Correlation between cognitive phenotype, neural morphology and molecular alterations in mouse models of Williams-Beuren syndrome: new therapeutic approaches

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"The way to get started is to quit talking and begin doing"

Walt Disney

"It's the possibility of having a dream come true that makes life interesting"

Paulo Coelho

Al Rubén,

a l'Eva i al Pep,

al papa, i a tu, mama.

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ABSTRACT

Williams-Beuren syndrome (WBS) is a rare neurodevelopmental disorder caused by a heterozygous deletion of 26-28 contiguous genes in the 7q11.23 region. So far, a great deal of attention has been focused on its unique and distinctive neurocognitive profile. Although important progress has been made with regards to clinical characterization or genotype-phenotype correlations, a much deeper insight into the neuropathological features of WBS would be of great interest. In this thesis project, we have used a WBS mouse model carrying a heterozygous deletion that mimics the most common deletion found in patients. We have characterized the cognitive and behavioral phenotype of these mice and we have indentified molecular and neuroanatomical alterations relevant for the disease. Moreover, we have attempted two novel therapeutic strategies: a gene therapy and a pharmacological approach. The results obtained highlight the utility of this animal model to study the mechanisms underlying the disease as well as to evaluate novel therapeutic strategies.

RESUM

La síndrome de Williams-Beuren (SWB) és una malaltia rara del neurodesenvolupament causada per una deleció heterozigota d'entre 26 i 28 gens contigus a la regió 7q11.23. Fins ara, una gran part de l'atenció s'ha centrat en el seu característic perfil neurocognitiu. Tot i que s'han fet progressos molt importants pel que fa a la caracterització clínica o a les correlacions genotip-fenotip, seria de gran interès aprofundir en les característiques neuropatològiques de la SWB. En aquesta tesi, hem utilitzat un model de ratolí del SWB amb una deleció heterozigota que mimetitza la deleció més comuna d'aquests pacients. Hem caracteritzat el fenotip cognitiu i comportamental d'aquests ratolins i hem identificat alteracions moleculars i neuroanatòmiques rellevants per a la malaltia. Per altra banda, hem dut a terme dues estratègies terapèutiques: una teràpia gènica i un tractament farmacològic. Els resultats obtinguts remarquen la utilitat d'aquest model animal per a l'estudi dels mecanismes subjacents a la malaltia així com també per a avaluar noves aproximacions terapèutiques.

PROLOGUE

The history of Williams-Beuren syndrome (WBS) began in the early 1950s when it was suggested to be a distinct syndrome with a wide range of clinical manifestations affecting mainly the cardiovascular, endocrine and nervous systems. During the last decades, an increasing number of studies have focused on the peculiar neurocognitive profile of WBS. Molecular analyses of individuals with atypical deletions along with the clinical phenotype have helped to link individual genes to clinical features. In addition, animal models of WBS are unquestionably providing relevant information about the contribution of some genes to the WBS phenotype. However, a better understanding of the neuropathological features of WBS would be of great importance to identify candidate targets and to develop novel therapeutic strategies.

The aim of this thesis project has been to contribute to the physiopathology of the syndrome by using an animal model of WBS. In addition, preclinical testing of novel therapeutic approaches has been explored.

This thesis is divided in several parts following the classical structure:

The **introduction** provides a general overview regarding the clinical characteristics of the WBS, the molecular causes, the tools for dissecting genotype-phenotype correlations and a brief description of the genes that have been related to the neurocognitive phenotype.

The **thesis body** contains the results obtained and is divided in five independent chapters. Each chapter presents in detail an introduction, the methodology used, the results obtained, a discussion and the bibliography related to the chapter.

The global **discussion** aims to integrate and interpret the obtained results relating them to previous existing knowledge. Following the discussion, the main **conclusions** of the thesis are summarized.

Finally, the **annexes** part includes other articles in which I have contributed during my PhD.

CONTENTS

LIST OF ACRONYMS

AAV	Adeno-associated Virus
aCGH	array Comparative Genomic Hybridization
aCSF	artificial Cerebral Spinal Fluid
ASD	Atriat-septal Defect
BDNF	Brain-derived Neurotrophic Factor
CD	Complete Deletion
ChIP-seq	Chromatin-immunoprecipitation and sequencing
CNV	Copy Number Variation
DD	Distal Deletion
DIV	Day In Vitro
DS	Down Syndrome
EGCG	Epigallocatechin-3-gallate
EPSC	Excitatory Postsynaptic Currents
FBS	Fetal Bovine Serum
fEPSP	field Excitatory Postsynaptic Potentials
FISH	Fluorescent In Situ Hybridization
IPSCs	Induced Pluripotent Stem Cells
IPSP	Inhibitory Postsynaptic Potential
IQ	Intelligence Quotient
LCR	Low Copy Repeat
LTP	Long-term Potentiation
MAPK	Mitogen-activated Protein Kinases
mEPSC	miniature Excitatory Postsynaptic Currents
MLPA	Multiplex Ligation-dependent Probe Amplification
MRI	Magnetic Resonance Imaging
NAHR	Non-Allelic Homologous Recombination
NADPH	Nicotinamide Adenine Dinucleotide Phosphatase
NMDA	N-methyl-D-aspartate
NOR	Novel Object Recognition
OFC	Orbitofrontal Cortex
PI3K	Phosphoinositide-3-kinase
PD	Proximal Deletion
PKC	Protein kinase C
PTP	Post-tetanic Potentiation

PPF	Paired-pulse Facilitation
SMC	Smooth Muscle Cell
SO	Stratum Oriens
SNP	Single Nucleotide Polymorphism
SR	Stratum Radiatum
SVAS	Supravalvular Aortic Stenosis
TBS	Theta-burst Stimulation
VSD	Ventrical-septal defect
WBS	Williams-Beuren Syndrome
WT	Wild-type
4-AP	4-aminopyridin

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INTRODUCTION

1. WILLIAMS-BEUREN SYNDROME PHENOTYPE

Williams-Beuren Syndrome (WBS, OMIM 194050) is a rare neurodevelopmental disorder that results from a heterozygous deletion of 26-28 genes at chromosome 7q11.23. It occurs sporadically with an incidence of 1 in 7500 individuals, although a few cases of autosomal dominant transmission have been reported[1]. It is characterized by multisystemic manifestations with a wide range of phenotypic variability.

1.1. History

The history of WBS began in the early 1950s when a complex form of hypercalcaemia was suggested by Fanconi and colleagues to be a distinct syndrome associated with infant mortality, developmental delay and dysmorphic facial features[2]. Later on, in 1961, Williams *et al*. reported four children from New Zealand without hypercalcaemia but with supravalvular aortic stenosis (SVAS) also associated with mental disabilities and distinct facial appearance. The authors proposed that this striking combination of findings might constitute a clinical unrecognized syndrome[3]. One year later, Beuren and colleagues also reported three children resembling those reported by Williams *et al*.[4] A "friendly nature", a remarkable feature of WBS patients, was already described in these patients. The similarities between the complex hypercalcaemia syndrome and the Williams and Beuren reported clinical characteristics, and the increasing number of similar cases in the following years, led to the theory that it should be a previously unrecognized syndrome and it was referred to as Williams-Beuren syndrome.

1.2. Clinical characteristics

WBS individuals present a characteristic pattern of clinical symptoms. The presence and severity of these features can vary across individuals. Some medical problems are more common during infancy than in adulthood, resulting in extra visits to the pediatrician than other children. Patients with WBS need medical attention throughout their life. Management includes medical monitoring, direct therapies, pharmacotherapy or surgery. However, none of the available treatments are curative[5,6].

1.2.1. Facial phenotype

WBS can be distinguished from other neurodevelopmental disorders by the facial gestalt. Infants and young children are often described as pixie-like or elfin. The classical facial features include a broad forehead, bitemporal narrowing, low nasal root, periorbital fullness, strabismus, bulbous nasal tip, malar flattening, long philtrum, full lips, wide mouth, full cheeks, small jaw, prominent earlobes and malocclusion with small widely spaced teeth (Figure 1). Older patients have coarser features, probably due to mild premature aging. These include: prominent supraorbital ridge, narrow nasal root, full nasal tip, malar flattening, wide mouth with full lips, small jaw, dental malocclusion and long neck[7].

Figure 1. WBS classical facial features. Young children are often described as pixie-like or elfin. Broad forehead, periorbital fullness, bulbous nasal tip, full lips and wide mouth are frequent facial features present in children with WBS. From[8].

1.2.2. Cardiovascular disease

An important clinical manifestation of WBS is cardiovascular disease, which is present in the majority of patients. Cardiovascular abnormalities are the major cause of death in WBS patients and are associated with a higher risk of sudden death, being anesthesia and sedation possible risk factors[5,9,10]. The most common cardiovascular defect is SVAS with an incidence of 70%, followed by pulmonary arterial stenosis (38-41%) and mitral valve prolapse (11-27%)[11,12]. Some of the patients suffer from multiple cardiovascular problems and males are usually more affected than females, presenting cardiovascular anomalies at an earlier age. Nearly the half of the patients need surgical or catheter interventions, with a perioperative mortality rate of 7%[11].

Numerous studies performed in cohorts of patients with WBS demonstrate that hypertension is also an important finding in WBS, being present in 40-

55% of the patients[12–15]. Broder *et al*. showed that not only adults but also children had higher frequency of hypertension compared to healthy controls (46% versus 6%) using 24-hour ambulatory blood pressure monitoring[13].

The deletion of one copy of the elastin gene is likely to be the cause for the cardiovascular abnormalities, since elastin plays an important role in stabilizing the arterial structure[16,17]. However, it has been demonstrated that the *NCF1* gene is a modifying factor of the cardiovascular phenotype. Specifically, a study shows that hypertension is significantly less prevalent in patients whose deletion also includes the *NCF1* gene[18].

1.2.3. Endocrinological phenotype

Endocrine-related problems such as hypercalcemia, diabetes, glucose intolerance or subclinical hypothyroidism are very frequent in WBS patients.

Hypercalcemia is one of the endocrinological features most discussed in WBS, as it was a key feature in the discovery of the disease. However, the prevalence of hypercalcemia is documented in only 15% of infants and young children, although it varies from 5 to 50% depending on the study[5,7,19,20]. There are also some reports with evidence of hypercalcemia in a proportion of adults, demonstrating that the risk for hypercalcemia is not restricted to infancy[21,22].

A high prevalence of impaired glucose metabolism has been described in WBS, with up to 75% of adults showing glucose intolerance or diabetes mellitus in response to a standard oral glucose tolerance test[16–18].

Structural and functional anomalies of thyroid gland have also been described. Thyroid gland hypoplasia has been detected in 60-75% of patients while subclinical hypothyroidism has been detected in a frequency ranging from 15 to 32%[19–23].

1.2.4. Growth and puberty

Short stature and growth deficiency are considered to be one of the leading diagnostic criteria in WBS. Longitudinal evaluation of growth and puberty has been performed in different cohorts of patients. Growth deficiency is already present in the prenatal stage[24,25]. During the first few years of life WBS

individuals present poor growth, with a growth rate of 1 to 2 cm/year bellow the norm. During childhood the mean growth for both sexes follows the 3rd percentile. Apart from growth deficiency, WBS individuals present a premature pubertal growth spurt, approximately 1 to 2 years earlier than normal. Menarche usually occurs at an early age than normal population. Regarding the height, approximately 70% of adults are below the 3rd percentile for both sexes[6,24,25].

1.2.5. Other frequent symptoms

Dental problems are quite frequent in WBS individuals. The most common finding is malocclusion, which occurs in 85% of the cases. However, it responds well to orthodontic treatment[26]. Other problems include microdontia, increased space between teeth and teeth malformation[27]. Dental evaluation usually shows poor oral hygiene, resulting in dental caries, halitosis and dental extractions[20,22,28]. These problems are likely due to the visual spatial deficits, which impedes the proper maintaining of dental hygiene[20].

Ophtalmologic findings are also associated with the disorder. Most of the patients have one or more ocular abnormalities. Strabismus and a typical stellate iris pattern are the most frequent findings (20-70%). Other abnormalities frequently found in WBS patients include hyperopia, myopia, cataracts, astigmatism or tortuosity of retinal vessels. Some of these findings such as strabismus require surgical correction[19,22,28–30].

The frequency of gastrointestinal problems varies a lot between studies, although they are relatively frequent at all ages. Constipation is one of the most common gastrointestinal findings in WBS, occurring in 25-40% of patients and affecting both children and adults[22,26,28]. There is an increased risk for diverticulosis and diverticulitis[7,22,31]. Other gastrointestinal problems include feeding problems during infancy, gastroesophageal reflux and chronic abdominal pain[7,21]. Prevalence of celiac disease is also higher in WBS individuals than in controls[28,32].

The incidence of renal and urinary tract anomalies in WBS is higher than in normal population (17.7% vs 1.5%), including bladder diverticula or renal aplasia/hypoplasia[33]. Renal dysfunction can also be found in WBS patients, sometimes secondary to hypercalcaemia[34]. Abdominal wall and external

genitalia anomalies are very frequent in males (73.1%) and females (44.7%). The most common anomalies include bilateral undescending testis, retractile testis or unilateral cryptochidism in the case of males and umbilical hernia and unilateral/bilateral inguinal hernia in the case of females[35].

Finally, orthopedic problems are also common in individuals with WBS, although they are not severe enough to impair their personal autonomy. Around 70% of individuals present hypotonia, resulting in a delay in learning to walk. Scoliosis, lordosis and feet problems are also common features in children or adults with WBS[19,28].

1.3. Neurocognitive phenotype

WBS individuals present some cognitive, behavioral and neurological problems that can affect their quality of life. In fact, independent living and competitive employment in WBS is not frequently reported[6].

1.3.1. Cognitive profile

Individuals with WBS present mild to moderate intellectual disability. The majority of cognitive studies have described a mean intelligence quotient (IQ) of 55-60, with a range of 40-100[36–38]. Many studies show that WBS individuals have a higher verbal than performance IQ[36,39,40]. In fact, WBS is typically defined by characteristic peaks and valleys including relative verbal strength and visuospatial weaknesses (Figure 2).

The onset of vocabulary acquisition and word production is delayed in children with WBS. Usually, children who are developing normally use gesturing skills before language development, such as pointing. WBS children use fewer gesturing skills, probably due to the visuospatial construction deficits[37,38]. Although language acquisition is delayed, expressive language capabilities are not affected. Older children show a fluent and grammatically correct language (Figure 2A). Adolescents and adults are usually very talkative to the point of being loquacious. They show strengths in concrete receptive and expressive vocabulary, but weaknesses in relational vocabulary (spatial, dimensional and temporal concepts). On the other hand, WBS children have difficulties with the pragmatics of language, such as maintaining the topic of a conversation[37,38,40,41].

The most severe deficit in WBS is the visuospatial construction, which is usually evaluated by either drawing or copying an illustration. Instead of constructing a pattern or a picture as a whole, they focus on the individual components, resulting in poor cohesion and lack of organization (Figure 2B)[38,40].

A

Experimenter question: What if you were a bird?	
WBS answers	**DS answers**
"You could fly, you could have babies, fly north or south, east or west"	"Bird seeds"
"Good question. I'd fly through the air being free"	"You'd be strong"
"I would fly through the air and soar like an airplane and dive through trees like a bird"	"I don't fly"
"I would fly where my parents could never find me. Birds want to be independent"	"I not a bird, you have wing"
"I would fly and if I liked a boy, I would land on his head and start chirping"	"Fly in the air"

B

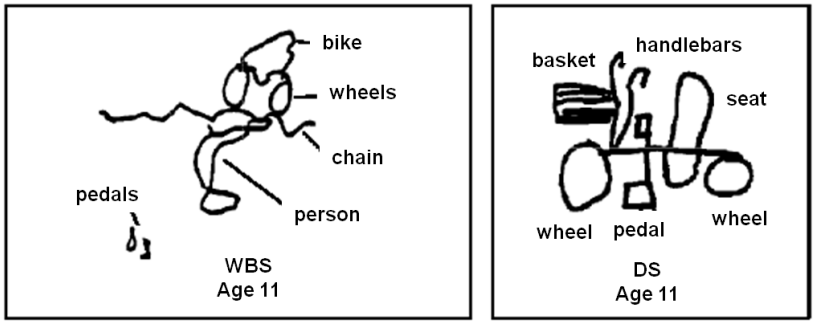

Figure 2. Relative verbal strength and visuospatial weaknesses in WBS compared to age- and IQ- matched Down Syndrome (DS) individuals. A) WBS individuals perform significantly better than those with DS on grammar and content when asked "What if you were a bird?" B) The child with WBS can identify all parts of a bicycle but cannot draw them in a single representation, whereas DS child produces a simplified but recognizable drawing of a bicycle. Adapted from[40].

Apart from visuospatial deficits, individuals with WBS also present poor skills in mathematics, affecting their everyday life in typically operations that can be easily performed by normally developing children, such as making change, cooking recipes, or estimating length, weight and similar concepts[40].

1.3.2. Behavioral profile

The main behavioral characteristic of WBS is hypersociability. Individuals with WBS present an overly friendly, gregarious, empathic and emotional personality[8,42,43]. They also tend to stare intently at human faces (particularly the eyes). This was demonstrated in a study in which an intense looking behavior at the mother, at a stranger or at the geneticist was evidenced by WBS infants and toddlers[44].

Doyle *et al*. demonstrated that hypersociability in WBS is not due to cognitive impairment[8]. However, despite the ease of social interaction they have difficulties with the establishment and maintenance of friendships[45]. Moreover, the excessive sociability to strangers can sometimes lead to situations of serious concern as they cannot resist the temptation to approach strangers regardless of the countenance expressed and they are unable to detect and respect social danger signals[46].

The prevalence of psychiatric disorders is also high in WBS individuals. The most common diagnoses are generalized anxiety disorder, specific phobias and attention deficit/hyperactivity disorder[47,48]. In a longitudinal study of anxiety disorders in children and adolescents with WBS, 82% of them received an anxiety diagnostic at some point in their lives[48]. Specific phobia is the most common form of anxiety found in children with WBS, being present in more than 90% of individuals[49,50]. Generalized and anticipatory anxiety about upcoming events and non-social anxiety are also quite frequent (50-60%). On the other hand, social anxiety about meeting strangers is absent in WBS individuals[49]. Finally, the presence of attention deficit hyperactivity disorder is not an uncommon finding in WBS children, being present in a 40-65% of individuals[47,50].

1.3.3. Neurological profile

A variety of neurological problems are common in WBS. Extrapyramidal signs and cerebellar abnormalities such as intention tremor, dysmetria or ataxia are often reported (40-70% of WBS individuals) irrespective of the age, although it seems they are more evident in adult subjects[51–53]. Both gross and fine motor coordination impairment has been documented either in young or adults individuals, indicating it is not simply a maturational problem[51,52]. These abnormalities result in difficulties with balance,

proprioception and motor planning. Other neurological problems include hyperreflexia, central hypotonia and peripheral hypertonia[53].

Some patients present neurologic symptoms secondary to Chiari malformation type I[54]. Although the exact frequency of this structural malformation is unknown as magnetic resonance imaging (MRI) is not routinely performed, around 12-14% of patients seem to be affected[22,55].

Hearing loss is very common in WBS, both in children and adults[56]. Hypersensitivity to sounds (hyperacusis) and extreme fear from sounds (phonophobia) are also present in most of the individuals[57]. Audiological testing of WBS individuals is highly recommended to prevent disturbances in their daily life.

1.3.4. Structural and functional brain abnormalities

A high number of studies using high MRI in individuals with WBS have revealed distinct brain morphology and functionality. However, some of the results obtained in these studies are different or even contradictory probably due to the study population or the methods used.

A consistent finding observed in WBS individuals is an overall reduction in total brain volume (10-15%), probably as a consequence of differences in relative gray and white matter tissue composition[58–61]. Cerebrospinal fluid is proportionally reduced to overall cerebral volume[59]. A relative preservation of cerebellar volume and a reduction in brainstem volume (20%) have also been described in these patients[59].

Surface complexity is significantly increased in WBS brains. Increased gyrification has been reported in parietal and occipital lobes, mainly in the left hemisphere[62,63]. Regionally specific alterations in cortical thickness have also been described[64].

Qualitative postmortem examination of gross neuroanatomy and studies using MRI have shown that cerebral shape differs between the brains of WBS patients and those of controls (Figure 3). Frontal lobe sparing and posterior (parietal-occipital) reductions in cerebral volume seems to be a feature of this syndrome[59,65,66]. Detailed differences in several brain areas are described below.

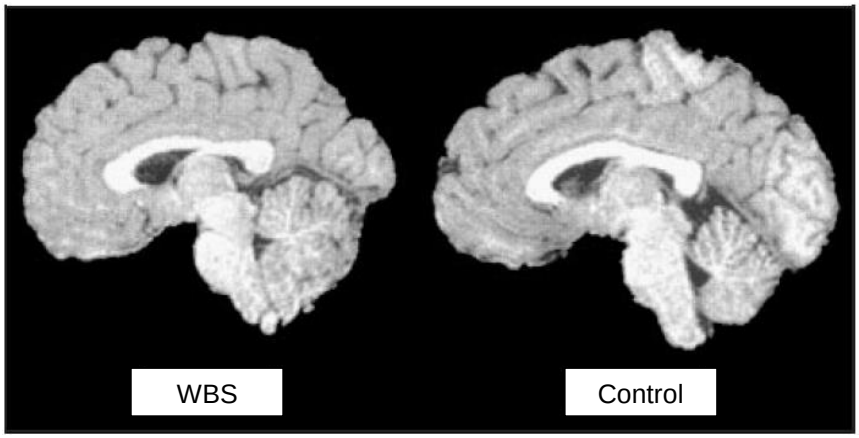

Figure 3. Qualitative cerebral shape differences between a WBS individual and an age- and sex-matched control using MRI. The image demonstrates the parieto-occipital lobe reduction. Note the reduction in total brain size. From[65].

Corpus Callosum

The corpus callosum, a structure which connects the left and right cerebral hemispheres facilitating interhemispheric communication, has been reported to be different in WBS patients regarding volume, shape and thickness. Specifically, the corpus callosum of WBS individuals is smaller in area and volume, shorter than normal and it presents a larger relative thickness. Its bending angle is significantly larger than in control subjects, being more curved in the posterior part. These features indicate an aberrant development of the corpus callosum in WBS[65,67,68].

Amygdala

The amygdala is a subcortical structure involved in emotional and social behavior as well as in the responses to stress and anxiety. It has also been implicated in threat detection and fear processing[69,70]. As individuals with WBS are socially disinhibited and have unusually non-social fears, some studies have focused on the anatomy and function of this structure. MRI scans reveal a relative increase in amygdala volume in WBS when compared to healthy controls, suggesting that abnormal functioning of this structure may underlie the hypersociability observed in WBS[71]. Individuals with WBS show reduced amygdala activation for threatening faces, which may contribute to

the decreased fear of strangers, and abnormally elevated amygdala activation when viewing threatening non-social stimuli, which is consistent with the elevated non-social fear of WBS (Figure 4)[72]. Neuroimaging studies suggest that the mechanisms involved in these abnormalities are related to modulation and regulation of the amygdala. In fact, a functional uncoupling of prefrontal-amygdala inhibition has been reported[72,73].

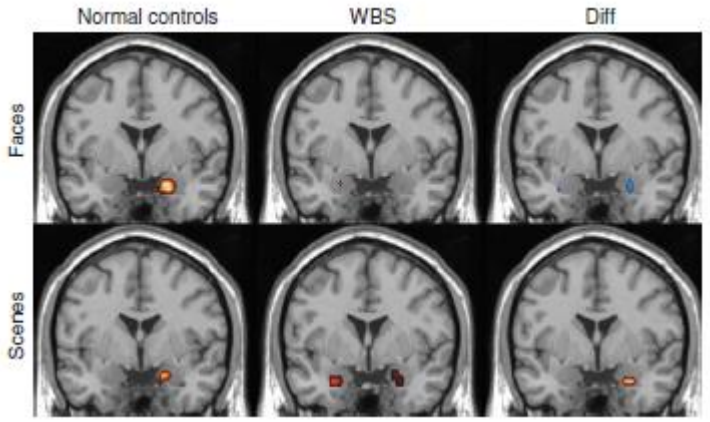

Figure 4. Amygdala activation in WBS. Amygdala activation when threatening faces or threatening non-social scenes are presented to WBS individuals and age- and sex-matched controls. From[72].

Orbitofrontal cortex

The orbitofrontal cortex (OFC) is a prefrontal cortex region involved in decision-making. Additionally, OFC sends projections to the amygdala producing an inhibitory regulation in the region. Therefore, the hyperactivity of the amygdala may result from a failure of the OFC to properly inhibit the amygdala responses during non-social fear processing. An analysis of functional interactions between OFC and amygdala suggested that OFC does not interact with amygdala in WBS, whereas in normal controls OFC has a significant negative correlation[72,74]. On the other hand, structural MRI studies have indicated reduced grey matter in this region[74].

Hippocampal formation

Hippocampal formation is involved in processing of spatial navigational information and verbal long-term memory[75,76], domains which are severely affected in WBS. A multimodal imaging study was performed in individuals with WBS to characterize the hippocampal formation. Patients presented a deficit in resting blood flow and a reduction of N-acetyl aspartate, a marker of cellular integrity and synaptic abundance, indicating an overall depression of hippocampal energy metabolism and synaptic activity in WBS[74,77]. Despite having hippocampal dysfunction, WBS individuals presented a normal overall volume of the hippocampus. Nevertheless, the midsection of the hippocampus presented subtle alterations in shape[77].

Visual cortex

Visuospatial tasks are impaired in WBS individuals. The visual cortex can be divided in two pathways: the ventral stream for object processing and the dorsal stream for spatial processing. Functional imaging experiments in WBS individuals indicate a specific deficit of dorsal stream functioning stream with relatively intact ventral stream function[74,78]. Histological examinations of brain have revealed some cytoarchitectonic anomalies in the primary visual cortex such as differences in neuronal cell size, abnormally clustered and oriented neurons and increased cell packing density. These findings suggest abnormal neuronal development and connectivity in this region[79,80].

Superior temporal lobe

The superior temporal region is an area of the brain involved in the perception and processing of music and has a key role in auditory and language processing[81]. This area is preserved in size in WBS and, curiously, individuals with WBS have musical and language abilities[59,82]. However, it is not known if they are good at music and language because this area is totally preserved in size or if the size is normal secondary to a greater use of these cognitive skills[83].

2. GENETIC BASIS OF WILLIAMS-BEUREN SYNDROME

WBS is classified as a recurrent genomic disorder, in which the clinical phenotype is a consequence of abnormal dosage of genes located within the rearranged genomic region[84]. Specifically, WBS is caused by the hemizygous deletion of 26-28 genes on chromosome 7q11.23.

2.1. Region structure

As WBS critical region has a complex genomic architecture, it has been extensively studied. The single copy region of WBS is flanked by three low copy repeats (LCR), also known as segmental duplications. LCR are defined as genomic DNA fragments of 10-400 kb of high sequence identity (>97%) which predispose the region to rearrangements[84]. The LCR are ordered in complexes, which are located on the centromeric (c), medial (m) and telomeric (t) segment of the WBS locus. Each LCR has a size of approximately 320 kb and is composed of three blocks named A, B and C[85]. The blocks of the centromeric and medial LCRs are in different order but in the same orientation, whereas the third LCR is more telomeric and is in the same order as the centromeric LCR, although in the opposite orientation[86,87]. The blocks contain mainly truncated copies of genes, although there are some genes transcriptionally active such as *POM121*, *NCF1*, *GTF2IRD2*, *NSUN5*, *TRIM50* and *FKBP6*[85,88]. The single copy gene region spans an area of ≈1.2 Mb and is located between the medial B and C blocks (Figure 5).

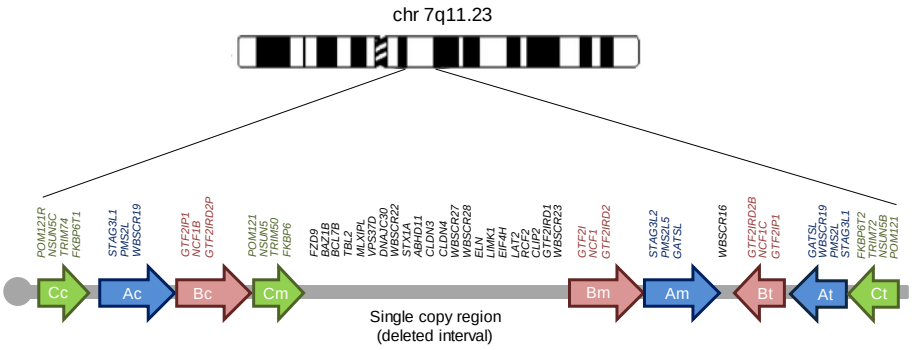

Figure 5. Schematic representation of the 7q11.23 region. The centromeric (c), middle (m) and telemoric (t) LCRs are shown as colored arrows with their relative orientation to each other. Modified from[87].

2.2. Mutational mechanisms

The high similarity between LCRs leads to a misalignment of these sequences during meiosis followed by unequal crossing over, giving rise to different genomic rearrangements by a mechanism known as non-allelic homologous recombination (NAHR)[84].

2.2.1. Deletion

Fine mapping of the WBS region has demonstrated that most of WBS individuals (around 90%) present a common deletion of approximately 1.55 Mb, which occur due to a crossing over between the centromeric and medial blocks B. However, approximately 10% of patients present a larger deletion of 1.83 Mb which occurs between centromeric and medial blocks A (Figure 6). Only 2-3% of patients carry atypical smaller deletions[89,90].

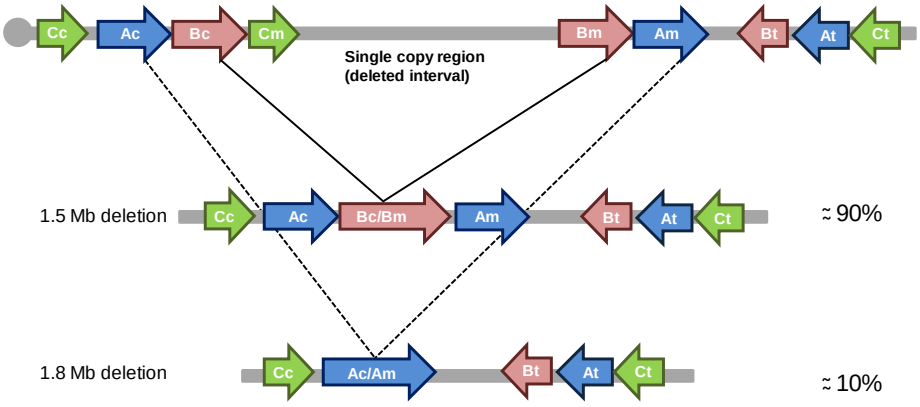

Figure 6. Schematic representation of the 7q11.23 deletions generated by non-allelic homologous recombination during meiosis between LCRs. Depending on between which blocks the recombination occurs, a deletion of 1.5 Mb or 1.8 Mb can occur.

Rearrangements are predominantly between centromeric and medial B blocks probably due to two main factors: the degree of sequence homology and the length of the genomic interval between blocks. On the one hand, blocks Bc and Bm have a 99.6% of identity with no large gaps while blocks Ac and Am have a 98.2% of identity with two large gaps. On the other hand,

the genomic interval length between blocks Bc and Bm is 1.55 Mb, shorter than between blocks Ac and Am (1.84 Mb approximately)[86].

Depending on where NAHR takes place, different situations can occur. When LCR are in the same orientation, interchromosomal or interchromatid recombination results in a deletion and a reciprocal duplication of the genomic region between the LCR. However, intrachromatid crossing-over produces a deletion and a circular DNA molecule without a centromere that will not segregate during cell division[91]. Haplotype analysis in families with an affected child has shown that two thirds of the cases result from interchromosomal recombination while one third of the cases result from an intrachromosomal recombination (either inter- or intra- chromatid)[92–94].

WBS usually occurs sporadically in a frequency of 1 in 7500 individuals[1]. However, a few cases of autosomal dominant transmission have been reported. In these families, the progenitor with WBS presented a mild pattern of WBS and was diagnosed after the identification of the syndrome in the affected child[95,96]. Some cases of concordant monozygotic twins have also been reported[97]. Finally, no association has been reported with regard to the parental origin of the deletion[86,98].

2.2.2. Duplication and triplication

Duplication of the WBS region should occur at the same frequency as deletions occurring through interchromosomal recombination. However, the first case of duplication was not described until 2005[99]. One possible explanation could be that the phenotype of these individuals is milder and clinically more heterogeneous than the WBS phenotype. Nevertheless, several new cases have been reported in the last years[100,101].

The clinical phenotype associated with the reciprocal duplication is not well defined, although speech delay seems to be the most common manifestation. Intellectual disability ranges from normal to moderate. Other findings associated with the duplication are hypotonia, epilepsy and an increased incidence of various congenital anomalies such as heart defects. Several patients present autistic features including repetitive interests and impairment in communication and social interactions[99–101]. No deficits in visuospatial integration have been described in these individuals. A facial phenotype

associated with the microduplication was described for the first time in 2009: straight eyebrows, high broad nose, thin upper lip, short philtrum and prominent forehead (Figure 7).

Figure 7. Patients with a 7q11.23 duplication. Note the prominent forehead, the straight placement of the eyebrows, the thin upper lip and the high broad nose. From[100].

Most of features associated with the duplication are in direct contrast with some of the features of WBS, including the facial phenotype or language development[100]. This suggests that genes within the WBS region are sensitive to dosage changes.

In several reported cases, the duplication was transmitted from one of the progenitors. Therefore, the frequency of parental transmission seems to be higher than in the WBS deletion. Some of the progenitors that carried the duplication did not show cognitive impairment or speech difficulties, although the majority of them had a history of difficulties in learning and/or language delay. This means that these parents would have never been diagnosed if it was not for their children and that there might be individuals carrying the duplication that have not been diagnosed[100,101].

In 2010, a case of a 7q11.23 triplication was described. The triplicated region included the commonly deleted genes in WBS except for *FZD9* and *FKBP6*, which were not triplicated. Therefore, the mechanism responsible for the triplication might be different from NAHR, since the breakpoint is not exactly located between the LCRs. The patient presented intellectual disability, severe expressive language delay, behavioral problems and dysmorphisms. The phenotype was remarkably similar to that observed in 7q11.23 duplication but more severe, supporting the idea that genes within the WBS region are sensitive to dosage changes[102].

2.3. Genetic risk factors

2.3.1. Inversion

In 2001, Osborne and colleagues identified a polymorphic inversion of the WBS region in families with affected children. The inversion occurs within a chromatid when a centromeric LCR misaligns with a telomeric LCR that is inverted relative to the centromeric block[103]. As the inversion breakpoints are externally to the WBS region, none of the genes commonly deleted in patients with WBS are disrupted, so these individuals do not present an abnormal phenotype[86].

The inversion is found in 25-33% of the transmitting progenitors, whereas in non-WBS population is found in only 5.8%[86,103,104]. Therefore, although inversion is considered a benign polymorphism, it increases the probability of having a child with WBS, since the inversion predisposes to unequal chromosome pairing in meiotic prophase which may lead to the WBS deletion. In fact, the recurrence risk for a parent heterozygous for the inversion is 1/1750, whereas without the inversion the risk is 1/9500 approximately[104].

2.3.2. Copy number variants

In 2008, Cusco *et al*. described a variety of genomic rearrangements mediated by NAHR between different LCR blocks leading to deletions and duplications of LCRs. These copy number variants (CNV) were found in 4.44% of the WBS-transmitting progenitors, whereas the prevalence among control individuals and non-transmitting progenitors was only 1%[98]. Therefore, the presence of these CNVs is another predisposing factor for the WBS deletion.

3. GENOTYPE-PHENOTYPE CORRELATIONS

Currently, determining which gene of the WBS region is contributing to a given phenotype has become of great interest. However, genotype-phenotype correlations are a big challenge due to the large number of genes that encompasses the deletion.

3.1. Tools for dissecting genotype-phenotype correlations

Different strategies have been adopted to examine genotype-phenotype correlations such as the study of patients with atypical deletions, the generation of mouse models with single and multiple gene deletion or the use of cellular models. However, each approach has inherent limitations.

3.1.1. Patients with atypical deletions

Molecular analyses of individuals with atypical deletions along with the clinical phenotype have helped to link individual genes to clinical features. To date, several WBS individuals with atypical deletions (smaller or larger) have been reported (Figure 8)[105]. So far, the strongest correlation reported is the link between the cardiovascular problems and the elastin haploinsufficiency[106,107].

Unfortunately, other correlations have been more difficult because there are few patients with atypical deletions and the sizes as well as the breakpoints of the deletions are different. The difficulty in comparing different individuals also lies in the high phenotypic variability. Several factors can play a role in this huge variability such as variants in the sequence of the non-deleted genes, parent-of-origin specific modulation of expression of the remaining alleles, the effect on the expression of neighboring genes which may be altered due to the deletion and, finally, the effect of genes located elsewhere whose expression may be affected by the transcriptional regulators encoded by genes in the deletion. Moreover, the comparison is even more complicated since patients are not evaluated by the same physician, so the battery of tests used to assess clinical, psychological or cognitive function is different[108,109]. Finally, there is a bias in the individuals studied as most of them have a deletion encompassing the elastin gene because it results in an easily distinguishable phenotype. Individuals with atypical deletions which does not include the elastin gene are more difficult to detect[109].

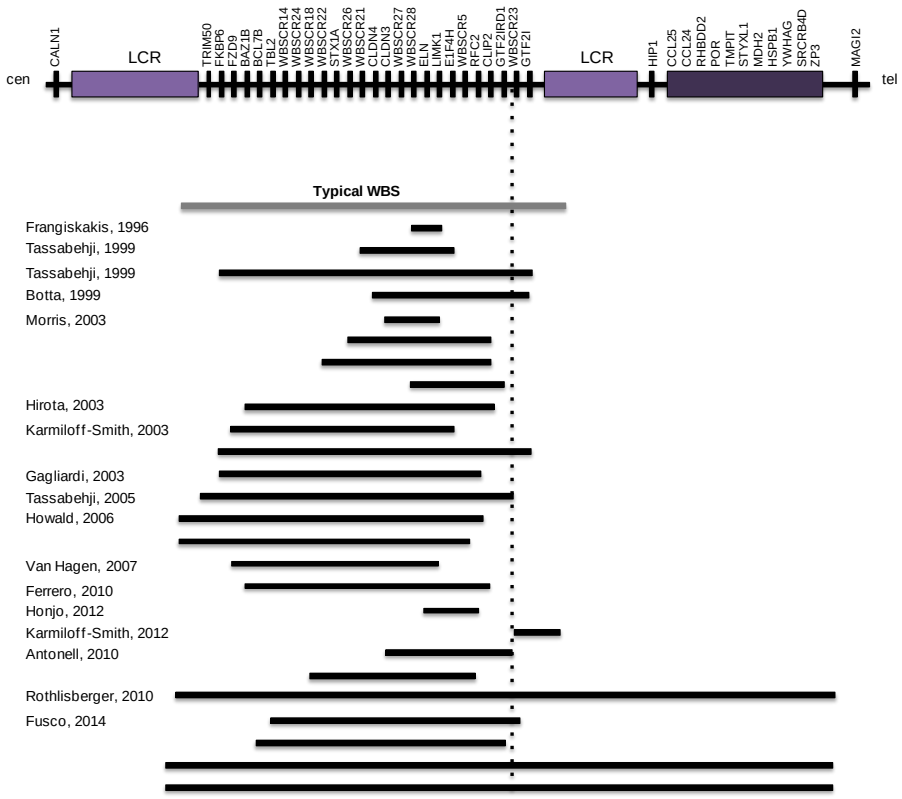

Figure 8. Schematic representation of typical and atypical 7q11.23 deletions. Each deletion encompasses different number of genes. Most of the deletions include the elastin gene as it results in an easily distinguishable phenotype. Adapted from[105].

3.1.2. Mouse models

Another strategy that allows the inference on genotype-phenotype correlations is the generation of mouse models. In mouse, the entire WBS region shows a high degree of synteny with the human region. It is located on the chromosome band 5G2 and has the full complement of genes, although in reverse orientation with respect to the centromere (**Figure 9**)[85,110]. However, the characteristic duplicated blocks flanking the region are not present in the mouse[110]. Such synteny allows the generation of single and multiple gene knock-out mouse models and provides a powerful tool for genotype-phenotype analyses. A part from genotype-phenotype correlations, animal models give the opportunity to perform molecular, biochemical and

genetic studies that are not possible in humans, which might help to elucidate the possible mechanisms underlying the disease. Finally, if the genetically modified mouse shows a similar phenotype observed in humans, pre-clinical testing of new therapeutic approaches can be performed to try to improve or even rescue the given phenotype[109].

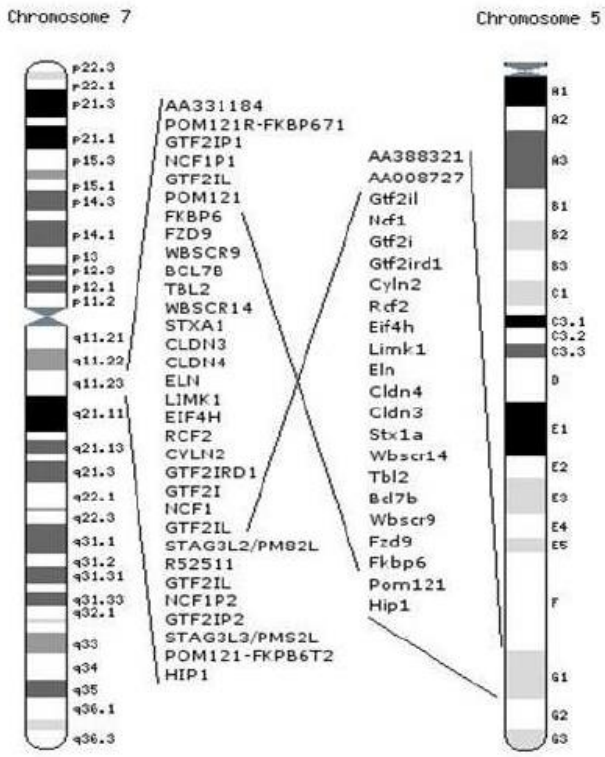

Figure 9. Comparative representation of WBS region at chromosomal band 7q11.23 in humans and in chromosomal band 5G2 in mice. The region in mouse is in reverse orientation with respect to the centromere. Adapted from[85].

So far, several single gene knock-out models have been generated to elucidate the contribution of individual genes to the complex phenotype associated with WBS. These models can be generated in the heterozygous or homozygous state. Although hemizygosity is equivalent to the WBS gene dosage, mouse models with deletion of the two alleles are also useful. Complete loss of gene dosage may produce more severe and easier to

identify phenotypes, providing hints of the underlying mechanisms, which may not be identified with heterozygous mouse models. Several mouse models with a single gene knocked-out have been generated for different genes within the WBS region (Table 1)[109].

Gene	Heterozygotes	Homozygotes	References
Baz1b	Mild craniofacial abnormalities, low frequency of cardiac malformations	High frequency of neonatal lethality, growth retardation, craniofacial abnormalities. Cardiac malformations such as ASD, VSD, and aortic coarctation	Ashe et al. 2008, Yoshimura et al. 2009
Clip2	Mild growth deficiency, hippocampal dysfunction, deficits in motor coordination	Mild growth deficiency, hippocampal dysfunction, deficits in motor coordination	Hoogenraad et al. 2002
Eif4h	Viable and fertile	Reduced body weight, growth retardation, smaller brain, reduced spine density and complexity of cortical neurons, impaired learning and memory	Capossela et al. 2012
Eln	Hypertension, decreased aortic compliance and mild cardiac hypertrophy	Perinatal embryonic lethality due to aortic obstruction by SMC proliferation	Li et al. 1998
Fkbp6	Fertile, with no phenotype observed	Males have abnormal spermatocyte pairing resulting in asperia and infertility	Crackower et al. 2003
Fzd9	Diminished seizure threshold, abnormal hippocampal structure	Splenomegaly, thymic atrophy, developing B-cell depletion; diminished seizure threshold, abnormal hippocampal structure, impaired spatial learning and memory	Ranheim et al. 2004, Zhao et al. 2005
Gtf2i	Hypersociability, crabifacial alterations, anxiety, lower threshold for sound intolerance	Craniofacial abnormalities, early embryonic lethality	Lucena et al. 2010, Sakurai et al. 2011, Enkhamandakh et al. 2009
Gtf2ird1	Mild growth deficiency, hypersociability, learning and memory deficits	Mild growth deficiency, craniofacial abnormalities, hypersociability, learning and memory deficits	Tassabehji et al. 2005, Young et al. 2008. Palmer et al. 2009
Lat2	No data reported	Abnormal T-cell activation and mast cell response resulting in an autoimmune syndrome	Volna et al, 2004, Zhu et al. 2004, 2006
Limk1	Synaptic plasticity deficits, deficits in spatial memory	Abnormal dendrite spine morphology, altered hippocampal function, mild deficit in spatial learning and memory	Todorovski et al. 2015, Meng et al. 2002
Mlxipl	-	Decreased lipogenesis, decreased ability to metabolize glucose leading to glycogen accumulation in liver	Iizuka et al. 2004
Ncf1	-	No hypertension when induced with angiotensin II, less oxidative stress production	Landmesser et al. 2002
Stx1a	No deficits in learning and memory, anxiety or locomotor activity observed or mild behavioral deficiencies	High frequency of embryonic lethality, impaired long-term potentiation and consolidation of conditioned fear memory	Fujiwara et al. 2006 and 2010, Ohara-Imaizumi et al. 2007, McRory te al. 2008, Nakamura et al. 2008

ASD, atrial-septal defect; VSD, ventrical-septal defect; SMC, smooth muscle cell.

Table 1. Single gene mouse models of WBS**.** The phenotypic features of several mouse models are described (either the heterozygote or the homozygote). Adapted from[109].

Although these single gene mouse models are useful to provide information about the function of an individual gene, contiguous gene deletion mice are better genetic models of WBS. The Francke's laboratory developed mice with deletions spanning either the proximal or the distal part of the region commonly deleted in individuals with WBS (Figure 10). The two half deletions were named proximal deletion (PD) and distal deletion (DD) mouse models. PD mice lack *Gtf2i* to *Limk1* while DD mice lack *Limk1* to *Trim50*. A model with both half deletions (named D/P) tries to model the complete human deletion, although *Limk1* is inactivated on both chromosomes and the two deletions are *in trans*[111].

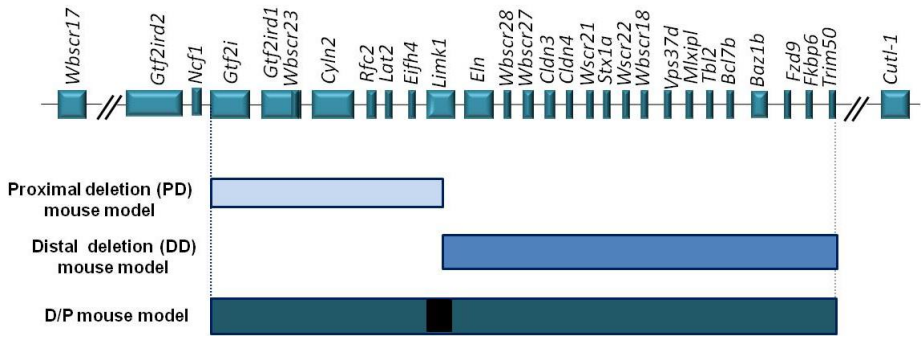

Figure 10. Partial deletion mice that model the WBS deletion. PD mice lack *Gtf2i* to *Limk1* while DD mice lack *Limk1* to *Trim50*. D/P model has the complete human deletion, although *Limk1* is inactivated on both chromosomes (in black) and the two deletions are *in trans*.

D/P mice show many of the classical symptoms of WBS such as hypersociability, cognitive defects, growth retardation or motor coordination abnormalities. In general, any phenotype observed in the D/P animal model can be assigned to the distal or proximal part of the deletion. For example, increased sociability is associated with genes deleted in PD mice whereas the craniofacial phenotype is associated with genes deleted in DD mice. Although the DD model includes more genes, it is associated with less phenotypes. In some features, such as motor coordination, D/P animals are

more affected than either of the half-deletion mice, indicating that genes in both regions may contribute to the phenotype[109,111].

Although these models have helped to gain insights into the specific contribution of different subsets of genes, they do not carry the same molecular defect present in humans. On 2014, a new animal model was created in our group. This model mimics the most common deletion found in WBS, as it carries the heterozygous deletion of the entire single copy region between *Gtf2i* and *Fkbp6* genes[112]. Although first studies on these mice have shown that it recapitulates some physical features such as postnatal growth delay, craniofacial abnormalities and a mild cardiovascular phenotype, an exhaustive characterization of the neurocognitive phenotype is still missing. This animal model is a useful tool to unravel the molecular mechanisms of the disease as well as to evaluate novel therapeutic approaches.

Unquestionably, the increasing number of WBS mouse models is helping to elucidate the contribution of some genes to the WBS phenotype. However, although their genome is very similar to the human genome, results should be interpreted carefully. Moreover, behavioral characterization of a particular phenotype in mouse, such as phobias, is sometimes difficult or even impossible. Finally, all studies are in a non-human context and require validation in relevant human cells.

3.1.3. Human cellular models

Some studies in human cells have focused on comparing the expression profile of WBS patient-derived tissues with unaffected controls. Antonell *et al*. performed comparative transcriptome profiling of lymphoblastoid cell lines from four WBS patients with the classic deletion and two patients with atypical deletion. The study revealed abnormal regulation of gene pathways related to relevant aspects of WBS such as glucose intolerance and visuospatial construction deficits[113]. Another study also using transcriptome profiling but in primary skin fibroblasts from WBS patients and sex- and age-matched controls identified 868 differentially expressed genes involved in the extracellular compartment, the immune response and the post-synaptic membrane[114]. However, although both analyses identified deregulated genes involved in neuronal functioning, they were performed in cell populations that are unrelated to brain development and function.

Obtaining live neurons or brain tissue is challenging when studying a rare disorder. Reprogramming human somatic cells to alternative fates, such as the conversion of human skin fibroblasts to induced pluripotent stem cells (iPSCs), has become a promising tool to study neurological disorders[115,116]. Adamo and colleagues studied transcriptional differences in a large cohort of WBS iPSCs lines. They found disruption of transcriptional circuits in pathways relevant for the disease as early as in the pluripotent state. These alterations were exacerbated after differentiation of the pluripotent cells into disease-relevant lineages[117]. Recently, Khattak *et al*. differentiated WBS patient-specific iPSC into cortical neurons for modeling WBS neuronal phenotype[118]. These differentiated neurons displayed normal neuronal phenotypes with 80-90% of cells expressing the mature neuronal markers, indicating that the protocol was efficient. Through electrophysiology experiments, WBS cortical neurons showed altered action potential repolarization and impaired voltage-gated potassium currents. Gene expression analyses identified alterations in genes related to neurotransmitter receptors, synapse assembly and potassium channel complexes[118]. These results suggest that patient-derived iPSC neurons represent another valid alternative for studying the mechanisms underlying WBS.

3.2. Genes related to the neurocognitive phenotype

The neurocognitive phenotype in WBS has been associated with several genes. *Gtf2i*, *Gtf2ird1* and *Gtf2ird2*, which are members of the TFII-I family, are the most studied genes linked to the phenotype. Other genes that have also been related to the neurocognitive phenotype are *Limk1*, *Fzd9, Clip2*, *Eif4h*, *Baz1b* and *Stx1a*.

3.2.1. Transcription factor II-I family

Transcription factor II-I (TFII-I) family is composed of the general transcription factor II-I (*GTF2I*, OMIM 601679), the GTF2I repeat domain-containing protein I (*GTF2IRD1*, OMIM 604318) and the GTF2I repeat domain-containing protein II (*GTF2IRD2*, OMIM 608899). These genes encode transcription factors with multiple helix-loop-helix-like domains, also known as I-repeats, which show a considerable sequence homology and are believed to play a role in DNA binding[119,120]. The TFII-I family is involved in important biological functions including gene transcription, signal transduction, cell

cycle and proliferation, and calcium and immune signaling[120]. All three genes are ubiquitously expressed, although higher levels are observed in neuronal tissues[121–123].

Although *GTF2I* and *GTF2IRD1* genes are invariably deleted in WBS patients, *GTF2IRD2* is only deleted when the deletion spans 1.84 Mb[86]. A growing body of evidence indicates that these genes are directly implicated in the craniofacial, cognitive and behavioral symptoms of WBS.

With the study of patients with atypical deletions, *GTF2I* has been linked to the neurocognitive deficits of WBS, especially to the hypersociability and facial dysmorphism[124–127]. On the other hand, study of the expression of the genes from the single normal copy revealed that expression of *GTF2I* in WBS individuals depends on the parental origin of the transmitted allele, pointing toward an epigenetic mechanism[128]. In 2010, a *Gtf2i* mouse model lacking the first 140 amino-acids of TFII-I was created in our group by gene targeting. Homozygous mutant mice presented highly compromised embryonic viability and fertility. Heterozygous mutant mice showed craniofacial alterations (also present in the homozygous condition), decreased exploratory activity, higher anxiety and a lower threshold for sound intolerance, resembling clearly to the WBS phenotype[129]. A second mouse model created by mutagenesis with a gene trap insertional vector presented similar characteristics. Homozygous deletion of *Gtf2i* caused lethality during embryonic development with neural tube closure defects and exencephaly (Figure 11). An interesting phenotype observed in heterozygous mutant mice was hypersociability. Mice showed increased social interaction with unfamiliar mice and did not show typical social habituation processes, indicating that *Gtf2i* is involved in the hypersociable phenotype of WBS[130]. Studies in human cells have also highlighted the role of *GTF2I* in this phenotype, as transcriptional dysregulation of pathways related to the phenotype were specifically caused by dosage imbalances in *GTF2I*[117].

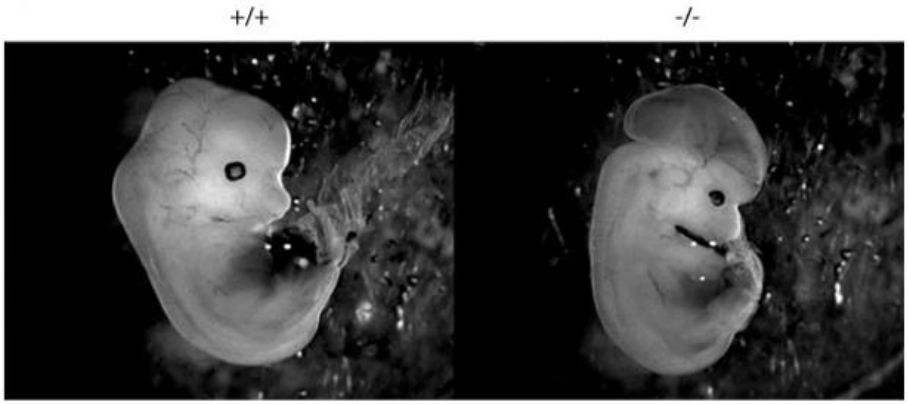

Figure 11. Exencephaly of *Gtf2i* knockout mice**.** Mutant mice at E14 showed exencephaly and distorted facial and eye morphology. From[130].

Regarding *GTF2IRD1*, several studies in patients with partial deletions have given strong evidence that this gene is also associated with some neurocognitive aspects[124,127,131,132]. Dai *et al.* studied a patient with an atypical deletion that included *GTF2IRD1* among others, but not *GTF2I*. The cognitive performance was better than typical WBS individuals, but presented marked visual-spatial construction deficits. The authors concluded that this result combined with previously published cases suggested that *GTF2IRD1* was associated with WBS facial features and the visuospatial construction deficits[126]. Several murine models have been created for *Gtf2ird1* to further study the specific role of this gene in the WBS phenotype. Mice with heterozygous or homozygous disruption of *Gtf2ird1* presented decreased fear and aggression and increased social interest in unfamiliar mice, features that are hallmarks of WBS. Increased levels of serotonin metabolites were found in several brain regions, suggesting that alteration of neurochemical pathways could be involved in the syndrome[133]. Another *Gtf2ird1* null mice showed craniofacial abnormalities, lower body weight and some neurological alterations such as decreased motor coordination and increased anxiety[134,135]. Similarly, a targeted *Gtf2ird1* mutation in mice that blocks normal GTF2IRD1 protein production revealed facial deformity, motor coordination deficits and alterations in exploratory activity[136]. Finally, a *Gtf2ird1* null mice generated by an insertion of a β-galactosidase gene trap cassette in intron 22 presented a more severe phenotype, including

embryonic lethality, brain hemorrhage and vascular, craniofacial and neural tube defects[137]. These phenotypic differences could be due to the different genetic background of the mice. Regarding the mechanisms underlying these phenotypes, as *GTF2IRD1* has a punctate nuclear localization and its interaction partners are involved in chromatin modification and transcriptional regulation, Carmona-Mora P *et al*. suggested that *GTF2IRD1* might act through a chromatin modification mechanism[138].

Although it has been less studied, some studies suggest that *GTF2IRD2* is also related to the neurocognitive phenotype. A study comparing cognitive, behavioral and psychological functioning between individuals with the typical deletion of 1.55 Mb with individuals with the larger deletion of 1.83 Mb (including *GTF2IRD2*) showed that those with the larger deletion were more cognitively impaired. Specifically, they presented alterations in areas of spatial functioning, social reasoning and cognitive flexibility[139]. Experimental analyses of function of *GTF2IRD2* suggest that it directly interacts with *GTF2IRD1* and *GTF2I*, inhibiting their function[140]. However, further studies are needed to clarify the role of *GTF2IRD2*.

3.2.2. LIM Domain Kinase I

LIM Domain Kinase I gene (*LIMK1*, OMIM 601329) belongs to a serine protein kinase involved in axon formation, membrane traffic and actin cytoskeletal organization[141,142]. It is strongly expressed in the nervous system, particularly at mature synapses[141,143]. Therefore, it is thought to be involved in neuronal development and brain function.

There are controversial results regarding the role of *LIMK1* in patients with atypical deletions. Frangiskakis *et al*. described two patients with atypical deletions including *ELN* and *LIMK1*. These patients presented the vascular disease caused by the hemizygozity of *ELN* and impaired visuospatial constructive cognition, which was related to the hemizygosity of *LIMK1*[144]. However, two other reports with similar partial deletions claim that *LIMK1* alone is not sufficient to cause the impairment of visuospatial abilities[145,146].

A knock-out mouse model for *Limk1* presented abnormalities in spine morphology and synaptic function, including enhanced hippocampal long-term potentiation (LTP), and some behavioral alterations[147]. Further

characterization of these mice in 2015, revealed that *Limk1* regulates synaptic plasticity via regulation of the transcription factor CREB, and demonstrated that the deficits in synaptic plasticity in these mice could be restored by enhancing CREB activity. On the other hand, heterozygous mice for *Limk1* displayed the same abnormalities in synaptic plasticity and spatial memory than the homozygous mice[148].

3.2.3. Frizzled 9

Frizzled 9 gene (*FZD9*, OMIM 601766) was first described in 1997 as a novel transmembrane receptor homolog of the Drosophila tissue polarity gene frizzled[149]. It interacts with Wnt ligands resulting in an inhibition of the β-catenin pathway[149,150].

At the beginning, little attention was paid to *FZD9*, as two patients with an atypical deletion which did not include this gene showed the full WBS phenotype[151]. However, null mutant mice for *Fzd9* generated in 2005 pointed to its potential involvement in the neurocognitive phenotype[152]. Mutant mice, either homozygous or heterozygous, showed an increased in apoptotic cell death with a moderate decrease in the number of adult granule cells in the dentate gyrus, suggesting an important role of this gene in hippocampal development. Functionally, null mice presented severe visuospatial and learning deficits and a higher predisposition of seizures[152]. Another mouse model of *Fzd9*, however, did not show any feature of WBS. Instead, they presented abnormal lymphoid development and maturation [153]. Further analyses on murine models figure out an additional phenotype of *Fzd9* in osteoblasts, as mice presented impaired bone formation by a cell-autonomous osteoblast defect[154]. Recently, experiments in cultured myotubes showed that *Fzd9*-mediated signaling through β-catenin has also a crucial role on the assembly of the vertebrate neuromuscular junction[155].

3.2.4. CAP-GLY Domain containing linker protein 2

CAP-GLY domain containing linker protein 2 (*CLIP2*, OMIM 603432), also known as *CYLN2*, is a cytoplasmic linker protein which regulates dynamic aspects of the cytoskeleton of the cell though the microtubule network[156]. It is highly expressed in neurons, especially in the hippocampus[156].

As in the case of *LIMK1*, there are controversial results regarding the role of *CLIP2* in patients with atypical deletions. Although cases of deletion of a single gene are very rarely, two healthy siblings having a hemizygous deletion of *CLIP2* were reported. They did not show any of the clinical features associated with WBS, suggesting that *CLIP2* is not related with the neurocognitive profile of WBS[157]. However, other reports with partial deletions suggest a role for *CLIP2* in the motor and cognitive characteristics of WBS[131]. Probably, *CLIP2* haploinsufficiency by itself does not lead to the WBS cognitive profile although may contribute to the phenotype in combination with other genes of the region.

Characterization of a mouse model for *Clip2* by electrophysiological experiments showed synaptic plasticity impairment in hippocampal CA1 area when compared to the wild-type (WT). Some behavioral alterations such as deficits in motor coordination were also present[156].

3.2.5. Eukaryotic Translation Initiation Factor 4H

Eukaryotic translation initiation factor 4H gene (*EIF4H*, OMIM 603431), identified in 1998, is ubiquitously expressed and it is involved in the initiation of protein synthesis at the level of mRNA[158].

There is a knock-out mouse model of *Eif4h* which has given clear evidence of the role of this gene in the neurocognitive profile of WBS. These mice had a significant reduction of body weight along with growth retardation. The brain of these mice was also smaller than WT mice and presented several neuroanatomical alterations. The number and complexity of cortical neurons was reduced in mutant mice and, as shown in Figure 12, spine density of these neurons was also reduced. Regarding the behavioral phenotype, spatial and associative learning and memory were severely impaired[159]. Research in patients with atypical deletions including *EIF4H* would be useful to establish the precise role of this gene in WBS.

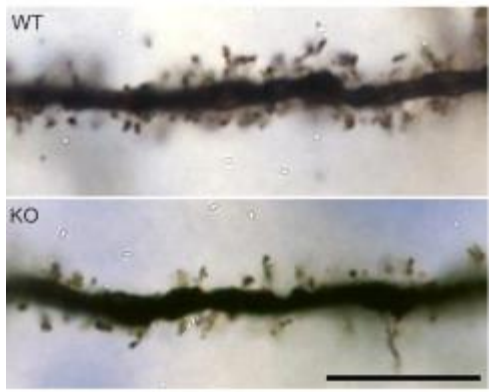

Figure 12. Golgi preparation showing a dendrite from a WT and a KO mouse model of *Eif4h*. As it can be note, spine density in the mutant mouse is significantly reduced when compared to the WT. From[159].

3.2.6. Bromodomain adjacent to zinc finger domain 1B

Bromodomain adjacent to zinc finger domain 1B (*BAZ1B*, OMIM 605681), also known as Williams syndrome transcription factor (WSTF), is a component of the multifunctional ATP-dependent chromatin-remodeling complex named WINAC (WSTF including nucleosome assembly complex). It is involved in the regulation of critical cellular events such as DNA replication and transcription and chromatin remodeling and maintenance[160,161]. *BAZ1B* is highly expressed in cranial neural crest-derived mesenchyme that drives facial morphogenesis[162].

Patients with an atypical deletion which does not include *BAZ1B* did not present any of the facial characteristics of WBS, suggesting that this gene could be a candidate for the facial phenotype[105]. A mouse model of *Baz1b* generated by random chemical mutagenesis (heterozygous L733R change in a highly conserved amino acid) resulted in a reduction of BAZ1B protein. These mice presented craniofacial abnormalities resembling those observed in WBS individuals. In particular, three-dimensional reconstruction of skull with micro-computed tomography revealed shorter skulls, with a bulbous appearance and a short, flattened or upwardly angulated nasal bone[162]. Hence, these results point to an involvement of this gene in the craniofacial features of WBS.

Recently, transcriptome analysis in patient-derived stem cells and neurons showed that 42% of the transcription dysregulation in neurons is due to *BAZ1B* haploinsufficiency. In addition, chromatin-immunoprecipitation and sequencing (ChIP-seq) experiments revealed that target genes of *BAZ1B* have relevant functions in neurogenesis, neuron differentiation and disease-relevant phenotypes[163].

3.2.7. Syntaxin 1A

Syntaxin 1A (*STX1A*, OMIM 186590) is highly expressed in neurons and is localized in the neuronal plasma membrane. It forms a complex called SNARE which is essential for synaptic vesicle exocytosis and, therefore, for synaptic transmission[164,165]. Polymorphisms in *STX1A* have been associated with some psychiatric disorders such as schizophrenia and autism[166,167].

McRory *et al*. generated a knock-out mouse model of *Stx1a* by deletion of exons 3 through 6. The majority of homozygous mice died in uterus and showed a drastic reduction in body size and development when compared to WT mice, indicating an important role of this gene for normal in utero development. However, heterozygous mice were viable and showed only mild behavioral deficiencies[168].

Another animal model generated by targeted gene disruption by Fujiwara *et al*. had no consequences on viability. Mice grew normally with no differences in appearance when compared to WT mice. Electrophysiological experiments in hippocampal neurons *in vitro* showed no alteration in basic synaptic transmission. However, LTP in hippocampal slices was impaired, suggesting abnormal synaptic plasticity in these mice[169]. Some behavioral abnormalities were observed in both homozygotes and heterozygotes models in a social interaction test, a novel object recognition test and a latent inhibition test. The abnormalities in the latent inhibition test were restored by administrating fluoxetine, a selective serotonin reuptake inhibitor. These results suggest that the abnormal behavior of these mice could be due to a disruption of the serotonergic transmission[170].

3.3. Genes related to other phenotypes

Some genes of the WBS region have been linked to clinical features such as cardiovascular abnormalities or metabolic alterations.

3.3.1. Elastin

Elastin (*ELN*, OMIM 130160) is the gene responsible for the SVAS and other connective tissue abnormalities related to WBS[107]. Elastin is the main component of the extracellular matrix of arteries and has an essential function in controlling the proliferation of smooth muscle and stabilizing arterial structure[16].

A mouse model lacking elastin, generated in 1998, died soon after birth (day 4.5) due to obstructive arterial disease secondary to smooth muscle proliferation and reorganization[16]. Heterozygous mice for elastin had a normal life span, although they were hypertensive from birth and presented mild heart hypertrophy[171].

3.3.2. Neutrophilic cytosolic factor 1

The neutrophilic cytosolic factor 1 gene (*NCF1*, OMIM 608512) encodes the p47phox subunit of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. It is present in the segmental duplications flanking the WBS region and is variably deleted depending on the breakpoints and gene conversion event[18].

In 2006, Del Campo *et al*. found out that hemizygosity at the *NCF1* gene is a protective factor against hypertension. WBS patients presenting only one functional copy of *NCF1* had a lower risk of hypertension when compared to patients with two or more copies, since angiotensin II-mediated oxidative stress was reduced[18].

DD mouse model, which carries a deletion that includes the *Eln* gene, presented generalized arteriopathy, hypertension, and cardiac hypertrophy. Levels of angiotensin II, oxidative stress and *Ncf1* expression were increased in these mice. These alterations were rescued by a pharmacological intervention reducing NADPH-oxidase-mediated oxidative stress, and also by a genetic approach crossing the DD model with a mutant of *Ncf1* and, therefore, reducing the *Ncf1* levels[172].

Mice deficient in p47phox, unlike WT mice, did not present hypertension when induced with angiotensin II. These mice showed less oxidative stress production when compared to the WT mice[173]. These results also indicate that deletion of *NCF1* is a protective factor against hypertension.

3.3.3. Mlx-interacting protein like

Mlx-interacting protein like (*MLXIPL*, OMIM 605678) is a member of the basic-helix-loop-helix leucine family of transcription factors[174]. *MLXIPL* has been related to the endocrinological phenotype of WBS, since its target genes are involved in glycolysis, lipogenesis and gluconeogenesis.

A single nucleotide polymorphism (SNP) at *MLXIPL* gene has been associated with hypotriglyceridemia[175]. Palacios-Verdú *et al*. studied this SNP in the non-deleted allele in a cohort of WBS patients to see if it was responsible for the variation in the lipid profile seen in those individuals. However, no significant differences were found between cases and controls[176].

Knock-out mouse for this gene had lower levels of triglycerides and increased levels of glycogen in the liver[177]. Therefore, *MLXIPL* haploinsufficiency might be involved in the glucose intolerance and diabetes mellitus of WBS.

3.3.4. Syntaxin 1A

STX1A not only has been related to the neurocognitive phenotype of WBS but also with the endocrinological phenotype. Particularly, it has been associated with the impaired glucose metabolism of WBS patients.

A transgenic mouse which expressed increased levels of *Stx1a* (30%) in pancreatic islets presented higher levels of fasting blood glucose levels when compared to WT animals. They also exhibited glucose intolerance after an intraperitoneal glucose tolerance test[178]. *Stx1a* knock-out mice, although did not present hyperglycemia, they did show impaired glucose tolerance with an oral glucose tolerance test[179]. Therefore, changes in dosage of *STX1A* might also contribute to the impaired glucose tolerance and diabetes of WBS.

OBJECTIVES

OBJECTIVES

The main objectives of this thesis were to unravel the molecular mechanisms underlying the WBS neurocognitive phenotype and to define novel therapeutic approaches. To conduct this work, we used the Complete Deletion (CD) mouse model, which carries a heterozygous deletion of the orthologous single copy genes, mimicking the most common deletion found in patients.

To achieve these global goals, we proposed the following specific objectives:

1. To characterize the cognitive and behavioral phenotype of CD mice model to determine whether they recapitulate the most relevant features of WBS, and therefore, could be appropriate models to study disease phenotype and novel therapeutic approaches.

2. To identify molecular and neuroanatomical alterations relevant for the disease which, in turn, could serve as markers to determine the efficiency of new possible treatments.

3. To define the status of synaptic function in WBS through electrophysiological studies in the hippocampus.

4. To evaluate the specific contribution of *Gtf2i* deletion to the specific molecular and neuroanatomical alterations associated to the WBS neurocognitive profile by: a) comparing several mouse models of the disorder with single-gene (*Gtf2i*), partial and complete deletions of the critical interval; and b) by restoring *Gtf2i* expression levels in the brain of CD mice through *in situ* adeno-associated virus-mediated *Gtf2i*-gene therapy.

5. To investigate the possible therapeutic effects of epigallocathechin-3-gallate (EGCG), the most abundant polyphenolic compound in green tea, in the WBS neurocognitive phenotype of CD mice.

CHAPTER 1

The CD mouse model presents relevant behavioral and neurological alterations related to Williams-Beuren syndrome

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Some of the results included in this chapter have been published in (Annex 1):

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Heterozygous deletion of the Williams-Beuren syndrome critical interval in mice recapitulates most features of the human disorder
Hum Mol Genet. 2014 Dec 15:23(24):6481-94

ABSTRACT

Williams-Beuren syndrome (WBS) is a rare neurodevelopmental disorder caused by a heterozygous deletion of 26-28 contiguous genes, manifesting a unique and distinctive neurocognitive profile. Mice heterozygous for a complete deletion (CD) of the single-copy syntenic interval of the most common deletion found in WBS individuals recapitulate relevant physical features of the disease and present differences in volume of several brain regions, together with increased immature neurons in the hippocampus. We performed an exhaustive characterization of the neurocognitive phenotype in these mice. CD mice revealed neurobehavioral alterations reminiscent of WBS such as hypersociability, impairment in motor coordination and altered levels of anxiety-like behavior. Neuroanatomical alterations were found in specific brain areas, including strikingly orientation of axons in motor cortex and a reduction in spine density of apical dendrites in CA1 neurons. In addition, alterations in calcium flux through NMDA receptors were observed in hippocampal primary neurons from CD mice. Collectively, these results suggest that these mice provide an excellent model to investigate the mechanisms involved in WBS as well as to evaluate novel therapeutic approaches.

INTRODUCTION

Williams-Beuren Syndrome (WBS, OMIM 194050) is a rare neurodevelopmental disorder caused by a heterozygous deletion of 26-28 contiguous genes and characterized by multisystemic manifestations with a wide range of phenotypic variability[1,2]. So far, a great deal of attention has been focused on its unique and distinctive neurocognitive profile. WBS individuals present mild to moderate intellectual disability with a specific pattern of strengths and weaknesses, including relative strengths in some aspects of language combined with extreme weakness in visuospatial and visuomotor skills[3,4]. In addition, WBS behavioral phenotype is characterized by an increase in general anxiety and phobias, and an overly friendly personality, which leads to inappropriate social behavior[3,5,6].

Studies using high magnetic resonance imaging in patients have revealed structural abnormalities in several brain areas such as amygdala, hippocampus or orbitofrontal cortex, which can be related to specific aspects of the WBS neurological phenotype[7,8]. In addition to macroscopic anomalies, Galaburda and colleagues reported several cytoarchitectonic anomalies by examining one single patient with WBS, such as reduced columnar organization throughout the cortex, abnormally clustered and oriented neurons and a generalized increase in cell packing density[9]. Several years later, a study in three WBS-affected brains revealed cell-packing density and neuronal size differences in primary visual cortex that was correlated with the visuospatial deficits present in these individuals[10]. Functional abnormalities have also been described, including depression of hippocampal energy metabolism and synaptic activity[11]. However, a much deeper insight into the neuropathological features of WBS is still needed.

The generation of mouse models can provide important information regarding the mechanisms underlying a disease. In mouse, the WBS region is located on the chromosome band 5G2 and shows high degree of synteny with the human region. In fact, it has the same genes although in reverse orientation with respect to the centromere[12,13]. Several single-gene knock-out mice have been generated to figure out the contribution of individual genes to the WBS complex phenotype. However, in most of the cases the consequences of the deleted gene have been studied in the homozygous mice, since phenotypes

in heterozygous mice have only been evident in few cases[14–17]. Mice with partial deletions spanning either the proximal (PD) or the distal (DD) part of the WBS region were generated to identify the specific contribution of different subsets of genes. In addition, in order to model the complete human deletion, the two partial deletions were crossed, creating the D/P mouse model[18]. Several phenotypes including alterations in overall brain growth, impaired social behavior and motor coordination, and some brain histological anomalies were described in these mice, linking subsets of genes of the WBS region to the specific phenotypes. However, although these models replicated several features of WBS, none of them carried the same molecular alteration present in humans, since in the D/P model *Limk1* is inactivated in both chromosomes and the two half deletions are *in trans*.

Recently, a new animal model carrying the most common deletion found in WBS individuals (heterozygous deletion from *Gtf2i* to *Fkbp6*) was generated in our group. First studies of this complete deletion (CD) mouse model demonstrated that it recapitulates some physical features of WBS, including cardiovascular alterations, postnatal growth delay or craniofacial abnormalities[19]. A general characterization of the neurocognitive profile revealed differences in volume of several brain regions, increased immature neurons in hippocampus, as well as increased startle response to acoustic simuli and deficits in the motility tonus strength. However, an exhaustive characterization of the neurocognitive phenotype to gain insight to the specific features implicated in the etiology of WBS was still missing.

In the present study, we have characterized the CD model by using behavioral and cognitive paradigms relevant to WBS phenotype, together with a more complex neuroanatomical analysis of brain. We have identified relevant features of WBS, including increased social interaction, motor abnormalities and altered anxiety-like behavior, accompanied by alterations in neural architecture in motor cortex and hippocampus. In addition, alteration in calcium flux through N-methyl-D-aspartate (NMDA) receptors was present in hippocampal primary cultures of CD mice. Since CD mice recapitulate most physical and cognitive features present in WBS patients, it will be an optimal model to unravel the molecular mechanisms of the disease as well as to evaluate novel therapeutic approaches.

MATERIALS AND METHODS

Ethics statement

All animal procedures were conducted in strict accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local Committee of Ethical Animal Experimentation (CEEA-PRBB). The PRBB also has Animal Welfare Assurance (#A5388-01, Institutional Animal Care and Use Committee approval date 05/08/2009), granted by the Office of Laboratory Animal Welfare (OLAW) of the US National Institutes of Health.

Animals' maintenance

CD mice (heterozygous deletion from *Gtf2i* to *Fkbp6*) were obtained as previously described[19] and were crossed with Thy1-YFP transgenic mice (B6.Cg-Tg(Thy1-YFPH)2Jrs/J, Jackson Laboratory) to label pyramidal neurons.

Mice were maintained on at least 97% C57BL/6J background. Animals were housed under standard conditions in a 12 hours dark/light cycle with access to food and water *ad libitum*. Genomic DNA was extracted from mouse tail to determine the genotype of each mouse using multiplex ligation-dependent probe amplification (MLPA) and appropriate primers (Table S1).

Behavioral tests

Behavioral testing was performed in adult males of 2-3 months of age. All the experiments were performed during the light phase of the dark/light cycle and were performed by researchers unaware of the different experimental groups.

Social interaction test: The test was adapted from a social approach/interaction test previously described[20]. It was conducted in an open field (50 x 50 cm) with a wire cup-like container large enough to hold a single mouse (7 cm diameter x 10 cm high). First, the subject was habituated to the open field for 10 minutes. Afterwards, an unfamiliar mouse (same strain) was introduced in the wire cup-like container and the amount of time spent by the subject investigating the novel mouse was scored in three 5 minutes segments followed by an additional 5 minutes segment with a second novel

mouse. Subject activity was scored manually (by at least two investigators) as the time that the subject was in nose-to-cage investigation of the novel stimulus in a 5 minutes segment.

Rotarod test: A commercially available rotarod apparatus (Rotarod LE8500, Panlab, Harvard Apparatus, Spain) was used. The experiment consisted of two trials: training criterion test, in which animals were trained until they could stay on the rod at the minimum speed (4 rpm) during 120 seconds; and the test session, in which motor coordination and motor skill learning were evaluated by measuring the latency to fall off the rod in consecutive trials of 4, 7, 10, 14, 19, 24, and 34 rpm. Animals were allowed to stay on the rod for a maximum period of 60 seconds per trial and to rest between trials during 5 minutes.

Marble-burying test: Polycarbonate rat cages were filled with bedding to a depth of 5 cm and lightly tamped down. A regular pattern of 20 glass marbles (five rows of four marbles) were placed on the surface of the bedding prior to each test. An individual animal was placed in each cage and the number of buried marbles (>2/3 marble covered) was counted every 5 minutes during 20 minutes.

Place preference test: The test was conducted in a custom-made three-chamber apparatus: two big chamber (20cm wide x 20cm deep x 20cm high each chamber) with a small neutral compartment in grey between. Mice were habituated to the apparatus with the two chambers in grey (both walls and floor) for 5 minutes 24 hours before the test. In the trial day, the color of the surfaces of the two big chambers was changed: one chamber had smooth black surfaces and the other chamber had black-and-white stripes in a square pattern (Figure 1). The neutral compartment remained in grey. The number of entries and the time spent in each compartment was

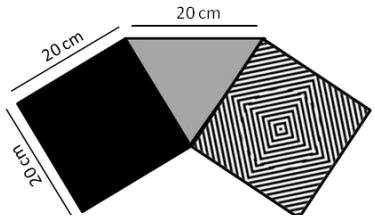

Figure 1. Custom-made three-chamber apparatus to test place preference. The design consisted of two equally sized chambers but in different color: one with smooth black surfaces (left) and the other one with black-and-white stripes in a square pattern surfaces (right). The two chambers were separated by a middle neutral compartment in grey.

manually measured during 4 segments of 5 minutes. After every single mouse the apparatus was cleaned with a diluted ethanol solution.

Histological preparation

Twelve-week-old mice were transcardially perfused with 1x PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and postfixed in the same buffer for 24 hours at 4°C, in PBS for 24 hours at 4ºC and, finally, crioprotected in 30% sucrose for 24 hours at 4ºC. For immunohistofluorescence, 40 µm coronal serial sections were collected in a cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% 0.1 M phosphate buffer [pH 7.4]). For neuroarchitecture analyses, 150 µm coronal serial sections were collected on a glass slide and mounted with Mowiol.

Immunohistofluorescence

Brain sections were blocked (1.5% blocking serum in PBS) and incubated overnight at 4ºC with a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) (1:500, MAB360, Millipore). Sections were incubated with an Alexa Fluor 555 conjugated secondary antibody (1:1000, A21424, Invitrogen) during 1 hour at room temperature. Finally, sections were collected on a glass slide and mounted with Mowiol.

Neuronal density and neuroarchitecture imaging

1024x1024 pixel confocal fluorescence image stacks were obtained using a HC PL FLUOTAR 20x 0.5 DRY objective in a TCS SP2 LEICA microscope. Multichannel imaging was done sequentially. Neuronal density was measured by staining 150 µm coronal tissue sections with the neuron-specific fluorescent Nissl stain Neurotrace 530/615 (1:250, Invitrogen). Only superficial (≤ 40 µm depth) tissue was imaged due to limited dye penetration. Counts from 5 tissue sections per animal (3-5 mice) were averaged for each brain region analyzed. For analysis of YFP expression patterns, and for P12-14 YFP^+ cell counting, 1249x956 pixel wide-field images of cerebral hemispheres were obtained using a 1.6x projection lens on a Leika epifluorescence microscope. Images were captured with a CCD camera (Leika). We used the ImageJ software for all image analysis.

For spine quantification in hippocampus, an HC PL FLUOTAR 63x (zoom 5) oil-immersion objective was used. Analysis of spine density was performed on basal proximal (30–120 µm from soma) and apical proximal (secondary apical dendrites from 50–150 µm from soma) dendrites. We randomly selected 8-15 dendritic segments from different neurons from a minimum of three animals per genotype. We counted the number of spines in 15-30 µm dendritic segments using ImageJ Cell Counter plugin. Spine counts included all type of dendritic protrusions. Spines located on the top or bottom surfaces of the dendrites were not counted, being the total number of spines an underestimation in all cases. Spine density was calculated by dividing the total spine count by the length of dendrite analyzed. Spine length was measured from the base of the spine neck to the end of the head of the spine.

For morphological analysis of neurons in the stratum radiatum (SR), stratum oriens (SO) or motor cortex, 1,360 × 1,024 images were obtained on a Leika epifluorescence microscopy at 4x magnification. Measures were made with ImageJ, and 4-6 sections per animal (3–6 mice) were averaged. In order to analyze the direction of axons of pyramidal neurons in the motorcortex, an arbitrary scale was used: 0 for axons which followed a straight direction to the pial surface, 1 for axons that were a bit displaced but recovered the normal direction, 2 for axons that were more displaced than in the category before, 3 for axons with meandering direction, and 4 for neurons extremely different form a normal pyramidal neuron (Figure 2).

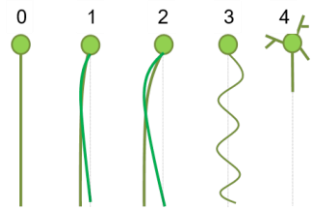

Figure 2. Arbitrary scale for axon orientation. 0 represents axons which follow a straight direction to the pial surface; 1 represents axons that are a bit displaced but recover the normal direction; 2 represents axons more displaced than in the category before; 3 represents axons with meandering direction; 4 represents axons extremely different from a normal pyramidal neuron.

Hippocampal primary cultures

E18-19 embryos were obtained from crosses between wild-type (WT) and CD mice and their brains were removed in HBSS medium (Invitrogen). A

piece of tail tissue was taken for genotyping and each embryo was handled individually. Using a dissecting microscope, the meninges of the cerebral hemispheres were removed and the hippocampus was dissected. After digesting the hippocampus for 17 minutes at 37ºC in trypsin 0.05%, cells were mechanically dissociated and plated at a density of $1x10^5$ cells/well in poly-L-lysine-treated coverslips in 24 well plates with DMEM containing 1x antibiotics (Penicillin-Streptomycin) and 10% fetal bovine serum (FBS). After two hours the medium was replaced by Neurobasal medium with 2% B27 (Invitrogen), 1x antibiotics (Penicillin-Streptomycin) and 1% L-Glutamine. On day 3 of culture, arabinoside (AraC) was added to the culture medium for 24 hours to eliminate proliferating non-neuronal cells.

Measurement of intracellular [Ca^{2+}] in mouse hippocampal neurons: Hippocampal cultures at day in vitro 10-12 were used. Cytosolic Ca^{2+} signal was determined at room temperature in cells loaded for 40 minutes with 4.5 µM FURA 2-AM (Life Technologies). Calcium measurements were performed on a NIKON inverted microscope with a 20x objective. Fura-2 (excitation: 340/380 nm, emission: 510 nm) ratiometric images were acquired every 2 seconds with a digital camera (Hamamatsu Photonics) and analysed with the AquaCosmos software. Cytosolic [Ca^{2+}] increases are presented as the ratio of emitted fluorescence (510 nm) after excitation at 340 and 380 nm, relative to the ratio measured prior to cell stimulation (F/F_o). During all experiments cells were bathed in an isotonic solution containing (in mM): 140 NaCl, 5 KCl, 1.2 CaCl2, 0.5 MgCl2, 5 glucose, 10 HEPES (300 mosmol/l, pH 7.4 with Tris). When indicated, bicuculline (50 µM), a $GABA_A$ receptor antagonist, and 4-aminopyridine (4-AP, 2.5 mM), a weak potassium-channel blocker, were co-applied for 3 minutes.

Statistical analyses

All data are presented as means ± SEM. For comparisons between two groups, the Student's t test or the Mann-Whitney U-test as a non-parametric test were used. A general linear model of repeated measures analysis of variance was used in some cases. All statistical tests were made under the SPSS environment. Values were considered significant when $p<0.05$. All graphs were made using GraphPad Prism software.

RESULTS

Behavioral alterations

Social behavior

Individuals with WBS have inappropriate social approach behavior, do not show stranger-anxiety as children and continue to display overly social and outgoing behavior towards strangers as adults[6]. To explore sociability in CD mice we performed an adaptation of a social approach/interaction test previously described[20]. WT mice exhibited a habituation effect, exploring the novel mouse for less time during the second 5-minute segment than during the first period of time (p=0.007). By contrast, CD mice showed less habituation with no significant difference in the amount of time spent investigating the unfamiliar mouse during the first and second 5-minute segments (p=0.101) (Figure 3a). The time spent in nose-to-cage contact by approach to the cage was significantly increased in CD respect to WT littermate mice (RS1, p=0.003 and RS3, p=0.004) (Figure 3b). Total interacting time with the first novel mouse showed a significant difference between WT and CD mice (p=0.001) (Figure 3c), mainly due to the lesser habituation in sessions 2 and 3 (Figure 3d). In the fourth and last trial, the subject was allowed to interact with another novel mouse. As expected for a social interest, an increase in the interaction time was observed in both genotypes (Figure 3d).

Motor function and exploratory activity

Motor problems along with hypotonia and some cerebellar signs are present in WBS patients[21]. Mice with partial deletions (PD and P/D) also showed poor performance on the rotarod test, indicative of motor problems[18]. In an initial approach to the motor phenotype of CD mice, a significant deficit in the motility tonus strength was observed, with no differences in gait or equilibrium. Regarding exploratory activity, no differences were observed in an open field test as measured by the total distance travelled in the center of the arena[19]. To further analyze possible motor coordination problems we assessed the accelerated rotarod test at different speeds (4, 7, 10, 14, 19, 24 and 34 rpm). First, animals were trained until they could stay on the rod at the minimum speed (4 rpm) during 120 seconds. Afterwards, the latency to fall

off the rod at each speed was measured. A significant reduction was found in CD mice as soon as 7 rpm (p=0.021). CD mice continued falling off the rod sooner than WT mice at 10 rpm (p=0.004), 14 rpm (p<0.001), 19 rpm (p<0.001), until 24 rpm (p=0.001) (Figure 4a).

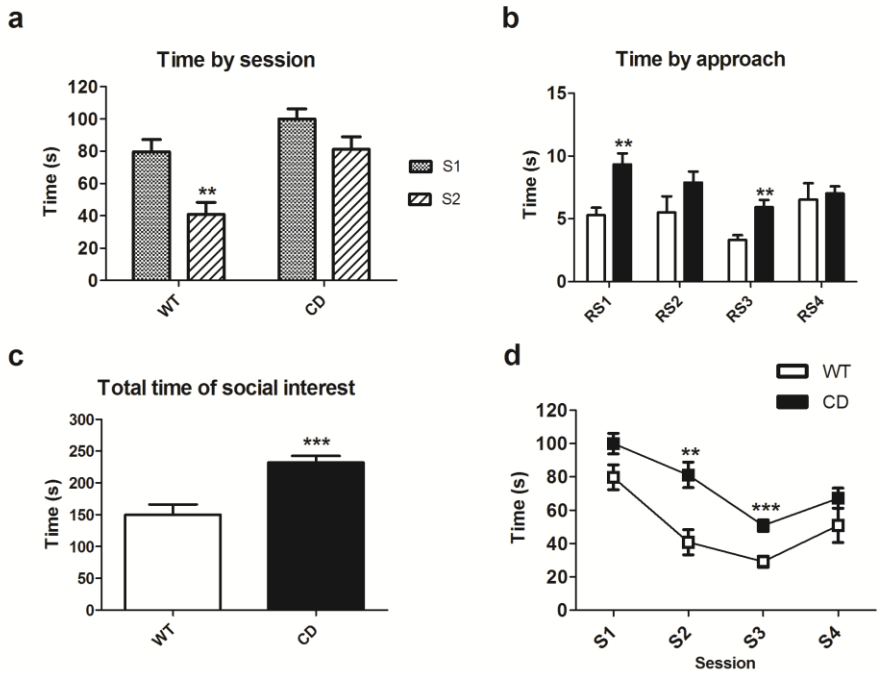

Figure 3. Social behavior is altered in CD mice. **a)** WT or CD mice were allowed to explore an unfamiliar mouse in a wire cup-like container placed in an open field. Time spent exploring the novel mouse was scored in two 5-minute segments (0–5, S1 and 5–10. S2). In contrast with CD mice, WT mice exhibited a habituation effect, exploring the novel mouse for less time during the second 5-minute segment than during the first (p=0.007). **b)** Significant differences between genotypes were observed in the time spent in nose-to-cage contact by cage approach in the first (RS1, p=0.003) and third (RS3, p=0.004) segments. **c)** Total exploration time with the first novel mouse was significantly higher in CD respect to WT littermates (p=0.001). **d)** A repeated measures ANOVA showed a significant difference of time-genotype ($F_{1,17}$=19,563, p≤0.001). WT: n=7, CD: n=12. Data are presented as the mean ± SEM. p values are shown with asterisks indicating values that are significantly different (General Linear Models of repeated measures analysis of variance or Mann-Whitney U-test). **p<0.01; ***p<0.001.

Anxiety-like behavior

Psychiatric disorders such as phobias, anxiety or obsessions are frequent in WBS. One of the most common findings is generalized anxiety disorder, which is prevalent in more than 80% of WBS individuals[1,22]. An anxiety-related response was found in PD and D/P animals by measuring the time spent in the centre of an open field test[18]. Controversially, CD mice did not show anxiety-like behavior in the open field test[19]. To further investigate anxiety-like behavior in CD mice, we used the marble-burying test to study anxiety or obsessive-compulsive traits, a test which is believed to depend, in part, on hippocampal function[23–25]. The number of marbles buried was counted every 5 minutes during 20 minutes. CD mice buried significantly less marbles than WT mice either after 5, 10, 15 or 20 minutes ($F_{1,8}$=24.163, p=0.001) (Figure 4b), indicative of abnormal levels of anxiety/obsessive behavior in this animal model.

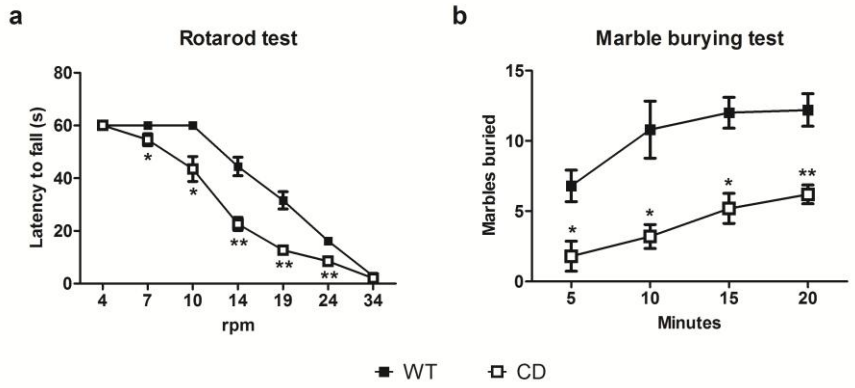

Figure 4. Motor function and anxiety-like behavior are altered in CD mice. **a)** The accelerated rotarod test was assessed to evaluate motor coordination and balance. A significant deficiency was observed as soon as 7 rpm (p=0.043) and continuing through 10 rpm (p=0.004), 14 rpm (p<0.001), 19 rpm (p<0.001), until 24 rpm (p=0.001). WT: n=13, CD: n=10. **b)** Anxiety-like behavior was evaluated in the marble-burying test. The number of buried marbles was counted every 5 minutes during 20 minutes. CD mice buried significantly less marbles after 5 (p=0.019), 10 (p=0.012), 15 (p=0.015) and 20 (p=0.009) minutes. WT: n=5, CD: n=5. Data are presented as the mean ± SEM. p values are shown with asterisks indicating values that are significantly different (General Linear Models of repeated measures analysis of variance or Mann-Whitney *U*-test). *p<0.05; **p<0.01.

Reluctance in changing the surface

Another interesting phenotype present in WBS children that was first described in 1996 is their great reluctance in changing the surface on which they are walking, especially if the new floor has a new pattern or color surface[26]. Sometimes they stop at the interface for several minutes and, in some cases, they refuse to proceed, reflecting a great fear of falling in the new surface. In several cases, a clear situation of agony and anxiety has been noted in these circumstances[26]. We developed an experimental test with the aim to see place preference or aversion to different patterns of surfaces. The design of the box consisted of two equally sized chambers (one with a black-and-white striped pattern with visual effects and another with smooth black surfaces, see Figure 1 in Materials and Methods), connected by a grey neutral space. Time exploring each chamber was measured during 4 segments of 5 minutes. Both genotypes preferred the smooth black chamber rather than the striped chamber (Figure 5a). During the first segment, CD mice significantly explored more the striped chamber rather than the black one (p=0.009). However, already on the second segment, CD mice significantly spent more time in the black compartment (p=0.006), showing a clear avoidance of the striped chamber (Figure 5b). Regarding WT mice, it was not until the third segment in which they showed a preference for the smooth black chamber (p=0.037) (Figure 5c). Number of entries in both chambers was also analyzed. Total number of entries in both compartments was significantly higher in CD mice (Figure 5d), which could be related to higher levels of anxiety probably due to the aversion of the striped pattern.

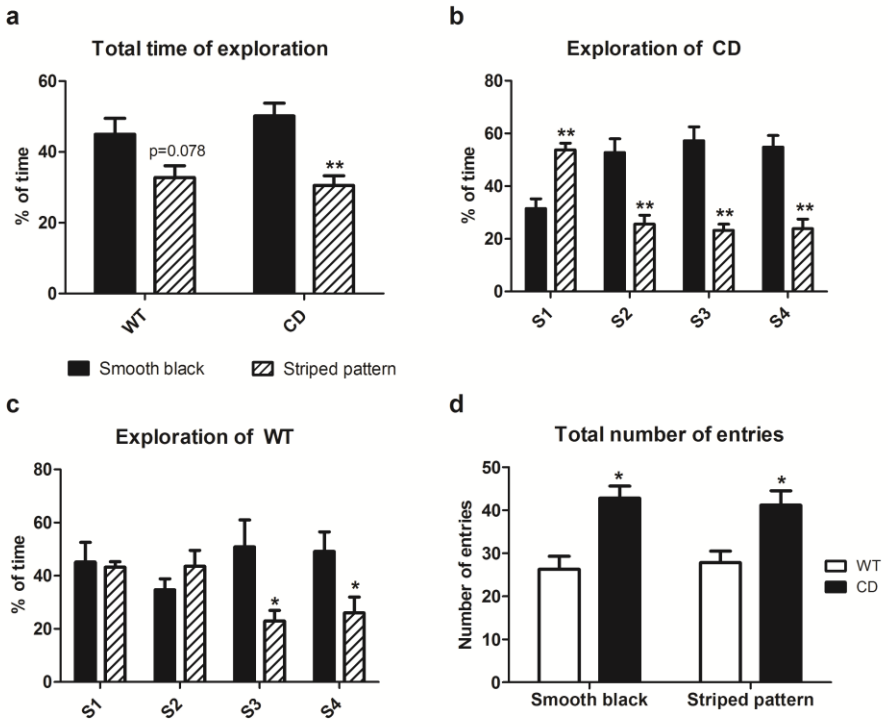

Figure 5. CD mice present reluctance in changing the surface. **a)** Total time of exploration was measured both in the smooth black compartment and the striped black-and-white compartment. Both genotypes spent more time in the black compartment rather than the striped one (WT: p=0.078; CD: p=0.006). **b)** During the first segment (S1) CD mice explored more the striped pattern chamber (p=0.009), while during the S2, S3 and S4 significantly preferred the smooth black one (S2: p=0.006; S3: p=0.004; S4: p=0.006). **c)** WT mice did not show any preference until the last two segments, in which they showed preference for the smooth black chamber (S3: p=0.037; S4: p=0.037) **d)** CD mice entered more times both in the smooth black (p=0.01) and the striped black-and-white (p=0.02) chamber than WT mice. WT: n=6, CD: n=6. Data are presented as the mean ± SEM. p values are shown with asterisks indicating values that are significantly different (General Linear Models of repeated measures analysis of variance or Mann-Whitney U-test). *p<0.05; **p<0.01.

Cellular alterations in glial and pyramidal cells

WBS individuals present an overall reduction in total brain volume (10-15%). Regionally specific differences in brain anatomy affecting volume or morphology of several brain regions such as amygdala, cortex, cerebellum, hippocampus or corpus callosum have also been reported[7,8,27,28]. CD mice

recapitulated the reduction in brain weight both in males and females. A volumetric and cytological analysis in CD mice revealed differences in some areas of the brain relevant to specific phenotypes of WBS[19].

Reduced GFAP+ cells in the basolateral amygdala and hippocampus of CD mice

Although the volume of the amygdala was preserved, a general decreased in cell density was observed in this area, especially in the basolateral area[19]. In order to clarify whether the decrease in cell density was due to local changes in cellular composition we evaluated the specific glial marker GFAP. Preliminary results obtained by qualitative analysis revealed a significant reduction of GFAP+ cells (p=0.022) in the basolateral amygdala of CD mice (Figure 6a). With regards to the hippocampus, previous studies showed a non significant reduction in cell density in CD mice[19]. We also evaluated GFAP+ cells in CA1 hippocampus and we found a significant reduction (p=0.003) of glial cells in this area (Figure 6b). Therefore, these results suggest that differences in the astroglial lineage could be implicated in WBS.

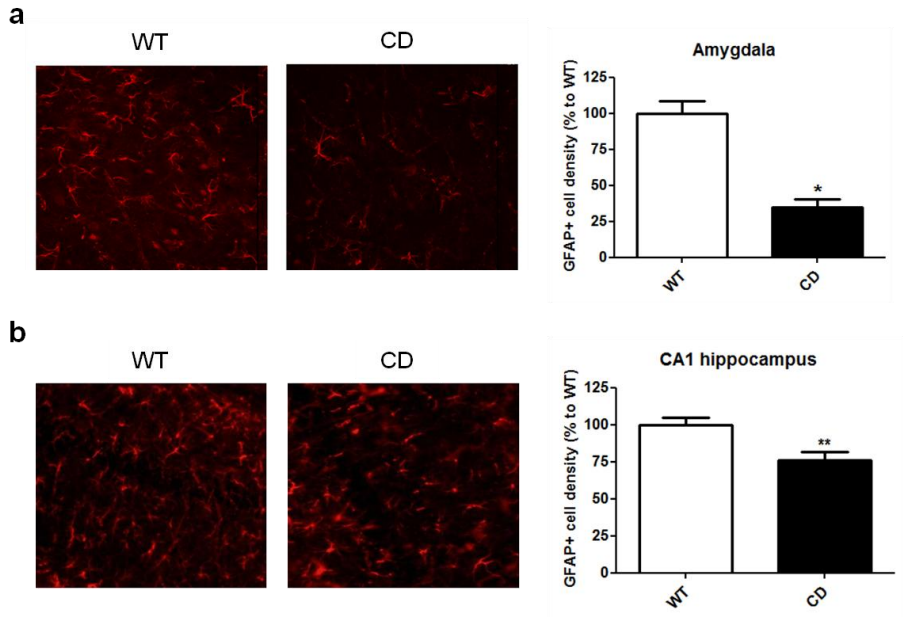

Figure 6. GFAP+ cells are reduced in the basolateral amygdala and CA1 hippocampus of CD mice. **a)** Representative immunofluorescence images of GFAP+ cells in the

basolateral amygdala of WT and CD mice and quantification of the images showing a significant reduction (p=0.022) in GFAP+ cell density in CD mice. WT: n=2; CD: n=2. **b)** Representative immunofluorescence images of GFAP+ cells in the CA1 hippocampus of WT and CD mice and quantification of the images showing a significant reduction (p=0.003) in GFAP+ cell density in CD mice (4 sections/mouse, 3 mice). Data are presented as the mean ± SEM. p values are shown with asterisks indicating values that are significantly different. *p<0.05.

Reduced number of YFP-expressing pyramidal neurons

We observed a remarkable and unexpected reduction in the number of YFP^+ pyramidal cells in CD mice relative to WT littermates (Figure 7a and 7b). We quantified the percentage of YFP^+ pyramidal cells in three areas (motor and somatosensory cortex and hippocampal region CA1) as a fraction of all fluorescent Nissl-stained neurons (Figure 7c). In all three regions CD mice had a drastic reduction of YFP^+ neurons. The fraction of YFP^+ neurons was decreased by 88% in motor and somatosensory cortex, and by 82% in CA1 (Figure 7c; p<0.001 in all pairs). The reduction in motor cortex occurred in contrast to the higher neuronal density (16.46%; p=0.009) observed with the Nissl-staining. Therefore, this reduction of YFP^+ cells does not correspond to the total number of cells and could result from overall down-regulation of YFP expression or transgene silencing in many cells of CD animals. In cortex, significant lower number of YFP^+ cells in mutant was present as early as P12-14 developmental stage (p=0,036), and nearly significant reduction was observed in hippocampus (p=0,057) (Figure 7d), suggesting that the reduced number of YFP^+ neurons in mutant mice results from transgene silencing.

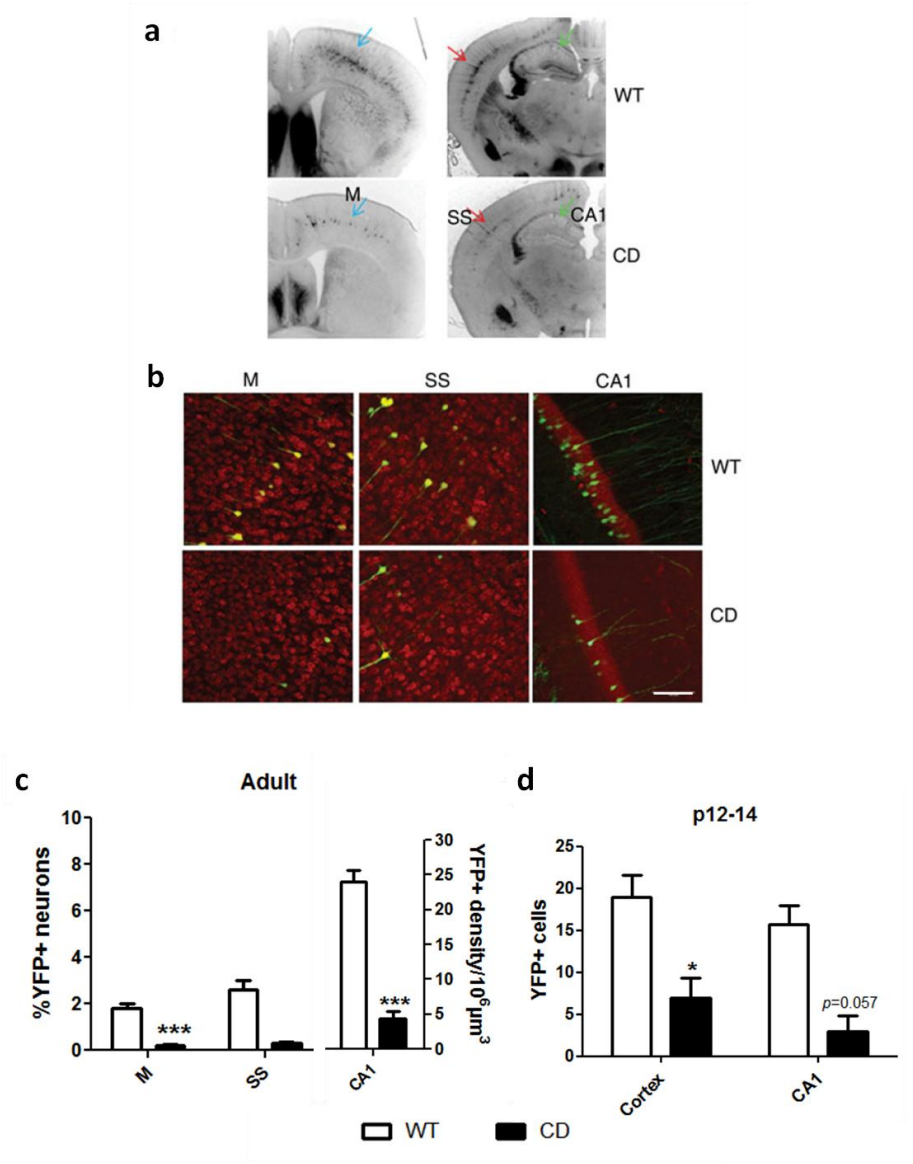

Figure 7. The number of YFP-expressing pyramidal neurons is reduced in CD mice. **a)** Reduced number of YFP+ cells was observed across the entire cortex and hippocampus in mutant mice (150 µm coronal sections, ≈ Bregma 0.74 and 21.28 mm in WT). Some regions were more dramatically affected than others, such as the somatosensory cortex (SS, red arrow), motor cortex (M, blue arrow) and CA1 region (CA1, green arrow). **b)** Fluorescent Nissl staining (red) of the L5 somatosensory and motor cortex and pyramidal neurons of the CA1 region. A limited subset of pyramidal neurons expressed YFP (scale bar = 100 µm). **c)** The percentage of YFP+ pyramidal neurons was reduced in motor and somatosensory cortical regions as well as YFP+ density in CA1

hippocampal region. **d)** The number of YFP+ pyramidal cells was significantly reduced as early as P12-P14 developmental stage in cortex (p=0.036) and almost in hippocampus (p=0.057). WT: n=3, CD: n=5. Data are presented as the mean ± SEM. p values are shown with asterisks indicating values that are significantly different (Student's t test). *p<0.05; ***p<0.001.

YFP+ CD neurons in motor cortex have shorter and disorganized axons

As CD mice presented clear motor coordination abnormalities, we analyzed general parameters of motor neurons, specifically in motor cortex. Axons of YFP+ neurons of CD mice were significantly shorter (p<0.001) when compared to the WT (Figure 8a). In addition, axons of CD mutant neurons were strikingly disorganized. While most of the axons in WT neurons were relatively vertically oriented with respect to the pial surface, axonal trajectories of CD neurons were quite variable, sometimes without a clear direction. An arbitrary scale from 0 to 4 was used in order to quantify this parameter (See Figure 2 in Material and Methods). The distribution of neurons into these categories was significantly different between WT and CD mice (p<0.001) (Figure 8b). Finally, we did not observe any difference with regards to the size of the somas between WT and CD mice (Supplementary Figure S1).

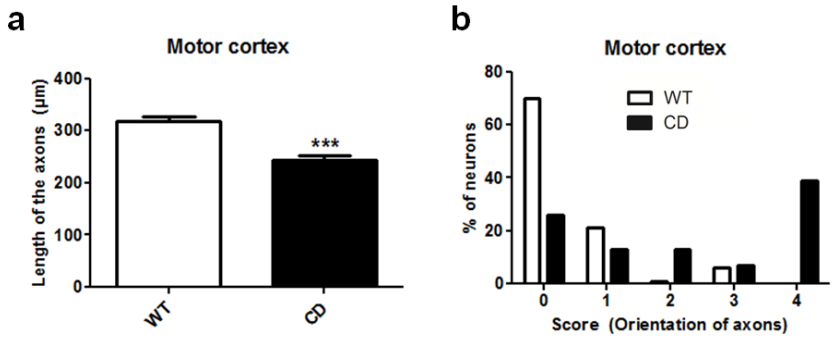

Figure 8. CD mice show neuroanatomical alterations in motor cortex. **a)** Length of YFP+ neurons in motor cortex was significantly shorter (p<0.001) in CD mice (n=30 neurons/mice ≥3 mice). **b)** Distribution of neurons into 4 categories depending on its orientation (0 the most well oriented and 4 the worst oriented neurons) was significantly different between WT and CD mice (p<0.001, Chi-squared test) (n=20 neurons/mouse, ≥3 mice). Data are presented as the mean ± SEM. p values are shown with asterisks indicating values that are significantly different (Student's t-test). ***p<0.001

YFP+ CD hippocampal neurons have reduced dendrite length and spine density in CA1 hippocampus

CD mice presented a volume reduction in the CA1 pyramidal region, although it was not significant[19]. As some of the behavioral tests used in this study were in part dependent on hippocampus we decided to further study this area to unravel the anatomical basis of the neurocognitive deficits observed in CD mice, specifically in CA1 region. Dendritic length of hippocampal neurons, both in the SR and in the SO, was shorter in CD mice (16% and 12%, respectively) (Figure 9a). Spine density in apical proximal dendrites of CD mice was significantly reduced by 17% (WT: 1.712 ± 0.049; CD: 1.422 ± 0.030) (Figure 9b and 9c). In addition, spines of CD mice were significantly shorter than WT spines (Figure 9d). Spine density and length in proximal basal dendrites presented similar values between WT and CD mice (Supplementary Figure S2).

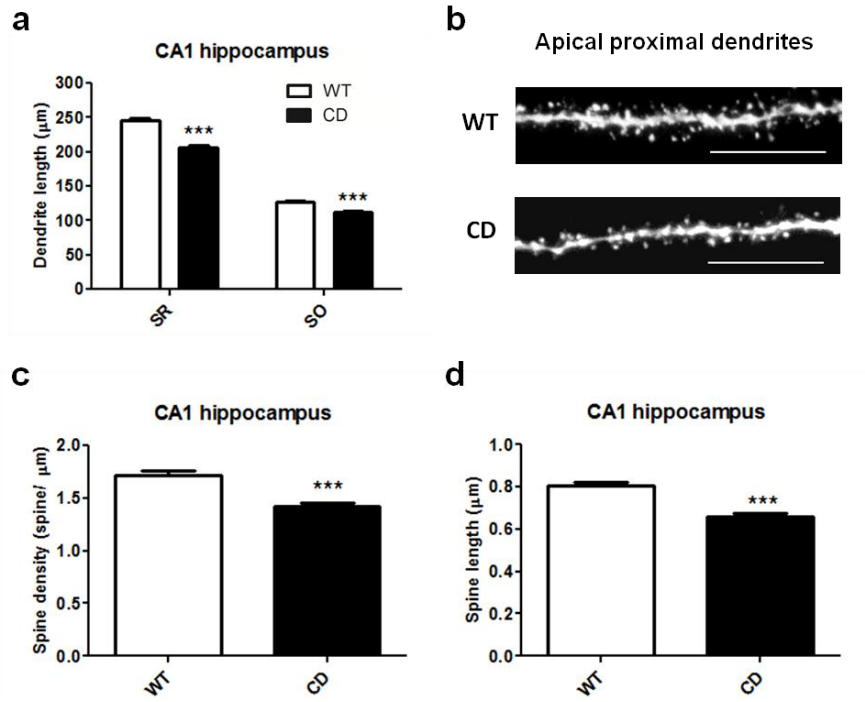

Figure 9. CD mice show neuroanatomical alterations in CA1 hippocampus. **a)** Width of SR (length of apical dendrites) and SO (length of basal dendrites) was measured in CA1 area of hippocampus. Apical and basal dendrites were significantly (p<0.001) shorter in CD mice (n=25–32 neurons/mouse, ≥3 mice). **b)** Representative photographs of

dendritic spines on apical proximal dendrites of CA1 pyramidal neurons in WT and CD mice (scale bar= 10 μm). **c)** A significant reduction (p<0.001) in spine density was found in apical dendrites of CD mice (n=8–16 dendrites/mouse, ≥3 mice). **c)** Spines in apical dendrites of CD mice were significantly shorter (p<0.001) when compared to WT mice (n=100 spines/mouse, ≥3 mice). Data are presented as the mean ± SEM. p values are shown with asterisks indicating values that are significantly different (Student's t-test). ***p<0.001.

Calcium flux through NMDA receptors is impaired in CD neurons

The alterations observed in hippocampal CA1 neurons together with the behavioral alterations observed in CD mice suggested alterations in the synaptic circuits of hippocampus. Calcium entry through NMDA receptors is thought to control important signaling pathways involved in synaptic transmission and plasticity among other synaptic functions[29,30]. Therefore, we decided to characterize this phenomenon by measuring intracellular calcium concentration in hippocampal primary cultures of CD mice. Since hippocampal cultures contain inhibitory interneurons, cells were treated with bicuculline, a $GABA_A$ receptor antagonist. This, induced synchronous bursts of firing activity associated with global calcium transients caused by calcium flux through synaptic NMDA receptors[31,32]. Both WT and CD hippocampal cultures exhibited highly synchronized calcium oscillations (Figure 10a). The mean frequency of calcium oscillations in CD neurons was significantly lower (p<0.001) when compared to the mean frequency of WT neurons (CD: 3.99 ± 0.21 peaks/min, n=130 cells from 6 animals, WT: 5.91 ± 0.28 peaks/min, n=103 cells from 4 animals) (Figure 10b). Both amplitude and duration of calcium transients were significantly higher in CD neurons when compared to WT neurons (Figure 10c and 10d). The alteration in the calcium flux through synaptic NMDA receptors may indicate alteration in glutamatergic synaptic activity by impairment of synaptic NMDA receptors or alteration in cellular calcium homeostatic systems.

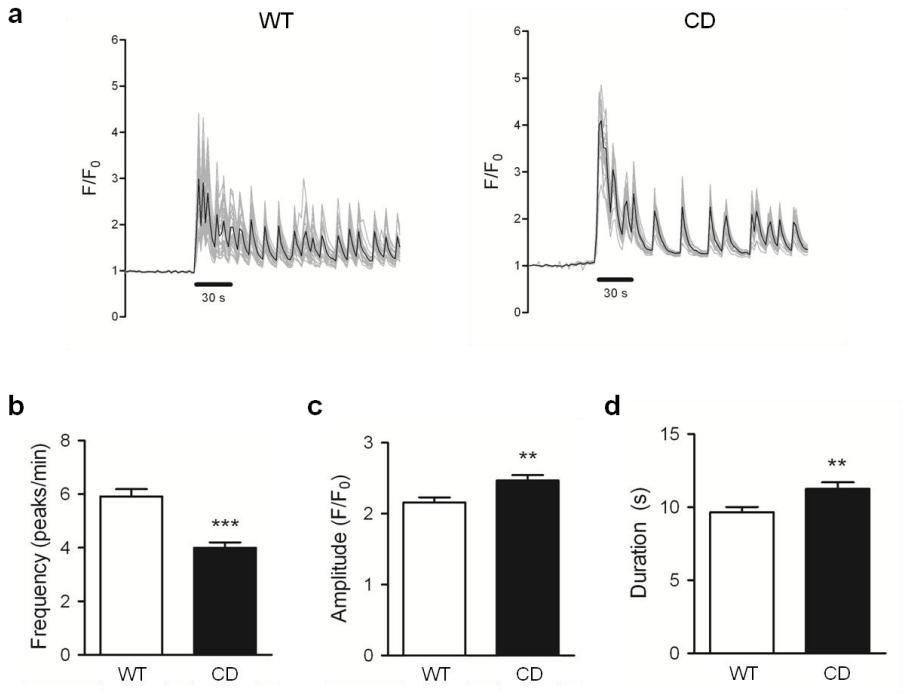

Figure 10. Impairment of calcium flux through synaptic NMDA receptors in CD mice. **a)** Imaging of global calcium transients in hippocampal neurons after bicuculline and 4-AP treatment. Both WT and CD hippocampal cultures exhibited highly synchronized calcium oscillations. **b)** Frequency of calcium signals in CD neurons was significantly reduced compared to WT neurons. **c)** Amplitude of calcium signals in CD neurons was significantly higher than WT neurons. **d)** Duration of calcium signals in CD neurons was significantly higher than WT neurons. WT: n=103 neurons from 4 mice; CD: n=130 neurons from 6 mice. Data are presented as the mean ± SEM. *p* values are shown with asterisks indicating values that are significantly different (Student's t-test). $^{**}p<0.01$; $^{***}p<0.001$

DISCUSSION

In addition to characteristic physical features and medical problems, WBS patients present a unique and distinctive cognitive and behavioral profile, including hypersociability, anxiety and phobias, visuospatial construction deficits and intellectual disability[33]. To date, most of neurological data in patients are limited to structural and functional brain imaging studies, but little is known about the pathophysiological mechanisms underlying the disease.

To better characterize the specific features implicated in the etiology of WBS, as well as to have an optimal model to evaluate novel therapeutic approaches, the CD model was generated. CD mice recapitulated several phenotypes present in WBS patients, but an exhaustive characterization of the neurocognitive phenotype was still missing. In the present report, the analyses of the behavioral characteristics in CD mice revealed a set of abnormalities strikingly similar to those manifested by WBS patients. In addition, some neuroanatomical findings in different brain regions were reported, together with alterations in calcium flux through synaptic NMDA receptors in hippocampus, which might be responsible for some of the behavioral alterations demonstrated in these mice.

Social disinhibition represents the most unique and intriguing behavioral characteristic of individuals with the WBS deletion. It has been proposed that WBS patients do not recognize social features such as facial expression, leading to hypersociability[1]. In a direct social interaction test, PD mice displayed more bouts of active social interactions relative to WT mice[18] and *Gtf2i* heterozygous animals showed lack of habituation[20]. In the paradigm we used, CD animals exhibited increased social interaction, spent more time with unfamiliar animals, and did not easily habituate to repeated stimuli, retaining social interest. Both WT and CD animals recovered their interest in the last session, when a new animal was exposed. Our results suggest that the observed phenotype in CD animals is due to increased sociability rather than memory problems, and the lack of habituation may be caused by impaired recognition of social partners. Also, hypersociability involves increased social interactions, in addition to less social anxiety which could explain the less social dominance observed by others in tube paradigm[18].

Anxiety is another problematic symptom endured by patients with WBS. Since CD mice did not show anxiety-like behavior in the open field test[19], we used the marble-burying test, which is believed to partially depend on hippocampal function[25,34]. CD mice performed significantly different from WT mice in this test, pointing to abnormal levels of anxiety-like behavior. In other mouse models of WBS controversial results have been obtained when evaluating anxiety. Heterozygous mice for *Gtf2i* displayed remarked increased levels of anxiety in two different paradigms: the elevated plus maze and the light-dark test[16]. PD and D/P mice showed an anxiety-related

response in the open-field test, measured by time spent in the centre of the field but no differences were obtained in other tests for anxiety such as the light-dark test or the marble-burying test. Differences in the anxiety-response could be due to methodological differences such as test protocols, housing or feeding or due to the different genetic alteration of the model.

When WBS children have to walk through a new floor with a new pattern or color, they feel stressed and they show reluctance in changing the surface on which they are walking[26]. In order to see the aversion to different surfaces, we design a two-chamber test: one chamber with smooth black surfaces and another chamber with black-and-white striped pattern. Both WT and CD mice clearly preferred the black chamber. However, the preference for the black chamber was evident sooner in CD than in WT mice. The higher number of entries of CD mice in both compartments could be due to higher levels of anxiety caused by the stripped pattern. In fact, even though CD mice had more entries in the stripped compartment, the total time spent inside the stripped box was the same as WT mice. These results are in line with the behavior seen in WBS children when faced in a changing surface, since reluctance in entering to the stripped chamber and higher levels of anxiety were evident.

The amygdala has been implicated in a variety of functions such as social cognition and anxiety processing[35,36]. Therefore, alterations in this brain area could be the underlying mechanism of the hypersociability and the anxiety-related response observed in CD mice. First insights into the neuroanatomical phenotype of CD mice showed no differences in the volume of the amygdala between WT and CD mice. However, CD mice presented a general decreased in cell density, especially in the basolateral area[19]. Our findings show there is a decrease in the number of GFAP+ cells in the basolateral amygdala of mutant mice, indicating there might be local changes in the cellular composition in this area, specifically in the astroglial lineage.

Dysfunction of hippocampus could also contribute to the behavioral alterations present in CD mice. Abnormal functionality of hippocampus detected by multimodal neuroimaging in patients has been described in WBS patients[11]. Previous results in CD mice showed a significant reduction in the CA3 pyramidal region with no differences in cell density in any compartment.

In addition, the subgranular zone of dentate gyrus presented a higher number of immature neurons when compared to WT mice[19]. Here, we focused on CA1 region of hippocampus. CD mice presented a reduction in the number of GFAP+ cells in this area. On the other hand, YFP^+ hippocampal pyramidal neurons in CD mice presented shorter dendrites in both apical (SR) and basal (SO) compartments, which is consistent with the decreased brain volume observed in these mice[19]. Apart from reduced dendritic length, mutant CA1 neurons had a significant decrease in spine density. In addition, these spines were shorter. It is now well known that spines receive the vast majority of excitatory synapses in the central nervous system and are key structures in learning and memory formation[37]. Therefore, changes in spine number and morphology might influence the functioning of neuronal circuits leading to behavioral impairment in CD animals. In fact, hippocampal primary neurons of CD mice showed impaired calcium flux through synaptic NMDA receptors, which may indicate alteration in glutamatergic synaptic activity and/or impaired cellular calcium homeostatic systems, leading to impairment of important synaptic circuits in hippocampus. These data open a new field of research which will contribute to further understand the mechanisms of the disease.

Abnormalities in motor coordination have been described both in young and adult individuals, resulting in difficulties with balance and motor planning[21,38]. First analysis of motor function in CD mice demonstrated significant alterations in the motility tonus strengths[19]. This time, we used the rotarod test to assess motor coordination abilities in CD mice. WT mice could adapt perfectly to the different speeds of the rod until 10 rpm, whereas CD mice performed poorly as soon as 7 rpm, demonstrating clear difficulties in motor coordination and balance. The main brain area involved in motor function is the motor cortex[39]. A general analysis of morphology in YPF^+ neurons of motor cortex revealed a significant decrease in the total length of axons in CD mice. In addition, these axons were strikingly disorganized. The presence of these findings may be correlated with abnormal neurotransmission between different neurons responsible for the corresponding motor phenotype. Therefore, to clarify the specific mechanisms involved, studies that address specifically the function of affected circuits as well as studies in other brain regions related to motor functions such as the cerebellum will be needed.

Finally, we also found a remarkable reduction (by 81-87%) of YFP-expressing cells in several brain regions of CD mice. In general, no YFP^+ cells can be visualized at birth. At P7, occasional labeled neurons can be detected but only by applying immunolabeling. By the end of the second postnatal week, the number of YFP^+ cells increases rapidly[40]. In both genotypes, we found individual YFP^+ neurons as early as P9. By P12-14, the number of YFP^+ neurons increased in WT, but remained low in CD mutants, suggesting that the reduced number of YFP^+ neurons in mutant mice results from transgene silencing. Similar findings have been previously described in a mouse model for another neurodevelopmental disorder, Rett Syndrome, caused by *Mecp2* knock-out[41]. More studies are needed to define whether the reduced number of YFP^+ neurons correlates with actual alterations in the proportion of certain subclasses of pyramidal neurons or it is due to aberrant developmental activation of the YPF transgene in CD mice.

In summary, our findings highlight the CD mouse model as a novel and powerful tool to investigate the mechanisms underlying the WBS neurocognitive profile. Since it recapitulates a wide range of behavioral and cognitive abnormalities seen in human, it will be a valuable model to evaluate novel therapeutic approaches.

REFERENCES

1. Pober, BR (2010). Williams-Beuren syndrome. *N. Engl. J. Med.* **362**: 239–52.

2. Pérez Jurado, AL (2003). Williams-Beuren syndrome: a model of recurrent genomic mutation. *Horm. Res.* **59 Suppl 1**: 106–13.

3. Morris, C (2010). The behavioral phenotype of Williams syndrome: A recognizable pattern of neurodevelopment. *Am. J. Med. Genet. C. Semin. Med. Genet.* **154C**: 427–31.

4. Bellugi, U, Lichtenberger, L, Jones, W, Lai, Z and St George, M (2000). I. The neurocognitive profile of Williams Syndrome: a complex pattern of strengths and weaknesses. *J. Cogn. Neurosci.* **12 Suppl 1**: 7–29.

5. Doyle, TF, Bellugi, U, Korenberg, JR and Graham, J (2004). 'Everybody in the world is my friend' hypersociability in young children with Williams syndrome. *Am. J. Med. Genet. A* **124A**: 263–73.

6. Järvinen-Pasley, A, Bellugi, U, Reilly, J, Mills, DL, Galaburda, A, Reiss, AL, *et*

al. (2008). Defining the social phenotype in Williams syndrome: a model for linking gene, the brain, and behavior. *Dev. Psychopathol.* **20**: 1–35.

7. Chiang, M, Reiss, AL, Lee, AD, Bellugi, U, Galaburda, AM, Korenberg, JR, *et al.* (2007). 3D pattern of brain abnormalities in Williams syndrome visualized using tensor-based morphometry. *Neuroimage* **36**: 1096–109.

8. Reiss, AL, Eliez, S, Schmitt, JE, Straus, E, Lai, Z, Jones, W, *et al.* (2000). IV. Neuroanatomy of Williams syndrome: a high-resolution MRI study. *J. Cogn. Neurosci.* **12 Suppl 1**: 65–73.

9. Galaburda, AM, Wang, PP, Bellugi, U and Rossen, M (1994). Cytoarchitectonic anomalies in a genetically based disorder: Williams syndrome. *Neuroreport* **5**: 753–7.

10. Galaburda, AM, Holinger, DP, Bellugi, U and Sherman, GF (2002). Williams syndrome: neuronal size and neuronal-packing density in primary visual cortex. *Arch. Neurol.* **59**: 1461–7.

11. Meyer-Lindenberg, A, Mervis, CB, Sarpal, D, Koch, P, Steele, S, Kohn, P, *et al.* (2005). Functional, structural, and metabolic abnormalities of the hippocampal formation in Williams syndrome. *J. Clin. Invest.* **115**: 1888–1895.

12. DeSilva, U, Elnitski, L, Idol, JR, Doyle, JL, Gan, W, Thomas, JW, *et al.* (2002). Generation and comparative analysis of approximately 3.3 Mb of mouse genomic sequence orthologous to the region of human chromosome 7q11.23 implicated in Williams syndrome. *Genome Res.* **12**: 3–15.

13. Valero, MC, de Luis, O, Cruces, J and Pérez Jurado, L a (2000). Fine-scale comparative mapping of the human 7q11.23 region and the orthologous region on mouse chromosome 5G: the low-copy repeats that flank the Williams-Beuren syndrome deletion arose at breakpoint sites of an evolutionary inversion(s). *Genomics* **69**: 1–13.

14. M, Tassabehji, Hammond P, Karmiloff-Smith A, Thompson P, Thorgeirsson SS, Durkin ME, Popescu NC, Hutton T, Metcalfe K, Rucka A, Stewart H, Read AP, Maconochie M, DD (2005). GTF2IRD1 in Craniofacial Development of Humans and Mice. *Science (80-.).* **310**: 1184–1187.

15. Hoogenraad, CC, Koekkoek, B, Akhmanova, A, Krugers, H, Dortland, B, Miedema, M, *et al.* (2002). Targeted mutation of Cyln2 in the Williams syndrome critical region links CLIP-115 haploinsufficiency to neurodevelopmental abnormalities in mice. *Nat. Genet.* **32**: 116–27.

16. Lucena, J, Pezzi, S, Aso, E, Valero, MC, Carreiro, C, Dubus, P, *et al.* (2010). Essential role of the N-terminal region of TFII-I in viability and behavior. *BMC Med. Genet.* **11**: 61.

17. Capossela, S, Muzio, L, Bertolo, A, Bianchi, V, Dati, G, Chaabane, L, *et al.* (2012). Growth defects and impaired cognitive-behavioral abilities in mice with knockout for Eif4h, a gene located in the mouse homolog of the Williams-Beuren syndrome critical region. *Am. J. Pathol.* **180**: 1121–35.

18. Li, HH, Roy, M, Kuscuoglu, U, Spencer, CM, Halm, B, Harrison, KC, *et al.* (2009). Induced chromosome deletions cause hypersociability and other

features of Williams-Beuren syndrome in mice. *EMBO Mol. Med.* **1**: 50–65.

19. Segura-Puimedon, M, Sahún, I, Velot, E, Dubus, P, Borralleras, C, Rodrigues, AJ, *et al.* (2014). Heterozygous deletion of the Williams-Beuren syndrome critical interval in mice recapitulates most features of the human disorder. *Hum. Mol. Genet.* **23**: 6481–94.

20. Sakurai, T, Dorr, NP, Takahashi, N, McInnes, LA, Elder, GA and Buxbaum, JD (2011). Haploinsufficiency of Gtf2i, a gene deleted in Williams Syndrome, leads to increases in social interactions. *Autism Res.* **4**: 28–39.

21. Gagliardi, C, Martelli, S, Burt, MD and Borgatti, R (2007). Evolution of Neurologic Features in Williams Syndrome. *Pediatr. Neurol.* **36**: 301–306.

22. Woodruff-Borden, J, Kistler, DJ, Henderson, DR, Crawford, N a. and Mervis, CB (2010). Longitudinal course of anxiety in children and adolescents with Williams syndrome. *Am. J. Med. Genet. Part C Semin. Med. Genet.* **154**: 277–290.

23. Takeuchi, H, Yatsugi, S and Yamaguchi, T (2002). Effect of YM992, a novel antidepressant with selective serotonin re-uptake inhibitory and 5-HT 2A receptor antagonistic activity, on a marble-burying behavior test as an obsessive-compulsive disorder model. *Jpn. J. Pharmacol.* **90**: 197–200.

24. Njung'e, K and Handley, SL (1991). Evaluation of marble-burying behavior as a model of anxiety. *Pharmacol. Biochem. Behav.* **38**: 63–7.

25. Deacon, RMJ and Rawlins, JNP (2005). Hippocampal lesions, species-typical behaviours and anxiety in mice. *Behav. Brain Res.* **156**: 241–9.

26. Withers, S (1996). A new clinical sign in Williams syndrome. *Arch. Dis. Child.*: 89–89.

27. Reiss, AL, Eckert, MA, Rose, FE, Karchemskiy, A, Kesler, S, Chang, M, *et al.* (2004). An experiment of nature: brain anatomy parallels cognition and behavior in Williams syndrome. *J. Neurosci.* **24**: 5009–15.

28. Schmitt, JE, Eliez, S, Bellugi, U and Reiss, AL (2001). Analysis of cerebral shape in Williams syndrome. *Arch. Neurol.* **58**: 283–7.

29. Léveillé, F, El Gaamouch, F, Gouix, E, Lecocq, M, Lobner, D, Nicole, O, *et al.* (2008). Neuronal viability is controlled by a functional relation between synaptic and extrasynaptic NMDA receptors. *FASEB J.* **22**: 4258–71.

30. Ghosh, A and Greenberg, ME (1995). Calcium signaling in neurons: molecular mechanisms and cellular consequences. *Science* **268**: 239–47.

31. Hardingham, GE, Fukunaga, Y and Bading, H (2002). Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat. Neurosci.* **5**: 405–14.

32. Hardingham, GE and Bading, H (2010). Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. *Nat. Rev. Neurosci.* **11**: 682–696.

33. Martens, M a, Wilson, SJ and Reutens, DC (2008). Research Review: Williams syndrome: a critical review of the cognitive, behavioral, and neuroanatomical phenotype. *J. Child Psychol. Psychiatry.* **49**: 576–608.

34. Deacon, RMJ (2006). Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. *Nat. Protoc.* **1**: 122–4.

35. Adhikari, A, Lerner, TN, Finkelstein, J, Pak, S, Jennings, JH, Davidson, TJ, *et al.* (2015). Basomedial amygdala mediates top-down control of anxiety and fear. *Nature* **527**: 179–85.

36. Amaral, DG (2003). The amygdala, social behavior, and danger detection. *Ann. N. Y. Acad. Sci.* **1000**: 337–47.

37. Bellot, A, Guivernau, B, Tajes, M, Bosch-Morató, M, Valls-Comamala, V and Muñoz, FJ (2014). The structure and function of actin cytoskeleton in mature glutamatergic dendritic spines. *Brain Res.* **1573**: 1–16.

38. Chapman, CA, du Plessis, A and Pober, BR (1996). Neurologic findings in children and adults with Williams syndrome. *J. Child Neurol.* **11**: 63–5.

39. Cheney, PD (1985). Role of cerebral cortex in voluntary movements. A review. *Phys. Ther.* **65**: 624–35.

40. Porrero, C, Rubio-Garrido, P, Avendaño, C and Clascá, F (2010). Mapping of fluorescent protein-expressing neurons and axon pathways in adult and developing Thy1-eYFP-H transgenic mice. *Brain Res.* **1345**: 59–72.

41. Stuss, DP, Boyd, JD, Levin, DB and Delaney, KR (2012). MeCP2 mutation results in compartment-specific reductions in dendritic branching and spine density in layer 5 motor cortical neurons of YFP-H mice. *PLoS One* **7**: e31896.

SUPPLEMENTARY MATERIAL

Gene	Sequence	Amplicon size	Location	Tm (ºC)
Gtf2i	L - gggttccctaagggttgga//gaatcatggcccaagtagtgatgtctgcct	101	Exon2	87,08
	R - tgcctgccgaagatgaagagtcttcagag//tctagattggatcttgctggcac		Exon2	85,64
Cyln	L - gggttccctaagggttgga//ctaatccacaaggtcatccgaattggcttccca	111	Exon 5	88,22
	R - tccaccagtccagccaaggccaagaagaccaaacgc//tctagattggatcttgctggcac		Exon 5	90,46
Rcf2	L - gggttccctaagggttgga//gccctcaggagaaccatggaaatctactcgaagacg	116	Exon 6	88,96
	R - cttcgcccttgcttgtaatgcttcggataagatcatcg//tctagattggatcttgctggcac		Exon 6	86,83
Baz1b	L - gggttccctaagggttgga//gctgaggaaagatacagtgaacgcatttggacgtgtaagag	114	Exon 2	87,47
	R - ggaagcagtcagctaacacacaaggagctgac//tctagattggatcttgctggca		Exon 2	86,19
Limk1	L - gggttccctaagggttgga//cgagtgcatgaggttgacgctactttgttgcacc	104	Exon 1	88,98
	R - tggagggaagaacgtatgggagaggaag//tctagattggatcttgctggcac		Exon 1	85,61
Fkbp6	L - gggttccctaagggttgga//ccggcgttatatcaaggccctcttcaggtgtggaca	113	Exon 6	90,59
	R - ggtgagttgagggtagtggggagatcgcgactgacg//tctagcttccatcttgctggca		Intron 6	91,11
Cutl1	L - gggttccctaagggttgga//caaaggaccattaagcccaatctatgtc	99	Exon 24	83,84
	R - gtgttttcaaggaagaaaacggaaatgtg//tctacattggatcttgctggcac		Exon 24	83,07
Wbscr17	L - gggttccctaagggttgga//gatcggggtgttgtttgatggcttcactgaggagagtcaaa	118	Exon 1	89,88
	R - tgttggtgttgaacttgatcgcggttgtgactgtac//tctagattggatcttgctggc		Exon 1	86,67
YFP	L - gggttccctaagggttgga//gccgccgacaagcagaagaacggcatc	93	cDNA 463-513	75,43
	R - aaggtgaacttcaagatccgccacaac//tctagattggatcttgctggcac			72,57

Table S1. Primer sequences for genotyping by MLPA

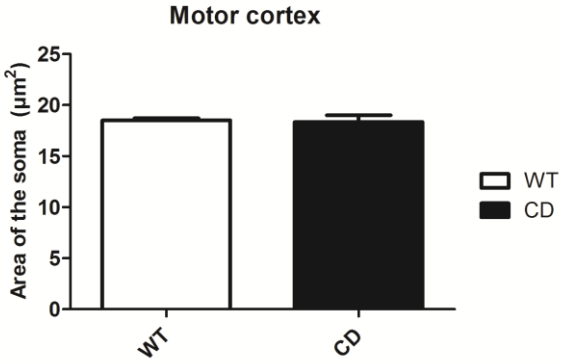

Figure S1. Motor cortex neurons in CD mice. No differences were observed regarding the area of the soma of YFP$^+$ neurons from motor cortex between WT and CD mice (n=80 neurons/mouse, ≥3 mice). Student's t-test. Data are presented as the mean ± SEM.

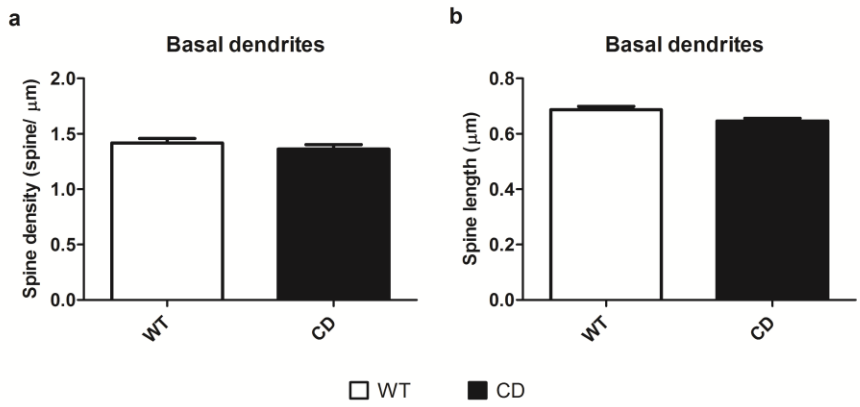

Figure S2. Basal proximal dendritic spine morphology in CA1 pyramidal neurons in CD mice. **a)** No differences were found in spine density between CD and WT basal dendrites (n=8-16 dendrites/mouse, ≥3 mice) **b)** No differences were found in spine length between CD and WT basal dendrites (n=100 spines/mouse, ≥3 mice). Student's t-test. Data are presented as the mean ± SEM.

CHAPTER 2

Borralleras C, Mato S, Amédée T, Matute C, Mulle C, Pérez-Jurado LA, et al. Synaptic plasticity and spatial working memory are impaired in the CD mouse model of Williams-Beuren syndrome. Mol Brain. 2016 Dec 2;9(1):76. DOI: 10.1186/s13041-016-0258-7

CHAPTER 3

Borralleras C, Sahun I, Pérez-Jurado LA, Campuzano V. Intracisternal Gtf2i Gene Therapy Ameliorates Deficits in Cognition and Synaptic Plasticity of a Mouse Model of Williams-Beuren Syndrome. Mol Ther. 2015 Nov;23(11):1691–9. DOI: 10.1038/mt.2015.130

CHAPTER 4

Epigallocatechin-3-gallate rescues short-term memory deficits and restores *Bdnf* levels in a Williams-Beuren syndrome mouse model

Cristina Borralleras, Guillermo Albericio, Mireia Viñals, Luis A. Pérez-Jurado, Victoria Campuzano

In preparation

ABSTRACT

Epigallocatechin-3-gallate (EGCG), the most abundant catechin found in green tea, has been associated with potential health benefits, including cognitive improvement in several neurological diseases such as Alzheimer disease or Down syndrome. The complete deletion (CD) mouse model of Williams-Beuren syndrome (WBS) mimics the most common deletion found in patients and recapitulates most of neurological features of the disease. Therefore, it is a potential and powerful model to study the effects of new therapeutic strategies. The purpose of this study was to investigate potential neuroprotective effects of tea extracts on WBS. After feeding CD animals with green tea in the drinking water, a set of behavioral tests and several neuroanatomical and molecular analyses were performed to unravel the effect of this diet. Although we could not appreciate any change in neuronal morphology, chronic administration of green tea was able to ameliorate short-term memory deficits of CD mice. In addition, levels of *Bdnf* in the hippocampus of CD mice were completely recovered. Therefore, memory improvements might be related to the upregulation of *Bdnf*, and/or modulation of BDNF-related pathways in the hippocampus of CD mice. Taken together, these results suggest that EGCG may be a promising therapeutic agent for WBS individuals.

INTRODUCTION

Accumulating evidence supports that the consumption of green tea is associated with potential health benefits, including prevention and treatment of cancer, inflammatory diseases, and cardiovascular diseases[1,2]. Most of the beneficial effects of green tea have been attributed to epigallocatechin-3-gallate (EGCG), the most abundant catechin found in green tea.

In the last years, an increasing number of studies have evidenced that EGCG is also associated with various neurological benefits. In fact, it has been confirmed that EGCG can easily cross the blood-brain barrier, and hence acting on the central nervous system[3,4]. The neuroprotective activity of EGCG has been mainly attributed to its potent antioxidant and radical scavenger-functions. Therefore, it has become particularly interesting in neurodegenerative diseases where oxidative stress plays an important role, such as Parkinson's and Alzheimer's diseases, and it has been shown to provide protective effects[5,6]. However, recent studies have suggested that not only the antioxidant and radical scavenger-properties are involved in the neuroprotective function of EGCG, but also the interaction with a wide range of cellular signaling pathways in the brain[7]. These include mitogen-activated protein kinases (MAPK)[8,9], protein kinase C (PKC)[10,11], and phosphoinositide-3-kinase (PI3K)[12–14] among others.

EGCG also promotes neuronal plasticity by enhancing long-term potentiation in mice from different genetic backgrounds[15]. In addition, EGCG has demonstrated to have a pro-survival and a pro-neurogenic role, since it increased neuronal survival in adult hippocampus *in vitro* and *in vivo,* and it increased proliferation of adult hippocampal neural precursor cells *in vitro*[12,16]. EGCG has been shown to increase cognitive function and learning ability in some behavioral studies. In a mouse model of Down syndrome, green tea polyphenols rescued major features of the transgenic phenotype such as long-term memory in the novel object test and normalized *Bdnf* levels in hippocampus[17]. EGCG also improved cognitive function in a pilot study in Down syndrome individuals, resulting in an improvement in the quality of life[18].

WBS is a neurodevelopmental disorder caused by a microdeletion of 26-28 genes on chromosome 7q11.23[19]. WBS individuals present mild to moderate intellectual disability, with a mean intelligence quotient (IQ) of 55-60, ranging from 40 to 100[20–22]. The syndrome is characterized by a unique and unusual cognitive functioning including relatively preserved expressive language and facial processing abilities but dramatic deficits in spatial cognition[23–25]. Another typical characteristic of the neurocognitive profile of WBS individuals is their personality, being socially fearless and disinhibited towards strangers[26]. The complete deletion (CD) mouse model of WBS replicated most of neurological features of the disease, including hypersociability, altered anxiety-related behavior, motor coordination abnormalities and cognitive impairment[27,28]. In addition to the behavioral phenotype, we found morphological alterations in hippocampal CA1 neurons, such as reduced dendritic length and decreased spine density in pyramidal neurons[27]. Functionally, we found a significantly reduced long-term potentiation in CA1 hippocampus (Chapter 2). Although data on potential cellular mechanisms are scarce, BDNF-related pathways, such as the PI3K/Akt/mTOR signaling pathway, have been proposed to be altered in CD animals[28,29]. Since this animal model mimics the most common deletion found in patients, it is a potential and powerful model to study the effects of new therapeutic agents.

In view of the neuroprotective properties of EGCG in several diseases, we decided to explore its effects on the CD model. CD animals were fed with green tea in the drinking water using different periods, from gestation to adulthood. To compare the effects of this diet on control and mutant mice, we performed a set of behavioral tests and several neuroanatomical and molecular analyses. We showed that chronic administration of green tea is able to ameliorate short-term memory deficits of CD mice, possibly through normalization of *Bdnf* mRNA levels in the hippocampus.

MATERIALS AND METHODS

Ethics statement

Animal procedures were conducted in strict accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research

and were approved by the local Committee of Ethical Animal Experimentation (CEEA-PRBB). The PRBB has Animal Welfare Assurance (#A5388-01, Institutional Animal Care and Use Committee approval date 05/08/2009), granted by the Office of Laboratory Animal Welfare (OLAW) of the US National Institutes of Health.

Animals' maintenance

CD mice (heterozygous deletion from *Gtf2i* to *Fkbp6*) were obtained as previously described[27] and were crossed with Thy1-YFP transgenic mice (B6.Cg-Tg(Thy1-YFPH)2Jrs/J, Jackson Laboratory)[30] to label pyramidal neurons. All mice used were males and were maintained on at least 97% C57BL/6J background. Animals were housed under standard conditions in a 12h dark/light cycle with access to food and water *ad libitum*. Genomic DNA was extracted from mouse tail to determine the genotype of each mouse using MLPA and appropriate primers[28].

Epigallocatechin-3-gallate (EGCG) treatment

EGCG was dissolved in drinking water and was prepared freshly from a green tea extract (Mega Green Tea Extract, decaffeinated, from Life Extension®, USA) every 3 days. We used a concentration of 1 mg/mL for a dose of 3-4 mg per day, resulting in 175 mg/kg/day, since mice drank 3-4 mL/day. The treatment was started in three different periods, using different cohorts of mice: at 5-6 weeks old (adulthood treatment), at postnatal day 8 (P8 treatment) or treating the pregnant female at post-coitum (PC treatment). Animals drank tea until its sacrifice at 8-16 weeks-old (young adult). The control group consisted of WT and CD mice drinking water without EGCG.

Behavioral tests

All the experiments were performed during the light phase of the dark/light cycle by researchers unaware of the different experimental groups. In all tests apparatus were cleaned with a diluted ethanol solution after each mouse.

Novel object recognition (NOR) test: Object-recognition memory was assessed in a V-maze made out of black Plexiglas with two corridors at a 90º angle (30 cm long x 4.5 cm wide, and 15 cm high walls) set at a 90° angle. At

the level of the task apparatus there was a constant illumination of about <10 lux. On the first day, each mouse was individually habituated for 10 minutes to the maze in the absence of objects. During the training session on the next day, two identical objects (A) were placed at the end of the corridors. The mouse was then placed in the middle of the maze and the total time spent exploring each object was recorded during 10 minutes. In the test session (10 minutes after the training session) the mouse was placed back in the maze, but a familiar object was replaced by a novel object (B). Time exploring each object was recorded again during 10 minutes. Object exploration was defined as time that the subject was in nose-to-object investigation. Throughout the experiments, the objects were used in a counterbalanced manner to avoid possible spatial-location bias. To measure cognitive function a preference index was measured, defined as a ratio of the amount of time exploring any of the two objects in the training session or the novel object in the test session over the total time spent exploring both objects. Therefore, a preference index above 50% indicates novel object preference, below 50% familiar object preference, and 50% no preference[31,32].

Spontaneous alternation test: The spontaneous alternation test was conducted in a T-maze. A central partition extending from the centre of the T into the start arm was included, allowing access to only one goal arm at a time and forcing the mouse to return to the starting arm each time[33]. The maze was equipped with one removal guillotine door separating a compartment at the beginning of the start arm. Mice were individually placed in the start compartment of the T-maze and after 5 seconds of confinement, the start arm door was lifted allowing mice to freely choose between the two goal arms (A or B). A total of 15 free choices made by the mice were annotated and the percentage of alternation during the free choice trials was calculated. The time to complete the 15 trials was also recorded and analyzed.

Social interaction test: We used the same test previously described[28]. The test was conducted in an open field. First, an empty wire cup-container was placed in the center of the arena. The subject mouse was allowed to explore the arena, and the amount of time sniffing the empty container was measured during 5 minutes. Next, an intruder mouse was hold in the container and,

again, the amount of time nose to nose sniffing was measured during 5 minutes.

Marble-burying test: The test was conducted in a polycarbonate rat cage filled with bedding to a depth of 5 cm and lightly tamped down. A regular pattern of 20 glass marbles (five rows of four marbles) were placed on the surface of the bedding prior to each test. An individual animal was placed in each cage. The number of buried marbles (>2/3 marble covered) was counted every five minutes during 20 minutes.

Histological preparation

Mice were transcardially perfused with 1x PBS followed by 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde for 24 hours at 4ºC, in PBS for 24 hours at 4ºC and, afterwards, crioprotected in 30% sucrose for 24 hours at 4ºC. Finally, serial coronal sections (150 µm) of brain were collected on a glass slide and mounted with Mowiol.

Imaging

For morphological analysis of the stratum radiatum (SR) and stratum oriens (SO), 1360x1024 images of CA1 hippocampus were obtained on a Leika epifluorescence microscopy at 4x magnification. Measures from six hippocampal sections per animal (3-6 mice) were averaged.

For spine density and spine length analyses we obtained 1024x1024 pixel confocal fluorescent image stacks from coronal tissue sections of using a TCS SP2 LEICA confocal microscope. Measurements were performed according to previously published method[27,28].

RNA preparation and gene expression quantification

RNA was extracted from hippocampal tissues of adult mice using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. cDNA was prepared from 1 µg of total RNA using random hexamers and SuperScript II RNase H reverse transcriptase (Invitrogen). The expression of *Bdnf*, *Pik3r1, Pik3cb, Limk1* and *Rps28* was evaluated by quantitative real-time PCR (qRT-PCR) and/or semi-quantitative RT-PCR as previously described[28].

Statistical analysis

All data are presented as means ± SEM. One-way, two-way or three-way ANOVA were used when needed, using Bonferroni's or Dunnett's *post hoc* test. Novel object recognition data were analyzed by one-sample *t*-tests to examine whether object exploration times were different from the 50% chance level. Values were considered significant when $p<0.05$. GraphPad Prism software was used for all statistical tests and graphs.

RESULTS

EGCG improves short-term memory but not working memory in CD mice

The NOR test evaluates the ability of rodents to recognize a novel object in the environment. It is very useful to study short- and long-term memory through manipulation of the retention interval. We studied short-term memory using an interval of 10 minutes between the training session and the test session. Both genotypes explored equally the two identical objects (A+A) during training (Figure 1a). In the test session, when a familiar object was replaced by a novel one (A+B), one-sample *t*-test showed that WT animals displayed preference for the novel object that was significantly different from the 50% chance level (t_7=5.0215, p=0.0015). In contrast, CD mice were unable to discriminate between the objects, and one-sample *t*-test showed no significant difference from chance levels (t_8=1.9209, p=0.091) (Figure 1a). Interestingly, CD mice treated with EGCG displayed increased exploration of the novel object equivalent to that observed in WT mice, which was significantly different from 50% chance level (t_6=3.7987, p=0.009) (Figure 1b and 1c). EGCG treatment in WT mice did not have any effect on their performance in the task, as they significantly discriminated between the two objects (t_5=4.0686, p=0.01) (Figure 1b and 1c). Regarding the total object exploration time, two-way ANOVA revealed a significant main effect of genotype ($F_{1,26}$=12.68, p=0.0015), since CD mice showed significantly more exploratory activity than WT mice ($p<0.05$, Bonferroni *post hoc* test) (Figure 1d).

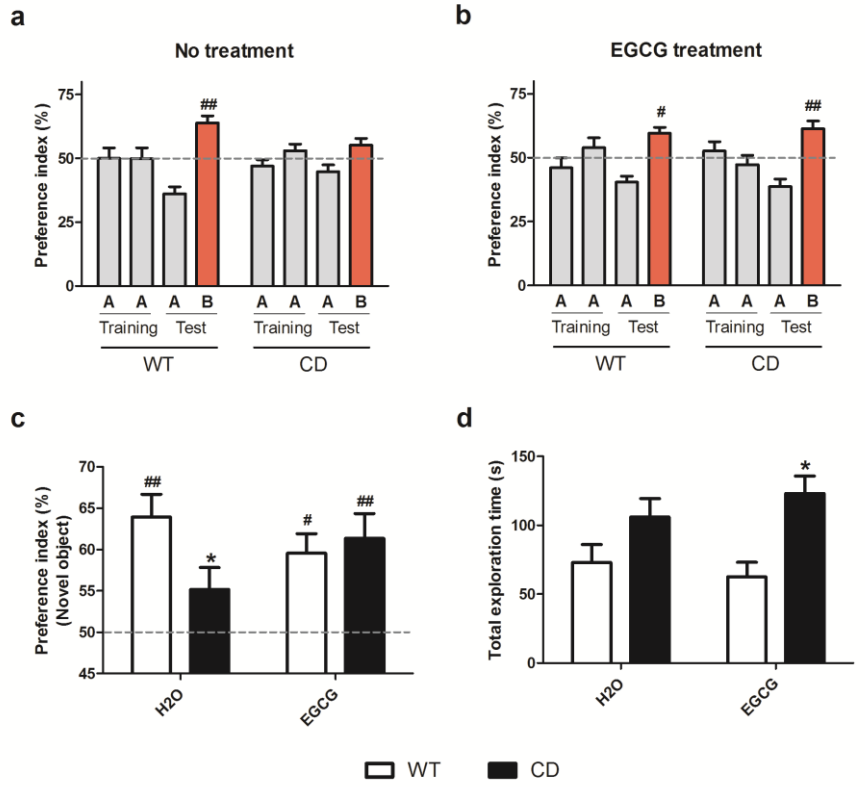

Figure 1. EGCG rescues short-term memory deficits in CD mice. Mice were trained in the NOR test with identical objects (A+A) for 10 minutes. The test session was performed after 10 minutes, and a familiar object was replaced by a novel one (A+B). Time spent exploring the objects was measured and a preference index was calculated (ratio of the amount of time exploring any of the two identical objects in the training session or the novel object in the test session over the total time spent exploring both objects) in animals water fed (H2O) **(a)** and in animals fed with EGCG **(b)**. **c)** Representation of the preference index for the novel object in the test session in animals fed with water (H2O) or EGCG. **d)** Total exploration time (time exploring the objects in the training and in the test session) was measured. A two-way ANOVA indicated a significant effect of genotype ($F_{1,26}$=12.68, p=0.0015) but no effect of treatment ($F_{1,26}$=0.06951, p=0.7941). WT-H2O: n=8, CD-H2O: n=9, WT-EGCG: n=6 and CD-EGCG: n=7 (PC treatment). Data are presented as the mean ± SEM. p values are shown with asterisks indicating values that are significantly different in a Two-way ANOVA with Bonferroni's *post hoc* test (*p<0.05, genotype effect) or shown with hash indicating values that are significantly different in one-sample t-test compared with 50% chance level . #p<0.05, ##p<0.01

In the spontaneous alternation test, the rate of spontaneous alternation (visiting each arm in turn) was greater in WT mice than in CD mice, indicating abnormalities in spatial working memory in CD mice (Chapter 2). As expected, we obtained similar findings in CD animals water-fed. Treatment with EGCG did not improve the performance of CD mice in this test (Figure 2a). Additionally, the total time to complete 15 trials did not differ between genotypes or treatment (Figure 2b).

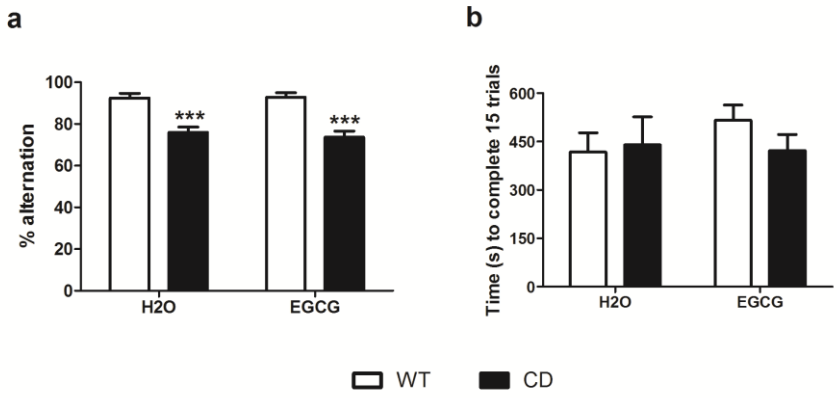

Figure 2. EGCG does not rescues spatial working memory deficits of CD mice. **a)** Percentage of alternation (number of alternations/total number of possible alternation x 100) in the spontaneous alternation test. A two-way ANOVA indicated a significant effect of genotype ($F_{1,39}$=47.76, p<0.0001) but no effect of treatment ($F_{1,39}$=0.1464, p=0.7041). **b)** Total time to complete 15 trials in the spontaneous alternation test. A two-way ANOVA indicated no differences in genotype or treatment. WT-H2O: n=11, CD-H2O: n=8, WT-EGCG: n=13 and CD-EGCG: n=12. Data are presented as the mean ± SEM. p values are shown with asterisks indicating values that are significantly different in a Two-way ANOVA with Bonferroni's *post hoc* test (***p<0.001, genotype effect).

EGCG does not influence sociability or anxiety-related behavior in CD mice

CD mice showed a hypersociable phenotype resembling the overfriendly and outgoing behavior towards strangers present in WBS individuals[27,28]. In addition, the performance of CD mice in the marble-burying test was completely different from WT, suggesting alterations related to anxiety-like behavior[28]. We wondered whether EGCG had any effect on sociability or

anxiety-like behavior. As shown in Figure 3a, CD mice fed with green tea explored significantly more time the container with the intruder mouse rather than the empty one (p<0.001), replicating the hypersociability previously obtained[28]. Regarding the marble-burying test, CD mice fed with EGCG performed as different from WT as mutant mice fed with water (Figure 3b). Therefore, these data indicate that EGCG does not influence sociability or anxiety-related behavior in CD mice, independently from the period in which EGCG was added to the water (Supplementary Figure S1).

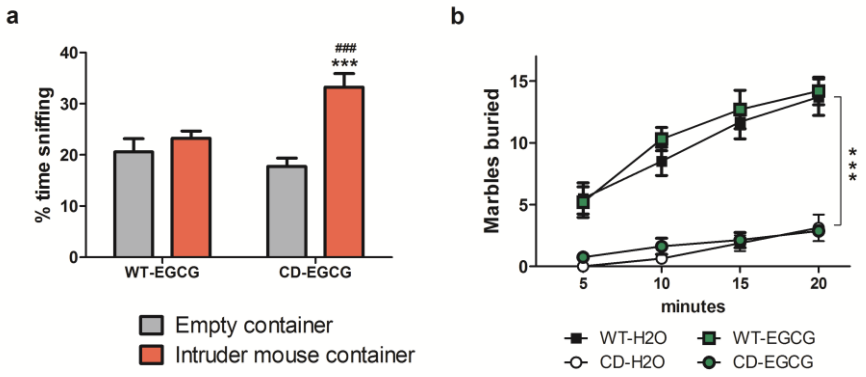

Figure 3. EGCG does not influence sociability or anxiety-related behavior in CD mice. **a)** In a direct social test, time spent exploring an empty container or an intruder mouse container was measured in WT (n=11) and CD (n=7) PC EGCG-fed mice. A two-way ANOVA indicated a significant interaction between genotypes and container ($F_{1,32}$=8.345, p=0.0069), with a significant effect of container ($F_{1,32}$=16.65, p=0.0003). Container effect ***p<0.001; Genotype effect ###p<0.001. **b)** Anxiety-like behavior was evaluated in the marble-burying test. The number of buried marbles was counted every 5 minutes during 20 minutes in WT (n=10) and CD (n=8) water fed (H2O) and in WT (n=10) and CD (n=8) PC EGCG-fed mice. A three-way ANOVA revealed no interaction between genotype, treatment and time ($F_{3,128}$=1.380, p=0.252), with a significant main effect of genotype ($F_{1,128}$=306.684, p<0.0001) and time ($F_{3,128}$=17.170, p<0.0001) but no effect of treatment ($F_{1,128}$=0.182, p=0.670). The same results were obtained when treatment was started at P8 or at adulthood (Supplementary Figure S1). Data are presented as the mean ± SEM. p values are shown with asterisks or hashes indicating values that are significantly different (Two-way or three-way ANOVA with Bonferroni's *post hoc* test). ***p<0.001

EGCG does not affect neuroanatomical features of CD mice

As it was previously described[27], brain weight of water-fed CD mice was significantly reduced when compared to water-fed WT mice (12.74%). Brains from CD mice fed with green tea were also smaller when compared to WT mice (Figure 4a), either the treatment was started at adulthood, P8 or PC (11.47%, 15.25% and 15.45%, respectively) (Supplementary Figure 2a). Therefore, the diet did not induce differences in the total brain weight in CD mice. In contrast, EGCG treatment in WT mice decreased the weight of the brain, reaching significance when treatment was started at P8 (Supplementary Figure S2b).

Next, we focused on the hippocampus, the main brain structure involved in learning and memory. Previous studies in CD mice showed a reduction in dendritic length in both SR and SO in the CA1 region of the hippocampus[27,28]. We could not appreciate any effect of EGCG on dendrite length in CD mice (Figure 4b and 4c, Supplementary Figure S3a and S3b). On the other hand, WT mice treated with EGCG presented a significant reduction in the length of dendrites in both SR and SO (Supplementary Figure S3c and S3d), consistent with the brain weight reduction. To explore the protective effect of EGCG on spine formation, the spine density in hippocampal CA1 neurons was measured. CD mice showed a significant reduction of about 17% in spine density in apical proximal dendrites of CA1 neurons[27,28]. In this study, CD mice fed with water did also show the reduction in spine density ($p<0.05$). EGCG treatment did not increase the number of spines present in apical dendrites of CD neurons (Figure 4d) either the treatment was started at adulthood, P8 or PC (Supplementary Figure S4a). Unexpectedly, WT animals treated with EGCG either at adulthood, P8 or PC, presented a reduction in spine density when compared to WT animals water-fed (Supplementary Figure S4b). Therefore, in light of these results it seems that EGCG not only does not have any protective effect in CD mice but also reduces the number of spines in apical dendrites of CA1 neurons in WT mice.

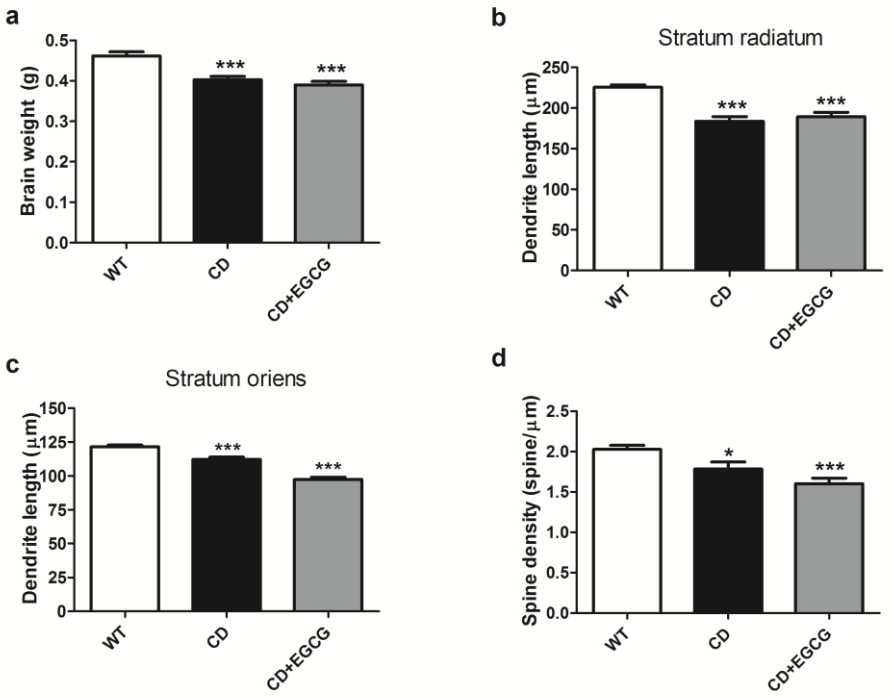

Figure 4. EGCG does not influence brain abnormalities present in CD mice. **a)** Total brain weight was determined after dissection in WT (n=8) and CD (n=8) water fed, and CD (n=7) PC EGCG-fed mice. **b and c)** Dendrite length of CA1 neurons was measured both in stratum radiatum and stratum oriens in all groups (n=25-30 neurons/mice, ≥3 mice/group). **d)** The number of spines was counted from 15–30 µm dendritic segments of randomly selected neurons. Spine density was calculated by dividing the total spine count by the length of dendrite analyzed (n=8-16 dendrites/mice, ≥3 mice/group). Data are presented as the mean ± SEM. *p* values are shown with asterisks indicating values that are significantly different from WT (One-way ANOVA with Dunnett's *post hoc* test). **p*<0.05, ****p*<0.001

Effect of EGCG on hippocampal synaptic plasticity markers

Synaptic plasticity is the main biological process involved in learning and memory. It has been linked to numerous molecular pathways and neurotrophic factors, such as BDNF[34–36]. Levels of *Bdnf* mRNA in hippocampus of CD mice were significantly reduced when compared to WT levels[28]. We compared *Bdnf* mRNA levels of hippocampus from CD mice with or without EGCG in the diet with WT mice. In line with previous results, CD

mice fed with water presented lower level of *Bdnf* in the hippocampus. Positively, this defect was completely corrected by EGCG treatment, since CD mice fed with green tea reached *Bdnf* levels similar to WT mice. Although the difference was not significant, we could also observe increased levels of *Bdnf* in WT mice fed with EGCG (Figure 5a).

The interaction of BDNF with its receptor activates the PI3K-Akt-mTor signaling pathway[37]. This pathway has been suggested to be deregulated in WBS, since the regulatory subunit of the PI3K (*Pik3r1*) is a direct target of GTF2I, a gene included in the deleted region in WBS[28,29]. In fact, CD mice presented upregulated levels of *Pik3r1* in the hippocampus[28]. We sought to determine if EGCG had any effect on this signaling pathway but, despite the recovery observed in the *Bdnf* levels in CD mice treated with EGCG, *Pi3kr1* levels remained as high as CD mice fed with water (Figure 5b). On the contrary, EGCG treatment in WT mice did show an effect, significantly increasing the levels of the *Pi3kr1* in these mice (Figure 5b) probably though the up-regulation of *Bdnf*. We could not observe any difference in the levels of the catalytic subunit of the PI3K (*Pi3kcb*) neither in WT nor CD mice with or without EGCG treatment (Figure 5c). BDNF regulates synaptic function by inducing local synthesis of numerous synaptic proteins, including LIMK1, in a PI3K/mTOR dependent mechanism[38,39]. *Limk1* is a gene included in the common deleted region of WBS, which is involved in neuronal development and brain function. Therefore, we studied the levels of *Limk1* in all groups of animals to see whether EGCG had any effect on its expression. *Limk1* levels of CD animals fed with water were reduced by ~ 50%, as expected for the genetic modification of the CD mouse. However, we could not appreciate any variation in *Limk1* levels in CD animals treated with EGCG. Interestingly, WT animals fed with green tea did show a significant increase in *Limk1* levels (Figure 5d), in line with the upregulation of *Bdnf* and *Pik3r1* levels.

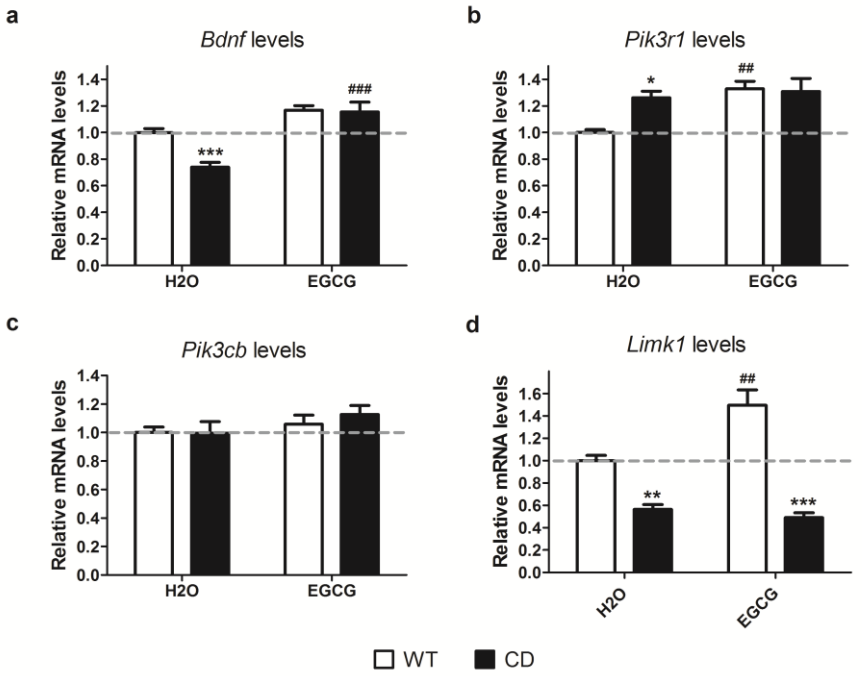

Figure 5. Effect of EGCG on hippocampal synaptic plasticity markers in WT and CD animals. *Bdnf*, *Pik3r1*, *Pik3cb* and *Limk1* mRNA levels were determined by quantitative PCR. **a)** *Bdnf* mRNA levels. A two-way ANOVA indicated a significant interaction between genotypes and treatment ($F_{1,41}$=5.732, p=0.0213), with a significant effect of genotype ($F_{1,41}$=6.863, p=0.0123) and treatment ($F_{1,41}$=31.69, p<0.0001). **b)** *Pik3r1* mRNA levels. A two-way ANOVA indicated a significant effect of treatment ($F_{1,24}$=7.406, p=0.0119). **c)** *Pik3cb* mRNA levels. A two-way ANOVA indicated no differences in genotype or treatment. **d)** *Limk1* mRNA levels. A two-way ANOVA indicated a significant interaction between genotypes and treatment ($F_{1,24}$=10.40, p=0.0036), with a significant effect of genotype ($F_{1,24}$=66.76, p<0.0001) and treatment ($F_{1,24}$=5.702, p=0.0252). n = 2–4 RT-PCRs/mice, ≥3 mice. Data are presented as the mean ± SEM. Genotype effect **p<0.05, **p<0.01 ***p<0.001; treatment effect ##p<0.01, ###p<0.001

DISCUSSION

WBS individuals present cognitive, behavioral and neurological problems that can affect their quality of life. In fact, independent living and competitive employment in WBS is not frequently reported[40]. Therefore, any potential

new treatment that has been shown to alleviate cognitive problems in other neurological disease with similar phenotypes deserves to be studied in WBS.

Natural polyphenols extracts have attracted increasing attention since they have shown diverse biological activities and health benefits in an increasing number of chronic pathological conditions, including cardiovascular, cancer, inflammatory and neurological diseases[1,2,6,18]. In Down syndrome, for instance, individuals treated with EGCG showed an improvement in visual memory recognition and spatial working memory[18]. Accordingly, administration of EGCG in different animal models of Down syndrome did also rescued hippocampal-dependent learning deficit[17,18]. In addition, EGCG improved cognitive function in other conditions, such as in alcohol-, oxidative stress-, age- or β-amyloid-mediated cognitive impairment[41–43]. Notably, administration of green tea ameliorated short-term memory deficits of CD mice in the NOR test. In contrast with another report[18], EGCG did not affect the performance of WT littermates in this test. It should be noted, though, that EGCG diet did not rescue the spatial working memory deficits observed in CD animals in the spontaneous alternation test. On the other hand, this study suggests that oral administration of EGCG, in the dose tested, does not have any effect on sociability or anxiety-related behavior in CD mice. To the best of our knowledge, no other reports have studied the influence of EGCG on sociability, but it has been proposed as an alternative therapeutic approach to treat anxiety, since it has been demonstrated to induce anxiolytic effects just like the benzodiazepine drug[44–46]. However, in these studies the doses of EGCG used were lower, and the effects on anxiety were studied in the elevated plus-maze test.

Studies using a favanol-rich diet have associated improvements in cognition with increases in neuronal spine density[47,48]. Van Praag *et al.* found that mice fed with (-)epicatechin presented improved spatial memory that was associated with increased angiogenesis and neuronal spine density[48]. In addition, EGCG *in vitro* demonstrated to promote neurite outgrowth[49]. We did not find any improvement with regards to spine density or dendrite length of CA1 neurons in CD animals. It should be mentioned, though, that we observed a visible effect of EGCG in WT mice: the diet induced a reduction of brain weight and a reduction in spine density and dendrite length of CA1 neurons. Ge *et al.* showed that EGCG had protective effects on spine

formation and maturation but only within a certain dose range: 10 and 25 mg/kg of EGCG reversed spine damage but not a dose of 50 mg/kg[47], pointing to a dose-dependent mechanism of EGCG. We used a higher dose of EGCG, since it has been proved to improve cognition in other genetic models[17,18]. Therefore, the dose used in this study could explain the lack of effects on neuroarchitecture in CD mice or even the deleterious effect of EGCG in WT mice. In fact, Guedj *et al.* did also report a reduction in the brain weight of WT animals fed with similar doses of green tea polyphenols[17]. Authors attributed this effect to a reduction in DYRK1A activity, one of the main genes involved in the cognitive phenotype of Down syndrome.

BDNF plays a critical role in learning and memory processes through regulation of synaptic plasticity mechanisms[50,51]. Indeed, genetically modified animals with decreased levels of BDNF are deficient in hippocampal-dependent behavioral tasks[35,52,53]. The interaction of BDNF with its receptor activates the PI3K/Akt/mTor signaling pathway[37]. *Gtf2i* and *Limk1* are genes located in the WBS region which have been related to the PI3K/Akt/mTOR pathway. GTF2I has been demonstrated to direct regulate the *Pik3r1* gene probably by inhibiting its expression[28,29]. On the other hand, LIMK1 has been reported to be upregulated in response to BDNF application in an mTOR-dependent mechanism[38]. Just as previously described[28], CD animals without treatment presented downregulated levels of *Bdnf* and upregulated levels of *Pik3r1*. In addition, CD levels of *Limk1* were downregulated, as expected for the genetic modification of the CD mouse. Several studies have supported the role of EGCG in the expression or activity of BDNF[17,54,55]. Moreover, EGCG has also been related to the PI3K pathway[9,13,14,56]. In accordance with these studies, WT animals fed with green tea did show an upregulation of *Bdnf*, *Pik3r1* and *Limk1* molecules. On the other hand, CD animals treated with EGCG completely recovered the levels of *Bdnf* in the hippocampus, suggesting either a direct or indirect effect of EGCG on the expression of *Bdnf*. However, despite the enhanced levels of *Bdnf* in CD animals fed with green tea, we could not appreciate any change in *Pik3r1* or *Limk1* levels in these mice. All together these results suggest that PI3K/Akt/mTOR pathway in CD mice might act independently of BDNF signaling and that the protective effects of EGCG on memory in CD animals might be linked to the modulation of BDNF and/or BDNF-related pathway other than the PI3K pathway.

In any case, these findings suggest that EGCG treatment can significantly improve short-term memory in CD mice, and this neuroprotective effect may be related to the upregulation of *Bdnf*, and/or modulation of BDNF-related pathways in the hippocampus. Therefore, EGCG may be a promising therapeutic agent for WBS individuals. Nevertheless, future research should be focused on the deep mechanism of EGCG, since despite encouraging results of this molecule in preclinical and clinical studies there are controversial results in some studies, probably due to differences in the dose, duration of treatment or route of administration of EGCG, or even differences in the behavioral tests used.

REFERENCES

1. Schneider, C and Segre, T (2009). Green tea: potential health benefits. *Am. Fam. Physician* **79**: 591–4.

2. Chacko, SM, Thambi, PT, Kuttan, R and Nishigaki, I (2010). Beneficial effects of green tea: a literature review. *Chin. Med.* **5**: 1–9.

3. Suganuma, M, Okabe, S, Oniyama, M, Tada, Y, Ito, H and Fujiki, H (1998). Wide distribution of [3H](-)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. *Carcinogenesis* **19**: 1771–6.

4. Nakagawa, K and Miyazawa, T (1997). Absorption and distribution of tea catechin, (-)-epigallocatechin-3-gallate, in the rat. *J. Nutr. Sci. Vitaminol. (Tokyo).* **43**: 679–84.

5. Levites, Y, Youdim, MBH, Maor, G and Mandel, S (2002). Attenuation of 6-hydroxydopamine (6-OHDA)-induced nuclear factor-kappaB (NF-kappaB) activation and cell death by tea extracts in neuronal cultures. *Biochem. Pharmacol.* **63**: 21–9.

6. Dragicevic, N, Smith, A, Lin, X, Yuan, F, Copes, N, Delic, V, *et al.* (2011). Green tea epigallocatechin-3-gallate (EGCG) and other flavonoids reduce Alzheimer's amyloid-induced mitochondrial dysfunction. *J. Alzheimers. Dis.* **26**: 507–21.

7. Mandel, S, Weinreb, O, Amit, T and Youdim, MBH (2004). Cell signaling pathways in the neuroprotective actions of the green tea polyphenol (-)-epigallocatechin-3-gallate: implications for neurodegenerative diseases. *J. Neurochem.* **88**: 1555–1569.

8. Schroeter, H, Boyd, C, Spencer, JPE, Williams, RJ, Cadenas, E and Rice-Evans, C. MAPK signaling in neurodegeneration: influences of flavonoids and of nitric oxide. *Neurobiol. Aging* **23**: 861–80.

9. Kim, H and Sakamoto, K (2012). (-)-Epigallocatechin gallate suppresses adipocyte differentiation through the MEK/ERK and PI3K/Akt pathways. *Cell Biol. Int.* **36**: 147–53.

10. Levites, Y, Amit, T, Mandel, S and Youdim, MBH (2003). Neuroprotection and neurorescue against Abeta toxicity and PKC-dependent release of nonamyloidogenic soluble precursor protein by green tea polyphenol (-)-epigallocatechin-3-gallate. *FASEB J.* **17**: 952–4.

11. Menard, C, Bastianetto, S and Quirion, R (2013). Neuroprotective effects of resveratrol and epigallocatechin gallate polyphenols are mediated by the activation of protein kinase C gamma. *Front. Cell. Neurosci.* **7**: 281.

12. Ortiz-López, L, Márquez-Valadez, B, Gómez-Sánchez, A, Silva-Lucero, M del C, Torres-Pérez, M, Téllez-Ballesteros, RI, *et al.* (2016). Green tea compound epigallo-cathechin-3-gallate (EGCG) increases neuronal survival in adult hippocampal neurogenesis in vivo and in vitro. *Neuroscience* **322**: 208–220.

13. Jang, S, Jeong, H-S, Park, J-S, Kim, Y-S, Jin, C-Y, Seol, MB, *et al.* (2010). Neuroprotective effects of (-)-epigallocatechin-3-gallate against quinolinic acid-induced excitotoxicity via PI3K pathway and NO inhibition. *Brain Res.* **1313**: 25–33.

14. Zhang, X, He, F, Yang, J and Chen, Z-S (2015). Protective effects of epigallocatechin-3-gallate on intestinal ischemia reperfusion injury through enhanced activation of PI3K/Akt pathway in rats. *J. Huazhong Univ. Sci. Technolog. Med. Sci.* **35**: 378–83.

15. Xie, W, Ramakrishna, N, Wieraszko, A and Hwang, Y-W (2008). Promotion of neuronal plasticity by (-)-epigallocatechin-3-gallate. *Neurochem. Res.* **33**: 776–83.

16. Wang, Y, Li, M, Xu, X, Song, M, Tao, H and Bai, Y (2012). Green tea epigallocatechin-3-gallate (EGCG) promotes neural progenitor cell proliferation and sonic hedgehog pathway activation during adult hippocampal neurogenesis. *Mol. Nutr. Food Res.* **56**: 1292–303.

17. Guedj, F, Sébrié, C, Rivals, I, Ledru, A, Paly, E, Bizot, JC, *et al.* (2009). Green tea polyphenols rescue of brain defects induced by overexpression of DYRK1A. *PLoS One* **4**: e4606.

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

19. Bayés, M, Magano, LF, Rivera, N, Flores, R and Pérez Jurado, LA (2003). Mutational mechanisms of Williams-Beuren syndrome deletions. *Am. J. Hum. Genet.* **73**: 131–51.

20. Pérez-García, D, Flores, R, Brun-Gasca, C and Pérez-Jurado, L a (2014). Lateral preference in Williams-Beuren syndrome is associated with cognition and language. *Eur. Child Adolesc. Psychiatry*doi:10.1007/s00787-014-0652-6.

21. Martens, M a, Wilson, SJ and Reutens, DC (2008). Research Review:

Williams syndrome: a critical review of the cognitive, behavioral, and neuroanatomical phenotype. *J. Child Psychol. Psychiatry.* **49**: 576–608.

22. Mervis, CB and Klein-Tasman, BP (2000). Williams syndrome: cognition, personality, and adaptive behavior. *Ment. Retard. Dev. Disabil. Res. Rev.* **6**: 148–58.

23. Mervis, CB, Robinson, BF, Bertrand, J, Morris, C a., Klein-Tasman, BP and Armstrong, SC (2000). The Williams Syndrome Cognitive Profile. *Brain Cogn.* **44**: 604–628.

24. Meyer-Lindenberg, A, Mervis, CB and Berman, KF (2006). Neural mechanisms in Williams syndrome: a unique window to genetic influences on cognition and behaviour. *Nat. Rev. Neurosci.* **7**: 380–93.

25. Bellugi, U, Lichtenberger, L, Jones, W, Lai, Z and St George, M (2000). I. The neurocognitive profile of Williams Syndrome: a complex pattern of strengths and weaknesses. *J. Cogn. Neurosci.* **12 Suppl 1**: 7–29.

26. Doyle, TF, Bellugi, U, Korenberg, JR and Graham, J (2004). 'Everybody in the world is my friend' hypersociability in young children with Williams syndrome. *Am. J. Med. Genet. A* **124A**: 263–73.

27. Segura-Puimedon, M, Sahún, I, Velot, E, Dubus, P, Borralleras, C, Rodrigues, AJ, *et al.* (2014). Heterozygous deletion of the Williams-Beuren syndrome critical interval in mice recapitulates most features of the human disorder. *Hum. Mol. Genet.* **23**: 6481–94.

28. Borralleras, C, Sahun, I, Pérez-Jurado, L a and Campuzano, V (2015). Intracisternal Gtf2i Gene Therapy Ameliorates Deficits in Cognition and Synaptic Plasticity of a Mouse Model of Williams-Beuren Syndrome. *Mol. Ther.* **23**: 1691–9.

29. Segura-Puimedon, M, Borralleras, C, Pérez-Jurado, L a. and Campuzano, V (2013). TFII-I regulates target genes in the PI-3K and TGF-β signaling pathways through a novel DNA binding motif. *Gene* **527**: 529–536.

30. Feng, G, Mellor, RH, Bernstein, M, Keller-Peck, C, Nguyen, QT, Wallace, M, *et al.* (2000). Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* **28**: 41–51.

31. Hammond, RS, Tull, LE and Stackman, RW (2004). On the delay-dependent involvement of the hippocampus in object recognition memory. *Neurobiol. Learn. Mem.* **82**: 26–34.

32. Wang, D, Noda, Y, Zhou, Y, Mouri, A, Mizoguchi, H, Nitta, A, *et al.* (2007). The allosteric potentiation of nicotinic acetylcholine receptors by galantamine ameliorates the cognitive dysfunction in beta amyloid25-35 i.c.v.-injected mice: involvement of dopaminergic systems. *Neuropsychopharmacology* **32**: 1261–71.

33. Deacon, RMJ and Rawlins, JNP (2006). T-maze alternation in the rodent. *Nat. Protoc.* **1**: 7–12.

34. Linnarsson, S, Björklund, A and Ernfors, P (1997). Learning deficit in BDNF

mutant mice. *Eur. J. Neurosci.* **9**: 2581–7.

35. Korte, M, Carroll, P, Wolf, E, Brem, G, Thoenen, H and Bonhoeffer, T (1995). Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proc. Natl. Acad. Sci. U. S. A.* **92**: 8856–8860.

36. Hall, J, Thomas, KL and Everitt, BJ (2000). Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. *Nat. Neurosci.* **3**: 533–5.

37. Troca-Marín, JA, Alves-Sampaio, A and Montesinos, ML (2011). An increase in basal BDNF provokes hyperactivation of the Akt-mammalian target of rapamycin pathway and deregulation of local dendritic translation in a mouse model of Down's syndrome. *J. Neurosci.* **31**: 9445–55.

38. Schratt, GM, Tuebing, F, Nigh, E a, Kane, CG, Sabatini, ME, Kiebler, M, *et al.* (2006). A brain-specific microRNA regulates dendritic spine development. *Nature* **439**: 283–289.

39. Schratt, GM (2004). BDNF Regulates the Translation of a Select Group of mRNAs by a Mammalian Target of Rapamycin-Phosphatidylinositol 3-Kinase-Dependent Pathway during Neuronal Development. *J. Neurosci.* **24**: 7366–7377.

40. Morris, CA, Demsey, SA, Leonard, CO, Dilts, C and Blackburn, BL (1988). Natural history of Williams syndrome: physical characteristics. *J. Pediatr.* **113**: 318–26.

41. Tiwari, V, Kuhad, A and Chopra, K (2010). Epigallocatechin-3-gallate ameliorates alcohol-induced cognitive dysfunctions and apoptotic neurodegeneration in the developing rat brain. *Int. J. Neuropsychopharmacol.* **13**: 1053–66.

42. Fukui, K, Omoi, N-O, Hayasaka, T, Shinnkai, T, Suzuki, S, Abe, K, *et al.* (2002). Cognitive impairment of rats caused by oxidative stress and aging, and its prevention by vitamin E. *Ann. N. Y. Acad. Sci.* **959**: 275–84.

43. Rezai-Zadeh, K, Arendash, GW, Hou, H, Fernandez, F, Jensen, M, Runfeldt, M, *et al.* (2008). Green tea epigallocatechin-3-gallate (EGCG) reduces beta-amyloid mediated cognitive impairment and modulates tau pathology in Alzheimer transgenic mice. *Brain Res.* **1214**: 177–87.

44. Vignes, M, Maurice, T, Lanté, F, Nedjar, M, Thethi, K, Guiramand, J, *et al.* (2006). Anxiolytic properties of green tea polyphenol (-)-epigallocatechin gallate (EGCG). *Brain Res.* **1110**: 102–15.

45. Adachi, N, Tomonaga, S, Tachibana, T, Denbow, DM and Furuse, M (2006). (-)-Epigallocatechin gallate attenuates acute stress responses through GABAergic system in the brain. *Eur. J. Pharmacol.* **531**: 171–5.

46. Park, K-S, Oh, JH, Yoo, H-S, Lee, Y-M, Lee, M-K, Hong, JT, *et al.* (2010). (-)-Epigallocatechin-3-O-gallate (EGCG) reverses caffeine-induced anxiogenic-like effects. *Neurosci. Lett.* **481**: 131–4.

47. Ge, M-M, Hu, F, Lou, Z-Y, Xue, W, Yu, H, Xu, L, *et al.* (2015). Role of Wnt/β-

catenin signaling in the protective effect of epigallocatechin-3-gallate on lead-induced impairments of spine formation in the hippocampus of rats. *RSC Adv.* **5**: 31622–31628.

48. Praag, H Van, Lucero, MJ, Yeo, GW, Stecker, K, Heivand, N, Zhao, C, *et al.* (2007). Plant-Derived Flavanol ( ) Epicatechin Enhances Angiogenesis and Retention of Spatial Memory in Mice **27**: 5869–5878.

49. Reznichenko, L, Amit, T, Youdim, MBH and Mandel, S (2005). Green tea polyphenol (-)-epigallocatechin-3-gallate induces neurorescue of long-term serum-deprived PC12 cells and promotes neurite outgrowth. *J. Neurochem.* **93**: 1157–67.

50. Yamada, K, Mizuno, M and Nabeshima, T (2002). Role for brain-derived neurotrophic factor in learning and memory. *Life Sci.* **70**: 735–44.

51. Cunha, C, Brambilla, R and Thomas, KL (2010). A simple role for BDNF in learning and memory? *Front. Mol. Neurosci.* **3**: 1.

52. Monteggia, LM, Barrot, M, Powell, CM, Berton, O, Galanis, V, Gemelli, T, *et al.* (2004). Essential role of brain-derived neurotrophic factor in adult hippocampal function. *Proc. Natl. Acad. Sci. U. S. A.* **101**: 10827–32.

53. Korte, M, Staiger, V, Griesbeck, O, Thoenen, H and Bonhoeffer, T (1996). The involvement of brain-derived neurotrophic factor in hippocampal long-term potentiation revealed by gene targeting experiments. *J. Physiol. Paris* **90**: 157–64.

54. Tian, W, Han, X-G, Liu, Y-J, Tang, G-Q, Liu, B, Wang, Y-Q, *et al.* (2013). Intrathecal epigallocatechin gallate treatment improves functional recovery after spinal cord injury by upregulating the expression of BDNF and GDNF. *Neurochem. Res.* **38**: 772–9.

55. Gundimeda, U, McNeill, TH, Fan, TK, Deng, R, Rayudu, D, Chen, Z, *et al.* (2014). Green tea catechins potentiate the neuritogenic action of brain-derived neurotrophic factor: role of 67-kDa laminin receptor and hydrogen peroxide. *Biochem. Biophys. Res. Commun.* **445**: 218–24.

56. Van Aller, GS, Carson, JD, Tang, W, Peng, H, Zhao, L, Copeland, R a, *et al.* (2011). Epigallocatechin gallate (EGCG), a major component of green tea, is a dual phosphoinositide-3-kinase/mTOR inhibitor. *Biochem. Biophys. Res. Commun.* **406**: 194–9.

SUPPLEMENTARY MATERIAL

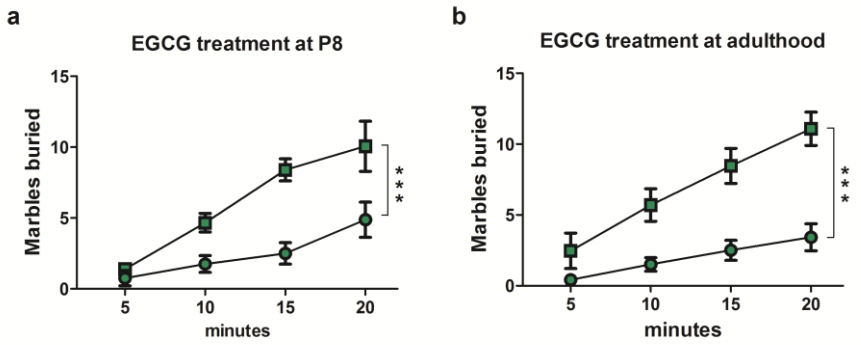

Figure S1. EGCG effect on anxiety-related behavior in CD mice. Anxiety-like behavior was evaluated in the marble-burying test either when EGCG treatment was started at P8 **(a)** or at adulthood **(b)**. The number of buried marbles was counted every 5 minutes during 20 minutes in WT (n=15) and CD (n=8) EGCG-fed at P8 and in WT (n=13) and CD (n=12) EGCG-fed at adulthood. Data are presented as the mean ± SEM. *p* values are shown with asterisks indicating values that are significantly different (General linear models of repeated measures analysis of variance). ***$p<0.001$

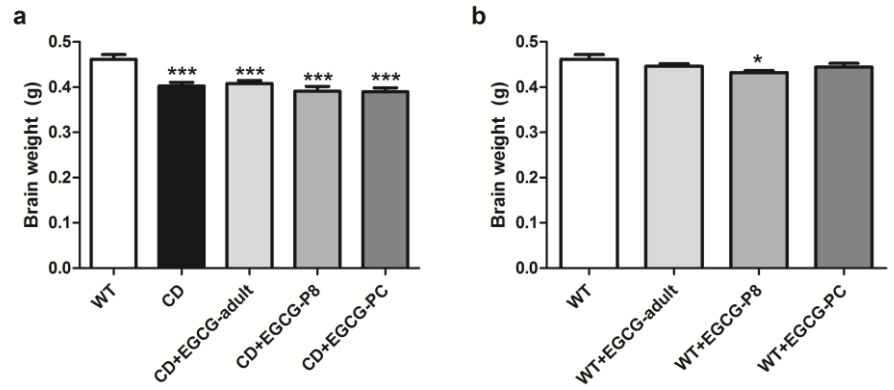

Figure S2. Effect of EGCG on brain weight. **a)** Total brain weight was determined after dissection in WT (n=8) and CD (n=8) water fed, CD (n=12) adulthood EGCG-fed mice, CD (n=11) P8 EGCG-fed mice, and CD (n=7) PC EGCG-fed mice. **b)** Total brain weight was determined after dissection in WT (n=8) water fed, WT (n=13) adulthood EGCG-fed mice, WT (n=19) P8 EGCG-fed mice, and WT (n=11) PC EGCG-fed mice. Data are presented as the mean ± SEM. *p* values are shown with asterisks indicating values that are significantly different from WT (One-way ANOVA with Dunnett's *post hoc* test). *$p<0.05$,***$p<0.001$

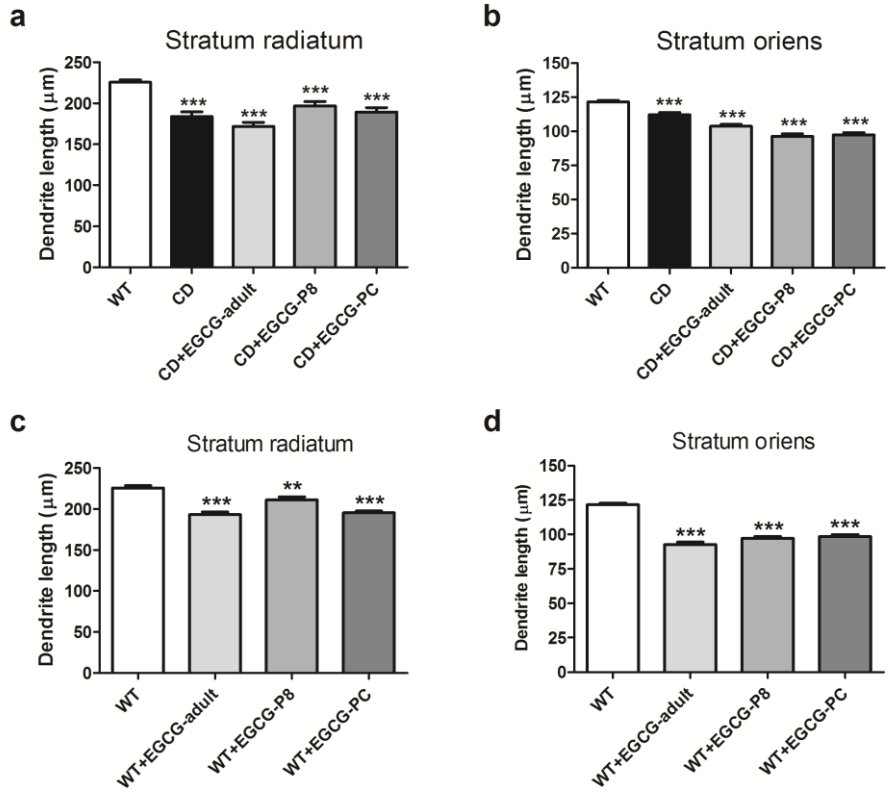

Figure S3. Effect of EGCG on dendrite length of CA1 neurons. **a-d)** Dendrite length of CA1 neurons was measured both in stratum radiatum and stratum oriens in all groups (n=25-30 neurons/mice, ≥3 mice/group). Data are presented as the mean ± SEM. *p* values are shown with asterisks indicating values that are significantly different from WT (One-way ANOVA with Dunnett's *post hoc* test). $^{**}p<0.01$,$^{***}p<0.001$

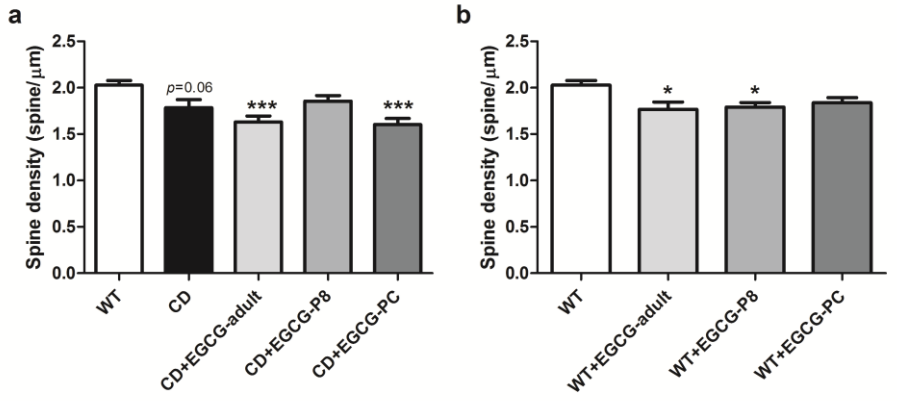

Figure S4. EGCG effects on spine density. **a and b)** The number of spines was counted from 15–30 µm dendritic segments of randomly selected neurons. Spine density was calculated by dividing the total spine count by the length of dendrite analyzed (n=8-16 dendrites/mice, ≥3 mice/group). Data are presented as the mean ± SEM. *p* values are shown with asterisks indicating values that are significantly different from WT (One-way ANOVA with Dunnett's *post hoc* test). **p*<0.05, ****p*<0.001

CHAPTER 5

Studying the involvement of the PI3K/Akt/mTOR pathway in Williams-Beuen syndrome mouse hippocampal primary cultures

Cristina Borralleras, Luis A. Pérez-Jurado, Victoria Campuzano

In preparation

ABSTRACT

The PI3K/Akt/mTOR signaling pathway has a relevant role in synaptic plasticity and dendritic morphology, and its dysregulation has been associated with several neurological diseases. The molecular alterations reported so far in the complete deletion (CD) mouse model of Williams-Beuren syndrome (WBS) point to a possible dysregulation of the PI3K pathway in the hippocampus. *In vitro* models, such as primary neuronal cultures, might provide important insights into the underlying mechanisms of a disease and represent a useful tool for the screening of potential pharmacological agents. Therefore, in this study we used hippocampal primary cultures from CD mice to investigate the activity of the PI3K/Akt/mTOR pathway in this model. We performed immunocytofluorescence analyses to assess the activity of several targets of the PI3K pathway. The results obtained suggest that the PI3K/Akt/mTOR signaling pathway is not altered in WBS hippocampal cultures and, hence, emphasizes the need for further research on *in vivo* models and on other potential cellular mechanisms of WBS.

INTRODUCTION

In neurons, brain-derived neurotrophic factor (BDNF) is a key upstream activator of the PI3K/Akt/mTOR signaling pathway. Among many cellular processes, this pathway regulates axon guidance, dendritic tree development, dendritic spine morphology and several forms of synaptic plasticity[1–3]. Involvement of PI3K/Akt/mTOR dysregulation in several human diseases, including different neurodevelopmental and neurodegenerative diseases, has become evident in the recent years[4,5]. For instance, hyperactivation of this pathway has been reported in mouse models of tuberous sclerosis, Down syndrome or Fragile syndrome[6–8]. On the other hand, the results obtained on another mouse model of intellectual disability, Rett syndrome, indicated reduced PI3K/Akt/mTOR pathway and protein synthesis dysregulation[9].

PI3K/Akt/mTOR pathway represents an interesting candidate that could be involved in the neuronal pathology of Williams-Beuren syndrome (WBS). WBS is caused by a heterozygous deletion of 26-28 contiguous genes on chromosome band 7q11.23[10] and is characterized by a distinctive neurocognitive profile, which has attracted special attention during the last decades. Individuals with WBS are in the mild to moderate intellectual disability range, and present a specific pattern of strengths and weaknesses, including relative strengths in selected aspects of language, combined with deficiency in visuospatial skills[11,12]. Most of patients are highly social and empathic and present excessive worry and fears, resulting in anxiety, preoccupations or obsessions[13]. Different animal models have been established to study WBS, most of them focusing on characterizing the behavioral phenotype along with its brain anatomical correlates[14–16]. Single gene mouse models have been useful to provide information about the function of an individual gene[17–21], and partial deletion mouse models have helped to gain insight into the specific contribution of different subsets of genes[15,22]. However, data on potential cellular mechanisms are scarce.

The complete deletion (CD) mouse model is the unique animal model which mimics the most common deletion found in patients, and it recapitulates most physical and cognitive features present in individuals with WBS[14]. These mice displayed significantly reduced long-term potentiation (see Chapter 2),

one of the main mechanisms underlying synaptic plasticity. In addition, CD mice present low levels of *Bdnf* in hippocampus, pointing to an alteration in BDNF-related pathways[16]. *Gtf2i* is a gene located on the region commonly deleted in WBS individuals and it has been reported to have a role in the PI3K/Akt/mTOR pathway[23,24]. In fact, it has been shown that GTF2I binds directly to the *Pik3r1* promoter in mouse embryonic fibroblasts[24]. Its effect on *Pik3r1* seems to be inhibitory, since the *Gtf2i* deletion lead to *Pik3r1* upregulation[16,24]. Therefore, the aim of this study was to perform a first approach to the activity of the PI3K/Akt/mTOR signaling pathway in the CD model to evaluate the possible involvement of this pathway in the cognitive impairment seen in CD mice. We used primary hippocampal neurons, since they have been widely used for defining the molecular mechanisms underlying other diseases. The results obtained suggest that PI3K/Akt/mTOR signaling pathway is not altered in WBS hippocampal cultures and, hence, it emphasizes the need for further research on *in vivo* models and on other potential cellular mechanisms of WBS.

MATERIALS AND METHODS

Primary mouse hippocampal neurons

E18-19 embryos were obtained from crosses between wild-type (WT) and CD mice and their brains were removed in HBSS medium (Invitrogen). A piece of tail tissue was taken for genotyping and each embryo was handled individually. Using a dissecting microscope, the meninges of the cerebral hemispheres were removed and the hippocampus was dissected. After digesting the hippocampus for 17 minutes at 37ºC in trypsin 0.05%, cells were mechanically dissociated and plated at a density of $1x10^5$ cells/well in poly-L-lysine-treated coverslips in 24 well plates with DMEM containing 1x antibiotics (Penicillin-Streptomycin) and 10% FBS. After two hours the medium was replaced by Neurobasal medium with 2% B27 (Invitrogen), 1x antibiotics (Penicillin-Streptomycin) and 1% L-Glutamine. On day 3 of culture, arabinoside (AraC) was added to the culture medium for 24 hours to eliminate proliferating non-neuronal cells. Cultures were used for the experiments at day in vitro (DIV) 12-14.

Immunocytofluorescence

Neurons were fixed in 4% paraformaldehyde in PBS, washed three times in PBS and blocked for 30 minutes at 37ºC in blocking solution (0.1% Triton X-100 and 10% fetal bovine serum in PBS). Cells were incubated overnight at 4ºC with the corresponding primary antibodies diluted in the same blocking buffer [Polyclonal antibiodies: BDNF (1:500, Santa Cruz Biotechnology), phospho-mTOR (Ser2448) and phospho-mTOR (Ser2481) (1:200, Millipore), mTOR (1:100, Millipore), p70S6K (1:250, Millipore), and GLUR1 (1:500, Millipore). Monoclonal antibody: MAP2 (1:1000, Sigma-Aldrich)]. Cells were then washed three times in PBS at room temperature and incubated in secondary antibodies in 3% fetal bovine serum in PBS for 1 hour at room temperature in the dark (Alexa Fluor 555 goat anti-mouse IgG (H+L) and Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Invitrogen) at concentration of 1:1000). After washing cells twice in PBS, they were incubated for 15 minutes in Hoechst33342 for DNA staining. Cells were mounted on a glass slide in a drop of Mowiol after washing twice in PBS, and were allowed to dry overnight before imaging.

Imaging

Images were acquired on a Leika epifluorescence microscopy at 100x magnification. For each tested antibody, at least three hippocampal WT and CD cultures were analyzed. In the figure legends, N represents the number of neurons measured per genotype. ImageJ was used to quantify images. First, on the MAP2 image (red channel) we manually delineated and eliminated the somatic region and then, the MAP2 immunofluorescence signal of dendrites was automatically detected using the color threshold function. We then applied the resulting mask to the green channel to determine the mean grey value of the corresponding region.

Statistical analyses

All data are presented as means ± SEM. We used an unpaired *t* test to compare between the two genotypes. Values were considered significant when $p<0.05$. GraphPad Prism software was used for all statistical tests and graphs.

RESULTS

Number of dendrites and soma size of CD neurons are not altered

The PI3K/Akt/mTOR pathway regulates the number of dendrites as well as dendritic complexity. In addition, hyperactivation of the PI3K pathway has been related with an increase in cell soma size[1]. The number of primary dendrites (defined as number of dendrites directly emanating from the soma) and the cell soma size (measured by the area of the cell body) were measured in CD neurons and they were unchanged with respect to WT neurons (p=0.8977 and p=0.2946, respectively. Figure 1).

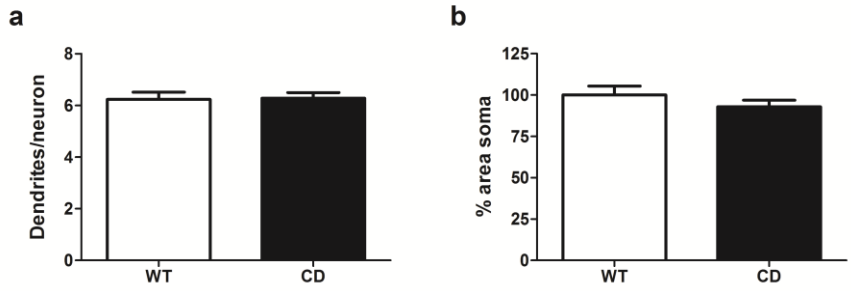

Figure 1. Number of dendrites and soma size are not altered in CD neurons. **a)** The number of primary dendrites (defined as number of dendrites directly emanating from the soma) was measured (WT: 6.234 ± 0.281, n=47; CD: 6.280 ± 0.223, n=50). **b)** Cell soma size (measured by area of the cell body) was measured (WT: 100.00 ± 5.439, n=47; CD: 92.87 ± 4.108, n=50). Data are presented as the mean ± SEM. An unpaired t-test was used to test for statistical significance.

CD hippocampal neurons do not show a reduction in BDNF levels

BDNF is a key upstream activator of several parallel signaling pathways, including the PI3K/Akt/mTOR pathway, through which it regulates the local translation of a series of mRNA involved in synaptic plasticity[25]. Expression analyses in hippocampal tissue of CD animals showed significantly decreased levels of *Bdnf* mRNA in the hippocampus[16]. We therefore sought to determine the levels of BDNF protein in hippocampal primary neurons of

CD mice. We failed to detect differences in the hippocampal BDNF levels between WT and CD neurons (p=0.2663, Figure 2).

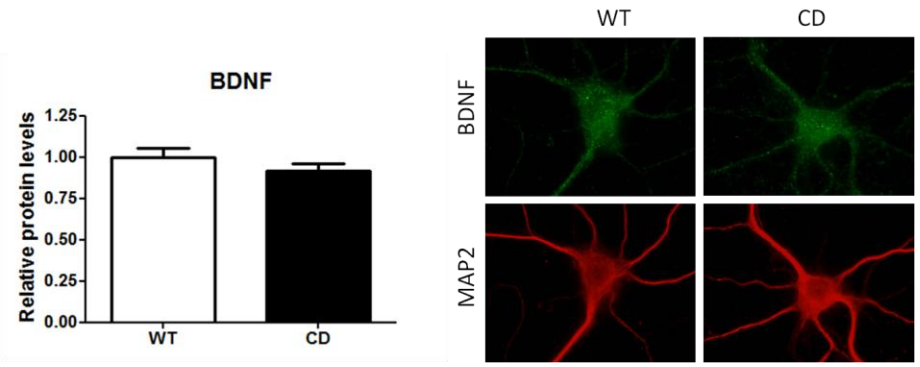

Figure 2. BDNF levels are not altered in CD hippocampal neurons. Immunofluorescence detection of BDNF proteins did not show any difference between WT and CD neurons (WT: 1.000 ± 0.057, n=17; CD: 0.918 ± 0.042, n=15). Data are presented as the mean ± SEM. An unpaired *t* test was used to test for statistical significance.

mTOR activity is not altered in primary hippocampal neurons of CD mice

mTOR is an important downstream target of the PI3K/Akt pathway and it regulates numerous cellular mechanisms by controlling regulation of protein synthesis[26]. We examined the phosphorylation state of the mTOR protein, since it is a biochemical indicator of its activation. mTOR can be phosphorylated at Ser2448 via the PI3K/Akt signaling pathway and autophosphorylated at Ser2481[26–28]. Therefore, we examined the levels of both phosphorylations. We could not observe any differences in the amount of phospho-mTOR(Ser2448) or phospho-mTOR(Ser2481) between WT and CD animals (p=0.5579 and p=0.9527, respectively. Figure 3a and 3b). We next examined total mTOR protein. CD animals presented a reduction of 15% in the total amount of protein, but this difference was not statistically significant (p=0.0965, Figure 3c).

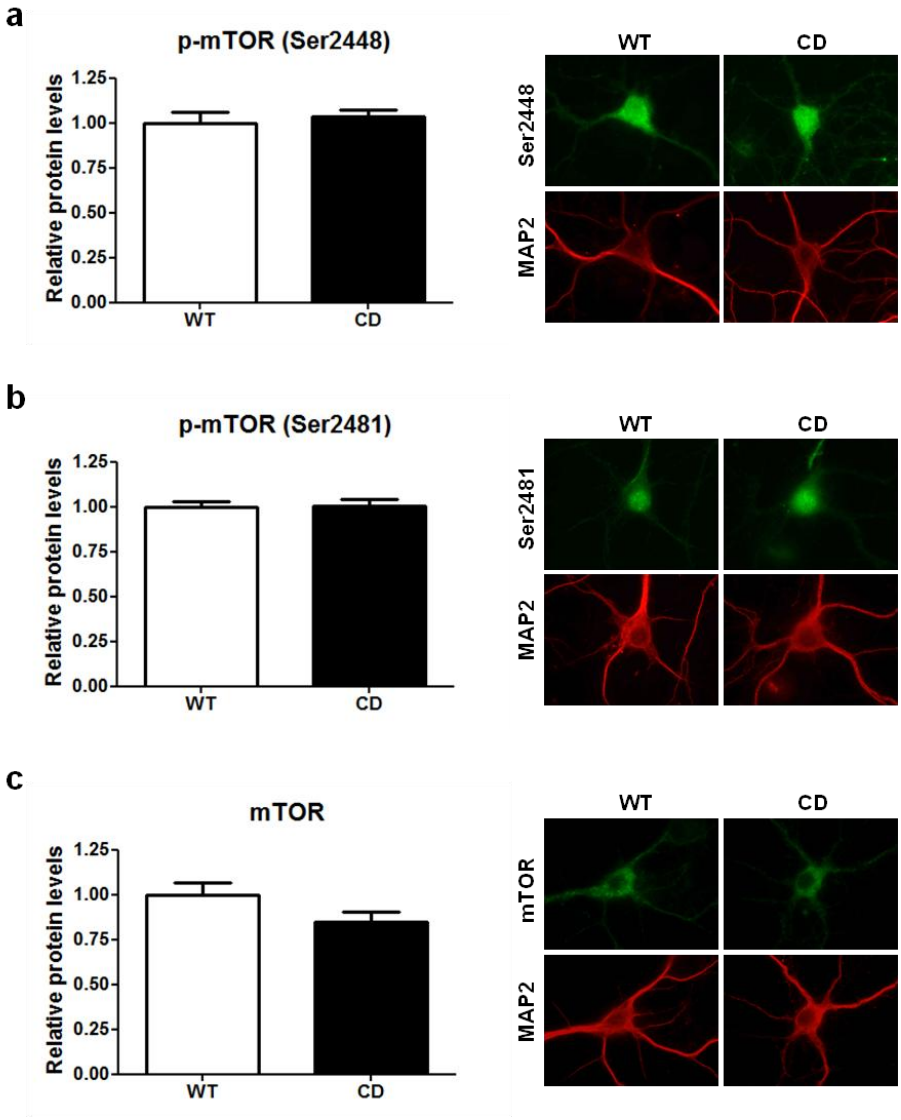

Figure 3. mTOR levels are not altered in CD hippocampal neurons. **a)** Immunofluorescence detection of phospho-mTOR(Ser2448) did not show any difference between WT and CD neurons (WT: 1.000 ± 0.061, n=14; CD: 1.038 ± 0.032, n=19). **b)** Immunofluorescence detection of phospho-mTOR(Ser2481) did not show any difference between WT and CD neurons (WT: 1.000 ± 0.032, n=15; CD: 1.003 ± 0.039, n=22). **c)** Immunofluorescence detection of total mTOR protein did not show any difference between WT and CD neurons (WT: 1.000 ± 0.069, n=15; CD: 0.849 ± 0.054, n=15). Data are presented as the mean ± SEM. An unpaired *t* test was used to test for statistical significance.

Downstream targets of mTOR signaling are not altered in CD neurons

To further analyze the activity of the PI3K pathway, we analyzed the levels of phospho-p70 S6 kinase (phospho-p70S6K), an important regulator of protein synthesis, since its main target is the 40S ribosomal protein S6, a major component of the machinery involved in protein synthesis[29]. Levels of phospho-p70S6K (Thr412/Thr389) in CD neurons were comparable to that of WT neurons (p=0.210, Figure 4a). Finally, we analyzed the levels of GluR1, a protein that has been shown to be locally translated in dendrites regulated by BDNF through the PI3K/Akt/mTOR pathway[30]. In line with the other results, levels of GluR1 in CD dendrites were unchanged (p=0.0611, Figure 4b).

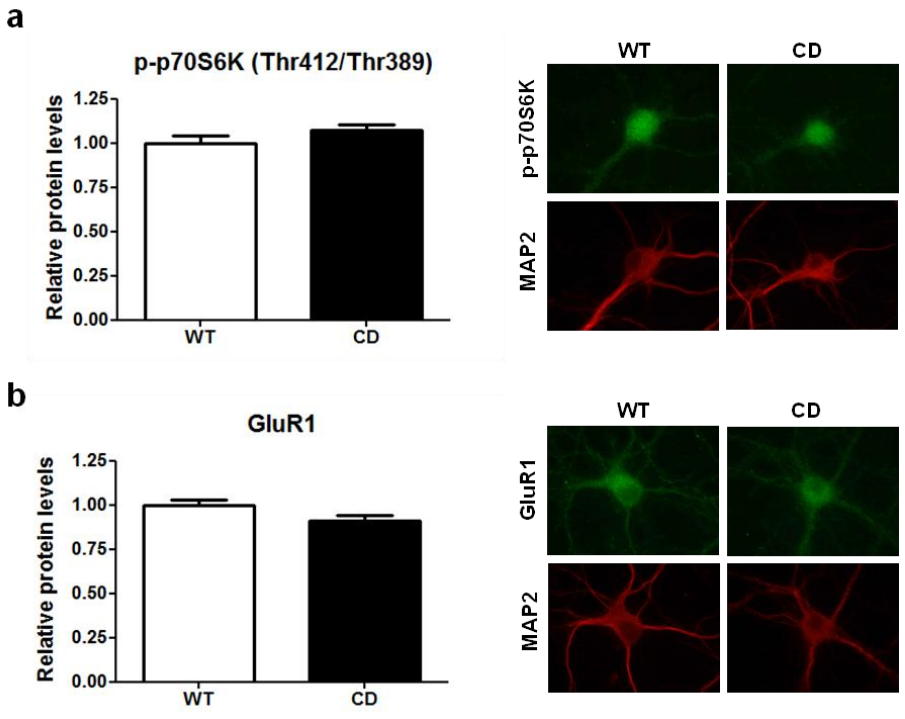

Figure 4. Downstream targets of mTOR signaling are not altered in CD hippocampal neurons. **a)** Immunofluorescence detection of phospho-p70S6K(Thr412/Thr389) did not show any difference between WT and CD neurons (WT: 1.000 ± 0.044, n=15; CD: 1.071 ± 0.034, n=20). **b)** Immunofluorescence detection of GluR1 did not show any difference between WT and CD neurons (WT: 1.000 ± 0.031, n=13; CD: 0.9113 ± 0.031, n=19). Data are presented as the mean ± SEM. An unpaired *t* test was used to test for statistical significance.

DISCUSSION

In vitro models, such as primary neuronal cultures, might provide important insights into the underlying mechanisms of a disease and represent a useful tool for the screening of potential pharmacological agents. In this study, we used hippocampal primary cultures to study the activity of the PI3K/Akt/mTOR pathway in the CD model and we did not find alteration in any of the targets tested.

Growing evidence indicates that dysfunction of the PI3K/Akt/mTOR pathway is associated with several neurological diseases, such as Fragile X syndrome, Down syndrome and Rett's syndrome[7–9]. In addition to alterations in this pathway, structural abnormalities of dendrites and spines are also a common feature in these diseases[31–33]. Accordingly, mouse models display alterations in synaptic plasticity which correlate with cognitive impairment[34–36]. Regarding WBS, CD animals do also present structural abnormalities of dendrites and decreased spine density in CA1 pyramidal neurons, which correlate with synaptic plasticity impairment and deficits in cognition[14,16,chapter2]. The molecular findings reported so far in WBS pointed to a possible dysregulation of the PI3K pathway (Figure 5)[16,23,24]. The upstream activator of the PI3K, BDNF, is downregulated in the hippocampus of CD mouse[16]. In addition, GTF2I has been shown to interfere with the PI3K pathway by binding directly to the *Pik3r1* promoter, probably inhibiting its expression since decreased levels of GTF2I in CD animals results in

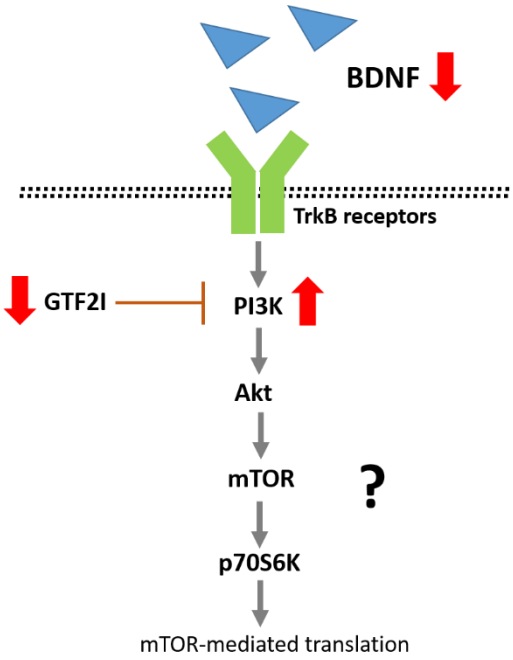

Figure 5. Putative dysregulation of PI3K pathway in WBS. CD animals present decreased mRNA levels of *Bdnf* and increased mRNA levels of *Pik3r1*. *Pik3r1* is a direct target of GTF2I (whose levels are reduced due to the

upregulation of the *Pik3r1*[16,23,24]. However, we could not appreciate any alteration in the PI3K pathway in hippocampal primary cultures of CD mice. We did not observe differences in the levels of BDNF nor in any of the targets tested of the PI3K pathway.

These results raise several possibilities. First, molecular pathways related to synaptic plasticity other than the PI3K might be affected in WBS. For instance, the Ras-ERK (extracellular signal-regulated kinase) pathway is also triggered by BDNF and has been shown to mediate LTP and structural remodeling of excitatory spine synapses[25,37]. Second, it is possible that the PI3K pathway is affected in WBS but only in certain conditions *in vitro*, for example when neurons are depolarized or exposed to exogenous BDNF. Hence, it would be interesting to test how the PI3K pathway in CD neurons responds in the presence of exogenous BDNF. Finally, hippocampal primary cultures might not be a suitable model in which to evaluate WBS mechanisms. Although the biological response of primary culture may be closer to an *in vivo* situation than the one obtained with cell lines, they are grown under controlled conditions, outside their natural environment and characteristics can change.

To sum up, the results obtained suggest that PI3K/Akt/mTOR signaling pathway is not altered in WBS hippocampal cultures and, hence, it emphasizes the need for further research on *in vivo* models and on other potential cellular mechanisms of WBS. Identifying the precise mechanism underlying synaptic plasticity and cognitive deficits of CD mice would help unraveling novel putative biomarkers of the pathological process and would provide a novel context of therapeutic intervention to ameliorate the neurocognitive phenotype of WBS.

REFERENCES

1. Kumar, V, Zhang, M-X, Swank, MW, Kunz, J and Wu, G-Y (2005). Regulation of dendritic morphogenesis by Ras-PI3K-Akt-mTOR and Ras-MAPK signaling pathways. *J. Neurosci.* **25**: 11288–99.

2. Jaworski, J and Sheng, M (2006). The growing role of mTOR in neuronal development and plasticity. *Mol. Neurobiol.* **34**: 205–19.

3. Jaworski, J, Spangler, S, Seeburg, DP, Hoogenraad, CC and Sheng, M

(2005). Control of dendritic arborization by the phosphoinositide-3'-kinase-Akt-mammalian target of rapamycin pathway. *J. Neurosci.* **25**: 11300–12.

4. Santini, E and Klann, E (2011). Dysregulated mTORC1-Dependent Translational Control: From Brain Disorders to Psychoactive Drugs. *Front. Behav. Neurosci.* **5**.

5. Troca-Marín, JA, Alves-Sampaio, A and Montesinos, ML (2012). Deregulated mTOR-mediated translation in intellectual disability. *Prog. Neurobiol.* **96**: 268–82.

6. Ehninger, D, Han, S, Shilyansky, C, Zhou, Y, Li, W, Kwiatkowski, DJ, *et al.* (2008). Reversal of learning deficits in a Tsc2+/- mouse model of tuberous sclerosis. *Nat. Med.* **14**: 843–8.

7. Troca-Marín, JA, Alves-Sampaio, A and Montesinos, ML (2011). An increase in basal BDNF provokes hyperactivation of the Akt-mammalian target of rapamycin pathway and deregulation of local dendritic translation in a mouse model of Down's syndrome. *J. Neurosci.* **31**: 9445–55.

8. Sharma, A, Hoeffer, C a, Takayasu, Y, Miyawaki, T, McBride, SM, Klann, E, *et al.* (2010). Dysregulation of mTOR signaling in fragile X syndrome. *J. Neurosci.* **30**: 694–702.

9. Ricciardi, S, Boggio, EM, Grosso, S, Lonetti, G, Forlani, G, Stefanelli, G, *et al.* (2011). Reduced AKT/mTOR signaling and protein synthesis dysregulation in a Rett syndrome animal model. *Hum. Mol. Genet.* **20**: 1182–96.

10. Pérez Jurado, AL (2003). Williams-Beuren syndrome: a model of recurrent genomic mutation. *Horm. Res.* **59 Suppl 1**: 106–13.

11. Bellugi, U, Lichtenberger, L, Jones, W, Lai, Z and St George, M (2000). I. The neurocognitive profile of Williams Syndrome: a complex pattern of strengths and weaknesses. *J. Cogn. Neurosci.* **12 Suppl 1**: 7–29.

12. Martens, M a, Wilson, SJ and Reutens, DC (2008). Research Review: Williams syndrome: a critical review of the cognitive, behavioral, and neuroanatomical phenotype. *J. Child Psychol. Psychiatry.* **49**: 576–608.

13. Davies, M, Udwin, O and Howlin, P (1998). Adults with Williams syndrome. Preliminary study of social, emotional and behavioural difficulties. *Br. J. Psychiatry* **172**: 273–6.

14. Segura-Puimedon, M, Sahún, I, Velot, E, Dubus, P, Borralleras, C, Rodrigues, AJ, *et al.* (2014). Heterozygous deletion of the Williams-Beuren syndrome critical interval in mice recapitulates most features of the human disorder. *Hum. Mol. Genet.* **23**: 6481–94.

15. Li, HH, Roy, M, Kuscuoglu, U, Spencer, CM, Halm, B, Harrison, KC, *et al.* (2009). Induced chromosome deletions cause hypersociability and other features of Williams-Beuren syndrome in mice. *EMBO Mol. Med.* **1**: 50–65.

16. Borralleras, C, Sahun, I, Pérez-Jurado, L a and Campuzano, V (2015). Intracisternal Gtf2i Gene Therapy Ameliorates Deficits in Cognition and Synaptic Plasticity of a Mouse Model of Williams-Beuren Syndrome. *Mol.*

Ther. **23**: 1691–9.

17. Todorovski, Z, Asrar, S, Liu, J, Saw, NMN, Joshi, K, Cortez, MA, *et al.* (2015). LIMK1 regulates long-term memory and synaptic plasticity via the transcriptional factor CREB. *Mol. Cell. Biol.* **35**: 1316–28.

18. Meng, Y, Zhang, Y, Tregoubov, V, Janus, C, Cruz, L, Jackson, M, *et al.* (2002). Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron* **35**: 121–33.

19. Fujiwara, T, Snada, M, Kofuji, T, Yoshikawa, T and Akagawa, K (2010). HPC-1/syntaxin 1A gene knockout mice show abnormal behavior possibly related to a disruption in 5-HTergic systems. *Eur. J. Neurosci.* **32**: 99–107.

20. Lucena, J, Pezzi, S, Aso, E, Valero, MC, Carreiro, C, Dubus, P, *et al.* (2010). Essential role of the N-terminal region of TFII-I in viability and behavior. *BMC Med. Genet.* **11**: 61.

21. Hoogenraad, CC, Koekkoek, B, Akhmanova, A, Krugers, H, Dortland, B, Miedema, M, *et al.* (2002). Targeted mutation of Cyln2 in the Williams syndrome critical region links CLIP-115 haploinsufficiency to neurodevelopmental abnormalities in mice. *Nat. Genet.* **32**: 116–27.

22. Goergen, CJ, Li, H-H, Francke, U and Taylor, CA (2011). Induced chromosome deletion in a Williams-Beuren syndrome mouse model causes cardiovascular abnormalities. *J. Vasc. Res.* **48**: 119–29.

23. Chimge, N-O, Makeyev, A V, Waigel, SJ, Enkhmandakh, B and Bayarsaihan, D (2012). PI3K/Akt-dependent functions of TFII-I transcription factors in mouse embryonic stem cells. *J. Cell. Biochem.* **113**: 1122–31.

24. Segura-Puimedon, M, Borralleras, C, Pérez-Jurado, L a. and Campuzano, V (2013). TFII-I regulates target genes in the PI-3K and TGF-β signaling pathways through a novel DNA binding motif. *Gene* **527**: 529–536.

25. Santos, AR, Comprido, D and Duarte, CB (2010). Regulation of local translation at the synapse by BDNF. *Prog. Neurobiol.* **92**: 505–16.

26. Hay, N and Sonenberg, N (2004). Upstream and downstream of mTOR: 1926–1945doi:10.1101/gad.1212704.hibiting.

27. Navé, BT, Ouwens, M, Withers, DJ, Alessi, DR and Shepherd, PR (1999). Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem. J.* **344 Pt 2**: 427–31.

28. Peterson, RT, Beal, PA, Comb, MJ and Schreiber, SL (2000). FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. *J. Biol. Chem.* **275**: 7416–23.

29. Berven, LA and Crouch, MF (2000). Cellular function of p70S6K: a role in regulating cell motility. *Immunol. Cell Biol.* **78**: 447–51.

30. Ju, W, Morishita, W, Tsui, J, Gaietta, G, Deerinck, TJ, Adams, SR, *et al.* (2004). Activity-dependent regulation of dendritic synthesis and trafficking of

AMPA receptors. *Nat. Neurosci.* **7**: 244–53.

31. Nimchinsky, EA, Oberlander, AM and Svoboda, K (2001). Abnormal development of dendritic spines in FMR1 knock-out mice. *J. Neurosci.* **21**: 5139–46.

32. Dierssen, M, Benavides-Piccione, R, Martínez-Cué, C, Estivill, X, Flórez, J, Elston, GN, *et al.* (2003). Alterations of neocortical pyramidal cell phenotype in the Ts65Dn mouse model of Down syndrome: effects of environmental enrichment. *Cereb. Cortex* **13**: 758–64.

33. Belichenko, P V, Wright, EE, Belichenko, NP, Masliah, E, Li, HH, Mobley, WC, *et al.* (2009). Widespread changes in dendritic and axonal morphology in Mecp2-mutant mouse models of Rett syndrome: evidence for disruption of neuronal networks. *J. Comp. Neurol.* **514**: 240–58.

34. Mercaldo, V, Descalzi, G and Zhuo, M (2009). Fragile X mental retardation protein in learning-related synaptic plasticity. *Mol. Cells* **28**: 501–7.

35. Siarey, RJ, Stoll, J, Rapoport, SI and Galdzicki, Z. Altered long-term potentiation in the young and old Ts65Dn mouse, a model for Down Syndrome. *Neuropharmacology* **36**: 1549–54.

36. Moretti, P (2006). Learning and Memory and Synaptic Plasticity Are Impaired in a Mouse Model of Rett Syndrome. *J. Neurosci.* **26**: 319–327.

37. Alonso, M, Medina, JH and Pozzo-Miller, L. ERK1/2 activation is necessary for BDNF to increase dendritic spine density in hippocampal CA1 pyramidal neurons. *Learn. Mem.* **11**: 172–8.

DISCUSSION

Elucidating Williams-Beuren syndrome neurocognitive phenotype through the CD mouse model

WBS is a complex neurodevelopmental disorder caused by the hemizygous deletion of 26-28 genes at 7q11.23[89]. It is characterized by a combination of physiological and cognitive deficits with a wide range of phenotypic variability[39,74]. To date, numerous studies have focused on its unique and distinctive neurocognitive profile, but most neurological data are limited to structural and functional brain imaging studies in patients. Therefore, a much deeper insight into the neuropathological features of WBS would be of great interest.

Animal models of WBS have become essential tools to understand the molecular basis underlying WBS neurological features, and are providing us valuable information about the neuroanatomy, neurophysiology and molecular pathways involved in the disorder[109]. Nevertheless, none of the WBS animal models created so far carried the same molecular alteration present in humans. Taking advantage of the high syntenic conservation between the human WBS locus at chr7q11.23 with the mouse genomic chr5G2 region[85,180], our group recently generated a new animal model (CD) that carries a heterozygous deletion of the entire single copy region between *Gtf2i* and *Fkbp6* genes, thereby mimicking the most common deletion found in WBS patients. We have then performed an exhaustive characterization of the neurocognitive profile in these mice. As CD mice recapitulate a wide range of behavioral and cognitive abnormalities seen in WBS, we can now conclude that these mice provide an excellent model to investigate the mechanisms involved in the disease as well to evaluate novel therapeutic approaches. In addition, we have compared several parameters in different mouse models of the disorder with single-gene (*ΔGtf2i*$^{+/-}$ and *ΔGtf2i*$^{-/-}$), partial (PD) and complete (CD) deletions of the critical interval to elucidate the function of individual genes or subsets of genes, allowing the inference on genotype-phenotype correlations.

First, we characterized the CD model by using behavioral and cognitive paradigms relevant to WBS phenotype. In line with the expectations, CD mice revealed some behavioral abnormalities strikingly similar to those manifested by WBS patients. One of the most unique and intriguing behavioral characteristic present in WBS individuals is the overly social and outgoing personality towards strangers[8,42]. Accordingly, in a social interaction test CD mice showed increased sociability with unfamiliar animals. While WT animals did easily habituate to the novel mouse, CD animals did retain social interest for more time, being the total interacting time with the unfamiliar mouse higher in CD mice. In contrast, without the presence of the unfamiliar mouse, we could not appreciate any difference between genotypes in the total interacting time with the empty container. In addition, we demonstrated that the increase of interaction for the unfamiliar mouse was due to increased sociability and not due to memory problems, as CD mice recovered the interest when a novel second unfamiliar animal was exposed at the last trial. PD mice also showed increased sociability in a variety of social interaction tests[111], providing evidence for gene(s) in the PD region as candidate(s) for the sociability. We demonstrated that *ΔGtf2i*$^{+/-}$ and *ΔGtf2i*$^{-/-}$ also showed a hypersociable phenotype in a direct social test, consistent with another *Gtf2i*-deficient-mouse model[130]. All together, these results suggest that *Gtf2i* gene is a main contributor to abnormal sociability phenotypes.

Regarding anxiety-like behavior, the majority of WBS individuals receive a diagnosis of anxiety at some point in their lives[48]. A wide range of behavioral testing paradigms exists to assess anxiety-related behavior in mice. PD mice showed an anxiety-related response in the open-field test, measured by time spent in the centre of the field, but no differences were obtained in other tests for anxiety such as the light-dark test or the marble-burying test[111]. On the other hand, *ΔGtf2i*$^{+/-}$ presented increased levels of anxiety in two different paradigms: the elevated plus maze and the light-dark test[129]. In a first approach to test anxiety in the CD model using the open-field test, no differences were appreciated[112]. Here, we chose another paradigm that depends in part on hippocampal function, the marble-burying test. Although it

is unclear whether an abnormal performance in this test reflects anxiety or obsessive-compulsive behavior[181–183], CD mice performance was completely different from WT, pointing to abnormal levels of anxiety/obsessive-like behavior in these mice. In the same line, PD and *ΔGtf2i*$^{+/-}$ mice showed altered levels of anxiety-like behavior in this test. However, we could not observe any alteration in the performance of *ΔGtf2i*$^{+/-}$, suggesting that not only *Gtf2i* is involved in the anxiety/obsessive behavior of WBS but other genes in the PD region might be involved. The discordant behavior in the marble-burying test previously reported for PD mice[111], with no differences respect to WT, could be due to methodological differences such as test protocols, housing or feeding. These results also strengthen the idea that sometimes it is necessary to conduct two or more anxiety-related tests to characterize the anxiety behavior of an animal model, since there are several forms of anxiety and it is still a challenge the interpretation of the results.

Visuospatial and visuomotor deficits are a consistent finding in WBS patients. Sometimes WBS children seem to have a problem in determining the depth perception when they have to proceed to a new surface, especially when the new surface has a different pattern or color. This reluctance in changing the surface on which they are walking may result in great distress or anxiety[184]. We were able to represent this interesting phenotype in a custom-made apparatus with a two equally sized chambers: one with smooth black surfaces and one with black-and-white stripes in a square pattern surfaces. The preference for the black chamber was evident sooner in CD than in WT mice. In addition, higher number of entries in both compartments in CD mice might indicate higher levels of anxiety probably due to the presence of the stripped compartment. These results suggest that the visuospatial deficits displayed by WBS individuals might also be present in the animal model.

As for motor abilities, a previous study in CD mice showed significant alterations in the motility tonus strength[112], which could be related to the hypotonia present in children with WBS[185]. We demonstrated that CD mice also present motor coordination abnormalities assessed with the accelerated rotarod test. Notably, CD mice had trouble walking on top of the rotating rod

at slower speed than WT mice. Similarly, individuals with WBS have difficulties with balance, proprioception and motor planning[51,52]. Li *et al.* also showed that PD mice performed worse than WT mice in this test[111], pointing to genes in the PD region to be involved in this phenotype. In our study, *ΔGtf2i*$^{+/-}$ and *ΔGtf2i*$^{/-}$ mice also showed motor coordination problems in the accelerating rotarod test, and *ΔGtf2i*$^{/-}$ mice required a significantly larger number of trials to learn the test. These results indicate that *Gtf2i* might be a major player in the motor phenotype of WBS.

Regarding learning and memory, a previous study showed impaired fear memory performance of CD mice in the fear conditioning test with no learning deficits in the water maze test, although a different strategy in their performance was shown, spending more time in the center of the pool[112]. To further investigate memory problems in this animal model, we switched our attention to the spatial working memory deficits present in WBS[186,187]. A suitable test to assess spatial working memory in rodents is the spontaneous alternation test. In addition to be a sensitive method to study cognitive deficits, the spontaneous alternation test has demonstrated to be useful in detecting hippocampal dysfunction. Therefore, the poor performance of CD mice in this test, not only indicates impairment in working memory but also an abnormal functioning of their hippocampus. In addition, CD mice presented deficits in short-term memory evaluated in the novel object recognition (NOR) test. NOR is a highly validated test for recognition memory which is based in the ability of rodents to recognize a novel object in the environment. In the test session, when a familiar object is replaced by a novel one, CD animals were unable to discriminate between the two objects. Taken together, these data provide evidence that memory deficits are clearly present in the CD model. It seems that genes in both PD and distal deletion (DD) regions might be involved in memory problems of WBS, since DD mice displayed fewer freezing responses relative to PD and WT mice in the fear conditioning test and an heterozygous mice for *Gtf2i* showed impaired recognition of novel object in the NOR test[111,130].

The complex neurological deficits present in CD mice might result from neuronal abnormalities in the brain. Several imaging studies investigating brain structure in WBS patients have revealed differences in whole-brain volume as well as volumetric differences in specific brain areas[58,59,65]. Likewise, CD animals recapitulated the reduction in total brain weight (9%)[112]. In addition, a more precise volumetric and cytological structural analysis in CD revealed specific differences in several brain regions. In the amygdala, for example, although the volume of this region was preserved, a general decreased in cell density was observed[112]. We found a significant reduction in the number of GFAP+ cells both in the amygdala and the hippocampus. The results from this study indicate that differences in the astroglial lineage could be implicated in WBS. In fact, there is increasing evidence in the literature that glia has key roles in various neurological diseases, since it is involved in the maintenance of healthy brain function[188,189]. Thus, future research should be focused on the study of this interesting lineage, unnoticed up to now in WBS.

Although many studies have described clinical details and brain anatomy and function, information about dendritic and axonal morphology in WBS subjects barely exists. Examination of WBS-affected brains have shown reduced columnar organization throughout the cortex, abnormally clustered and oriented neurons and a generalized increase in cell packing density[79,80]. Mouse models promise to enhance our understanding of dendritic and axonal morphology. An important advance has been the generation of transgenic mouse lines expressing a fluorescent protein product in specific neuron subpopulations. Crossing these lines with other genetically modified mice facilitates the study of the analysis of the somatodendritic morphologies or axon distributions. In this thesis, we used the Thy1-YFP-H line, which has been widely used because it selectively labels projection neocortical and hippocampal pyramidal neurons[190,191]. Surprisingly, we found a remarkable and unexpected reduction in the number of YFP-expressing cells throughout the brain in CD animals. Occasional individual YFP+ neurons were present both in WT and CD mice as early as P9. As expected, by the end of the

second postnatal week the number of YFP+ cells increased rapidly in WT mice. However, YFP+ cells in CD mice remained low from early postnatal stages to late maturity, probably as a consequence of transgene silencing. Similar findings have been previously described in a mouse model for Rett syndrome[192]. It has been reported that the Thy-1 promoter expression cassette is highly sensitive to chromosomal context, and identical constructs can produce highly variable patterns across transgenic lines. In addition, the neuron subpopulations labeled in Thy1-YPF-H mice are still not fully understood[190–192]. Definitely, more studies are needed to define whether the reduced number of YFP+ neurons reflects alterations in the proportions of certain subclasses of pyramidal neurons, or it is due to aberrant development activation of the YFP transgene in CD mice.

A general analysis of morphology in YFP+ neurons in motor cortex revealed a significant decrease in the total length of axons in the CD model. In addition, these axons were strikingly disorganized. While most of the axons were relatively vertically oriented with respect to the pial surface, axonal trajectories of CD neurons were quite variable, sometimes without a clear direction. These alterations might cause abnormal neurotransmission between neurons responsible for the corresponding motor phenotype and, in consequence, might be responsible for the motor coordination abnormalities observed in the rotarod test in these mice. However, not only the motor cortex is involved in motor functions but also other brain areas such the cerebellum might be involved.

Since CD mice displayed cognitive deficits in different behavioral paradigms dependent on the function of the hippocampus, we focused on the neuroarchitecture in this structure. Most disorders associated with intellectual disability, such as Fragile X or Down syndrome, are characterized by structural abnormalities of dendrites, especially at the level of the dendritic spines[193,194]. Accordingly, CD mice presented shorter dendrites in both apical and basal compartments of CA1 area, which is consistent with the decreased brain volume observed in these mice. In addition, apical proximal dendrites of

these YFP+ neurons had less and shorter dendritic spines. *Clyn2* and *Limk1* are two synaptic genes that regulate cytoskeletal dynamics, either via the actin filament system (LIMK1) or through the microtubule network (CYLN2)[195]. Therefore, abnormalities in the number and morphology of dendritic spines in CD mice could be due to alterations in gene dosage of these molecules. Accordingly, PD mice (heterozygous for *Limk1* and *Cyln2*) also presented a reduction in spine density similar to CD mice. In addition, GTF2I may also have a relevant role in the developmental regulation of dendritic morphology, since hippocampal CA1 neurons of *ΔGtf2i*$^{-/-}$ presented decreased spine density and shorter dendrites as CD mice. It should be noted, though, that the spine density in CD mice was only reduced by 17%. This slight reduction might be in line with the degree of intellectual disability present in WBS individuals, which is described to be in the mild to moderate range[37,38]. Anyway, changes in spine number and morphology have been shown to influence the functioning of neuronal circuits whose dysfunction could lead to the behavioral alterations of CD animals. In fact, a first approach to synaptic functioning in hippocampal primary cultures of CD mice showed impaired calcium flux through synaptic NMDA receptors, which may indicate alteration in glutamatergic synaptic activity and/or impaired cellular calcium homeostatic systems. Interestingly, GTF2I, in addition to function as a transcription factor in the nucleus, it has been described as an inhibitor of agonist-induced calcium entry in the cytoplasm, thus regulating calcium homeostasis[196]. Several neurological diseases have been associated with defects in calcium signaling[197,198]. Hence, calcium signaling may be affected in WBS and open a new field of research which will contribute to further understand the mechanisms of the disease.

To further elucidate the synaptic function of CD mice, electrophysiological experiments using hippocampal slices were performed. Synaptic plasticity is considered the main cellular mechanism underlying learning and memory. The most widely studied form of synaptic plasticity is the LTP at the synapses between Schaffer collaterals and commissural neurons in area CA1 of the hippocampus[199]. In line with other genetic models of neurological diseases

with cognitive deficits[200–202], CD mice presented deficits in LTP: while theta-burst stimulation induced a persistent enhancement of synaptic transmission that remained stable during the entire period of recording in WT mice, synaptic potentiation decayed slowly in CD mice. These results are consistent with previous reports of LTP deficits in *LIMK1* and *CLYN2* knockout mice[148,156], and are correlated with the memory problems present in CD mice. Additionally, it can be extrapolated that part of the complex neurocognitive phenotype present in human with WBS might be due to deficits in hippocampal LTP.

In this electrophysiological study, we could not elucidate the mechanism underlying the LTP deficits, since it was not associated with changes in presynaptic function, LTP induction nor AMPA and NMDA receptor function. Nevertheless, some hypotheses can be raised. On the one hand, LTP is accompanied by changes in cytoskeletal organization and morphology of dendritic spines. Since dendritic spines contain the majority of excitatory synapses on CA1 pyramidal neurons, changes in spine density or in morphology have been associated with aberrations in synaptic plasticity[147,203,204]. For instance, LIMK1 knockout mice presented abnormal morphology of dendritic spines of pyramidal neurons and correlated with alterations in LTP[147]. As mentioned before, CD mice present a reduction in spine density in apical proximal dendrites of CA1 pyramidal neurons. It is thus possible that spine abnormalities contribute to disrupt the production of LTP.

On the other hand, CD mice presented specific molecular alterations that could be associated with the LTP deficits. BDNF levels (both mRNA and protein levels) were significantly reduced in the hippocampus of CD mice. Evidence is accumulating that BDNF plays a fundamental role in synaptic plasticity[205,206]. LTP deficits of CD mice could thus be related to a reduction in *Bdnf* levels. In fact, several cognitive diseases have associated deficits in *Bdnf* levels with LTP and memory disturbance. Even more interestingly, synaptic plasticity deficits were rescued by BDNF in murine models of

Huntington's disease, Angelman syndrome and Fragile X syndrome[207–209], pointing to this neurotrophin a logical candidate for treatment of neurological disorders. Therefore, an important and unquestionable issue to be performed that arises from this study is trying to rescue LTP deficits of CD mice by restoring the levels of *Bdnf*. BDNF is a key upstream activator of several parallel signaling pathways, including the PI3K/AKT/mTOR pathway, through which it regulates the local translation of a series of mRNA involved in synaptic plasticity[210]. Growing evidence indicates that dysfunction of this pathway is associated with several neurological diseases, such as Fragile X syndrome, Down syndrome and Rett syndrome[211–213]. Interestingly, WBS has also been related to the PI3K pathway[214,215]. We demonstrated increased mRNA levels of the regulatory subunit of the PI3K (*Pik3r1)* in the hippocampus of CD mice, pointing to a possible dysregulation of the pathway in WBS. The PI3K/AKT/mTOR pathway also plays key roles in the regulation of many aspects of dendrite formation, including formation of dendritic spines. Therefore, dysregulation of this pathway would be compatible with the reported abnormalities in spine density and morphology, as well as with the deficits in synaptic plasticity of CD mice.

Bdnf and *Pik3r1* synaptic plasticity markers were also dysregulated in *ΔGtf2i*$^{+/-}$ and PD mice, suggesting that *Gtf2i* dosage influences the regulation of these molecules. Since GTF2I has been shown to bind directly to the *Pik3r1* promoter in mouse embryonic fibroblasts[214], these results suggest that its effect on *Pik3r1* is probably inhibitory, since *Gtf2i* deletion lead to *Pik3r1* upregulation. Similarly, Adamo and colleagues reported that GTF2I levels were inversely correlated with the levels of several other genes such as BEND4, suggesting a repressive effect of GTF2I on those genes[117]. Regarding *Bdnf*, although we can now establish that there must be a link between *Gtf2i* and *Bdnf*, the mechanism through which *Gtf2i* effects the expression of *Bdnf* remains to be elucidated yet.

To go in depth into the dysregulation of the PI3K pathway we attempt to use hippocampal primary cultures, since they have provided important insights into the mechanisms underlying other diseases and represent a useful tool for the screening on potential pharmacological agents. We could not observe any alteration in the activity of the PI3K pathway in these conditions. In addition, levels of BDNF in hippocampal primary neurons were normal, in contrast to the results obtained *in vivo*. This study shows that although the biological response of primary cultures may be closer to an *in vivo* situation than the one obtained with cell lines, they are grown under controlled conditions, outside their natural environment and therefore, characteristics can change. Thus, we can not draw any conclusion with regards to the dysregulation of the PI3K pathway. Other studies *in vivo* will be necessary to determine the role of this pathway in the neurocognitive phenotype of WBS.

Novel therapeutic approaches

The cognitive, behavioral and neurological problems present in WBS patients directly affect their quality of life. In fact, independent living and competitive employment in WBS is not frequently reported[6]. Since the genetically modified mouse shows a similar neurocognitive phenotype to that observed in humans, pre-clinical testing of new therapeutic approaches deserves to be performed to ameliorate or even rescue the specific phenotypes. In this thesis we attempted two different therapeutic approaches: a genetic and a pharmacological treatment.

Gtf2i gene therapy

Given the role of GTF2I in the neurocognitive profile of WBS, we sought to determine if the neurocognitive defects could be rescued in the adult CD mouse through *in situ Gtf2i* replacement by gene therapy in the brain. Interestingly, CD-*mGtf2i*–injected mice had a better performance in all the tests studied: social behavior was completely normalized; motor learning and coordination in the accelerating rotarod was nearly as good in treated CD mice as in WT mice; and finally, in the marble-burying test, anxiety-like behavior of CD mice was improved, although WT levels were not reached until the end of the test. We also demonstrated that *Bdnf* expression was

completely normalized in CD-*mGtf2i*–injected mice, without changing the levels of *Pik3r1*. However, the documented increase of *Gtf2i* mRNA was not enough to reverse the morphological phenotypes observed in the adult brain of CD mice.

In this study we used recombinant adeno-associated virus (AAV) serotype 9, which has been shown to have one of the highest expression in brain with rapid-onset kinetics compared to other serotypes[216]. In addition, we injected the vectors into the cistern magna since we wanted the vector to spread throughout the brain. Accordingly, spread AAV9 transduction throughout the brain was confirmed in mice brains by immunofluorescence assays. Specifically, the efficiency in hippocampus was documented by a significant increase of *Gtf2i* expression (57.5%) in injected animals when compared to controls. Therefore, this study further supports the use of the AAV9 for the study and treatment of neurological diseases.

Several conclusions can be drawn from this study. First, these results reinforce the concept that *Gtf2i* is a major player in the neurocognitive phenotype of WBS. We could not completely rescue the performance of CD mice in the marble-burying test but, as mentioned before, not only *Gtf2i* might be involved in the anxiety/obsessive behavior of WBS but also other genes in the region. Second, the cognitive improvement observed in CD-*mGtf2i*–injected mice might be probably due to the normalization of *Bdnf* expression and/or normalization of *Bdnf*-related pathways other than the PI3K pathway. Finally, the fact that we could not appreciate any improvement with regards to the neuroanatomical findings suggests that an early treatment or reaching physiological levels of GTF2I might be required. However, we hypothesized that some functional reversal must have taken place despite the absence of relevant structural changes, possibly through remodeling of already existing spines.

All together, these results indicate that therapeutic intervention by increasing *GTF2I* expression might be a good strategy to ameliorate WBS neurocognitive phenotype in patients. However, further research is needed since GTF2I is a general transcription factor which regulates the transcription of numerous genes involved in multiple pathways, some of them still unknown. In addition, the overexpression of *GTF2I* has been associated with

autism spectrum disorders[217], suggesting that it is necessary a rigorous regulation of this gene.

Epigallocatechin-3-gallate (EGCG) treatment

Due to the increasing evidence that green tea is associated with potential health benefits, including in neurological diseases, we decided to explore its effects on WBS. Interestingly, green tea diet was able to rescue the short-term memory deficits present in CD mice when evaluated in the NOR test. Curiously, EGCG did not reverse the spatial working memory deficits of CD mice, pointing to specific effects of EGCG on memory. Despite the reported anxiolytic effects of EGCG[218], we were not able to improve the performance of CD animals in the marble-burying test. A possible explanation for this lack of effect is that different forms of anxiety exist, and EGCG anxiolytic effects were described using another test, the elevated plus-maze test. As it was mentioned, CD animals do not exhibit anxiety-like behavior in the elevated plus-maze, so there was no point in using this test. Therefore, these results highlight the fact that sometimes we should perform several tests to measure a specific phenotype such as memory or anxiety, since depending on the test chosen the results and conclusions drawn can be different.

EGCG had no effect on the neuroarchitecture of CD mice. However, WT animals treated with EGCG presented shorter dendrites and decreased spine density in CA1 neurons of the hippocampus. In addition, total brain weight of these mice tended to be smaller. Guedj *et al.* did also report a reduction in the brain weight of WT animals using similar doses of green tea polyphenols[219]. Some studies have suggested that the effects of EGCG are highly dependent on the dose. For example, Ge *et al.* demonstrated that low doses of EGCG reversed spine damage but not higher doses of EGCG[220]. Therefore, the high doses of EGCG used in this study could explain the lack of effects on neuroarchitecture in CD mice or even the deleterious effect of EGCG in WT mice. In accordance with other studies which have related EGCG to BDNF expression or activity and to the PI3K pathway, WT animals treated with green tea presented an increase of *Bdnf*, *Pik3r1* and *Limk1* levels. On the contrary, CD EGCG fed animals presented normalized levels of *Bdnf* without changes in the levels of *Pik3r1* or *Limk1*. These results suggest that PI3K pathway in CD mice might act independently of *Bdnf*

levels. In addition, the complete recovery of *Bdnf* correlates with the improvement of short-term memory in CD mice.

In addition to the promising results of EGCG in WBS and in other diseases, the safety of green tea extracts should easy the translation from preclinical to human clinical trials. Nevertheless, further research is needed to elucidate the precise mechanism through which EGCG is acting as well as to establish the effective dosage of EGCG.

To sum up, both *Gtf2i* gene therapy and EGCG treatment were able to rescue aspects of the neurocognitive profile of CD mice. Increasing the levels of *Gtf2i* improved sociability, anxiety and motor coordination and EGCG rescued short-term memory deficits. Either by increasing the levels of *Gtf2i* or by an unknown mechanism of EGCG, both treatments recovered the levels of *Bdnf* without changing the levels of *Pik3r1*. These results suggest that the neurocognitive improvements seen in CD mice might be related to the upregulation of *Bdnf* and/or modulation of BDNF-related pathways. Therefore, increasing the levels of this neurotrophin in the brain may be a promising therapeutic agent for WBS.

Concluding remarks

The unique and distinctive neurocognitive phenotype of WBS has been attracting attention for the last decades. Important progress has been made with regards to clinical characterization of WBS individuals, genotype-phenotype correlations by studying patients with atypical deletions, or anatomical and functional characterization using neuroimaging studies. However, a deepen characterization of the neurocognitive phenotype to gain insight into the specific features implicated in the etiology of WBS was still missing.

Unquestionably, animal models are essential tools to understand the molecular basis underlying diseases as well as to evaluate novel therapeutic approaches. In this thesis project, we used the CD mouse model to deeper insight into the neuropathological features of WBS. Figure 1 shows a summary of the main findings observed in the CD mouse model. Globally, the CD model clearly recapitulated relevant behavioral and cognitive phenotypes

present in WBS individuals. In addition, we were able to enhance our knowledge of WBS neurocognitive phenotype by identifying molecular and neuroanatomical alterations relevant for the disease, as well as by studying synaptic function using electrophysiological techniques. On the other hand, we have confirmed the role of *Gtf2i* as the main player in the neurocognitive phenotype of WBS by comparing several mouse models of the disorder with single-gene (*Gtf2i),* partial and complete deletions of the critical interval. Furthermore, we demonstrated that a *Gtf2i*-gene therapy in CD mice using adeno-associated virus is able to rescue some aspects of the neurocognitive profile of these mice. Finally, chronic administration of green tea was able to ameliorate short-term memory of CD mice. Therefore, we can conclude that the CD mouse model is a novel and powerful tool which is helping to elucidate the mechanisms involved in the disease as well as to evaluate novel therapeutic approaches.

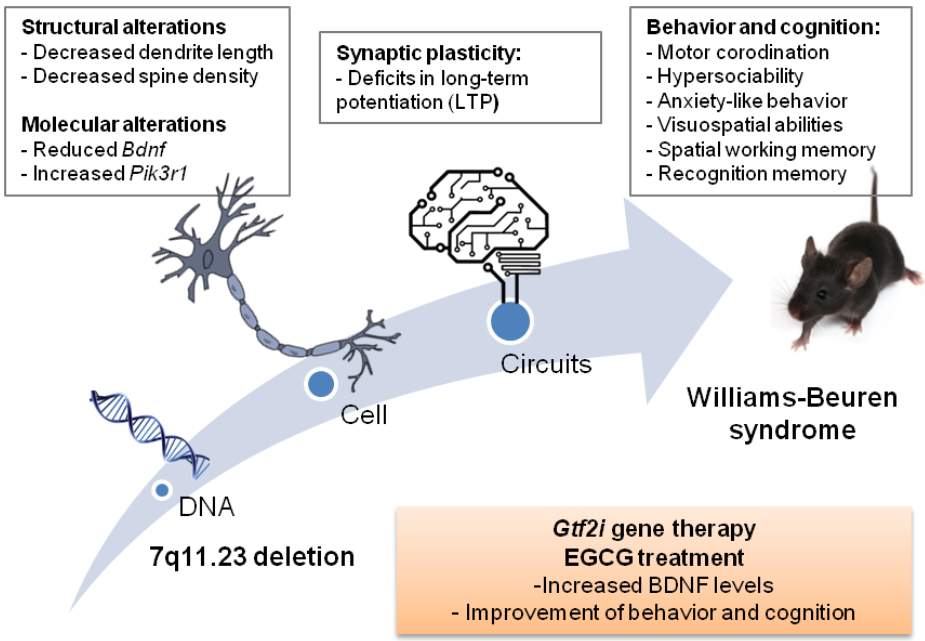

Figure 1. Summary of the main findings present in the CD animal model of WBS.

CONCLUSIONS

CONCLUSIONS

1. The CD mouse model recapitulates relevant aspects of the neurobehavioral phenotype of WBS, such as hypersociability, altered levels of anxiety-like behavior, motor coordination abnormalities, visuospatial deficits and impairment in spatial working memory and recognition memory. Thus, CD mice provide a very good model to investigate the mechanisms involved in the disease as well as to evaluate novel therapeutic approaches.

2. The astroglial lineage could be implicated in the pathology of WBS, since the amygdala and the hippocampus of CD mice present a reduction in the number of GFAP+ cells. Thus, future research should focus on the study of this lineage, unnoticed up to now in WBS.

3. The number of YPF-expressing neurons is remarkably reduced throughout the brain of CD animals, probably as a consequence of transgene silencing.

4. Axons of YFP+ neurons in motor cortex are significantly shorter and strikingly disorganized, which might contribute to motor coordination abnormalities observed in these mice.

5. YFP+ neurons in the CA1 region of the hippocampus of CD mice present shorter dendrites in both apical and basal compartment. In addition, apical proximal dendrites of these neurons present shorter spines and reduced spine density. These alterations might contribute to the behavioral alterations of the CD model.

6. Levels of *Bdnf,* an important neurotrophin involved in synaptic plasticity, are downregulated in the hippocampus of CD mice. In addition, levels of the regulatory subunit of the PI3K (*Pik3r1*) are upregulated. These molecular alterations suggest that BDNF-related pathways, such as the PI3K/Akt/mTOR pathway, might be implicated in the neurocognitive phenotype of WBS.

7. We have used hippocampal primary cultures to study the PI3K/Akt/mTOR pathway, and no differences have been observed on its

activity in these conditions. Other studies *in vivo* will be necessary to determine the role of this pathway in the neurocognitive profile of WBS.

8. Functional analyses in CD hippocampal primary cultures suggest alterations in glutamatergic synaptic activity and/or defects in calcium signaling. These data open a new field of research which will contribute to further understand the mechanisms of the disease.

9. CD mice present deficits in LTP at hippocampal CA3-CA1 synapses, which correlate with the cognitive dysfunction present in these mice.

10. The LTP deficit was not associated with changes in presynaptic function, LTP induction mechanisms or AMPA and NMDA receptor function, but might be associated with the deficits in *Bdnf* levels and spine abnormalities present in CD mice.

11. We have strengthened the idea that *Gtf2i* is one of the main players in the neurocognitive phenotype of WBS by comparing different mouse models of the disorder with single-gene (*Gtf2i)*, partial and complete deletions of the critical interval.

12. Replacement of GTF2I by intracisternal gene therapy was able to rescue some aspects of the neurocognitive phenotype of CD mice, such as sociability, motor coordination and anxiety-related behavior, along with normalization of *Bdnf* levels. However, we could not appreciate any improvement with regards to the neuroanatomical findings.

13. Chronic administration of green tea was able to ameliorate short-term memory deficits of CD mice. This improvement might be related to the upregulation of *Bdnf* and/or modulation of BDNF-related pathways. The therapy did not have any effect on the neuroanatomical defects of CD mice.

14. Two independent therapies conducted in this thesis project indicate that upregulation of *Bdnf* by different mechanisms is associated with cognition improvement in CD mice. Therefore, increasing the levels of *Bdnf* in the brain might be a promising therapeutic approach for WBS.

REFERENCES

1. Stromme, P, Bjomstad, PG and Ramstad, K (2002). Prevalence Estimation of Williams Syndrome. *J. Child Neurol.* **17**: 269–271.

2. Fanconi, G, Girardet, P, Schlesinger, B, Butler, N and Black, J (1952). Chronic hyperglycemia, combined with osteosclerosis, hyperazotemia, nanism and congenital malformations. *Helv. Paediatr. Acta* **7**: 314–349.

3. WILLIAMS, JC, BARRATT-BOYES, BG and LOWE, JB (1961). Supravalvular aortic stenosis. *Circulation* **24**: 1311–8.

4. Beuren, a J, Apitz, J and Harmjanz, D (1962). Supravalvular aortic stenosis in association with mental retardation and a certain facial appearance. *Circulation* **26**: 1235–1240.

5. Pober, BR (2010). Williams-Beuren syndrome. *N. Engl. J. Med.* **362**: 239–52.

6. Morris, CA, Demsey, SA, Leonard, CO, Dilts, C and Blackburn, BL (1988). Natural history of Williams syndrome: physical characteristics. *J. Pediatr.* **113**: 318–26.

7. Morris, CA and Mervis, CB (2000). Williams syndrome and related disorders. *Annu. Rev. Genomics Hum. Genet.* **1**: 461–84.

8. Doyle, TF, Bellugi, U, Korenberg, JR and Graham, J (2004). 'Everybody in the world is my friend' hypersociability in young children with Williams syndrome. *Am. J. Med. Genet. A* **124A**: 263–73.

9. Burch, TM, McGowan, FX, Kussman, BD, Powell, AJ and Dinardo, J a. (2008). Congenital supravalvular aortic stenosis and sudden death associated with anesthesia: What's the mystery? *Anesth. Analg.* **107**: 1848–1854.

10. Wessel, A, Gravenhorst, V, Buchhorn, R, Gosch, A, Partsch, C-J and Pankau, R (2004). Risk of sudden death in the Williams-Beuren syndrome. *Am. J. Med. Genet. A* **127A**: 234–237.

11. Bruno, E, Rossi, N, Thüer, O, Córdoba, R and Alday, LE (2003). Cardiovascular findings, and clinical course, in patients with Williams syndrome. *Cardiol. Young* **13**: 532–536.

12. Eronen, M, Peippo, M, Hiippala, A, Raatikka, M, Arvio, M, Johansson, R, *et al.* (2002). Cardiovascular manifestations in 75 patients with Williams syndrome. *J. Med. Genet.* **39**: 554–558.

13. Broder, K, Reinhardt, E, Ahern, J, Lifton, R, Tamborlane, W and Pober, B (1999). Elevated ambulatory blood pressure in 20 subjects with Williams syndrome. *Am. J. Med. Genet.* **83**: 356–360.

14. Wessel, A, Motz, R, Pankau, R and Bürsch, JH (1997). Arterial hypertension and blood pressure profile in patients with Williams-Beuren syndrome. *Z. Kardiol.* **86**: 251–257.

15. Bouchireb, K, Boyer, O, Bonnet, D, Brunelle, F, Decramer, S, Landthaler, G, *et al.* (2010). Clinical features and management of arterial hypertension in children with Williams-Beuren syndrome. *Nephrol. Dial. Transplant.* **25**: 434–438.

16. Li, DY, Brooke, B, Davis, EC, Mecham, RP, Sorensen, LK, Boak, BB, *et al.* (1998). Elastin is an essential determinant of arterial morphogenesis. *Nature* **393**: 276–280.

17. Karnik, SK, Brooke, BS, Bayes-Genis, A, Sorensen, L, Wythe, JD, Schwartz, RS, *et al.* (2003). A critical role for elastin signaling in vascular morphogenesis and disease. *Development* **130**: 411–423.

18. Del Campo, M, Antonell, A, Magano, LF, Muñoz, FJ, Flores, R, Bayés, M, *et al.* (2006). Hemizygosity at the NCF1 gene in patients with Williams-Beuren syndrome decreases their risk of hypertension. *Am. J. Hum. Genet.* **78**: 533–42.

19. Amenta, S, Sofocleous, C, Kolialexi, A, Thomaidis, L, Giouroukos, S, Karavitakis, E, *et al.* (2005). Clinical manifestations and molecular investigation of 50 patients with Williams syndrome in the Greek population. *Pediatr. Res.* **57**: 789–95.

20. Pober, BR and Morris, CA (2007). Diagnosis and management of medical problems in adults with Williams-Beuren syndrome. *Am. J. Med. Genet. Part C Semin. Med. Genet.* **145**: 280–290.

21. Morris, CA, Leonard, CO, Dilts, C and Demsey, SA (1990). Adults with Williams syndrome. *Am. J. Med. Genet. Suppl.* **6**: 102–7.

22. Cherniske, EM, Carpenter, TO, Klaiman, C, Young, E, Bregman, J, Insogna, K, *et al.* (2004). Multisystem study of 20 older adults with Williams syndrome. *Am. J. Med. Genet. A* **131**: 255–264.

23. Cambiaso, P, Orazi, C, Digilio, MC, Loche, S, Capolino, R, Tozzi, A, *et al.* (2007). Thyroid morphology and subclinical hypothyroidism in children and adolescents with Williams syndrome. *J. Pediatr.* **150**: 62–5.

24. Pankau, R, Partsch, CJ, Gosch, A, Oppermann, HC and Wessel, a (1992). Statural growth in Williams-Beuren syndrome. *Eur. J. Pediatr.* **151**: 751–755.

25. Partsch, CJ, Dreyer, G, Gosch, A, Winter, M, Schneppenheim, R, Wessel, A, *et al.* (1999). Longitudinal evaluation of growth, puberty, and bone maturation in children with Williams syndrome. *J. Pediatr.* **134**: 82–9.

26. Morris, C a (2010). Introduction: Williams syndrome. *Am. J. Med. Genet. C. Semin. Med. Genet.* **154C**: 203–8.

27. Hertzberg, J, Nakisbendi, L, Needleman, HL and Pober, B. Williams syndrome--oral presentation of 45 cases. *Pediatr. Dent.* **16**: 262–7.

28. Bedeschi, MF, Bianchi, V, Colli, AM, Natacci, F, Cereda, A, Milani, D, *et al.* (2011). Clinical follow-up of young adults affected by Williams syndrome: Experience of 45 Italian patients. *Am. J. Med. Genet. Part A* **155**: 353–359.

29. Viana, MM, Frasson, M, Galvão, H, Leão, LL, Stofanko, M, Gonçalves-Dornelas, H, *et al.* (2014). Ocular Features in 16 Brazilian Patients with Williams-Beuren Syndrome. *Ophthalmic Genet.*doi:10.3109/13816810.2013.873941.

30. Winter, M, Pankau, R, Amm, M, Gosch, A and Wessel, A (1996). The spectrum of ocular features in the Williams-Beuren syndrome. *Clin. Genet.* **49**: 28–31.

31. Partsch, CJ, Siebert, R, Caliebe, A, Gosch, A, Wessel, A and Pankau, R (2005). Sigmoid diverticulitis in patients with Williams-Beuren syndrome: relatively high prevalence and high complication rate in young adults with the syndrome. *Am. J. Med. Genet. A* **137**: 52–4.

32. Simşek-Kiper, PO, Sahin, Y, Arslan, U, Alanay, Y, Boduroğlu, K, Orhan, D, *et al.* Celiac disease in Williams-Beuren syndrome. *Turk. J. Pediatr.* **56**: 154–9.

33. Pankau, R, Partsch, CJ, Winter, M, Gosch, A and Wessel, A (1996). Incidence and spectrum of renal abnormalities in Williams-Beuren syndrome. *Am. J. Med. Genet.* **63**: 301–4.

34. Pober, BR, Lacro, R V, Rice, C, Mandell, V and Teele, RL (1993). Renal findings in 40 individuals with Williams syndrome. *Am. J. Med. Genet.* **46**: 271–4.

35. Sammour, ZM, Gomes, CM, de Bessa, J, Pinheiro, MS, Kim, CAE, Hisano, M, *et al.* (2014). Congenital genitourinary abnormalities in children with Williams-Beuren syndrome. *J. Pediatr. Urol.* **10**: 804–9.

36. Pérez-García, D, Flores, R, Brun-Gasca, C and Pérez-Jurado, L a (2014). Lateral preference in Williams-Beuren syndrome is associated with cognition and language. *Eur. Child Adolesc. Psychiatry*doi:10.1007/s00787-014-0652-6.

37. Martens, M a, Wilson, SJ and Reutens, DC (2008). Research Review: Williams syndrome: a critical review of the cognitive, behavioral, and neuroanatomical phenotype. *J. Child Psychol. Psychiatry.* **49**: 576–608.

38. Mervis, CB and Klein-Tasman, BP (2000). Williams syndrome: cognition, personality, and adaptive behavior. *Ment. Retard. Dev. Disabil. Res. Rev.* **6**: 148–58.

39. Mervis, CB, Robinson, BF, Bertrand, J, Morris, C a., Klein-Tasman, BP and Armstrong, SC (2000). The Williams Syndrome Cognitive Profile. *Brain Cogn.* **44**: 604–628.

40. Bellugi, U, Lichtenberger, L, Jones, W, Lai, Z and St George, M (2000). I. The neurocognitive profile of Williams Syndrome: a complex pattern of strengths and weaknesses. *J. Cogn. Neurosci.* **12 Suppl 1**: 7–29.

41. Mervis, CB and John, AE (2010). Cognitive and behavioral characteristics of children with Williams syndrome: implications for intervention approaches. *Am. J. Med. Genet. C. Semin. Med. Genet.* **154C**: 229–48.

42. Järvinen-Pasley, A, Bellugi, U, Reilly, J, Mills, DL, Galaburda, A, Reiss, AL, *et al.* (2008). Defining the social phenotype in Williams syndrome: a model for linking gene, the brain, and behavior. *Dev. Psychopathol.* **20**: 1–35.

43. Klein-Tasman, BP and Mervis, CB (2003). Distinctive personality characteristics of 8-, 9-, and 10-year-olds with Williams syndrome. *Dev. Neuropsychol.* **23**: 269–90.

44. Mervis, CB, Morris, CA, Klein-Tasman, BP, Bertrand, J, Kwitny, S, Appelbaum, LG, *et al.* (2003). Attentional characteristics of infants and toddlers with Williams syndrome during triadic interactions. *Dev. Neuropsychol.* **23**: 243–68.

45. Klein-Tasman, BP, Li-Barber, KT and Magargee, ET (2011). Honing in on the social phenotype in Williams syndrome using multiple measures and multiple raters. *J. Autism Dev. Disord.* **41**: 341–51.

46. Bellugi, U, Adolphs, R, Cassady, C and Chiles, M (1999). Towards the neural basis for hypersociability in a genetic syndrome. *Neuroreport* **10**: 1653–1657.

47. Leyfer, OT, Woodruff-Borden, J, Klein-Tasman, BP, Fricke, JS and Mervis, CB (2006). Prevalence of psychiatric disorders in 4 to 16-year-olds with Williams syndrome. *Am. J. Med. Genet. Part B Neuropsychiatr. Genet.* **141**: 615–622.

48. Woodruff-Borden, J, Kistler, DJ, Henderson, DR, Crawford, N a. and Mervis, CB (2010). Longitudinal course of anxiety in children and adolescents with Williams syndrome. *Am. J. Med. Genet. Part C Semin. Med. Genet.* **154**: 277–290.

49. Dykens, EM (2003). Anxiety, fears, and phobias in persons with Williams syndrome. *Dev. Neuropsychol.* **23**: 291–316.

50. Kennedy, JC, Kaye, DL and Sadler, LS (2006). Psychiatric Diagnoses in Patients with Williams Syndrome and Their Families. *J. Psychiatry* **20**: Article 4.

51. Chapman, CA, du Plessis, A and Pober, BR (1996). Neurologic findings in children and adults with Williams syndrome. *J. Child Neurol.* **11**: 63–5.

52. Gagliardi, C, Martelli, S, Burt, MD and Borgatti, R (2007). Evolution of Neurologic Features in Williams Syndrome. *Pediatr. Neurol.* **36**: 301–306.

53. Morris, C (2010). The behavioral phenotype of Williams syndrome: A recognizable pattern of neurodevelopment. *Am. J. Med. Genet. C. Semin. Med. Genet.* **154C**: 427–31.

54. Pober, BR and Filiano, JJ (1995). Association of Chiari I malformation and Williams syndrome. *Pediatr. Neurol.* **12**: 84–8.

55. Ferrero, GB, Biamino, E, Sorasio, L, Banaudi, E, Peruzzi, L, Forzano, S, *et al.* (2007). Presenting phenotype and clinical evaluation in a cohort of 22 Williams-Beuren syndrome patients. *Eur. J. Med. Genet.* **50**: 327–37.

56. Marler, J a, Sitcovsky, JL, Mervis, CB, Kistler, DJ and Wightman, FL (2010). Auditory function and hearing loss in children and adults with Williams syndrome: cochlear impairment in individuals with otherwise normal hearing. *Am. J. Med. Genet. C. Semin. Med. Genet.* **154C**: 249–65.

57. Zarchi, O, Attias, J and Gothelf, D (2010). Auditory and visual processing in Williams syndrome. *Isr. J. Psychiatry Relat. Sci.* **47**: 125–131.

58. Chiang, M, Reiss, AL, Lee, AD, Bellugi, U, Galaburda, AM, Korenberg, JR, *et al.* (2007). 3D pattern of brain abnormalities in Williams syndrome visualized using tensor-based morphometry. *Neuroimage* **36**: 1096–109.

59. Reiss, AL, Eliez, S, Schmitt, JE, Straus, E, Lai, Z, Jones, W, *et al.* (2000). IV. Neuroanatomy of Williams syndrome: a high-resolution MRI study. *J. Cogn. Neurosci.* **12 Suppl 1**: 65–73.

60. Faria, AV, Landau, B, O'Hearn, KM, Li, X, Jiang, H, Oishi, K, *et al.* (2012). Quantitative analysis of gray and white matter in Williams syndrome. *Neuroreport* **23**: 283–9.

61. Reiss, AL, Eckert, MA, Rose, FE, Karchemskiy, A, Kesler, S, Chang, M, *et al.* (2004). An experiment of nature: brain anatomy parallels cognition and behavior in Williams syndrome. *J. Neurosci.* **24**: 5009–15.

62. Schmitt, JE, Watts, K, Eliez, S, Bellugi, U, Galaburda, AM and Reiss, AL (2002). Increased gyrification in Williams syndrome: evidence using 3D MRI methods. *Dev. Med. Child Neurol.* **44**: 292–5.

63. Gaser, C, Luders, E, Thompson, PM, Lee, AD, Dutton, R a, Geaga, J a, *et al.* (2006). Increased local gyrification mapped in Williams syndrome. *Neuroimage* **33**: 46–54.

64. Thompson, PM, Lee, AD, Dutton, R a, Geaga, J a, Hayashi, KM, Eckert, M a, *et al.* (2005). Abnormal cortical complexity and thickness profiles mapped in Williams syndrome. *J. Neurosci.* **25**: 4146–58.

65. Schmitt, JE, Eliez, S, Bellugi, U and Reiss, AL (2001). Analysis of cerebral shape in Williams syndrome. *Arch. Neurol.* **58**: 283–7.

66. Galaburda, AM and Bellugi, U (2000). V. Multi-Level Analysis of Cortical Neuroanatomy in Williams Syndrome. *J. Cogn. Neurosci.* **12**: 74–88.

67. Sampaio, A, Bouix, S, Sousa, N, Vasconcelos, C, Férnandez, M, Shenton, ME, *et al.* (2013). Morphometry of corpus callosum in Williams syndrome: shape as an index of neural development. *Brain Struct. Funct.* **218**: 711–20.

68. Tomaiuolo, F, Di Paola, M, Caravale, B, Vicari, S, Petrides, M and Caltagirone, C (2002). Morphology and morphometry of the corpus callosum in Williams syndrome: a T1-weighted MRI study. *Neuroreport* **13**: 2281–4.

69. Adhikari, A, Lerner, TN, Finkelstein, J, Pak, S, Jennings, JH, Davidson, TJ, *et al.* (2015). Basomedial amygdala mediates top-down control of anxiety and fear. *Nature* **527**: 179–85.

70. Amaral, DG (2003). The amygdala, social behavior, and danger detection. *Ann. N. Y. Acad. Sci.* **1000**: 337–47.

71. Capitão, L, Sampaio, A, Sampaio, C, Vasconcelos, C, Férnandez, M, Garayzábal, E, *et al.* (2011). MRI amygdala volume in Williams Syndrome. *Res. Dev. Disabil.* **32**: 2767–72.

72. Meyer-Lindenberg, A, Hariri, AR, Munoz, KE, Mervis, CB, Mattay, VS, Morris, C a, *et al.* (2005). Neural correlates of genetically abnormal social cognition in Williams syndrome. *Nat. Neurosci.* **8**: 991–3.

73. Avery, SN, Thornton-Wells, T a, Anderson, AW and Blackford, JU (2012). White matter integrity deficits in prefrontal-amygdala pathways in Williams syndrome. *Neuroimage* **59**: 887–94.

74. Meyer-Lindenberg, A, Mervis, CB and Berman, KF (2006). Neural mechanisms in Williams syndrome: a unique window to genetic influences on cognition and behaviour. *Nat. Rev. Neurosci.* **7**: 380–93.

75. Shrager, Y, Bayley, PJ, Bontempi, B, Hopkins, RO and Squire, LR (2007). Spatial memory and the human hippocampus. *Proc Natl Acad Sci U S A* **104**: 2961–2966.

76. Grasby, PM, Frith, CD, Friston, K, Frackowiak, RS and Dolan, RJ (1993). Activation of the human hippocampal formation during auditory-verbal long-term memory function. *Neurosci. Lett.* **163**: 185–8.

77. Meyer-Lindenberg, A, Mervis, CB, Sarpal, D, Koch, P, Steele, S, Kohn, P, *et al.* (2005). Functional, structural, and metabolic abnormalities of the hippocampal formation in Williams syndrome. *J. Clin. Invest.* **115**: 1888–1895.

78. Paul, BM, Stiles, J, Passarotti, A, Bavar, N and Bellugi, U (2002). Face and place processing in Williams syndrome: evidence for a dorsal-ventral dissociation. *Neuroreport* **13**: 1115–9.

79. Galaburda, AM, Wang, PP, Bellugi, U and Rossen, M (1994). Cytoarchitectonic anomalies in a genetically based disorder: Williams syndrome. *Neuroreport* **5**: 753–7.

80. Galaburda, AM, Holinger, DP, Bellugi, U and Sherman, GF (2002). Williams syndrome: neuronal size and neuronal-packing density in primary visual cortex. *Arch. Neurol.* **59**: 1461–7.

81. Liégeois-Chauvel, C, Peretz, I, Babaï, M, Laguitton, V and Chauvel, P (1998). Contribution of different cortical areas in the temporal lobes to music processing. *Brain* **121**: 1853–1867.

82. Hickok, G, Bellugi, U and Jones, W (1995). Asymmetrical ability. *Science* **270**:

219–20.

83. Pantev, C, Oostenveld, R, Engelien, A, Ross, B, Roberts, LE and Hoke, M (1998). Increased auditory cortical representation in musicians. *Nature* **392**: 811–4.

84. Stankiewicz, P and Lupski, JR (2002). Genome architecture, rearrangements and genomic disorders. *Trends Genet.* **18**: 74–82.

85. Valero, MC, de Luis, O, Cruces, J and Pérez Jurado, L a (2000). Fine-scale comparative mapping of the human 7q11.23 region and the orthologous region on mouse chromosome 5G: the low-copy repeats that flank the Williams-Beuren syndrome deletion arose at breakpoint sites of an evolutionary inversion(s). *Genomics* **69**: 1–13.

86. Bayés, M, Magano, LF, Rivera, N, Flores, R and Pérez Jurado, LA (2003). Mutational mechanisms of Williams-Beuren syndrome deletions. *Am. J. Hum. Genet.* **73**: 131–51.

87. Merla, G, Brunetti-Pierri, N, Micale, L and Fusco, C (2010). Copy number variants at Williams-Beuren syndrome 7q11.23 region. *Hum. Genet.* **128**: 3–26.

88. Merla, G, Unit, MG, Casa, I and Hospital, S (2012). Molecular Genetics of Williams – Beuren Syndromedoi:10.1002/9780470015902.a0022436.

89. Pérez Jurado, AL (2003). Williams-Beuren syndrome: a model of recurrent genomic mutation. *Horm. Res.* **59 Suppl 1**: 106–13.

90. Howald, C, Merla, G, Digilio, MC, Amenta, S, Lyle, R, Deutsch, S, *et al.* (2006). Two high throughput technologies to detect segmental aneuploidies identify new Williams-Beuren syndrome patients with atypical deletions. *J. Med. Genet.* **43**: 266–73.

91. Turner, DJ, Miretti, M, Rajan, D, Fiegler, H, Carter, NP, Blayney, ML, *et al.* (2008). Germline rates of de novo meiotic deletions and duplications causing several genomic disorders. *Nat. Genet.* **40**: 90–5.

92. Baumer, A, Dutly, F, Balmer, D, Riegel, M, Tükel, T, Krajewska-Walasek, M, *et al.* (1998). High level of unequal meiotic crossovers at the origin of the 22q11. 2 and 7q11.23 deletions. *Hum. Mol. Genet.* **7**: 887–94.

93. Urbán, Z, Helms, C, Fekete, G, Csiszár, K, Bonnet, D, Munnich, A, *et al.* (1996). 7q11.23 deletions in Williams syndrome arise as a consequence of unequal meiotic crossover. *Am. J. Hum. Genet.* **59**: 958–62.

94. Schubert, C (2009). The genomic basis of the Williams-Beuren syndrome. *Cell. Mol. Life Sci.* **66**: 1178–97.

95. Morris, CA, Thomas, IT and Greenberg, F (1993). Williams syndrome: Autosomal dominant inheritance. *Am. J. Med. Genet.* **47**: 478–481.

96. Sadler, LS, Robinson, LK, Verdaasdonk, KR and Gingell, R (1993). The Williams syndrome: evidence for possible autosomal dominant inheritance. *Am. J. Med. Genet.* **47**: 468–70.

97. Castorina, P, Selicorni, A, Bedeschi, F, Dalprà, L and Larizza, L (1997). Genotype-phenotype correlation in two sets of monozygotic twins with Williams syndrome. *Am. J. Med. Genet.* **69**: 107–11.

98. Cuscó, I, Corominas, R, Bayés, M, Flores, R, Rivera-Brugués, N, Campuzano, V, *et al.* (2008). Copy number variation at the 7q11.23 segmental duplications is a susceptibility factor for the Williams-Beuren

syndrome deletion. *Genome Res.* **18**: 683–694.

99. Somerville, MJ, Mervis, CB, Young, EJ, Seo, E-J, del Campo, M, Bamforth, S, *et al.* (2005). Severe expressive-language delay related to duplication of the Williams-Beuren locus. *N. Engl. J. Med.* **353**: 1694–701.

100. Van der Aa, N, Rooms, L, Vandeweyer, G, van den Ende, J, Reyniers, E, Fichera, M, *et al.* (2009). Fourteen new cases contribute to the characterization of the 7q11.23 microduplication syndrome. *Eur. J. Med. Genet.* **52**: 94–100.

101. Berg, JS, Brunetti-Pierri, N, Peters, SU, Kang, S-HL, Fong, C, Salamone, J, *et al.* (2007). Speech delay and autism spectrum behaviors are frequently associated with duplication of the 7q11.23 Williams-Beuren syndrome region. *Genet. Med.* **9**: 427–41.

102. Beunders, G, van de Kamp, JM, Veenhoven, RH, van Hagen, JM, Nieuwint, AWM and Sistermans, EA (2010). A triplication of the Williams-Beuren syndrome region in a patient with mental retardation, a severe expressive language delay, behavioural problems and dysmorphisms. *J. Med. Genet.* **47**: 271–5.

103. Osborne, LR, Li, M, Pober, B, Chitayat, D, Bodurtha, J, Mandel, A, *et al.* (2001). A 1.5 million-base pair inversion polymorphism in families with Williams-Beuren syndrome. *Nat. Genet.* **29**: 321–5.

104. Hobart, HH, Morris, CA, Mervis, CB, Pani, AM, Kistler, DJ, Rios, CM, *et al.* (2010). Inversion of the Williams syndrome region is a common polymorphism found more frequently in parents of children with Williams syndrome. *Am. J. Med. Genet. C. Semin. Med. Genet.* **154C**: 220–8.

105. Fusco, C, Micale, L, Augello, B, Teresa Pellico, M, Menghini, D, Alfieri, P, *et al.* (2014). Smaller and larger deletions of the Williams Beuren syndrome region implicate genes involved in mild facial phenotype, epilepsy and autistic traits. *Eur. J. Hum. Genet.* **22**: 64–70.

106. Metcalfe, K, Rucka, AK, Smoot, L, Hofstadler, G, Tuzler, G, Mckeown, P, *et al.* (2000). Elastin : mutational spectrum in supravalvular aortic stenosis: 955–963.

107. Ewart, AK, Morris, CA, Atkinson, D, Jin, W, Sternes, K, Spallone, P, *et al.* (1993). Hemizygosity at the elastin locus in a developmental disorder, Williams syndrome. *Nat. Genet.* **5**: 11–16.

108. Francke, U (1999). Williams-Beuren syndrome: Genes and mechanisms. *Hum. Mol. Genet.* **8**: 1947–1954.

109. Osborne, LR (2010). Animal models of Williams syndrome. *Am. J. Med. Genet. C. Semin. Med. Genet.* **154C**: 209–19.

110. DeSilva, U, Massa, H, Trask, BJ and Green, ED (1999). Comparative mapping of the region of human chromosome 7 deleted in Williams syndrome. *Genome Res.* **9**: 428–436.

111. Li, HH, Roy, M, Kuscuoglu, U, Spencer, CM, Halm, B, Harrison, KC, *et al.* (2009). Induced chromosome deletions cause hypersociability and other features of Williams-Beuren syndrome in mice. *EMBO Mol. Med.* **1**: 50–65.

112. Segura-Puimedon, M, Sahún, I, Velot, E, Dubus, P, Borralleras, C, Rodrigues, AJ, *et al.* (2014). Heterozygous deletion of the Williams-Beuren syndrome critical interval in mice recapitulates most features of the human disorder. *Hum. Mol. Genet.* **23**: 6481–94.

113. Antonell, A, Vilardell, M and Pérez Jurado, LA (2010). Transcriptome profile in Williams-Beuren syndrome lymphoblast cells reveals gene pathways implicated in glucose intolerance and visuospatial construction deficits. *Hum. Genet.* **128**: 27–37.

114. Henrichsen, CN, Csárdi, G, Zabot, M-T, Fusco, C, Bergmann, S, Merla, G, *et al.* (2011). Using transcription modules to identify expression clusters perturbed in Williams-Beuren syndrome. *PLoS Comput. Biol.* **7**: e1001054.

115. Yu, J, Vodyanik, MA, Smuga-Otto, K, Antosiewicz-Bourget, J, Frane, JL, Tian, S, *et al.* (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**: 1917–20.

116. Qiang, L, Fujita, R and Abeliovich, A (2013). Remodeling neurodegeneration: somatic cell reprogramming-based models of adult neurological disorders. *Neuron* **78**: 957–69.

117. Adamo, A, Atashpaz, S, Germain, P-L, Zanella, M, D'Agostino, G, Albertin, V, *et al.* (2014). 7Q11.23 dosage-dependent dysregulation in human pluripotent stem cells affects transcriptional programs in disease-relevant lineages. *Nat. Genet.*doi:10.1038/ng.3169.

118. Khattak, S, Brimble, E, Zhang, W, Zaslavsky, K, Strong, E, Ross, PJ, *et al.* (2015). Human induced pluripotent stem cell derived neurons as a model for Williams-Beuren syndrome. *Mol. Brain* **8**: 77.

119. Hinsley, TA, Cunliffe, P, Tipney, HJ, Brass, A and Tassabehji, M (2004). Comparison of TFII-I gene family members deleted in Williams-Beuren syndrome. *Protein Sci.* **13**: 2588–99.

120. Roy, AL (2001). Biochemistry and biology of the inducible multifunctional transcription factor TFII-I. *Gene* **274**: 1–13.

121. Tipney, HJ, Hinsley, TA, Brass, A, Metcalfe, K, Donnai, D and Tassabehji, M (2004). Isolation and characterisation of GTF2IRD2, a novel fusion gene and member of the TFII-I family of transcription factors, deleted in Williams–Beuren syndrome. *Eur. J. Hum. Genet.* **12**: 551–560.

122. Danoff, S., Taylor, H., Blackshaw, S and Desiderio, S (2004). TFII-I, a candidate gene for Williams syndrome cognitive profile: parallels between regional expression in mouse brain and human phenotype. *Neuroscience* **123**: 931–938.

123. Franke, Y, Peoples, RJ and Francke, U (1999). Identification of GTF2IRD1, a putative transcription factor within the Williams-Beuren syndrome deletion at 7q11.23. *Cytogenet. Genome Res.* **86**: 296–304.

124. Edelmann, L, Prosnitz, A, Pardo, S, Bhatt, J, Cohen, N, Lauriat, T, *et al.* (2007). An atypical deletion of the Williams-Beuren syndrome interval implicates genes associated with defective visuospatial processing and autism. *J. Med. Genet.* **44**: 136–43.

125. Antonell, A, Del Campo, M, Magano, LF, Kaufmann, L, de la Iglesia, JM, Gallastegui, F, *et al.* (2010). Partial 7q11.23 deletions further implicate GTF2I and GTF2IRD1 as the main genes responsible for the Williams-Beuren syndrome neurocognitive profile. *J. Med. Genet.* **47**: 312–20.

126. Dai, L, Bellugi, U, Chen, X-N, Pulst-Korenberg, AM, Järvinen-Pasley, A, Tirosh-Wagner, T, *et al.* (2009). Is it Williams syndrome? GTF2IRD1 implicated in visual-spatial construction and GTF2I in sociability revealed by high resolution arrays. *Am. J. Med. Genet. A* **149A**: 302–14.

127. Ferrero, GB, Howald, C, Micale, L, Biamino, E, Augello, B, Fusco, C, *et al.* (2010). An atypical 7q11.23 deletion in a normal IQ Williams-Beuren syndrome patient. *Eur. J. Hum. Genet.* **18**: 33–8.

128. Collette, JC, Chen, X-N, Mills, DL, Galaburda, AM, Reiss, AL, Bellugi, U, *et al.* (2009). William's syndrome: gene expression is related to parental origin and regional coordinate control. *J. Hum. Genet.* **54**: 193–198.

129. Lucena, J, Pezzi, S, Aso, E, Valero, MC, Carreiro, C, Dubus, P, *et al.* (2010). Essential role of the N-terminal region of TFII-I in viability and behavior. *BMC Med. Genet.* **11**: 61.

130. Sakurai, T, Dorr, NP, Takahashi, N, McInnes, LA, Elder, GA and Buxbaum, JD (2011). Haploinsufficiency of Gtf2i, a gene deleted in Williams Syndrome, leads to increases in social interactions. *Autism Res.* **4**: 28–39.

131. van Hagen, JM, van der Geest, JN, van der Giessen, RS, Lagers-van Haselen, GC, Eussen, HJFMM, Gille, JJP, *et al.* (2007). Contribution of CYLN2 and GTF2IRD1 to neurological and cognitive symptoms in Williams Syndrome. *Neurobiol. Dis.* **26**: 112–24.

132. Antonell, A, de Luis, O, Domingo-Roura, X and Pérez-Jurado, LA (2005). Evolutionary mechanisms shaping the genomic structure of the Williams-Beuren syndrome chromosomal region at human 7q11.23. *Genome Res.* **15**: 1179–88.

133. Young, EJ, Lipina, T, Tam, E, Mandel, A, Clapcote, SJ, Bechard, AR, *et al.* (2008). Reduced fear and aggression and altered serotonin metabolism in Gtf2ird1-targeted mice. *Genes, Brain Behav.* **7**: 224–234.

134. Tassabehji, M, Hammond, P, Karmiloff-smith, A, Thompson, P, Thorgeirsson, SS, Durkin, ME, *et al.* (2005). GTF2IRD1 in Craniofacial Development of Humans and Mice **310**: 1184–1188.

135. Schneider, T, Skitt, Z, Liu, Y, Deacon, RMJ, Flint, J, Karmiloff-Smith, A, *et al.* (2012). Anxious, hypoactive phenotype combined with motor deficits in Gtf2ird1 null mouse model relevant to Williams syndrome. *Behav. Brain Res.* **233**: 458–473.

136. Howard, ML, Palmer, SJ, Taylor, KM, Arthurson, GJ, Spitzer, MW, Du, X, *et al.* (2012). Mutation of Gtf2ird1 from the Williams-Beuren syndrome critical region results in facial dysplasia, motor dysfunction, and altered vocalisations. *Neurobiol. Dis.* **45**: 913–22.

137. Enkhmandakh, B, Makeyev, A V., Erdenechimeg, L, Ruddle, FH, Chimge, N-ON-O, Tussie-Luna, MI, *et al.* (2009). Essential functions of the Williams-Beuren syndrome-associated TFII-I genes in embryonic development. *Proc. Natl. Acad. Sci. U. S. A.* **106**: 181–6.

138. Carmona-Mora, P, Widagdo, J, Tomasetig, F, Canales, CP, Cha, Y, Lee, W, *et al.* (2015). The nuclear localization pattern and interaction partners of GTF2IRD1 demonstrate a role in chromatin regulation. *Hum. Genet.* **134**: 1099–115.

139. Porter, MA, Dobson-Stone, C, Kwok, JBJ, Schofield, PR, Beckett, W and Tassabehji, M (2012). A role for transcription factor GTF2IRD2 in executive function in Williams-Beuren syndrome. *PLoS One* **7**: e47457.

140. Palmer, SJ, Taylor, KM, Santucci, N, Widagdo, J, Chan, Y-KA, Yeo, J-L, *et al.* (2012). GTF2IRD2 from the Williams-Beuren critical region encodes a mobile-element-derived fusion protein that antagonizes the action of its related family

members. *J. Cell Sci.* **125**: 5040–50.

141. Pröschel, C, Blouin, MJ, Gutowski, NJ, Ludwig, R and Noble, M (1995). Limk1 is predominantly expressed in neural tissues and phosphorylates serine, threonine and tyrosine residues in vitro. *Oncogene* **11**: 1271–81.

142. Rosso, S, Bollati, F, Bisbal, M, Peretti, D, Sumi, T, Nakamura, T, *et al.* (2004). LIMK1 regulates Golgi dynamics, traffic of Golgi-derived vesicles, and process extension in primary cultured neurons. *Mol. Biol. Cell* **15**: 3433–49.

143. Scott, RW and Olson, MF (2007). LIM kinases: function, regulation and association with human disease. *J. Mol. Med. (Berl).* **85**: 555–68.

144. Frangiskakis, JM, Ewart, AK, Morris, CA, Mervis, CB, Bertrand, J, Robinson, BF, *et al.* (1996). LIM-kinase1 hemizygosity implicated in impaired visuospatial constructive cognition. *Cell* **86**: 59–69.

145. Gray, V, Karmiloff-Smith, A, Funnell, E and Tassabehji, M (2006). In-depth analysis of spatial cognition in Williams syndrome: A critical assessment of the role of the LIMK1 gene. *Neuropsychologia* **44**: 679–85.

146. Tassabehji, M, Metcalfe, K, Karmiloff-Smith, A, Carette, MJ, Grant, J, Dennis, N, *et al.* (1999). Williams syndrome: use of chromosomal microdeletions as a tool to dissect cognitive and physical phenotypes. *Am. J. Hum. Genet.* **64**: 118–25.

147. Meng, Y, Zhang, Y, Tregoubov, V, Janus, C, Cruz, L, Jackson, M, *et al.* (2002). Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron* **35**: 121–33.

148. Todorovski, Z, Asrar, S, Liu, J, Saw, NMN, Joshi, K, Cortez, MA, *et al.* (2015). LIMK1 regulates long-term memory and synaptic plasticity via the transcriptional factor CREB. *Mol. Cell. Biol.* **35**: 1316–28.

149. Wang, YK, Samos, CH, Peoples, R, Pérez-Jurado, LA, Nusse, R and Francke, U (1997). A novel human homologue of the Drosophila frizzled wnt receptor gene binds wingless protein and is in the Williams syndrome deletion at 7q11.23. *Hum. Mol. Genet.* **6**: 465–72.

150. Karasawa, T, Yokokura, H, Kitajewski, J and Lombroso, PJ (2002). Frizzled-9 is activated by Wnt-2 and functions in Wnt/beta -catenin signaling. *J. Biol. Chem.* **277**: 37479–86.

151. Botta, A, Novelli, G, Mari, A, Novelli, A, Sabani, M, Korenberg, J, *et al.* (1999). Detection of an atypical 7q11.23 deletion in Williams syndrome patients which does not include the STX1A and FZD3 genes. *J. Med. Genet.* **36**: 478–80.

152. Zhao, C, Avilés, C, Abel, R a, Almli, CR, McQuillen, P and Pleasure, SJ (2005). Hippocampal and visuospatial learning defects in mice with a deletion of frizzled 9, a gene in the Williams syndrome deletion interval. *Development* **132**: 2917–27.

153. Ranheim, EA, Kwan, HCK, Reya, T, Wang, Y-K, Weissman, IL and Francke, U (2005). Frizzled 9 knock-out mice have abnormal B-cell development. *Blood* **105**: 2487–94.

154. Albers, J, Schulze, J, Beil, FT, Gebauer, M, Baranowsky, A, Keller, J, *et al.* (2011). Control of bone formation by the serpentine receptor Frizzled-9. *J. Cell Biol.* **192**: 1057–72.

155. Avilés, EC, Pinto, C, Hanna, P, Ojeda, J, Pérez, V, De Ferrari, G V, *et al.* (2014). Frizzled-9 impairs acetylcholine receptor clustering in skeletal muscle

cells. *Front. Cell. Neurosci.* **8**: 110.

156. Hoogenraad, CC, Koekkoek, B, Akhmanova, A, Krugers, H, Dortland, B, Miedema, M, *et al.* (2002). Targeted mutation of Cyln2 in the Williams syndrome critical region links CLIP-115 haploinsufficiency to neurodevelopmental abnormalities in mice. *Nat. Genet.* **32**: 116–27.

157. Vandeweyer, G, Van der Aa, N, Reyniers, E and Kooy, RF (2012). The contribution of CLIP2 haploinsufficiency to the clinical manifestations of the Williams-Beuren syndrome. *Am. J. Hum. Genet.* **90**: 1071–8.

158. Richter-Cook, NJ, Dever, TE, Hensold, JO and Merrick, WC (1998). Purification and characterization of a new eukaryotic protein translation factor. Eukaryotic initiation factor 4H. *J. Biol. Chem.* **273**: 7579–87.

159. Capossela, S, Muzio, L, Bertolo, A, Bianchi, V, Dati, G, Chaabane, L, *et al.* (2012). Growth defects and impaired cognitive-behavioral abilities in mice with knockout for Eif4h, a gene located in the mouse homolog of the Williams-Beuren syndrome critical region. *Am. J. Pathol.* **180**: 1121–35.

160. Cavellán, E, Asp, P, Percipalle, P and Farrants, A-KO (2006). The WSTF-SNF2h chromatin remodeling complex interacts with several nuclear proteins in transcription. *J. Biol. Chem.* **281**: 16264–71.

161. Xiao, A, Li, H, Shechter, D, Ahn, SH, Fabrizio, LA, Erdjument-Bromage, H, *et al.* (2009). WSTF regulates the H2A.X DNA damage response via a novel tyrosine kinase activity. *Nature* **457**: 57–62.

162. Ashe, A, Morgan, DK, Whitelaw, NC, Bruxner, TJ, Vickaryous, NK, Cox, LL, *et al.* (2008). A genome-wide screen for modifiers of transgene variegation identifies genes with critical roles in development. *Genome Biol.* **9**: R182.

163. Lalli, MA, Jang, J, Park, JC, Wang, Y, Guzman, E, Zhou, H, *et al.* (2016). Haploinsufficiency of BAZ1B contributes to Williams syndrome through transcriptional dysregulation of neurodevelopmental pathways: 1–38.

164. Zhang, R, Maksymowych, AB and Simpson, LL (1995). Cloning and sequence analysis of a cDNA encoding human syntaxin 1A, a polypeptide essential for exocytosis. *Gene* **159**: 293–4.

165. McNew, JA, Parlati, F, Fukuda, R, Johnston, RJ, Paz, K, Paumet, F, *et al.* (2000). Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature* **407**: 153–9.

166. Wong, AHC, Trakalo, J, Likhodi, O, Yusuf, M, Macedo, A, Azevedo, M-H, *et al.* (2004). Association between schizophrenia and the syntaxin 1A gene. *Biol. Psychiatry* **56**: 24–9.

167. Nakamura, K, Anitha, A, Yamada, K, Tsujii, M, Iwayama, Y, Hattori, E, *et al.* (2008). Genetic and expression analyses reveal elevated expression of syntaxin 1A (STX1A) in high functioning autism. *Int. J. Neuropsychopharmacol.* **11**: 1073–84.

168. McRory, JE, Rehak, R, Simms, B, Doering, CJ, Chen, L, Hermosilla, T, *et al.* (2008). Syntaxin 1A is required for normal in utero development. *Biochem. Biophys. Res. Commun.* **375**: 372–7.

169. Fujiwara, T, Mishima, T, Kofuji, T, Chiba, T, Tanaka, K, Yamamoto, A, *et al.* (2006). Analysis of knock-out mice to determine the role of HPC-1/syntaxin 1A in expressing synaptic plasticity. *J. Neurosci.* **26**: 5767–76.

170. Fujiwara, T, Snada, M, Kofuji, T, Yoshikawa, T and Akagawa, K (2010). HPC-

1/syntaxin 1A gene knockout mice show abnormal behavior possibly related to a disruption in 5-HTergic systems. *Eur. J. Neurosci.* **32**: 99–107.

171. Faury, G, Pezet, M, Knutsen, RH, Boyle, WA, Heximer, SP, McLean, SE, *et al.* (2003). Developmental adaptation of the mouse cardiovascular system to elastin haploinsufficiency. *J. Clin. Invest.* **112**: 1419–1428.

172. Campuzano, V, Segura-Puimedon, M, Terrado, V, Sánchez-Rodríguez, C, Coustets, M, Menacho-Márquez, M, *et al.* (2012). Reduction of NADPH-oxidase activity ameliorates the cardiovascular phenotype in a mouse model of Williams-Beuren Syndrome. *PLoS Genet.* **8**: e1002458.

173. Landmesser, U, Cai, H, Dikalov, S, McCann, L, Hwang, J, Jo, H, *et al.* (2002). Role of p47phox in Vascular Oxidative Stress and Hypertension Caused by Angiotensin II. *Hypertension* **40**: 511–515.

174. Cairo, S (2001). WBSCR14, a gene mapping to the Williams-Beuren syndrome deleted region, is a new member of the Mlx transcription factor network. *Hum. Mol. Genet.* **10**: 617–627.

175. Kooner, JS, Chambers, JC, Aguilar-Salinas, CA, Hinds, DA, Hyde, CL, Warnes, GR, *et al.* (2008). Genome-wide scan identifies variation in MLXIPL associated with plasma triglycerides. *Nat. Genet.* **40**: 149–51.

176. Palacios-Verdu, MG, Segura-Puimedon, M, Borralleras, C, Flores, R, Del Campo, M, Campuzano, V, *et al.* (2015). Metabolic abnormalities in Williams-Beuren syndrome. *J. Med. Genet.*: 1–8doi:10.1136/jmedgenet-2014-102713.

177. IIZUKA, K and HORIKAWA, Y (2008). ChREBP: A Glucose-activated Transcription Factor Involved in the Development of Metabolic Syndrome. *Endocr. J.* **55**: 617–624.

178. Lam, PPL, Leung, Y-M, Sheu, L, Ellis, J, Tsushima, RG, Osborne, LR, *et al.* (2005). Transgenic Mouse Overexpressing Syntaxin-1A as a Diabetes Model. *Diabetes* **54**: 2744–2754.

179. Ohara-Imaizumi, M, Fujiwara, T, Nakamichi, Y, Okamura, T, Akimoto, Y, Kawai, J, *et al.* (2007). Imaging analysis reveals mechanistic differences between first- and second-phase insulin exocytosis. *J. Cell Biol.* **177**: 695–705.

180. Peoples, R, Franke, Y, Wang, YK, Pérez-Jurado, L, Paperna, T, Cisco, M, *et al.* (2000). A physical map, including a BAC/PAC clone contig, of the Williams-Beuren syndrome--deletion region at 7q11.23. *Am. J. Hum. Genet.* **66**: 47–68.

181. Thomas, A, Burant, A, Bui, N, Graham, D, Yuva-Paylor, LA and Paylor, R (2009). Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology (Berl).* **204**: 361–373.

182. Takeuchi, H, Yatsugi, S and Yamaguchi, T (2002). Effect of YM992, a novel antidepressant with selective serotonin re-uptake inhibitory and 5-HT 2A receptor antagonistic activity, on a marble-burying behavior test as an obsessive-compulsive disorder model. *Jpn. J. Pharmacol.* **90**: 197–200.

183. Njung'e, K and Handley, SL (1991). Evaluation of marble-burying behavior as a model of anxiety. *Pharmacol. Biochem. Behav.* **38**: 63–7.

184. Withers, S (1996). A new clinical sign in Williams syndrome. *Arch. Dis. Child.*: 89–89.

185. Voit, T, Kramer, H, Thomas, C, Wechsler, W, Reichmann, H and Lenard, HG

(1991). Myopathy in Williams-Beuren syndrome. *Eur. J. Pediatr.* **150**: 521–6.

186. Vicari, S, Bellucci, S and Carlesimo, GA (2003). Visual and spatial working memory dissociation: Evidence from Williams syndrome. *Dev. Med. Child Neurol.* **45**: 269–273.

187. O'Hearn, K, Courtney, S, Street, W and Landau, B (2009). Working memory impairment in people with Williams syndrome: effects of delay, task and stimuli. *Brain Cogn.* **69**: 495–503.

188. Fields, RD (2010). Central role of glia in disease research. *Neuron Glia Biol.* **6**: 91–2.

189. Almad, AA and Maragakis, NJ (2012). Glia: an emerging target for neurological disease therapy. *Stem Cell Res. Ther.* **3**: 37.

190. Porrero, C, Rubio-Garrido, P, Avendaño, C and Clascá, F (2010). Mapping of fluorescent protein-expressing neurons and axon pathways in adult and developing Thy1-eYFP-H transgenic mice. *Brain Res.* **1345**: 59–72.

191. Feng, G, Mellor, RH, Bernstein, M, Keller-Peck, C, Nguyen, QT, Wallace, M, *et al.* (2000). Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* **28**: 41–51.

192. Stuss, DP, Boyd, JD, Levin, DB and Delaney, KR (2012). MeCP2 mutation results in compartment-specific reductions in dendritic branching and spine density in layer 5 motor cortical neurons of YFP-H mice. *PLoS One* **7**: e31896.

193. Nimchinsky, EA, Oberlander, AM and Svoboda, K (2001). Abnormal development of dendritic spines in FMR1 knock-out mice. *J. Neurosci.* **21**: 5139–46.

194. Dierssen, M, Benavides-Piccione, R, Martínez-Cué, C, Estivill, X, Flórez, J, Elston, GN, *et al.* (2003). Alterations of neocortical pyramidal cell phenotype in the Ts65Dn mouse model of Down syndrome: effects of environmental enrichment. *Cereb. Cortex* **13**: 758–64.

195. Hoogenraad, CC, Akhmanova, A, Galjart, N and De Zeeuw, CI (2004). LIMK1 and CLIP-115: linking cytoskeletal defects to Williams syndrome. *Bioessays* **26**: 141–50.

196. Caraveo, G, van Rossum, DB, Patterson, RL, Snyder, SH and Desiderio, S (2006). Action of TFII-I outside the nucleus as an inhibitor of agonist-induced calcium entry. *Science* **314**: 122–5.

197. Splawski, I, Timothy, KW, Sharpe, LM, Decher, N, Kumar, P, Bloise, R, *et al.* (2004). Ca(V)1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. *Cell* **119**: 19–31.

198. Lidow, MS (2003). Calcium signaling dysfunction in schizophrenia: a unifying approach. *Brain Res. Brain Res. Rev.* **43**: 70–84.

199. Malenka, RC and Bear, MF (2004). LTP and LTD : An Embarrassment of Riches **44**: 5–21.

200. Mercaldo, V, Descalzi, G and Zhuo, M (2009). Fragile X mental retardation protein in learning-related synaptic plasticity. *Mol. Cells* **28**: 501–7.

201. Siarey, RJ, Stoll, J, Rapoport, SI and Galdzicki, Z. Altered long-term potentiation in the young and old Ts65Dn mouse, a model for Down Syndrome. *Neuropharmacology* **36**: 1549–54.

202. Moretti, P, Levenson, JM, Battaglia, F, Atkinson, R, Teague, R, Antalffy, B, *et al.* (2006). Learning and memory and synaptic plasticity are impaired in a mouse model of Rett syndrome. *J. Neurosci.* **26**: 319–27.

203. Sala, C, Piëch, V, Wilson, NR, Passafaro, M, Liu, G and Sheng, M (2001). Regulation of dendritic spine morphology and synaptic function by Shank and Homer. *Neuron* **31**: 115–130.

204. Gomez, AM, Froemke, RC and Burden, SJ (2014). Synaptic plasticity and cognitive function are disrupted in the absence of Lrp4. *Elife* **3**: 1–16.

205. Thoenen, H (1995). Neurotrophins and neuronal plasticity. *Science* **270**: 593–8.

206. Minichiello, L (2009). TrkB signalling pathways in LTP and learning. *Nat. Rev. Neurosci.* **10**: 850–860.

207. Cao, C, Rioult-Pedotti, MS, Migani, P, Yu, CJ, Tiwari, R, Parang, K, *et al.* (2013). Impairment of TrkB-PSD-95 Signaling in Angelman Syndrome. *PLoS Biol.* **11**.

208. Lauterborn, JC, Rex, CS, Kramár, E, Chen, LY, Pandyarajan, V, Lynch, G, *et al.* (2007). Brain-Derived Neurotrophic Factor Rescues Synaptic Plasticity in a Mouse Model of Fragile X Syndrome. *J Neurosci* **27**: 10685–94.

209. Lynch, G, Kramar, EA, Rex, CS, Jia, YS, Chappas, D, Gall, CM, *et al.* (2007). Brain-derived neurotrophic factor restores synaptic plasticity in a knock-in mouse model of Huntington's disease. *J. Neurosci.* **27**: 4424–4434.

210. Santos, AR, Comprido, D and Duarte, CB (2010). Regulation of local translation at the synapse by BDNF. *Prog. Neurobiol.* **92**: 505–16.

211. Sharma, A, Hoeffer, C a, Takayasu, Y, Miyawaki, T, McBride, SM, Klann, E, *et al.* (2010). Dysregulation of mTOR signaling in fragile X syndrome. *J. Neurosci.* **30**: 694–702.

212. Troca-Marín, JA, Alves-Sampaio, A and Montesinos, ML (2011). An increase in basal BDNF provokes hyperactivation of the Akt-mammalian target of rapamycin pathway and deregulation of local dendritic translation in a mouse model of Down's syndrome. *J. Neurosci.* **31**: 9445–55.

213. Ricciardi, S, Boggio, EM, Grosso, S, Lonetti, G, Forlani, G, Stefanelli, G, *et al.* (2011). Reduced AKT/mTOR signaling and protein synthesis dysregulation in a Rett syndrome animal model. *Hum. Mol. Genet.* **20**: 1182–96.

214. Segura-Puimedon, M, Borralleras, C, Pérez-Jurado, L a. and Campuzano, V (2013). TFII-I regulates target genes in the PI-3K and TGF-β signaling pathways through a novel DNA binding motif. *Gene* **527**: 529–536.

215. Chimge, N-O, Makeyev, A V, Waigel, SJ, Enkhmandakh, B and Bayarsaihan, D (2012). PI3K/Akt-dependent functions of TFII-I transcription factors in mouse embryonic stem cells. *J. Cell. Biochem.* **113**: 1122–31.

216. Büning, H, Perabo, L, Coutelle, O, Quadt-Humme, S and Hallek, M (2008). Recent developments in adeno-associated virus vector technology. *J. Gene Med.* **10**: 717–33.

217. Malenfant, P, Liu, X, Hudson, ML, Qiao, Y, Hrynchak, M, Riendeau, N, *et al.* (2012). Association of GTF2i in the Williams-Beuren syndrome critical region with autism spectrum disorders. *J. Autism Dev. Disord.* **42**: 1459–69.

218. Vignes, M, Maurice, T, Lanté, F, Nedjar, M, Thethi, K, Guiramand, J, *et al.* (2006). Anxiolytic properties of green tea polyphenol (-)-epigallocatechin

gallate (EGCG). *Brain Res.* **1110**: 102–15.

219. Guedj, F, Sébrié, C, Rivals, I, Ledru, A, Paly, E, Bizot, JC, *et al.* (2009). Green tea polyphenols rescue of brain defects induced by overexpression of DYRK1A. *PLoS One* **4**: e4606.

220. Ge, M-M, Hu, F, Lou, Z-Y, Xue, W, Yu, H, Xu, L, *et al.* (2015). Role of Wnt/β-catenin signaling in the protective effect of epigallocatechin-3-gallate on lead-induced impairments of spine formation in the hippocampus of rats. *RSC Adv.* **5**: 31622–31628.

ANNEXES

Segura-Puimedon M, Sahún I, Velot E, Dubus P, Borralleras C, Rodrigues AJ, Valero M, Valverde O, Sousa N, Herault Y, Dierssen M, Pérez-Jurado LA, Campuzano V.

Heterozygous deletion of the Williams-Beuren syndrome critical interval in mice recapitulates most features of the human disorder

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Metabolic abnormalities in Williams-Beuren Syndrome

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Segura-Puimedon M, Borralleras C, Pérez-Jurado LA, Campuzano V.

TFII-I regulates target genes in the PI-3K and TGF-β signaling pathways through a novel DNA binding motif

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Utilidad de los modelos animales en el estudio del Síndrome de Williams

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Utilidad de los modelos animales en el estudio del Síndrome de Williams

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Aunque existe una cierta variabilidad interindividual, una de las características específicas de las personas con Síndrome de Williams (SW) es su perfil cognitivo y de personalidad. El perfil cognitivo se caracteriza por un cociente intelectual medio de 50-60, indicativo de una discapacidad intelectual entre leve y moderada, asociada a claros dificultades evidentes en las funciones visuo-espaciales y en las actividades psicomotoras relacionadas, con una preservación relativa de las funciones relacionadas con el lenguaje. En cuanto a la personalidad, son personas muy amigables y desinhibidas que presentan con frecuencia diversos problemas conductuales como la ansiedad generalizada, las múltiples fobias, algunas obsesiones y compulsiones o el déficit de atención e hiperactividad (1).

Figura 1. Modelo animal de ratón.

Actualmente existe un gran número de estudios de diversos grupos de investigación que indican que *GTF2I*, un gen que se encuentra dentro de la región delecionada (y por tanto en una sola copia en lugar de dos, lo normal, en las personas con SW), es uno de los principales implicados en este perfil cognitivo y los problemas relacionados. Para conocer mejor la función de *GTF2I*, los efectos de perder una copia y sus acciones a nivel del cerebro, en nuestro grupo utilizamos modelos animales de ratón. Los modelos de ratón son muy útiles para el estudio de enfermedades humanas por su similitud genética y la posibilidad de observar en los ratones toda su trayectoria vital desde el desarrollo en útero hasta la edad adulta. Gracias a la manipulación genética de ratones se ha podido reproducir una enorme cantidad de enfermedades para entender los mecanismos alterados y probar posibles tratamientos.

La región genética que se deleciona en el SW está altamente conservada en el ratón, por lo que se han podido generar varios modelos para su estudio. Uno de ellos presenta únicamente la deleción o pérdida de *Gtf2i*, permitiendo así el estudio de la función de este gen. Estos ratones presentan alteraciones craniofaciales así como algunas de las características cognitivas presentes en los pacientes como la ansiedad, hecho que verifica que este gen está claramente involucrado en el perfil neuroconductual del SW (2). El mejor modelo es un animal con la deleción completa de la región para mimetizar al máximo la alteración genética que ocurre en humanos. Estos ratones recapitulan la mayor parte de características tanto físicas como cognitivas que presentan las personas con SW, siendo así una herramienta clave para el estudio del síndrome (3).

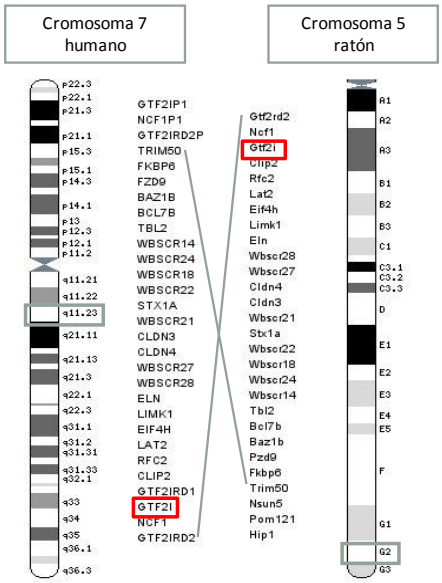

Figura 2. Región del SW en humano y en ratón. Posición de *Gtf2i* marcada en rojo.

Un estudio completado recientemente ha demostrado que el incremento de *Gtf2i* en el cerebro de los ratones mediante terapia génica mejora su comportamiento (4). La terapia génica consiste en la transferencia de material genético a las células de un individuo mediante un vector. Normalmente la finalidad de esta terapia es restaurar la función de un gen defectuoso o deficiente, en este caso la función de *Gtf2i*. Para ello, se utilizó como vector un virus inocuo denominado AAV9 al que se le insertó el gen de *Gtf2i*. El virus AAV9 se inyectó en la cisterna magna del cerebro de ratones adultos jóvenes, para intentar restaurar los niveles de *Gtf2i* en todas las regiones del cerebro de manera temporal (duración aproximada de la

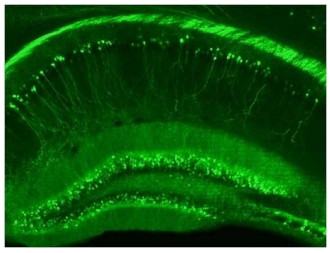

Figura 3. Neuronas hipocampales de un modelo de ratón.

acción un mes). El resultado de este estudio fue una mejora ostensible de estos ratones a nivel de ansiedad y en la coordinación motora, con una disminución también de la sociabilidad. También aumentaron los niveles de *Bdnf* en el cerebro, una molécula con un papel muy importante en la plasticidad sináptica, que es la capacidad de las neuronas para adaptarse y establecer nuevas comunicaciones entre ellas. Sin embargo, no se pudieron restaurar por completo todos los defectos ya descritos en estos ratones, persistiendo las alteraciones en la morfología neuronal y el número de dendritas (4).

Aunque estos resultados son prometedores, queda mucho trabajo por hacer. Los datos indican que efectivamente *GTF2I* es un gen muy importante para el SW y que si se logra restituir su función se puede mejorar la sintomatología, aunque no se corrijan del todo las secuelas debidas a alteraciones del desarrollo. No obstante, este tipo de terapia génica para restaurar la función cerebral en humanos aún está en fase de investigación y todavía es necesario profundizar en el conocimiento sobre su seguridad y viabilidad. Además, el gen *GTF2I* es un factor regulador de otros genes que tiene muchas funciones en diversas células del organismo, algunas todavía desconocidas, y se sabe que el exceso de función puede causar también problemas por lo que se precisa una regulación muy rigurosa. De manera alternativa o complementaria, se investiga sobre posibles fármacos que permitan de otra manera regular la función y dosis de GTF2I. La investigación sigue siendo necesaria para entender más a fondo los mecanismos patológicos y abrir así nuevas perspectivas terapéuticas.

Referencias

1. Pérez-García D, et al. (2011). Res Dev Disabil 32:643-652
2. Lucena J, et al. (2010). BMC Med Genet 11:61
3. Segura-Puimedon M, et al. (2014). Hum Mol Genet 23:6481-94
4. Borralleras C, et al. (2015). Mol Ther. 23(11):1691-9