous mutant mice and can be rescued by exogenous BDNF (Korte et al., 1996; Patterson et al., 1996). Moreover, the effect of exogenous BDNF upon hippocampal LTP is dependent on a proper adenosinergic tonus via activation of A2ARs (Diogenes et al., 2011; Fontinha et al., 2008). Therefore, whenever the endogenous activation of A2ARs is compromised, BDNF loses the ability to potentiate LTP (Diogenes et al., 2011; Fontinha et al., 2008). Here we describe that the effect of BDNF upon LTP induced by θ-burst stimulation is completely abolished in hippocampal slices taken from *Mecp2* KO animals. Thus, one could ask whether this could be explained by changes on adenosine signaling pathways.

A2AR mRNA quantification in cortical tissue from a girl with RTT suggested a reduction in A2ARs levels. As previously mentioned, several important actions of BDNF are dependent on A2ARs activation. Since BDNF and TrkB-FL receptors levels and the A2ARs expression are reduced in symptomatic stages of the RTT model, we postulated that the activation of $A_{2A}R$, together with the exogenous administration of BDNF, could rescue BDNF actions upon

LTP. The selective A2AR agonist, CGS2168 (Jarvis et al., 1989) was used to test this hypothesis. Indeed, we found that it could restore BDNF facilitatory effect upon LTP, similar to what is observed in hippocampal slices from WT animals.

These findings open up a therapeutic window in RTT, through the modulation of adenosine signaling. Further studies are needed to consolidate our knowledge about adenosine dysfunction in RTT. However, in view of the present results, increasing adenosine tonus or acting directly at the specific receptors could have a potential beneficial role in this disorder. Increasing A_1R activity would prevent pathological excitability and epilepsy susceptibility. On the other hand, activation of A2ARs, could bypass the impairment of TrkB-FL signaling, restoring BDNF actions upon LTP, with potential benefic effects on cognition. Combining BDNF administration with increased adenosine availability or direct activation of A2ARs could be beneficial for cognitive impairment in RTT.

In conclusion, we found that BDNF actions upon LTP are impaired in RTT animal model. This dysfunction could be explained by the changes in TrkB receptors levels, described for the first time in the present study. Adenosinergic system is also compromised in this RTT model, according to our results, in favour of a reduction of an impaired expression of adenosine A2ARs. Since robust evidence suggests that BDNF and TrkB receptors levels are impaired in RTT models, BDNF signaling modulation, through A2ARs activation, potentiating TrkB-FL signaling pathway, is a promising therapeutic approach for RTT.

Chapter 5 Neural Commitment of Human Pluripotent Stem Cells under Defined Conditions Recapitulates Neural Development and Generates Patient-Specific Neural Cells

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*Co-first authors

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In this work, Sofia Duarte participated in the conception of experiments, the establishment of fibroblast cultures and reprograming of RTT patients' iPSCs, Cell Culture experiments including iPSC feeder free culture, iPSC neural induction and neuronal differentiation and immunofluorescence analysis. Sofia Duarte also contributed to the manuscript writing.

ABSTRACT

Standardization of culture methods for human pluripotent stem cell (PSC) neural differentiation can greatly contribute to the development of novel clinical advancements through the comprehension of neurodevelopmental diseases. Here, we report an approach that reproduces neural commitment from human iPSCs using dual-SMAD inhibition under defined conditions in a vitronectin-based monolayer system. By employing this method it was possible to obtain neurons derived from both control and RTT patients' pluripotent cells. During differentiation mutated cells displayed alterations in the number of neuronal projections, and production of Tuj1 and MAP2-positive neurons. Although investigation of a broader number of patients would be required, these observations are in accordance with previous studies showing impaired differentiation of these cells. Consequently, our experimental methodology was proved useful not only for the generation of neural cells, but also made possible to compare neural differentiation behavior of different cell lines under defined culture conditions. This study thus expects to contribute with an optimized approach to study the neural commitment of human PSCs, and to produce patient-specific neural cells that can be used to gain a better understanding of disease mechanisms.

5.1 Introduction

Pluripotent stem cells (PSCs) are cells capable to differentiate and give rise to every tissue cell in the human body (Mitalipov and Wolf, 2009). Until recently, these cells could only be isolated from the inner cell mass of the blastocyst, being designated ESCs (Keller and Snodgrass, 1999; Thomson et al., 1998). However, in 2007, Shinya Yamanaka and co-workers were able to reprogram human somatic cells into the pluripotent stem cell state using transfection of four transcription factors (Oct-3/4, Sox2, KLF4 and c-Myc). These human iPSCs brought many expectations into the biomedical field due to their potential applications in disease modeling, drug and toxicity screening, patient-tailored therapies and engineered tissues (Takahashi et al., 2007), potentially preventing immunosuppression and graft rejection, and paving the way for the next generation of personalized medicine (Yamanaka, 2009).

Countless protocols for *in vitro* expansion and differentiation of human PSCs have been developed since then, which typically comprise the use of undefined culture components (Chambers et al., 2009; Zhang et al., 2001) (Kennedy et al., 2007; Koivisto et al., 2004; Laflamme et al., 2007; Yang et al., 2008). Problematically, this presents many difficulties to the translation of these protocols from basic research to tangible applications (Abbasalizadeh and Baharvand, 2013). For example, the use of products from animal origin renders the produced cells unsuitable for clinical applications due to the risk of cross-species contamination and immunogenic reactions (Martin et al., 2005; Oh and Choo, 2006). Furthermore, the use of undefined culture conditions poses barriers to the understanding of the biological processes involved in pluripotency and lineage specification and compromises the reproducibility of these culture systems. Therefore, there is an urgent need to develop methods and protocols that use defined culture conditions and allow reproducibility when generating specific cell populations from PSCs.

In particular, neural specification can be induced by the synergistic inhibition of both Activin/Nodal and BMP signaling pathways with small molecules resulting in blockage of mesoderm, endoderm and trophoblast lineages while allowing neuroectodermal specification (Chambers et al., 2009; Shi et al.,

2012b). Despite being relatively efficient and straightforward, this method uses substrates and medium components of animal origin and of undefined nature (*e.g.* matrigel and serum replacements). Chemically defined substrates based on proteins, polymers and hybrids of polymers with active biomolecules have been developed, exhibiting low lot-to-lot variability and presenting high reproducibility (Azarin and Palecek, 2010) (Jin et al., 2012; Rodin et al., 2010). Truncated, recombinant vitronectin (VTN) conjugates, in particular, have been used as a support for robust expansion and differentiation of human PSCs under standardized and totally defined culture conditions(Chen et al., 2011; Jin et al., 2012; Lippmann et al., 2014; Melkoumian et al., 2010). Such system would greatly benefit neural commitment protocols, allowing the foundation of defined settings for *in vitro* studies of neural development and ensuring reliable methods for the anticipated biomedical applications.

In this study, we have validated the use of defined culture conditions to achieve an efficient and reproducible neural commitment of human PSCs. A defined culture medium supplemented with chemical inhibitors of BMP and TGFβ signaling, together with vitronectin for adherent growth, was shown to be sufficient to generate neural precursors (NPs) expressing *SOX1, PAX6*, and *NESTIN*. As a proof-of-concept, we have validated the neural commitment protocol using different cell lines, including patient-specific RTT-derived iPSCs. This neurological disorder is characterized by autistic-like behaviors and is commonly caused by mutations in the X-linked *MECP2* gene, which is vital for proper functioning of the brain and acts as one of the many biochemical switches for gene expression (Marchetto et al., 2010; Walsh and Hochedlinger, 2010). Consequently, abnormal expression of this gene causes atypical brain function, leading to many disease processes that are mostly uncharacterized (Laccone et al., 2001; Li et al., 2013). Thus, this study also expects to contribute with a novel methodology capable to provide patient-specific neural cells that could be used to gain a better understanding of the disease. Furthermore, the ability to recapitulate the development of the human nervous system *in vitro* will provide important insights on the mechanisms involved in the maturation of specific neural cell types, making

this approach transversal to other related areas in neurodevelopmental research.

5.2 Materials and Methods

5.2.1 Cell lines

Two human iPSC lines obtained from healthy donors (46, XY) were used: WT-Évora F0000B13 and iLB-C1-30 m-r12. WT-Évora was kindly provided by TCLab (Évora, Portugal), and iLB-C1-30 m-r12 cells were derived at the University of Bonn, Germany. These cell lines were reprogrammed from fibroblasts through retroviral transduction of human genes *OCT4, SOX2, c-MYC and KLF4* (Takahashi et al., 2007), and were used as wild type controls to verify the robustness of the neural commitment method. The EMC23i cell line was generated at the iPSC Facility, Erasmus Medical Center, Rotterdam, using engineered color-coded lentiviral vectors (as described by Warlich and coworkers (Warlich et al., 2011)), and was derived from a patient (46, XX) with a *MECP2* mutation (R306C). Cells were routinely evaluated for karyotype abnormalities by conventional cytogenetics using the services of Genomed SA (Lisbon, Portugal). Biological samples collected for this work were obtained following rigorous national and European ethical guidelines and informed consent from donor or patient's legal guardian.

5.2.2 Feeder-free culture of human iPSCs

Human iPSCs were thawed and cultured in mTeSR™1 medium (StemCell Technologies™), on Matrigel™ (BD)-coated, Synthemax™ (Corning®)-coated, or Vitronectin (VTN-N, Gibco®)-coated plates. Medium was changed daily and cells were passaged every 3-4 days, using EDTA dissociation buffer 0.5mM (Gibco®, diluted in sterile PBS) with a split-ratio of 1:4 (Chen et al., 2011).

5.2.3 Neural induction of human iPSCs

When human iPSC cultures were nearly confluent, two different strategies were followed for neural induction, based on Chambers, et al (Chambers et al., 2009) and Shi, et al (Shi et al., 2012b). The first protocol was performed

using DMEM/serum replacement (SR)-based differentiation medium (KO-DMEM medium (Gibco®) supplemented with 20% (v/v) KO-SR (Gibco®), 1% (v/v) non-essential aminoacids (Gibco®), 1 mM L-glutamine (Gibco®), 0.1 mM β-mercaptoethanol (Sigma®) and 1% (v/v) Penicillin/Streptomycin (PenStrep, Gibco®)), while the other was performed in N2B27 medium (50% (v/v) DMEM/F12/N2 medium (DMEM-F12 (Gibco®), supplemented with 1% (v/v) N2 (Gibco®), 1 mM L-glutamine, 1.6 g/L Glucose (Sigma®), 1% (v/v) PenStrep, and 20 µg/mL Insulin (Sigma®)) and 50% (v/v) of B27 medium (Neurobasal (Gibco®) supplemented with 2% (v/v) B27-supplement (Gibco®), 1 mM glutamine and 1% (v/v) PenStrep)). In both cases, media formulation was supplemented with 10 µM SB-431542 (SB) and 100 nM LDN-193189 (Wernig et al.) (both from Stemgent®), as outlined in Figure 5.2.A.

5.2.4 Neuronal differentiation

At day 12 of differentiation, cells were passaged with EDTA dissociation buffer (0.5mM) and re-plated onto laminin (Sigma®)-coated plates (split ratio of 1:3). When neural rosettes were observable (around day 14), N2B27 was supplemented with 10 ng/mL of bFGF (Peprotech®) for two to four days. At day 17, cells were passaged using EDTA to new laminin-coated plates (split ratio of 1:3) (Shi et al., 2012a). Cells were then cultured until day 28-30 in N2B27 medium without the addition of small molecules or any other factors. Finally, cells were split with accutase (Sigma®) and plated at a density of 100,000 cells/cm^2. Cells were further cultured until day 150 with medium replacement every other day (Figure 5.1).

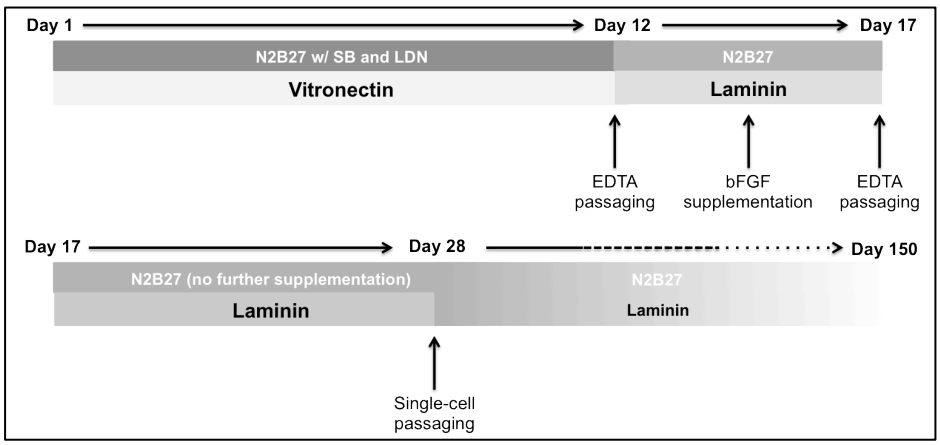

Figure 5.1 Schematic representation of neural commitment and subsequent neural differentiation of human pluripotent stem cells.

Neural commitment is initiated under defined culture conditions using dual SMAD inhibition for 12 days. At this stage cells are passed as clumps using EDTA dissociation to laminin-coated plates. At around day 14 bFGF is added to facilitate proliferation of neural rosettes until day 17. Subsequent replating onto laminin is done using EDTA dissociation, and further differentiation occurs in the absence of growth factors or chemical inhibitors. A final single-cell passaging step is done at day 28-30 and cells are allowed to differentiate until day 150 with periodic medium change and laminin supplementation.

5.2.5 Immunofluorescence staining of intracellular markers

We performed immunofluorescence staining as previously described (Rodrigues et al., 2014). Briefly, cells were fixed in paraformaldehyde (PFA, Gibco®) 4% for 30 minutes, followed by blocking for 60 minutes at room temperature. Primary antibodies were then added and left at 4ºC overnight. Secondary antibodies (goat anti-mouse or goat anti-rabbit IgG, Alexa Fluor® –488 or –546, 1:500 (v/v) dilution, Molecular Probes®) were prepared and incubated with cells for 1 hour at room temperature. DAPI (Sigma®) was used to counterstain cell nuclei. Primary antibodies used were FoxG1 (1:100, Abcam®), GFAP (1:100, Millipore), Ki-67 (1:100, BD), MAP2 (1:400, Abcam®), NANOG (1:5000, Millipore), NESTIN (1:400, R&D™), OCT4 (1:500, Millipore), OTX1/2 (1:100, Millipore), PAX6 (1:400, Covance®), SOX2 (1:200, R&D™), Tuj1 (1:5000, Covance®), VGLUT1 (1:300, Synaptic Systems) and ZO-1 (1:100, Novex®).

5.2.6 Quantification of immunofluorescence images

Quantification of *OCT4* and *PAX6*-expressing cells was performed using Fiji® software (for ImageJ). A macro was developed to perform conversion of the original image to 8 bits, adjustment of a threshold, watershed treatment, and definition of parameters for particle analysis. Firstly, total cell nuclei were counted using DAPI images. Then, PAX6 and OCT4 positive nuclei were counted in the same way and the ratio between positive cells and the total number of cells was calculated.

5.2.7 Flow Cytometry Analysis

For staining, approximately 500,000 cells were resuspended in FACS buffer with the diluted primary antibody, and incubated for 15 min at room temperature in the dark. Cells were then washed and resuspended in PBS for later analysis by flow cytometry (FACSCalibur, Becton Dickinson). Antibodies used for flow cytometry were SSEA-4-PE (1:10), and Tra-1-60-PE (1:10) (Stemgent®).

5.2.8 Quantitative real-time PCR

Total RNA was isolated from cells at different stages of neural commitment (day 0, 3, 6, 9 and 12) using a high pure RNA isolation kit (Roche) according to manufacturer's instructions. cDNA was synthetized from RNA, starting amount of 1 µg, using a transcriptor first strand cDNA synthesis kit (Roche). Taqman® Gene Expression Assays (20X) were selected for *PAX6*, *SOX1*, *NANOG*, *OCT4/POU5F1* and *GAPDH* (Applied Biosystems, supporting Table S2). All other genes (*FGF5*, *NESTIN*, *β-Tubulin III*) were analyzed using SYBR® green chemistry (primers are presented in Supporting Table 5.S1). PCR-reactions were run in duplicate, using the StepOne™ RT-PCR System (Applied BioSystems). Reactions were normalized to the housekeeping gene *GAPDH* and results analyzed with StepOne software.

5.2.9 Electrophysiology

Whole-cell recordings were performed at room temperature in artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$, 10 Glucose, pH 7.4 (gassed with 95% O_2, 5% CO_2). Patch pipettes (4-9 MΩ) were pulled from borosilicate glass electrodes (1.5 mm outer diameter, 0.86 mm inner diameter, Harvard Apparatus) with PC-10 Puller (Narishige Group) and filled with an intracellular solution containing (in mM): 125 K-gluconate, 11 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 2 MgATP, 0.3 NaGTP, 10 phosphocreatine, pH 7.3, adjusted with KOH (1 M), 280-290 mOsm. Cells were viewed using a Carl Zeiss Axioskop 2FS upright microscope equipped with a differential interference contrast-infrared (DIC-IR) CCD video camera (VX44, Till Photonics) and screen and recorded with an EPC-7 electrical amplifier (List

Biologic). Signals were low-pass filtered using a 3- and 10-kHz three-pole Bessel filter of an EPC-7 amplifier, digitized at 10 kHz using a Digidata 1322A board, and registered by the Clampex software version 10.2 (Molecular Devices). Series resistance was not compensated during voltage-clamp recordings, but was regularly monitored throughout each experiment with a -5 mV, 50 ms pulse, and cells with >20% change in series resistance were excluded from the data. Data were not corrected for junction potentials. GABA-evoked postsynaptic currents (GABA-PSCs) were evoked through a micropipette (2-4 MΩ) containing GABA (30 μM in aCSF) coupled to a pressure application system (Picopump PV820, World Precision Instruments) and positioned close to the soma of the recorded cell. Single pulses of 100-150 ms and 6-8 psi were applied every 2 min. Action potential firing was systematically evoked in current-clamp mode by injecting current pulses of -50 to +300 pA, in 50 pA increments for 1000ms from an initial holding potential of −70 mV.

5.2.10 Statistical Analysis

Results are presented as mean ± standard error of mean (Semyanov et al.). Comparisons between experimental results were determined by Mann-Whitney test for independent samples. Unless stated otherwise, three replicates (n=3) were performed and a p-value less than 0.05 was considered significant.

5.3 Results

5.3.1 Human iPSCs efficiently commit to the neuroectoderm lineage without the need for serum replacement-based medium

The dual-SMAD inhibition protocol is a procedure for the rapid commitment of confluent human PSCs into early PSC-derived neural precursors (NPs)(Chambers et al., 2009; Rodrigues et al., 2014; Shi et al., 2012a) (Figure 5.2.A). Cells are first plated as cell colonies in conditions that support pluripotency, and allowed to reach confluence (Figure 5.2.B-I and -II). Culture conditions are then changed to include chemical inhibitors of BMP and Activin/Nodal signaling pathways, causing the emergence of a neuroepithelial cell sheet (Figure 5.2.B-III). This swift induction is due to the blocking of SMAD signaling transduction by SB and LDN small molecules (Boergermann et al., 2010; Inman et al., 2002). When combined, these inhibitors repress mesoendodermal fates, directing the differentiation towards neuroectoderm (Smith et al., 2008).

In this work we first compared different strategies to achieve human NPs based on two different methods (Chambers et al., 2009; Shi et al., 2012a) (Figure 5.2.A). In both cases human PSCs were first plated in matrigel-coated plates as clumps using a non-enzymatic passaging procedure (Beers et al., 2012). This yielded compact colonies of cells (Fgure 5.2.B-I) that stained positively for pluripotency markers OCT4 and SOX2 (Figure 5.2.C and Figure 5.2.D, respectively). At this stage, a high percentage (> 95%) of cells were positive for these markers (Figure 5.2.E). Cells were then allowed to reach confluence (Figure 5.2.B-II), at which point the two neural induction strategies were tested.

The first consisted in the use of serum replacement (SR)-based medium for the first 4 days of commitment supplemented with both SB and LDN. From day 5 to 11, N2 supplementation was gradually added to the medium, while LDN-mediated inhibition of BMP was maintained (Chambers et al., 2009). In the second protocol, neural commitment was induced in N2B27 medium supplemented with SB and LDN for 12 days (Shi et al., 2012a). In both cases a neuroephitelial sheet of cells was obtained, but some qualitative differences

in the efficiency of the neural commitment process were immediately visualized using immunofluorescence (Figure 5.2.F and Figure 5.2.G).

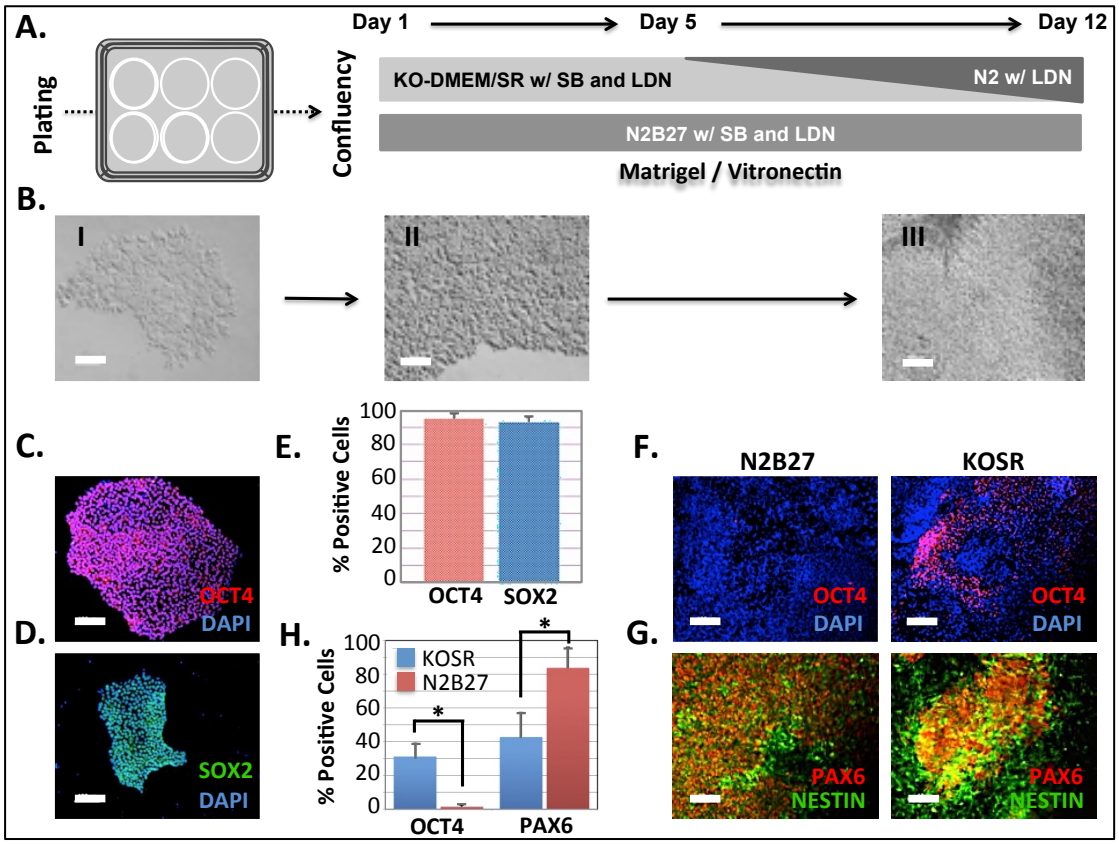

Figure 5.2 Neural commitment of human PSCs.

(A) Neural induction was initiated when cells reached confluence and two strategies were tested in parallel. The first strategy involved the use of serum replacement-based medium (KO-DMEM/SR) for the first four days and a gradual increase in N2 medium thereafter. In the second case, N2B27 defined medium was used for 12 days. Small molecule inhibitors of SMAD signaling (SB and LDN) were used as described in the scheme. **(B)** Human PSCs were cultured in adherent conditions (I) until confluence (II). A neuroepithelial sheet of cells could be observed after neural induction (III). **(C)** and **(D)** PSC colonies stained for pluripotency markers OCT4 and SOX2 (scale bars: 100µm). **(E)** At the start of neural commitment, high percentages (> 95%) of cells positive for OCT4 and SOX2 could be measured. **(F)** After 12 days of differentiation, immunofluorescence staining revealed that OCT4-positive cells were still present in serum replacement (KOSR) conditions (scale bars: 100µm). **(G)** PAX6 and NESTIN immunostaining for differentiated cells in N2B27 or KOSR-based medium (scale bars: 50µm). **(H)** Quantification of OCT4- and PAX6-positive cells for the two medium compositions (KOSR or N2B27) used for neural commitment. *p-

value < 0.05. Results presented in this figure were obtained using iLB-C1-30 m-r12 PSCs and matrigel coating.

While in the case of SR-based medium OCT4-positive cells still persisted in culture 12 days after neural induction with chemical inhibitors, for N2B27-based induction these cells were virtually absent (Figure 5.2.F). Additionally, the distribution of PAX6- and NESTIN-positive cells was more homogeneous in the case of N2B27 when compared with SR-based medium, which presented islands of PAX6-positive cells surrounded by NESTIN expressing cells instead of a homogeneous monolayer (Figure 5.2.G). Using image-processing software we then quantified *OCT4*- and *PAX6*-expressing cells for both protocols (Figure 5.2.H). Neural commitment in N2B27 medium resulted in more than 80% of NPs positive for PAX6 and very low numbers of OCT4-positive PSCs (< 2%), while in KO-SR medium, an average of 40% PAX6-positive cells was obtained, with more than 20% of OCT4-positive cells still in culture (Mann-Whitney test, p-value < 0.05).

These results highlight the importance of medium formulation in maximizing differentiation yield (Lippmann et al., 2014). By comparing data from both differentiation media it is possible to conclude that the chemically defined, serum-free composition of N2B27 yields a more efficient neural conversion into NPs. This medium was particularly optimized for neural cell culture, and contains insulin and retinoids, which have been shown to be crucial for the neuroepithelial induction of human PSCs (Shi et al., 2012b). The relatively high percentage of pluripotent cells and potential non-neural differentiated cells obtained in SR-based medium (Figure 5.3) is not desirable since these cells may interfere with the outcome of *in vitro* differentiation protocols (Rodrigues et al., 2014).

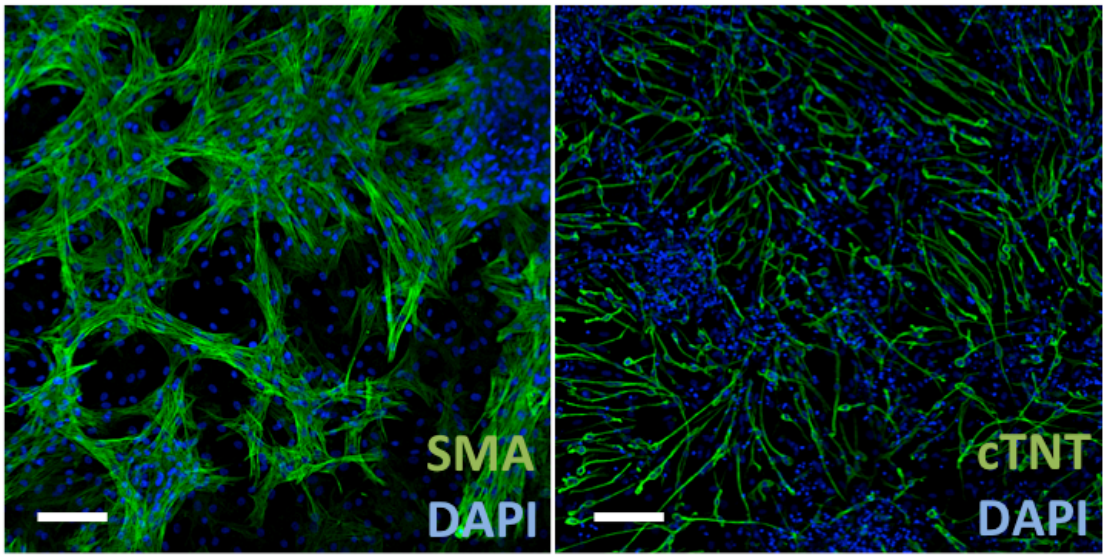

Figure 5.3 Immunofluorescence staining of human iPSC-derived cells at day 120 of differentiation after neural commitment for 12 days using matrigel and serum replacement-based medium.

In addition to typical neural phenotypes, cells marking positive for alpha smooth muscle actin (SMA) and cardiac troponin T (cTNT) could be identified at this stage of differentiation. DAPI was used to counterstain the cell nuclei. Scale bar: 100 µm.

5.3.2 Human PSCs generate neural precursor cells using dual SMAD signaling inhibition under defined culture conditions

In addition to medium formulation, the composition of the extracellular matrix to which cells adhere is also a potential source of ill-defined components. This is the case of matrigel, whose constituents include an heterogeneous mixture of structural proteins of animal origin (Hughes et al., 2010). However, non-xenogeneic, defined substrates that support the culture of human PSCs have also been proposed in the literature (Azarin and Palecek, 2010). In particular, vitronectin (VTN) peptide sequences have shown to support both expansion and differentiation of human PSCs under totally defined culture conditions (Chen et al., 2011; Jin et al., 2012; Lippmann et al., 2014; Melkoumian et al., 2010). Our next goal was to compare two different extracellular matrixes in supporting neural commitment from human iPSCs in combination with a fully defined culture medium, allowing the foundation of defined settings for *in vitro* studies of neural development.

A side-by-side comparison was made using N2B27 medium together with matrigel or VTN (Figure 5.4). The dual SMAD inhibition yielded similar

outcomes in both cases, and cells presented typical neuroectodermal markers (Figure 5.4.A). These included the neuroectoderm fate determinant PAX6 (Zhang et al., 2010), the forebrain marker FoxG1 (Chambers et al., 2009), and the midbrain/forebrain marker OTX1/2 (Pankratz et al., 2007), in addition to SOX2 and NESTIN. Cells differentiated for 12 days in both substrates showed up-regulation of *PAX6* and *SOX1* mRNA, with corresponding down-regulation of pluripotency transcription factors (*OCT4*/*POU5F1* and *NANOG*) (Figure 5.4.B). Additionally, real-time PCR analysis indicated that for both substrates expression of the pluripotency markers sharply decreased in the first three days of neural induction (Figure 5.4.C). After this point, both *OCT4/POU5fF1* and *NANOG* retain minor levels of expression when compared to day 0. Also, *PAX6* and *SOX1* expression increased rapidly from day 0, reaching maximum levels after day 3 in both matrigel and VTN (Figure 5.4.D). At the same time, when human PSCs entered differentiation, the presence of cell surface markers characteristic of the pluripotent state (Tra-1-60 and SSEA-4) was reduced, and the percentage of cells positive for these markers dropped progressively during the 12 days of neural commitment (Figure 5.5.A). The simultaneous up-regulation of *FGF5* expression (Figure 5.5.B), together with down-regulation of pluripotency genes (Figure 5.4.C) and up-regulation of *PAX6* and *SOX1* (Figre 5.4.D), most likely resulted from the emergence of an initial population of NPs after neural induction (Abranches et al., 2009).

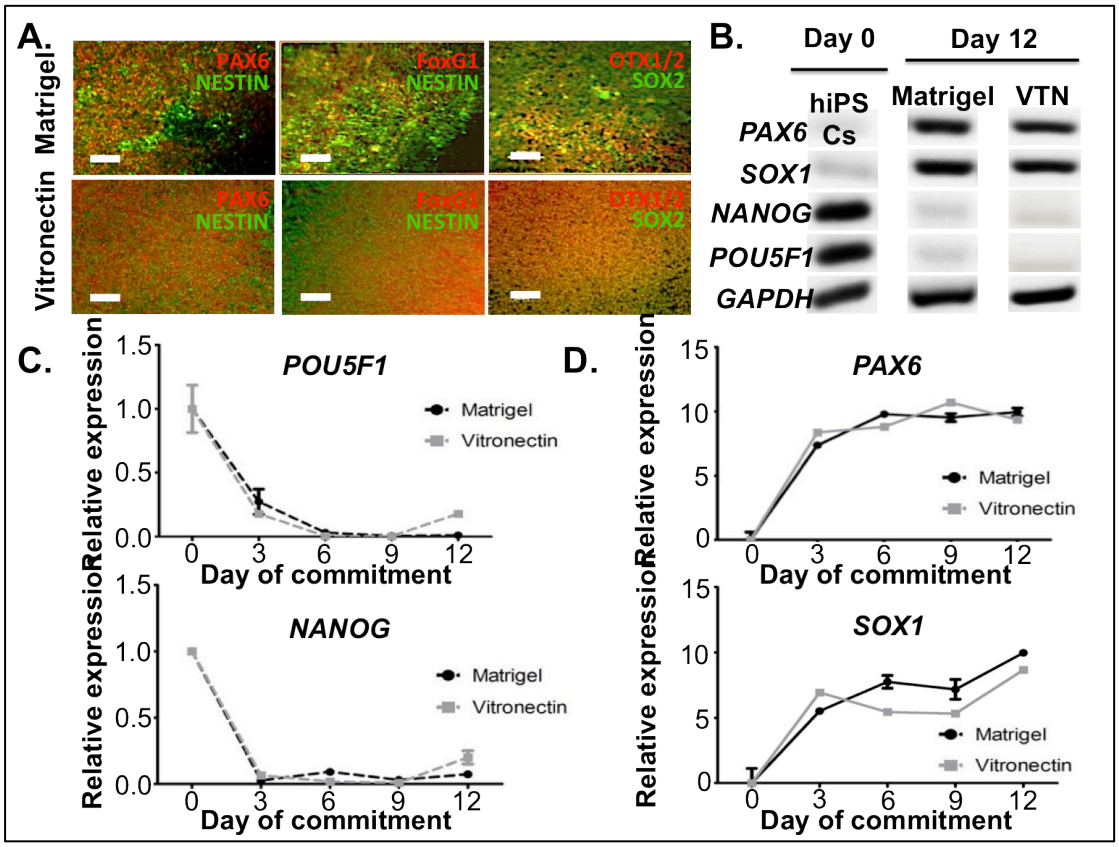

Figure 5.4 Efficient neural commitment of human PSCs using defined medium and vitronectin coating.

(A) Immunofluorescence staining of PSC-derived NPs after 12 days of induction with chemical inhibitors in matrigel or VTN. Cells were stained for PAX6, NESTIN, FoxG1, OTX1/2 and SOX2 (scale bars: 50µm). **(B)** RT-PCR analysis of NPs generated in matrigel and VTN. After 12 days of commitment down-regulation of pluripotency genes (*POU5F1*, *NANOG*) was observed whereas neuroectodermal markers (*PAX6*, *SOX1*) were up-regulated regardless of the surface coating used. **(C)** and **(D)** Temporal gene expression analysis during neural commitment of human PSCs in matrigel and VTN. Pluripotency genes (*POU5F1*, *NANOG*) and neuroectodermal markers (*PAX6*, *SOX1*) were evaluated by quantitative real-time PCR at different time points. *GAPDH* expression was used as internal control. Results presented in this figure were obtained using the iLB-C1-30 m-r12 cell line.

Taken together, these outcomes demonstrate that human iPSCs can effectively differentiate to NPs using the dual SMAD inhibition protocol under defined culture conditions composed of N2B27 and VTN as adhesion substrate.

To further confirm the generation of competent NPs after 12 days of dual SMAD inhibition, cells were re-plated onto laminin-coated plates as small clumps, and cultured in N2B27 medium without chemical inhibitors SB and LDN. Two to five days after re-plating, a substantial number of neural rosette-like structures were observed (Figure 5.5.C). Rosettes were radially arranged like neuroepithelial cells, which were positive for PAX6, NESTIN, and SOX2, and already showed signs of polarization, with apical ZO1 expression. When neural rosettes were first visible, bFGF was added to the medium, to induce proliferation of progenitors, and around day 17 of differentiation cells were re-plated onto new laminin-coated plates and cultured until day 28-30 without the addition of small molecules or any other factors. Substantial maturation of NPs within rosettes was observed (Figure 5.5.D and Figure 5.5.E). As expected, after peaking during the initial stage of commitment, expression of typical neural markers (*PAX6*, *SOX1* and *NESTIN*) declined during further differentiation (Figure 5.5.D). This was followed by up-regulation of the neuronal marker *β-Tubulin III*. Using immunofluorescence staining we were able to see a proliferative (Ki67-positive) population of progenitors within polarized rosettes (apical expression of ZO1), and to confirm a reduction of the number of PAX6-positive cells. At this stage of differentiation, Tuj1-positive neurons outgrew from neural rosettes (Figure 5.5.E).

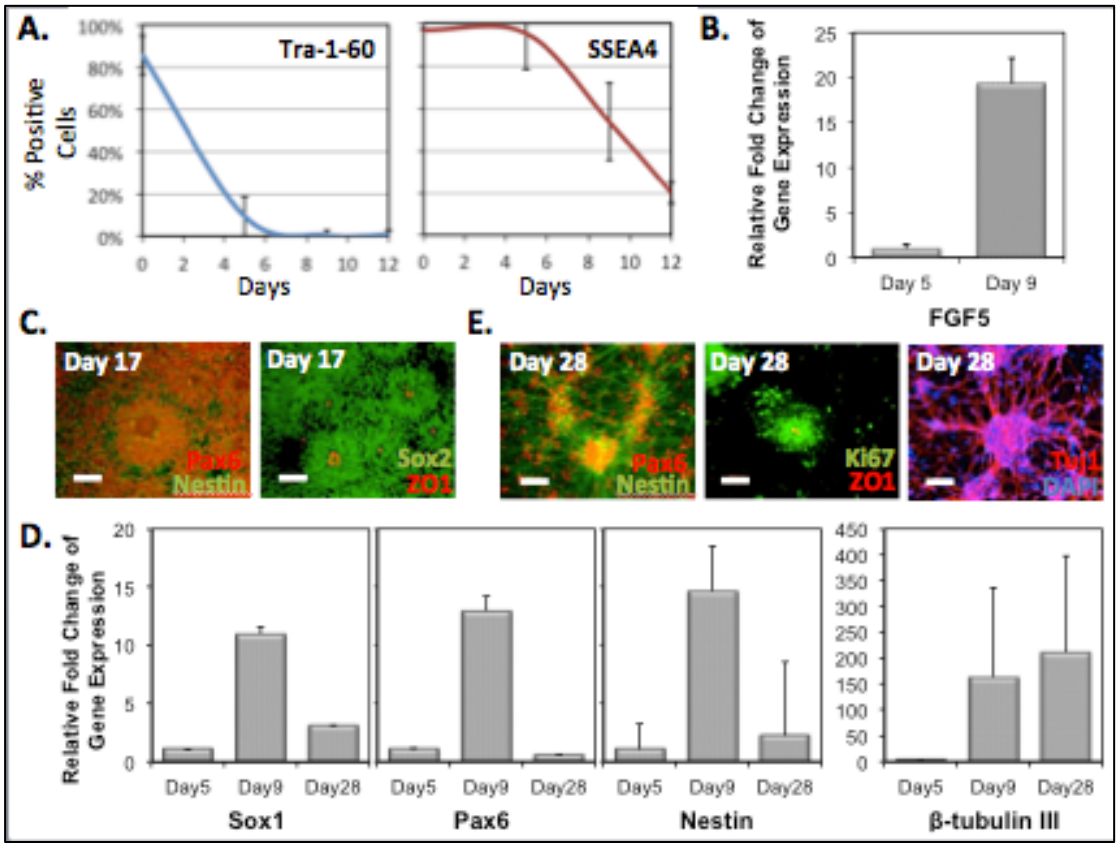

Figure 5.5 Neural maturation capability of cells derived under defined conditions using vitronectin coating.

(A) During neural commitment the percentage of cells positive for surface antigens Tra-1-60 and SSEA4 gradually decreased. Tra-1-60- and SSEA4-positive cells were evaluated by flow cytometry. **(B)** Gene expression analysis by quantitative real-time PCR showed an increase of the early specification marker *FGF5*. **(C)** After re-plating onto laminin-coated surfaces, immunostaining analysis demonstrated that cells formed neural rosettes. Cells within these structures were radially arranged and positive for PAX6, NESTIN, and SOX2. Rosettes showed signs of polarization marking ZO1 at the center (scale bars: 50μm). **(D)** Expression of typical neural markers (*PAX6*, *SOX1* and *NESTIN*) declined after further differentiation, as assessed by quantitative real-time PCR. This was followed by up-regulation of the neuronal marker *β-Tubulin III*. **(E)** By day 28, Ki67-positive cells could be seen within polarized rosettes (ZO1-centered). These cells still marked positive for neural markers (PAX6, NESTIN), but Tuj1-positive neuronal projections started to develop (scale bars: 50μm). Results presented in this figure were obtained using iLB-C1-30 m-r12 PSCs.

Taken together, these results demonstrate and validate the capacity to generate functional NPs using the dual SMAD inhibition protocol under defined culture conditions. Our results show efficient (> 80%) generation of PAX6-positive NPs, and the capacity of these cells to self-organize into neural rosettes upon passaging. Further differentiation into more mature cells was achieved within rosettes, which can be correlated with events leading to the neural tube formation *in vivo* (Chambers et al., 2009; Shi et al., 2012a). As previously demonstrated in similar systems (Chen et al., 2011; Jin et al., 2012; Lippmann et al., 2014; Melkoumian et al., 2010; Pennington et al., 2015) peptide conjugates can support robust expansion and differentiation of human PSCs under defined conditions, and in our case VTN supported neural commitment of human PSCs in conjugation with N2B27 medium supplemented with SMAD inhibitors.

5.3.3 Generation of patient-specific neural precursors and further neural maturation

At this stage we have established conditions that allowed the study of neural specification of human PSCs without interference of undetermined components stemming from serum replacements (Chambers et al., 2009) or extracellular matrix protein mixtures (Hughes et al., 2010; Shi et al., 2012a). This is an important feature of our system since undisclosed constituents may interfere with different signaling pathways, and thus affect cell fate decisions. Therefore, to further explore and demonstrate the usefulness of our method for neural specification studies, we have taken advantage of the potential of iPSC technology to investigate the neural commitment of patient-specific iPSCs, in particular RTT patient-derived cells (Dajani et al., 2013). This disorder is caused by mutations in the *MECP2* gene of the X chromosome and affects 1 in 10,000-20,000 girls worldwide (Walsh and Hochedlinger, 2010). Hallmarks of this condition include impaired motor function, seizures, autistic behavior (Amir et al., 1999), and changes in neuronal density and in brain size (Kim et al., 2011; Marchetto et al., 2010).

Control and RTT-derived fibroblasts were reprogrammed to a pluripotent state as described elsewhere (Takahashi et al., 2007). The iPSC lines used in this work showed typical characteristics of pluripotent stem cells,

and could be maintained in culture for dozens of passages without signs of differentiation or atypical karyotype (Supporting Table 5.1, Figure 5.6).

Table 5.1: Flow cytometry analysis of pluripotency markers. Human iPSC lines used in this work were analyzed using flow cytometry for transcription factors OCT4, SOX2, and NANOG, and surface antigens Tra-1-60 and SSEA4. All cell lines presented high percentages of cells marking positive for these markers.

Cell Line	OCT4	SOX2	NANOG	Tra-1-60	SSEA4
iLB-C1-30 m-r12 (***wild type***)	99 ± 0.2%	96 ± 1.0%	97 ± 2.1%	93 ± 1.2%	97 ± 1.8%
WT-Évora F0000B13 (***wild type***)	96 ± 1.1%	94 ± 1.5%	98 ± 0.9%	92 ± 1.5%	94 ± 2.6%
EMC23i (***RTT, mutated***)	98 ± 0.4%	97 ± 1.2%	98 ± 0.8%	94 ± 2.4%	94 ± 4.0%

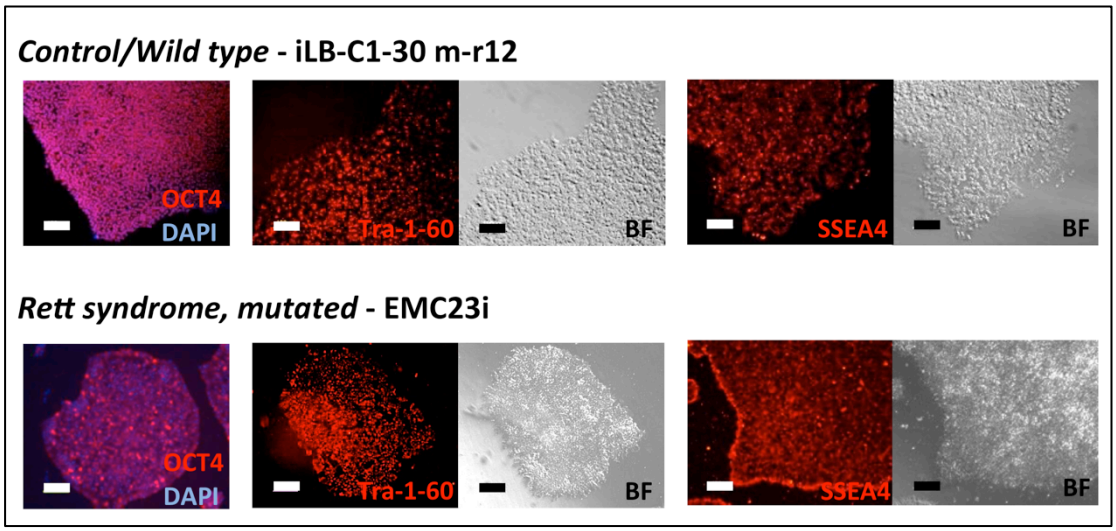

Figure 5.6 Immunofluorescence staining of human iPSC colonies derived from a control/wild type individual (top panel) and a RTT mutated patient (bottom panel).

Cells were plated in VTN-coated plates and expanded in pluripotency maintenance conditions, as described in the *Material and Methods* section. Cells were stained for the pluripotency markers OCT4, Tra-1-60, and SSEA4. In the case of OCT4 staining, DAPI was used to counterstain the cell nuclei.

For surface markers Tra-1-60 and SSEA4, bright field (BF) images are shown for comparison. Scale bar: 100 µm.

These cells were then adapted to our culturing conditions using a non-enzymatic passaging procedure and plated in VTN-coated surfaces (Chen et al., 2011). Both RTT and control-derived iPSCs were cultured in pluripotency maintenance conditions using mTESR, and stained for typical pluripotency markers (Figure 5.7.A and Figure 5.7.C, Figure 5.6). After reaching confluence, medium was changed to N2B27 and small molecule inhibitors of SMAD signaling (LDN and SB) were added to culture for 12 days. Both normal and patient-derived cells were able to differentiate and presented typical neuroectodermal markers such as PAX6, NESTIN, FoxG1, OTX1/2 and SOX2 (Figure 5.7.B and Figure 5.7.D). Also, at this time point, pluripotency markers like OCT4 were virtually absent.

After 12 days of dual SMAD inhibition, NPs were re-plated onto laminin-coated plates as small clumps, and cultured in N2B27 medium without chemical inhibitors. At day 17, after stimulation with bFGF, both normal and mutant cells were able to form neural rosettes without any noticeable differences (Figure 5.7.E and Figure 5.7.G). Substantial maturation of NPs within rosettes was observed with cells expressing neural markers (NESTIN, PAX6, SOX2) within polarized structures (ZO1). At this stage, cells were again re-plated onto laminin to allow further neural differentiation. Proliferative, Ki67-positive cells within rosettes could still be identified in both cultures, and polarization could also be seen with apical localization of ZO1 (Figure 5.7.F and Figure 5.7.H). Nevertheless, while in normal cells Tuj1-positive neurons started to migrate outwards of rosettes, forming neuronal projections and connections, RTT derived-neurons were substantially less frequent, and neurites less complex (Figure 5.7.F and Figure 5.7.H).

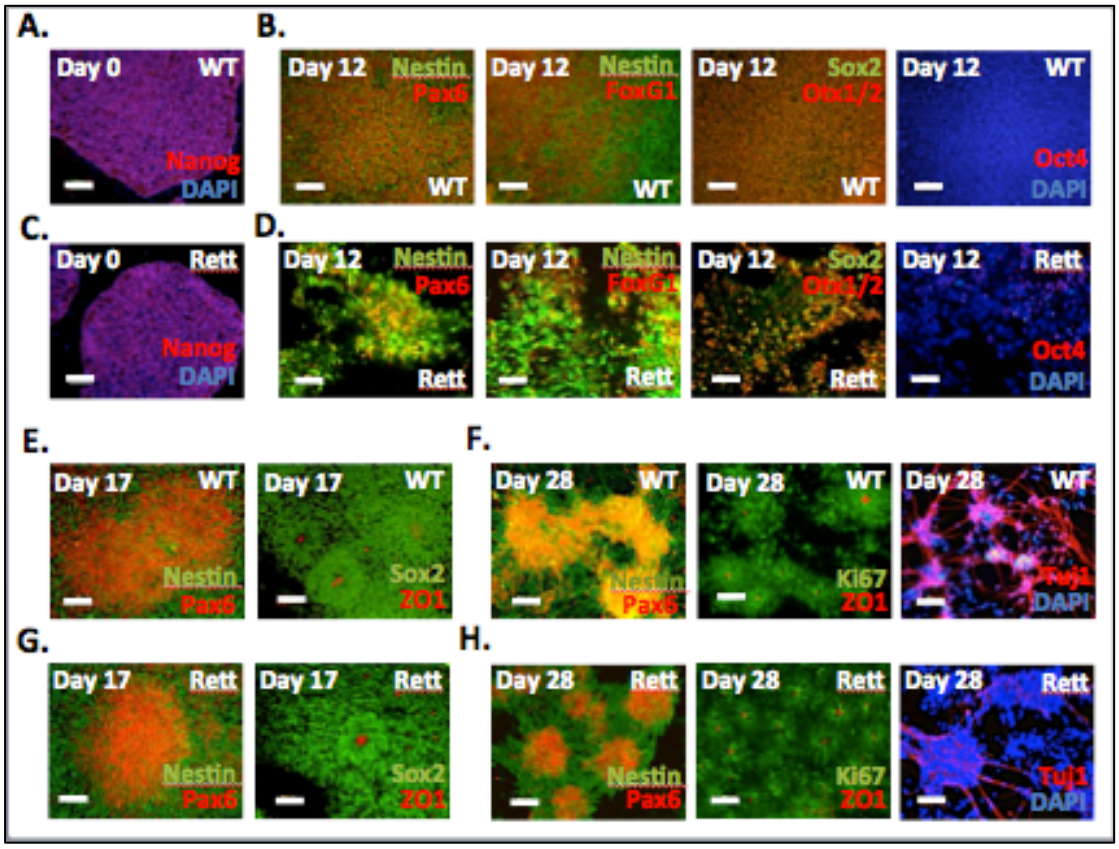

Figure 5.7 Neural commitment and further neural differentiation of both normal and RTT patient specific iPSCs.

(A) and **(C)** Immunofluorescence staining of both normal or RTT patient-derived iPSCs showing typical expression of pluripotency marker NANOG (scale bars: 100μm). **(B)** and **(D)** Immunofluorescence staining of neural markers (PAX6, NESTIN, FoxG1, OTX1/2, SOX2, scale bars: 50 μm) and of pluripotency marker OCT4 (scale bars: 100 μm) after twelve days of dual SMAD inhibition using a defined culture system. **(E)** and **(G)** After re-plating onto laminin, immunostaining analysis demonstrated that both normal and affected cells were capable to form neural rosettes. Cells within these structures were positive for PAX6, NESTIN, and SOX2. Rosettes showed signs of polarization, marking ZO1 at the center (scale bars: 50μm). **(F)** and **(H)** Direct comparison of further neural maturation revealed that both cell types marked for neural markers (PAX6, NESTIN), and Ki67-positive cells could be seen within polarized rosettes (ZO1-centered). However, Tuj1-positive neuronal projections were more frequent and pronounced in 'wild type' cells (scale bars: 50μm). Results presented in this figure were obtained using iLB-C1-30 m-r12, WT-Évora F0000B13, and EMC23i PSCs.

To further assess the neuronal maturation state, cells were finally passed as single-cells at day 30 onto laminin-coated plates and maintained in N2B27 medium. This procedure was expected to generate cortical projection neurons in about 80 days (Shi et al., 2012a). At day 75 of differentiation, we evaluated not only neuronal maturation, but also differentiation into glial cells, like astrocytes, in two non-affected 'wild type' human iPSCs and one RTT-derived mutant line (Figure 5.8.A). A substantial number of widespread neuronal projections was observed in both normal cell lines. Furthermore, extensive gliogenesis had occurred in such cultures and GFAP-positive astrocytes could be seen interspersed with Tuj1-positive neurons. This process was minimally reproduced in affected cells, showing less neurons, similarly to what had been visualized at day 28 of differentiation. Similar conclusions could be taken from MAP2 staining at day 100 (Figure 5.8.B). These observations are in accordance with other reports in the literature (Kim et al., 2011; Marchetto et al., 2010), and did not seem to result from reduced proliferation, since the number of DAPI stained nuclei within the microscope field of view was similar among samples. By day 120, control neurons developed to glutamatergic cortical neurons presenting the vesicular transporter VGLUT1 (Figure 5.8.C), which could not be observed in mutated cells.

Finally, electrophysiological recordings performed at day 120 of differentiation, revealed neuronal functional properties characteristic of mature neurons. Values of neuronal resting membrane potential, determined immediately after establishing whole-cell configuration, averaged -45.0 ± 2.2 mV (n=17). Action-potentials were then evoked in current-clamp mode by injecting depolarizing current pulses. Most cells tested showed firing of at least one action potential during current injection steps, revealing a mature neuronal state (n=10, Figure 5.8.D). In addition, GABA-evoked postsynaptic currents were observed (voltage-clamp mode, Vh = -70 mV) in response to pressure application of GABA (30μM) directly to the soma of the cell, indicating the expression of functional neurotransmission receptors in these cells (n=11, Figure 5.8.E).

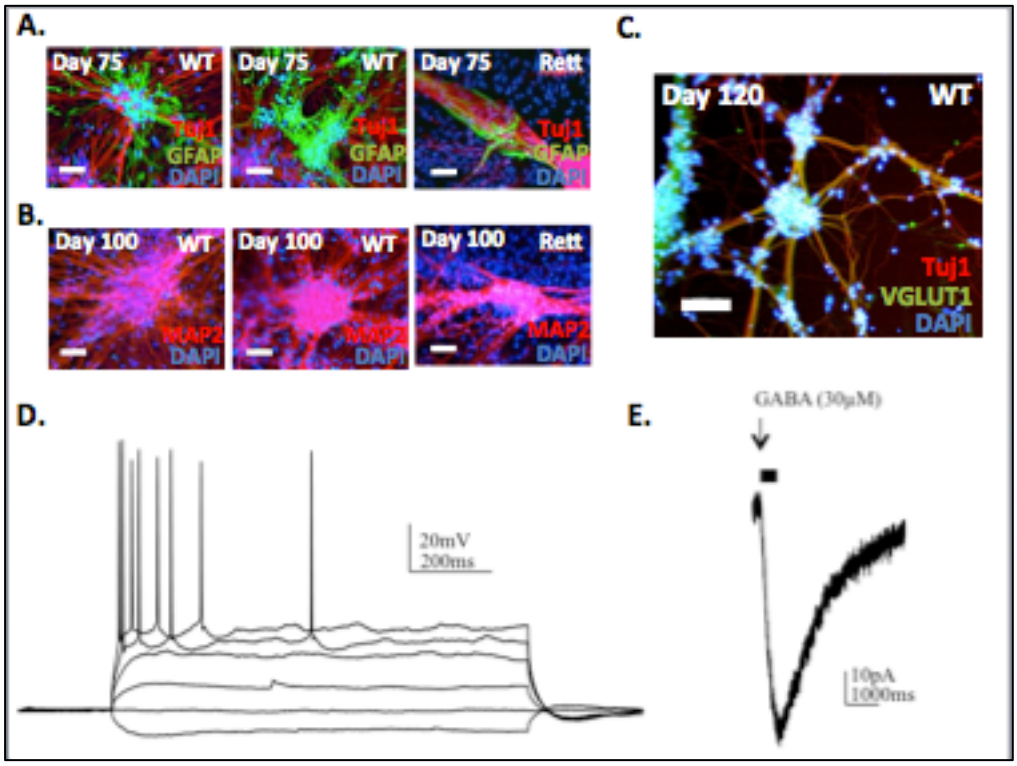

Figure 5.8 Neuronal differentiation of normal and RTT-derived neural progenitors.

(A) At day 75 of differentiation neuronal maturation of 'wild type' cells originated extensive neuronal projections (Tuj1-positive) together with astrocytes (GFAP-positive). This phenomenon was less pronounced in mutated cells (scale bars: 50μm). **(B)** Further differentiation and maturation of normal neurons resulted in MAP2-positive mature dendrites at day 100. The frequency of such structures in *MECP2* mutated cells was reduced when compared to control ones (scale bars: 50μm). **(C)** 'Wild type' Tuj1 neurons at 120 days of differentiation were immunoreactive for glutamatergic neuronal protein VGLUT1 (scale bar: 100μm). **(D)** and **(E)** show electrophysiological properties of 'wild type' cells proving maturation and cortical nature of generated neurons. **(D)** Increased depolarizing voltage steps (1000ms) in whole-cell current-clamp mode show the firing of action potentials with strong adaptation, characteristic of a mature neuron. **(E)** Representative GABA (30μM)-evoked postsynaptic currents (GABA-PSC) from a neuron recorded in whole-cell voltage-clamp (-70mV) showing responsiveness to GABA. Black bar corresponds to GABA application. Electrophysiological recordings have been performed by DM Rombo, co-author of the paper.

5.4 Discussion

We herein identify defined culture conditions to achieve efficient and reproducible neural commitment of human PSCs. A defined neural maintenance medium supplemented with chemical inhibitors of both BMP and TGFβ signaling pathways, together with VTN for adherent growth, was shown to be sufficient to induce neuronal commitment, generating a homogeneous population of NPs expressing typical neural markers (Figure 5.2, Figure 5.4 and Figure 5.5). The results obtained herein are comparable with other reported data in terms of neural conversion efficiency (Shi et al., 2012b), when using N2B27 medium, demonstrating that VTN-coating does not affect neural commitment negatively.

In fact, VTN-based conjugates have been previously used for PSC derivation, expansion (Chen et al., 2011; Melkoumian et al., 2010) and neural specification studies (Lippmann et al., 2014; Yan et al., 2013), and our results also validate the usage of VTN as a defined substrate for neural commitment. Another important feature of this system is the use of retinoid rich medium, which have been shown to increase NPs output (Shi et al., 2012b). When combined with VTN adhesion motifs, the use of defined medium was thought to facilitate progression through neural maturation, particularly during re-plating of NPs to laminin surfaces, by promoting survival and functionality of differentiated cells (Xie et al., 2000). In fact, when compared with serum replacement-based medium, we have been able to significantly increase the number of PAX6-positive NPs in N2B27 (Figure 5.2). However, other studies have reported minimally constituted monolayer systems that could achieve similar efficiencies in generating PAX6-positive neuroectoderm (Lippmann et al., 2014), questioning the role of retinoids in neural commitment. Nevertheless, monolayer neural commitment protocols still require a high local cell density for generation of NPs with high efficiencies (Chambers et al., 2009). It would be interesting to engage other methods (Peerani et al., 2007; Ungrin et al., 2008), such as micropatterning or forced aggregation of cells, to evaluate the local dynamics of high-density cell-to-cell contacts in the neural specification of human PSCs. Still, one shortcoming of monolayer systems is

the fact that for later stages of neural development, NPs require laminin to adhere and differentiate (Heaton and Swanson, 1988). Laminins are extracellular matrix trimeric glycoproteins and at least a dozen of different isoforms have been identified in nature. Commonly used laminin is isolated from a murine source and represents an undisclosed mixture of several of these natural isoforms. To fully define neural commitment, neural maturation, and neuronal differentiation of human PSCs in adherent conditions, specific laminin isoforms should be investigated and subsequently used. The Tryggvason's group has been able to produce recombinant laminins and evaluate their specific role in several biological functions (Domogatskaya et al., 2012), but the exact isoforms involved in neural differentiation remain unknown. However, potential future therapeutic strategies for neurological disorders might implicate the use of progenitors (Liu et al., 2013), since mature cells are less capable of establishing the adequate connections without being continuously exposed to the combination of growth factor and signaling molecules that orientate synaptogenesis and neuronal wiring. Obtaining NPs in xeno-free conditions can be a relevant first step to establish a safe approach for future transplant based therapies.

As a proof-of-concept, we have validated our neural commitment protocol using different cell lines, including patient-specific, RTT-derived iPSCs. Although investigation of a broader number of patients would be required, we were able to replicate some of the reported RTT defects in NPs derived in our defined system (Figure 5.7 and Figure 5.8). Interestingly, given the fact that *MECP2* is located on the X-chromosome and that RTT patients present mosaicism (Walsh and Hochedlinger, 2010), X-chromosome activation/inactivation during reprogramming, expansion and differentiation could lead to difficulties in reproducing the pathology of the disease *in vitro*. We have not confirmed these events during reprogramming of our cells, but Marchetto and coworkers have previously shown the capacity to recapitulate X-inactivation during neural differentiation, producing mosaic neuronal cultures with different *MECP2* expression levels (Marchetto et al., 2010). Isogenic human iPSC lines from RTT patients can therefore be used as *in vitro* models of the disease (Ananiev et al., 2011), and our results (Figure 5.8)

are in accordance with the published literature (Ananiev et al., 2011; Kim et al., 2011; Marchetto et al., 2010). Since several studies have reported similar findings, and taking into consideration the different levels of *MECP2* expression in mutated cells, the contribution of non-neural cells, like astrocytes, to the onset of the disease should not be excluded. In fact, this influence was already described, indicating that glial cells carrying *MECP2* mutations can also distress healthy neurons (Ballas et al., 2009; Maezawa et al., 2009).

In conclusion, our defined culture system provides a way to recapitulate some of the temporal and regional patterning events that occur during *in vivo* cortical neurogenesis (van den Ameele et al., 2014). Also, by deconstructing the natural complexity of neural development into a simpler experimental approach, we could mimic several aspects of RTT pathology potentially contributing to a better understanding of cortical development and disease.

5.5 Acknowledgements

T.G.F., G.M.C.R. and D.M.R. acknowledge support from Fundação para a Ciência e a Tecnologia (SFRH/BPD/86316/2012, SFRH/BD/89374/2012 and SFRH/BD/60386/2009, respectively). S.T.D. integrates the Program for Advanced Medical Education of Calouste Gulbenkian Foundation and Fundação para a Ciência e Tecnologia (SFRH/BDINT/51548/2011) and D.M.R. the PhD Program of Centro Académico de Medicina (Neurosciences) of the Faculty of Medicine, University of Lisbon. D.M.R is also the recipient of a grant from Fondo BioRett. This work was further financially supported by the European Community's 7th Framework Programme through projects Neurostemcell (#22943) and NeuroStemcellRepair (#602278), by FCT, through the MIT-Portugal Program, Bioengineering Systems Focus Area and Grants PTDC/SAU-NMC/110838/2009, PTDC/EBB-BIO/122504/2010, EXPL/BBB-EBI/0294/2013 and EXPL/bim-mec/0009/2013.

Supporting information

Supporting Table 5. S1: Primers for quantitative real time PCR analysis using SYBR® green chemistry.

Target genes	Amplicon Size (bp)	Annealing Temperature (ºC)	Sequence
GAPDH	101	60.2 61.7	(fwd) 5'-ACA ACT TTG GTA TCG TGG AAG G-3' (rev) 5'- GCC ATC ACG CCA CAG TTT C-3'
FGF5	196	61 60.5	(fwd) 5'-CAC TGA TAG GAA CCC TAG AGG C-3' (rev) 5'-CAG ATG GAA ACC GAT GCC C-3'
NESTIN	200	56.8 54.9	(fwd) 5'-GCC CTG ACC ACT CCA GTT TA-3' (rev) 5'-GGA GTC CTG GAT TTC CTT CC-3'
β-Tubulin III	126	56.7 49.6	(fwd) 5'-CCA TCT TGC TGC CGA CAC-3' (rev) 5'-CAA TAA GAC AGA GAC AGG AG-3'

Supporting Table S2: Taqman® assays used for quantitative real time PCR analysis.

Target genes	ID numbers
PAX6	Hs00240871_m1
SOX1	Hs01057642_s1
OCT4/POU5F1	Hs04260367_gH
NANOG	Hs0460366_g1
GAPDH	Hs99999905_m1

S.2. Supporting Results

Supporting Table S3: Flow cytometry analysis of pluripotency markers. Human iPSC lines used in this work were analyzed using flow cytometry for transcription factors OCT4, SOX2, and NANOG, and surface antigens Tra-1-60 and SSEA4. All cell lines presented high percentages of cells marking positive for these markers.

Cell Line	OCT4	SOX2	NANOG	Tra-1-60	SSEA4
iLB-C1-30 m-r12 (*wild type*)	99 ± 0.2%	96 ± 1.0%	97 ± 2.1%	93 ± 1.2%	97 ± 1.8%
WT-Évora F0000B13 (*wild type*)	96 ± 1.1%	94 ± 1.5%	98 ± 0.9%	92 ± 1.5%	94 ± 2.6%
EMC23i (*RTT syndrome, mutated*)	98 ± 0.4%	97 ± 1.2%	98 ± 0.8%	94 ± 2.4%	94 ± 4.0%

Chapter 6 GABAergic dysfunction in neurons derived from induced pluripotent stem cells of patients with Rett Syndrome

Sofia Duarte conceived the work, performed part of the experiments and wrote the first draft of the Chapter. Mehrnaz Gahzvini performed part of the experiments to obtain iPSCs and supervised the work at the iPSC facility of Erasmus Medical School.

Other contributers to the experiments described in this chapter (yet unpublished) are identified in the Methods and Results sections.

Abstract

Objective: iPSCs are a promising tool for the study of neurological diseases. Disruption of GABAergic activity in RTT can be related, at least in part, with a failure to complete the GABA functional switch that occurs during normal development, from excitation in immature neurons to inhibition in mature neurons. This switch is mainly mediated by a developmental increase in KCC2 expression, a cotransporter that drives Cl- efflux outside neurons, with the consequent decrease in intracellular Cl- concentration and GABA-mediated hyperpolarization in mature neurons. We documented reduced KCC2 levels in the cerebrospinal fluid of RTT patients and aimed to test GABAergic function in neurons derived from RTT patients' IPSC cells.

Methods: Human skin biopsies were collected in accordance with European and National ethical regulation and human iPSCs were generated from fibroblast cells upon infection with a retroviral vector expressing the four canonical transcription factors (Oct4, Sox2, Klf4, and Myc). iPSC lines were obtained from patients carrying *MECP2* mutations (R306C and R255X) and controls. Neuronal cortical populations were derived in a monolayer culture system using the dual-SMAD inhibition protocol. Perforated patch recordings were performed in these neurons and GABA-evoked postsynaptic currents were measured to evaluate GABAAR equilibrium potential. Additionally, we performed molecular and neurophysiology experiments in a RTT mouse model (Mecp2tm1.1Bird/J), to search for the same disturbances.

Results: Recordings from RTT cells exhibit a GABAAR equilibrium potential that is more positive than in recordings from control cells, favoring changes in Cl- gradient characteristic of an immature state. The level of KCC2 protein expression is lower in *Mecp2*-KO mice hippocampi when compared to control littermates, as addressed by western blot analysis of 6 week old hippocampi.

Conclusion: Results form the RTT animal model, iPSC-based models and human cerebrospinal fluid studies suggest a GABAergic dysfunction in RTT that includes alterations in KCC2 expression and/or activity.

6.1 Introduction

RTT (OMIM: #312750) is a severe neurodevelopmental disorder and leading cause of severe intellectual disability and autistic-like symptoms in females. Clinical features include hand stereotypes, developmental stagnation, social withdrawal, motor abnormalities, autonomic disturbances and seizures (Dolce et al., 2013). RTT is caused primarily by mutations in the widely expressed X-linked gene *MECP2* (Amir et al., 1999), which encodes a multifunctional protein involved not only in transcriptional silencing but also in transcriptional activation, chromatin remodeling, RNA splicing (Damen and Heumann, 2013) and other posttranscriptional regulatory mechanisms (Cheng et al., 2014). MeCP2 is highly expressed in the brain and MeCP2 levels are critical for CNS development (Shahbazian et al., 2002). The timing of *MECP2* expression, in mouse and human, correlates with CNS maturation, with the ontogenetically older structures such as the spinal cord and brainstem starting expression before newer structures such as the hippocampus and cerebral cortex. The level of MeCP2 is also up-regulated during postnatal neuronal development (Ding, 2015).

Several studies have shown that partial phenotypical rescue can be attained upon restoration of *Mecp2* expression in postmitotic neurons of *Mecp2*-deficient mice (Guy et al., 2007) (Robinson et al., 2012) (Lang et al., 2014), revealing that MeCP2 deficiency does not lead to permanent and irreversible alterations. However, gene therapy is not a valid approach for RTT, since the slightest perturbation in MeCP2 levels is deleterious for brain function (Cheng and Qiu, 2014). Thus, alternative treatment approaches should rely on the identification of downstream effectors of MeCP2, and its associated dysfunctions. One reported dysfunction involves a compromised synaptic maturation in RTT patients, associated, at least in part, to disruption of GABA activity during cortical development (Smrt et al., 2007). There is evidence for the impact of *MECP2* conditional deletion in cortical excitability and GABAergic circuit formation, both in GABAergic neurons (Chao et al., 2010) and forebrain excitatory neurons (Zhang et al., 2014), indicating that MeCp2 is relevant for GABA metabolism and presynaptic release, and also for

appropriate GABAergic postsynaptic actions.

GABA is the main inhibitory (hyperpolarizing) neurotransmitter in the adult brain, but has excitatory (depolarizing) action in the developing brain. The magnitude and direction of the ionic current through GABAARs exquisitely depends on its driving force, defined as the difference between the electrochemical equilibrium potential of Cl- anions (reversal potential, Ecl) and the resting membrane potential (RMP) of the neuron. If this difference is positive or negative, there will be a net flux of Cl- anions through the plasma membrane following GABAAR opening, and this will result in a change in the membrane potential of the neuron. In particular, the net flux of Cl- anions through GABAAR (i.e., toward inside or outside the cell) critically depends on its intracellular concentration ([Cl-]i). (Deidda et al., 2014). A shift in GABAergic function therefore occurs during development (Ben-Ari, 2002). Failure to complete this shift might result in network hyperexcitability and seizure activity in the early post-natal brain. [Cl-]i is mainly regulated by two ion transporters: NKCC1 (allows chloride to accumulate inside the cell), and KCC2 (the major chloride extruder in mature neurons), encoded in the gene *SLC12A5.* The higher [Cl-]i in young neurons depends on high expression of NKCC1 (Dzhala et al., 2005), and results in depolarizing GABAA transmission. As development proceeds, KCC2 becomes expressed at higher levels than NKCC1 (Dzhala et al., 2005; Rivera et al., 1999), and hyperpolarizing GABAergic transmission takes over (Ben-Ari, 2002). (Cancedda et al., 2007) (Sernagor et al., 2010). Balanced activity of NKCC1 and KCC2 is therefore determinant for neuronal response to GABA.

RTT neurons display reduced spine density, motility, impaired synaptic plasticity and deficits in hippocampal-dependent learning and memory, which could ultimately account for intellectual disability. Interestingly, genetic ablation of KCC2 prevents spinogenesis and excitatory synapse formation in immature neurons, through a mechanism independent of KCC2 transporter function (Li et al., 2007).

In Chapter 2, we described a **lower abundance of KCC2 in RTT patients'** CSF, suggesting lower cerebral *KCC2* expression levels (Duarte et al., 2013). This observation prompted us to search for additional evidence to document a

specific defect in GABAergic function, with potential impact in impaired learning and cognition, epilepsy, and eventually also behavioral disturbances of RTT.

To pursue this aim, as described in this chapter, we reprogrammed fibroblasts from RTT patients to generate iPSCs, an important tool for the study of neurological diseases. By offering an unlimited source of patient-specific disease-relevant neuronal and glial cells, iPSC-based disease models hold enormous promise for identification of disease mechanisms, discovery of molecular targets and development of phenotypic screens for drug discovery (Saporta et al., 2011). After successful reprogramming of patient's iPSC and controls, a multistep protocol for cortical neuronal directed differentiation was optimized (see Chapter 5). This system enables functional studies of human cerebral cortex development, and the generation of patient-specific cortical networks *ex vivo* for disease modeling and therapeutic purposes (Shi et al., 2012b). To further document KCC2 impaired expression in RTT, we performed protein expression studies in tissue from a RTT mouse model (*Mecp2*tm1.1Bird/J), in order to characterize *Kcc2* expression and neuronal excitability during postnatal development in this model.

6.2 Methods

6.2.1 Human Skin Samples

Human skin punch biopsy samples were collected using the standard technique. A small 3 mm skin biopsy was obtained from patients carrying two different *MECP2* mutations (R306C and R255X) and, for control samples, a protocol was established with the Paediatric Surgery Department at Centro Hospitalar de Lisboa Norte (CHLN) to collect skin samples when minor surgeries were performed. Biopsy tissue was incubated in cell-culture medium and transported to the Instituto de Medicina Molecular (IMM) for *in vitro* expansion. HSJD and CHLN Ethic Committees approved the study and written informed consent was obtained from patient's legal guardians.

6.2.2 Establishment of human fibroblast cultures

Skin biopsies were washed with phosphate-buffered saline (PBS) and subcutaneous tissue and epidermis were excised. The dermal sample was placed in a new dish and cut into small squares. Fragments were transferred to a 6-well plate and after attachment were cultivated with complete growth medium that included DMEM (Life Technologies) supplemented with 20% FBS, 1% nonessential amino acid mixture (Life technologies), 1% penicillin/streptomycin (Life Technologies) and 1% sodium pyruvate (Life Technologies). After fibroblast expansion and migration, cells were passaged to a 25 cm^2 using trypsin/EDTA (10%, Invitrogen). Medium was changed every 3 to 4 days until the culture becomes confluent. Fibroblasts were harvested and ressuspended in 10% DMSO/90% complete DMEM at $1x10^5$ per ml for cryopreservation.

6.2.3 iPSC generation and culture

To generate human iPSCs, $1x10^5$ human fibroblasts from lines R1 (female patient, R306C *MECP2* mutation), R2 (female patient, R255X *MECP2* mutation) and F7 (female, age-matched control) were seeded per well of a 6-well plate. The next day, cells were transduced with a polycistronic lentiviral reprogramming vector (Warlich et al., 2011) for 16 hours in the presence of 4 µg/ml polybrene (Millipore). Cells were further cultured in fibroblast medium (as in 7.2.2) at 20% O2. At day 4 post-transduction, $5x10^4$ cells were seeded on irradiated Mouse Embryonic Fibroblasts (MEFs) in fibroblast medium. One day later, medium was changed to standard hESC medium. From day 2 to 9 medium was supplemented with 2 mmol/l valproic acid (Calbiochem) as described by Warlich et al. (Warlich et al.). iPSC colonies were picked between day 22 to 25 and further propagated on MEFs. Human iPSC lines growing on conventional/standard medium were passaged weekly using collagenase IV (1 mg/ml, Invitrogen) on γ-irradiated MEFs. All analysis (pluripotency markers quantification and immunohistochemistry, embryoid bodies characterization, chromosome count, mycoplasma testing) for characterization of iPSC was performed prior to cryopreservation.

All human iPSCs were maintained in hESC culture medium, consisting of DMEM/F12 (Gibco-Invitrogen) supplemented with 20% knock-out serum replacement (Gibco-Invitrogen), 1% L-glutamine, 1% penicillin/streptomycin, 1% MEM-non-essential amino acids (PAA Laboratories GmbH), 0.1mM β-mercaptoethanol (Sigma), and 10 ng/ml bFGF (Invitrogen) filtered through a 0.22μm filter (Corning). To generate Embryoid Bodies (EBs), iPSCs were differentiated in suspension culture for 1 week in EB medium, consisting of IMDM-glutamax, 15% foetal calf serum, 100 U ml penicillin, 100 mg ml streptomycin, 1% non-essential amino acids (all Invitrogen), 37.8 ml l monothioglycerol (Sigma) and 50 mg/ml ascorbic acid (Sigma).

An additional iPSC line from a male carrying a different *MECP2* mutation (Q83X) was kindly provided by Alysson Muotri (UCSD, USA). A control line, WT-Évora was kindly provided by TCLab (Évora, Portugal). This line was obtained from a male control with 3 years old. Moreover, fibroblasts from the 7 years old control were also reprogramed at Prof. Luis Pereira de Almeida Lab, Centro de Neurociências de Coimbra (CNC) and characterized at Stem Cell Bioengineering Laboratory, Centre for Biological and Chemical Engineering, Instituto Superior Técnico (IST) and IMM (see table 6.1 and Figure 6.5).

Cell Line	Age	Sex	Mutation	iPSC Facility	iPSC generation method
EMC25i/WT-R/F7 (F7)	7 years old	F	Wild Type	Erasmus Medical School	Fluorescence-based lentiviral reprogramming technique (Warlich et al., 2011)
EMC23i/R1 (R1)	6 years old	F	*MECP2* R306C	Erasmus Medical School	Fluorescence-based lentiviral reprogramming technique (Warlich et al., 2011)
EMC24i/R2 (R2)	8 years old	F	*MECP2* R255X	Erasmus Medical School	Fluorescence-based lentiviral reprogramming technique (Warlich et al., 2011)
COIMBRA/F7	7 years old	F	Wild Type	Luis Pereira de Almeida Lab, CNC	Fluorescence-based lentiviral reprogramming technique (Warlich et al., 2011)
Rett Male	3 years old	M	*MECP2* Q83X	Alysson Muotri LAB (UCSD, USA)	Retroviral reprogramming vectors (Takahashi et al., 2007)
WT-Évora F0000B13	3 years old	M	Wild Type	TCLab	Retroviral reprogramming vectors

Table 6.1. iPSC lines (*MECP2* mutated and controls).

6.2.4 Feeder-free culture of human iPSCs

iPSCs were thawed on mitotically inactivated MEFs. Cells were ressuspended in hESC medium, prepared as described in section 6.2.3. After initial expansion, iPSCs were dissociated with EDTA dissociation buffer 0.5mM (Gibco®, diluted in sterile PBS) and cultured in mTeSR™1 medium (StemCell Technologies™), on Matrigel™ (BD)-coated plates. Medium was changed daily and cells were passaged every 3-4 days, using with a split-ratio of 1:4 (Chen et al., 2011).

6.2.5 Neural induction of human iPSCs

When human iPSC cultures were nearly confluent (Shi et al., 2012b), neuronal induction was started in N2B27 medium (50% (v/v) DMEM/F12/N2 medium (DMEM-F12 (Gibco®), supplemented with 1% (v/v) N2 (Gibco®), 1 mM L-glutamine, 1.6 g/L Glucose (Sigma®), 1% (v/v) PenStrep, and 20 µg/mL Insulin (Sigma®)) and 50% (v/v) of B27 medium (Neurobasal (Gibco®) supplemented with 2% (v/v) B27-supplement (Gibco®), 1 mM glutamine and 1% (v/v) PenStrep)). Media formulation was supplemented with 10 µM SB-431542 (SB) and 100 nM LDN-193189 (Wernig et al.) (both from Stemgent®) for 12 days.

6.2.6 Neuronal differentiation

At day 12 of differentiation, cells were passaged with EDTA dissociation buffer (0.5mM) and re-plated onto laminin (Sigma®)-coated plates (split ratio of 1:3). Neural induction was confirmed as explained in Chapter 5. When neural rosettes were observable (around day 14), N2B27 was supplemented with 10 ng/mL of bFGF (Peprotech®) for two to four days. At day 17, cells were passaged using EDTA to new laminin-coated plates (split ratio of 1:3) (Shi et al., 2012a). Cells were then cultured until day 28-30 in N2B27 medium without the addition of small molecules or any other factors. Finally, cells were split with accutase (Sigma®) and plated at as single cells at a density of 200,000 cells/cm^2. Cells were further cultured in laminin coated plates until day 120 with N2B27 medium replacement every other day (Figure 6.6)

6.2.7 Electrophysiology experiments

Electrophysiology experiments in iPSC derived neurons were performed by Diogo Rombo and Raquel Dias at Prof. Ana Sebastião Lab. Cells were recorded in culture medium, in order to reduce osmotic pressure.
Whole-cell recordings were performed as described in 5.2.9. For perforated patch clamp recordings, gramicidin was diluted in the pippete solution to a final concentration of 25 µg/mL immediately before use. GABA (30µM)-evoked postsynaptic currents (GABA-PSC) were measured to evaluate $GABA_A$ receptor equilibrium potential (E_{GABAA}). Recordings were performed under voltage clamp at multiple holding potentials. Peak current amplitude and holding potential were ploted to yield the chloride equilibrium potential. Electrophysiology experiments in mice hippocampi were performed by Raquel Dias. Whole cell patch-clamp recordings were obtained from CA1 pyramidal cells. The resting membrane potential was measured immediately on establishing whole cell configuration. For each neuron, the threshold for action potential generation was determined as the difference between the resting membrane potential and the membrane potential at which phase plot slope reached 10mV/ms (Felix-Oliveira et al., 2014).

6.2.8 Animals

The expression studies of KCC2 was done in hippocampi taken from 6 week old wild type and *Mecp2* Knockout (KO) (B6.129P2 (C)-*Mecp2*tm1.1Bird/J) animals. Animals were handled according to the Portuguese law on Animal Care and European Union guidelines (86/609/EEC). Care was taken so to reduce the number of used animals to the absolutely necessary. The genotype of the mice was determined by polymerase chain reaction (Guy et al., 2007) using supplier instructions.

6.2.9 Western blot analysis

Protein extracts for western blot analysis were prepared from snap-frozen hippocampi or human cell lysates. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Zeng et al.) was used to evaluate the levels of KCC2. Brain tissue was disrupted in sucrose-Tris solution with a Teflon pestle and the protein content in the supernatants was determined

using a commercial Bradford assay (Sigma, MO, United States of America). Total proteins (70 µg) were separated on 10% SDS-polyacrylamide electrophoresis gels and blotted onto a polyvinylidene fluoride membrane according to the standard procedures. The blots were probed overnight at 4ºC either with the polyclonal rabbit anti-KCC2 (1/500) in 3% BSA (Bovine Serum Albumin). After washing (3 x 5 min in TBST [10 mM Tris, 150 mM NaCl, 0,05% Tween 20 in H_2O]), blots were then with secondary antibodies conjugated with horseradish peroxidase and bands were visualized with a commercial enhanced chemiluminescence detection method (ECL) kit (PerkinElmer Life Sciences, MA, United States of America). Values were normalized to the β-actin or α-Tubulin or GAPDH loading control and the relative intensities were normalized to WT (set as 1). Densitometry of the bands was performed using the Image J processing software (NIH, MD, United States of America).

6.3 Results

6.3.1 Fibroblasts from RTT patients with different *MECP2* mutations (R306C and R255X) and age-matched controls were reprogrammed into iPSC lines.

To generate iPSC lines, fibroblasts from RTT patients and age-matched controls were transduced with a polycistronic lentiviral vector expressing *OCT4*, *SOX2*, *KLF4*, and *MYC*, and a dTomato reporter, under the control of a retroviral promoter (SFFV) that is rapidly silenced during the reprogramming process (Warlich et al., 2011) (Figure 6.1).

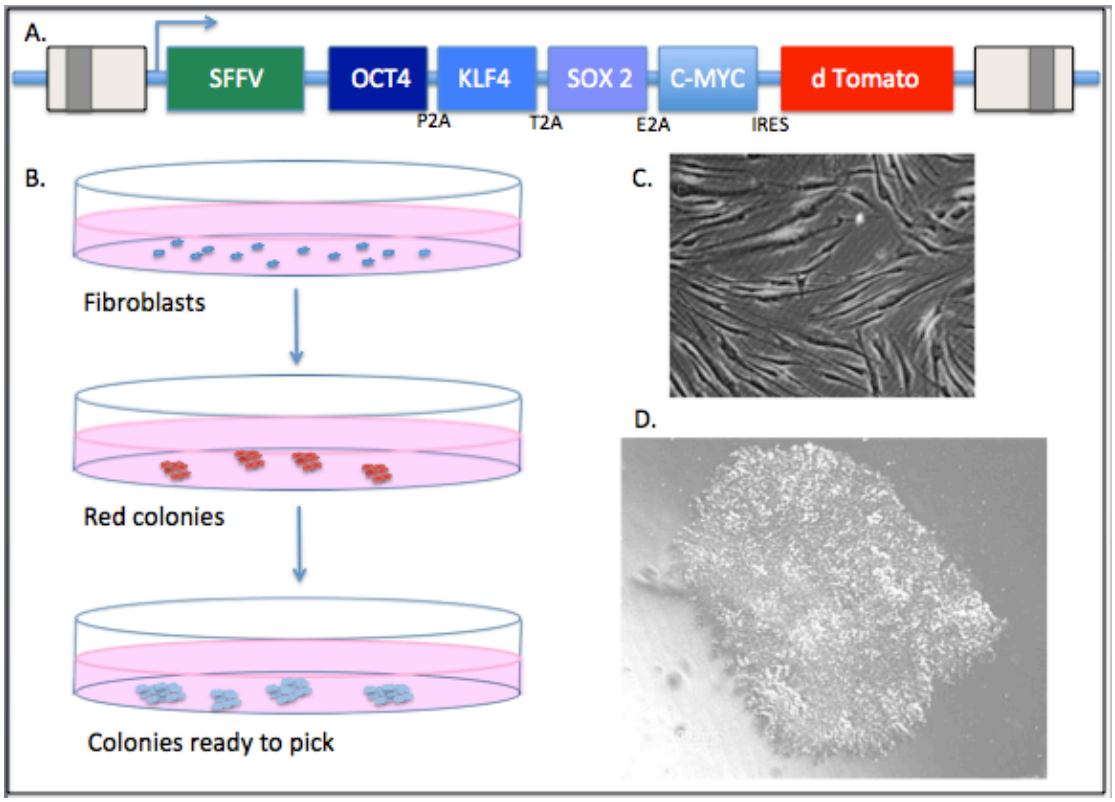

Figure 6.1 Generation of human iPSC lines from fibroblasts

A. Schematic representation of the lentiviral reprogramming cassete (Warlich et al., 2011) used to establish F7, R1 and R2 human iPSC lines. B. Transduced cell lines express dTomato and red colonies are observed at around 10 days and after 22 days, colonies are ready to pick. C. Human fibroblasts in culture. D. Human iPSC colony.

Fibroblasts were plated on mouse embryonic fibroblasts and cultured in the presence of standard hESC medium. After approximately 10 days, small

clusters of cells appeared that started to develop hESC morphology. These clusters gradually lost the expression of the dTomato reporter, which indicated proper silencing of the lentiviral transgene, required to establish fully reprogrammed human iPSCs.

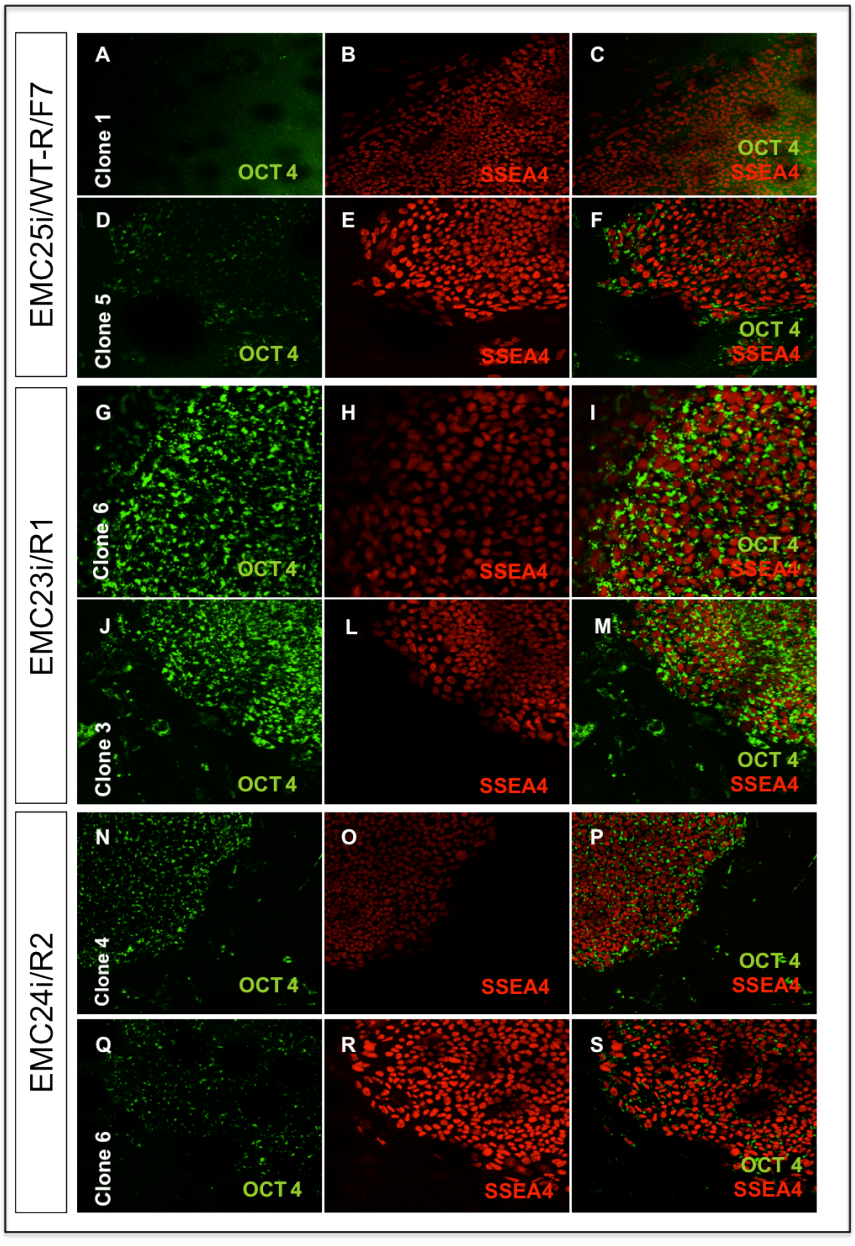

Figure 6.2 Characterization of EMC lines with immunohistochemistry

Imunohistochemistry analysis of representative clones with pluripotency markers.

For each one of the different donors, we obtained several iPSC clones. All these iPSC clones showed morphology resembling hESCs and expression of

key endogenous pluripotency factors, including *NANOG* and *REX1*, *KLF4*, *cMYC, OCT3/4, SOX2, GDF3, FGF4, ESG1*, at different passages after establishment (Figure 6.2 and Figure 6.3).

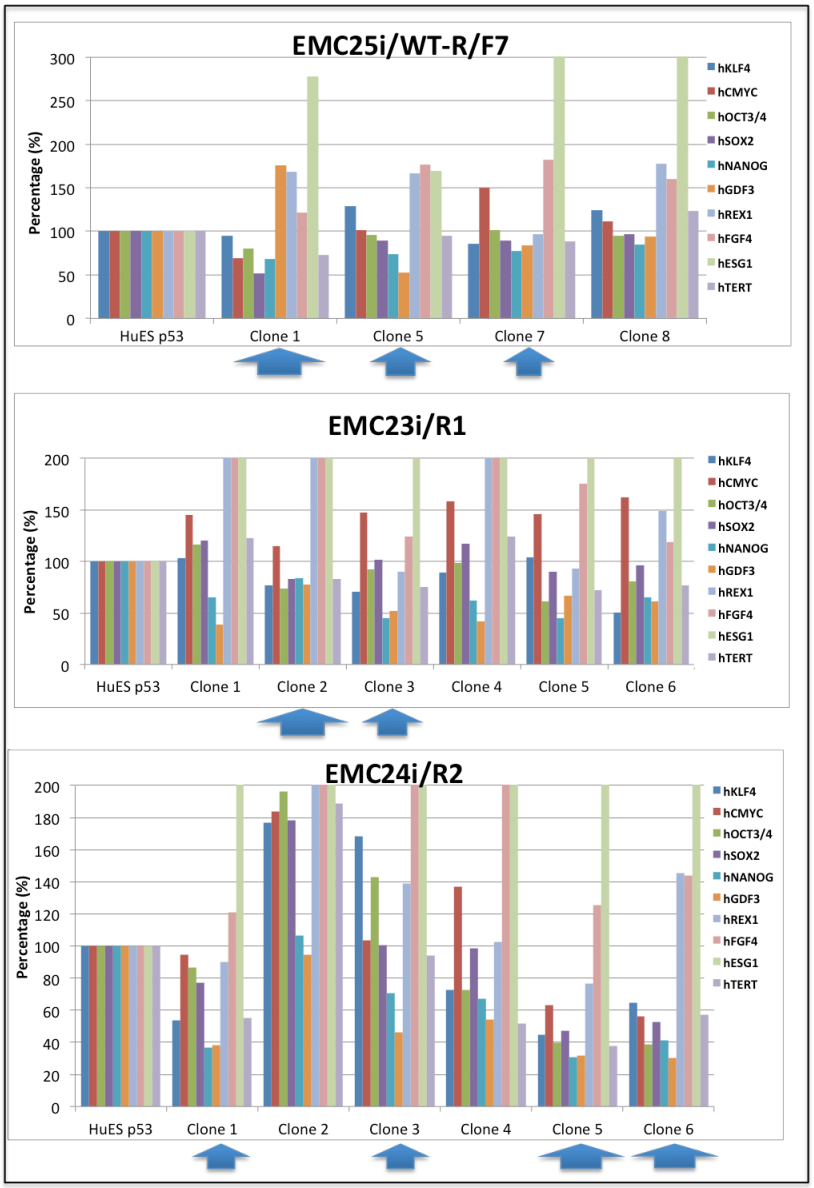

Figure 6.3 Characterization of EMC lines with qRT-PCR analysis

qRT-PCR analysis of pluripotency factors in EMC25I/WT-R/F7 line. Expression of the same factors in hESC line H9 (Amit et al., 2000) served as a control and was set at 100%. Clones with higher *NANOG* expression and reduced c-myc expression are pointed with arrows.

EB differentiation was also performed and qPCR analysis indicated that most human iPSC lines showed expression of endodermal (*FLK1, AFP*), mesodermal (*GATA2*), and ectodermal (*PAX6*) marker genes (Figure 6.4).

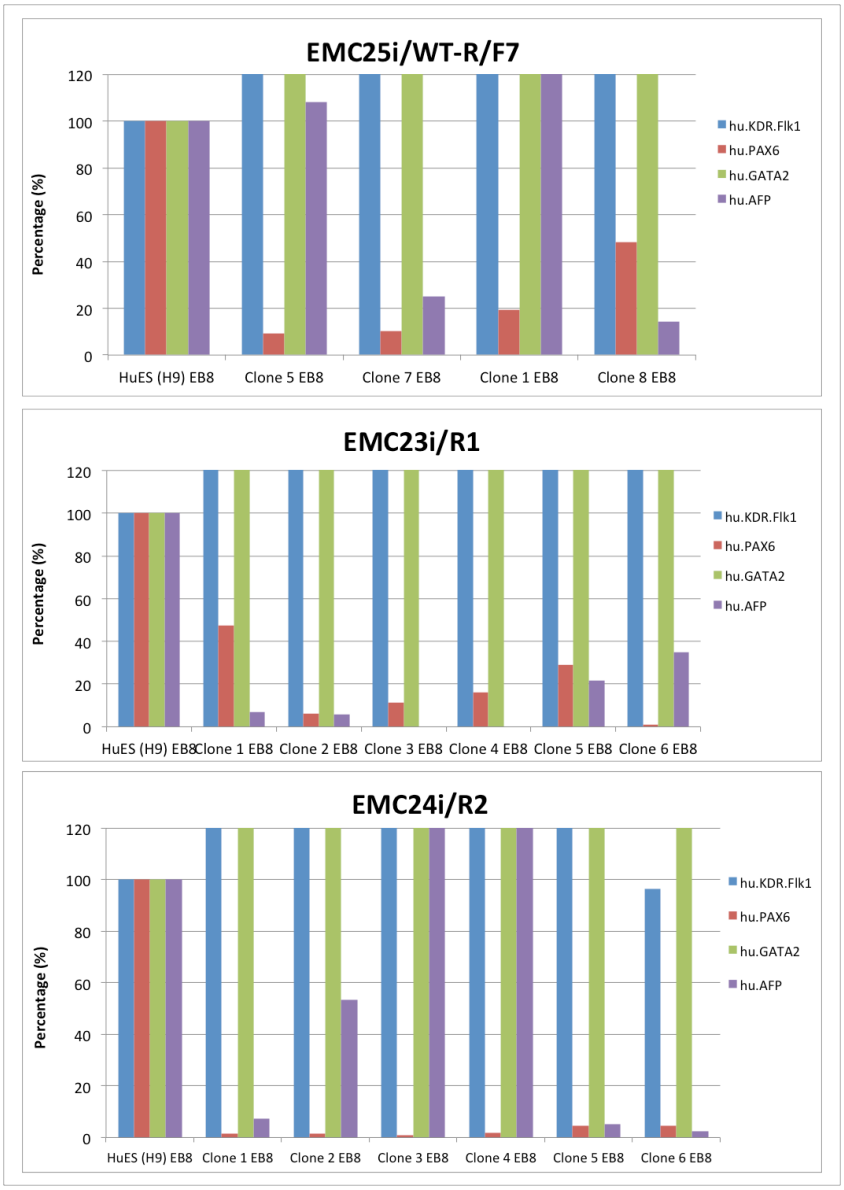

Figure 6.4 Characterization of embryoid bodies derived from EMC lines.

Differentiation markers mRNA quantification at day 8: *PAX-6* (ectoderm), *FLK1* and *GATA2* (mesoderm) and *AFP* (endoderm).

In order to select iPSC clones to differentiate into neurons, several factors were considered: i) expression of pluripotency factors- clones with higher *NANOG* expression and lower c-*MYC* expression were selected. ii) karyotype: clones with aberrant number of chromosomes were excluded.

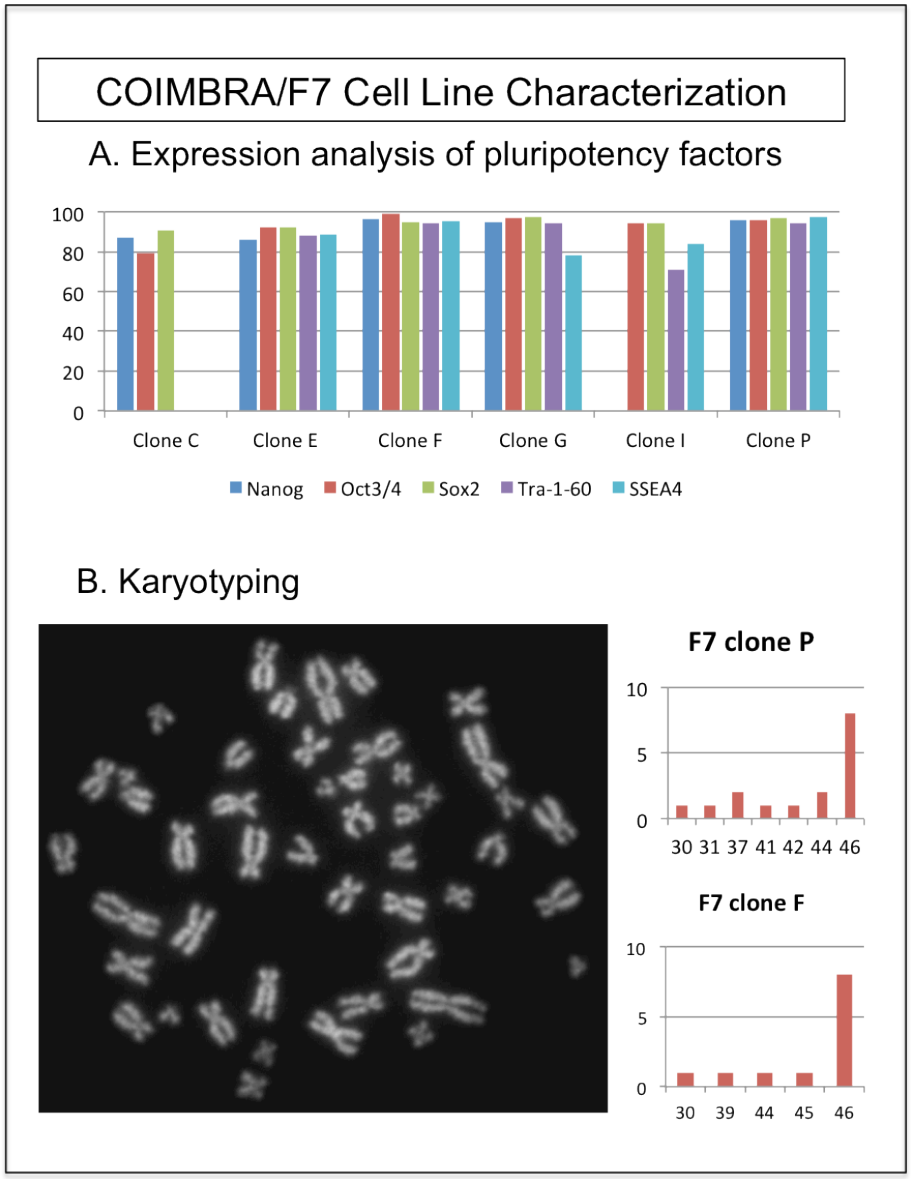

Figure 6.5 Characterization of COIMBRA/F7line

A. Flow cytometry analysis of pluripotency markers. COIMBRA/F7clones were analyzed using flow cytometry for transcription factors OCT4, SOX2, and NANOG, and surface antigens Tra-1-60 and SSEA4. All cell lines presented high percentage of cells expressing these markers. B. Karyotype photo. Experiments performed by Claudia Gaspar and Tiago Fernandes.

Regarding chromosome count, all iPSC clones were tested. Clones with a correct number of chromosomes were clone 2 and 3 of EMC23i/R1 (R1), clone 3 of EMC24i/R2 (R2) and clone 1, 5 and 7 of EMC25i/WT-R/F7 (F7). Clones initially selected for neuronal differentiation were R1 – clone 2, R2 -

clone 3 and F7/Coimbra clone P.

6.3.2 Functional neurons derived from RTT and wild type lines using a protocol for directed differentiation into cortical neurons.

In order to model a neurodevelopmental disorder with cognitive impairment and epilepsy like RTT, with clinical features associated with cortical dysfunction, a human neuronal differentiation protocol designed to direct neuronal differentiation to cortical neurons (Shi et al., 2012a; Shi et al., 2012b) was optimized with wild-type iPSCs, as described in chapter 5. This system was designed to obtain neurons from different cortical layers with functional synapses, after a period of prolonged neuronal maturation that resembles the process of human cortex development *in utero*. Moreover, since we aimed to study RTT and, more precisely, effects of MeCP2 impairment that are thought to be dependent of BDNF deregulation, this protocol was convenient because it does not require the addiction of exogenous BDNF. A schematic figure of the protocol is shown in Figure 6.6, and representative examples are shown in Figure 6.6 and 6.7, for different stages of differentiation.

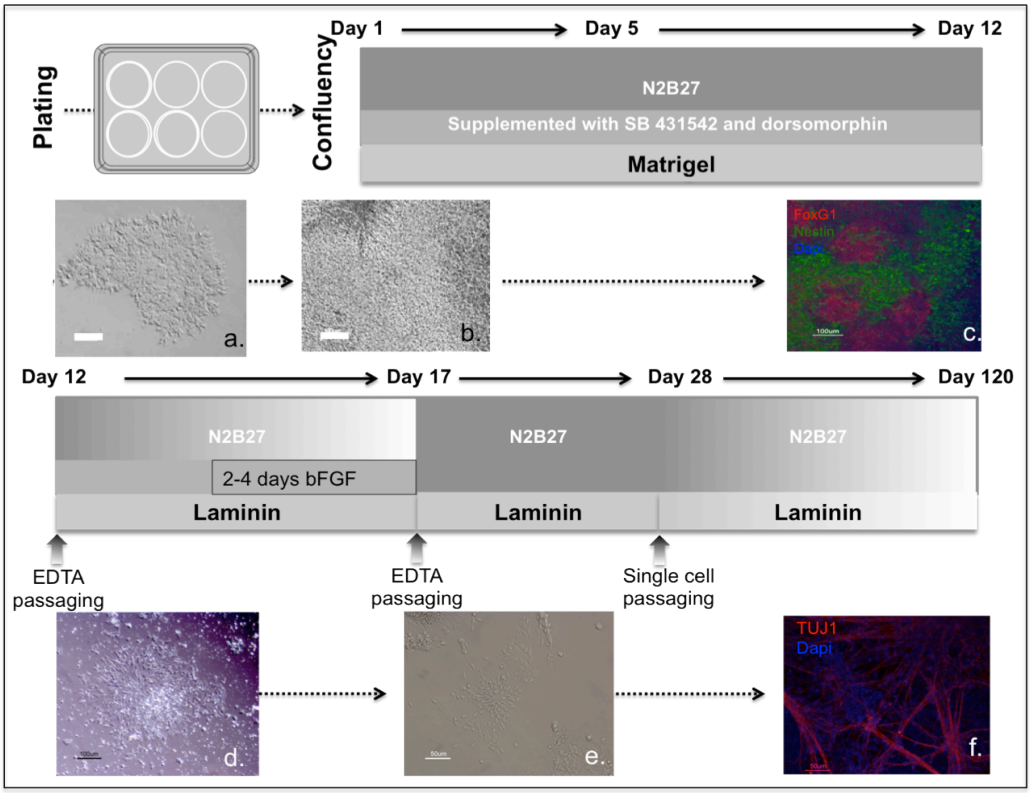

Figure 6.6 Schematic representation of neural commitment and subsequent neural differentiation of human pluripotent stem cells.

Neural commitment is initiated under defined culture conditions using dual SMAD inhibition for 12 days. At this stage cells are passed as clumps using EDTA dissociation to laminin-coated plates. At day 14, bFGF is added to facilitate proliferation of neural rosettes until day 17. Subsequent re-plating onto laminin is done using EDTA dissociation, and further differentiation occurs in the absence of growth factors or chemical inhibitors. A final single-cell passaging step is done at day 28-30, and cells are allowed to differentiate until day 120 with periodic medium change and laminin supplementation. a. iPSCs colony; b. confluent iPSCs colonies, ready to start neural induction; c. Foxg1+ neural precursors; d. rosettes appear after re-plating. e. polarized rosettes (day 25); f. Tuj 1 neurons. Adapted from *Fernandes TG, Duarte ST, et al. Neural commitment of human pluripotent stem cells under defined conditions recapitulates neural development and generates patient-specific neural cells. Biotechnol J. 2015 Jun 30* (Fernandes et al., 2015)

iPSCs from lines F7, R1, R2, Rett Male and WT Évora were thawed and expanded. Several vials of different clones of the EMC F7 line were thawed, but we were not successful in expanding these cells. For this reason, we used COIMBRA/F7 as wild-type iPSCs, reprogramed at Prof. Luis Pereira de Almeida Lab with the same reprograming technique.

After 12 days of neuronal commitment, combining retinoids and dual SMAD inhibition, a downregulation of pluripotency markers like *OCT4*, *NANOG* and *POU5F1* was observed, and an upregulation of neuroectodermal markers (*PAX 6* and *SOX 1*) was also detected (Figure 5.4). Imunostaining confirmed that these cells do not express *OCT4* but express *SOX4, FOXG1, PAX 6* and *OTX1/2* (Figure 5.4). Thus, neuronal progenitors were successfully derived from iPSC lines.

Rosettes were observable two to five days after the first passage at day 12 (Figure 6.6.D), and at day 18 imunostaining confirmed that rosettes were radially arranged like neuroepithelial cells, being positive for PAX6, NESTIN (Figure 6.7.B), and *SOX2* expression, and showing already signs of polarization, with apical ZO1 expression (Figure 6.7.C, D). When neural rosettes were first visible, bFGF was added to the medium, to induce proliferation of progenitors, and around day 17 of differentiation cells were re-plated onto new laminin-coated plates and cultured until day 28-30 without the

addition of small molecules or any other factors. Substantial maturation of NPs within rosettes was observed.

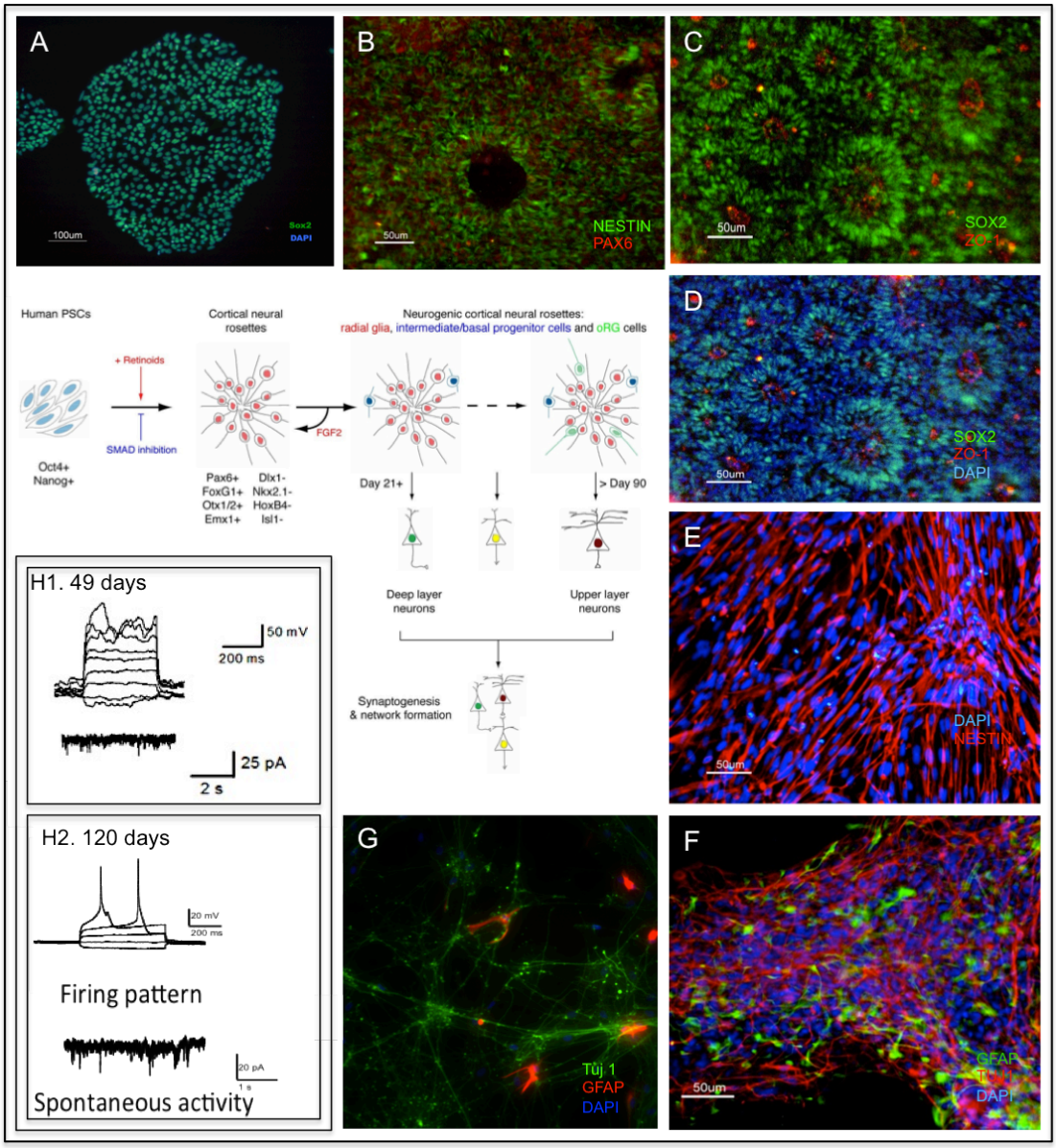

Figure 6.7 Neuronal differentiation steps

iPSCs were thawed and expanded and pluripotency markers expression like SOX2 were reanalyzed with immunohistochemistry (A). At day 12 of neural induction, rosettes were observed. At day 18 immunohistochemistry revealed rosettes positive for NESTIN, PAX6, SOX2 and apical ZO1 expression (B, C, D). At day 77 of neuronal differentiation, homogeneous of NESTIN positive neuronal precursors were observed (E) and also positive cells for TUJ1 and GFAP, identifying neurons and glial cells, respectively (F). These cells remained in culture for synaptic maturation until day 120-130 (G). Whole cell patch clamp experiments were performed early, at

day 49, showing spontaneous activity that indicated the presence of synapses. Firing pattern was immature at this stage (H1). After 120 days in culture, electrophysiological recordings revealed mature neurons (H2). Patch Clamp experiments were performed by Raquel Dias.

The presence of glial cells was detected around day 75 of diferentiation, and confirmed with imunostaining for GFAP (Figure 6.7.F). The presence of synapses was confirmed with imunostaining for VGLUT and SNAP25 (Figure 6.8.A). Finally and as mentioned in chapter 5, cells at dat 120 of differentiation displayed excitability parameters compatible with a mature neuronal state, as assessed by the resting membrane potential, the action potential firing pattern and response to GABA (see also Figure 6.7.H2 and Figure 6.8.B1-B2).

6.3.3 RTT neurons exhibit a GABAAR equilibrium potential characteristic of an immature state.

After obtaining mature neurons from iPSC lines derived from patients with RTT mutations and aged matched controls, functional studies were performed to characterize GABAAR equilibrium potential in these neurons. Perforated whole-cell patch-clamp is a variant of the patch-clamp technique used to measure the sum activity of ion channels in the plasma membrane of a single cell (Linley, 2013). Its defining feature is that electrical access to the cell is obtained through inclusion of a pore-forming antibiotic in the patch pipette which perforates the sealed patch of membrane in contact with the patch pipette. The antibiotic pores allow equilibration of small monovalent ions between the patch pipette and the cytosol whilst maintaining endogenous levels of divalent ions such as Ca(2+) and signalling molecules such as cAMP. Therefore, the perforated patch-clamp technique is ideal for studying ion dynamics and this technique was selected to study GABAAR chloride dynamics in patient derived neurons. GABA (30μM)-evoked postsynaptic currents (GABA-PSC) were measured to evaluate $GABA_A$ receptor equilibrium potential (E_{GABAA}). Recordings were performed under voltage clamp at multiple holding potentials. Peak current amplitude and holding potential were ploted to yield the chloride equilibrium potential.

As shown in Figure 6.8.B3a and B3b, preliminary data indicates that RTT patient derived neurons exhibit a GABAAR equilibrium potential that is more positive than in control cells, suggesting changes in chloride gradient characteristic of an immature state.

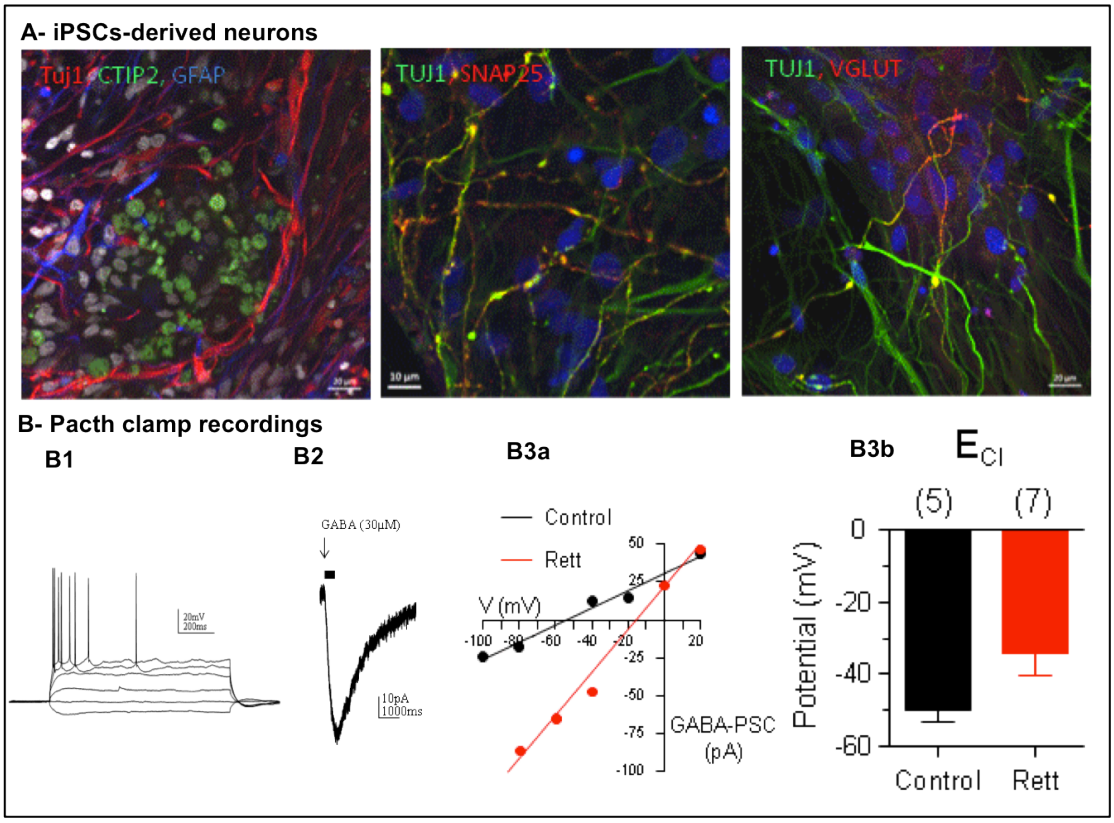

Figure 6.8 GABAAR equilibrium potential in RTT patients' derived neurons is suggestive of an immature state.

A. Immunofluorescence analysis of iPS-derived neuronal cells using the dual-SMAD inhibition protocol. Cortical deep layer neurons (CTIP2), Glial cells (GFAP), differentiated neurons (Tuj1) glutamatergic neurons (Vglut) and synapses (SNAP25). **B.** Derived neurons are functional. **B1** Changes in membrane potential and AP were evoked under current clamp mode by injection current pulses from an initial holding potential of −70 mV. **B2** Representative GABA (30µM)-evoked postsynaptic currents (GABA-PSC) from a neuron recorded in whole-cell voltage-clamp (-70mV) showing responsiveness to GABA. **B3** Changes in chloride equilibrium potential (Ecl) in RTT cells characteristic of an immature state. Gramicidin-perforated patch recordings were performed and GABA (30µM)-evoked postsynaptic currents (GABA-PSC) were measured. **B3a** Two representative experiments showing the peak amplitude of GABA-PSC (pA) as a function of voltage (mV). Lines represent linear regression curves fitting the data. **B3b** Interpolated E_{Cl} from linear regression curves.

All values are mean ± standard error of mean. *P<0.05 (paired Student's t-test). WT is represented in black and *MECP2* mutated in red. Note: Action potential threshold generation was measured as the beginning of the upward rise of the AP when dV/dT exceeded 10 mV/ms. Neurons studied in these experiments were derived from Rett Male iPSC line. Patch Clamp experiments were performed by Diogo Rombo. Traces in B1 and B2 are the same as those depicted in Figure 5.8 and are herein repeated for the sake of clarity.

6.3.4 KCC2 expression is reduced in *Mecp2*-KO mice hippocampi, and intrinsic neuronal excitability is increased in CA1 pyramidal neurons

Taking advantage of the possibility of performing molecular and neurophysiological studies in the RTT animal model brain, we decided to investigate KCC2 levels in the hippocampi of symptomatic (6-8 weeks old) male mice, using imunoblot analysis. A significant reduced level of KCC2 expression was found (0,5457 ± 0,1227, n=7; p= 0.0115) (Student's t test) (Figure 6.9).

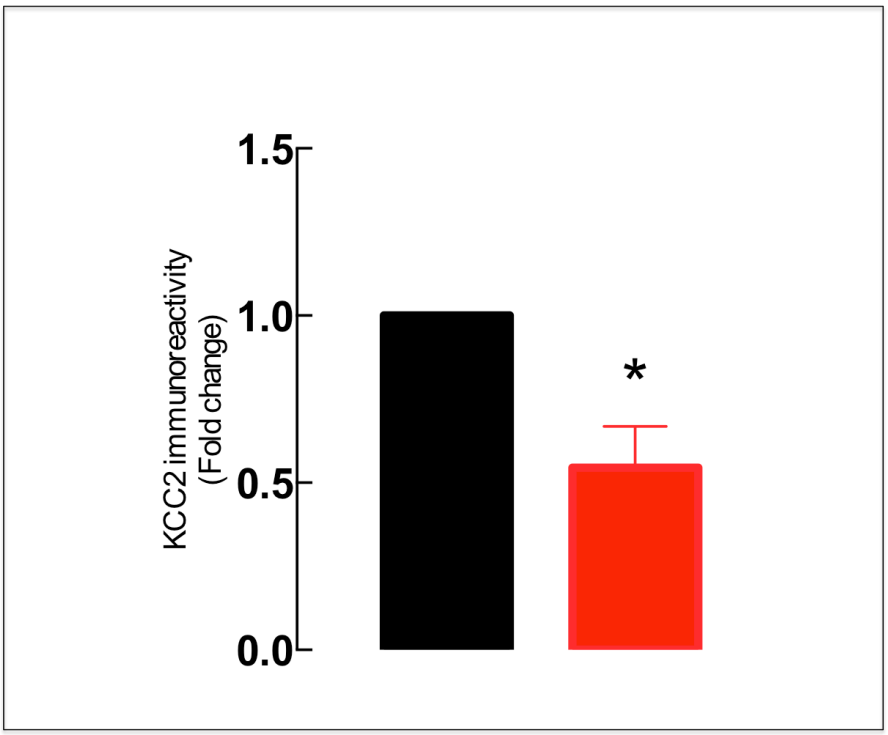

Figure 6.9 KCC2 hippocampal protein level expression in the MECP2 KO mouse model

KCC2 density evaluated by western blot analysis in hippocampal homogenates prepared from hippocampi taken from WT (red bar) and KO (black bar). Value is

mean ± standard error of mean. *P<0.05 (Student's t-test). The values obtained for WT samples were considered 1.

Moreover, to address changes in intrinsic excitability whole cell patch clamp recordings under current clamp mode were performed in neurons from hippocampi of control and *Mecp2* KO mice. A significant difference was found regarding the resting membrane potentilal (RPM) of neurons from controls and *Mecp2* KO mice (Figure 6.10) and also regarding action potential (AP) firing threshold (difference between the RMP of each neuron and the membrane potential at which they first fired an AP), thus showing an increased intrinsic neuronal excitability of *Mecp2* KO neurons.

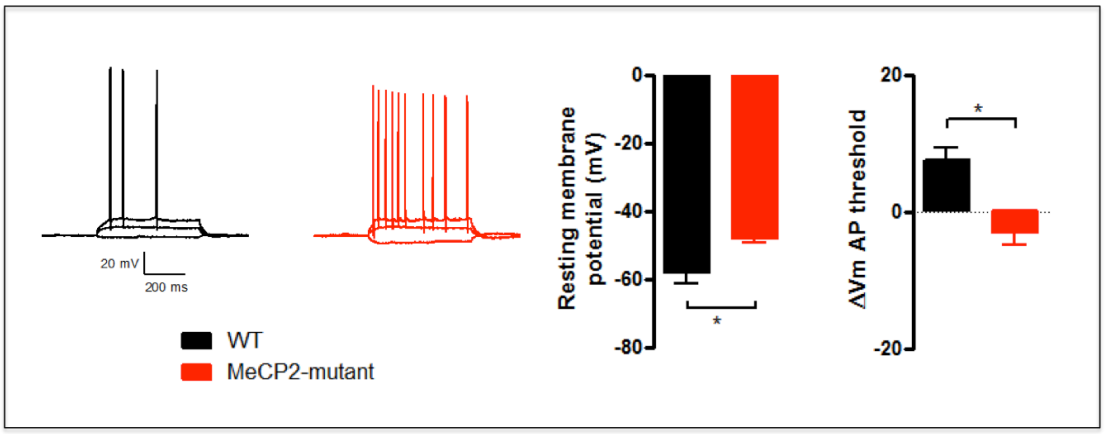

Figure 6.10 Increased intrinsic neuronal excitability from CA1 Hippocampal

Changes in membrane potential and action potentials (AP) were evoked under current clamp mode by injection current pulses from an initial holding potential of −70 mV. The difference between the resting membrane potential (RMP) of each neuron and the threshold potential (Vt) at which they first fired an AP (ΔVm) was significantly different between conditions. Wild type (WT) *Mecp2* KO (red). Recordings performed by Raquel Dias.

6.4 Discussion

Mutations in *MECP2* cause RTT, an X-linked neurodevelopmental disorder associated with synaptic dysfunction, intellectual disability, and autism symptoms (Chahrour and Zoghbi, 2007). MeCP2 controls the transcription of several genes relevant for CNS development and function, with *BDNF* being one of the genes most consistently reported as being under MeCP2 transcriptional control (Chen et al., 2003). RTT is a synaptic disorder with changes in synaptic structure and morphology with documented decreased dendritic arborization and synaptic immaturity (Kaufmann et al., 2000) and also functional disturbances of synaptic development and plasticity (Boggio et al., 2010) (Weng et al., 2011). In this chapter, we have explored the hypothesis that KCC2 expression is deregulated in RTT.

KCC2 is exclusively expressed in neurons and *Kcc2* genetically modified models have revealed KCC2 determinant functions for neuronal, and particularly synaptic functions (Blaesse et al., 2009). Genetic inactivation of KCC2 expression is lethal at birth (Hubner et al., 2001), while genetic inactivation of only the KCC2b isoform leads to spontaneous seizures and death, 2–3 weeks postnatally (Woo et al., 2002) (Uvarov et al., 2009). However, KCC2 exhibits several transport-independent properties at excitatory synapses: (1) it binds scaffolding proteins within dendritic spines to promote spine development (Li et al., 2007) (2) it affects dendritic spine morphology (Fiumelli et al., 2013); (Kelleher and Bear) (Kelleher and Bear) it influences the lateral membrane diffusion of AMPA receptors (Gauvain et al., 2011); and (4) it forms complexes with kainate receptors (Mahadevan et al., 2014). Moreover, the relevance for both inhibitory and excitatory synapses places KCC2 as a potential candidate to contribute to the balance between excitatory and inhibitory synapse formation.

An initial clinical observation of KCC2 reduced levels in the CSF of RTT patients prompted us to search for functional neuronal changes in RTT patients' derived neurons that could translate this molecular change. In the present study, we have derived iPSCs from patients with two different *MECP2* mutations: R306C (missense) and R255X (nonsense), and from an age

matched female control.

The results described in this Chapter suggest that neurons derived from RTT iPSCs have an immature response to GABA. This functional finding is in accordance with the reduced levels of KCC2 detected in the CSF, and also with the significant reduced KCC2 protein expression in *Mecp2* mice hippocampus, also described for the first time in this Chapter. This finding is relevant for:

I. Understanding RTT pathophysiology: the alteration in GABA reversal potential in RTT iPSC derived neurons indicates that these have higher Cl- intracelular concentration, which likely reflects a lower activity of KCC2 and contributes to higher excitability. Previous reports described that specific inactivation of *Mecp2* in mouse GABAergic neurons leads to a presynaptic reduction in GABA release, causing similar neurological phenotypes to those shown by *Mecp2* null mice (Chao et al., 2010). Our results address mainly post-synaptic effects of GABA dysfunction and our finding of an abnormal response to GABA in RTT-derived neurons adds another perspective on the complexity of GABAergic dysfunction in RTT. Moreover, it allows us to focus on a particular mechanism involved in the post-synaptic response to GABA - KCC2 downregulation, which has been previously associated with epilepsy (Dzhala et al., 2005) and autism (Tyzio et al., 2014). In fact, KCC2 downregulation is a common characteristic to other neurodevelopmental disorders like Tuberous sclerosis (Talos et al., 2012) and Fragile X syndrome (He et al., 2014), all exhibiting epilepsy and autistic traits as key clinical features, and abnormal synaptic plasticity as a key underlying neurobiology dysfunction. KCC2 dysfunction, with impact in Cl- transport or in ion transport independent properties, is becoming a new common link between these disorders. Targeting a common node in the complex mechanisms underlying such disorders would allow us to interfere with a series of related neurodevelopmental disorders at once. The fact that results from different models, the murine RTT model and human iPSC derived neurons, are convergent in this particular mechanism is relevant for the robustness of this finding.

II. The search of potential pharmacological strategies for RTT and related disorders. The balance between KCC2 and NKCC1 expression is determinant for GABAergic actions upon GABAAR activation. The unbalance of cation chloride cotransporters can be pharmacologically addressed through NKCC1 inhibitors like the FDA-approved drug bumetanide (Deidda et al., 2015), or through direct and indirect strategies to enhance KCC2-mediated neuronal chloride extrusion (Silayeva et al., 2015) (Puskarjov et al., 2014). A possible way to manipulate cation chloride cotransporters activity is by targeting their upstream regulatory molecules. For example, N-terminal threonine phosphorylation of NKCC1, required for the activity of this transporter, is catalyzed by the WNK/SPAK (with no lysine/Ste20-related proline alanine-rich kinase) kinase cascade. This is in contrast to the situation with KCC2, where activation of WNK kinases appears to have a suppressing effect on KCC2-mediated K-Cl cotransport (de Los Heros et al., 2014). Specific manipulation of such kinase pathways with apparently opposing effects on KCC2 versus NKCC1 may configure a novel pharmacotherapeutic strategy (Kahle et al., 2013). The fast membrane recycling of KCC2 (Lee et al., 2007) could be exploited by generating KCC2-activating drugs that would act to modulate KCC2 membrane insertion and functionality. On this line, a recent high-throughput screening identified the compound CLP257 and its carbamate prodrug derivative CLP290 as KCC2-activating molecules (Gagnon et al., 2013).

III. Optimization of a system to test pharmacological approaches in human neurons. After identifying this particular GABAergic dysfunction, the effect of pharmacological approaches can be tested easily in iPSC derived neurons, with the advantage of performing this test in human neurons with identified pathogenic mutations.

In mammals, X chromosome inactivation (XCI) is a process in which one of the two X chromosomes is silenced. Human iPSCs can change their X inactivation state during reprograming (Bruck and Benvenisty, 2011). This process could hamper the interpretation of our results, since the mutated

allele can be silenced if the correspondent X chromosome is randomly inactivated after reprograming. RTT iPSCs derived from female patients give origin to a mixed population of neurons, some expressing the mutated *MECP2* and others the non-mutated one (Marchetto et al., 2010). This mixed population reproduces what happens in the brain, but when experiments are performed at the single cell level, an increased number of cells would have to be studied. In order to overcome this difficulty, we have used iPSCs derived from a male RTT patient (kindly provided by Prof. Alyson Moutry), and all the reported neurophysiology experiments in this chapter were done on neurons derived from this iPSC line.

6.5 Conclusions

Hippocampal KCC2 protein content is reduced in a symptomatic RTT mice model (*Mecp2*-mutant), when compared to wild-type animals. Furthermore, electrophysiological recordings show reduction of membrane resting potential and threshold potential in *Mecp2*-mutant hippocampal slices. In cortical neurons generated from RTT patient-derived iPSCs, we observed a shift in the equilibrium potential for chloride towards more depolarized values, as usually observed in more immature neurons when compared to normal IPSCs-derived neurons. RTT is a disorder characterized by synaptic immaturity and altered network excitability. Considering the relevant role of KCC2 for GABAergic physiologic actions in the mature brain, and also for a proper synaptic structure and function, deregulation of this cation chloride cotransporter expression can have a significant contribution for RTT pathophysiology. Searching for strategies to mitigate this dysfunction can bring advances in treatment strategies for RTT and for several other neurodevelopmental disorders in which the same dysfunction has been identified.

Chapter 7
Summary and Conclusions

The experiments and results described in this thesis reflect different approaches to study synaptic dysfunction in early encephalopathies, in particular in Rett Syndrome. In this chapter, I will summarize the main findings of my work and outline the main conclusions that can be reached.

1. The CSF studies reported in this thesis can reflect disturbances of CNS physiology in disease and are thus useful for the diagnosis of several child neurology disorders. The advantages of this approach include the possibility of obtaining results *in vivo*, specific for each patient, contributing to a "personalized medicine" strategy. Moreover, these studies can contribute to biomarker research, which can improve directly the diagnosis and also bring cues for the development of more refined research studies in disease models.

   Our aim was to explore the possibility of detecting synaptic proteins in the CSF, to characterize a group of child neurology disorders, from a "synaptic perspective". We were able to conclude that synaptic proteins can be detected in the CSF and are more abundant during the first months of life, a period of intense synaptogenesis, pruning and circuit remodelling. This finding is relevant for the study of early encephalopathies, offering the possibility of using synaptic proteins as biomarkers of disease states to obtain information about their pathophysiology. However, in spite of providing a global perspective of what is going on in the child CNS, this type of proteomic study raises the question of the origin of these proteins, i.e, which neuronal populations are responsible for their production and shedding into the CSF. Also, it is not yet clear how the presence of synaptic proteins in the CSF might be related to specific neuronal dysfunctions. Since it is a study that can be performed *in vivo*, and due to the difficulties of obtaining human tissue samples to study, this is a relevant approach to study child neurology disorders.

   Besides the original publication (Duarte et al., 2011), this work contributed to further research in RTT, described in Chaper 3 (Duarte et al., 2013), and in other child neurology disorders (Ortez et al., 2015).

2. GABAergic system is highly relevant during development, contributing for synaptogenesis, wiring and neuronal circuit formation, allowing the neurons to fire together and consequently wire together. Modulation of GABAergic transmission is achieved in diverse ways, ranging from regulation of GABAAR expression/activity and GABA metabolism to regulation of intracellular chloride concentration. GABAergic system impairment is associated with several neurological and psychiatric disorders and neurodevelopmental disorders like RTT are increasingly being noted as examples of diseases where this impairment is determinant. We focused on intracellular chloride concentration regulation in different models of RTT. We described a reduction in KCC2 levels in RTT patient's cerebrospinal fluid (Chapter 3), (Duarte et al., 2013) and our studies demonstrate that hippocampal KCC2 protein content is drastically reduced in symptomatic RTT mice model (*Mecp2*-mutant) comparing to wild type animals (Chapter 6). Furthermore, electrophysiological recordings show reduction of membrane resting potential and threshold potential in *Mecp2*-mutant hippocampal slices. Synaptic transmission evaluated by Input/Output curves reveals an increased excitatory synaptic transmission. To further characterize GABAAR chloride dinamics, we obtained neurons from RTT patients' iPSCs. Remarkably, in cortical neurons generated from RTT patient-derived IPSCs, we observed a shift in the equilibrium potential for chloride towards more depolarized values, as usually observed in more immature neurons when compared to normal iPSCs-derived neurons. All together, these findings point to the same disturbed mechanism, deregulation of cation chloride cotransporters expression, and particularly KCC2 downregulation, as underlying various neurodevelopmental disorders that have as common features epilepsy, autistic traits and cognitive impairment, as originally hipothesyzed.
   Cation Chloride Cotransporters have very relevant roles for neuronal structure and function (reviewed in (Blaesse et al., 2009). One of the main tasks of Cation Chloride Cotransporters is to maintain a constant [Cl–]i in the face of channel-mediated fluxes that tend to dissipate the

transmembrane electrochemical Cl– gradient, i.e., the driving force of GABAAR-mediated Cl– currents. KCC2 has also a relevant role in spine formation, a role that is independent of its ion transport actions (Li et al., 2007). Therefore, KCC2 downregulation can contribute to an abnormal inhibitory action of GABA and also to an abnormal glutamatergic synaptic communication. Pharmacological targeting of this dysfunction can have impact in epilepsy, autistic traits and eventually also in the intellectual impairment described in RTT. The human *in vitro* neuronal differentiation model that we have developed can be an interesting tool to test these pharmacological approaches and also to understand how KCC2 downregulation interferes with synaptogenesis. In addition, it can help in testing whether a modulation of this process can influence synaptic structure and function.

3. MeCP2 is a multifunctional protein that acts as a transcriptional repressor and also as a transcriptional activator. Several additional regulatory roles, including at the posttranscriptional level, have been described for this protein. A more recent view is that MeCP2 acts as a fine-tune regulator of gene transcription, during neuronal development and also during normal neuronal function. One of best studied transcriptional targets of MeCP2 is the *BDNF* gene. We studied *BDNF* expression on the animal model during development, and also the expression of the relevant TrkB receptors. Interestingly, we have preliminary data that points to higher levels of BDNF during the first week of life, and TrkB-FL receptors levels are reduced during the same period. It is possible that the initially higher levels of BDNF can contribute to the TrkB-FL downregulation that we observed in symptomatic mice. When exogenous BDNF was added to cultured brain slices and LTP was induced, the facilitatory effect of BDNF, observed in WT animals, was lost in symptomatic mice. This lack of effect can be related, at least in part, with the reduced TrkB receptor levels.

Based on previous work from Professor Ana Sebastião Lab, which

provided evidence for a positive modulation of adenosine upon BDNF-TrkB signaling (through A2ARs), we tested if activation of A2AR would be a strategy to recover BDNF-TrkB effects. We found that, when the selective A2AR agonist CGS2168 was applied in combination with BDNF, the BDNF effect upon LTP was restored, similar to what was observed in hippocampal slices from WT animals with BDNF alone. Together, our data highlight A2ARs as a new possible therapeutic target for boosting BDNF effects in RTT. Results from molecular studies described in the thesis, and unpublished data from the Professor Ana Sebastião Lab, point to a reduction of A2ARs expression and increased expression of A1Rs, and also decreased adenosine levels in symptomatic RTT mouse model. Pharmacological targeting of A2ARs, with an excitatory effect, could increase excitability and reduce seizure threshold in RTT patients. However, an additional rational therapeutical approach would be the use of strategies to increase adenosine levels, in order to act on both A1Rs, with inhibitory effects, useful to control epilepsy, and also on A2ARs, with facilitatory effects on BDNF actions.

4. During the optimization of the neuronal differentiation protocol, we have shown that neural commitment from human iPSCs using dual-SMAD inhibition can be performed under defined conditions in a vitronectin-based monolayer system. These progenitors were further diferentiatiated to functional neurons. Obtaining neuronal progenitors in xeno-free conditions can be a relevant first step to establish a safe approach for future transplant based therapies. Compared with mature neurons, neuronal progenitors transplanted to a lesioned region of the CNS have the potential to differentiate *in situ*, with local environmental cues like neurotrophins and signaling molecules that can facilitate their integration. On the other hand, neuronal progenitors can be genetically modified to produce neurotrophic factors or other molecules that can stimulate the regeneration of the original neuronal population.

Chapter 8 Future Directions

Intellectual disability, autistic traits, and epilepsy are important features of a group of neurodevelopmental disorders that collectively affect a significant percentage of the world population (Braat and Kooy, 2015a). GABAARmodulation is increasingly being pointed as a new pharmacological strategy for different neurodevelopmental disorders like fragile X (Braat and Kooy, 2015b), Down Syndrome (Deidda et al., 2015), Neurofibromatosis type 1 (Violante et al., 2013), Tourette Syndrome , Schizophrenia, and monogenic epilepsy or ASD (reviewed in (Ramamoorthi, 2011 #656) and (Deidda et al., 2014)). Finding a common molecular pathway for heterogeneous disorders with clinical similarities can be relevant for future therapeutic strategies that target these symptoms.

In the present thesis work, we started from a clinical observation and set up to perform functional studies in human neurons that could better characterize GABAARs dysfunction in RTT. After establishing the possibility of detecting synaptic proteins in the CSF, an abnormally reduced KCC2 level in the CSF of RTT patients was found. Inspired by these results, we searched for the same mechanism in the RTT animal model, and documented the predicted hyper excitability, along with a reduced expression of KCC2 in the hippocampus of these animals. Using perforated patch clamp, we also have preliminary results that point to an abnormal functional pattern in neurons derived from RTT patients' iPSCs. These neurons display changes in GABAAR chloride equilibrium potential characteristic of an immature state.

This work paves the way for further experimental approaches and brings additional questions that can be addressed in the future. I discuss below several of these approaches and questions:

1. **Delineating strategies to characterize chloride dinamics in neurons derived from RTT patients' iPSCs.** The ultimate aim will be to address whether the depolarizing/hyperpolarizing shift does occur in RTT-derived neurons, and if chloride homeostasis is altered. To achieve this aim, an interesting approach will involve genetic manipulation of iPSCs to express a "chloride sensor", i.e., a fluorescent optogenetic sensor (Grimley et al., 2013) that monitors $[Cl^-]_i$ and therefore can report on this synaptic action of GABA

on GABAAR. A first-generation Cl^- sensor, Clomeleon, has been used to monitor such dynamic changes in $[Cl^-]_i$ in cultured neurons (Kuner and Augustine, 2000) and in brain tissue (Berglund et al., 2006). Clomeleon is a sensor that has been optimized for optimal time resolution, and consists of a fusion of the yellow fluorescent protein (YFP), which contains a serendipitous Cl^- binding site adjacent to its chromophore, and the chloride-insensitive cyan fluorescent protein (CFP) (Grimley et al., 2013). Halide binding to YFP quenches fluorescence emission, altering fluorescence resonance energy transfer (FRET) between the CFP donor and the YFP acceptor (Kuner and Augustine, 2000). The use of FRET enables ratiometric determination of $[Cl^-]_i$, which is unaffected by variations in fluorescence emission intensity associated with differences in indicator concentration, optical path length, or excitation intensity (Miyawaki, 2005). Alterations in chloride homeostasis can thus be tested by chloride sensor live imaging, and can be further confirmed by measuring EGABA using gramicidin perforated-patch recordings of currents evoked by GABA, improving the quantification of GABAAR chloride gradients. In parallel, chloride homeostasis can be addressed in the mouse model by measuring EGABA in *Mecp2*-deficient hippocampal slices at different developmental stages.

The aim will be to have a clearly defined developmental window for the GABAergic switch in normal controls and in RTT models. With this information and the functional tools in practice, we hope to better define the timings and molecular characteristics of this process. In addition, we should also be able to test how different pharmacological strategies con contribute to a normalization of this pathological process.

2-Another relevant step to understand the downstream effect of MeCP2 impairment on Cation Chloride Cotransporters deregulation would be to study the impact of KCC2 downregulation on spine development, excitatory synapses and LTP. Although KCC2 function primarily influences the efficacy of GABAergic signaling through chloride transport, several other ion transport independent properties are relevant for excitatory synapse formation and function. KCC2 has also been considered relevant for the

balance between inhibitory and excitatory synaptogenesis. I thus propose to explore whether RTT-derived neurons display defects in spine maturation, excitatory synaptic transmission plasticity, through morphological studies and using electrophysiology paradigms for neuronal plasticity. Different paradigms and different techniques can be applied for the study of plasticity at different levels, from extracellular field recordings and LTP paradigms at the neuronal population level, to glutamate uncaging at the single spine level, using two photon microscopy. This last technology shall allow us to study individual synaptic responses to glutamatergic stimulation, and the dynamics between neighboring spines in normal and pathological conditions.
KCC2 can also be genetically targeted with a reporter and in this way facilitate the understanding of how changes in KCC2 expression or phosphorylation can impact on spine morphology and function.

3. In order to better characterize the impact of KCC2 in neuronal function, a pharmacological approach to target Cation Chloride Cotransporters is proposed in order to determine their impact on RTT phenotype. **Phenotypical rescue of various RTT features with this pharmacological approach will be addressed in both iPSC-derived neurons (intracellular chloride levels, spine number and morphology), and in *Mecp2*-deficient mouse models.** Bumetanide is a drug that blocks the activity of NKCC1 resulting in decreased intracellular chloride concentration, reinforcing GABAergic inhibition. It has been demonstrated to improve neonatal seizures (Dzhala et al., 2008). CLP257 is a drug that restores impaired chloride transport in neurons by increasing KCC2 activity (Gagnon et al., 2013). To restore chloride homeostasis in RTT neurons, systemic administration of the NKCC1 antagonist bumetanide (or analogs) and KCC2 activity enhancer CLP257 will be performed in both human iPSCC-derived neurons and in the mouse model. These strategies can be applied to neurons derived from another iPSC line, already available, from a patient with mutations in the KCC2 gene- *SLC12A5*, who showed severe epileptic encephalopathy (unpublished data). This approach might offer a promising model to pharmacologically target KCC2 dysfunction.

4. There is evidence that upregulation of KCC2 expression is induced by BDNF (Aguado et al., 2003; Carmona et al., 2006). However, depending on the stage of neuronal maturation, BDNF can either up-regulate or down-regulate KCC2 expression (Rivera et al., 2004). Also, there is some evidence that BDNF plays a role in the observed KCC2 upregulation after a seizure (Shulga et al., 2008). I therefore propose to test whether boosting BDNF effects through A2ARs activation could have an impact in KCC2 expression and function.

IGF1, a compound undergoing clinical trials for RTT, has similar effects to BDNF but crosses the blood brain barrier. This compound, or other BDNF mimetics, can thus be tested in our RTT and *SLC12A5* mutated models to reestablish KCC2 actions.

5. In order to characterize GABAergic signaling *in vivo* in different brain areas, brain imaging techniques can be applied to study the RTT animal model and also human patients with RTT. Due to the scarcity of postmortem materials, imaging techniques are a relevant tool to characterize brain synaptic changes in disease. If we can perform the same studies in human patients and in the animal model, potentially relevant information can be obtained about how we can interpret data from animal model and if it translates into similar results in patients.

To study and monitor GABA levels, we can apply GABA spectroscopy. Magnetic resonance spectroscopy (MRS) is a powerful methodology that allows the direct detection of endogenous metabolites in the human body non-invasively *in vivo* (Puts and Edden, 2012). J-edited PRESS-based methods, such as MEGA-PRESS, have been applied in a number of different brain regions – occipital, parietal and frontal regions (including the anterior cingulate). It is probably fair to say that edited methods in general, and the MEGA-PRESS method specifically, are currently the most widely used methods to quantify GABA at 3T (Puts and Edden, 2012). Using MRS to measure GABA levels in patients with neurofibromatosis type 1, Violante et al (Violante et al., 2013) demonstrated reduced GABA in the visual cortex. It is also possible to couple magnetic resonance GABA spectroscopy

measurement with functional magnetic resonance, to assess the functional correlates of the magnetic resonance spectroscopy signal.

Positron Emission Tomography (PET) uses trace amounts of short-lived radioactive material to map functional processes in the brain. When the material undergoes radioactive decay a positron is emitted, which can be picked up by the detector. Areas of high radioactivity are associated with brain activity. [¯11C]Flumazenil, a highly selective benzodiazepine antagonist is the most extensively used GABAAR ligand for PET. This technique was used to demonstrate a reduced binding of GABA to its receptor in patients with succinic semialdehyde dehydrogenase deficiency, an autosomal recessive disorder of GABA metabolism clinically characterized by intellectual impairment, hypotonia, hyporeflexia, hallucinations, autistic behaviors, and seizures (Pearl et al., 2009). It was also used to study patients with epilepsy, Angelman Syndrome and Prader-Willi Syndrome (Sundaram et al., 2005). We aim to perform similar studies in RTT patients and to compare the results with autoradiographic labeling in the murine model.

In conclusion, the present thesis work allowed the establishment of several fruitful collaborations and I shall pursue the aim of better understanding GABAergic system maturation in neurodevelopmental disorders. Current medications that systemically potentiate GABA-mediated inhibition can be effective at suppressing seizures, but prolonged drug treatment can have unwanted cognitive or neurobehavioral side effects, particularly during the maturation of the CNS. The challenge is to find harmless ways to modulate GABA actions adequate to each developmental stage.

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ORIGINAL ARTICLE

Analysis of synaptic proteins in the cerebrospinal fluid as a new tool in the study of inborn errors of neurotransmission

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Abstract In a few rare diseases, specialised studies in cerebrospinal fluid (CSF) are required to identify the underlying metabolic disorder. We aimed to explore the possibility of detecting key synaptic proteins in the CSF, in particular dopaminergic and gabaergic, as new procedures that could be useful for both pathophysiological and diagnostic purposes in investigation of inherited disorders of neurotransmission. Dopamine receptor type 2 (D2R), dopamine transporter (DAT) and vesicular monoamine transporter type 2 (VMAT2) were analysed in CSF samples from 30 healthy controls (11 days to 17 years) by western blot analysis. Because VMAT2 was the only protein with intracellular localisation, and in order to compare results, GABA vesicular transporter, which is another intracellular protein, was also studied. Spearman's correlation and Student's *t* tests were applied to compare optical density signals between different proteins. All these synaptic proteins could be easily detected and quantified in the CSF. DAT, D2R and GABA VT expression decrease with age, particularly in the first months of life, reflecting the expected intense synaptic activity and neuronal circuitry formation. A statistically significant relationship was found between D2R and DAT expression, reinforcing the previous evidence of DAT regulation by D2R. To our knowledge, there are no previous studies on human CSF reporting a reliable analysis of these proteins. These kinds of studies could help elucidate new causes of disturbed dopaminergic and gabaergic transmission as well as understanding different responses to L-dopa in inherited disorders affecting dopamine metabolism. Moreover, this approach to synaptic activity in vivo can be extended to different groups of proteins and diseases.

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Introduction

Specialised investigations in cerebrospinal fluid can be crucial to identify some inborn errors of metabolism. Defects of biogenic amines (dopamine and serotonine), GABA and glycine make up the group of diseases that are currently considered to be inborn errors of neurotransmitters. In these disorders, quantification of some particular CSF metabolites (biogenic amines, GABA or glycine) is necessary in the diagnostic process. However, although growing evidence suggests that genetic or pathologic alterations of

proteins involved in synaptic transmission may underlie a number of neurological and psychiatric disorders (Südhof and Malenka 2008; Südhof 2008; Witzmann et al. 2005; Corradini et al. 2009; Kauer and Malenka 2007), the study of these proteins is not normally included in the array of investigations carried out in disorders of this kind. In vivo measurement of synaptic activity is difficult to carry out in humans. CSF samples are easily accessible by standard lumbar puncture techniques. Additionally, biochemical changes in CSF may reflect pathological alteration of CNS physiology, as previously demonstrated (Ormazabal et al. 2005). Accordingly, CSF receives a wide variety of molecules released by different CNS cell populations (Thouvenot et al. 2008). Several authors, including some from our group, have shown that there is a strong variability of CSF biogenic amines with age in children not suffering neurologic disease (Ormazabal et al. 2005), which suggests that the same variability may be expected when analysing synaptic proteins related to classical neurotransmitter function.

Synaptic transmission depends on neurotransmitter pools stored within vesicles that undergo regulated exocytosis. In the case of dopaminergic transmission, the vesicular monoamine transporter-2 (VMAT2) is responsible for the loading of dopamine (DA) and other monoamines into synaptic vesicles (Cartier et al. 2010). The DA transporter (DAT) carries DA across the plasmalemmal membrane from the synaptic cleft into the cytoplasm (Volz et al. 2009). The central actions of DA are mediated by five distinct receptors that belong to the G-protein receptor family. They are classified as D1-like (D1 and D5) and D2-like (D2, D3 and D4), and their interaction with dopamine translates into activation/inhibition of specific neurons and circuitries (De Mei et al. 2009). Those suffering D2 receptor (D2 R) genetic mutations exhibit postural abnormalities, bradykinesia with delayed initiation of movements, impaired coordination and prolonged periods of immobility (Glickstein and Schmauss 2001). These symptoms are also characteristic of patients suffering from dopaminergic neurotransmission disorders.

In spite of the critical role of these proteins in dopaminergic transmission (Fig. 1), information about procedures for studying them in CSF is very scarce, to our knowledge. However, such knowledge would be useful to increase understanding of the mechanisms of disturbed dopaminergic transmission in primary (inherited disorders of dopamine) and secondary deficiencies.

We aimed to analyse, in the CSF of a control population, the synaptic proteins VMAT2, DAT and D2 R. Because VMAT2 was the only protein with intracellular localisation, GABA vesicular transporter (GABA VT), which is another intracellular protein, was also included in the study. We evaluated the correlation of these proteins with each other and with the age of controls. Additionally, because total CSF protein concentration is known to decrease with age, we introduced this variable in the age correlation studies.

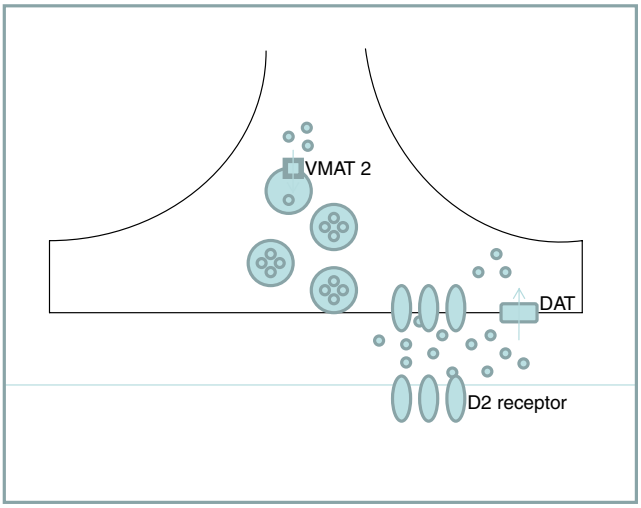

Fig. 1 Schematic representation of the dopaminergic synapse, illustrating the link between *VMAT2* (vesicular monoamine transporter 2), *DAT* (dopamine transporter) and D2 R (*D2 receptor*)

Methods

CSF samples

The study was performed in 30 subjects (age range: 11 days–17 years; average: 1.8 years) whose CSF samples were submitted to Hospital San Joan de Déu laboratory for analysis under suspicion of viral or bacterial meningitis or encephalitis. Lumbar puncture was performed in the emergency room, and the first ten drops were used for routine cytochemical/microbiological studies. Inflammatory markers (C-reactive protein and procalcitonin) were also analysed. Exclusion criteria were diagnosis of viral or bacterial meningitis, a chronic neurological condition, and hematic or xanthochromic CSF (blood contamination). Samples from patients were obtained in accordance with the Helsinki Declaration of 1964, as revised in 2000. The ethical committee of the Hospital Sant Joan de Déu approved the study. After collection, samples were stored at −80°C up to the moment of the analysis.

Western blot

Western blot analysis was performed for each protein (DAT, D2 R, VMAT2, GABA VT). A sample of 20 μL of CSF was loaded into the gel and proteins were separated on 10% sodium dodecyl sulphate-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham™ Hybond™–ECL; GE Healthcare). The mem-

branes were incubated in TBST buffer (0.02 M Tris-base, pH7.6, 0.8% NaCl, 0.1% Tween 20) supplemented with 5% dried skimmed milk for 60 min to block non-specific binding. Anti-DAT extracellular loop (1:1,000; Sigma®), anti-D2 R (1:1,000; Millipore®), anti-VMAT 2 (1:1,000; Santa Cruz Biotechnology®), and anti-GABA VT (1:500; Millipore®) antibodies were added, and the preparations were incubated at 4°C overnight. The membranes were washed three times with TBST buffer and then incubated with appropriate anti-rabbit (1:3,000; Promega®) or anti-mouse (1:5,000; Promega®) IgG secondary antibodies at room temperature for 1 h. The blot was then washed six times with TBST and prepared with ECL (Pierce® ECL Western Blotting Substract; Thermo Scientific) for developing. Relative levels of each protein were quantified by measuring optical densities (OD) of the corresponding bands with Quantity One® V 4.3.1. software.

CSF total protein concentration, procalcitonin and C-reactive protein determination

CSF total protein and C-Reactive Protein concentrations were measured by standard automated procedures in an Architect ci8200 analyser (Abbott, USA). Procalcitonin was measured by fluorimetric detection, with Brahams-Kryptor-Atom® analyser.

Statistical analysis

Statistical analysis was performed with the SPSS 17.0 program. Spearman's correlation test was applied to search for associations among the OD signals of the different proteins and with the age of controls. Multivariate linear regression analysis was used to adjust the results by total CSF protein concentration. Student's *t* test was applied to compare OD signals of the different proteins according to gender. Statistical significance was considered as $p<0.05$.

Results

DAT, D2R, VMAT2 and GABA vesicular transporter were clearly detectable at different ages using conventional western blot analysis in all the CSF samples studied (Fig. 2).

VMAT2 expression measured as OD showed a statistically significant negative correlation with age (Table 1.). Correlation between DAT expression and age showed a strong tendency to significance (p value=0.05). D2R expression and GABA vesicular transporter expression did not show statistical association with age when all the CSF samples were included in the statistical study. However, since our sample population was predominantly younger than 200 days (for age distribution, see Fig. 3), we searched for a correlation between D2R and GABA VT expression with age in this group. A significant negative correlation was found for D2R and GABA VT. In order to rule out interference of the expected decrease in total CSF protein concentration with age, we adjusted these results by total CSF protein concentration (range, 7–77 g/l; mean, 34.7; median, 31; standard deviation, 17.2. All values were within normal limits according to different age ranges (Biou et al. 2000)). Except for VMAT2, the relation between age and expression of these synaptic proteins remained statistically significant (Table 1).

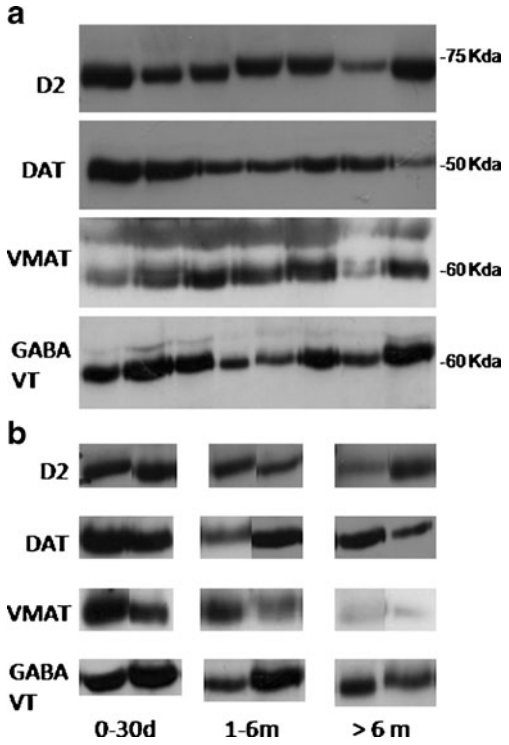

Fig. 2 D2, DAT, VMAT and GABAVT detection in the CSF by western blot in different control samples (**a**), and a representation of them according to age ranges (**b**)

No differences were observed when we compared DAT, D2, VMAT2 and GABA VT protein expression according to gender.

Concerning association studies among the different proteins, OD signal of CSF, D2R, and DAT showed a statistically significant positive correlation (Spearman's rho correlation test $r=0.016$; $p=0.443$), while no correlation was found between D2 R and VMAT, or between DAT and VMAT.

Because VMAT2 is also involved in adrenaline and noradrenaline transport, and its release can be triggered by acutely stressful situations, we also tried to correlate VMAT2 expression with acute phase reactants (C-reactive protein and procalcitonin), in order to rule out the possible influence of fever or acute disease in the expression of this protein, since our control population was selected from a group of children under suspicion of an active infectious process. C-reactive protein and procalcitonin levels were

Table 1 Statistical data of the results for the synaptic proteins studied

	D2 R	DAT	VMAT2	GABA VT
Mean (OD measure)	580,755.5	825,805.8	654,848.24	945,490.3
Standard deviation (OD measure)	302,541.9	598,210.0	385,135.9	356,870.5
Upper Limit (OD measure)	112,6336	211,5196	50,255	1,582,609
Lower Limit (OD measure)	99,224.4	87,053.87	1,574,680	395,287.83
Spearman's Rho correlation test between age and OD measure	$r=-0.591$; $p=0.01$ (if age under 200 days)	$r=-0.329$; $p=0.05$ $r=-0.482$; $p=0.015$ (if age under 200 days)	$r=-0.532$; $p=0.002$	$r=-0.681$; $p=0.002$ (if age under 200 days)
Coefficients of a multivariate linear regression model adjusted by total protein concentration	$\beta=-6139.738$; $p=0.007$ (if age under 200 days)	$\beta=16.294$; $p=0.834$ $\beta=-8{,}119.042$; $p=0.016$ (if age under 200 days)	$\beta=-28.736$; $p=0.358$	$\beta=-5{,}886.624$; $p=0.001$ (if age under 200 days)

Optic densities are expressed in arbitrary units

D2R D2 receptor, *DAT* dopamine transporter, *VMAT2* vesicular monoamine transporter 2, *GABA VT* GABA vesicular transporter, *OD* optic density

not related to total CSF protein concentration, nor with VMAT 2 expression.

Discussion

We aimed to explore the possibility of detecting key dopaminergic and gabaergic synaptic proteins in a control population of children. To our knowledge, this is the first report of detection of these proteins in CSF in a paediatric population.

In spite of their low abundance compared to global CSF proteome (Thouvenot et al. 2008), the intensity and definition of the different bands obtained with the present procedure (Fig. 1) lends strong support to the applicability of this analysis in neurochemical research.

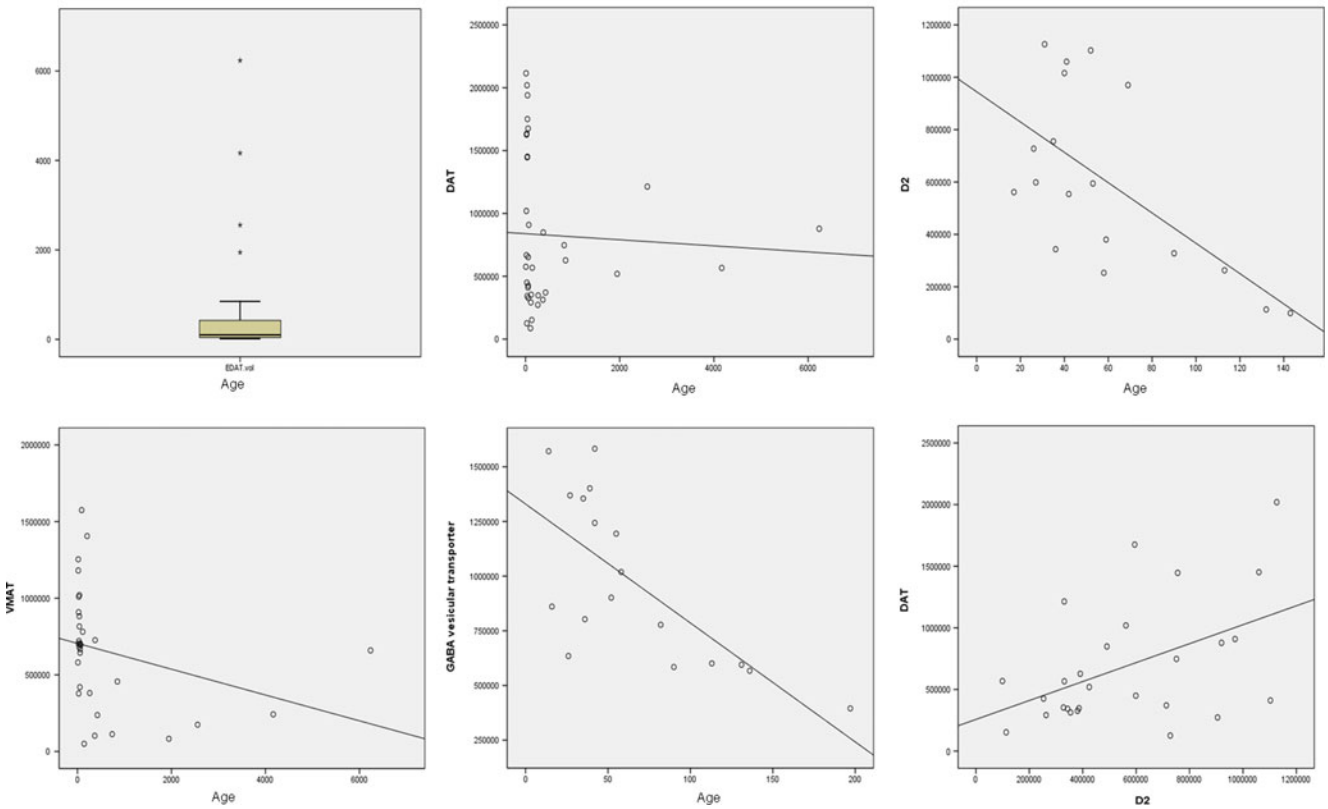

Fig. 3 *Upper left* Population age distribution. *Upper middle, upper right, lower left, lower middle* Correlation between age and synaptic proteins. *Lower right* Correlation between DAT and D2

The presence of transmembrane synaptic proteins in CSF can be explained in several ways. It has been suggested that membrane protein detection is the consequence of extensive protease actions that cleave the fragments from proteins that are embedded in membranes, and that these fragments enter the CSF (Egaña et al. 2009; Zougman et al 2008). However, our study demonstrates that these proteins are detected at the expected molecular weight, indicating that there is no fragmentation, or at least that they are intact in part. Harrington et al. (2009) identified the presence of CSF membranous nanostructures, thought to play a physiologically active role, and not merely the result of blebbing, apocrine secretion, or apoptosis events, or cellular debris. These structures can provide an appropriate environment for transmembrane proteins which are hydrophobic in nature. Their morphology is similar to that of synaptic vesicles and exosomes; their structure resembles that of nanotubules, cell-to-cell interactions that facilitate the selective transfer of membrane vesicles and organelles but which seem to impede the flow of small molecules (Rustom et al. 2004). In the first months of life, a period of intense synaptogenesis and neuronal circuitry formation, cell-to-cell exchange of synaptic machinery is thought to be carried out by small dense core vesicles (~80 nm) (Sorra et al. 2006). However, extracellular localisation of these structures is not well documented.

Based on our previous experience with the quantification of biogenic amines in a population of children without neurological disease (Ormazabal et al. 2005) who exhibited a decrease from the first days of life until reaching adult age, we aimed to learn whether the same tendency was observed with the synaptic proteins included in the study. For DAT, D2R and GABA VT, it was possible to establish a statistically significant relationship, particularly in the group of children under 6 months of age from whom most of our sample was drawn. Age-related changes in DAT functionally active protein have been described in rats (Volz et al. 2009), showing a decrease between young adolescent rat levels and adult levels. Interestingly, in the same report, no difference between VMAT 2 immunoreactivity in the two groups was found, although conflicting data had previously been published (Volz et al. 2006). Furthermore, because VMAT2 is involved in fast neurotransmission, its regulation could appear to be related to rapid communication needs and not to age. Another result that deserves discussion is the relationship discovered between D2 and DAT expression. Numerous studies have supported the notion that DAT is subjected to dynamic regulation in the plasma membrane, and this regulation has been extensively reviewed by Eriksen et al. (2010). There is strong evidence that D2 R causes an increase in dopamine uptake through an increase in DAT surface expression. Additionally, loss of D2R/DAT co-immunoprecipitation has been described in schizophrenia, suggesting a role for the loss of the interaction in the disease process (Bolan et al. 2007).

Currently, investigations are underway of CSF in relation to disorders of suspected neurometaolic origin; these include examination of cytochemistry (cells, glucose, proteins), lactate, amino acids, biogenic amine metabolites, pterins and 5-methyltetrahydrofolate. However, only a few disorders can be detected by means of these analyses (García-Cazorla et al. 2010). Furthermore, the diagnosis of monogenic defects of dopaminergic neurotransmission is almost exclusively based on the quantitative determination of their metabolites in CSF (Marín-Valencia et al. 2008). Secondary deficiencies of dopamine are found in about 10% of patients who undergo a lumbar puncture in the diagnostic work-up of a suspected neurometabolic disorder (García-Cazorla et al. 2007; Van Der Heyden et al. 2003). Disorders such as mitochondrial diseases (García-Cazorla et al. 2008) and Lesch-Nyhan disease (Serrano et al. 2008) are amongst the main known causes of secondary deficiencies. The analysis of these and other synaptic proteins is expected to be useful to both increased understanding of the mechanisms of disturbed neurotransmission and identification of new causes of dopaminergic defects. Concerning primary deficiencies such as tyrosine hydroxylase deficiency, this approach could be useful to improving the understanding of the variable response that patients have to L-dopa therapy (from normalisation to almost absent effect) (Willemsen et al. 2010), but we need further data to document this hypothesis.

To conclude, we report the detection of transmembrane synaptic proteins in the CSF of a population of children without neurological disorders. This approach was made as a means of monitoring neurotransmitter genetic disease outcome and response to treatment, but its applications can be extended to the investigation of a growing group of neurological and psychiatric disorders related to neurotransmission. The proteins included in this study were chosen because of their relevance for dopaminergic and gabaergic transmission, but there are many other proteins of interest that can be selected for analysis with this methodology.

The findings reported here deserve further investigation, especially in the field of CSF-neuron and inter-cellular interactions, in order to better understanding of their physiological implications in the developing brain and their pathophysiological role in disease states. Significant correlation with age was found with D2R, DAT and GABA VT expression, as expected from the previously reported decrease of biogenic amines in a paediatric population. Coordinated regulation of DAT by stimulation of D2 R is further reinforced by our results.

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Abnormal Expression of Cerebrospinal Fluid Cation Chloride Cotransporters in Patients with Rett Syndrome

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Abstract

Objective: Rett Syndrome is a progressive neurodevelopmental disorder caused mainly by mutations in the gene encoding methyl-CpG-binding protein 2. The relevance of MeCP2 for GABAergic function was previously documented in animal models. In these models, animals show deficits in brain-derived neurotrophic factor, which is thought to contribute to the pathogenesis of this disease. Neuronal Cation Chloride Cotransporters (CCCs) play a key role in GABAergic neuronal maturation, and brain-derived neurotrophic factor is implicated in the regulation of CCCs expression during development. Our aim was to analyse the expression of two relevant CCCs, NKCC1 and KCC2, in the cerebrospinal fluid of Rett syndrome patients and compare it with a normal control group.

Methods: The presence of bumetanide sensitive NKCC1 and KCC2 was analysed in cerebrospinal fluid samples from a control pediatric population (1 day to 14 years of life) and from Rett syndrome patients (2 to 19 years of life), by immunoblot analysis.

Results: Both proteins were detected in the cerebrospinal fluid and their levels are higher in the early postnatal period. However, Rett syndrome patients showed significantly reduced levels of KCC2 and KCC2/NKCC1 ratio when compared to the control group.

Conclusions: Reduced KCC2/NKCC1 ratio in the cerebrospinal fluid of Rett Syndrome patients suggests a disturbed process of GABAergic neuronal maturation and open up a new therapeutic perspective.

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Introduction

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder with an incidence of 1:10000 live female births and is one of the leading causes of mental retardation and autistic behavior in females [1]. Loss-of-function mutations in the gene encoding methyl-CpG binding protein 2 (MeCP2) cause most cases of RTT. Individuals affected with RTT experience normal development up to the age of 6–18 months, at which time they fail to acquire new skills and enter a period of motor regression [2]. Autistic features are a hallmark of this disorder and epilepsy is frequent [3]. RTT patient brain does not show obvious signs of neurodegeneration, atrophy, gliosis, demyelination, or neuronal migration defects [4,5], suggesting that neurological symptoms may primarily stem from subtle defects of subcellular compartments such as dendrites, axons, or synaptic structures [6]. MeCP2 is a transcriptional regulatory protein, and in its absence, a large number of genes exhibit abnormal expression with implications in the balance between synaptic excitation and inhibition [7,8]. MeCP2 might be particularly important to GABAergic function and there is evidence that the expression of MeCP2 is approximatelly 50% higher in GABAergic neurons when compared to non GABAergic neurons. Mice with conditional deletion of *Mecp2* in GABAergic neurons initially show normal behavior but in the course of development start displaying forepaw stereotyped movements, compulsive grooming, impaired motor coordination, learning/memory deficits, abnormal EEG hyperex-

citability, severe respiratory dysrhythmias and premature lethality [8].

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the adult brain. During early development, activation of the chloride- permeable, postsynaptic, GABAA receptors (GABAA-R) can induce depolarization and the basal intracellular chloride concentration is determinant for the action of GABA in the developing neurons [9]. Two major contributors to intracellular chloride concentration are NKCC1 (Na^+, K^+, $2Cl^-$ cotransporter, that accumulates chloride in the cell), and KCC2 (K^+, Cl^- cotransporter, that extrudes chloride). Several lines of research correlate epileptogenesis with altered function of NKCC1 and KCC2 [10,11]. *In vitro*, experiments suggest that bumetanide, a potent NKCC1 inhibitor, can increase GABAergic inhibition, in combination with phenobarbital [12]. Bumetanide has also been reported useful in a neonatal patient with seizures [13] and in autistic children [14].

Moreover, the brain of MeCP2 deficient animal models shows deficits in brain-derived neurotrophic factor [15] (BDNF), which is thought to contribute to the pathogenesis of RTT. BDNF can also promote the functional maturation of GABAA-R mediated responses by inducing upregulation of KCC2 [16,17,18].

Human age related changes in GABAA-R physiology remain controversial, although neuropathological studies have already identified postnatal developmental changes of NKCC1 and KCC2 cortical expression [18]. The detection of synaptic proteins in the cerebrospinal fluid (CSF) gives us the possibility to indirectly access synaptic composition and alterations, using the CSF of patients with disorders related to neurotransmission, with the advantage of performing these studies *in vivo* [19].

We hypothesize that changes in BDNF expression levels or the direct effect of the underlying genetic mutation can interfere with the normal expression of NKCC1 and KCC2 leading to a reduction in the KCC2/NKCC1 ratio, characteristic of the immature GABAergic system. A comparison of NKCC1 and KCC2 protein levels in the CSF of patients affected with RTT and a control population was made in order to address this question.

Patients and Methods

Patients and Controls

Sixteen patients with RTT were recruited to this study, aged between 2 to 19 years at the moment of CSF collection. Patients' clinical characteristics are summarized in table 1. Patients without a documented mutation fulfilled clinical criteria for RTT according to the last updated revision [20]. The control study was performed in 67 subjects (age range: 1 day - 14 years; mean: 740 days; female: 27; male: 40) whose CSF samples were submitted to Hospital San Joan de Deu (HSJD) laboratory under suspicion of viral or bacterial meningitis or encephalitis. Exclusion criteria were: diagnosis of viral or bacterial meningitis, neurologic disease, and hematic or xantocromic CSF (blood contamination).

CSF Samples

CSF samples were collected by lumbar puncture as previously described [21]. They were obtained after parent's written informed consent and in accordance with the Helsinki Declaration of 1964, as revised in 2000. The ethical committee of HSJD approved the study. After lumbar puncture, the first ten drops were used for routine cytochemical/microbiological studies and then CSF was immediately stored in 4 aliquots at $-80^{\circ}C$ until the moment of analysis. Biogenic amines metabolites and synaptic proteins were studied using the following 20 drops.

NKCC1 and KCC2 expression levels were analyzed by western blot. Twenty μL of CSF were loaded on gel and proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (AmershamTM HybondTM –ECL, GE Healthcare). Membranes were blocked in TBST buffer (0.02 M Tris-base, pH7.6, 0.8% NaCl, 0.1% Tween 20) with 5% dry skimmed milk for 60 min at room temperature. Anti-NKCC1 (1:500; Santa Cruz Biotechnology®) and anti-KCC2 (1:500; Millipore®) antibodies were added and incubated at 4 °C overnight. Membranes were washed three times with TBST buffer followed by incubation with appropriate anti-rabbit (1:3000, Promega®) IgG secondary antibody at room temperature for 1 h. The blot was then washed six times with TBST and signal was revealed with ECL (Pierce® ECL Western Blotting Substract, Thermo Scientific). Relative levels of each protein were quantified by measuring optical densities (OD) of the corresponding bands with Quantity One® V 4.3.1.software.

Statistical Analysis

Statistical analysis was performed using IBM Statistical Package for the Social Sciences (IBM SPSS Statistics Version 19.0, SPSS Inc: Chicago, IL). A significance level of.05 was used in all analyses. Outlier analysis was done taking into account the primary variable in the study – healthy/RTT. Outliers (defined as values 1.5 times lower than the 1st quartile or 1.5 times higher than the 3rd quartile) [22] were found in 8 cases (1 Rett patient, 7 healthy controls) for KCC2/NKCC1 ratio, 4 cases for NKCC1 (1 Rett patient, 3 healthy controls) and in 1 case (healthy control) for KCC2. Outliers were excluded from the respective analyses.

Non-parametric tests were applied when possible for age, NKCC1 and KCC2, since the assumption of normal distribution was not fulfilled for these variables.

Results

Total CSF protein concentration values ($M = 33.27$, $SD = 19.04$, range: 7–73 g/l) were within normal limits according to different age ranges [23]. Clinical and genetic features of RTT patients are described in table 1. NKCC1 and KCC2 western blot analysis were performed on the CSF of controls and RTT patients. CCCs were detected in the CSF of this population, at the expected molecular weight (Figure 1A). Considering the reported sexually dimorphic expression of KCC2 and GABA function in the substantia nigra [24] it was decided to control for gender in all reported analysis.

Furthermore, the homogenous distribution of the demographical variable age was verified with Mann-Whitney Test (U). Controls ($M = 746.00$ days, $SD = 1075.89$) were significantly ($U = 932.00$, $p < .001$) younger than RTT patients ($M = 3444.69$ days, $SD = 2173.59$) therefore we have controlled for this variable in all reported analysis (table S1).

KCC2 Expression is Decreased in the CSF of Rett Syndrome Patients

As patients grew older their OD signal of KCC2 in the CSF decreased. Partial correlation analysis was performed to clarify the relationship between age and the OD signal of the different synaptic proteins (NKCC1, KCC2) in each group (RTT, Controls), controlling for the effect of gender. A negative correlation between age and the OD signal of KCC2 ($r(74) = -.292$, $n = 80$, $p < .05$) has been clearly identified in our data, i.e. as the participants grew older their OD signal of KCC2 decreased, even when controlling for the effect of gender. Meaning that, KCC2 levels decrease in CSF throughout aging. However,

Table 1. Clinical and laboratory features of Rett Syndrome patients included in the study.

	Age (Years at CSF collection)	Genetic screening	Epilepsy	Medication (when LP was performed)	Respiratory anomalies	KCC2/NKCC1 (optical densities)
1	2	MECP2 screened, no alteration found	No	NO AED	No	36795/310023
2	2	P.Y141X	Refractory Epilepsy Generalized seizures	NO AED	No	0/242968
3	2	P.R270X	Refractory epilepsy	No AED	Severe syperventilation bursts and apneas	0/323889
4	4	P302H, 905C>T	Generalized seizures from 2 years of life	No AED	Hyperventilation bursts	167071/228307
5	5	P.R306C	Epileptic status Generalized seizures from 4 years of life	VPA	Hyperventilation bursts and apneas	0/169395
6	6	MECP2, CDKL5 screened, no alteration found	Generalized seizures since 8 years of life	CBZ	Hyperventilation bursts	177102/257110
7	7	MECP2, CDKL5 screened, no alteration found	Reflex seizures Abcences Atonic seizures	No AED	No	28646/296735
8	8	P.R255X	Generalized seizures from 2 years of life	VPA	Hyperventilation bursts and apneas	15584/342592
9	9	MECP2, CDKL5 screened, no alteration found	Refractory epilepsy	VPA TPM	Severe Hyperventilation and apneas	0/54010
10	10	P.R306C	Absences and partial seizures from 8 years	CBZ	Hyperventilation bursts	155391/621440
11	11	DEL EX.1–2	Generalized seizures from 8 years	No AED	Hyperventilation	91959/527745
12	16	MECP2, CDKL5 screened, no alteration found	Generalized seizures from 11 years of life	No AED (VPA was withdrawn 2 years before LP)	Hyperventilation	156883/445649
13	16	MECP2, CDKL5screened, no alteration found. Polymorfism in NTNG1	Generalized and absence seizures from 14 years of life	VPA CBZ	Hyperventilation bursts	164531/259927
14	16	P.R294X	Generalized Seizures from 6 years of life	CBZ LEV	Hyperventilation bursts	219795/281395
15	18	MECP2, CDKL5 screened, no alteration found	Partial seizures	CBZ	Hyperventilation	7000/535292
16	19	P.R294X	Partial, secondarily generalized and absences	CBZ	Hyperventilation bursts and apneas	604155/28867

DEL: Deletion. LP: Lumbar Puncture. AED: Anti epileptic drugs. VPA: Valproic Acid. CBZ: Carbamazepine. LEV: Levetiracetam. TPM: Topiramate.
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concerning the OD signal of NKCC1 this correlation did not reach statistical significance ($r\,(74) = -.207$, $p > .05$). Interestingly, if the effect of gender was not taken into consideration, a strong, negative correlation between age and the OD signal of KCC2 ($r_s\,(80) = -.509$, $p < .0001$) and NKCC1 ($r_s\,(78) = -.472$, $p < .0001$) would have been identified in our data, i.e. as the participants grew older their OD signals of KCC2 and NKCC1 decrease.

Rett Patients Present a Significantly Lower OD Signal of KCC2 than Healthy Controls (Figure 1B)

To determine whether there were statistically significant differences in the OD signal of the different synaptic proteins (NKCC1, KCC2) between healthy controls and RTT patients, a between-subjects MANCOVA was performed, controlling for the effects of age and gender. This analysis revealed a statistically significant difference between healthy controls and RTT patients, $F\,(1, 73) = 6.99$, $p < .01$, $\eta_p^2 = .087$ even when controlling for the possible confounding effects of age and gender. The subsequent follow-up Univariate ANCOVAs run to specify the characteristics of this finding revealed that RTT patients presented a significantly lower OD signal of KCC2 ($M = 81383.80$, $SD = 82370.99$; $F\,(1, 76) = 12.28$, $p < .001$, $\eta_p^2 = .139$) than healthy controls ($M = 692663.52$, $SD = 425753.91$). Notwithstanding, NKCC1 expression does not differ significantly between patients ($M = 307834.25$, $SD = 162592.59$) and healthy controls, ($M = 608703.27$, $SD = 416157.33$; $F\,(1, 74) = 1.87$, $p > .05$).

KCC2/NKCC1 Ratio is Decreased in the CSF of Rett Syndrome Patients

To determine whether there were significant differences in the ratio of the two synaptic proteins (KCC2/NKCC1) on the CSF of the healthy population and RTT patients a between-subjects

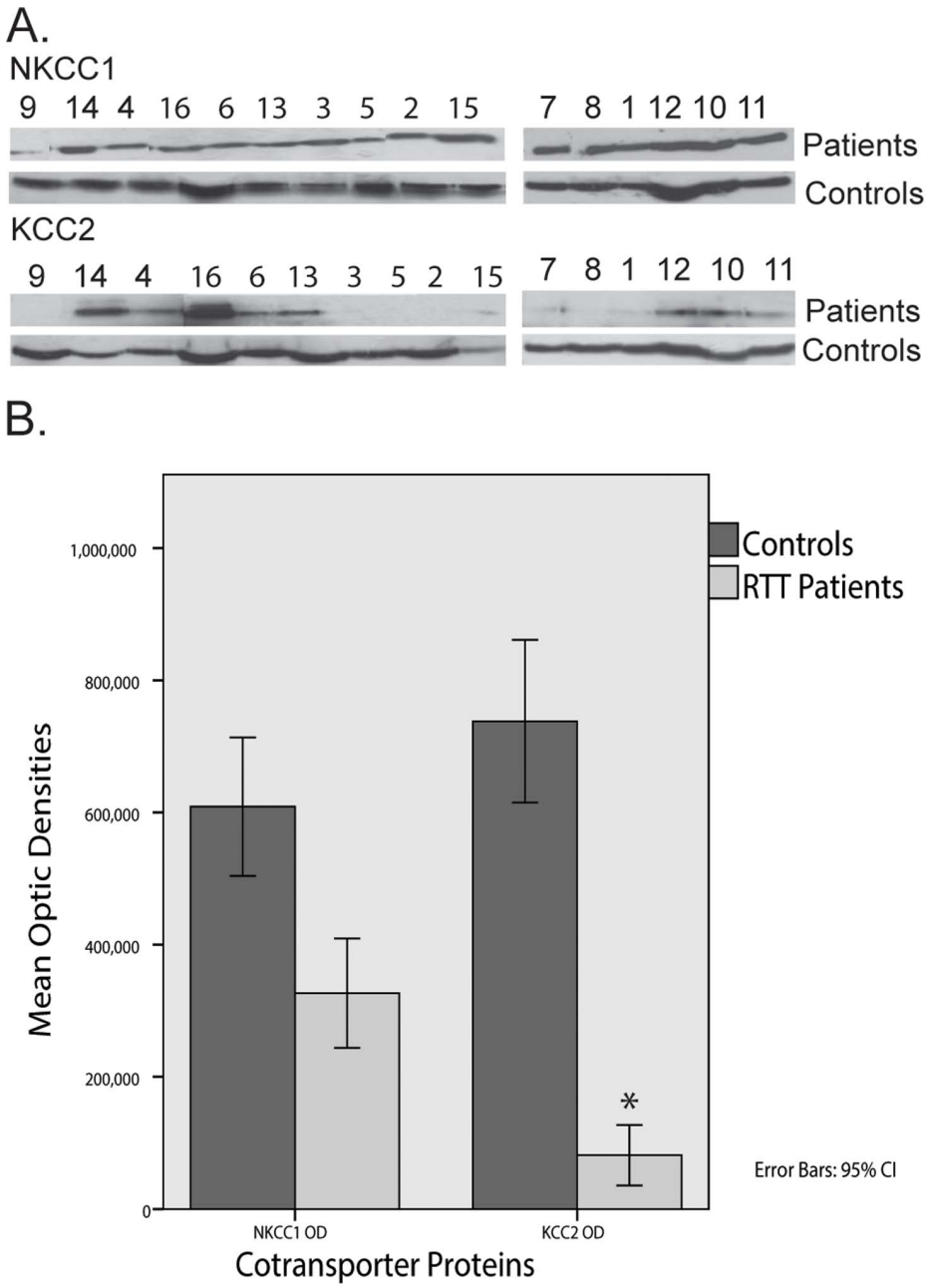
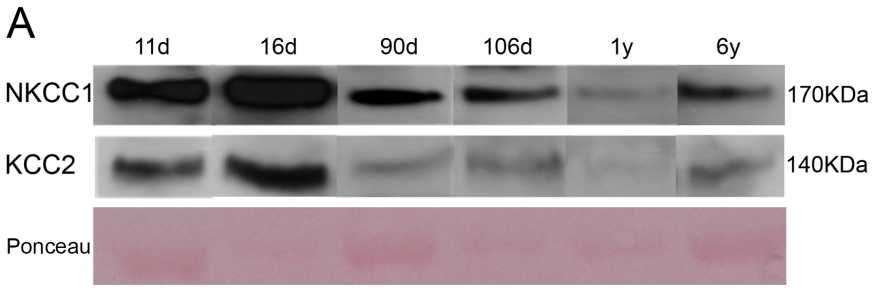


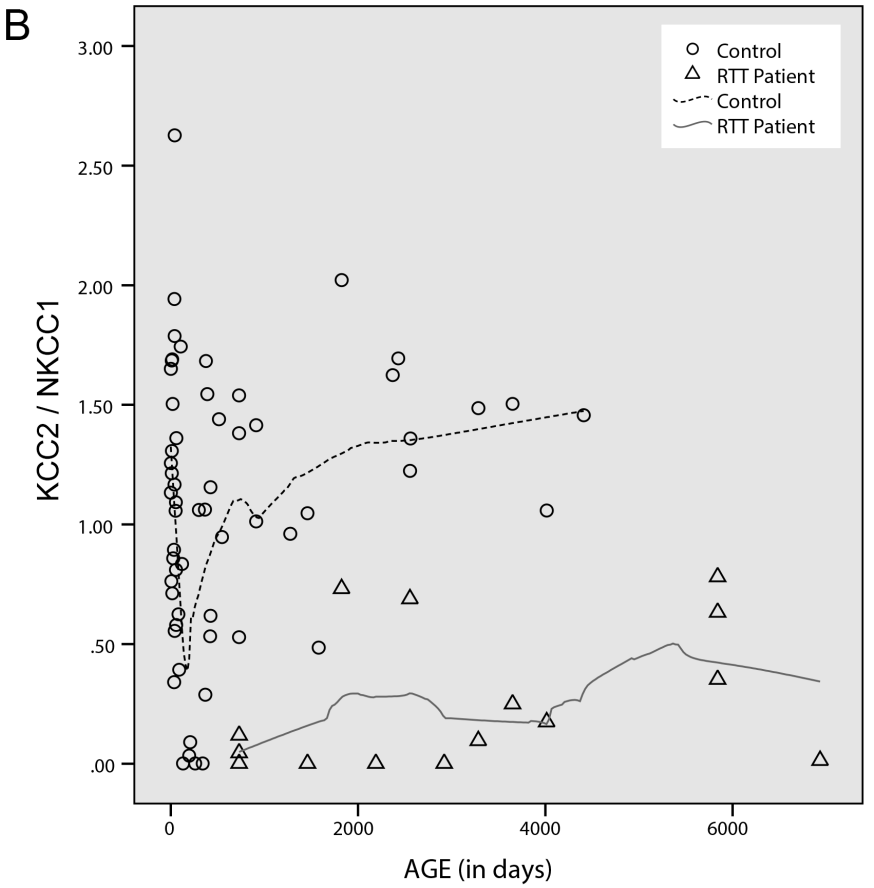
Figure 1. NKCC1 and KCC2 Cotransporters in the CSF of Rett Syndrome (RTT) Patients and Controls. (A) Immunoblot results in RTT patients and controls. Numbers refer to the patient ID numbers of Table 1. Comparison of patients and age matched controls. (B) Mean Optic Densities of NKCC1 and KCC2 Cotransporter Proteins for Rett Patients and Controls suggesting discrepant cotransporter levels between Patients and Controls supported by the respective MANCOVA (F (1, 73) = 6.99, $p<.01$, $\eta_p^2 = .087$). Error bars represent 95% Confidence Interval. *$p<.01$.
doi:10.1371/journal.pone.0068851.g001

ANCOVA was performed, controlling for the effect of age and gender. This analysis revealed a statistically significant difference between Healthy controls and RTT patients, F (1, 70) = 26.56, $p<.001$, $\eta_p^2 = .28$ even when controlling for the possible confounding effect of age and gender (figure 2B and 2C). RTT patients presented a significantly lower KCC2/NKCC1 ratio ($M = .26$, $SD = .30$) than Healthy controls ($M = 1.08$, $SD = .56$). Even if the effects of age and gender would not have been controlled for in the analysis the decrease in KCC2/NKCC1 ratio was still significant (F (1, 70) = 30.08, $p = .001$, $\eta_p^2 = .29$).

Discussion

In this study, we demonstrate reduced KCC2 levels and KCC2/NKCC1 ratio in the CSF of RTT patients. These findings suggest that altered inhibitory GABA function can underlie the pathophysiology of RTT and also play a role in the epileptogenesis of this neurodevelopmental disorder, in which epilepsy is present in around 70% of patients [3]. Detection of transmembrane synaptic proteins in the CSF is a useful tool in the study of neurotransmission disorders, as recently reported by our group [19]. Despite their low abundance compared to the global CSF


A
11d
16d
90d
106d
1y
6y
NKCC1
170KDa
KCC2
140KDa
Ponceau


B
Control
RTT Patient
Control
RTT Patient
KCC2 / NKCC1
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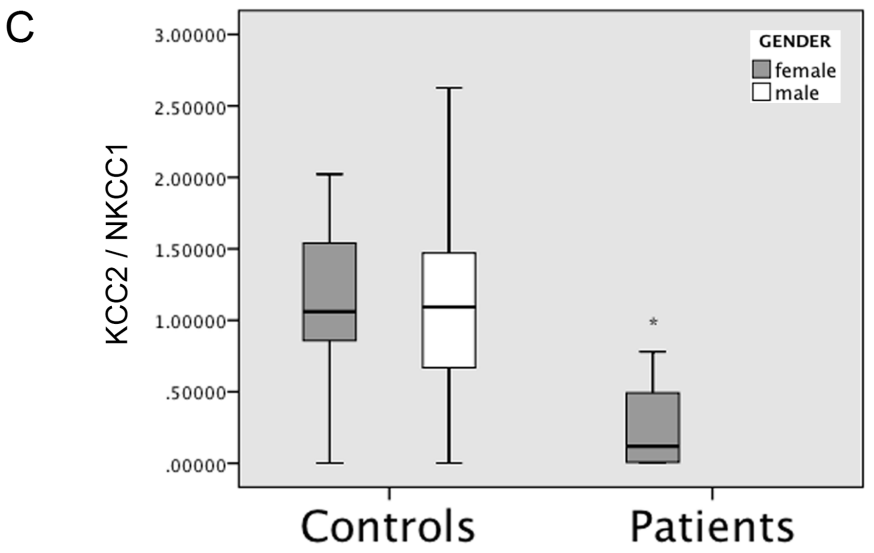
AGE (in days)


C
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female
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1.50000
1.00000
.50000
.00000
Controls
Patients

Figure 2. Cation Chloride Cotransporters ratio. (A) Immunoblot analysis of NKCC1 and KCC2. (B) Scatterplot of the relationship between Cotrasporters ratio (KCC2/NKCC1) and age for Rett Patients and Controls. Lines show a LOWESS smooth (locally-weighted polynomial regression - nonparametric smooth) suggesting a discrepant cotransporter ratio between Patients and Controls supported by the respective ANCOVA (F (1, 70) = 30.08, $p = .001$, $\eta_p^2 = .29$). (C) Boxplot of Cotransporters ratio (KCC2/NKCC1) for Rett Patients and Controls (males and females). *$p = 0.001$.
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proteome [25], the proteins here studied (NKCC1 and KCC2) were readily detected and at the expected molecular weight. Harrington and co-workers [26] identified the presence of CSF membranous nanostructures that can provide an appropriate environment for transmembrane proteins, which are hydrophobic in nature. Their morphology is similar to that of synaptic vesicles and exosomes; their structure resembles that of nanotubules, cell-to-cell interacting structures that facilitate the selective transfer of membrane vesicles and organelles but which seem to impede the flow of small molecules [27]. The intensity and resolution of the different bands obtained with the immunoblot procedure (Figures 1A and 2A) strongly supports the applicability of this analysis in neurochemical research. To our knowledge, this is the first report of detection of cation chloride cotransporters in CSF.

CSF turnover ratio and the extent of central nervous system cell death and synaptic pruning can influence circulating protein levels but these factors are likely to affect both proteins, NKCC1 and KCC2, equally. During the first months of postnatal life there is a period of intense synaptogenesis that subsequentially decreases. This is probably the cause for the reduction of protein levels in CSF observed during the first year of life. In fact, regarding transmembrane proteins like NKCC1 and KCC2, we have observed the same tendency that also was detected with other synaptic proteins [19]. The same phenomena could explain the fact that, in the CSF, both cation chloride cotransporters exhibit a reduction and KCC2 does not increase in the CSF, as was expected from previous studies in brain tissue [28,29]. Experimental limitations in humans have been an obstacle in obtaining direct evidence of age-related changes in GABAA-R physiology [28].

In mice, loss of MeCP2 leads to reduced expression of BDNF after birth [15] and in humans evidence of BDNF reduction in RTT has also been detected [29]. The effects of BDNF on neurotransmission in developing and mature neurons have been partly associated with the regulation of GABAergic transmission. Apart from its effects on GABAergic innervation [30], BDNF can also promote the functional maturation of GABAA-R mediated responses by inducing upregulation of KCC2 [16,17]. The imbalance between excitatory and inhibitory functions in RTT has been associated with reduced BDNF [31] and GABA levels, decreased expression of GABA receptor subunits [32], reduced expression of the enzymes glutamic acid decarboxylase 67 and glutamic acid decarboxylase 65 [8], reduced number of glutamatergic synapses [33] and reduced strength of basal inhibitory rhythms [34]. Moreover, exogenous BDNF has been shown to rescue synaptic dysfunction in Mecp-2 null mice. However, the mechanism by which reduced levels of BDNF contribute to disease and also to the phenotypical rescue is not completely understood [31].

Our results suggest an immature pattern of GABAergic neurotransmission in RTT patients, by revealing a dysregulation on the KCC2/NKCC1 ratio (the two major contributors to intracellular chloride concentration) and this evidence in humans is in accordance with the relevance of MeCP2 for GABAergic function described in animal models [8]. An imbalance between excitatory and inhibitory synaptic events, in the brain of children with neurodevelopmental disorders that have epilepsy and autism as key features, is a postulated general mechanism. Moreover, KCC2/NKCC1 ratio dysregulation is a particularly interesting specific molecular change, already described for diseases like tuberous sclerosis [35].

Conclusions

We describe a significant decrease of KCC2 in the cerebrospinal fluid of Rett patients. A major advantage of doing these *in vivo* studies in children with severe neurologic disorders like RTT, is that it allows to search for disturbances in the normal developmental pattern. Therefore, our findings might have implications for the understanding of RTT pathophysiology, considering that KCC2 is a neuronal specific protein with a key role for neuronal electrical function and structure, properties that are known to be altered in *Mecp2* mutated neurons. Moreover, these results could bring light to new therapeutic approaches, particularly through the pharmacological manipulation of the cation chloride cotransporters. Further studies in the MECP2 knockout model and other models to study the disease process are needed to explore these possibilities.

Supporting Information

Table S1 Socio-demographic variable: mean values (and standard deviations) of age. Significant differences among the groups were assessed with Mann-Whitney Test (U).
(DOCX)

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Author Contributions

Conceived and designed the experiments: SD AR CO MP JA RA FS AG. Performed the experiments: SD AR CO AP AG. Analyzed the data: SD MO AP AO RA MP AG. Contributed reagents/materials/analysis tools: SD AR MO AO RA MP FS JA AG. Wrote the paper: SD AR CO RA MP AG.

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Research Article

Neural commitment of human pluripotent stem cells under defined conditions recapitulates neural development and generates patient-specific neural cells

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Standardization of culture methods for human pluripotent stem cell (PSC) neural differentiation can greatly contribute to the development of novel clinical advancements through the comprehension of neurodevelopmental diseases. Here, we report an approach that reproduces neural commitment from human induced pluripotent stem cells using dual-SMAD inhibition under defined conditions in a vitronectin-based monolayer system. By employing this method it was possible to obtain neurons derived from both control and Rett syndrome patients' pluripotent cells. During differentiation mutated cells displayed alterations in the number of neuronal projections, and production of Tuj1 and MAP2-positive neurons. Although investigation of a broader number of patients would be required, these observations are in accordance with previous studies showing impaired differentiation of these cells. Consequently, our experimental methodology was proved useful not only for the generation of neural cells, but also made possible to compare neural differentiation behavior of different cell lines under defined culture conditions. This study thus expects to contribute with an optimized approach to study the neural commitment of human PSCs, and to produce patient-specific neural cells that can be used to gain a better understanding of disease mechanisms.

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Abbreviations: ESCs, embryonic stem cells; **iPSCs,** induced pluripotent stem cells; **KO,** knock Out ; **LDN,** LDN-193189; **MECP2,** methyl CpG binding protein 2; **NPs,** neural precursors; **PSCs,** pluripotent stem cells; **RTT,** Rett syndrome; **SB** SB-431542; **SR,** serum replacement; **VTN,** vitronectin

1 Introduction

Pluripotent stem cells (PSCs) are cells capable to differentiate and give rise to every tissue cell in the human body [1]. Until recently, these cells could only be isolated from the inner cell mass of the blastocyst, being designated

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embryonic stem cells (ESCs) [2, 3]. However, in 2007, Shinya Yamanaka and co-workers were able to reprogram human somatic cells into the pluripotent stem cell state using transfection of four transcription factors (OCT4, SOX2, KLF4, c-MYC). These human induced pluripotent stem cells (iPSCs) brought many expectations into the biomedical field due to their potential applications in disease modeling, drug and toxicity screening, patient-tailored therapies and engineered tissues [4], potentially preventing immunosuppression and graft rejection, and paving the way for the next generation of personalized medicine [5, 6].

Countless protocols for in vitro expansion and differentiation of human PSCs have been developed since then, which typically comprise the use of undefined culture components [7–12]. Problematically, this presents many difficulties to the translation of these protocols from basic research to tangible applications [13]. For example, the use of products from animal origin renders the produced cells unsuitable for clinical applications due to the risk of cross-species contamination and immunogenic reactions [14, 15]. Furthermore, the use of undefined culture conditions poses barriers to the understanding of the biological processes involved in pluripotency and lineage specification and compromises the reproducibility of these culture systems. Therefore, there is an urgent need to develop methods and protocols that use defined culture conditions and allow reproducibility when generating specific cell populations from PSCs.

In particular, neural specification can be induced by the synergistic inhibition of both Activin/Nodal and BMP signaling pathways with small molecules resulting in blockage of mesoderm, endoderm and trophoblast lineages while allowing neuroectodermal specification [7, 16]. Despite being relatively efficient and straightforward, this method uses substrates and medium components of animal origin and of undefined nature (e.g. matrigel and serum replacements). Chemically defined substrates based on proteins, polymers and hybrids of polymers with active biomolecules have been developed, exhibiting low lot-to-lot variability and presenting high reproducibility [17–19]. Truncated, recombinant vitronectin (VTN) conjugates, in particular, have been used as a support for robust expansion and differentiation of human PSCs under standardized and totally defined culture conditions [19–22]. Such system would greatly benefit neural commitment protocols, allowing the foundation of defined settings for in vitro studies of neural development and ensuring reliable methods for the anticipated biomedical applications.

In this study, we have validated the use of defined culture conditions to achieve an efficient and reproducible neural commitment of human PSCs. A defined culture medium supplemented with chemical inhibitors of BMP and TGFβ signaling, together with vitronectin for adherent growth, was shown to be sufficient to generate neural precursors (NPs) expressing SOX1, PAX6, and NESTIN. As a proof-of-concept, we have validated the neural commitment protocol using different cell lines, including patient-specific Rett syndrome-derived iPSCs. This neurological disorder is characterized by autistic-like behaviors and is commonly caused by mutations in the X-linked methyl CpG binding protein 2 (MECP2) gene, which is vital for proper functioning of the brain and acts as one of the many biochemical switches for gene expression [23, 24]. Consequently, abnormal expression of this gene causes atypical brain function, leading to many disease processes that are mostly uncharacterized [25, 26]. Thus, this study also expects to contribute with a novel methodology capable to provide patient-specific neural cells that could be used to gain a better understanding of the disease. Furthermore, the ability to recapitulate the development of the human nervous system in vitro will provide important insights on the mechanisms involved in the maturation of specific neural cell types, making this approach transversal to other related areas in neurodevelopmental research.

2 Materials and methods

2.1 Cell lines

Two human iPSC lines obtained from healthy donors (46, XY) were used: WT-Évora F0000B13 and iLB-C1-30 m-r12. WT-Évora was kindly provided by TCLab (Évora, Portugal), and iLB-C1-30 m-r12 cells were derived at the University of Bonn, Germany. These cell lines were reprogrammed from fibroblasts through retroviral transduction of human genes OCT4, SOX2, c-MYC and KLF4 [4], and were used as wild type controls to verify the robustness of the neural commitment method. The EMC23i cell line was generated at the iPSC Facility, Erasmus Medical Center, Rotterdam, using engineered color-coded lentiviral vectors (as described by Warlich and coworkers [27]), and was derived from a patient (46, XX) with a MECP2 mutation (R306C). Cells were routinely evaluated for karyotype abnormalities by conventional cytogenetics using the services of Genomed SA (Lisbon, Portugal). Biological samples collected for this work were obtained following rigorous national and European ethical guidelines and informed consent from donor or patient's legal guardian.

2.2 Feeder-free culture of human iPSCs

Human iPSCs were thawed and cultured in mTeSR™1 medium (StemCell Technologies™), on Matrigel™ (BD)-coated, Synthemax™ (Corning®)-coated, or Vitronectin (VTN-N, Gibco®)-coated plates. Medium was changed daily and cells were passaged every three to four days, using EDTA dissociation buffer 0.5 mM (Gibco®, diluted in sterile PBS) with a split-ratio of 1:4 [21].

2.3 Neural induction of human iPSCs

When human iPSC cultures were nearly confluent, two different strategies were followed for neural induction, based on Chambers et al [7] and Shi et al [28]. The first protocol was performed using DMEM/serum replacement (SR)-based differentiation medium (KO-DMEM medium (Gibco®) supplemented with 20% v/v KO-SR (Gibco®), 1% v/v non-essential aminoacids (Gibco®), 1 mM L-glutamine (Gibco®), 0.1 mM β-mercaptoethanol (Sigma®) and 1% v/v Penicillin/Streptomycin [PenStrep, Gibco®]), while the other was performed in N2B27 medium (50% v/v DMEM/F12/N2 medium (DMEM-F12 (Gibco®), supplemented with 1% v/v N2 (Gibco®), 1 mM L-glutamine, 1.6 g/L Glucose (Sigma®), 1% v/v PenStrep, and 20 µg/mL Insulin [Sigma®]) and 50% v/v of B27 medium (Neurobasal (Gibco®) supplemented with 2% v/v B27-supplement (Gibco®), 1 mM glutamine and 1% v/v [PenStrep]). In both cases, media formulation was supplemented with 10 µM SB-431542 (SB) and 100 nM LDN-193189 (LDN) (both from Stemgent®), as outlined in Fig. 1A.

2.4 Neuronal differentiation

At day 12 of differentiation, cells were passaged with EDTA dissociation buffer (0.5 mM) and re-plated onto laminin (Sigma®)-coated plates (split ratio of 1:3) [28]. When neural rosettes were observable (around day 14), N2B27 was supplemented with 10 ng/mL of bFGF (Peprotech®) for two to four days. At day 17, cells were passaged using EDTA to new laminin-coated plates (split ratio of 1:3). Cells were then cultured until day 28–30 in N2B27 medium without the addition of small molecules or any other factors. Finally, cells were split with accutase (Sigma®) and plated at a density of 100 000 cells/cm^2. Cells were further cultured until day 150 with medium replacement every other day (Supporting information, Fig. S1).

2.5 Immunofluorescence staining of intracellular markers

We performed immunofluorescence staining as previously described [29]. Briefly, cells were fixed in paraformaldehyde (PFA, Gibco®) 4% for 30 min, followed by blocking for

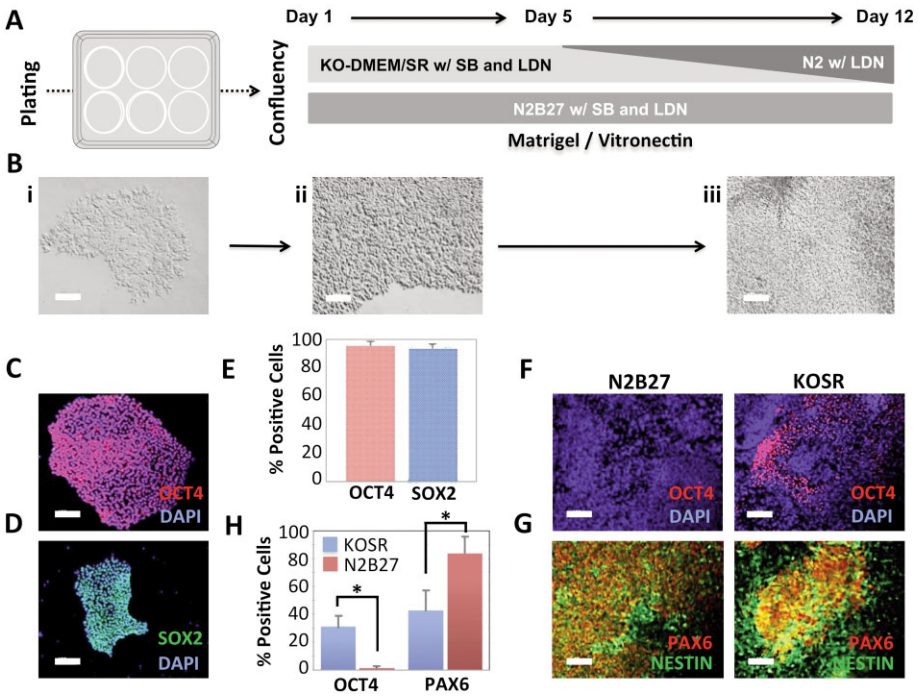

Figure 1. Neural commitment of human PSCs. (**A**) Neural induction was initiated when cells reached confluence and two strategies were tested in parallel. The first strategy involved the use of serum replacement-based medium (KO-DMEM/SR) for the first four days and a gradual increase in N2 medium thereafter. In the second case, N2B27 defined medium was used for 12 days. Small molecule inhibitors of SMAD signaling (SB and LDN) were used as described in the scheme. (**B**) Human PSCs were cultured in adherent conditions (i) until confluence (ii). A neuroepithelial sheet of cells could be observed after neural induction (iii). (**C**) and (**D**) PSC colonies stained for pluripotency markers OCT4 and SOX2 (scale bars: 100 µm). (**E**) Quantification of immunofluorescence images for pluripotency markers OCT4 and SOX2 at the start of neural commitment. (**F**) Immunofluorescence staining for pluripotency marker OCT4 after 12 days of differentiation in N2B27 medium and in serum replacement (KO-SR) conditions (scale bars: 100 µm). (**G**) PAX6 and NESTIN immunostaining for differentiated cells in N2B27 or KOSR-based medium (scale bars: 50 µm). (**H**) Quantification of OCT4- and PAX6-positive cells for the two medium compositions (KOSR or N2B27) used for neural commitment. *p-value<0.05. Results are presented in this figure as mean ± SEM of three independent experiments, and were obtained using iLB-C1-30 m-r12 PSCs and matrigel coating.

60 minutes at room temperature. Primary antibodies were then added and left at 4°C overnight. Secondary antibodies (goat anti-mouse or goat anti-rabbit IgG, Alexa Fluor® –488 or –546, 1:500 v/v dilution, Molecular Probes®) were prepared and incubated with cells for 1 h at room temperature. DAPI (Sigma®) was used to counterstain cell nuclei. Primary antibodies used were FoxG1 (1:100, Abcam®), GFAP (1:100, Millipore), Ki-67 (1:100, BD), MAP2 (1:400, Abcam®), NANOG (1:5000, Millipore), NESTIN (1:400, R&D™), OCT4 (1:500, Millipore), OTX1/2 (1:100, Millipore), PAX6 (1:400, Covance®), SOX2 (1:200, R&D™), Tuj1 (1:5000, Covance®), VGLUT1 (1:300, Synaptic Systems) and ZO-1 (1:100, Novex®).

2.6 Quantification of immunofluorescence images

Quantification of OCT4, SOX2 and PAX6-expressing cells was performed using Fiji® software (for ImageJ). A macro was developed to perform conversion of the original image to eight bits, adjustment of a threshold, watershed treatment, and definition of parameters for particle analysis. Firstly, total cell nuclei were counted using DAPI images. Then, PAX6, SOX2 and OCT4 positive nuclei were counted in the same way and the ratio between positive cells and the total number of cells was calculated.

2.7 Flow cytometry analysis

For staining, approximately 500 000 cells were resuspended in FACS buffer with the diluted primary antibody, and incubated for 15 min at room temperature in the dark. Cells were then washed and resuspended in PBS for later analysis by flow cytometry (FACSCalibur, Becton Dickinson). Antibodies used for flow cytometry were SSEA-4-PE (1:10), and Tra-1-60-PE (1:10) (Stemgent®).

2.8 Quantitative real-time PCR

Total RNA was isolated from cells at different stages of neural commitment (day 0, 3, 6, 9 and 12) using a high pure RNA isolation kit (Roche) according to manufacturer's instructions. cDNA was synthetized from RNA, starting amount of 1 µg, using a transcriptor first strand cDNA synthesis kit (Roche). Taqman® Gene Expression Assays (20X) were selected for *PAX6*, *SOX1*, *NANOG*, *OCT4/POU5F1* and *GAPDH* (Applied Biosystems, Supporting information, Table S2). All other genes (*FGF5*, *NESTIN*, *β-Tubulin III*) were analyzed using SYBR® green chemistry (primers are presented in Supporting information, Table S1). PCR-reactions were run in duplicate, using the StepOne™ RT-PCR System (Applied BioSystems). Reactions were normalized to the housekeeping gene *GAPDH* and results analyzed with StepOne software.

2.9 Electrophysiology

Whole-cell recordings were performed at room temperature in artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1 $MgCl_2$, 2 $CaCl_2$, 10 Glucose, pH 7.4 (gassed with 95% O_2, 5% CO_2). Patch pipettes (4–9 MO) were pulled from borosilicate glass electrodes (1.5 mm outer diameter, 0.86 mm inner diameter, Harvard Apparatus) with PC-10 Puller (Narishige Group) and filled with an intracellular solution containing (in mM): 125 K-gluconate, 11 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 2 MgATP, 0.3 NaGTP, 10 phosphocreatine, pH 7.3, adjusted with KOH (1 M), 280–290 mOsm. Cells were viewed using a Carl Zeiss Axioskop 2FS upright microscope equipped with a differential interference contrast-infrared (DIC-IR) CCD video camera (VX44, Till Photonics) and screen and recorded with an EPC-7 electrical amplifier (List Biologic). Signals were low-pass filtered using a 3- and 10-kHz three-pole Bessel filter of an EPC-7 amplifier, digitized at 10 kHz using a Digidata 1322A board, and registered by the Clampex software version 10.2 (Molecular Devices). Series resistance was not compensated during voltage-clamp recordings, but was regularly monitored throughout each experiment with a -5 mV, 50 ms pulse, and cells with >20% change in series resistance were excluded from the data. Data were not corrected for junction potentials. GABA-evoked postsynaptic currents (GABA-PSCs) were evoked through a micropipette (2–4 MO) containing GABA (30 µM in aCSF) coupled to a pressure application system (Picopump PV820, World Precision Instruments) and positioned close to the soma of the recorded cell. Single pulses of 100–150 ms and 6–8 psi were applied every 2 min. Action potential firing was systematically evoked in current-clamp mode by injecting current pulses of –50 to +300 pA, in 50 pA increments for 1000 ms from an initial holding potential of -70 mV.

2.10 Statistical analysis

Results are presented as mean ± standard error of mean (SEM). Comparisons between experimental results were determined by Mann-Whitney test for independent samples. Unless stated otherwise, three replicates ($n = 3$) were performed and a p-value less than 0.05 was considered significant.

Additional methods and practical information can be found in the Supporting information section of the paper.

3 Results

3.1 Human PSCs efficiently commit to the neuroectoderm lineage without the need for serum replacement-based medium

The dual-SMAD inhibition protocol is a procedure for the rapid commitment of confluent human PSCs into early PSC-derived neural precursors (NPs) [7, 16, 28, 29] (Fig. 1A). Cells are first plated as cell colonies in conditions that support pluripotency, and allowed to reach confluence (Fig. 1Bi and ii). Culture conditions are then changed to include chemical inhibitors of BMP and Activin/Nodal signaling pathways, causing the emergence of a neuroepithelial cell sheet (Fig. 1Biii). This swift induction is due to the blocking of SMAD signaling transduction by SB and LDN small molecules [30, 31]. When combined, these inhibitors repress mesoendodermal fates, directing the differentiation towards neuroectoderm [32].

In this work we first compared different strategies to achieve human NPs based on two different methods [7, 16] (Fig. 1A). In both cases human PSCs were first plated in matrigel-coated plates as clumps using a non-enzymatic passaging procedure [33]. This yielded compact colonies of cells (Fig. 1Bi) that stained positively for pluripotency markers OCT4 and SOX2 (Fig. 1C and 1D, respectively). At this stage, a high percentage (>95%) of cells were positive for these markers (Fig. 1E). Cells were then allowed to reach confluence (Fig. 1Bii), at which point the two neural induction strategies were tested.

The first consisted in the use of serum replacement (SR)-based medium for the first four days of commitment supplemented with both SB and LDN. From day 5 to 11, N2 supplementation was gradually added to the medium, while LDN-mediated inhibition of BMP was maintained [7]. In the second protocol, neural commitment was induced in N2B27 medium supplemented with SB and LDN for 12 days [28]. In both cases a neuroephitelial sheet of cells was obtained, but some qualitative differences in the efficiency of the neural commitment process were immediately visualized using immunofluorescence (Fig. 1F and 1G). While in the case of SR-based medium OCT4-positive cells still persisted in culture 12 days after neural induction with chemical inhibitors, for N2B27-based induction these cells were virtually absent (Fig. 1F). Additionally, the distribution of PAX6- and NESTIN-positive cells was more homogeneous in the case of N2B27 when compared with SR-based medium, which presented islands of PAX6-positive cells surrounded by NESTIN expressing cells instead of a homogeneous monolayer (Fig. 1G). Using image-processing software we then quantified OCT4- and PAX6-expressing cells for both protocols (Fig. 1H). Neural commitment in N2B27 medium resulted in more than 80% of NPs positive for PAX6 and very low numbers of OCT4-positive PSCs (<2%), while in KO-SR medium, an average of 40% PAX6-positive cells was obtained, with more than 20% of OCT4-positive cells still in culture (Mann-Whitney test, p-value <0.05).

These results highlight the importance of medium formulation in maximizing differentiation yield [22]. By comparing data from both differentiation media it is possible to conclude that the chemically defined, serum-free composition of N2B27 yields a more efficient neural conversion into NPs. This medium was particularly optimized for neural cell culture, and contains insulin and retinoids, which have been shown to be crucial for the neuroepithelial induction of human PSCs [16]. The relatively high percentage of pluripotent cells and potential non-neural differentiated cells obtained in SR-based medium (Supporting information, Fig. S2) is not desirable since these cells may interfere with the outcome of in vitro differentiation protocols [29].

3.2 Human PSCs generate neural precursor cells using dual SMAD signaling inhibition under defined culture conditions

In addition to medium formulation, the composition of the extracellular matrix to which cells adhere is also a potential source of ill-defined components. This is the case of matrigel, whose constituents include an heterogeneous mixture of structural proteins of animal origin [34]. However, non-xenogeneic, defined substrates that support the culture of human PSCs have also been proposed in the literature [17]. In particular, vitronectin (VTN) peptide sequences have shown to support both expansion and differentiation of human PSCs under totally defined culture conditions [19–22]. Our next goal was to compare two different extracellular matrixes in supporting neural commitment from human iPSCs in combination with a fully defined culture medium, allowing the foundation of defined settings for in vitro studies of neural development.

A side-by-side comparison was made using N2B27 medium together with matrigel or VTN (Fig. 2). The dual SMAD inhibition yielded similar outcomes in both cases, and cells presented typical neuroectodermal markers (Fig. 2A). These included the neuroectoderm fate determinant PAX6 [35], the forebrain marker FoxG1 [7], and the midbrain/forebrain marker OTX1/2 [36], in addition to SOX2 and NESTIN. Cells differentiated for 12 days in both substrates showed up-regulation of *PAX6* and *SOX1* mRNA, with corresponding down-regulation of pluripotency transcription factors (*OCT4*/*POU5F1* and *NANOG*) (Fig. 2B). Additionally, real-time PCR analysis indicated that for both substrates expression of the pluripotency markers sharply decreased in the first three days of neural induction (Fig. 2C). After this point, both *OCT4/POU5fF1* and *NANOG* retain minor levels of expression when compared to day 0. Also, *PAX6* and *SOX1* expression increased rapidly from day 0, reaching maximum levels after day 3 in both matrigel and VTN (Fig. 2D). At the

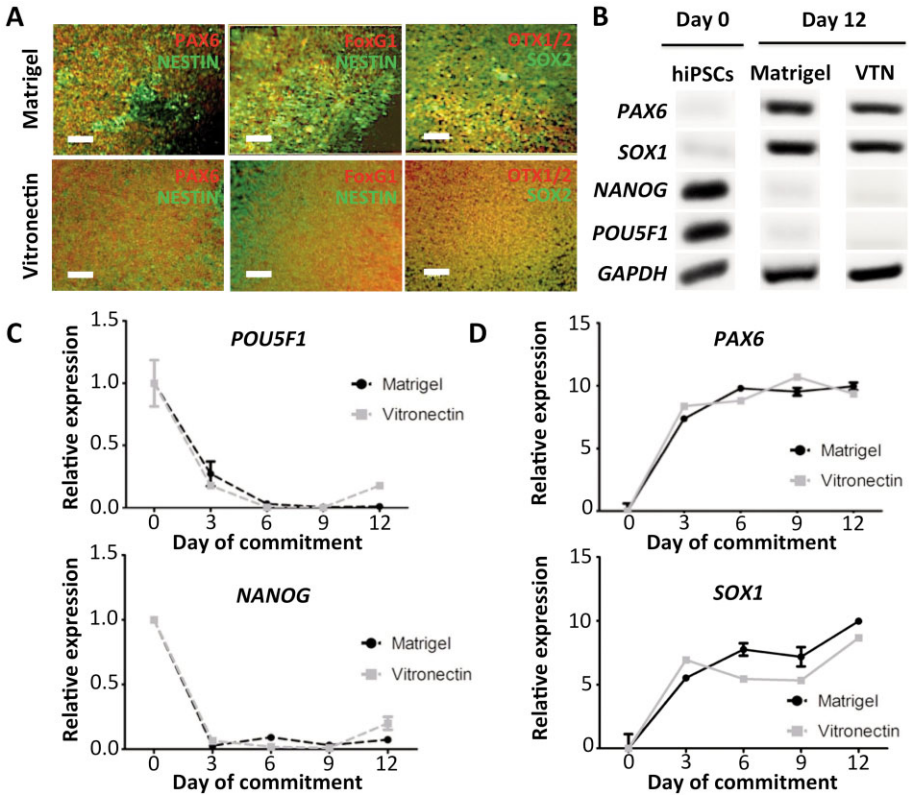

Figure 2. Efficient neural commitment of human PSCs using defined medium and vitronectin coating. (**A**) Immunofluorescence staining of PSC-derived NPs after 12 days of induction with chemical inhibitors in matrigel or VTN. Cells were stained for PAX6, NESTIN, FoxG1, OTX1/2 and SOX2 (scale bars: 50 μm). (**B**) RT-PCR analysis of NPs generated in matrigel and VTN. Pluripotency genes (*POU5F1*, *NANOG*) and neuroectodermal markers (*PAX6*, *SOX1*) are shown for comparison. (**C**) and (**D**) Temporal gene expression analysis during neural commitment of human PSCs in matrigel and VTN. Pluripotency genes (*POU5F1*, *NANOG*) and neuroectodermal markers (*PAX6*, *SOX1*) were evaluated by quantitative real-time PCR at different time points. *GAPDH* expression was used as internal control. Results are presented in this figure as mean ± SEM of three independent experiments and were obtained using the iLB-C1-30 m-r12 cell line.

same time, when human PSCs entered differentiation, the presence of cell surface markers characteristic of the pluripotent state (Tra-1-60 and SSEA-4) was reduced, and the percentage of cells positive for these markers dropped progressively during the 12 days of neural commitment (Fig. 3A). The simultaneous up-regulation of *FGF5* expression (Fig. 3B), together with down-regulation of pluripotency genes (Fig. 2C) and up-regulation of *PAX6* and *SOX1* (Fig. 2D), most likely resulted from the emergence of an initial population of NPs after neural induction [37]. Taken together, these outcomes demonstrate that human iPSCs can effectively differentiate to NPs using the dual SMAD inhibition protocol under defined culture conditions composed of N2B27 and VTN as adhesion substrate.

To further confirm the generation of competent NPs after 12 days of dual SMAD inhibition, cells were re-plated onto laminin-coated plates as small clumps, and cultured in N2B27 medium without chemical inhibitors SB and LDN. Two to five days after re-plating, a substantial number of neural rosette-like structures were observed (Fig. 3C). Rosettes were radially arranged like neuroepithelial cells, which were positive for PAX6, NESTIN, and SOX2, and already showed signs of polarization, with apical ZO1 expression. When neural rosettes were first visible, bFGF was added to the medium, to induce proliferation of progenitors, and around day 17 of differentiation cells were re-plated onto new laminin-coated plates and cultured until day 28–30 without addition of small molecules or any other factors. Substantial maturation of NPs within rosettes was observed (Fig. 3D and 3E). As expected, after peaking during the initial stage of commitment, expression of typical neural markers (*PAX6*, *SOX1* and *NESTIN*) declined during further differentiation (Fig. 3D). This was followed by up-regulation of the neuronal marker *β-Tubulin III*. Using immunofluorescence staining we were able to see a proliferative (Ki67-positive) population of progenitors within polarized rosettes (apical expression of ZO1), and to confirm a reduction of the number of PAX6-positive cells. At this stage of differentiation, Tuj1-positive neurons outgrew from neural rosettes (Fig. 3E).

Taken together, these results demonstrate and validate the capacity to generate functional NPs using the dual SMAD inhibition protocol under defined culture conditions. Our results show efficient (>80%) generation of PAX6-positive NPs, and the capacity of these cells to self-organize into neural rosettes upon passaging. Further differentiation into more mature cells was achieved within rosettes, which can be correlated with events leading to the neural tube formation in vivo [7, 16]. As previously demonstrated in similar systems [19–22, 38], peptide conjugates can support robust expansion and differentiation of human PSCs under defined conditions, and in our case VTN supported neural commitment of human PSCs in conjugation with N2B27 medium supplemented with SMAD inhibitors.

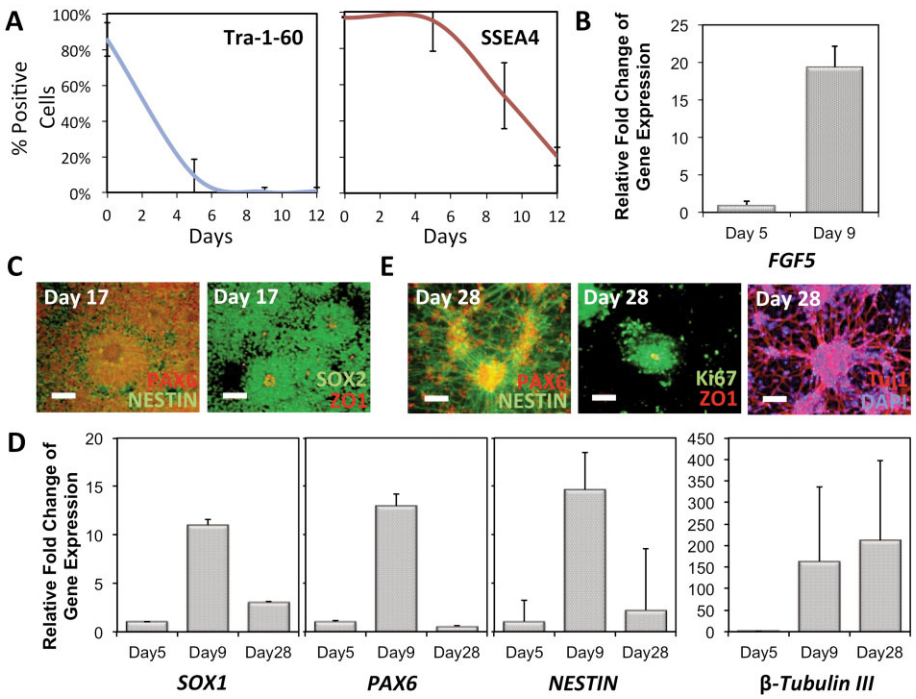

Figure 3. Neural maturation capability of cells derived under defined conditions using vitronectin coating. (**A**) Tra-1-60- and SSEA4-positive cells were evaluated by flow cytometry during neural commitment. (**B**) Gene expression analysis by quantitative real-time PCR of the early specification marker *FGF5*. (**C**) After re-plating onto laminin-coated surfaces, immunostaining analysis was performed on cells at day 17 of differentiation. PAX6, NESTIN, and SOX2 markers were used to identify neural rosettes, and ZO1 was used to show polarization within rosettes (scale bars: 50 μm). (**D**) Expression of typical neural markers (*PAX6*, *SOX1* and *NESTIN*) and of neuronal marker *β-Tubulin III*, as assessed by quantitative real-time PCR at different stages of neural differentiation. (**E**) At day 28 of differentiation, Ki67 and ZO1 immunostaining were used to identify polarized rosettes (ZO1-centered). At this stage cells were also marked for neural markers (PAX6, NESTIN), and Tuj1-positive neuronal projections (scale bars: 50 μm). Results are presented in this figure as mean ± SEM of three independent experiments and were obtained using iLB-C1-30 m-r12 PSCs.

3.3 Generation of patient-specific neural precursors and further neural maturation

At this stage we have established conditions that allowed the study of neural specification of human PSCs without interference of undetermined components resulting from serum replacements [7] or extracellular matrix protein mixtures [28, 34]. This is an important feature of our system since undisclosed constituents may interfere with different signaling pathways, and thus affect cell fate decisions. Therefore, to further explore and demonstrate the usefulness of our method for neural specification studies, we have taken advantage of the potential of iPSC technology to investigate the neural commitment of patient-specific iPSCs, in particular Rett syndrome (RTT) patient-derived cells [39]. This disorder is caused by mutations in the MECP2 gene of the X chromosome and affects 1 in 10 000–20 000 girls worldwide [23]. Hallmarks of this condition include impaired motor function, seizures, autistic behavior [40], and changes in neuronal density and in brain size [24, 41].

Control and RTT-derived fibroblasts were reprogrammed to a pluripotent state as described elsewhere [4]. The iPSC lines used in this work showed typical characteristics of pluripotent stem cells, and could be maintained in culture for dozens of passages without signs of differentiation or atypical karyotype (Supporting information, Table S3 and Fig. S3). These cells were then adapted to our culturing conditions using a non-enzymatic passaging procedure and plated to VTN-coated surfaces [21]. Both RTT and control-derived iPSCs were cultured in pluripotency maintenance conditions using mTESR, and stained for typical pluripotency markers (Fig. 4A and 4C, and Supporting information, Fig. S3). After reaching confluence, medium was changed to N2B27 and small molecule inhibitors of SMAD signaling (LDN and SB) were added to culture for 12 days. Both normal and patient-derived cells were able to differentiate and presented typical neuroectodermal markers such as PAX6, NESTIN, FoxG1, OTX1/2 and SOX2 (Fig. 4B and 4D). Also, at this time point, pluripotency markers like OCT4 were virtually absent.

After 12 days of dual SMAD inhibition, NPs were replated onto laminin-coated plates as small clumps, and cultured in N2B27 medium without chemical inhibitors. At day 17, after stimulation with bFGF, both normal and mutant cells were able to form neural rosettes without any noticeable differences (Fig. 4E and 4G). Substantial mat-

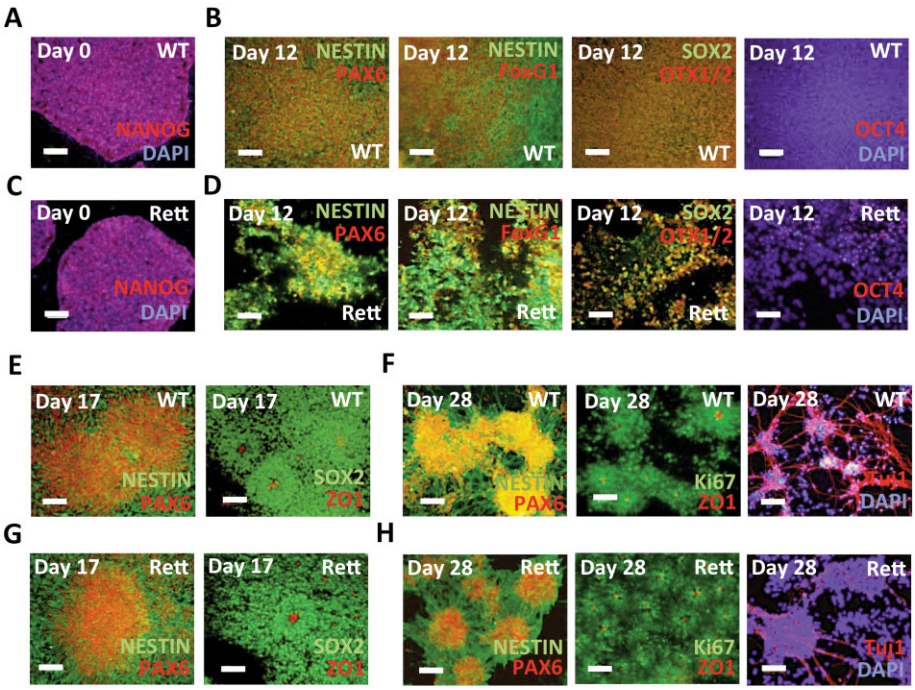

Figure 4. Neural commitment and further neural differentiation of both normal and Rett syndrome patient specific human iPSCs. (**A**) and (**C**) Immunofluorescence staining of pluripotency marker NANOG for both normal and Rett syndrome patient-derived iPSCs (scale bars: 100 μm). (**B**) and (**D**) Immunofluorescence staining of neural markers (PAX6, NESTIN, FoxG1, OTX1/2, SOX2, scale bars: 50 μm) and of pluripotency marker OCT4 (scale bars: 100 μm) after twelve days of dual SMAD inhibition using a defined culture system. (**E**) and (**G**) After re-plating onto laminin, immunostaining analysis was performed to demonstrate potential of both normal and affected cells to form neural rosettes. Cells were stained for PAX6, NESTIN, SOX2 and ZO1 (scale bars: 50 μm). (**F**) and (**H**) Direct comparison of both cell types during further neural maturation. Cells were marked for neural markers (PAX6, NESTIN), Ki67, ZO1, and Tuj1-positive neuronal projections (scale bars: 50 μm). Results presented in this figure were obtained using iLB-C1-30 m-r12, WT-Évora F0000B13, and EMC23i PSCs.

uration of NPs within rosettes was observed with cells expressing neural markers (NESTIN, PAX6, SOX2) within polarized structures (ZO1). At this stage, cells were again re-plated onto laminin to allow further neural differentiation. Proliferative, Ki67-positive cells within rosettes could still be identified in both cultures, and polarization could also be seen with apical localization of ZO1 (Fig. 4F and 4H). Nevertheless, while in normal cells Tuj1-positive neurons started to migrate outwards of rosettes, forming neuronal projections and connections, RTT derived-neurons were substantially less frequent, and neurites less complex (Fig. 4F and 4H).

To further assess the neuronal maturation state, cells were finally passed as single-cells at day 30 onto laminin-coated plates and maintained in N2B27 medium. This procedure was expected to generate cortical projection neurons in about 80 days [28]. At day 75 of differentiation, we evaluated not only neuronal maturation, but also differentiation into glial cells, like astrocytes, in two non-affected 'wild type' human iPSCs and one Rett syndrome-derived mutant line (Fig. 5A). A substantial number of widespread neuronal projections was observed in both normal cell lines. Furthermore, extensive gliogenesis had occurred in such cultures and GFAP-positive astrocytes could be seen interspersed with Tuj1-positive neurons. This process was minimally reproduced in affected cells, showing less neurons, similarly to what had been visualized at day 28 of differentiation. Similar conclusions could be taken from MAP2 staining at day 100 (Fig. 5B). These observations are in accordance with other reports in the literature [24, 41], and did not seem to result from reduced proliferation, since the number of DAPI stained nuclei within the microscope field of view was similar among samples. By day 120, control neurons developed to glutamatergic neurons presenting the vesicular transporter VGLUT1 (Fig. 5C), which could not be observed in mutated cells.

Finally, electrophysiological recordings performed at day 120 of differentiation, revealed neuronal functional properties characteristic of mature neurons. Values of neuronal resting membrane potential, determined immediately after establishing whole-cell configuration, averaged –45.0 ± 2.2 mV (n = 17). Action-potentials were then evoked in current-clamp mode by injecting depolarizing current pulses. Most cells tested showed firing of at least one action potential during current injection steps, revealing a mature neuronal state (n = 10, Fig. 5D). In addition, GABA-evoked postsynaptic currents were observed (volt-

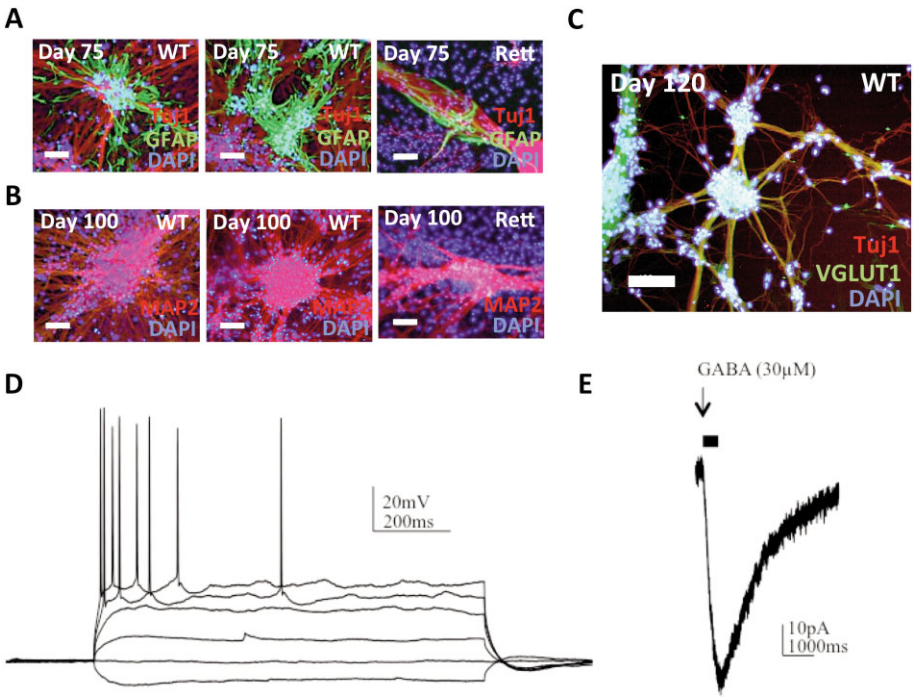

Figure 5. Neuronal differentiation of normal and Rett syndrome-derived neural progenitors. (**A**) Immunostaining analysis was performed at day 75 of differentiation to evaluate neuronal maturation (Tuj1-positive) and astrocyte differentiation (GFAP-positive) in both normal and mutated cells (scale bars: 50 μm). (**B**) After further differentiation and maturation of neurons, immunostaining was performed to identify MAP2-positive mature dendrites at day 100. The frequency of such structures in *MECP2* mutated cells was compared to the control cells (scale bars: 50 μm). (**C**) Immunostaining for glutamatergic neuronal protein VGLUT1 of 'wild type' Tuj1 neurons at 120 days of differentiation (scale bar: 100 μm). (**D**) and (**E**) show electrophysiological properties of 'wild type' cells after 120 days of differentiation. (**D**) Increased depolarizing voltage steps (1000 ms) in whole-cell current-clamp mode. A representative firing of action potential characteristic of mature neurons is shown. (**E**) Representative GABA (30 μM)-evoked postsynaptic currents (GABA-PSC) from a neuron recorded in whole-cell voltage-clamp (–70 mV). Black bar corresponds to GABA application.

age-clamp mode, Vh = -70 mV) in response to pressure application of GABA (30 μM) directly to the soma of the cell, indicating the expression of functional neurotransmitter receptors in these cells (n = 11, Fig. 5E).

4 Discussion

We herein identify defined culture conditions to achieve efficient and reproducible neural commitment of human PSCs. A defined neural maintenance medium supplemented with chemical inhibitors of both BMP and TGFβ signaling pathways, together with VTN for adherent growth, was shown to be sufficient to induce neuronal commitment, generating a homogeneous population of NPs expressing typical neural markers (Fig. 1, 2 and 3). The results obtained herein are comparable with other reported data in terms of neural conversion efficiency [16], when using N2B27 medium, demonstrating that VTN-coating does not affect neural commitment negatively.

In fact, VTN-based conjugates have been previously used for PSC derivation, expansion [20, 21] and neural specification studies [22, 42], and our results also validate the usage of VTN as a defined substrate for neural commitment. Another important feature of this system is the use of retinoid rich medium, which have been shown to increase NPs output [16]. When combined with VTN adhesion motifs, the use of defined medium was thought to facilitate progression through neural maturation, particularly during re-plating of NPs to laminin surfaces, by promoting survival and functionality of differentiated cells [43]. In fact, when compared with serum replacement-based medium, we have been able to significantly increase the number of PAX6-positive NPs in N2B27 (Fig. 1). However, other studies have reported minimally constituted monolayer systems that could achieve similar efficiencies in generating PAX6-positive neuroectoderm [22], questioning the role of retinoids in neural commitment. Nevertheless, monolayer neural commitment protocols still require a high local cell density for generation of NPs with high efficiencies [7]. It would be interesting to engage other methods [44, 45], such as micropatterning or forced aggregation of cells, to evaluate the local dynamics of high-density cell-to-cell contacts in the neural specification of human PSCs. Still, one shortcoming of monolayer systems is the fact that for later stages of neural development, NPs require laminin to adhere and differentiate [46]. Laminins are extracellular matrix trimeric gly-

coproteins and at least a dozen of different isoforms have been identified in nature. Commonly used laminin is isolated from a murine source and represents an undisclosed mixture of several of these natural isoforms. To fully define neural commitment, neural maturation, and neuronal differentiation of human PSCs in adherent conditions, specific laminin isoforms should be investigated and subsequently used. The Tryggvason's group has been able to produce recombinant laminins and evaluate their specific role in several biological functions [47], but the exact isoforms involved in neural differentiation remain unknown. However, potential future therapeutic strategies for neurological disorders might implicate the use of progenitors [48], since mature cells are less capable of establishing the adequate connections without being continuously exposed to the combination of growth factor and signaling molecules that orientate synaptogenesis and neuronal wiring. Obtaining NPs in xeno-free conditions can be a relevant first step to establish a safe approach for future transplant based therapies.

As a proof-of-concept, we have validated our neural commitment protocol using different cell lines, including patient-specific, Rett syndrome-derived iPSCs. Although investigation of a broader number of patients would be required, we were able to replicate some of the reported Rett syndrome defects in NPs derived in our defined system (Fig. 4 and 5). Interestingly, given the fact that MECP2 is located on the X-chromosome and that Rett syndrome patients present mosaicism [23], X-chromosome activation/inactivation during reprogramming, expansion and differentiation could lead to difficulties in reproducing the pathology of the disease in vitro. We have not confirmed these events during reprogramming of our cells, but Marchetto and coworkers have previously shown the capacity to recapitulate X-inactivation during neural differentiation, producing mosaic neuronal cultures with different MECP2 expression levels [24]. Isogenic human iPSC lines from Rett syndrome patients can therefore be used as in vitro models of the disease [49], and our results (Fig. 5) are in accordance with the published literature [24, 41, 49]. Since several studies have reported similar findings, and taking into consideration the different levels of *MECP2* expression in mutated cells, the contribution of non-neural cells, like astrocytes, to the onset of the disease should not be excluded. In fact, this influence was already described, indicating that glial cells carrying *MECP2* mutations can also distress healthy neurons [50, 51].

In conclusion, our defined culture system provides a way to recapitulate some of the temporal and regional patterning events that occur during in vivo cortical neurogenesis [52]. Also, by deconstructing the natural complexity of neural development into a simpler experimental approach, we could mimic several aspects of Rett syndrome pathology potentially contributing to a better understanding of cortical development and disease.

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All co-authors have agreed to the submission of the manuscript. Oliver Brüstle is co-founder of and has stock in LIFE & BRAIN GmbH. All other authors declare no financial or commercial conflict of interest.

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