Neuroplasticity-targeted therapy for Down syndrome: a translational approach

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Knowing is not enough; we must apply.
Willing is not enough; we must do.

Maxims and reflections of J. W. von Goethe

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Abstract

At the present time, there is no clinically accepted available intervention to ameliorate intellectual disability (ID) in individuals with Down syndrome (DS), that has been, until recently, considered as a permanent and intractable hallmark of the disorder. However, extensive research along the last years has identified a number of altered molecular pathways, and neurobiological processes putatively involved with ID in DS that could be targeted to intervention for the improvement of brain and cognitive function in this population. One of the most critical neuronal mechanisms underlying ID are the defects in brain neuroplasticity, the ability of neurons and neuronal networks to change structurally and functionally in response to the environment and experience, which is intimately associated to learning and memory capabilities. So far, non-pharmacological interventions aimed at enhancing intellectual capacities in people with DS, such as special education programs, have shown limited although positive outcomes. Accordingly, it has been suggested that a reduced remodelling neuroplasticity potential could explain the little impact of cognitive stimulation and experience on DS brains and cognition. Therefore, normalizing neuroplasticity in DS could improve the neurobiological interplay between environmental experience, and learning and memory. Accumulative evidence has shown that in DS, among the approximately 500 triplicated genes located on Hsa21, there is a reduced number of dosage-sensitive candidate genes that play a critical role in the pathogenesis of the disorder. Two of these genes encode the dual specificity tyrosine-phosphorylation regulated kinase 1A (Dyrk1A) and the amyloid precursor protein (APP), which are tightly involved in the altered neuroplasticity and neurodegeneration processes that take place in DS.

In this Thesis we have examined the effects of a multimodal therapy consisting of the use of (-)-Epigallocatechin-3-gallate (EGCG), a catechin found in green tea, which inhibits the kinase activity of Dyrk1A and modulates the proteolytic processing of APP, in

combination with environmental cognitive stimulation. We have used a translational research approach involving preclinical studies, in a DS mouse model, and clinical trials, with DS humans.

Our preclinical studies showed positive effects of combined environmental enrichment (EE) with EGCG treatment on visuospatial learning and memory deficits, both in young and middle-age Ts65Dn mice, suggesting its efficacy despite the age-dependent cognitive impairments and their underlying pathophysiological mechanisms. An additional improvement in contextual learning was detected in EE-EGCG treated middle-age Ts65Dn mice. Histological and molecular experiments revealed that combined EE-EGCG treatment promotes hippocampal neuroplasticity changes by increasing dendritic spine density in CA1 and restoring the balance between excitatory and inhibitory synaptic proteins in CA1 and DG.

Our clinical trials showed that EGCG treatment is safe in young adult individuals with DS and induces a memory improvement when administered alone for a short period. Administration for a longer period of combined treatment with cognitive training and EGCG, improved memory and adaptive behavior, increased functional connectivity as measured by fMRI and normalized excitability in TMS studies, in a more efficient way than cognitive training combined with placebo.

Altogether our results show, for the first time, that a multimodal therapy consisting of combined environmental cognitive stimulation and EGCG significantly ameliorates cognitive deficits in Ts65Dn mice and young adult individuals with DS, by modifying neuronal network structure and function.

Resumen

En la actualidad, no hay disponible ninguna intervención terapéutica para tratar la discapacidad intelectual (DI) en personas con síndrome de Down (SD), y hasta hace poco, esta se consideraba una característica permanente e intratable de la enfermedad. Sin embargo, diversas investigaciones a lo largo de los últimos años han identificado alteraciones en una serie de vías moleculares y procesos neurobiológicos, posiblemente involucrados con la DI en el SD, que podrían ser sometidos a intervención, para la mejora de las funciones cerebrales y cognitivas en esta población. Uno de los mecanismos neuronales subyacentes más críticos se basa en defectos en la neuroplasticidad cerebral, que es la capacidad de las neuronas y redes neuronales de cambiar estructuralmente y funcionalmente en respuesta al medio ambiente y la experiencia, y está íntimamente asociada a las capacidades de aprendizaje y memoria. Hasta ahora, las intervenciones no farmacológicas destinadas a mejorar capacidades intelectuales en personas con SD a través de programas de educación especial, han mostrado resultados limitados, aunque positivos. Por consiguiente, se ha sugerido que una reducción en el potencial neuroplástico de remodelación podría explicar el limitado impacto de la estimulación cognitiva y la experiencia sobre el cerebro y la cognición en SD. Por lo tanto, la normalización de la neuroplasticidad en el SD podría mejorar la interacción neurobiológica entre la experiencia y el ambiente, y el aprendizaje y la memoria. Numerosas evidencias sugieren que, entre los aproximadamente 500 genes localizados en Hsa21, que están triplicados en SD, hay un número reducido de genes candidatos dosis-sensible que juegan un papel crítico en la patogénesis de la enfermedad. Dos de estos genes codifican la quinasa de especificidad dual, regulada por autofosforilación de tirosina (Dyrk1A) y la proteína precursora del amiloide (APP), que están estrechamente implicados con alteraciones en procesos de neuroplasticidad y neurodegeneración que tienen lugar en el SD.

En esta Tesis se han examinado los efectos de una terapia multimodal que consiste en el uso de la (-)-Epigalocatequina-3-galato (EGCG), una catequina encontrada en el té verde, que inhibe la actividad quinasa de Dyrk1A y modula el procesamiento proteolítico de APP, en combinación con estimulación cognitiva. Hemos utilizado un enfoque de investigación traslacional que implica la realización de estudios preclínicos, un modelo murino de SD, y ensayos clínicos, con personas con SD.

Nuestros estudios preclínicos demostraron efectos positivos del tratamiento combinado con enriquecimiento ambiental (EE) y EGCG en los déficits de aprendizaje y memoria visuoespacial, tanto en ratones Ts65Dn jóvenes y de mediana edad, lo que sugiere su eficacia a pesar de las deficiencias cognitivas dependientes de la edad y de los distintos mecanismos fisiopatológicos subyacentes. En ratones Ts65Dn de mediana edad tratados con EE-EGCG hemos detectado adicionalmente una mejora en el aprendizaje contextual.. Nuestros experimentos histológicos y moleculares demostraron que el tratamiento combinado EE-EGCG promueve cambios neuroplásticos en el hipocampo, aumentando la densidad de espinas dendríticas en CA1 y restableciendo el equilibrio entre proteínas sinápticas excitatorias e inhibitorias en CA1 y DG.

Nuestros ensayos clínicos demostraron que el tratamiento único con EGCG es seguro en individuos adultos jóvenes con SD e induce una mejora en la memoria, cuando se administra durante un período corto. La administración del tratamiento combinado con estimulación cognitiva y EGCG, durante un período más largo, promovió una mejora en memoria y en conducta adaptativa, aumentó la conectividad funcional en estudios de fMRI y normalizó patrones de excitabilidad en estudios de TMS, de una manera más eficiente que la estimulación cognitiva combinada con placebo.

En resumen, nuestros resultados muestran, por primera vez, que una terapia multimodal que consiste en la estimulación cognitiva ambiental combinado y EGCG mejora los déficits cognitivos significativamente en ratones Ts65Dn y jóvenes individuos adultos

con DS, mediante la modificación de la estructura de red neuronal y función.

Preface

This Thesis emanates from the main interest of the Cellular and Systems Neurobiology Group at the Center for Genomic Regulation that is understanding the neuropathological basis of intellectual disability (ID) and developing new possible therapeutic avenues to ameliorate cognition in those disorders. In the last years we have made important contributions demonstrating that despite the broad spectrum of genetic and environmental aetiologies, alterations in neural plasticity are a common neuropathological finding in ID disorders that underlines overlapping molecular networks and correlates with cognitive impairments. Over time, abnormal neuroplasticity leads to a cognitive impairment regardless of the particular molecular cause. Neural plasticity is an umbrella term that includes structural and functional changes in neural systems, that have been consistently related to learning and memory abilities. Alterations in the physical structure and the functional efficiency of communication elements such as dendrites and spines would be expected to adversely affect the information storage capacity of neural networks by reducing the number of potential sites for plasticity to occur. Consistent with this idea and the observed deficits in cognition associated with Down syndrome (DS), examination of postmortem brain tissue from DS individuals reveals profound alterations in dendritic and neuronal densities and morphology across many regions of the brain beginning in utero and persisting throughout life.

Our hypothesis is that drugs targeting core molecules in neuroplasticity cascades will set the brain in a favourable state for cognitive function and will be disease-modifying treatments in individuals with ID, and specifically in DS. Therefore, we have explored a novel systems approach that combines non-pharmacological therapeutic strategies, such as cognitive stimulations, that would induce physiological activity-dependent plasticity, in combination with a pharmacological tool that enables plasticity-related cascades. The aim of this Thesis project was to

assess the effects of a combined therapy, consisting of an environmental cognitive enhancing intervention and the coadjuvant administration of the green tea catechin (-)-epigallocatechin-3-gallate (EGCG). The rationale is that these two treatments enhance neuroplasticity through common molecular mechanisms including the modulation of two Hsa21 candidate genes, the dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) and the amyloid precursor protein (APP), that play a major role in DS pathogenesis across lifetime and thus represent strategic targets for intervention. The concept behind was that normalizing DYRK1A kinase activity and APP proteolytic pathway, would enable cognitive stimulation to more stably and potently affect neural circuit reconfiguration.

The work I present here explores to what extent a combined therapy with environmental enrichment (EE) and EGCG can ameliorate cognitive deficits in DS mouse models and humans, focusing on their hippocampal-dependent functional and structural alterations. Given the truly translational nature of our approach, during my Thesis work, I have managed a plethora of frameworks and theoretical perspectives keeping in mind the challenge of using consistent cross-species terms and concepts, one over another.

In Chapter I, I present *in vivo* preclinical studies aimed at examining the effects of a combined relatively chronic/ long-term treatment with EE-EGCG on learning deficits and structural alterations of a partial trisomic DS mouse model (Ts65Dn). Specifically, we investigated whether a combined EE-EGCG treatment had differential effects at different ages characterized by distinct pathophysiological processes. Beyond the behavioral experiments, we aimed at addressing questions regarding specific treatment effects in precise brain regions that are compromised both in mouse models and humans with DS but cannot be tackled in clinical populations. All the behavioral experiments and treatments were carried out in the PRBB animal facility, and the histology and molecular biology approaches in the Cellular and Systems

Neurobiology lab of the CRG. Those studies are presented in three papers (see below) on which I am the first author.

In Chapter II, I present the results of two clinical trials (phase I and phase II) in which I had the privilege to participate, that were carried out by the TESDAD study group coordinated by Dr. Rafael de la Torre at the Institut Hospital del Mar d'Investigacions Mèdiques (IMIM). These clinical trials involved young adult individuals with DS (14-30 years in the phase I and II) and were aimed at evaluating the effects of EGCG alone (phase I) and the combination of cognitive training with EGCG (phase II), on different clinically relevant parameters including cognitive abilities, neurophysiological and neuroanatomical measures, every-day adaptive functionality and quality of life. My specific contribution involved the performance of longitudinal neuropsychological assessments of the volunteers using a new standardized clinical trial battery that was developed in the context of the TESDAD clinical trial but can also be used in future clinical trials with individuals with DS. The work in humans, even if having a different framework and challenges, has greatly profited from the outcomes of the preclinical studies. As such, a bidirectional interplay between human and mouse studies guided the directions towards which the preclinical studies should be oriented in order to be relevant to the clinical trials.

The results presented here have provided pivotal data in the field of ID that will certainly promote the development of a novel therapeutic framework for intervention in people with DS. Further investigation in a multicenter (phase III) study will be needed including a larger sample of individuals and different ages. Additionally, the outcomes of this Thesis have set the basis for further research lines in Dierssen´s lab involving mechanistic studies that will address the molecular effects of the treatments at a connectomic, proteomic and epigenetic levels.

During my Thesis I have co-authored seven papers, related to different aspects of my work. From those, three of them are original contributions from the preclinical work of which I am first author (one is already published in Frontiers Behavioral Neuroscience in 2015, one is under revision in E-neuro and one will be submited to Neurobiology of Disease in September 2016), three are contributions to the clinical trials, in which I am among the main authors (the phase I study, De la Torre et al., 2014, a methodological paper, De Sola et al., 2015 and the phase II study De la Torre et al., 2016), and a review paper (Lepeta et al., 2016). In this Thesis I only present original pieces of work in which my contribution has been principal.

List of published articles (in backwards chronological order):

1. Lepeta K, Lourenco MV, Schweitzer BC, Martino Adami PV, Banerjee P, **Catuara-Solarz S**, de La Fuente Revenga M, Guillem AM, Haidar M, Ijomone OM, Nadorp B, Qi L, Perera ND, Refsgaard LK, Reid KM, Sabbar M, Sahoo A, Schafer N, Sheean RK, Suska A, Verma R, Vicidomini C, Wright D, Zhang XD, Seidenbecher C. Synaptopathies: synaptic dysfunction in neurological disorders. *J Neurochem*. 2016 Jun 22. doi: 10.1111/jnc.13713. Review. PMID: 27333343

2. De la Torre R, De Sola S, Hernandez G, Farré M, Pujol J, Rodriguez J, Espadaler JM, Langohr K, Cuenca-Royo A, Principe A, Xicota L, Janel N, **Catuara-Solarz S**, Sanchez-Benavides G, Bléhaut H, Dueñas-Espín I, Del Hoyo L, Benejam B, Blanco-Hinojo L, Videla S, Fitó M, Delabar JM, Dierssen M Safety and efficacy of cognitive training plus epigallocatechin-3-gallate in young adults with Down's syndrome (TESDAD): a double-blind, randomised, placebo-controlled, phase 2 trial. *The Lancet Neurology* 2016 Volume 15, No. 8, p801–810 DOI: http://dx.doi.org/10.1016/S1474-4422(16)30034-5

3. **Catuara-Solarz S**, Espinosa-Carrasco J, Erb I, Langohr K, Notredame C, Gonzalez JR, Dierssen M. Principal Component Analysis of the Effects of Environmental Enrichment and (-)-epigallocatechin-3-gallate on Age-Associated Learning Deficits in a Mouse Model of Down Syndrome. *Front Behav Neurosci.* 2015 Dec 11;9:330. PMID: 26696850

4. De Sola S, De la Torre R, Sanchez-Benavides G, Benejam B, Cuenca-Royo A, Del Hoyo L, Rodriguez J, **Catuara-Solarz S**, Sanchez-Gutiérrez J, Dueñas-Espin I, Hernandez G, Peña-Casanova J, Langohr K, Videla S, Bléhaut H, Farré M, Dierssen M and the TESDAD Study Group. A new cognitive evaluation battery for Down syndrome and its relevance for clinical trials. *Front. Psychol*. 2015 doi: 10.3389/fpsyg.2015.00708

5. De la Torre R, De Sola S, Pons M, Duchon A, de Lagran MM, Farré M, Fitó M, Benejam B, Langohr K, Rodriguez J, Pujadas M, Bizot JC, Cuenca A, Janel N, **Catuara S**, Covas MI, Blehaut H, Herault Y, Delabar JM, Dierssen M. Epigallocatechin-3-gallate, a DYRK1A inhibitor, rescues cognitive deficits in Down syndrome mouse models and in humans. *Mol Nutr Food Res*. 2014 58(2):278-88. doi: 10.1002/mnfr.201300325.

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1. INTRODUCTION

1.1 Intellectual disability as neuroplasticity disorders

Intellectual disability (ID) is the term used to define developmental disorders characterized by both cognitive and adaptive functioning deficits. This term replaced the use of "mental retardation" in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V). There are currently 4.2 million individuals affected by ID in Europe (Wittchen et al., 2011). IDs are highly prevalent, chronic non-communicable diseases that severely compromise quality of life and that are associated to higher rates of comorbidities such as dementia, depression, autism or epilepsy than the general population, carrying a huge medical, social, and educational burdens.

Recently, a new approach for the classification of mental disorders emerged. It is called the Research Domain Criteria (RDoC) project and attempts to transform diagnosis by building on the findings of Neuroscience and Cognitive Science, rather than relying solely on symptoms, as in the past century (Cuthbert and Insel, 2013). According to this new approach, mental disorders are now addressed as brain disorders, or more specifically as brain circuit disorders. In the case of neurodevelopmental anomalies such as IDs, they are not limited to cognitive systems, but rather affect widely distributed neural networks involved in a broad range of behaviors. One important implication of the RDoC conceptualization is that it encourages the examination of differences (of nature or degree) in common neural circuit disruptions and the study of developmental, environmental, and epigenetic factors underlying phenotypic differences among neurodevelopmental disorders (Borsboom, 2008).

Our group has contributed to the notion that despite the broad spectrum of genetic and environmental aetiologies of ID disorders, there are common neuropathological alterations affecting different

cognitive domains across ID conditions that converge on overlapping underlying molecular networks. We proposed that ID is the consequence of both abnormal postnatal neurodevelopment and incorrect remodeling of the brain circuitry, being neuroplasticity dysfunction the critical underlying cause of those alterations (Dierssen et al., 2003; Dierssen and Ramakers, 2006). Neuroplasticity is the capacity of neurons and neuronal networks to change structurally and functionally in response to the environment and experience. These changes include modifications in the strength of synaptic function, and can induce both transient fluctuations in the efficacy of neurotransmission or long-term changes in the morphology and number of synapses. Different forms of neuroplasticity include, but are not limited to, neurogenesis, synaptogenesis, changes in dendritic arborization and complexity. In multiple ID conditions, dendritic pathology is a common and consistent feature that has been linked to abnormal neuroplasticity. It consists of different alterations affecting dendritic complexity and morphology, and spine density and shape in brain regions such as cerebral cortex and the hippocampus (Kaufmann and Moser, 2000; Dierssen and Ramakers, 2006). These disruptions lead to a suboptimal number of efficient synaptic connections associated to information processing and storage, giving rise to ID.

Accumulative studies have revealed that a large number of genes, that contribute to ID, encode proteins that play an important role in synaptic protein synthesis and neuronal network development signaling pathways (Ramakers, 2000, 2002; Chelly and Mandel, 2001) suggesting that different aetiological factors converge in a common pathogenetic mechanism affecting the communication systems in the brain in IDs. Therefore, we have proposed that neuroplasticity-targeted pharmacologic interventions that increase the brain responsiveness to environment and experience, could exert a significantly favorable biologic effect in different forms of IDs (Benavides-Piccione et al., 2004).

Given that disorders with ID, are lifelong conditions, even relatively small improvements in functional abilities or reductions in co-morbidities such as dementia, would have a significant impact on the well-being of these individuals and the associated care burden and sanitary costs.

1.2 Down syndrome intellectual disability and neuropathology

1.2.1 Intellectual disability in DS.

Down syndrome (DS) is the most common genetic aneuploidy leading to ID. DS results from an extra (full or partial) copy of chromosome 21 (HSA21), which produces the abnormal expression of hundreds of genes and a global genetic imbalance in the brain, leading to suboptimal intellectual functioning.

Virtually all individuals with DS present intellectual disability, although the severity of the cognitive deficits varies from mild to severe, among the DS population (Nadel, 2003). In fact, intellectual quotient (IQ) in people with DS usually falls in the moderate to severe range (IQ = 30–70) (Chapman and Hesketh, 2000; Vicari, 2004; Liogier d'Ardhuy et al., 2015). However, the cognitive deficits in DS are not constant during life but rather appear during early childhood, as a result of maldevelopment, and become more pronounced in adolescence and adulthood, due to cognitive decline (Brown et al., 1990; Carr and Carr, 1995; Vicari, 2004).

Indeed, 3-month-old-infants with DS show contingencies learning abilities equivalent to euploid infants (Ohr and Fagen 1991, 1993), but during early childhood their cognitive capacities go through a linear deceleration associated to developmental delay. Specifically, children with DS exhibit incomplete and delayed acquisition of motor, linguistic, cognitive, and adaptive functions, compared with typically developing children of the same mental age (Hesketh and Chapman, 1998; Chapman and Hesketh, 2001; Silverman, 2007). Although some of these developmental impairments may only reflect delays that are compensated with time, others will affect DS

adults. The cognitive symptoms in adulthood could also be the consequence of malfunction of specific molecular cascades, since gene overexpression is maintained throughout life.

Language ability shows special weaknesses in both children and adults with DS, affecting morphosyntax, explicit verbal working, short-term and long-term memory, although some individuals present rather good vocabulary skills (Tager-Flusberg et al., 1990; Fidler et al., 2005; Dykens et al., 2006; Lott and Dierssen, 2010).

In relation to the memory profile, several studies have reported that children and adults with DS show rather preserved visuospatial working memory (by Corsi block span) as opposed to verbal working memory (by digit span) (Edgin et al., 2010; Conners et al., 2011). This has been attributed to a specific deficiency in the capacity to process verbal information, possibly related to alterations in the phonological loop (short-term phonological store or articulatory rehearsal component) while preserving a rather intact visuospatial sketchpad (Laws 2002). However, during the recent years, the strength of visuospatial memory abilities in DS has been called to question (Yang et al., 2014).

Beyond immediate memory, children and adults with DS also show deficits in their ability to create and retain new lasting memories for facts and events (declarative memory) including visuospatial and contextual information (Carlesimo et al., 1997; Pennington et al., 2003; Visu-Petra et al., 2007; Lavenex et al., 2015). Difficulties in both the acquisition of information (learning), and the long-term storage and retrieval of information (memory) are a critical part of the phenotype in DS (Nadel, 2003). These deficits are critically incapacitating in everyday life as it is involved with a large number of activities from self-care to socialization and independent functioning. Neuropsychological assessment tools operationalize these memory deficits in individuals with DS, through the use of standardized tests in which striking difficulties are specifically shown for example in in immediate memory for patterns (CANTAB Pattern Recognition Memory, PRM) and paired associative learning of object and location (CANTAB PAL). The deficits on these tasks

have been linked to the function of the medial temporal lobe (MTL) including brain structures adjacent to the hippocampus (Heuer and Bachevalier, 2011a, 2011b).

In addition to the characteristic cognitive impairment during development and early adult life, individuals with DS present a higher incidence and earlier onset of Alzheimer's disease (AD)-like cognitive decline and dementia than the general population (Ballard et al., 2016). Despite the striking difficulties in diagnosing dementia in people with premorbid ID, several studies have reported that individuals with DS older than 40 years show a rapid and progressive cognitive decline resembling the cognitive profile found in sporadic AD. Individuals with DS aged 40–49 years present a particularly marked cognitive impairment, with a prevalence of up to 55%, while in people aged 60–69 years, prevalence raises up to 77% and virtually all individuals aged 70 years or older (Hartley et al., 2014; Ballard et al., 2016). The age-associated symptoms include specific decays in attention, recall, explicit memory and receptive language, confusion, visuospatial disorganization and disorientation. Further non-cognitive symptoms affect personality and behavioral traits and involve apathy, lack of motivation, stubbornness, impulsivity, and executive dysfunction (Holland et al., 1998, 2000; Wiseman et al., 2015). These over-imposing cognitive and behavioral deficits result in additive difficulties in their self-management or in complete dependency on their caregivers (Visser et al., 1997; Holland et al., 1998, 2000; Coppus et al., 2006; Lott and Dierssen, 2010).

1.2.2 Neuropathology in DS

The underlying neurobiological alterations that give rise to cognitive impairment in people with DS are diverse and while some aspects are present throughout the lifespan, others appear at specific temporal windows.

During the gestation period, early neurodevelopmental alterations include reductions in brain size that are already present in 4–5 month old DS fetuses (Guihard-Costa et al., 2006). Additional

disruptions in late gestational stages comprise reductions in neurogenesis, defects in neuronal maturation and migration associated to defective neocortical lamination, abnormalities in neurotransmitter systems, mitochondrial function and protein expression (Bar-Peled et al., 1991; Golden and Hyman, 1994; Busciglio et al., 1995; Contestabile et al., 2007).

Despite those early neurodevelopmental defects, at the time of birth gross neuroanatomical and neuroarchitectural aspects, such as brain shape and weight, proportion between cerebral lobes, size of cerebellum and brainstem, and neuronal dendritic branching, are relatively indistinguishable between DS and euploid brains (Takashima et al., 1981; Wisniewski, 1990; Vuksić et al., 2002). In fact, even greater dendritic branching has been reported in newborn babies (younger than 6 months of age) (Becker et al., 1986). However, as early as 3-5 months of age clear alterations start appearing in DS brains involving brachycephaly (shorter antero-posterior diameter and broader parietal lobe), myelination delay, reduction in neocortical neuronal densities, synaptic density distribution and synaptic length (Wisniewski 1990). During early development and childhood, DS brains show a steady reduction in neuronal dendritic number, dendritic branching complexity and dendritic spine density below euploid levels in the cortex and the hippocampus (Marin-Padilla, 1976; Suetsugu and Mehraein, 1980; Becker et al., 1986; Schulz and Scholz, 1992). The development of dendritic abnormalities follow a complex temporal sequence, being acquired at early stages and progressively increasing with age towards a more pronounced dendritic simplification of density and morphology (Takashima et al., 1989, 1994; Ferrer and Gullotta, 1990). Due to the rather (although not completely) normal brain phenotype in DS at birth, and the subsequent accumulation of brain alterations with age, some researchers have claimed that DS main dysfunctions are due to alterations in the postnatal development (Sheppard, 1987) or to a premature neurodegenerative process (Hardy and Selkoe, 2002) in systems such as the prefrontal cortex, the hippocampus and the cerebellum.

The dendritic atrophy seen in DS is of particular importance since dendrites, and especially dendritic spines, represent the main receptive structures of neurons and constitute the postsynaptic site for glutamatergic neuronal contacts. These are essential structures for brain connectivity and experience-dependent neuroplasticity and play a critical role in learning and memory processes (Sorra and Harris, 2000; Kasai et al., 2003; Newpher and Ehlers, 2009).

The characteristic memory deficits found in individuals with DS have been particularly associated to disruptions of the functional integrity of the hippocampus and related structures of the medial temporal lobe (MTL) across their whole lifespan. These brain areas have been long known to be responsible of processes that involve information acquisition, encoding, and retrieved (Burgess et al., 2002). Once information has been processed by the hippocampus, it is transferred to neocortical association areas for further processing and permanent storage (Kandel et al., 2014). The hippocampus is extensively innervated by diverse modulatory inputs that originate in several neuronal populations including basal forebrain cholinergic neurons (BFCNs), norepinephrine (NE)–containing neurons of the locus coeruleus (LC), serotoninergic neurons of the raphe nuclei, and calretinin-positive neurons of the supramammillary area. In DS brain, neuroimaging studies using structural magnetic resonance imaging (MRI) have revealed disproportionately smaller volumes in temporal areas with specific decrease in the size of the hippocampus and a converse increase in the parahippocampal gyrus (i.e., perirhinal and entorhinal cortices) (Kesslak et al., 1994; Raz et al., 1995; Aylward et al., 1999; Pinter et al., 2001; Teipel et al., 2003; White et al., 2003). The reduced hippocampal volumes are associated to decreased neuronal densities and less dendritic branching, length, and spine densities within the hippocampus (Suetsugu and Mehraein, 1980; Guidi et al., 2008). Recently, functional MRI studies have reported additional disruptions in neuronal network synchrony in DS brains (Anderson et al., 2013) that may also affect the functional connectivity of the hippocampus with other brain regions.

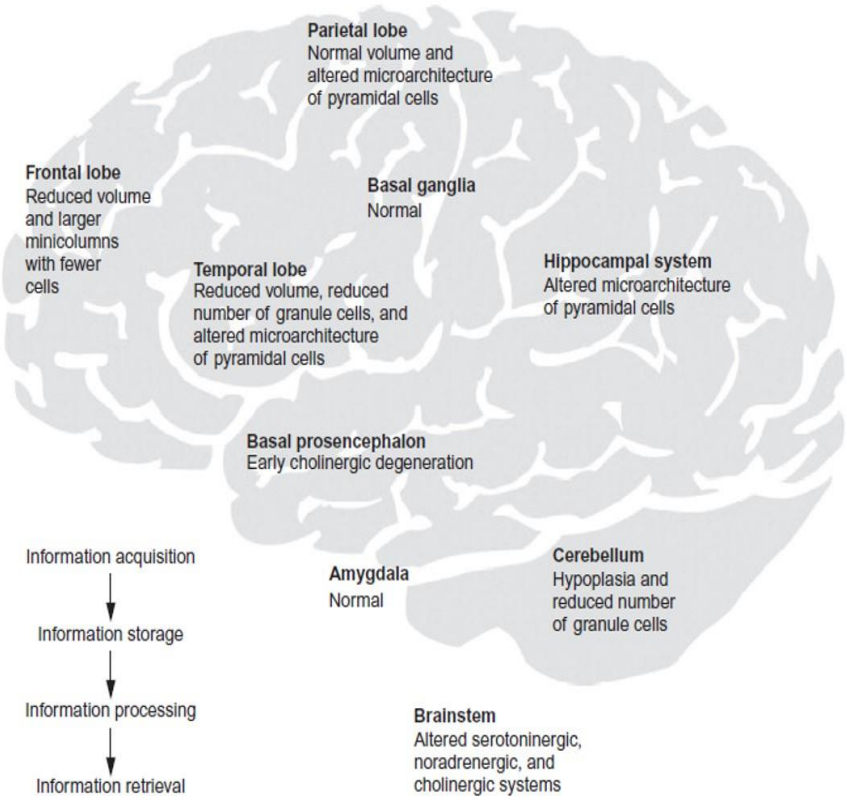


Fig. 1 Structures involved in dysfunction in individuals with DS (Lott and Dierssen, 2010)

Furthermore, widespread alterations in various neurotransmitter systems have been found in DS brains, including the serotonergic, noradrenergic, cholinergic, glutamatergic and GABAergic systems, suggesting the existence of profound alterations of neuronal network activity (Mann et al., 1985; Yates et al., 1986; Godridge et al., 1987; Risser et al., 1997; Whittle et al., 2007). During the recent years special attention has been devoted to the excitatory-inhibitory systems, since cumulative data suggest that disruptions in their balance in different brain regions can be associated to alterations in neuroplasticity and lead to cognitive impairment in different forms of ID including DS (Baroncelli et al., 2011). The fact that individuals with DS present increased seizure activity supports the

idea that the excitatory-inhibitory systems (Menéndez, 2005; De Simone et al., 2010). At the molecular level reduced γ-aminobutyric acid (GABA) levels in fetal frontal cortex (Whittle et al., 2007) and in temporal lobes of children with DS (Śmigielska-Kuzia et al., 2010) have been reported. Such decreased GABA levels have also been detected in postmortem adult brain studies along with reductions in the number of calbindin and parvalbumin interneurons in the cerebral cortex (Reynolds and Warner, 1988; Kobayashi et al., 1990; Seidl et al., 2001). Additionally, an *in vitro* study with human neural progenitor cells found increased α2 and decreased α5 and β3 GABAAR-subunit expression (Bhattacharyya et al., 2009). Regarding the levels of glutamate and other excitatory neurotransmitters, findings appear to show a decrease in adult DS brains although with some inconsistencies in different subregions and at different ages (Yates et al., 1986; Risser et al., 1997; Seidl et al., 2001; Tan et al., 2014). Thus, it has been hypothesized that DS brains bear general alteration in excitatory-inhibitory signaling, although it is not clear yet whether this alteration is due to a decrease or an increase in glutamatergic or GABAergic transmission in different brain regions or a dynamic imbalance between both systems.

This hypothesis is sustained by data coming from the AD field that suggest that increased oxidative stress can lead to excessive glutamatergic tone inducing glutamate-mediated excitotoxicity, which can contribute to neuronal loss in AD (Rissman and Mobley, 2011). In DS the overdosage of the HSA21 gene that encodes the Cu/Zn superoxide dismutase (SOD1) leads to oxidative stress, which is the imbalance between the production and elimination of toxic species of free oxygen and nitrogen radicals and their reactive metabolites. In conditions of oxidative stress the excessive reactive metabolites lead to the oxidation of biomolecules such as lipids, proteins and nucleic acids, resulting in the damage or change of the function of different cells in the organism. Indeed prenatal DS brains present higher levels of reactive oxygen species accompanied by consequent cellular damage (Lott, 1982, 2012; Busciglio et al.,

1995; Muchová et al., 2014). The increased activity of SOD1 leads not only to excessive production of hydrogen peroxide but also to an imbalance in the concentration of metal ions, especially Cu and Zn. Mitochondria are critically susceptible to oxidative stress, since they contain enzymes for ATP production, calcium homeostasis, and apoptotic signaling, and, in fact, different DS cell types have shown mitochondrial dysfunction both at early in life and during the AD-like aging process (Valenti et al., 2011; Coskun and Busciglio, 2012). Therefore, increased oxidative stress is a relevant phenotype in DS and involves a currently open field of investigation in the search for genotype–phenotype correlations for this disorder (Tiano and Busciglio, 2011.; Perluigi et al., 2011; Helguera et al., 2013; Butterfield et al., 2014; Valenti et al., 2014).

Most of the above brain alterations, are present across the whole life span of DS individuals, but they also present a number of lesions and molecular disruptions, including the development and accumulation of amyloid-β plaques and neurofibrillary tangles (NFTs), that resemble the neuropathology found in AD patients. These lesions become more pronounced as individuals age and are associated to the characteristic progressive cognitive decline displayed by aged people with DS. The Hsa21 gene encoding for the amyloid precursor protein (APP) is thought to play a key role in the development of these neuropathological alterations by increasing the levels of amyloid-β (Aβ), a cleavage product of APP, that misfolds and accumulates in the brain of both people with DS and AD (Prasher et al., 1998; Selkoe, 2001). However this accumulation occurs much earlier in DS. As early as 21 weeks of gestational age, soluble Aβ42 is detected in DS brains (Teller et al., 1996) while during childhood, diffuse deposits of Aβ are already displayed (Lemere et al., 1996). In adult DS patients, plasma levels of Aβ42 are increased (Schupf et al., 2007) and approximately by the age of 30-40, all DS subjects present amyloid-β plaques and neurofibrillary tangles (NFTs) (Wiseman et al., 2015). Intraneuronal amyloid-β can be detected by radiolabelled Pittsburgh compound-B

(PiB) through positron emission tomography (PET) neuroimaging neuroimaging (Handen et al., 2012). As in AD, its toxic accumulation could trigger a cascade of neurodegeneration by increasing the generation of free radicals which expands the burden of oxidative stress, disrupting calcium homeostasis, which contributes to the phosphorylation of tau protein and the formation of NFT, and eventually leading to neuronal death (Hardy and Higgins, 1992; Hardy and Selkoe, 2002; Zigman and Lott, 2007). The formation of amyloid-β requires APP to be cleaved by the two proteases β- and ɣ-secretase. A third protease, α-secretase, contributes to the non-amyloidogenic processing of APP by cleaving APP within the amyloid-β domain thus avoiding amyloid-β generation, and by generating an inhibitor of ɣ-secretase (Tian et al., 2010). The activity of α-secretase is mediated by ADAM10 (Kuhn et al., 2010), whose overexpression has shown to induce increased the cortical synaptogenesis (Prinzen et al., 2009). Therefore, an increase in α-secretase activity has been proposed as therapeutic strategy for DS and AD (Lichtenthaler, 2011). Some gene polymorphisms have been shown to modulate the risk for AD neuropathology and dementia, such as the gene encoding for apolipoprotein E (APOE), which is a lipid and cholesterol transporter (Herz and Beffert, 2000). The APOE ε4 allele is associated with greater Aβ deposition, as well as with earlier onset and increased risk of AD dementia both in the general population and in individuals with DS, whereas the APOE ε2 allele leads to reduced Aβ deposition and a lower risk of disease (Herz and Beffert, 2000; Schupf and Sergievsky, 2002; Wiseman et al., 2015).
Additionally, aged individuals with DS show a superimposing dendritic atrophy that increases the pre-existing developmental dendritic abnormalities (Takashima et al., 1989, 1994; Ferrer and Gullotta, 1990; Teipel and Hampel, 2006). Indeed, dystrophic neurites have been found in the hippocampus among other brain regions (Iyer et al., 2013).
A gross neuroanatomical reduction has also been reported by MRI studies in aged non-demented DS patients showing volume loss of

the medial temporal lobe, including hippocampus amygdala (Kesslak et al., 1994; Krasuski et al., 2002; Teipel et al., 2004; Haier et al., 2008; Beacher et al., 2010) and neocortical areas such as corpus callosum, parietal, frontal and occipital cortices (Teipel et al., 2003, 2004), consistent with prodromal stages of Alzheimer-type pathology (Teipel and Hampel, 2006). Similarly to the neurodegenerative profile in AD, brains from demented individuals with DS have shown a considerable atrophy in total brain and a greater volume loss specifically in hippocampus, orbitofrontal cortex and the parietal cortex, accompanied by an enlargement of the ventricles (Kesslak et al., 1994; Koran et al., 2014). Additionally, detection of fluorodeoxyglucose (FDG), a marker for glucose uptake, by PET has also demonstrated a global decrease in cerebral glucose utilization and parietal hypometabolism in demented individuals with DS (Devinsky, 1990).Furthermore, despite the fact that brains from young children with DS display no alterations in cholinergic enzymes (such as choline-acetyltransferase and acetylcholinesterase) (Becker et al., 1991; Lubec et al., 2001), during late adolescence and adulthood the basal forebrain cholinergic neurons (BFCN) that project to the hippocampus, become susceptible to atrophy and degeneration, along with a decreased activity of choline-acetyltransferase (ChAT), as happens in AD (Yates et al., 1983; Casanova et al., 1985; Mann et al., 1985; Mufson et al., 2003; Contestabile et al., 2008). As acetylcholine is involved in attention, learning, and synaptic plasticity (Everitt and Robbins, 1997; Hasselmo, 2006; Micheau and Marighetto, 2011), the degeneration of BFCN is thought to critically contribute to cognitive decline and memory deficits in both DS and AD, since it leads to the withdrawal of cholinergic input to the hippocampus, disrupting its neuromodulation. Accordingly, different interventions targeting the cholinergic system, such as acetylcholinesterase inhibitors, have been used for the development of cognitive enhancers to combat dementia in individuals with DS and AD and also in cases of sporadic AD (De la Torre and Dierssen, 2012).

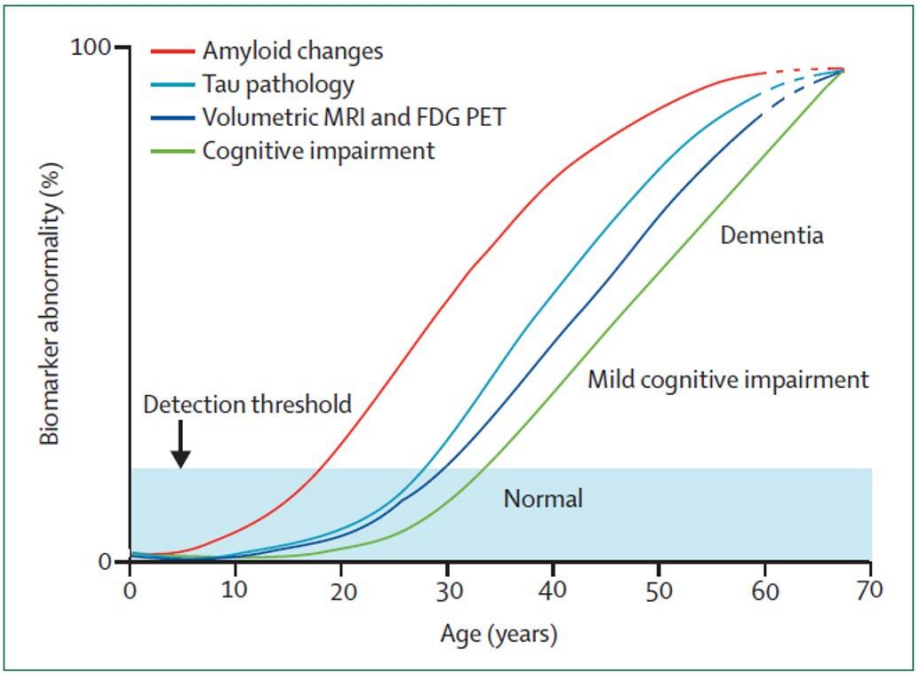


Fig. 2: Hypothetical model for amyloid changes, tau pathology, and appearance of biomarkers on volumetric MRI and fluorodeoxyglucose (FDG), and development of cognitive decline and dementia in people with DS. Of note, the occurrence of these symptoms is dramatically earlier that in sporadic AD (Ballard et al., 2016).

Altogether, DS encompasses the emergence of multiple cognitive and neuropathological alterations presented in a complex temporal sequence with distinct dynamics starting at the initiation of development and continuing throughout life (illustrated in Figs 1 and 2).

1.3 Mouse models to study DS and elucidate targets for intervention

Initially, the predominant research strategy in the field of DS was to analyze postmortem human tissue, or biochemical indicators in blood. This approach only provided a snapshot of the problem with with no opportunity to manipulate the system. Subsequently, the generation of mouse models with genetic triplications of different length of murine chromosomal regions homologous to Hsa21 (shown in Table 1), and also transgenic mice overexpressing specific candidate genes enabled the study of genotype-phenotype relationships and putative pathogenic mechanisms of the disorder. The sequencing of mouse and human genomes by the beginning of the 21st century confirmed syntenic conservation between mouse and human, and revealed that Hsa21 is homologous to three different regions of the mouse genome, in three different chromosomes (Mmu16, Mmu17, and Mmu10).

Mouse model	Mouse chromosomes affected	HSA21 syntenic regions
Ts65Dn (Davisson et al., 1990)	MMU16, subcentromeric region of MMU17	Region spanning from *Mrpl39* to *Zfp295*; MMU 17 trisomic region not syntenic to any HSA21 region
Ts1Cje (Sago et al., 1998)	MMU16	Region spanning from 21q22.1 to 22.3
Ts1Rhr (Olson et al., 2004a)	MMU16	"Down Syndrome Critical Region" (DSCR); spanning from *cbr3* gene to *mx2* gene on chr21 (~21q22.2–22.3)
Ts1Yah (Pereira et al., 2009)	MMU17	*Abcg1-U2af1* telomeric region
Tc1 (O'Doherty et al., 2005)	N/A	Most of HSA21 inserted through microcell-mediated chromosome transfer; regions inserted bound by markers CXADR and D21S1922, and IFNAR1 and RUNX1
Dp(16)1Yu (also see Ts1Yu, Dp(16)1Yey) (Li et al., 2007)	MMU16	Large region spanning from 21q11.2 to 22.3
Dp(16)1Yey/+, Dp(17)1Yey/+, Dp(10)1Yey/+ (Yu et al., 2010)	MMU16, MMU17, MMU10	Distal portion of 21q22.3 (MMU10); proximal portion of 21q22.3 (MMU17); region spanning from 21q11.2 to 22.3 (MMU16)

Table 1: Mouse models of Down syndrome and descriptions of their genetic alterations (Edgin et al., 2012)

These models mimic specific aspects of the disease and their genetic dependency, enabling to evaluate the phenotypic and mechanistic similarity to humans with DS. However, none of them fully reproduce the human disorder.

1.3.1 The Ts65Dn mouse model of DS

In the early 1990s, Murien Davisson created the first genetic mouse model for DS, the Ts65Dn strain. A radiation-induced chromosomal rearrangement generated a spontaneous and unplanned reciprocal translocation of the telomere proximal region of Mmu16 to the centromere and pericentromeric region of Mmu17 (17^{16}). Thereafter missegregation of this translocated chromosome in female mice gave rise to partial trisomic pups carrying the extra 17^{16} chromosome (Davisson et al., 1990; Reeves et al., 1995). It demonstrated that a similar trisomy in mouse and human provides similar structural and functional outcomes (Reeves et al., 1995). The Ts65Dn mouse bears segmental trisomy for a distal region of Mmu16 that contains approximately 55% of Hsa21 conserved genes (Davisson et al., 1990) (illustrated in Fig. 3).

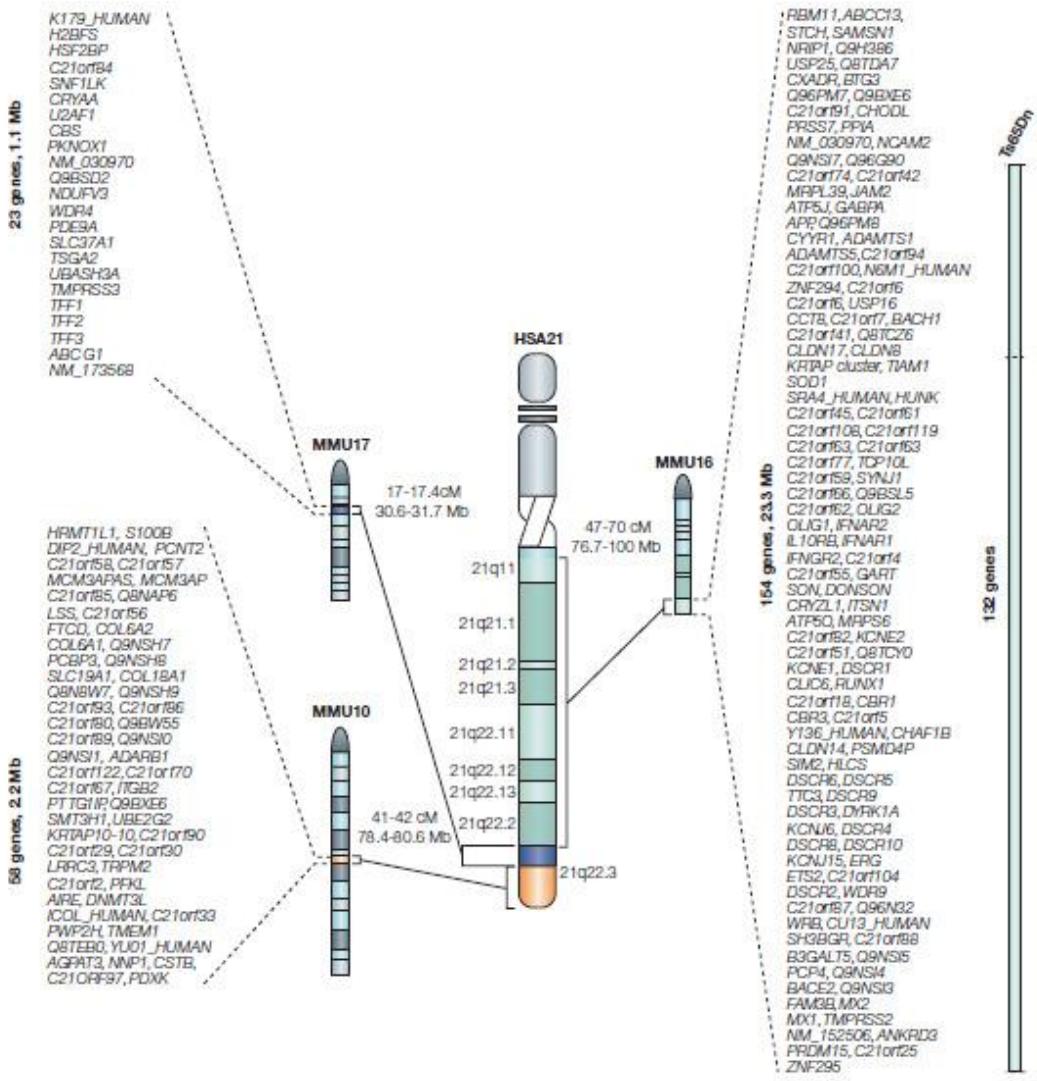


Fig. 3: Comparative genetic maps of degree of conservation between Hsa21 and Mmu 16, 17 and 10 (Adapted from Antonarakis et al., 2004).

However, this model is also trisomic for approximately 60 Mmu17 genes that are non-orthologous to Hsa21 (Duchon et al., 2011) and lacks a number of Hsa21 orthologous genes from Mmu10 and Mmu17, which has questioned its construct validity[1]. Additionally, as males are sterile, mice are generated from Ts65Dn dams which causes problems in the pups due to inadequate fostering, independently of pups genotype. Being aware of its limitations, the Ts65Dn mouse model is still a highly valuable tool both for the investigation of genotype-phenotype relationships and for the identification of potential therapeutic interventions for DS, and its behavioral and structural phenotype has been thoroughly described along the last decades, with special emphasis on learning and memory.

Extensive research has demonstrated its face validity[2]. Like DS individuals, Ts65Dn mice present early learning and memory impairment and age-related cognitive decline associated to cholinergic neurodegeneration that arises approximately at the age of 6-8 months. Young adult Ts65Dn mice have shown impaired performance in a behavioral test for hippocampal-dependent spatial learning and long-term spatial memory in rodents, the Morris water maze (MWM), both during the learning phase and in the recall test (Escorihuela et al., 1995; Reeves et al., 1995; Holtzman et al., 1996). Young Ts65Dn also present spatial working and reference memory impairment in the radial arm maze (RAM), a task that requires both working memory (WM) when retaining information for a very short time and reference memory when retaining memory for longer times. This task takes place in complex spatial environment and animal performances are measured by counting

[1] Construct validity points to the degree of similarity between the mechanisms underlying behavior in the model and that underlying the behavior in the condition, which is being modeled.

[2] Face validity is the degree of descriptive similarity between, for example, the behavioral dysfunction seen in an animal model and in the human affected by a particular neurobehavioral disorder.

errors. Ts65Dn mice make fewer correct choices than WT mice and perform at (or near) chance levels (Demas et al., 1996). In the passive avoidance test, a one trial fear-motivated avoidance task in which the mouse learns to refrain from stepping down from a platform or stepping through a door to an apparently safer but previously punished dark compartment, Ts65Dn mice are able to learn the task (Coussons-Read and Crnic, 1996; Holtzman et al., 1996), although this strain presents a high variability among individual mice (Holtzman et al., 1996). Between 4 and 8 months of age, Ts65Dn mice show a decrease in performance in spatial learning and reversal, but not visual discrimination learning and reversal (Granholm et al., 2000a; Hunter et al., 2003), and at 6 months old they exhibited impairments in working and reference memory as assessed on a water radial-arm maze (Bimonte-Nelson et al., 2003; Hunter et al., 2003).

Accumulative data indicates that these cognitive deficits in Ts65Dn are associated with a number of neuro-morphological and synaptic alterations in learning and memory brain regions, which are trisomy driven. At the macroscopic level, Ts65Dn mice show craniofacial dysmorphology and brachycephaly (Richtsmeier et al., 2000; Reeves et al., 2001). Reduced volumes have been reported in regions similar to those affected in DS, such as the cerebellum, but other regions are preserved. For example, Ts65Dn mice present normal total hippocampal volume (Insausti et al., 1998; Lorenzi and Reeves, 2006). However, they show an age-dependent reduction in CA2[3], the hilus and granule cellular layer of DG[4] (Insausti et al., 1998; Lorenzi and Reeves, 2006; Contestabile et al., 2007).

[3] Cornus Ammonis 2 is a relatively small area between CA3 and CA1 that forms the nexus of the disynaptic circuit linking EC input with CA1 output. Recent data suggest the it is involved with social memory (Hitti and Siegelbaum, 2014)

[4] Dentate Gyrus (DG) plays a key reole in spatial memory participating in pattern completion and separation processes. It encompasses granule cells that receive input from the entorhinal cortex and project their axons to CA3 forming the the mossy fibers (Deng et al., 2010).

Neuronal density is also compromised, being increased in CA3[5] and decreased in CA1[6] and DG (Insausti et al., 1998; Kurt et al., 2004). Despite the rather intact hippocampal volume at initial stages, the neuro-architecture is altered both in hippocampal and neocortical pyramidal and granule neurons in Ts65Dn mice. Specifically, these neurons display shorter and less branched dendrites and reduced spine density and increased spine size, which is paralleled by an increased size of presynaptic terminals (Dierssen et al., 2003; Belichenko et al., 2004, 2007). CA3 hippocampal region shows a hyperconnectivity of active associational connections (Hanson et al., 2007).

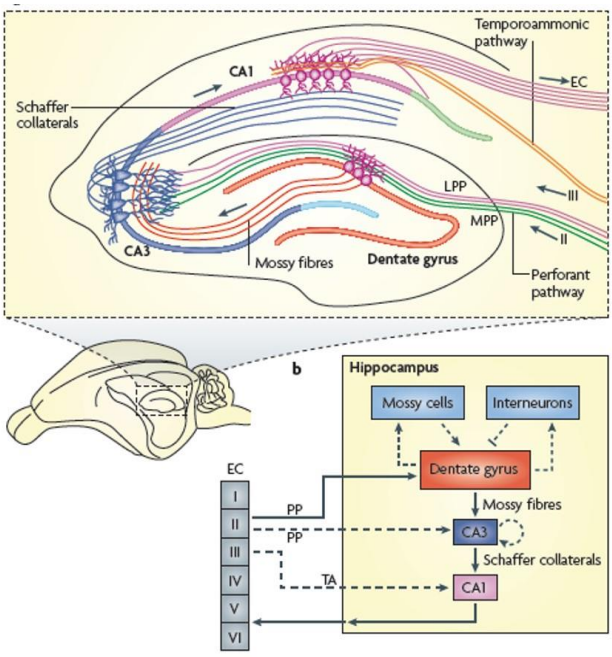


Fig. 4. Diagram of the neural circuitry in the rodent hippocampus depicting the performant pathway, including the trisynaptic flux of information [entorhinal

[5] Cornus Ammonis 3 is thought to encode the memory trace through its auto-associative networks. It relays information to CA1 pyramidal neurons through Schaffer collaterals (Deng et al., 2010).

[6] Cornus ammonis 1 pyramidal neurons send back-projections into deep-layer neurons of the entorhinal cortex (EC) and receives direct input from EC layer III neurons, through the temporoammonic pathway (Deng et al., 2010)

cortex (EC)–dentate gyrus (DG)–CA3–CA1–EC] and the temporoammonic pathway [EC layer III–CA1–EC layer V, VI] (Deng et al., 2010).

These alterations at the neuromorphological and connectivity levels, possibly lead to dysfunctions in the flow of information (Fig. 4) and in computational processes required for learning and memory, such as pattern completion and separation[7]. In agreement with that, Ts65Dn also display disruptions in experience-dependent structural neuroplasticity, suggesting a compromise in the flexibility of neuronal and behavioral shaping as a function of varying environments (Martínez-Cué et al., 2002; Dierssen et al., 2003).
Those microstructural defects in Ts65Dn mice are accompanied by alterations in the proportion of excitatory-inhibitory synapses, being the density of asymmetric (excitatory) synapses reduced in DG, CA1 and CA3 regions, while the symmetric (inhibitory) synapses are decreased only in the DG (Kurt et al., 2004). Also the synaptic apposition length in symmetric (inhibitory) synapses in the DG is increased, suggesting enhancement of inhibition in specific brain regions (Belichenko et al., 2009), that seems partially in contradiction with the findings in humans described above. However, it has to be considered that human studies are mainly in neocortical regions, while the Ts65Dn has been mainly studied at the hippocampal level that may explain the discrepancies. The above imbalance in excitatory-inhibitory synapses has functional consequences at the electrophysiological level, that affect GABAergic and glutamatergic transmission, and lead to defects in long-term potentiation (LTP) and long-term depression (LTD) (Siarey et al., 1999; Kleschevnikov et al., 2004; Costa and Grybko, 2005).

[7] Pattern separation and completion are complementary processes that have been proposed to take place in the hippocampus and other brain regions in order to encode and retrieve information in the form of memories or engrams. The system encodes similar input patterns into separated orthogonal representations, so that unique non-overlapping memories are created. At the time of retrieval, completion enables to recall stored activity patterns from partial or degraded cues (Hunsaker and Kesner, 2013; Josselyn et al., 2015).

In addition, the activity levels of reactive oxygen species (ROS) are increased in the cortex from male 7,5 months old Ts65Dn mice and correlate with working memory deficits observed in the MWM (Lockrow et al., 2009), and adult Ts65Dn hippocampal neural progenitor cells (NPCs) present alterations in the mitochondrial biogenesis and bioenergetics (respiration and ATP production) leading to a reduced cell energy status (Valenti et al., 2016). This highlights the role of oxidative stress and mitochondrial dysfunction in the memory decline in the Ts65Dn mouse.

Furthermore, like in humans with DS and AD, Ts65Dn mice show age-dependent cognitive decline (Granholm et al., 2000b; Hyde and Crnic, 2001) that is linked to abnormal neuronal processes such as enlarged early endosomes (Cataldo et al., 2003), dysfunction of retrograde transport of neural growth factor (NGF) from the hippocampus to the basal forebrain (BF) (Salehi et al., 2006), and gradual cholinergic neuronal loss in the basal forebrain (Holtzman et al., 1996; Granholm et al., 2000b; Cooper et al., 2001; Seo and Isacson, 2005). BFCN degeneration that is initiated at 5-6 months of age and progress up to 20 months of age (Holtzman et al., 1996; Granholm et al., 2000a; Hunter et al., 2004; Lockrow et al., 2009). As a compensatory mechanism of this loss of BFCN 10-month old Ts65Dn mice show increased ChAT activity in the cortex and hippocampus (Cooper et al., 2001; Seo and Isacson, 2005; Contestabile et al., 2006; Chen et al., 2009).

Due to the large overlap of features between Ts65Dn mouse model and humans with DS (as partially shown in Fig. 5), this mouse is, at the moment, the only model used in preclinical studies to assess therapies for DS (Gardiner, 2015).

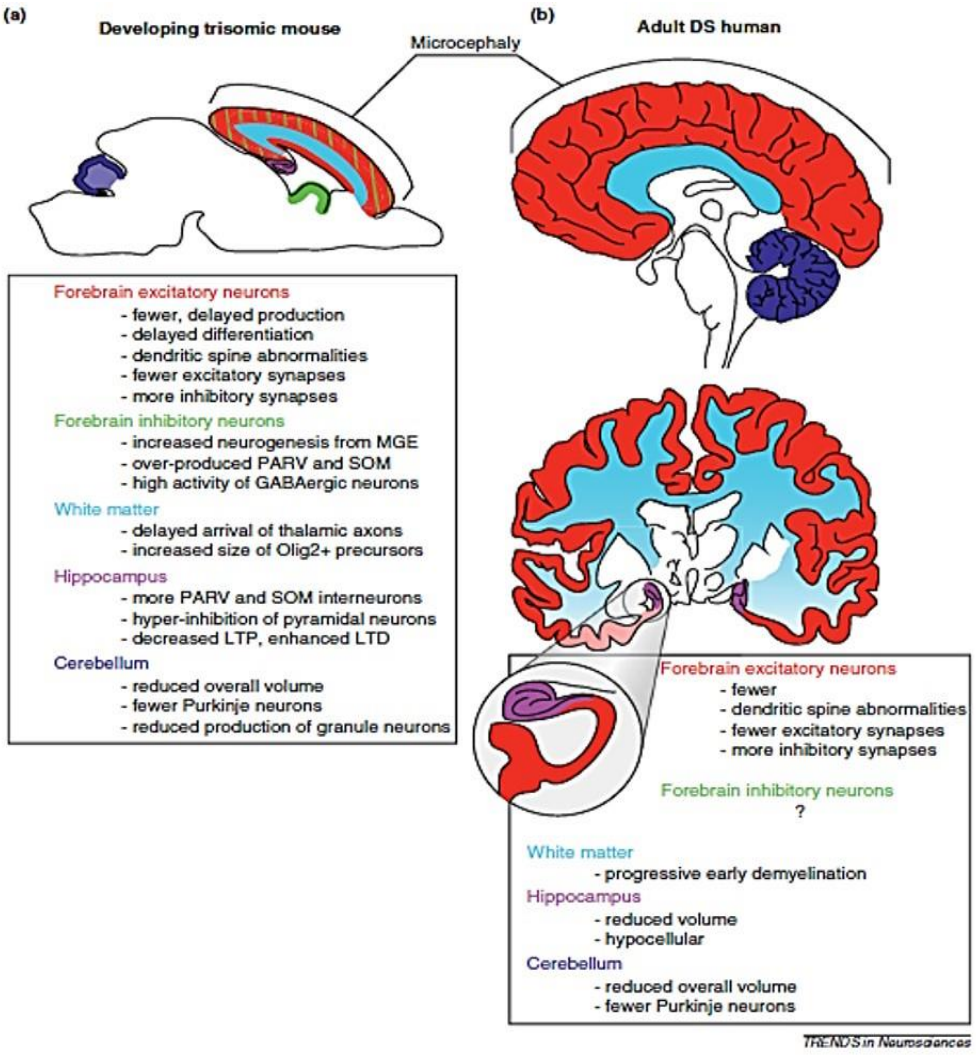


Fig 5: Comparison of altered cellular and anatomical alterations observed in prenatal brain from Ts65Dn mouse model and postnatal human DS brain (Haydar and Reeves, 2012).

1.4 Candidate genes to explain the neurological and cognitive phenotypes in DS

Over the last years substantial research has revealed the biological relevance of specific Hsa21 genes that play a fundamental role in

the pathogenesis of neurological and cognitive phenotypes of DS across life, which have been proposed as targets for therapeutic intervention for the disorder. Two of these genes encode the dual specificity tyrosine-phosphorylation regulated kinase 1A (Dyrk1A) and the amyloid precursor protein (APP).

1.4.1 Dyrk1A gene

Dyrk1A gene is located in the long arm of Hsa21 (Guimerá et al., 1996; Song et al., 1996) and is orthologous to the drosophila minibrain (MNB) gene. DYRK1A is a kinase that catalyzes both its autophosphorylation on a tyrosine residue in the activation loop (Himpel et al., 2001) and the phosphorylation of serine and threonine residues in its substrates (Kentrup et al., 1996; Becker et al., 1998). Through the phosphorylation of multiple targets DYRK1A is implicated in diverse biological processes that are critical in DS and AD such as neurodevelopment, neuroplasticity and neurodegeneration. In fact, Dyrk1A expression and activity are increased in cerebral cortex, white matter and hippocampus in both individuals with DS and sporadic AD, and also in other neurodegenerative diseases including Parkinson's, Huntington's, and Pick's disease (Ferrer et al., 2005; Dowjat et al., 2007; Kimura et al., 2007; Ryoo et al., 2007; Liu et al., 2008; Wegiel et al., 2011). In the euploid human brain, DYRK1A is expressed both in the nucleus and in the cytoplasm of neurons and astrocytes with a reduction across life (Wegiel et al., 2004; Kida et al., 2011). In vertebrates DYRK1A is expressed since prenatal brain development in a specific sequence of distinct temporal and subcellular patterns starting in neural progenitor cells and finishing in neuronal dendritic tree and synapses (Hämmerle et al., 2008). During neuronal differentiation DYRK1A expression translocates from the neuronal cytoplasm to the nucleus and differential expression of Dyrk1A is also found across the cell cycle, suggesting its involvement in neuronal differentiation. Indeed, DYRK1A modulates different signaling pathways by phosphorylating substrates that trigger effects in cell proliferation, cell cycle progression of progenitor

cells and neuronal differentiation (Tejedor and Hämmerle, 2011). For instance DYRK1A regulates the transcriptional activity of glioma-associated oncogene 1, a major effector of sonic hedgehog (SHH) signaling, which is a key pathway in the regulation of proliferation during vertebrate central nervous system development (Ruiz i Altaba et al., 2002). In addition, DYRK1A phosphorylates REST/NRSF chromatin remodeling complex and NOTCH receptor, contributing to cell fate definition of pluripotent embryonic stem cells and neuronal progenitors (Canzonetta et al., 2008; Fernandez-Martinez et al., 2009).

DYRK1A also plays a key role in structural and synaptic plasticity processes such as neurite formation, dendritic growth and synaptic vesicle trafficking through the phosphorylation of proteins such as the transcription factor cAMP responsive element binding (CREB) (Yang et al., 2001), cytoskeleton-related proteins, MAP1B, GSK3β, N-WASP and β-tubulin (Scales et al., 2009; Park et al., 2012; Ori-McKenney et al., 2016), and components of the endocytic protein complex machinery, amphiphysin, dynamin 1, endophilin 1 and synaptojanin 1 (Chen-Hwang et al., 2002; Hammerle et al., 2003; Murakami et al., 2006, 2009).

The role of Dyrk1A in neuronal differentiation and neuroplasticity is supported by the fact the truncation of its sequence in humans causes microcephaly (Møller et al., 2008) while Dyrk1A knockout mice are embryonically lethal, and Dyrk1A heterozygous mice show decreased viability and region-specific reductions in brain size. In addition, cortical pyramidal neurons from both Dyrk1A heterozygous and transgenic mice overexpressing Dyrk1A (TgDyrk1A) are smaller, less branched and have a reduced number of spines than control littermates suggesting its dosage-dependent function (Altafaj et al., 2001; Benavides-Piccione et al., 2005; Martinez de Lagran et al., 2012). Furthermore, TgDyrk1A mice exhibit altered hippocampal LTP and LTD associated with learning and memory defects (Ahn et al., 2006). Interestingly, inhibition of DYRK1A activity using harmine rescues the neurite outgrowth

impairment in TgDyk1A primary cortical cultures (Martínez de Lagran et al 2012).

Furthermore, DYRK1A is involved with neurodegenerative processes through its participation in the phosphorylation and alternative splicing regulation of key AD-associated proteins such as tau and APP. DYRK1A directly contributes to NFT formation by the phosphorylation of tau (Woods et al., 2001) and also has an indirect role through the promotion of GSK-3β activity upon tau (Liu et al., 2008; Azorsa et al., 2010). Moreover, Dyrk1A phosphorylates RCAN1 (regulator of calcineurin-1) thereby enhancing its ability to inhibit the phosphatase activity of calcineurin (Caln), leading to reduced NFAT transcriptional activity and enhanced tau phosphorylation (Jung et al., 2011). DYRK1A also regulates tau alternative splicing through the phosphorylation of alternative splicing factor (ASF) which controls splicing of many transcripts. Accordingly, when Dyrk1A is overexpressed it can lead to disruptions in splicing, which causes an imbalance between 3R and 4R isoforms of tau and promotes NFT formation, as seen in AD (Deshpande et al., 2008; Toiber et al., 2010). This is supported by the several-fold increases in the number of DYRK1A-positive and 3R-tau-positive NFTs in DS (Wegiel et al., 2011). On the other hand, DYRK1A phosphorylates APP (Ryoo et al., 2008) and presenilin 1 (PS1), a key component of the ɣ-secretase (Ryu et al., 2010) contributing to increases in the proteolytic cleavage of amyloid precursor protein (APP) and elevated Aβ40 and Aβ42 in DS and AD. Additional research suggests a positive feedback mechanism through which Aβ stimulates the expression of DYRK1A, thereby further accelerating the synthesis of neurotoxic Aβ peptides (Ryu et al., 2010).

Extensive research has repeatedly associated the overexpression of DYRK1A with cognitive deficits in both people with DS and AD. In mouse models, Dyrk1A overdosage is sufficient to recapitulate learning and memory deficits found in individuals with DS (Altafaj et al., 2001). Accordingly, normalization of Dyrk1A expression levels by genetic engineering rescues motor and learning and

memory phenotypes in both TgDyrk1A and Ts65Dn mice (Ortiz-Abalia et al., 2008; Altafaj et al., 2013). In addition, environmental enrichment, an intervention boosting "physiological" plasticity, modulates Dyrk1A expression and activity in TgDyrk1A and Ts65Dn mice (Golabek et al., 2011; Pons-Espinal et al., 2013), suggesting that Dyrk1A is involved in activity-dependent neuroplasticity.

As a result, DYRK1A inhibitors may provide a therapeutically exploitable venue to ameliorate neurodevelopmental, neuroplasticity-related and neurodegenerative phenotypes in DS.

1.4.2 APP gene

APP gene is located in Hsa21 and encodes the core protein of the amyloid cascade hypothesis of AD (Glenner and Wong, 1984; Hardy and Higgins, 1992; Hardy and Selkoe, 2002). This is currently the most widely accepted paradigm of AD pathogenesis and suggests that abnormal APP metabolism triggers a set of sequential events that result in Aβ accumulation in extracellular amyloid plaques, formation of intracellular NFTs and eventually loss of synapses and neuronal death (Selkoe and Hardy, 2016). These sequential events are initiated by the processing of APP by several (α-, β-, and ɣ-) secretases that lead to the generation of different carboxyl-terminal fragments (CTFs): C83, C99, and APP-intracellular carboxyl domain (AICD) and N-terminal soluble peptides. APP is thus metabolized by two distinct pathways. The major pathway is driven by the α- secretase cleavage, performed by two disintegrin metalloproteases (ADAM 10 and ADAM 17), which release the soluble N-terminal ectodomain (sAPPα) (Vincent and Govitrapong, 2011). Both DS and AD, this proteolytic pathway is attenuated as shown by a reduced co-expression of ADAM10 and/or ADAM17 with nardilysin, which is a peptidase that enhances α-secreatase activity (Bernstein et al., 2009). The alternative pathway, generates Aβ through the sequential APP cleavage by β-secretase and then ɣ-secretase in a complex with presenilin.

Cleavage by β- and ɣ- secretases generates the soluble APP-β protein (sAPPβ) that gives rise to toxic soluble amyloid-β peptides (Aβ40/42) that oligomerize and aggregate (Selkoe et al., 1996). In DS, the increased gene dose of APP leads to accelerated accumulation of C99, C83, AICD, and Aβ40/42, in addition to the full length APP protein (Choong et al., 2015; Wiseman et al., 2015). The role of APP in DS and AD neurodegeneration is supported by the fact that mouse models with APP mutations present dysfunction of BFCN, and changes at the synaptic and behavioral levels (Yamaguchi et al., 1991; Moran et al., 1995; Mucke et al., 2000), although overexpression of wild-type APP is not sufficient to cause AD neuropathology in mice (Balducci and Forloni, 2011). Conversely, humans with rare hereditary duplications of small regions of Hsa21 including APP gene (Dup-APP) develop early onset AD (Sleegers et al., 2006; Kasuga et al., 2009; McNaughton et al., 2012), while exceptional cases of partial trisomies excluding APP do not show neuropathology or dementia (Prasher et al., 1998; Korbel et al., 2009).

The dyshomeostasis of APP has been linked to degeneration of BFCN in DS and Ts65Dn mice. In both DS and AD, Aβ peptides are accumulated in neuronal early endosomes that appear enlarged (Cataldo et al., 2000, 2004; Nixon, 2005) and which has been shown in Ts65Dn mice to interfere with the retrograde transport of neural growth factor (NGF) from the hippocampus to the BFCN somas (Cooper et al., 2001; Salehi et al., 2006, 2007), thus contributing to their degeneration. Interestingly, interventions aimed at lowering amyloid-β levels in the Ts65Dn mouse, such as genetic engineering (APP deletion), γ-secretase inhibitors (DAPT) and antibody delivery, correct learning deficits and revert BFCN atrophy (Salehi et al., 2006; Netzer et al., 2010; Belichenko et al., 2016). Nevertheless, several *in vitro* studies have shown that drugs/compounds that specifically target and inhibit the ɣ-secretase have a negative impact such as accumulation of ɣ-secretase cleavage-dependent peptides, inhibition of cell-cell aggregation and migration in neuroblastoma cells and ovary cells wound healing

assay (Lee et al., 2002; Kim et al., 2005; Yagishita et al., 2006). The fact that ɣ-secretase processes many other signaling molecules such as receptors (e.g., VEGFR-1, Notch, ErbB-4, IGFI-R) (Wolfe, 2008; Xia, 2008; Guardia-Laguarta et al., 2010; Haapasalo and Kovacs, 2011) could lead to undesired effects of its inhibition. On the other hand, Aβ production is not deleterious *per se*. Aβ monomers share similar properties with sAPPα, the soluble proteolytic product of α-secretase. These include neurotrophic and neuroprotective functions, as well as stimulation of neural-progenitor proliferation, suggesting beneficial effects of the promotion of the non-amyloidogenic proteolytic pathway of APP (Chasseigneaux and Allinquant, 2012). Indeed, sAPPα has been shown to increase both synaptic density, LTP and, learning and memory in mice, indicating its role in synaptic plasticity (Meziane et al., 1998; Taylor et al., 2008; Klevanski et al., 2015). Additionally, sAPPα has exerts potent neuroprotective actions against glutamate neurotoxicity, Aβ peptide-induced oxidative injury, glucose deprivation or UV irradiation (Mattson et al., 1993; Goodman and Mattson, 1994; Copanaki et al., 2010).
However, increasing sAPPα levels could shift proliferating cells towards tumorigenesis and lead to neurotoxicity (Hansel et al., 2003; Takayama et al., 2009). Additionally, the expression of non-amyloidogenic peptides (Aβ9–42 and Aβ17–42) in human cortical neurons have been reported to form toxic mobile ion channels that allow calcium uptake possibly inducing neurite degeneration in DS and AD (Jang et al., 2010).

Therefore, the therapeutic modulation of APP proteolysis is an intricate although promising approach to target neuropathology in DS and AD.

1.5 Towards a therapeutic intervention for DS

As DS is caused by a full or partial extra copy of a chromosome including hundreds of genes, it is generally viewed as a too complex genetic perturbation to be amenable to postnatal interventions.

Thus, until relatively recently, DS was considered an "incurable" disease by most people and, what is more, it was viewed as a disorder that hindered individuals from acquiring education (Smith et al., 1976). However, a number of therapies of different nature have been implemented or examined in the attempt to attenuate the cognitive impairments in individuals with DS.

1.5.1 Non-pharmacological interventions in DS

The only available therapy at the moment for DS consists on non-pharmacological early intervention programs that are primarily focused on infants and young individuals. Early intervention programs were developed rather recently, as fifty years ago there were no formalized interventions of any type, and were initially based on experimental programs, impulsed by the advocacy of parents and researchers (Rondal et al., 2011). They are aimed at providing cognitive stimulation and special education to promote children´s development of skills and support them to fully participate in family, school and community life (Odom and Diamond, 1998). They consist on specific programs that emphasize education and training, targeted to cognitive domains that are especially affected in individuals with DS, such as speech, language and nonverbal communication, motor and problem-solving skills, attention, learning and memory. Training strategies involve reinforcement principles and stimulus-response learning models and behavior modification in relevant aspects for self-development, peer interactions and integration in society.

Several studies have shown that early intervention programs induce beneficial effects on children with DS, including acceleration of skill acquisition, prevention of abnormal patterns of functioning, promotion of better parent-child interactions and encouragement of inclusion (Bailey et al.,1997; Meisels and Shonkoff, 1990; Guralnick, 1997, 2001; Rondal et al., 2011; Engevik et al., 2016). Additionally, cognitive and physical exercise programs improved health status and wellbeing in adults with DS (Moni and Jobling, 2001; Heller et al., 2004b). However, although these improvements

made a huge impact in the way individuals with DS are integrated in society, they are limited since the learnt skills through these programs are rarely generalized to everyday situations (Moni and Jobling, 2001; Mahoney et al., 2006; Bonnier, 2008), suggesting that intervention programs are still insufficient to mitigate cognitive impairment and provide only moderate relief in DS.

In the healthy elderly population and patients with mild-to-moderate AD, cognitive therapies, consisting of activities enhancing cognitive and social functioning, have also shown some improvements and attenuation of risk for cognitive decline (Buschert et al., 2010). For instance, the SIMA (Maintaining and supporting Independent Living in old Age) study demonstrated that a combination of memory and psychomotor training significantly improved cognitive status in healthy elderly people (75–89 years) after 1 year of training (Oswald et al., 1996). In addition, the ACTIVE (Advanced Cognitive Training for Independent and vital Elderly) study showed that 2 years of cognitive intervention therapy promoted significant improvements in memory, reasoning, problem solving and speed of processing in all participants (aged 65–94 years) (Ball et al., 2002). In patients with mild cognitive impairment (MCI) and dementia associated to AD, cognitive training interventions alone or in combination with medication, have shown to be efficient to delay cognitive decline (Spector et al., 2003; Requena et al., 2004; Bottino et al., 2005; Belleville, 2008; Troyer et al., 2008; Buschert et al., 2010).

The observed beneficial effects of cognitive training are in agreement with the brain reserve hypothesis. This hypothesis is based on the observation that, in the general population, individuals with higher levels of education and/or more-active social and intellectual lifestyles have a lower risk of developing dementia as they preserve brain's ability to adequately perform cognitive tasks despite neuropathological damage (Stern, 2012). However, this hypothesis not only predicts that highly cognitively active individuals would be protected from cognitive decline but also that individuals with more-severe premorbid cognitive impairment, as

the case of DS, will have an increased risk of developing dementia. The underlying mechanisms of cognitive therapy both in DS and AD may be related to the regular activation of brain neuronal networks by cognitive stimulation, which trigger neuroplasticity processes that contribute to brain health and cognitive status.

From the biological perspective environmental cognitive stimulation was initially studied in the context of availability of sensory information and its effects on brains from kittens. Pivotal studies from Hubel and Wiesel on the visual system demonstrated that modifications in the availability of visual inputs were able to modify the synaptic organization of the visual cortex during certain critical periods in postnatal early life (Hubel and Wiesel, 1970). After that, multiple studies mainly in rodents but also in humans have strengthen the idea that experience is able to regulate the structure and function of different areas of brain both in young and adult individuals (Watanabe et al., 1992; Maguire et al., 2000; Bermudez et al., 2009; May, 2011).

Along the past fifty years the preponderant paradigm to study the effects of experience and environmental stimulation in experimental settings with rodents has been called environmental enrichment (EE), which consists of housing conditions involving a complex combination of social, cognitive, and physical stimulation. In a classical study by Hebb (1947), housing rodents in EE comprising a large cage with varying sets of toys, such as balls, tunnels, and ladders, improved learning and memory (Ghassemzadeh et al., 2013). Beneficial effects of EE on behavior and brain function have ever since been reported in a multitude of studies using rodent spatial memory, neuroanatomical, cellular, and molecular assays (Greenough et al., 1973; Rosenzweig and Bennett, 1996). In particular, changes such as increased brain weight, neurotransmitter content, gliogenesis, synaptic plasticity, and dendritic spine growth, as well as upregulation of neuronal signaling molecules, neurotrophin levels, and adult hippocampal neurogenesis have been associated with cognitive enhancement (for reviews Praag et al.,

2000; Nithianantharajah and Hannan, 2006; Baroncelli et al., 2010; Voss et al., 2013).
The experimental setting of EE provided a powerful tool for the study of cognitive stimulation in mouse models of different neurological disorders including intellectual disabilities and neurodegenerative diseases.

1.5.2 Pharmacological interventions in DS

The other approach that has been used in the attempt to mitigate intellectual disability and cognitive decline in DS involves pharmacological treatments. So far, pharmacological interventions have been mainly targeted to restore the neurotransmitter imbalance found in the disorder.
Due to the high prevalence of AD-like neuropathology in DS subjects, and the overlapping molecular pathways between DS and AD, most of the therapies tried in the DS population have repurposed drugs, currently in use as AD therapeutics. Some of these drugs include acetylcholinesterase inhibitors, such as donepezil and rivastigmine (Heller et al., 2004a, 2010; Prasher and Ad, 2004; Spiridigliozzi et al., 2007; Kishnani et al., 2010), nicotine (Seidl et al., 2000), Acetyl-L-carnitine (Pueschel, 2006) and N-methyl-D-aspartate (NMDA) receptor antagonist memantine, (Hanney et al., 2012).
In addition, the potential of diverse compounds, vitamins and mineral supplements, has been assessed to ameliorate DS symptoms. For instance, different antioxidants have been addressed to counteract increased oxidative stress resulting from the overactivity of CuZnSOD1. Also folate supplementation has been used to try to normalize the folate deficiency derived from Cystathionine b-synthase (Ellis et al., 2008). Although some of these interventions promoted positive outcomes for some singular participants, most of them have yielded a big disappointment due to

their limited efficacy or complete failure to provide improvement in DS cognition (reviewed in detail in de la Torre and Dierssen, 2012).

As a result, in order to develop novel and more efficient therapies, another approach has been the translation of scientific evidence from mouse preclinical studies into the clinical practice. The translational research approach consists of two main "translational blocks" (Fig. 6). The first block comprises the transfer of new understandings of disease mechanisms gained from basic science into the development of new therapies and their first testing in humans. In this process, the transition from the late preclinical phases to phase II and III clinical trials is the most critical step since only 10–15% of therapeutic agents eventually become approved products (Lesko, 2007). The optimal execution of this stage implies a large bidirectional interplay between the laboratory and the clinic and multidisciplinary research. The second block consists of the translation of results from clinical studies into everyday clinical practice (Woolf, 2008; Rubio et al., 2010).

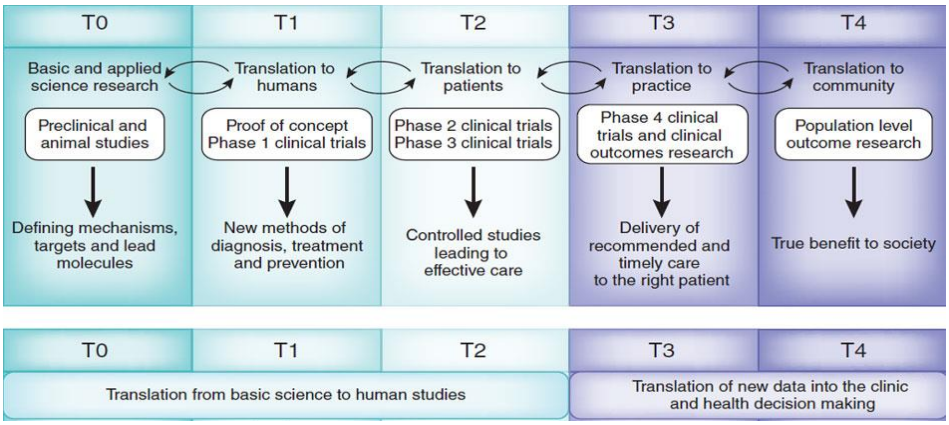


Fig. 6: The classic T0-T4 translational pathway (adapted from Blumberg et al., 2012).

All the preclinical studies carried out in order to address the effects of potential therapeutic interventions for DS have been performed in the Ts65Dn mouse. Over the last few years, multiple studies have

shown that more than 20 drugs/small molecules are able to successfully rescue learning and memory deficits and hippocampal defects in adult Ts65Dn mice (reviewed in Gardiner, 2015).
These efficient pharmacological interventions comprise diverse drugs with multiple targets and mechanisms. Many of them have been examined on the basis of their beneficial effect in other mouse models on a relevant phenotype for Ts65Dn impairments (eg, neurodegeneration in an AD mouse model). Most of these drugs tackle specific altered processes in DS. Concretely, neuroplasticity deficits have been addressed by serotonin reuptake inhibitor (fluoxetine) and lithium to increase neurogenesis (Clark et al., 2006; Bianchi et al., 2010; Contestabile et al., 2013), by sonic hedgehog agonist to revert neurodevelopmental alterations (Roper et al., 2006) and by β-adrenergic receptor agonists (L-DOPS and xamoterol) to normalize norepinephrine input from the Locus Coeruleus to the hippocampus (Salehi et al., 2009). On the other hand, excitation/inhibition imbalance has been targeted through $GABA_A$ receptor antagonists (picrotoxin, bilobalide, and pentylenetetrazole) or uncompetitive antagonist of the N-methyl-d-aspartate (NMDA) (memantine) (Fernandez et al., 2007; Costa et al., 2008; Rueda et al., 2008; Braudeau et al., 2011; Lockrow et al., 2011; Colas et al., 2013). Neurodegeneration has also been tackled in the Ts65Dn mouse by the use of acetylcholinesterase and γ-secretase inhibitors, and acetylcholine precursors among other agents (Chang and Gold, 2008; Netzer et al., 2010; de Souza et al., 2011; Ash et al., 2014).

Therefore, it seems that many different drugs converging on related pathways that contribute to overlapping biological processes can rescue behavioral and structural defects in the Ts65Dn mouse. The problem about these drugs is that most of them can have secondary undesirable effects, such as promoting increased risk for epilepsy and mood instability among others.

1.5.2.1 (-)-Epigallocatechin-3-gallate (EGCG)

In the last decades, (-)-Epigallocatechin-3-gallate (EGCG), the most abundant catechin found in green tea (Camellia sinensis) has gained a lot of attention because in the early 2000 it was discovered that it is a potent inhibitor of DYRK1A kinase activity with in vitro IC50 of 0.33 μM (Bain et al., 2003) as recently confirmed (Wang et al., 2012a). A study in mouse embryonic fibroblast immortalized NIH-3T3 cells, showed that the mechanism by which EGCG acts on DYRK1A kinase activity involves a non-competitive inhibition against ATP binding site (Adayev et al., 2006). As DYRK1A had been shown to be a good candidate gene for many DS related phenotypes, considerable interest grew around the therapeutic potential of EGCG as it provided the means to rescue DS phenotypic features with a natural and apparently safe polyphenolic compound.

The effects of EGCG on Ts65Dn mice were initially studied in hippocampal slices and demonstrated that pre-incubation with 10 μM EGCG induced a normalization of long-term potentiation (LTP) in Schaffer collaterals-CA1 synapses (CA3-CA1 LTP), after a high frequency stimulation (HFS) protocol, but did not alter the degree of paired-pulse inhibition (PPI) suggesting a synaptic plasticity mechanism other than the attenuation of GABAergic inhibitory circuit (Xie et al., 2008). Another *in vitro* study showed that in hippocampal neuronal cultures derived from Dyrk1A overexpressing mice (bacterial artificial chromosome BACTgDyrk1A) presented a slower rate of synaptic vesicle endocytosis, which was reverted by EGCG treatment (Kim et al., 2010). Additional neuroplasticity effects of EGCG were also shown in euploid conditions, increasing neurogenesis in adult hippocampal neural progenitor cell (NPC) cultures and in the DG of adult mice (Wang et al., 2012).

As EGCG had been reported to be able to cross both the blood brain barrier in conscious and freely moving rats (Lin et al., 2007) and the placental barrier in gestating rats (Chu et al., 2007), subsequent

studies examined the effects of EGCG on neurodevelopment by its administration to pregnant mice bearing a small Hsa21 region duplication containing Dyrk1A (human Yeast Artificial Chromosome YACTg152F7) and their litters up to adult age. The results showed that oral treatment with EGCG (50 mg/Kg), beginning prenatally and through adulthood, rescued brain volume alterations assessed by *in vivo* MRI, cognitive deficits in the object recognition test, and reductions in hippocampal levels of BDNF neurotrophin and its plasma membrane receptor TRKB in their offspring (Guedj et al., 2009).

Our group also assessed the effects of one month oral treatment with EGCG (30 mg/Kg) on post-weaning TgDyrk1A mice and found a normalization of the excessive proliferating cells and their accelerated cell cycle exit in the granular cellular layer of the DG, a phenotype that possibly contributes to deficient spatial learning and memory in these mice (Pons-Espinal et al., 2013). These changes were accompanied by a normalization of hippocampal DYRK1A kinase activity levels (Pons-Espinal et al., 2013), suggesting a potential pharmacological role of EGCG to tackle DS altered neurodevelopment and neuronal differentiation, at least partially due to its ability to normalize DYRK1A kinase activity. The procedure used to assess DYRK1A kinase activity involved first immunoprecipitating hippocampal DYRK1A protein and subsequently measuring DYRK1A catalytic activity through the quantification of the incorporation of radiolabeled ^{32}P to an artificial DYRK1A substrate (DYRKtyde) (Himpel et al., 2000). This method implies that the inhibitory effects of EGCG on DYRK1A remains after protein isolation, which may suggest an additional mechanism to the previously described non-competitive inhibition against ATP binding site (Adayev et al., 2006). This alternative mechanism would possibly involve a modification of the molecular structure of DYRK1A protein that may be reversible or not.

A more recent study by Souchet and colleagues (2015) showed that the oral administration of EGCG to BACTgDyrk1A and Ts65Dn mice had beneficial effects on both GABAergic and glutamatergic

components in different brain regions. Both BACTgDyrk1A and Ts65Dn mice presented increased levels of GABAergic markers (GAD67,GAD65 and VGAT) and decreased levels of glutamatergic markers (GLUR1, NR1, NR2a, and VGLUT1) in the cortex, hippocampus, and cerebellum, (with the exception of hippocampal VGLUT1 levels which were increased in BACTgDyrk1A, and hippocampal GLUR1 and GLUR2 levels that were unchanged in Ts65Dn). EGCG (60 mg/Kg) treatment reverted the altered GABAergic and glutamatergic levels in both BACTgDyrk1A and Ts65Dn mouse models, mainly in the cortex and the hippocampus (except for VGLUT1 in the hippocampus which remained increased in BACTgDyrk1A and decreased in Ts65Dn), while the correction was weaker in the cerebellum. Moreover, EGCG treatment rescued behavioral deficits in short-term spatial working memory in both mouse models (Souchet et al., 2015).

Additionally, a recent work showed that treatment with EGCG (~9 mg/Kg/day) in post-weaning Ts65Dn mice improves some skeletal abnormalities such as femoral bone mineral density (BMD) by reducing femoral bone-associated DYRK1A kinase activity (Blazek et al., 2015). However both the dose and composition of supplements containing EGCG can affect its ability to improve skeletal deficits in Ts65Dn mice (Abeysekera et al., 2016).

In most of the above studies, EGCG was delivered orally, hence it can either be absorbed directly into the bloodstream or go through metabolic transformations, such as formation of glucuronide, sulfate and methyl derivatives, which can have different physical and chemical properties than the parent compound (Lotito and Frei, 2006; Lambert et al., 2007). Additionally, it has been shown that EGCG displays a low bioavailability and is rapidly cleared from the blood with an elimination half-life of 2.0–3.5 h (Zini et al., 2006; Yang et al., 2009). Therefore, further research is still needed to fully understand the *in vivo* mechanisms of EGCG when administered orally.

Collectively, the above data indicate that EGCG exerts effects on synaptic neuroplasticity, brain and skeletal development, neuronal cell cycle and differentiation and, hippocampal and cortical excitation/inhibition balance, at least partially through the modulation of DYRK1A kinase activity. However, it is rather unlikely that the benefits of EGCG treatment in Ts65Dn mice are limited to the inhibition of DYRK1A kinase activity. As pointed by Gardiner (2014), the reduction of DYRK1A kinase activity by EGCG occurs in the Ts65Dn mouse in a context of elevated expression of other multiple Hsa21 genes. Among those other overexpressed Hsa21 genes, some proteins are phosphorylation substrates of DYRK1A, such as APP, SYNJ1 (a phosphoinositide phosphatase), and RCAN1. Thus, if EGCG optimally and specifically normalized DYRK1A activity in the context of elevated expression of those Hsa21-encoded substrates, additional imbalances relevant to DS phenotypic features could arise.

EGCG, is a pleiotropic agent and participates in multiple signaling pathways that could contribute to the beneficial effects observed in Ts65Dn mice. For instance, EGCG has been shown to reduce Aβ generation in neuroblastoma N2a cells overexpressing Swedish mutant APP (SweAPP N2a) (Rezai-Zadeh et al., 2005) by promoting the non-amyloidogenic enzymatic processing of APP via ADAM10 activation, inducing α-secretase proteolytic function (Obregon et al., 2006). It was shown that EGCG-mediated enhancement of APP non-amyloidogenic processing is mediated by an estrogen receptor-α (ERα)/phosphoinositide 3-kinase (PI3K)/Protein kinase B (AKT)- dependent signaling pathway (Fernandez et al., 2010). Another mechanism found regarding the reduction of Aβ accumulation induced by EGCG, involves the enhancement of Aβ clearance through increasing the expression of Aβ-degrading peptidase neprilysin (NEP) (Iwata et al., 2000; Chang et al., 2015). Additionally, in the human neuroblastoma cell line SH-SY5Y, EGCG was shown to induce a neuroprotective effect by the down-regulation of Bad protein levels mediated by rapid PKC-dependent mechanism (Kalfon et al., 2007). In several mouse

models of AD and accelerated senescence, EGCG reduced brain Aβ levels, resulting in mitigation of cerebral amyloidosis, and promoted other beneficial effects involving reduction in oxidative stress, prevention of neuroinflammation and neurodegeneration (Rezai-Zadeh et al., 2008; Li et al., 2009b; Biasibetti et al., 2013; Lee et al., 2013; Chang et al., 2015). The activity of EGCG over neurodegenerative phenotypes is also relevant for DS as to prevent the progression to AD.

Moreover, EGCG as a polyphenol, is a potent natural antioxidant able to augment endogenous antioxidant defense systems and improve the oxidative stress linked to the triplicated gene CuZnSOD1. It was demonstrated that the administration of EGCG (20 μM) in human DS lymphoblast and fibroblast cultures, was efficient to counteract oxidative stress and restoring mitochondrial energy deficit by promoting mitochondrial biogenesis and rescuing mitochondrial complex I and ATP synthase catalytic activities (Valenti et al., 2013). Besides, a recent case study showed that combined nutraceutical supplementation with EGCG (10 mg/Kg/day) and fish oil omega-3 fatty acids (8 mg/Kg/day) in a 10-year and 3-month-old child with DS, was safe, counteracts deficits in mitochondrial respiratory chain (MRC) complex activities in lymphocytes from peripheral blood and improved scores in neuropsychological evaluation for ADHD, including auditory attention and verbal strategic tests (Vacca and Valenti, 2015). Furthermore, in hippocampal neural progenitor cells (NPCs) from Ts65Dn mice, EGCG (20 μM) improved NPCs proliferation, and restored mitochondrial biogenesis and ATP production (Valenti et al., 2016). Conversely, it has been shown that EGCG has also a pro-oxidant role in some cellular contexts. Indeed, in the presence of Fe (III), treatment with high concentrations (>50 μM) of EGCG results in production of hydrogen peroxide and hydroxyl radicals, contributing to cytotoxicity (Nakagawa et al., 2002, 2004). This highlights that EGCG has dosage-dependent pro-oxidant and anti-oxidant effects (Kim et al., 2014), which must be accounted for the development of potential therapies for DS.

The elucidation of the exact underlying mechanisms of EGCG will be complex due to its multifactorial pharmacology, involving the modulation of a plethora of proteins participating in diverse biochemical pathways (for a detailed review Xicota et al., 2015).

1.5.3 Multimodal therapies as an approach for multifactorial disorders

In the last years, multimodal therapy approaches, are being considered as a potential way to enhance clinical outcomes for patients with different nervous system disorders. They consist on the combination of different interventions, for example, a prescribed drug or dietary supplement along with a device, cognitive intervention or lifestyle adjustment, aimed at modifying distinct aspects of disease. There are still important open questions and challenges regarding whether these interventions are more efficient than monotherapies, how these interventions will interact, how they should be used, in what type of patients they should be applied (with which nervous system disorder, age, gender), when it would be best to start the intervention (e.g., earlier versus later in disease progression) and for how long. In cancer and viral infections therapy, a similar approach, using cocktails of different drugs is becoming common (Honda et al., 2013; Jaynes et al., 2013) but this type of therapies have not been applied yet for nervous system disorders.

Multimodal therapy approaches have already been recommended for sporadic AD since, so far, monotherapies using agents targeting single pathogenic factors (Aβ/NFT production or clearance) have led to disappointment in clinical trials. It has been proposed that early intervention with combinations of safe and inexpensive pleiotropic agents, may be effective even to prevent later stages symptoms of AD (Frautschy and Cole, 2010).

In the case of DS, multimodal therapies would be particularly useful as it is a disorder that affects several systems with a specific temporal dynamics and pronounced deficits in neuroplasticity-related processes that impair proper brain and behavioral

modifications driven by environment. A multimodal therapy combining environmental and pharmacological interventions targeting neuroplasticity and neurodegeneration may thus provide the means to simultaneously counteract several of the altered processes in DS.

2. HYPOTHESIS and OBJECTIVES

2.1 Hypothesis

A growing number of studies have consistently linked intellectual disability in Down syndrome (DS) with alterations in brain morphology, synaptic connectivity, activity-dependent neuroplasticity, excitation-inhibition balance and Alzheimer disease (AD)-like age-related neurodegeneration. Accumulating data indicate that cognitive stimulation, through early intervention programmes in humans or by environmental enrichment (EE) in mice, induces multiple beneficial effects on cognition. However, these effects are limited and temporary, possibly due to inadequate neuroplasticity mechanisms responsible for the translation of transient changes into more stable memory traces. A number of Hsa21 dosage-sensitive genes, among which, the dual specificity tyrosine-phosphorylation-regulated kinase 1A (Dyrk1A) gene and the gene encoding the amyloid precursor protein (APP), have been proposed to play a role in the failure of those neuroplasticity mechanisms and represent good targets for intervention in DS.

During the recent years several studies have demonstrated overlapping molecular mechanisms of EE and specific drug treatments such as (-)-epigallocatechin-3-gallate (EGCG), a catechin found in green tea. Those include the promotion of neuroplasticity and neuroprotection, antioxidant activity, anti-inflammatory function, enhancement of the non-amyloidogenic proteolytic pathway of APP and inhibition of the kinase activity of Dyrk1A.

The working hypothesis in this Thesis is that pharmacologically targeting neuroplasticity and neurodegeneration-related molecules by EGCG may enhance the beneficial effects of EE and thus improve physiological learning and neuroprotection in DS. The secondary hypothesis is that these effects could be beneficial across different life stages.

2.2 Objectives

The main objective of this Thesis is to assess the potential ability of combined EE-EGCG treatment to ameliorate two distinct pathophysiological processes that give rise to cognitive impairment in mouse models and humans with DS and occur in specific timeframes across the lifespan: 1) the cognitive deficits associated to impaired hippocampal neuroplasticity and excitation-inhibition imbalance, and 2) the AD-like age-associated cognitive decline related to hippocampal dysfunction and cholinergic neurodegenerative process in the basal forebrain in Ts65Dn mice. Additionally, we aimed to translate our findings to clinical trials with DS individuals.

Specific objectives:

Preclinical studies

1. To examine the effect of combined EE and EGCG treatment on spatial learning and memory, and hippocampal structural neuroplasticity and excitation-inhibition balance in young adult Ts65Dn mice.

2. To assess the effect of EE, EGCG and combined EE and EGCG treatments on spatial learning and memory, recent and long-term associative memory, and basal forebrain cholinergic neurodegeneration in middle age adult Ts65Dn mice, at the age of the onset of cognitive decline.

Clinical trials

3. To evaluate the effects of combined cognitive training and EGCG treatment on cognitive ability, adaptive functionality and quality of life of young adults with DS and on prodromal dementia signs.

3. CHAPTER I. PRECLINICAL STUDIES

3.1 Preface

This chapter comprises the preclinical studies in mice that represent the core of my PhD Thesis.

Since previous data of the lab had already indicated that both EE and green tea extract containing EGCG had beneficial effects on young adult Ts65Dn mice, the first experiments (Article I), specifically addressed the effects of the combined EE-EGCG treatment at this age. This work studied the spatial learning and memory deficits, hippocampal structural neuroplasticity and excitation/inhibition imbalance. We focused on these aspects because, like DS individuals, Ts65Dn mice show disruptions in dendritic complexity and dendritic spines in brain regions involved in learning and memory. These are partially due to developmental abnormalities but also to alterations in experience-dependent neuronal shaping. Furthermore, accumulating evidence suggest that intellectual disability in DS is tightly associated with a disruption of the excitation-inhibition balance. Both of these aspects are modulated by EE and Dyrk1A overexpression thus we expected to see changes with the combined EE-EGCG treatment.

At the age of the onset of cognitive decline, there were no previous data about the effects of these treatments on DS. Thus, the second set of experiments (Articles II and III) addressed the effects of the treatments (EE, EGCG, combined EE-EGCG) on Ts65Dn mice at six months of age. We investigated the effects of the treatments on spatial learning and memory deficits and cholinergic neurodegeneration at the basal forebrain because they represent the anatomic substrates of memory and attention, and Ts65Dn mice present a gradual atrophy and loss of these population of neurons which is associated to the onset of cognitive decline, as happens in DS and AD humans. This cholinergic neurodegeneration has been linked to the abnormal expression of APP (full length and proteolytic derivatives) and the abnormal retrograde transport of

neural growth factor (NGF) from the hippocampus to the BFCN somas. Both APP proteolytic processing and neurotrophins secretion have been shown to be modulated by EE, EGCG and Dyrk1A through different mechanisms. Therefore, we predicted to detect changes with the EE-EGCG treatment.

Collectively, the results of this preclinical studies showed that combined EE-EGCG treatment induced an amelioration in spatial learning deficits in young and middle-age Ts65Dn mice. This cognitive improvement was accompanied in young mice by a rescue of dendritic spine density in CA1 and a normalization in the density and size of excitatory and inhibitory synaptic puncta in DG and CA1, while in middle-age mice it was associated by a moderate although not significant restoration of cholinergic neuronal density in the medial septum of the basal forebrain.

3.2 Paper I: Combined treatment with environmental enrichment and (-)-epigallocatechin-3-gallate ameliorates learning deficits and hippocampal alterations in a young adult mouse model of Down syndrome

In this work we examined the effects of combined EE-EGCG treatment on hippocampal-dependent visuo-spatial learning and memory deficits in young adult Ts65Dn mice using the Morris water maze (MWM). One of the added values of this work, is the analysis we proposed. The cognitive effects of the treatment were first examined by a standard single-variate analysis of the behavioral data. However, this type of analysis provides a fragmented vision of the behavioral effects of the treatment. Thus, we performed a novel multidimensional analysis of the data based on principal component analysis (PCA) which enabled us to better discriminate the global treatment effects on mice behavioral response along the different sessions of the test. Principal component analysis is a variable reduction procedure. It is useful when dealing with a number of variables with some degree of correlation among them, as happens in the MWM. Because of this correlation, it is possible to reduce the number of variables into a smaller number of principal components (artificial variables) that account for most of the variance. Indeed, in our analysis the two obtained principal components, contributed by learning-related and unrelated variables accounted for most of the between group variability. Furthermore, we addressed the treatment effects on hippocampal alterations by studying dendritic spine density, and excitatory and inhibitory synaptic molecules in order to shed light on the possible underlying mechanisms of the treatment.

This work was performed in collaboration with Jose Antonio Espinosa-Carrasco and Ionas Erb from the Comparative Bioinformatics lab (Cedric Notredame CRG), Klaus Langohr from Rafael de la Torre lab (IMIM) and Juan Ramón González

(CREAL). My specific contribution consisted in performing all the experiments including the administration of the treatments to the mice, the behavioral tests, contributing to the single-variate analysis of the behavioral data, the design and execution and analysis of neuronal and synaptic experiments and the elaboration of the manuscript.

Catuara-Solarz S, Espinosa-Carrasco J, Erb I, Langohr K, Gonzalez JR, Notredame C, et al. Combined Treatment With Environmental Enrichment and (-)-Epigallocatechin-3-Gallate Ameliorates Learning Deficits and Hippocampal Alterations in a Mouse Model of Down Syndrome. eNeuro. 2016 Nov 10;3(5). DOI: 10.1523/ENEURO.0103-16.2016

3.3 Paper II: Principal Component Analysis of the Effects of Environmental Enrichment and (-)-epigallocatechin-3-gallate on Age-Associated Learning Deficits in a Mouse Model of Down Syndrome

The following work, was aimed at examining the effects of treatments with EE, EGCG and combined EE-EGCG treatment on the performance of Ts65Dn and WT mice on the MWM, at the age of onset of cognitive decline previously reported in trisomic mice. Again, the cognitive effects of the treatments were analyzed by a classical single-variate analysis of the behavioral data and a multidimensional analysis of the data based on principal component analysis (PCA).

As for Paper I, this work was performed in collaboration with Jose Antonio Espinosa-Carrasco and Ionas Erb from Cedric Notredame lab (CRG), Klaus Langohr from Rafael de la Torre group (IMIM) and Juan Ramón González (CREAL). My specific contribution consisted in performing all the experiments including the administration of the treatments to the mice, the behavioral tests, contributing to the single-variate analysis of the behavioral data, and the elaboration of the manuscript.

Catuara-Solarz S, Espinosa-Carrasco J, Erb I, Langohr K, Notredame C, Gonzalez JR, et al. Principal Component Analysis of the Effects of Environmental Enrichment and (-)-epigallocatechin-3-gallate on Age-Associated Learning Deficits in a Mouse Model of Down Syndrome. Front Behav Neurosci. 2015 Dec 11;9:330. DOI: 10.3389/fnbeh.2015.00330

3.4 Paper III: Combined therapy with environmental enrichment and (-)-epigallocatechin-gallate (EGCG) mitigates long-term contextual memory deficits in a mouse model of DS at the age of initiation of cognitive decline

As we had previously demonstrated that combined EE-EGCG treatment ameliorated spatial learning and memory deficits more efficiently than EE or EGCG in Ts65Dn mice at the age of the initiation of cognitive decline (Paper II), we hypothesized that this therapy would also have effects on cholinergic-dependent memory and on the degenerative process that occurs in the basal forebrain cholinergic neurons.

This work extends the assessment of the effects of EE, EGCG and combined EE-EGCG treatments on recent and long-term associative memory, and on the cholinergic neuronal degeneration that takes place within the medial septum of the basal forebrain in Ts65Dn mice. The treatments effects were addressed by the step-down passive avoidance test and by unbiased stereological quantifications of medial septum cholinergic neurons.

Catuara-Solarz S, Ayala-Ruiz C, Langohr K, Dierssen M. Combined therapy with environmental enrichment and (-)-epigallocatechin-gallate (EGCG) mitigates long-term memory deficits in a mouse model of DS at the age of initiation of cognitive decline. (Manuscript in preparation)

Combined therapy with environmental enrichment and (-)-epigallocatechin-gallate (EGCG) mitigates long-term memory deficits in a mouse model of DS at the age of initiation of cognitive decline.

Abstract

Down syndrome (DS) is the most common genetic cause of intellectual disability and is associated with increased risk of early onset Alzheimer´s disease (AD)-like neuropathology and dementia. In this work we assessed the effects of a combined treatment with environmental enrichment (EE) and green tea extracts containing (-)-epigallocatechin-3-gallate (EGCG) in the Ts65Dn mouse model of DS, at the age of initiation of cognitive decline. We examined recent and long-term contextual memory and cholinergic neurodegenerative process in the medial septum from the basal forebrain. We found that both EE and combined EE-EGCG treatments ameliorate Ts65Dn long-term memory deficits but only moderately mitigates cholinergic neuronal loss. Our results suggest that combined EE and EGCG represents a promising therapeutic intervention to prevent age-related cognitive decline and neurodegeneration in DS and AD individuals.

Introduction

Down syndrome (DS), the trisomy of chromosome 21, is the most common genetic cause of intellectual disability affecting 5,8 million people worldwide (Centers for Disease Control and Prevention, 2006). In addition to the characteristic cognitive impairment during development and adult life, individuals with DS present a higher incidence and earlier onset of Alzheimer´s disease (AD)-like neuropathology and dementia than the general population (for a review see Ballard et al., 2016). Despite de striking difficulties in diagnosing dementia in people with intellectual disability, several

studies have reported that individuals with DS older than 40 years present a progressive cognitive decline with specific decays in attention, recall and explicit memory that resembles the one found in sporadic AD (Visser et al., 1997; Holland et al., 1998, 2000; Coppus et al., 2006). These cognitive symptoms are associated to a number of neuropathological changes that are common between DS with AD and sporadic AD including extracellular deposition of amyloid-ß (Aβ) plaques, intracellular accumulation of hyperphosphorylated tau in the form of neurofibrillary tangles, dysregulation of the endocytic pathway, oxidative damage, neuroinflammation and progressive atrophy and loss of neurons mainly from the hippocampus and basal forebrain (Wisniewski et al., 1985; Mann, 1988; Leverenz and Raskind, 1998; Cataldo et al., 2000b, 2003; Sendera et al., 2000; Nixon, 2005; Hartley et al., 2014). Accumulating evidence indicates that overexpression or dysregulation of certain genes, such as amyloid precursor protein (APP) and dual specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), play a key role in the neuropathology found both in DS with AD and in sporadic AD (Ferrer et al., 2005; Wiseman et al., 2015).

Currently, there is no available therapy to efficiently delay or revert age-associated cognitive decline and neurodegeneration in DS or AD. In the last decades, AD-pharmacological agents, such as memantine, donepezil, rivastigmine and galantamine have been used in DS showing little or no success (De la Torre 2012). On the other hand the use of non-pharmacological therapeutic intervention such as cognitive training, and treatment with (-)-epigallocatechin-3-gallate (EGCG), the most abundant polyphenol found in green tea, have gained attention due to their multiple and overlapping beneficial effects in the central nervous system (for reviews see Sale et al., 2014; Xicota et al., 2015) and their capacity to regulate the proteolytic products of APP (Rezai-Zadeh et al., 2005; Obregon et al., 2006; Fernandez et al., 2010) and the activity of Dyrk1A (Bain et al., 2003; Pathways et al., 2006; Wang et al., 2012). Recently, we demonstrated that combined treatment with environmental

enrichment (EE) and EGCG is more efficient than EE or EGCG alone to ameliorate spatial learning deficit in the Ts65Dn mouse model of DS at the age of the onset of cognitive decline (Catuara-Solarz et al., 2015). The Ts65Dn bears a segmental trisomy for MMU16 (syntenic region to HSA21) including APP and DYRK1A (reviewed in Dierssen, 2012). Even though Ts65Dn lacks the trisomy of a subset of genes from the distal end of HSA21 and it includes the trisomy of a small portion of MMU17 that is not homologous to HSA21 (Davisson et al., 1993), it is a useful DS model since it recapitulates many DS cognitive and neuroanatomical phenotypes across life. As in humans with DS, Ts65Dn mice show age-dependent cognitive decline (Granholm et al., 2000b; Hyde and Crnic, 2001) that is linked to abnormal neuronal processes such as enlarged early endosomes (Cataldo et al., 2003), dysfunction of neural growth factor retrograde transport from the hippocampus to the basal forebrain (BF) (Salehi et al., 2006) and gradual cholinergic neuronal loss in the basal forebrain (Holtzman et al., 1996; Granholm et al., 2000b; Cooper et al., 2001; Seo and Isacson, 2005).

Here, we examined the effects of these treatments on the performance of Ts65Dn at age of 6-7 months in a step-down inhibitory "passive" avoidance test (PAT) that is based on classical associative (pavlovian) conditioning and has been shown to be influenced by cholinergic function (Rush and Streit, 1992; Yoshida and Suzuki, 1993; Wilson and Cook, 1994; Cole and Jones, 1995; Kojima et al., 1997). Thus, we also assessed the effects of the treatments on the cholinergic neurodegeneration process that takes place in the (MS) nucleus of the BF in Ts65Dn.

Materials and methods

Ts65Dn mouse colony

Ts65Dn and wild type (WT) littermate mice were obtained through repeated crossings of B6EiC3Sn a/A-Ts(17^{16})65Dn (Ts65Dn) females to B6C3F1/J males purchased from The Jackson

Laboratory (Bar Harbor, ME). The mouse colony was bred in the Animal Facilities of the Barcelona Biomedical Research Park (PRBB, Barcelona, Spain, EU). Mice were housed in standard or enriched conditions (see below) under a 12:12 hour light–dark schedule (lights on at 8:00 a.m.) in controlled environmental conditions of humidity (60%) and temperature (22 °C±2 °C) with food and water *ad libitum*. Both the Ts65Dn and euploid mice were genotyped by qPCR (Liu et al., 2003). Experiments were conducted using 5-6 months old female mice. We used females since Ts65Dn males show high levels of stress in EE conditions that could mask the effect of the treatments (Martínez-Cué et al., 2002). The age of mice was selected since it corresponds to the initiation of gradual degeneration of basal forebrain cholinergic neurons and decline in cognitive function (Granholm et al., 2000b). All animal procedures met the guidelines of European Community Directive 2010/63/EU and the local guidelines (Real Decreto 53/2013) and were approved by the Local Ethics Committee (Comité Ético de Experimentación Animal del PRBB (CEEA-PRBB); procedure numbers MDS-08-1060P2 and MDS-14-1611).

(-)-Epigallocatechin-3-gallate (EGCG) and environmental enrichment treatment

Treatments are described in detail in Catuara-Solarz (2015). Briefly, Ts65Dn and WT mice were randomly assigned to one of the 4 experimental groups: control conditions, environmental enrichment (EE), EGCG or a combination of EE and EGCG. Mice received the treatments for 30 days. In the control conditions, animals were reared in conventional cages (20 × 12 × 12 cm height, Plexiglas cage) in groups of 2–3 animals. EE consisted of spacious (55 × 80 × 50 cm height) Plexiglas cages where 6–8 mice were housed and were provided with diverse environmental stimuli that were changed every 2 days to keep novelty conditions. Green tea extract containing 45% EGCG was administered in drinking water by preparing fresh EGCG solution (Mega Green Tea Extract, Decaffeinated, Life Extension®, Florida, USA; dosage: 0.326

mg/ml, 0.9 mg per day; 30 mg/Kg per day) every 2 days from a green tea leaf extract.

Step-down inhibitory "passive" avoidance test (PAT)

Single-trial step-down PAT was carried out on an automatically operated apparatus (Panlab SL, Barcelona, Spain) that consisted on transparent Plexiglas circular cage (40 cm in height, 30 cm in diameter) with a grid floor and a white circular non-conductive platform (4 cm diameter) in the center. During the task animals were gently placed on the platform from which they tended to step down. Immediately after stepping down, animals received an electric shock (0.7 mA, 2 s) and after that, were removed from the apparatus. The latency to step down from the platform with all four paws was measured with a cut-off time of 300 s. The whole task consisted on 3 trials: a training session, a 24 h (recent memory) retention test and a 6 days (long-term memory) retention test. In order to avoid behavioral extinction all animals received a shock at the end of the 24h trial. The calculation of the number of mice per experimental group was based on effect sizes observed our previous experiments using this test (Azkona 2010) and were the following: WT=22, TS=23, WT-EE=23, TS-EE=16, WT-EGCG=21, TS-EGCG=13, WT EE-EGCG=20, TS EE-EGCG=17.

Immunohistochemical labeling of MS cholinergic neurons

BFCN were labeled by immunohistochemistry using choline acetyltransferase (ChAT) antibody. Animals were perfused transcardially with 0.1 M phosphate buffer solution (PBS) and with a 4% solution of paraformaldehyde in PBS (pH=7,4). Brains were kept in the same fixative for 24 h and were afterwards transferred for 48 h to PBS 30% sucrose for cryoprotection and frozen at -80 ºC. Coronal sections 40 µm thick were cut with a cryostat and maintained in cryoprotective solution at -20 ºC until use. Brain section series 1:5 of containing basal forebrain nuclei (Bregma 1.34 mm; 0.14 mm according to Franklin and Paxinos mouse brain atlas, 1997) were rinsed in PBS and then incubated in PBS with 3% H_2O_2

and 10% MetOH at room temperature (RT) to quench endogenous peroxidases. After that, the tissue was rinsed in PBS and washed with PBS 0,2% Triton X-100 and, in order to inhibit non-specific binding, it was incubated for 1 h in blocking solution with PBS, 0,2% Triton X-100, 5% fetal bovine serum (FSB), 0,25% gelatine at RT. Tissue was then incubated overnight at 4ºC in goat anti-choline acetyltransferase (1:750 AB144P Chemicon International) in 2,5% FBS 0,25% gelatine in PBS-T 0,2%. After washing with PBS 0,2%Triton X-100 tissue was incubated during 1 h at RT in the secondary biotinylated antibody anti-goat (Vector laboratories, Burlingame, CA, USA) in 2,5% FSB 0,25% gelatine in PBS 0,2% Triton X-100. After washings in PBS the brain sections were incubated for 1 h 30 min in the streptavidin–biotin complex (VECTASTAIN Elite ABC complex, Vector laboratories, Burlingame, CA, USA) with 2,5% FSB 0,25% gelatine in PBS 0,2% Triton X-100. After washing, peroxidase reaction was visualized by adding a solution containing 0,05% 3,3'-Diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, USA) and 0,01% H_2O_2 in PBS for 2 min. Finally tissues were dehydrated in a graded series of ethanol, cleared in xylenes and cover-slipped. The number of mice per experimental group was determined based on effect size observed in our previous studies: WT=9, TS=8, WT-EE=6, TS-EE=7, WT-EGCG=8, TS-EGCG=7, WT EE-EGCG=8, TS EE-EGCG=9.

Medial Septum volume and cholinergic neuronal quantification

The landmarks used for the circumscription of the MSN were the fusion of both the corpus callosum (rostral) and of the anterior commissure (caudal). MS volumes (μm^3) were estimated by planimetry as the summation of the MS area (μm^2) outlined from each section and calculating the total width based on the used sampling criteria (1/5 section series) and the section thickness (Mazonakis et al., 2004). The volumetric shrinkage of the tissue derived from the immunohistochemical procedure was similar in WT and Ts65Dn mice. The number of cholinergic neurons, within

the MS, was estimated using the optical fractionator, a stereological probe that enables unbiased sampling (CAST stereological software package [Visiopharm, Hørsholm, Denmark] adapted to an OLYMPUS BX51 microscope). MS region and cholinergic neuronal nuclei from brain sections were examined using 4X and 40X magnification lenses, counting frames area of 2139 μm^2 and a dissector height of 20 μm. Cholinergic neuronal number (N) was estimated by the equation:

$$N = \frac{1}{ssf} x \frac{1}{asf} x \frac{1}{hsf} x Q$$

Where ssf is the section sampling fraction (number of sections sampled/number of total sections), asf is the area sampling fraction (counting frames area/grid frame area) and hsf is the dissector height / thickness of the tissue. The coefficient of error (CE) was calculated according to Howard and Reed (2005) in order to evaluate the precision of the estimates for each brain. Only brains with neuronal number estimations associated with CE ≤ 0.10 were considered for the statistical analysis.

Statistical analysis

The latency times of the step-down inhibitory "passive" avoidance test after both one and six days were analyzed, as a function of genotype-treatment combination, with Weibull regression models, which take into account that the times of interest may be right-censored because of cut-off times in the case of this test for 3000 s (Klein et al., 2003). For the analysis of the cholinergic neuronal density, one-way ANOVA models were used being the genotype-treatment combination the only factor. Since the stereological estimations of neuronal densities had an associated coefficient of error, the observations were given weights inverse proportional to the square root of the measurement of this error. In the framework of both types of models, several contrasts of interest were tested: WT vs. TS; TS vs. TS-EE; TS vs. TS-EGCG; TS vs. TS EE-EGCG; WT vs. TS EE-EGCG; WT vs. WT-EE; WT vs. WT-EGCG; WT vs. WT EE-EGCG; WT vs. TS EE. To control the false discovery rate due to multiple post-hoc comparisons, the Benjamini-Hochberg

method was used (Benjamini and Hochberg, 1995). Statistical analysis were carried out with R (The R Foundation for Statistical Computing, Vienna, Austria), version 3.2.1. In particular, the contributed package multcomp (Hothorn et al., 2008) was used for the post-hoc comparisons.

Results

Effects of EE, EGCG and combined EE-EGCG treatments on the performance of the inhibitory "passive" avoidance test

Untreated WT mice showed a significant increment of the step-down latency both at 24 h (estimated median difference = 22 s; 95% CI [9.2; 59.2], Fig 1A), and 6 days (estimated median difference = 28.9 s; 95% CI [8.9; 108]; Fig 1A) after the training trial, indicating that the conditions of the test were appropriate to induce associative learning and retrieval of the aversive experience.

The differences among experimental groups in the step-down latency in the 24h and the 6 days retention trials were estimated using a survival censored model that accounted for the cut-off time of 300 s. In the 24 h retention trial Ts65Dn mice did not show significantly different step-down latencies than WT mice (Fig. 1, Table 1) and the different treatments did not show changes in performance neither in WT nor in Ts65Dn mice (Fig. 1, Table 1). Conversely, Ts65Dn mice presented a significantly reduced step-down latency in the 6 days retention trial in comparison to WT mice (Fig. 1E, Table 1). Both EE and EE-EGCG treated Ts65Dn mice showed an amelioration of this memory impairment as they presented an increased step-down latency in comparison to untreated Ts65Dn, rescuing Ts65Dn mice performance to WT levels (Fig. 1G, 1H, Table 1).

On the other hand, combined EE-EGCG, but not EE or EGCG, treated WT mice showed a significantly increased step-down latency in comparison to the untreated WT. (Fig. 1 Table 1).

Effects of EE, EGCG and EE-EGCG treatments on MS cholinergic neurodegeneration of Ts65Dn mice

The differences among experimental groups in volume and number of basal forebrain cholinergic neurons (BFCN) in the MS were estimated using a mixed model that accounted for the coefficient of error that arose from the stereological quantifications.

Ts65Dn showed no difference in the volume or rostro-caudal extension of the MS nucleus in comparison to WT mice (Table 2). Stereological analysis showed that 6-7 months old Ts65Dn mice presented a significant reduction in the number of cholinergic neurons within the MS (Fig. 2, Table 2) as previously described. No statistical differences were detected in the number of cholinergic neurons of EE or EGCG treated Ts65Dn mice in comparison to their untreated counterparts nor in EE-EGCG treated Ts65Dn mice although a non-significant tendency to an increase was detected in this group (Fig. 2, Table 2). Neither of the treatments showed differences in this parameter in WT mice (Fig. 2, Table 2).

Discussion

Individuals with DS present a higher incidence and earlier onset of AD-like neuropathology and dementia than the general population. They undergo a progressive age-associated neurodegenerative process and cognitive decline that resembles that of AD patients (Ballard et al., 2016) and these alterations are recapitulated in the partial trisomy Ts65Dn mouse model of DS (Holtzman et al., 1996; Granholm et al., 2000b).

This study extends our previous work (Catuara-Solarz et al., 2015) showing the effects of a combined treatment with EE and a green tea extract containing EGCG on contextual learning and memory deficits of Ts65Dn mice at the age of 6-7 months. Here we used the passive avoidance task (PAT), since its performance has been shown to rely on the correct functionality of the septo-hippocampal system. However, we did not found altered retention in the recent memory trial (24h), in the Ts65Dn mice. This is in accordance with

earlier reports in Ts65Dn mice male of 3-4.5 months (Coussons-Read and Crnic, 1996), nor 6-8 months old Ts65Dn (Holtzman et al 1996), in a multi-trial step-through PAT.

Previous studies have shown that performance in the recent memory trial (24 h) of PAT depends directly on specific transient changes of cholinergic activity and NGF at the basal forebrain (Wilson and Cook, 1994; Cole and Jones, 1995; Kojima et al., 1997). For instance, administration of nonselective muscarinic receptor antagonists such as scopolamine prior to PAT training causes impairment in the 24 h retention test (Meyers, 1965; Rush and Streit, 1992; Wilson and Cook, 1994; Cole and Jones, 1995). Conversely, pretraining administration of cholinomimetics such as AChE inhibitors (physostigmine, tacrine, donepezil) antagonizes the scopolamine-induced impairment of PAT retention (Dierssen et al., 1992; Rush and Streit, 1992; Yoshida and Suzuki, 1993; Kojima et al., 1997).

On the light of these results, our findings may suggest that cholinergic-dependent cognitive function is still preserved at age 6-7 months in Ts65Dn mice. However, in our experiments Ts65Dn mice showed poor performance on the 6 days retention test of the PAT. The performance in the delayed retention session of the PAT requires a more complex set of neurobiological processes, involving not only the basal forebrain, but also other brain regions such as the hippocampus, the amygdala and the perirhinal, postrhinal, and entorhinal cortices. These processes involve neural circuit reorganization, epigenetic modifications and metabotropic receptor signaling pathways (Kandel et al., 2014), which have been shown to be altered in Ts65Dn mice. We previously showed hippocampal disruptions in post-receptor signal transduction, in particular those mediated by adenylyl cyclase (Dierssen et al., 1997; Baamonde et al., 2011), that could lead to abnormal downstream activities of cAMP-dependent kinases and downstream CREB, which is critical for learning and memory (Kandel, 2012). In fact, it has been demonstrated that activation of adenylyl cyclase by infusion of forskolin into the hippocampus improved long-term memory in a

step-down avoidance task in rats (Bernabeu et al., 1997). Interestingly, both EE and EE-EGCG treated Ts65Dn presented improved long-term memory in the 6 days trial, in comparison to untreated Ts65Dn. This may suggest that both EE and EE-EGCG induced an improvement in the above neurobiological processes involved either with consolidation or retrieval of information in order to generate the appropriate behavioral response.

We examined the effects of the different treatments on the medial septum BFCN within the MS since they supply most of the cholinergic innervation to the hippocampus (Mufson et al., 2003), are anatomic substrates of memory and attention (Bartus, 2000) and had been shown to undergo a process of age-related degeneration in Ts65Dn mouse model (Salehi et al., 2006). In accordance with previous findings (Holtzman et al., 1996; Granholm et al., 2000b; Cooper et al., 2001; Seo and Isacson, 2005; Salehi et al., 2006) we found that 6-7 months old Ts65Dn mice presented a reduction in the number of BFCN in MS. The fact that this BFCN reduction did not reflect a behavioral impairment in the 24h retention trial of the PAT supports the idea that the neuropathological damage precedes detectable cognitive manifestations, as it has already been shown in DS and AD individuals (Ballard et al., 2016). This discrepancy between the cognitive and neuronal levels may be a result of a synaptic compensation by an increase of ChAT activity, as it has been previously reported (Seo 2005, Cooper 2011).

Interestingly, although EE and EGCG treatments did not prevent the loss of cholinergic neurons, combined EE-EGCG moderately ameliorated the BFCN degenerative phenotype, although the effects were not significant ($p = 0.09$). This suggests that combined EE-EGCG treatment may preserve the BFCNs modulatory inputs to the hippocampus which could, at least partially, explain the improvement in the long-term memory trial in the PAT. One possible explanation of the EE-EGCG-driven improvement in cognition and prevention of BFCN degeneration in Ts65Dn mice lies on the fact that both EE and EGCG treatments promote the secretion and activity of neurotrophins (BDNF and NGF) (Ickes et

al., 2000; Gundimeda et al., 2014; Renno et al., 2016) and modulate the kinase activity of Dyrk1A (Bain et al., 2003; Adayev et al., 2006; Golabek et al., 2011; Pons-Espinal et al., 2013) which phosphorylates both APP and tau proteins (Wegiel et al., 2011b) and influences choline acetyltransferase function (Hijazi et al., 2013). It has been demonstrated that one of the mechanisms that play a critical role in the BFCN degeneration seen in Ts65Dn involves abnormal expression of APP, which accumulates in early endosomes (Salehi et al., 2006, Cataldo et al., 2003), and interferes with the retrograde transport of neural growth factor (NGF) from the hippocampus to the BFCN somas (Salehi et al., 2006).

Taken together our results show that combined EE-EGCG treatment ameliorates age-related deficits in long-term memory in Ts65Dn mice and that this cognitive improvement is accompanied by a tendency towards an amelioration of the cholinergic degenerative process that takes place in the MS nucleus of the BF. In conclusion, our results suggest that the combination of EGCG and EE could be an efficient therapeutic strategy in older DS individuals and AD patients.

Figures and tables

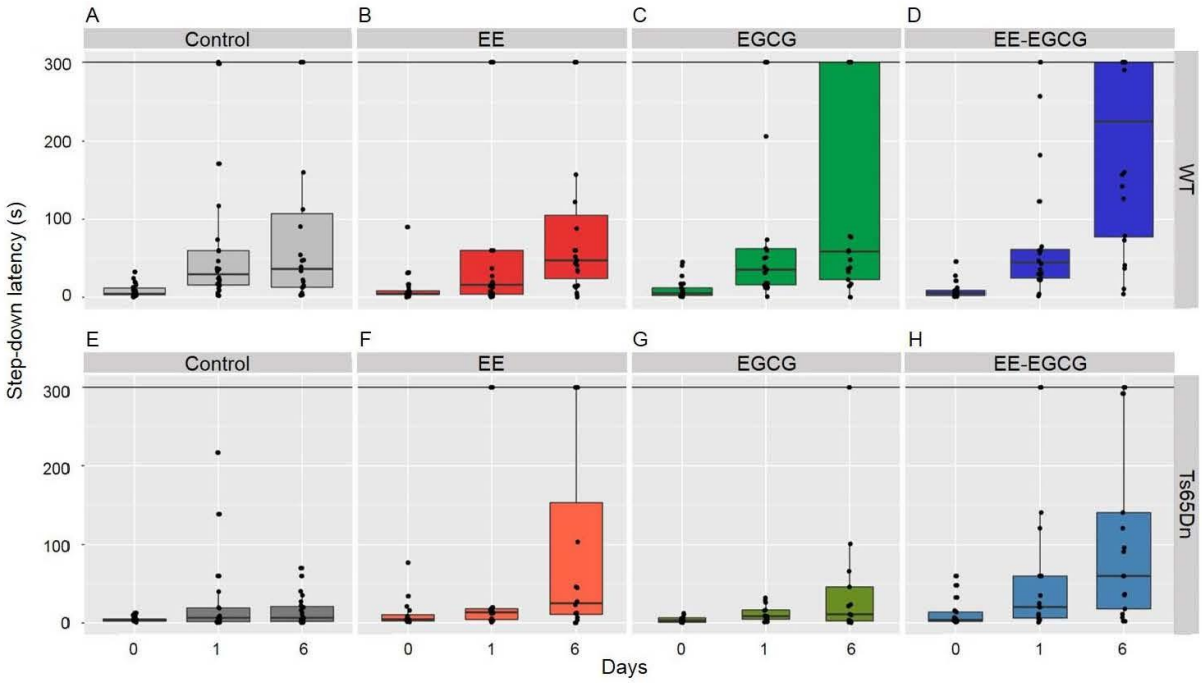
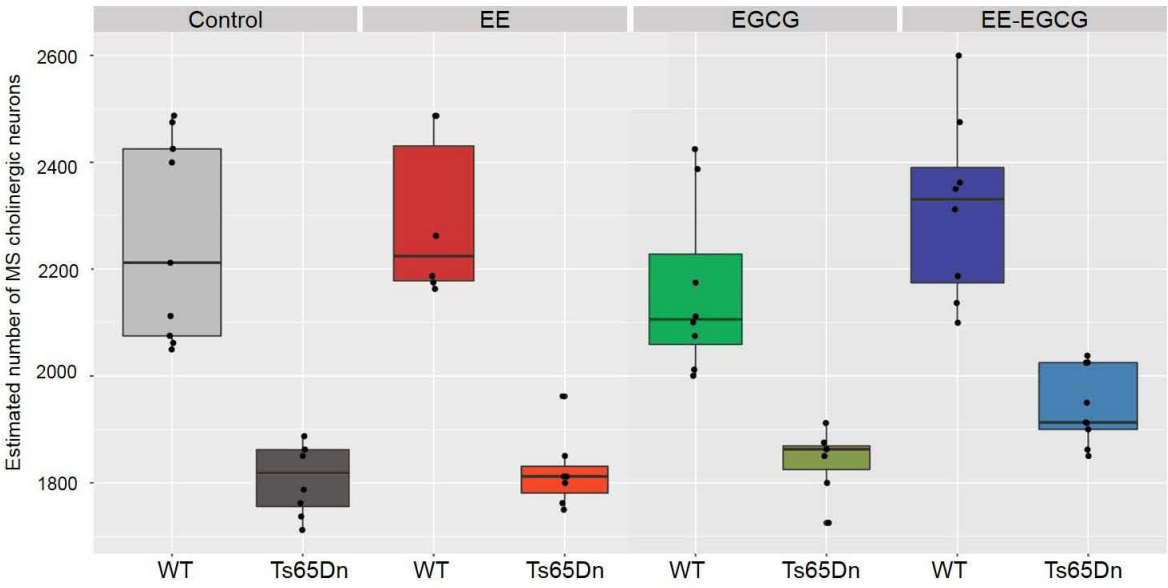


Figure 1: Step-down inhibitory passive avoidance test performance in WT and Ts65Dn mice, and EE, EGCG and EE-EGCG treatment effects. The figure shows boxplots of the distribution of the step-down latency in the passive avoidance test at training day, 1 day after and 6 days after, of each experimental group. In each boxplot, the dots indicate the values of each individual mouse, the box edges gives the 25th and 75th percentiles and the whiskers depict minimum and maximum values to a maximum of 1.5 times the interquartile distance from the box, the horizontal line corresponds to group median, and more extreme values are individually plotted. The cut-off time at 300 s is indicated by a continuous line. The number of mice per experimental group was the following: WT=22, TS=23, WT-EE=23, TS-EE=16, WT-EGCG=21, TS-EGCG=13, WT EE-EGCG=20, TS EE-EGCG=17. Data were analyzed with a censored model which accounted for the cut-off time. In the 6 days retention trial, Ts65Dn mice presented a reduced step-down latency than WT mice. EE and EE-EGCG treated Ts65Dn showed an increased latency than untreated Ts65Dn. EE-EGCG treated WT presented higher latency than their untreated counterparts. Statistical data and multiple comparisons are shown in Table 1.

Table 1: Post-hoc multiple comparisons of step-down latency among groups using the Weibull regression model

Trial	Contrast	Statistic	Sd error	p-value
1 day	WT vs. TS	1,096	0,46	0,154
1 day	TS vs. TS EE	-0,706	0,5156	0,513
1 day	TS vs. TS EGCG	0,5251	0,529	0,685
1 day	TS vs. TS EE_EGCG	-0,8796	0,4957	0,342
1 day	WT vs. TS EE_EGCG	0,2171	0,5047	0,751
1 day	WT vs. TS EE	0,3908	0,5252	0,685
1 day	WT vs. WT EE	0,1387	0,4817	0,773
1 day	WT vs. WT EGCG	-0,3858	0,4887	0,685
1 day	WT vs. WT EE_EGCG	-0,2146	0,4817	0,751
6 days	**WT vs. TS**	2,0077	0,5243	**0,0006**
6 days	**TS vs. TS EE**	-2,1521	0,5931	**0,0009**
6 days	TS vs. TS EGCG	-1,0092	0,5929	0,1597
6 days	**TS vs. TS EE_EGCG**	-2,1929	0,5529	**0,0006**
6 days	WT vs. TS EE_EGCG	-0,1853	0,5820	0,8508
6 days	WT vs. TS EE	-0,1445	0,6204	0,8508
6 days	WT vs. WT EE	-0,1030	0,5476	0,8508
6 days	WT vs. WT EGCG	-0,7573	0,5939	0,3035
6 days	**WT vs. WT EE_EGCG**	-1,6586	0,6413	**0,0218**

Figure 2



Stereological estimation of MS cholinergic neurons in WT and Ts65Dn mice, and EE, EGCG and EE-EGCG treatment effects. The figure shows boxplots of the distribution of the MS cholinergic neuronal density (number of neurons/MS volume) of each experimental group. In each boxplot, the dots indicate the values for each individual mouse, the box edges gives the 25th and 75th percentiles and the whiskers depict minimum and maximum values to a maximum of 1.5 times the interquartile distance from the box, the horizontal line corresponds to group median, and more extreme values are individually plotted. The number of mice per experimental group was the following: WT=9, TS=8, WT-EE=6, TS-EE=7, WT-EGCG=8, TS-EGCG=7, WT EE-EGCG=8, TS EE-EGCG=9. Data were analyzed with a model which weighted the coefficient of error associated to each cholinergic neuronal number estimation. Ts65Dn mice presented a reduced number of cholinergic respect to WT mice. EE-EGCG treated Ts65Dn showed a trend towards an increased number of cholinergic neurons in comparison to untreated Ts65Dn. Statistical data and multiple comparisons are shown in Table 2.

Table 2: Analysis of MS cholinergic neuronal density among groups. Analysis was performed using one-way ANOVA accounting the coefficient of error associated to each estimation. Post-hoc multiple comparisons performed with Benjamini-Hochberg correction to control for false discovery rate

Variable	Contrast	Statistic	Sd error	p-value
volume MS	WT vs. TS	-0,0078	0,005	0,813
volume MS	TS vs. TS EE	0,00066	0,005	0,904
volume MS	TS vs. TS EGCG	0,00319	0,005	0,813
volume MS	TS vs. TS EE_EGCG	0,0034	0,005	0,813
volume MS	WT vs. TS EE_EGCG	-0,0044	0,004	0,813
volume MS	WT vs. WT EE	0,00369	0,005	0,813
volume MS	WT vs. WT EGCG	0,00258	0,005	0,813
volume MS	WT vs. WT EE_EGCG	-0,00181	0,005	0,824
BFCN number	**WT vs. TS**	459,55	62,83	**<0,0001**
BFCN number	TS vs. TS EE	-17,34	68,79	0,802
BFCN number	TS vs. TS EGCG	-34,72	66,77	0,778
BFCN number	TS vs. TS EE_EGCG	-130,7	63,17	**0,09**
BFCN number	**WT vs. TS EE_EGCG**	328,85	60,96	**<0,0001**
BFCN number	**WT vs. TS EE**	442,21	66,75	**<0,0001**
BFCN number	WT vs. WT EE	-26,61	68,03	0,784
BFCN number	WT vs. WT EGCG	106,47	62,99	0,174
BFCN number	WT vs. WT EE_EGCG	-47,36	63,46	0,688

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3.5 Unpublished observations

In the preclinical studies presented here, we examined, among other phenotypes, the spatial learning and memory ability of young adult (2-3 months old) and middle age (6-7 months old) Ts65Dn female mice by the Morris water maze (MWM) and the potential therapeutic effects of combined treatment with EE and EGCG.

Our results confirmed previous work showing that Ts65Dn mice present significant learning and memory impairments in the MWM at both ages tested. However, we did not compare Ts65Dn learning and memory deficits between ages, since each timeframe was examined in separate pieces of work. Furthermore, we showed that combined EE-EGCG treatment ameliorates spatial learning deficits in middle-age Ts65Dn mice more efficiently than EE or EGCG alone, and also mitigates spatial deficits and hippocampal alterations in younger Ts65Dn mice.

These aspects, regarding age-associated phenotype and treatment effects, are particularly important in the context of translational research because they could be informative about both the underlying pathophysiological processes occurring across the lifespan and the putative modification by the treatment at each timeframe. As such, having insights about these aspects may shed light about a potential therapeutic window in which interventions would be optimal to treat intellectual disability in humans with DS. Therefore, as the conditions were practically identical between experiments, it is worthwhile to make here some qualitative observations about the cognitive impairments and the differential effects of EE-EGCG treatment at each timeframe.

3.5.1 Age-dependent spatial learning and memory deficits in Ts65Dn mice in the Morris water maze (MWM)

In the MWM, a number of learning and memory processes need to be executed to successfully navigate and locate the hidden platform to escape. During the acquisition sessions, efficient learning reflects the ability of mice to progressively create explicit operant-like associative memory of the spatial configuration of the visual extra-maze cues and their relation to the position of the platform. To display effective reference memory at the probe test, the previously learnt spatial information needs to be consolidated, retained, and retrieved. It has been known for long that the acquisition, storage and retrieval of spatial information is highly dependent on the dorsal hippocampus (Tolman, 1948; Nadel, 1991; Josselyn et al., 2015), being the neuronal populations within CA1 and DG subregions particularly important for these cognitive processes (Tsien et al., 1996; Deng et al., 2010).

When comparing the results accross different ages, younger mice regardless of the genotype, showed less efficient learning and cognitive flexibility than middle-age mice (Fig. 7). These results are in line with previous findings from Seo and collaborators (2005) that showed that 1 month old Ts65Dn and WT mice showed longer escape latencies compared to their 4 and 12 months old counterparts (Seo and Isacson, 2005). Hyde and colleagues (2001) also reported a developmental delay in Ts65Dn mice younger than 3 months of age, affecting context discrimination (Hyde and Crnic, 2001). They show that these deficits disappeared during a period spanning between 3 and 5 months of age, and were present again after 5 months of age. In our results, at both ages Ts65Dn mice showed thigmotactic behavior (swimming next to the wall of the pool doing circles), but in younger mice thigmotaxis was more pronounced, contributing to the increased learning and cognitive flexibility impairment (Fig. 7). Some authors have argued that thigmotactic

behavior is due to the use of inefficient learning strategies (non-searching or non-spatial) (Shichiri et al., 2011; Altafaj et al., 2013; García-Cerro et al., 2014) while others propose that it reflects sensorimotor and/or emotional issues that are independent from spatial learning and memory deficits (Simon et al., 1994; Holmes et al., 2002; Vorhees and Williams, 2014). This tendency to peripheral swimming is also present in transgenic mice overexpressing Dyrk1A, suggesting that Dyrk1A may contribute in the development of thigmotactic behavior (Altafaj et al., 2001). One possible explanation for the more pronounced cognitive impairment in younger Ts65Dn mice is that they have a delayed developmental maturation, as happens in individuals with DS (Takashima et al., 1994). Consistent with this interpretation, Seo et al (2005) found that, in comparison to older mice, 1–2 months old Ts65Dn mice presented higher levels of hippocampal NGF, which is a critical neurotrophin for both neurodevelopment and adult neuronal maintenance, that appears to be abnormally regulated throughout the lifespan in both mice and humans with DS (Sofroniew et al., 2001; Iulita et al., 2014). On the other hand, younger WT learning performance was also less efficient and presented higher thigmotaxis than that of their older counterparts. This has been attributed to developmental consequence from abnormal Ts65Dn maternal fostering (Liu et al., 2000).

In the probe session, there was a clear improvement in WT mice with age, but this age-associated progress does not take place in Ts65Dn mice (Fig 7). Younger and middle age Ts65Dn mice showed a similar level of impairment in specific reference memory parameters, such as the average distance to the platform (Gallagher index) and the time spent in the target quadrant. The fact that both younger and middle-age Ts65Dn mice presented an impairment in the acquisition and retrieval of spatial information suggests a disruption in the hippocampal processes needed for encoding and consolidation of associative memory. These disruptions could arise from developmental delay in younger mice or basal forebrain cholinergic neurodegeneration in middle-age mice.

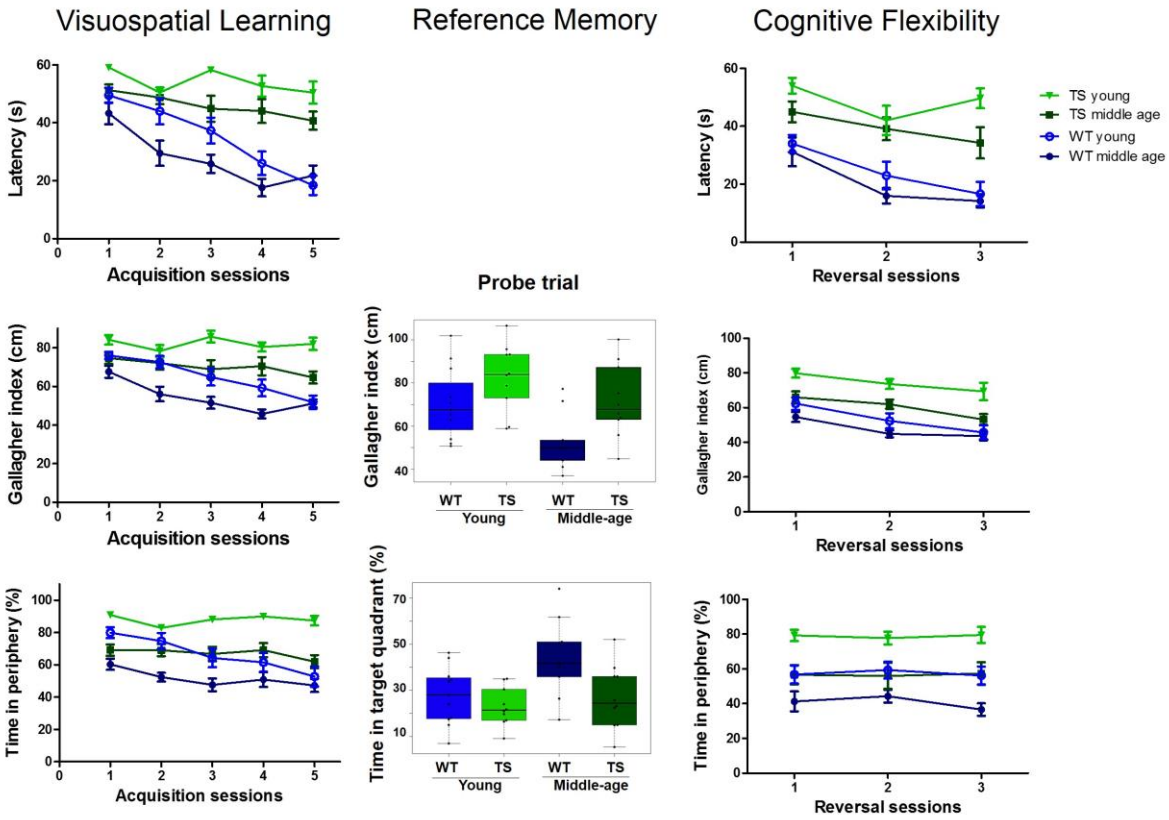

Fig 7. Visuospatial learning, reference memory and cognitive flexibility in the MWM in young (2-3 months old) and middle-age (6-7 months old) WT and Ts65Dn mice. For the visuospatial learning and cognitive flexibility phases, the mean ± SEM of the variables latency (s) to reach the escape platform, Gallagher index (mean distance to the goal in cm) and thigmotaxis (percentage of time spent on the periphery) are presented during the sessions. For the reference memory session (probe trial), boxplots of the distribution of the Gallagher index and the time spent in the target quadrant of the four experimental groups are presented, being the dots the values of each individual mouse. The purpose of showing this data is to make qualitative observations.

Additionally, our multidimensional analysis using PCA revealed that the act of learning in the MWM induced an increment in within-group behavioral heterogeneity in both young and middle-age mice. Middle-age mice showed a higher behavioral variability before and after learning in comparison to younger mice, regardless of the genotype (Fig. 8). This highlights an increased phenotypic

variability induced by experience and learning, even in a relatively isogenic population of mice.

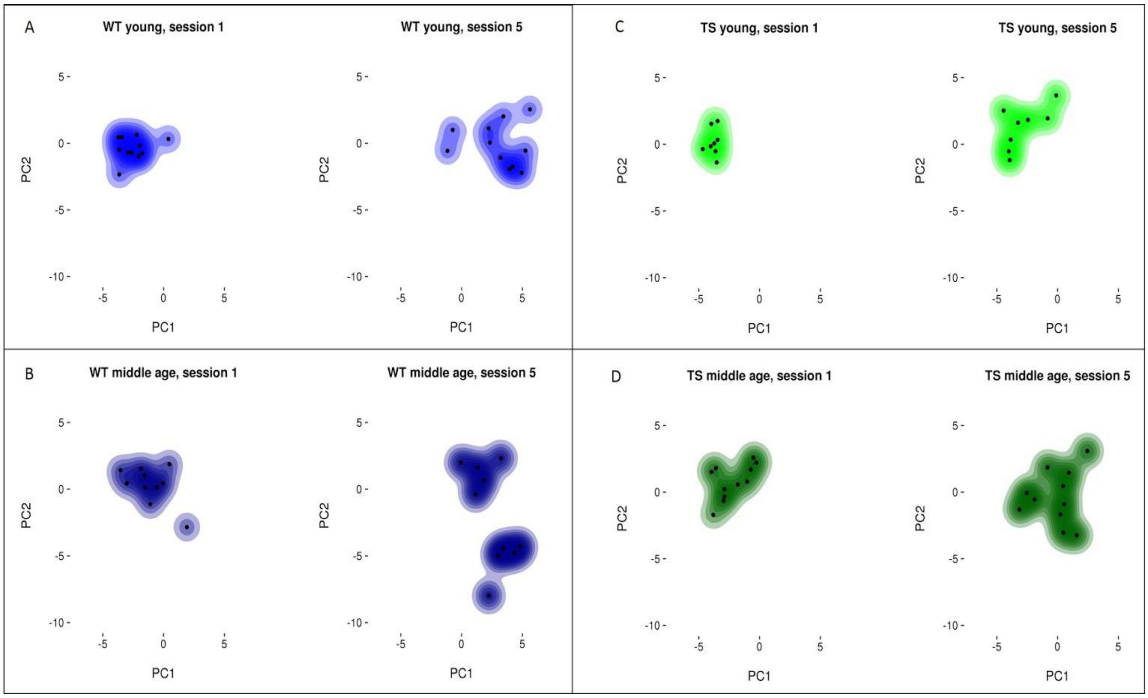

Fig. 8: Learning induces within group behavioral heterogeneity. Density distribution of young and middle-age WT (A-B) and Ts65Dn (C-D) groups for PC1 (learning composite variable) and PC2 (mainly swimming speed) at the first and the last acquisition learning sessions of the MWM.

Noteworthy, given the fact that we used female mice to perform the experiments, since in males EE produces a shift towards a more territorial organization increasing stress and aggressiveness (Haemisch and Gärtner, 1997; Martínez-Cué et al., 2002), and that female Ts65Dn mice showed a more pronounced visuo-spatial learning impairment compared to male Ts65Dn mice (Martinez-Cué st al., 2002), all the visuo-spatial learning data presented here should be considered gender-specific.

3.5.2 EE-EGCG treatment effects on young and middle-age Ts65Dn mice performance in the MWM

When comparing the EE-EGCG treatment effects at each ages, while the treatment resulted in learning improvement in Ts65Dn mice at both ages, middle-age mice were more benefited from the treatment than younger mice (Fig 9). The combined treatment had a stronger impact on reference memory in middle-age mice than in younger mice (Fig 9).

The fact that younger mice were less benefitted by the EE-EGCG treatment than middle-age mice suggests that the molecular pathways triggered by the treatment may not be able to fully reverse their developmental delay. However, if EE-EGCG treatment was able to reverse, at least partially, the developmental delay in young Ts65Dn mice, potentially their cognitive impairment later in life would also be ameliorated. On the other hand, despite the fact that middle-age Ts65Dn mice have already gone through the initiation of cholinergic neurodegeneration and cognitive decline, EE-EGCG treatment was fairly beneficial, which may suggest its ability to slow down the neurodegenerative process.

Conversely, according to the statistical analysis shown in the papers presented above, EE-EGCG treatment improved cognitive flexibility in younger mice but not in middle-age mice. During the reversal sessions, mice have to extinguish their initial learning of the platform position and acquire new spatial information regarding the current location of the platform. It is considered a measure of executive function and cognitive flexibility and depends both on the prefrontal cortex and the prefrontal functional integrity (de Bruin et al., 1994). The fact that EE-EGCG partially restored cognitive flexibility in younger mice may suggest that it can enhance the functionality of the prefrontal cortex despite their developmental delay.

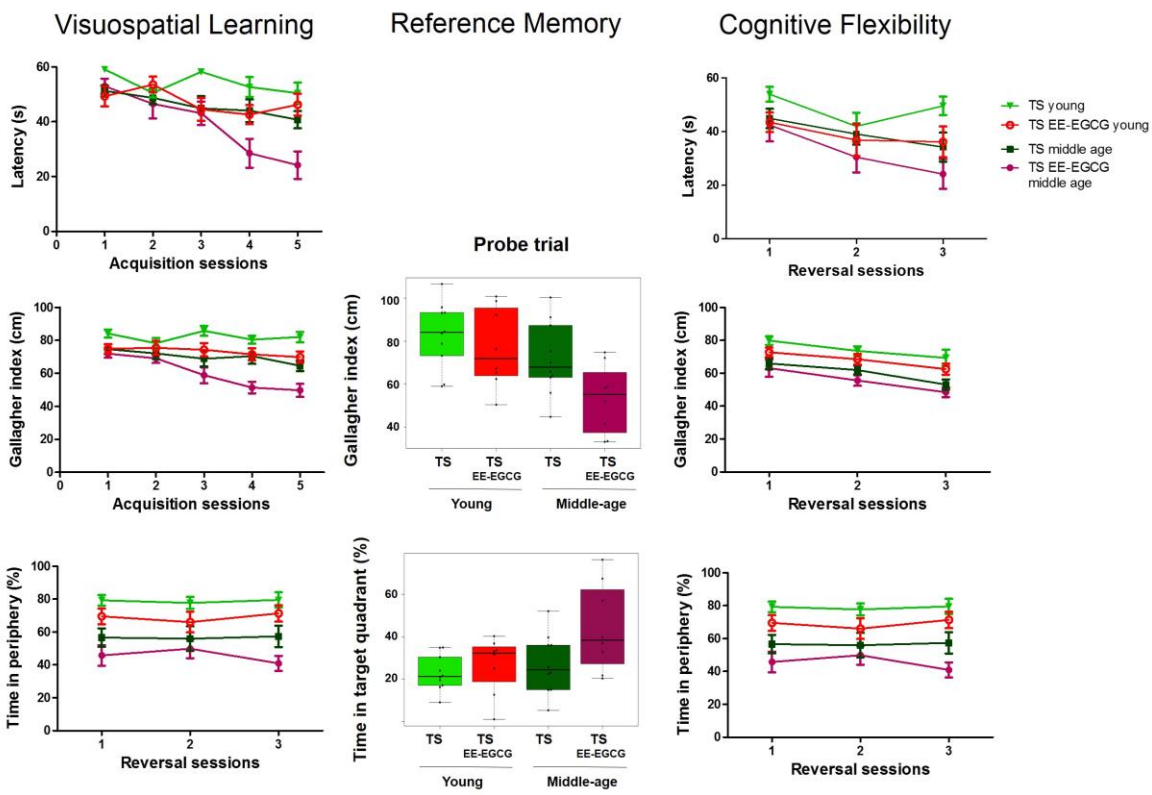

Fig 9. EE-EGCG treatment effects on visuospatial learning, reference memory and cognitive flexibility in the MWM in young (2-3 months old) and middle-age (6-7 months old) Ts65Dn mice. For the visuospatial learning and cognitive flexibility phases, the mean ± SEM of the variables latency (s) to reach the escape platform, Gallagher index (mean distance to the goal in cm) and thigmotaxis (percentage of time spent on the periphery) are presented during the sessions. For the reference memory session, boxplots of the distribution of the Gallagher index and the time spent in the target quadrant of the four experimental groups are presented, being the dots the values of each individual mouse. The purpose of showing this data is to make qualitative observations.

In fact, this could be explained by the EE-EGCG treatment modulation of Dyrk1A activity, as its overexpression has been shown to play a role in prefrontal cortex dysfunction by altering NMDAR-mediated long term potentiation, increasing oblique dendrites spine density and enlarging miniature EPSCs (Thomazeau et al., 2014), and also by disrupting gamma frequency power and

inducing a selective disinhibition of interneurons (Ruiz-Mejias et al., 2016). On the other hand, the finding that EE-EGCG treatment failed to improve cognitive flexibility in middle age mice is consistent with findings from Granholm et al (2000) that show an increasing impairment in behavioral flexibility in Ts65Dn mice over 6 months of age. Inflexibility of learning is an early feature of AD dementia (Albert, 1996; Traykov et al., 2007) and thus perhaps cognitive flexibility is more vulnerable than learning, and the prefrontal cortex may not be modulated by the treatment at this age due to AD-like degeneration.

Additionally, multidimensional analysis showed that the learning-dependent heterogeneity was higher in middle-age untreated WT and EE-EGCG treated Ts65Dn mice (Fig. 10). Among Ts65Dn mice, some extent of genetic variability is expected because they are maintained as F1 hybrids of female C57BL/6JEi and male C3H/HeSnJ (Davisson 1993) and thus, the three alleles of the trisomic segment in each mouse may all derive from C57BL/6JEi or be combinations of C57BL/6JEi and C3H/HeSnJ (Gardiner 2004). Therefore, different sources of the behavioral variability that we found in both untreated WT and treated Ts65Dn mice, may include this allelic variation in trisomic and euploid genes in the case of Ts65Dn mice, and other stochastic or environmental factors, in mice of both genotypes. The higher behavioral variability in EE-EGCG treated middle-age Ts65Dn mice could be attributed to the interaction between allelic variations, age and EE-EGCG treatment, giving rise to differential behavioral responses. The reasons of this behavioral heterogeneity are still uncertain however this could have clinical consequences since DS population may as well have heterogeneous outcomes after EE-EGCG treatment.

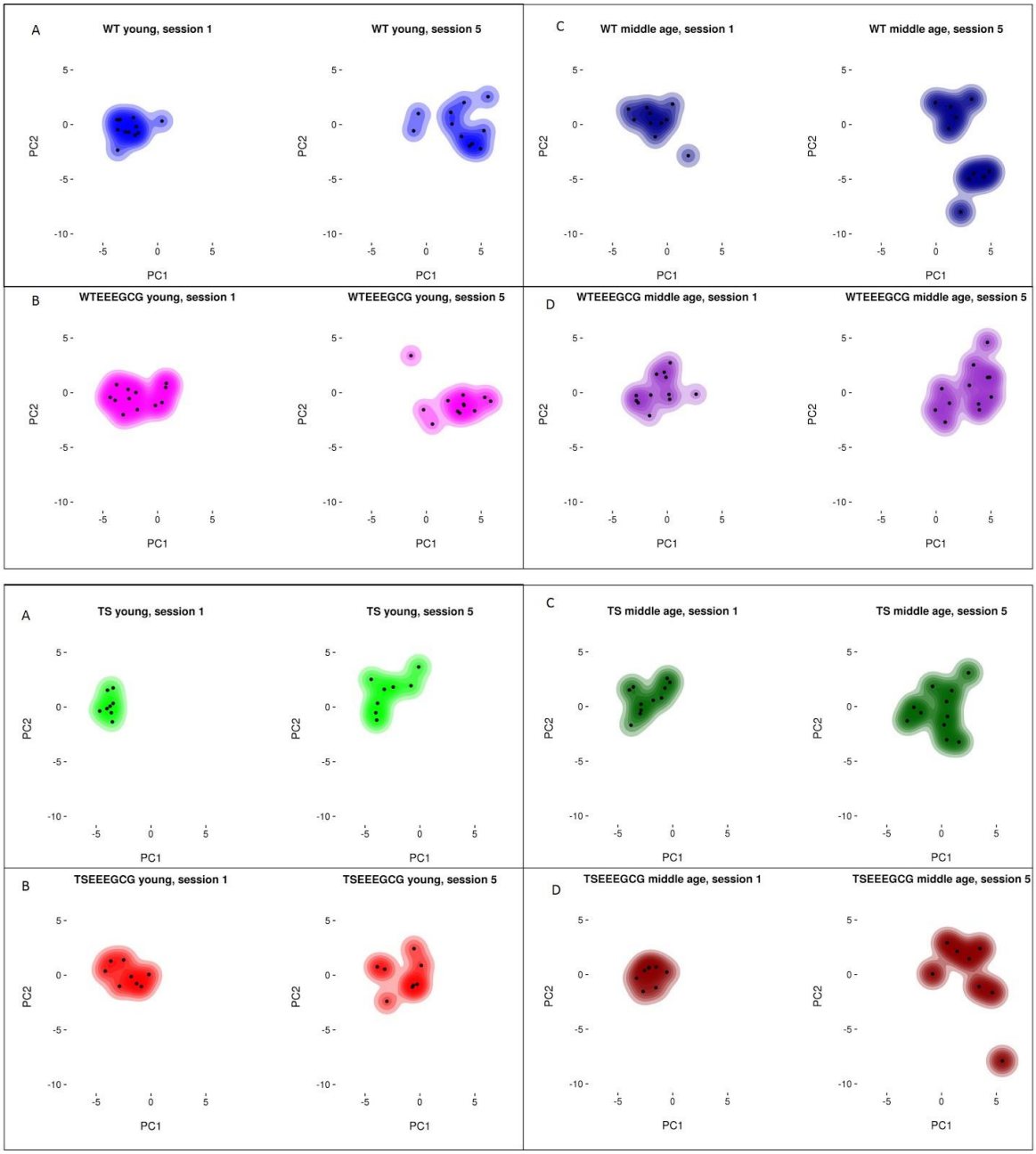

Fig 10: EE-EGCG treatment effects on learning-dependent behavioral heterogeneity in young and middle age WT and Ts65Dn mice. Density distribution of untreated and EE-EGCG treated WT and Ts65Dn mice at 2-3 and 6-7 months of age, for PC1 (learning composite variable) and PC2 (mainly swimming speed) at the first and the last acquisition learning sessions of the MWM.

Collectively, our results suggest that spatial learning and memory impairment is more pronounced in younger in comparison to middle-age Ts65Dn mice, possibly due to a developmental delay. We also found that in all mice learning induced an increment in behavioral heterogeneity. Regarding the effects of the combined EE-EGCG treatment our data suggest that middle-age Ts65Dn mice showed a stronger improvement in learning and memmory than younger mice and presented a greater heterogeneity after learning, meaning that some mice were more benefited by treatment than others. However, EE-EGCG treatment improved cognitive flexibility in younger but not in middle age Ts65Dn mice, suggesting a different effect of the treatment in prefrontal cortex accross different ages.

4. CHAPTER II. CLINICAL STUDIES

4.1 Preface

During my PhD studies I participated in the performance of two clinical trials (phase I and II) that I present in this chapter.

The first work (clinical trial phase I) includes the study of the effects of the oral administration of green tea extract containing EGCG in both animal models and young adult individuals with DS. Studies with mice were focused on the effects of EGCG on cognitive performance, hippocampal Dyrk1A kinase activity and plasma homocysteine (Hcy) levels as an efficacy biomarker of Dyrk1A normalization. In the pilot clinical study in humans, the main aspects that were addressed were the safety and toxicity of the EGCG compound, its effects on prefrontal and hippocampal-dependent cognitive function, plasma Hcy levels and quality of life. The dosage of the EGCG compound was matched between mice and humans studies. However, humans with DS only received a short-term EGCG treatment in the clinical trial since the most important outcome measure was safety. This implies a relevant difference between the duration and administration type of the EGCG treatment in the mouse preclinical studies and the human clinical studies, being relatively chronic/ long-term in the former and acute/ short-term (3 months) in the latter. The results from this pilot clinical trial were promising, showing that, besides being safe, EGCG treatment was able to promote improvements in cognitive function in both animal models and individuals with DS, and that these cognitive effects were accompanied by a transient normalization of Dyrk1A kinase activity.

We then hypothesized that EGCG treatment would potentially be more beneficial if paired with interventions that also increase neuroplasticity, such as cognitive stimulation. The second work (clinical trial phase II) was aimed at testing the hypothesis elicited by the pilot clinical trial. Besides safety, it primarily addressed the

efficacy of the combined treatment with EGCG and cognitive training vs. cognitive training with placebo, and consisted of a much longer longitudinal study (13 months) with a larger population of individuals with DS. The effects of the treatments were examined on cognitive ability, neurophysiological measures of brain function and quality of life. The results showed that the combined treatment significantly improved memory, executive functions and adaptive behaviour. Additionally, neuroimaging and neurophysiology showed increased functional connectivity and normalized cortical excitability. Interestingly, the positive effects of EGCG and cognitive training on memory and executive functions persisted 6 months after treatment discontinuation.

4.2 Paper I. Epigallocatechin-3-gallate, a DYRK1A inhibitor, rescues cognitive deficits in Down syndrome mouse models and in humans

The results from this work, published in the journal of Molecular Nutrition and Food Research in 2014, settled an inflection point and represented the grounds for the rest of the evidence-based translational study presented in this Thesis.

In this paper we combined mouse and human experiments to demonstrate that EGCG produces its cognitive effects, at least partially, through inhibition of DYRK1A kinase activity in the hippocampus. Furthermore, we validated the use of and plasma homocysteine (Hcy) levels as an efficacy biomarker of Dyrk1A activity normalization.

My specific contribution consisted on the performance of preclinical behavioral experiments comparing the effects of two different formulas of green tea extracts containing 45% EGCG (Mega green tea extract, Life Extension) or 94.2% EGCG (Teavigo).

De la Torre R, De Sola S, Pons M, Duchon A, de Lagran MM, Farré M, et al. Epigallocatechin-3-gallate, a DYRK1A inhibitor, rescues cognitive deficits in Down syndrome mouse models and in humans. Mol Nutr Food Res. 2014 Feb;58(2):278–88. DOI: 10.1002/mnfr.201300325

4.3 Paper II. A new cognitive evaluation battery for Down syndrome and its relevance for clinical trials

In this paper we propose a new neuropsychological assessment tool, the TESDAD battery, designed to evaluate DS therapies in the context of clinical trials, and we provide the baseline scores of the volunteers that participated in the phase II clinical trial.

The main advantage of the method of the TESDAD battery is that instead of comparing DS individuals to matched healthy controls of the same mental age, as it has been traditionally done, it relates performance of DS individuals to age-matched typically developed adults. As a result it confers a clear definition of the cognitive gap that is addressed by therapies aiming at restoring intellectual and adaptive functioning in individuals with DS.

My specific contribution to this work was involved with the performance of the neuropsychological assessment of the volunteers and the interviews with the care-givers of the volunteers.

De Sola S, de la Torre R, Sánchez-Benavides G, Benejam B, Cuenca-Royo A, Del Hoyo L, et al. A new cognitive evaluation battery for Down syndrome and its relevance for clinical trials. Front Psychol. 2015 Jun 4;6:708. DOI: 10.3389/ fpsyg.2015.00708

4.4 Paper III. Safety and efficacy of cognitive training plus epigallocatechin-3-gallate for cognitive improvement in young adults with Down's syndrome (TESDAD): a double-blind, randomised controlled, phase II trial

This work is the first long-term randomized controlled clinical trial using a dietary supplement (green tea extract containing 45% of EGCG) combined with a computerized cognitive training in a population of young adults with DS.

My specific contribution was involved in the performance of the longitudinal neuropsychological assessment of the volunteers using the TESDAD battery and the performance of interviews with parents or caregivers to gather information of their perception of the volunteers' progress along the clinical trial.

De la Torre R, de Sola S, Hernandez G, Farré M, Pujol J, Rodriguez J, et al. Safety and efficacy of cognitive training plus epigallocatechin-3-gallate in young adults with Down's syndrome (TESDAD): a double-blind, randomised, placebo-controlled, phase 2 trial. Lancet Neurol. 2016 Jul;15(8):801–10. DOI: 10.1016/S1474-4422(16)30034-5

5. DISCUSSION

Until few years ago, intellectual disability associated with DS was considered a permanent and intractable hallmark of the disorder. As such, during decades, DS had been relegated as a disease orphan of treatment, since a trisomy was considered a too complex scenario for granting pharmacological intervention. This derived in improvements in general health in individuals with DS that were not accompanied by the amelioration of their cognitive impairment and its related clinical, social and economic burden. Along the last years, extensive preclinical research has identified a number of altered molecular pathways, and neurobiological processes that may be involved in the cognitive deficits of DS. Increasing available evidence in preclinical models has shown the likelihood of improving cognitive deficits and mitigating structural and functional brain alterations in DS through the administration of diverse therapies targeting altered neurodevelopment and neuroplasticity, excitation–inhibition imbalance and neurodegeneration. Nevertheless, at the present time, only some of those possibilities are being tested in individuals with DS.

In this Thesis preclinical and clinical studies have validated the first therapeutic intervention to improve cognitive performance in DS. The results reported here show, for the first time, that combined environmental cognitive stimulation and EGCG therapy significantly ameliorates cognitive deficits in Ts65Dn mice and young adult individuals with DS, by modifying neuronal network structure and function.

The preclinical studies showed that the treatment is effective in both in young and middle-age Ts65Dn mice, despite the disparities underlying pathophysiological mechanisms in age-dependent cognitive impairments. However, the behavioral experiments also showed that phenotypic heterogeneity, which is a typical feature in DS, increases after learning and upon treatment in both Ts65Dn and WT mice. This was observable in both young and aged adults, and could reflect environmental and developmental differences that may

also account for the inter-subject variability of treatment efficacy. Histological and molecular experiments revealed that combined environmental enrichment (EE)-EGCG treatment promotes hippocampal structural and synaptic neuroplasticity changes, by increasing dendritic spine density in CA1 and restoring the balance between excitatory and inhibitory synaptic proteins in CA1 and DG. These preclinical data shed some light on the underlying mechanisms of the treatment and the best timing for its administration.
The main findings of the clinical trials showed that EGCG treatment is safe in young adult individuals with DS and induces a cognitive improvement when administered alone for a short period of time. Moreover, the administration, for a longer period, of combined treatment with cognitive training and EGCG, improved cognitive function by modifying patterns of brain functional connectivity and excitability, in a more efficient way than cognitive training as a monotherapy. These effects had a significant impact in adaptive behavior.

Besides the discussion of the specific results that is provided in each of the paper, here I provide a more general view including also aspects that were not addressed before.

5.1 Bridging preclinical and clinical results

5.1.1 Cognitive phenotype of mice and humans with DS: a matter of face validity and cognitive assessment tools

In the past decades, a remarkable progress has been made on the understanding of DS cognitive function. We now know that the cognitive profile of humans with DS primarily comprises deficits in learning, memory, language and executive functions, reflecting alterations on aspects of medial temporal lobe, cerebellar and prefrontal function (Pennington et al., 2003; Fidler and Nadel, 2007; Vicari et al., 2007). The study of cognitive deficits in DS has

benefitted substantially from the use of mouse models, which have provided data highlighting the neural mechanisms that may underscore cognitive difficulties. Beyond offering testable mechanisms of drug treatment in this population, mouse models that prove sensitive to the same treatment as humans may also help us understand the reasons for treatment success, the projected time-course of treatment effects, and in the identification of biomarkers to establish treatment efficacy. However, it is still challenging to assess equivalent cognitive capabilities in mouse models and humans, to be able to directly translate the findings from one to another.

The preclinical studies presented here confirmed a strong deficit in hippocampal-dependent visuospatial learning and memory in Ts65Dn mice, as shown by the Morris water maze (MWM). The experimental procedure of the MWM ensures that, in order to efficiently perform the test, mice have to create a coordinate map holding the relationships among the location of the cues and the platform. These representations are independent of the mice position since each trial is initiated from random sites around the pool. The deficits in this task were shown to be more pronounced in Ts65Dn mice at 2-3 months than at 6-7 months of age, possibly due to maturational delay, as discussed in the unpublished observations section. Within the hippocampus, the dentate gyrus and CA3 are involved in memory tasks that require spatial pattern separation (Bakker et al., 2008) and the CA1 and the subiculum have been shown to be involved in novelty detection and spatial navigation (Kesner and Goodrich-Hunsaker, 2010).
Conversely, in both the phase I and II clinical trials, the basal cognitive profile of the volunteers with DS presented clear deficits in language and executive functions but, although clinically relevant, the deficits in spatial memory (spatial span, SSP, forward recall, CANTAB) were not so robust, suggesting a better preservation of hippocampal-dependent memory processes compared to frontal-mediated processes (De la Torre et al., 2014; De Sola et al., 2015). These results are in agreement with early

studies showing that individuals with DS perform at the level of mental age matched controls on tasks requiring immediate memory for spatial locations (Corsi block-tapping task) (Wang and Bellugi, 1994; Jarrold et al., 1999; Numminen et al., 2001; Laws, 2002), which may have been somewhat hastily interpreted as a rather spared visuospatial memory capacities. In fact, in early studies of the visual-spatial processing of global and local elements of a visual display, Wang et al., (1995) presented data suggesting individuals with DS had an abnormal tendency towards globally oriented visual perception. In addition, Pennington et al. (2003) and Visu-Petra et al (2007) reported deficits on binding between object and location (CANTAB PAL) and spatial memory through navigation learning (a virtual MWM). These findings suggest deficits on tasks dependent on medial temporal lobe (MTL) structures adjacent to the hippocampus. Indeed, MRI studies showed consistent evidence for reductions in gray matter density in the hippocampus in children and young adults with DS (Pinter et al., 2001; Menghini et al., 2011). Alterations in MTL microstructure are also apparent, with levels of dendritic branching in the temporal cortex, CA1, CA2 and CA3 in the hippocampus particularly affected in patients with DS (Ferrer and Gullotta, 1990). White et al., (2003) also found reductions in gray matter density in non-demented adults with DS, including specific reductions in CA2 and CA3.

Thus the discrepancy between preclinical and clinical studies may reflect the fact that the neuropsychological tests used in the clinical trial to measure spatial memory, involving the spatial location of 2D objects are not the optimal tools to measure hippocampal-dependent function. In fact, these tests can be solved using egocentric (view-point dependent) strategies and do not require allocentric (view-point independent) learning and memory representations, which depend on the functional integrity of the hippocampus. In support of this argument, meta-analysis of previous neuropsychological data and also recent studies implementing more sophisticated assessment tools with 3D spatial tasks and real-world orientation, have found

alterations in hippocampal-dependent spatial memory in individuals with DS (Yang et al., 2014; Lavenex et al., 2015).
By this observation we do not intend to assume that the visuospatial impairments in mice and humans with DS would be fully equivalent. In fact, cross-species differences may involve still unknown particularities in the way spatial information is processed and which could be differently affected by aneuploidy. This discussion highlights the importance of the sensitivity of the behavioral and neuropsychological methods used in preclinical and clinical populations and how different approaches in their design could lead to divergent conclusions about the nature of intellectual disabilities.

5.1.2 Cognitive improvements derived from combined EE-EGCG treatment on mice and humans with DS

We have to take into account the previous discussion on the experimental tools when comparing the effects of the treatments in mice and humans. The results from mouse studies showed that combined EE-EGCG improved spatial learning in the MWM, and long-term contextual memory in the step-down "passive" avoidance test (PAT), both being cognitive functions contributed by the hippocampus. Even though a direct comparison to humans has to be taken with caution, these results are consistent with those obtained in the clinical trial showing that both EGCG alone, and combined cognitive training with EGCG specifically improved visual episodic memory (pattern recognition memory-immediate recall, PRM, CANTAB), which depends on hippocampus but also the surrounding entorhinal, perirhinal, and parahippocampal cortices. Moreover, the combined EE-EGCG therapy improved cognitive flexibility (reversal learning) observed in young adult Ts65Dn mice, which is in line with the improvements in executive function in humans that received cognitive training and EGCG treatment, as shown by the "cats and dogs" inhibitory control test.

Interestingly, in the phase I clinical trial, the cognitive effects of EGCG were temporary but, on the phase II clinical trial, the improvements on memory and executive function by the combined cognitive training and EGCG lasted longer and persisted after treatment discontinuation. In the preclinical studies, we did not examine the duration of the treatments effects at the behavioral and cellular levels or whether they persisted after intervention withdrawal. Noteworthy, while the length of the treatment in preclinical studies was 1 month (which accounts as a relatively chronic/ long-term treatment considering the mouse lifespan), in the phase I study the length of EGCG treatment was only 3 months whereas in the phase II clinical trial the combined treatment lasted 13 months (accounting as short-term interventions). The fact that the effects of the combined treatment (and not EGCG alone) persisted after discontinuation suggests that the combined treatment could have intrinsically different effects, potentially translating transient changes into more long-lasting structural modifications and thus persist over time. In fact both the preclinical histology and the clinical fMRI studies suggest structural plasticity related changes that in humans were long-lasting.

Finally, the determination of the most appropriate age for treatment administration and the most suitable treatment length is a very relevant aspect that could be advised by preclinical studies. In fact, qualitative comparison between the preclinical studies in young (2-3 months) and middle-age (6-7 months) mice suggest that the effects of the combined EE-EGCG treatment were more robust in middle-age mice (see Chapter I: Unpublished Observations).

However, our preclinical and the clinical studies presented subtle differences in the age of mice and humans. The age of the volunteers with DS that participated in the clinical trials (14-30 years old) corresponded to an intermediate stage between the groups of mice of the preclinical studies (2-3 and 6-7 months old) considering the approximation by Flurkey and colleagues (2007) (Fig. 11) (De la Torre et al., 2016).

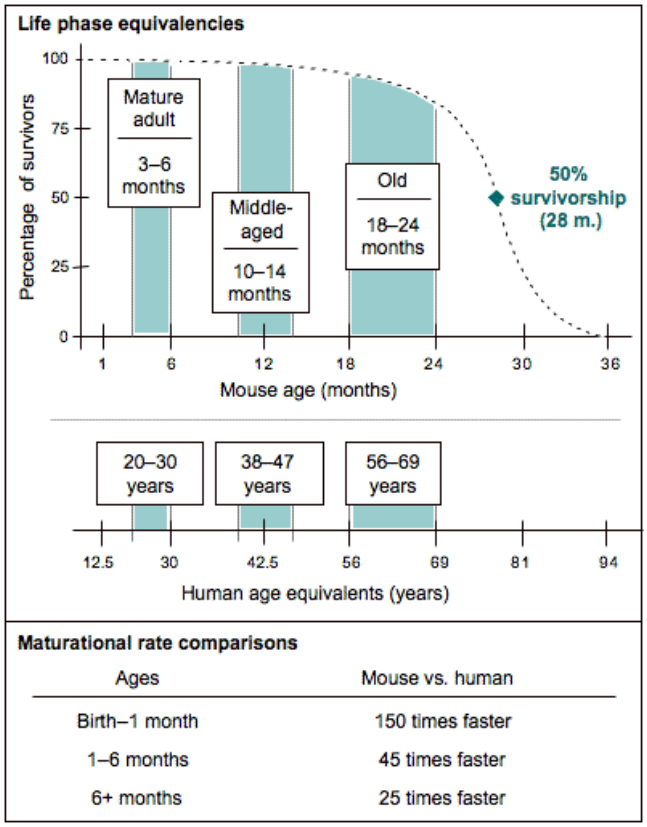

Fig 11. Representative age ranges for mature life history stages in C57BL/6J mice; comparison to human beings. (Adapted from Flurkey, 2007)

Thus, unfortunately, these issues still remain to be defined. Considering the substantial evidence that the cognitive profile in individuals with DS evolves across the lifespan, with periods of developmental delay and cognitive decline, these aspects will possibly be of crucial importance for the achievement of the maximum effectiveness of the treatment and will be essential for the potential standardization of combined cognitive training with EGCG as a therapy for DS. The expectation is that the best timeframe to start a neuroplasticity-targeted treatment is the earliest possible, since at earlier stages of life, neuroplasticity potential is at a higher peak enabling maximum effects of therapy (Bartesaghi et al., 2015; Stagni et al., 2015).

5.1.3 Neuro-structural and functional correlates of cognitive improvements derived from the treatment in mice and humans with DS

The pattern of findings of brain dysfunction in DS is suggestive of compromised development and function in late-developing systems. The prefrontal cortex, hippocampus and cerebellum are regions with relatively protracted neural development, including post-natal generation of neurons and synapses, and myelination of the tracts connecting these regions and the rest of the brain persisting into later childhood and early adulthood. Recent evidence suggests that there may be dissociations in the developmental trajectory of functional subregions within these structures as well, and that the later developing components are again at greatest risk. Why having an extra copy of Hsa21 differentially affects late-developing structures remains unclear, but the pattern of differences in brain structure and function observed in these regions seems relatively well established in both humans and mouse models, and would possibly be a relevant target for therapy.

The phase II clinical trial showed that the cognitive improvement associated to the combined cognitive training and EGCG was at least partially mediated by an increase in connectivity in the frontal, somatosensory, and occipito-temporal cortices, as shown by a marked enhancement in the functional integration of distributed networks in these cortical and subcortical regions, through resting state functional MRI (fMRI). This is in line with the results at the neuro-morphological level from young Ts65Dn mice studies showing that EE-EGCG treatment induced a partial recovery in structural neuroplasticity, as shown by an increment in dendritic spine density at CA1 region, but not in DG, in the hippocampus. Even being local, it could be speculated that the region-dependent structural neuroplasticity changes detected in the preclinical studies may have consequences in the functionality of more global neuronal

networks, as shown in the large-scale results from the clinical studies. Some of the mechanisms that may underlie the observed neuroplasticity changes induced by the combined treatment both in humans and mice include increases in BDNF expression (Young et al., 1999; Li et al., 2009a, 2009b), increased phosphorylation of CREB and Akt (Jia et al., 2013; Ramírez-Rodríguez et al., 2014; Ortiz-López et al., 2016) and also a reduction of Dyrk1A kinase activity (Bain et al., 2003; Golabek et al., 2011; Pons-Espinal et al., 2013). However, further studies will be needed to elucidate the exact signaling pathways responsible for the effects of the combined treatment.

On the other hand, in the clinical trial we detected intracortical facilitation by transcranial magnetic stimulation (TMS) in our DS population. This increased facilitation is in agreement with previous findings showing higher frequency of seizures and epilepsy in DS population (Menéndez, 2005; De Simone et al., 2010), suggesting an overall hyperexcitability. However, it is difficult to reconcile with previous results from Ts65Dn mice that have suggested over-inhibition in hippocampal circuitry as shown by reductions in the number of asymmetric (excitatory) synapses along with enlargement of symmetric (inhibitory) synaptic active zones in cortex and hippocampus (Kurt et al., 2000, 2004; Belichenko et al., 2009), increased immunoreactivity of inhibitory synaptic proteins (Belichenko et al., 2009), increment of inhibitory inputs onto spine necks (Belichenko et al., 2007), decrease in LTP which is revered by $GABA_A$ and $GABA_B$ receptors antagonists (Kleschevnikov et al., 2004, 2012; Martinez-Cue et al., 2013). In fact, our results from young Ts65Dn mice showed an increment in the density with a reduction in the size of excitatory (Vglut1) puncta while inhibitory (Vgat) puncta remained the same or was increased in size in hippocampal DG and in CA1 regions, likely affecting probability of neurotransmitters release. Interestingly, a recent work by Deidda and colleagues (2015) found that $GABA_A$ receptor signaling exerted excitatory rather than inhibitory function in adult Ts65Dn hippocampus and neocortex, suggesting that GABA

neurotransmitter may induce neuronal depolarization. This shift in neurotransmitter effects could account for synaptic plasticity defects, including a lower threshold for action-potential generation that contribute to cognitive impairment in DS.

Of course these data in mouse and human cannot be directly compared since in humans the hyperexcitability is detected at the medial temporal and motor cortices, the mouse studies are directed to the hippocampal areas. It may well be that different regions could have differential excitation-inhibition disbalance or that species differences account for the observed discrepancies.

Interestingly, the combined EE-EGCG treatment induced a restoration of the excitatory and inhibitory balance in both mice and humans. In mice, EE-EGCG treatment normalized the density and size of excitatory (Vglut1) puncta in DG and in CA1 regions. This is in agreement with previous data showing that EE and EGCG alone can partially restore excitation/inhibition imbalance in the cortex and the hippocampus of Ts65Dn mice, either by reversing the decreased expression levels of glutamatergic markers and the increased expression levels of GABAergic markers (Souchet et al., 2015) or by reducing GABAergic release (Begenisic et al., 2011). In our clinical trial, the combined treatment promoted normalization in the hyperexcitability of the motor cortex after 12 months of treatment.

The restoration of excitation-inhibition imbalance by the EE-EGCG treatment may be mediated partially by the reduction of Dyrk1A kinase activity, since it has been previously reported that Dyrk1A overexpression contributes to the excitation-inhibition imbalance by regulating NMDA receptors through phosphorylation of GluN2A subunit (Grau et al., 2014), disrupting NMDAR-mediated LTP and increasing pyramidal neurons spine density in prefrontal cortex (Thomazeau et al., 2014), increasing the number and signal intensity of GAD67 positive hippocampal neurons (Souchet et al., 2014), and reducing inhibitory-inhibitory contacts circuits leading to increased inhibition in prefrontal cortex (Ruiz-Mejias et al.,

2016). However, other mechanisms could not be discarded considering the pleiotropic effects of both EE and EGCG.
Additionally, EE-EGCG treatments showed a cognitive improvement in middle-age Ts65Dn mice accompanied by a moderate neuroprotective (although not statistically significant) effect on basal forebrain cholinergic neurodegeneration which suggests that combined therapy with CT and EGCG could contribute also be beneficial in older DS individuals that present age-associated Alzheimer disease (AD)-like cognitive decline.

Collectively, the work presented here supports the possibility that intellectual disability in Down syndrome can be ameliorated by multimodal interventions, still when administered in adulthood, and specifically that a combined therapy consisting of cognitive stimulation and EGCG is able to modulate several altered systems, improving cognitive, structural and functional brain parameters both in mouse models and people with DS.

5.2 Clinical studies: learned lessons

There are some aspects that need to be considered in the context of the clinical trials presented here and also for future clinical trials that attempt to explore the effects of novel therapies for intellectual disability in DS. Since this has been the first clinical trial performed in a randomized double-blind placebo controlled manner, we faced important challenges: 1/ even though we knew that our treatment targeted neuroplasticity, and thus, the pediatric population would have been the best for an efficacy study, the trial had to be first performed in young adults, a period with limited plasticity. 2/ There is no reference treatment in Down syndrome. 3/ There are no reference (normative) values for some explorations (neuroimaging, neurophysiology). 4/ We had to define “treatment efficacy”: does it mean increases in IQ? Does it mean cognitive changes that translate into functional changes? Those aspect were not previously defined and thus our study had to provide a framework for advancing the field.

A very important issue that we encountered is the sensitivity of the neuropsychological assessment tools to evaluate the impact of treatments on cognitive and functional abilities in DS individuals. Since the individuals with DS present a highly heterogeneous range of severity in the impaired cognitive domains, it is still a great challenge to develop a methodological tool sensitive enough to be able to evaluate subtle cognitive functioning changes. Additionally, despite that the trajectory of cognitive decline followed by DS is very similar to the one in AD, current neuropsychological evaluation tools still lack the power to accurately detect cognitive decline and dementia in DS population due also to the highly variable pre-existing intellectual disabilities. Accordingly, current methods also have limitations to address the potential effects of therapies aimed at the prevention of age-dependent cognitive decline. Considering that in DS individuals, some aspects such as morpho-syntax, allocentric visuospatial memory, and explicit long-term memory, are more affected than others (like visual-spatial short term memory, associative learning, and implicit long term memory) (Lott and Dierssen, 2010), the optimal neuropsychological assessment methods should take into account the baseline variability in the capacities across cognitive domains. Besides they should consider the different factors that could modulate the cognitive enhancing effects of a certain treatment, such as basal intellectual quotient, age, gender, socio-cultural background, lifestyle factors like diet and exercise, etc.

In the clinical studies presented here, the TESDAD battery, a customized neuropsychological assessment tool suitable for clinical trials, was developed and used to address the effects of the treatments. It compares individual's performance with typically developed controls in order to define the gap among them, which is the target of the intervention. The analysis of the data provided by the TESDAD battery was designed to address the different factors that could modulate the cognitive effects of the treatments. However, there are still limitations that the method could not

overcome like the phenotypic variability in the DS population and the potential practice effect (test-retest) across the longitudinal study. Another aspect that represents a drawback is the use of parent/caregiver-proxy measures for obtaining relevant information about the individual's quality of life and adaptive functionality. These measures, although valuable, are based on subjective perceptions and thus could be biased, potentially having consequences on the final clinical trial outcomes.

Regarding the use of EGCG as a co-adjuvant nutraceutical intervention for DS, despite that previous (*in vitro* and *in vivo*) studies and also the clinical studies presented here, have shown its safety and tolerability (Isbrucker et al., 2006; De la Torre et al., 2014; 2016), the pharmacology data still awaits. Indeed, a full characterization of EGCG half-life still remains to be done which will be relevant to better understand EGCG metabolism and therapeutic mechanisms.
In relation with this subject we detected that cognitive training and EGCG induced an increase in plasma levels of homocysteine that vanished after 6 months of treatment discontinuation, suggesting that the treatment modulates DYRK1A activity. However we are aware that the composition of green tea compounds, and especially the concentration of EGCG content, can slightly vary in the commercial supplements used in the clinical trials presented here, which may represent an issue for future replication studies. On the other hand, available data from a case study suggests that the application of EGCG in combination with other supplements, such as Omega 3 fatty acids, could also have beneficial effects (Vacca and Valenti, 2015), opening new potential research opportunities. Accordingly, we have advised in the development of a compound that can overcome the dosification difficulties while also improving the organoleptic properties, such as purified EGCG in the form of a milkshake with polyunsaturated fatty acids. Certainly, further studies using purified EGCG compounds, will be an important next step in translating their employment into the clinical practice.

5.3 Environmental enrichment (EE) in mice and cognitive training (CT) in humans: similarities and limitations

The environmental enrichment paradigm, providing a complex combination of social, cognitive, and physical stimulation, has been extensively used for the study of experience-dependent neuroplasticity in rodents (Rosenzweig and Bennett, 1969; Kempermann et al., 1997; Nithianantharajah and Hannan, 2006; Sale et al., 2014). Many studies have shown that EE enhances learning and memory through the induction of biochemical, morphological and functional changes in the adult brain. However, some authors have argued that experimental environmental enrichment promotes simply a normalization of the natural stimulatory conditions in wildlife, which are generally lacking in the environmentally impoverished standard laboratory settings (Cummins et al., 1977; Praag et al., 2000). In the preclinical studies presented here, control groups with standard housing were not in isolation, but in groups of 2-3 mice. Nevertheless, it could be claimed that they were environmentally impoverished as they had no stimulatory input apart from food and water and a few cage-mates. Therefore, we need to be cautious in the interpretation of our results as we are comparing extreme conditions that are not completely equivalent to real world conditions with humans. Most likely, the optimal degree of environmental enrichment for both mice and humans will achieve an equilibrium of social and sensorimotor stimulation, otherwise deviations would probably affect the organism either through impoverishment or stress.

It has been proposed that the physical exercise component of EE alone, can elicit many of the changes induced by full EE, such as increases in hippocampal neurogenesis, spine density, synaptic plasticity, neurotrophin levels, and spatial memory function in mice (van Praag, 2008; Voss et al., 2013). In fact, Llorens-Martín et al. (2010) showed that long-term running in 10–12 months old Ts65Dn male mice improved performance in the MWM, without rescuing altered hippocampal neurogenesis. Additionally, Kida et al. (2013)

found that long-term running starting after weaning and at adulthood has beneficial effects on both cognition and motor skills in Ts65Dn females accompanied by changes in the expression levels of some proteins in the brain. In post-weaning runner Ts65Dn mice, SOD1 levels were increased and total APP levels were decreased, while adult runner Ts65Dn mice showed moderately lower levels of α-cleaved C-terminal fragment of APP. However, they detected no changes on Dyrk1A expression upon running. Given that full EE with social, cognitive and sensorimotor components has shown to regulate the expression and kinase activity of Dyrk1A (Golabek et al., 2011, Pons-Espinal et al., 2013), it appears that these combined components of EE are intrinsically different than physical exercise alone, they may play a relevant role in the effects on Dyrk1A, and thus likely in neuroplasticity and cognition in Ts65Dn mice.

In the context of human research, we cannot control the whole environment of individuals, and thus the most equivalent experimental setting of EE in mouse is a CT intervention. In this field, over the last decades, a growing number of non-pharmacological recommendations and interventions aimed at modifying brain function, sharpen cognitive skills and improving mental capacities both for healthy individuals and for people with cognitive impairment, have emerged. They range from physical exercise to computerized CT by electronic games, devices and applications, such as the online exercise platform (Feskits) that was used in the phase II clinical trial presented in this Thesis.

In the recent years computerized CT programs have been targeted to diverse cognitive capabilities such as memory (Mahncke et al., 2006; Schmiedek et al., 2010; Zelinski et al., 2011; Clemenson and Stark, 2015), attention (Smith et al., 2009), executive function, and processing speed (Nouchi et al., 2012; Subramaniam et al., 2012), especially in elderly people, but recently working memory has gained particular attention since it strongly correlates with global intelligence and thus its improvements could be translated into augmentation in general cognitive capacities also in young people

(Jaeggi et al., 2008; Borella et al., 2010). From a biological perspective CT could be compared to some extent to cognitive demanding environments or everyday experience, since both stimulate activity-dependent neuroplasticity through learning and practice and are expected to improve cognitive abilities. However, despite the fact that numerous studies have reported benefits from CT in healthy and aging populations (for a review Kueider et al., 2012; for meta-analysis Karbach and Verhaeghen, 2014; Au et al., 2015), there are still some inconclusive evidence about their effectiveness (Owen et al., 2010; Redick et al., 2013; Melby-Lervåg and Hulme, 2016). One of the main issues of CT refers to the concept of "distance transfer", which is related to the degree of transferability of the learnt skills into meaningful, real-world increase in cognitive capabilities (Noack et al., 2014). In fact, due to the growing number of companies and advertisements offering CT "brain games" to promote intellectual improvements and prevention of cognitive decline to the general public, a group of cognitive psychologists and neuroscientists from the Stanford Center for Longevity and Berlin Max Planck Institute for Human Development recently published a consensus letter warning that there is a lack of conclusive research showing effectiveness of this type of interventions (http://longevity3.stanford.edu/blog/2014/10/15/the-consensus-on-the-brain-training-industry-from-the-scientific-community/). The authors claim that many popular computerized training programs only induce a gain in the trained task with limited skill transfer to real life activities due to a strategy-based training as opposed to a "core" CT (Morrison and Chein, 2011).

This emphasizes the relevance of the fact that, in the phase II clinical trial presented here, the combined treatment with CT and EGCG showed significant effects in the outcome measures of adaptive functionality and quality of life, as reported by the parents and caregivers of the individuals with DS. This measures may not accurately reflect global cognitive functioning as intellectual quotient (IQ) but would represent a valuable proxy of transferability

of learnt skills to real life situations as opposed to a mere improvement in the performance of exercises provided by Feskits.
In this regard, whether cognitive training programs have the same (or different) effects on healthy people than in individuals with intellectual disability, still remains an open question since different studies have shown promising results (Van der Molen et al., 2010; Söderqvist et al., 2012; Ottersen and Grill, 2015). However, in the phase II clinical trial presented here, the group with cognitive training and placebo showed no significant positive cognitive or functional effects suggesting that it was the interaction between the cognitive training and EGCG, which promoted synergistic effects that lead to cognitive improvements.

In the attempt to make the maximum efficiency in a potential combined therapy of CT with EGCG, there are still some aspects, regarding the best CT program that could be optimized. For example there is no criteria to define the best type of cognitive exercises regime regarding the cognitive domains of the tasks, the frequency and the length of the sessions to ensure full potential beneficial effects. Furthermore, as DS individuals present a high variability in the severity of the symptoms, the best CT would possibly be implemented as a personalized intervention specifically tailored according to individual skills strengths and needs but current methods are still not sufficiently adapted to each person.
Additionally, it is still an open question whether it would be more beneficial to include physical exercise as part of the cognitive enhancing intervention in DS. Although physical exercise programs have shown some improvements in working performance variables in individuals with DS, so far cognitive improvement has not been demonstrated as a result of physical exercise in this population (Andriolo et al., 2010; Shields et al., 2010).

5.4 Future perspectives on evidence-based clinical translational research in Neuroscience

During the last decades, the field of Neuroscience has undergone a period of explosive growth in the development of major technical and conceptual breakthroughs in Genetics, and Cellular and Molecular Neurobiology that have led to the progress of Neuroscience basic knowledge (Insel and Landis, 2013). However, the extraordinary progress in basic Neuroscience over the past decades does not reflect a proportional progress in clinical research and clinical care for people with brain disorders associated to cognitive impairment such as DS. In fact, many neurological disorders including intellectual disability and neurodegenerative diseases among others, still have not met efficient therapeutic interventions (Insel and Landis, 2013; Pankevich et al., 2014). Basic Neuroscience has proposed several new molecular targets that have become the basis of new therapies, like the one of the focus of this Thesis project. Nevertheless, to fully understand and optimally treat these brain disorders and especially neurodevelopmental disorders like DS, we still need a deeper knowledge of how the genetic alterations perturb the brain. This knowledge will be achieved by the use of new technical methods and strategies in the clinical translational process.

So far, the use of mouse models has brought significant information although sometimes spatially and temporally fragmented. Even if mouse models of disease have represented a critically valuable tool in the study of the pathophysiology of brain disorders, there are a number of species-associated neural and cognitive differences that could be obstacles in the way to clinical translation (Ericsson et al., n.d.; Insel, 2007). Accordingly, modern Neuroscience is evolving to the use of other approaches that can complement or add new types of brain information. These approaches include the use of computational neuroscience in silico models of brain function (Kotaleski and Blackwell, 2010; Deco et al., 2015; Markram et al., 2015), tools for more precise monitoring and manipulation of

neuronal networks in awake behaving animals (Harris and Thiele, 2011; Boyden, 2015; Hamel et al., 2015; Yuste, 2015), techniques for the structural and molecular examination of intact biological systems (Chung et al., 2013) and methods for the analysis of single cells within a population (Usoskin et al., 2014; Henikoff, 2015; Tasic et al., 2016). Furthermore, some authors have claimed that nowadays humans are the best model organism (Brenner, 2003) since current progress in technologies, enhanced by the BRAIN Initiative in the U.S. and Human Brain Project in Europe, enables to directly study human samples or subjects preventing the cross-species bridge. New cell reprogramming techniques, such as induced pluripotent stem (iPS) cells from fibroblasts, allows the use of human cellular models as platforms for screening potential mechanisms and therapies for specific brain disorders (Yu et al., 2013). Additionally, neuroimaging and neurophysiology techniques such as functional and structural Magnetic Resonance Imaging (MRI), Diffusion Tensor Imaging (DTI) Transcranial Magnetic Stimulation (TMS) are gaining more resolution and giving insights into brain circuits' organization and function (Deco and Kringelbach, 2014; Fritz, 2014; Fornito et al., 2015). On the other hand, the assessment of patients during ongoing translational clinical trials will soon be optimized and enhanced by wearable monitoring devices that will provide data that may be useful to understand the clinical outcomes and the factors underlying inter-subject response variability.

Future Neuroscience thus holds the promise of a more integrative perspective, able to encompass data from different levels of analysis to provide a more global and solid picture of brain structure and function. Most likely, the future approach in translational neuroscience research will integrate human, animal and in silico data (Stam, 2014; Ritchie et al., 2015) in an interactive process with feedback loops within and across levels, and will be more efficient and productive on the route towards the development of therapies for brain disorders.

6. CONCLUSIONS

1. Combined treatment with EE and EGCG ameliorates hippocampal-dependent spatial learning and cognitive flexibility deficits in young adult Ts65Dn mice.

2. The cognitive improvement in young Ts65Dn mice is accompanied by a mitigation of structural hippocampal alterations, as shown by an increase in dendritic spine density in CA1, and a restoration of the balance between excitatory and inhibitory synaptic puncta in CA1 and DG.

3. In young WT mice the combined treatment with EE and EGCG did not affect spatial learning but reduced dendritic spine density, and induced an imbalance between excitatory and inhibitory synaptic puncta in CA1 and DG.

4. Combined treatment with EE and EGCG is more efficient than EE or EGCG alone to improve hippocampal-dependent spatial learning in middle-age Ts65Dn mice.

5. Both EE alone and combined treatment with EE and EGCG improve long-term associative memory in middle-age Ts65Dn and WT mice.

6. The cognitive improvement in middle-age Ts65Dn mice is accompanied by a moderate effect on medial septum cholinergic neurons from the basal forebrain.

7. Short-term treatment with EGCG in young adult individuals with DS is safe and induces transient improvement in memory and quality of life.

8. Longer-term combined treatment with CT and EGCG in young adult individuals with DS is more efficient than CT

alone to ameliorate cognitive function, functional adaptability and quality of life, having persisting effects after treatment discontinuation.

9. The cognitive improvements by the combined treatment with CT and EGCG in young adult individuals with DS is accompanied by increases in functional connectivity and a normalization of cortical excitability shown by fMRI and TMS measures.

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