ROLE AND THERAPEUTIC POTENTIAL OF THE ENDOCANNABINOID SYSTEM IN ANIMAL MODELS OF INTELLECTUAL DISABILITY

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A mi familia,

Caminante no hay camino,
se hace camino al andar

Antonio Machado (Proverbios y cantares)

Abstract

Intellectual disability encompasses several neurodevelopmental disorders characterized by impairments in cognitive and adaptive functioning with onset during the developmental period. Currently, treatment of intellectual disability is restricted to early intervention programs and individualized education, which have limited efficacy. Several preclinical studies indicate that intellectual disability may be improved by pharmacological interventions. This thesis is focused on the role and therapeutic potential of the endocannabinoid system in intellectual disability disorders. The relevance of the endocannabinoid system in this context is determined by a major role of this neuromodulatory system in memory and social behavior, both impaired in intellectual disability disorders. Using well-characterized mouse models of Down syndrome and Williams-Beuren syndrome and combining behavioral, pharmacological, electrophysiological and biochemical studies, we have described the improvement of memory deficits and social behavior of these models by modulating the endocannabinoid system.

Resumen

La discapacidad intelectual engloba diversas enfermedades del neurodesarrollo caracterizadas por déficits en el funcionamiento cognitivo y en la conducta adaptativa que se inician durante el período de desarrollo. Actualmente, el tratamiento de la discapacidad intelectual se limita a programas de intervención temprana y una educación personalizada los cuales tienen una eficacia limitada. Diversos estudios preclínicos indican que la discapacidad intelectual podría ser mejorada con intervenciones farmacológicas. Esta tesis se centra en el papel y el potencial terapéutico del sistema endocannabinoide en enfermedades de discapacidad intelectual. La relevancia del sistema endocannabinoide en este contexto viene determinada por el importante papel de este sistema de neuromodulación en la memoria y el comportamiento social, habilidades que se encuentran alteradas en la discapacidad intelectual. Utilizando modelos murinosbien caracterizados del síndrome de Down y del síndrome de Williams-Beuren y combinando estudios de comportamiento, farmacológicos, electrofisiológicos y bioquímicos hemos descrito mejoras en los déficits en memoria y comportamiento social de estos modelos a través de la modulación farmacológica del sistema endocannabinoide.

Abbreviations

2-AG: 2-arachidonoylglycerol
2-LG: 2-linoleoylglycerol
2-OG: 2-oleoylglycerol
2-PG: 2-palmitoylglycerol
AAV9: adeno-associated viral serotype 9
AEA: N-arachidonoylethanolamine
AMPA: methyl-4-isoxazolepropionic acid
APP: amyloid precursor protein
BDNF: brain-derived neurotrophic factor
CA: *cornu ammonis*
CB1R: cannabinoid type-1 receptor
CB2R: cannabinoid type-2 receptor
CNS: central nervous system
CV: coefficient of variation
D/P: double heterozygous
DAG: 1,2-diacylglycerol
DAGL: 1,2-diacylglycerol lipase
DD: distal deletion
DEA: N-docosatetraenoylethanolamine
DHEA: docosahexaenoylethanolamine
DS: Down syndrome
DSCR: Down syndrome critical region
DSE: depolarization-induced suppression of excitation
DSI: depolarization-induced suppression of inhibition
DYRK1A: dual specificity tyrosine-phosphorylation-regulated kinase 1A
eCB-LTD: endocannabinoid-mediated long-term depression
eCB-STD: endocannabinoid-mediated short-term depression
ECS: endocannabinoid system
eEPSCs: evoked excitatory postsynaptic currents
eIPSCs: evoked inhibitory postsynaptic currents
E-LTP: early phase long-term potentiation

EPSP: excitatory postsynaptic potential
FAAH: fatty acid amide hydrolase
fEPSP: field excitatory postsynaptic potential
FXS: fragile X syndrome
GPCR: G-protein-coupled receptors
GPR55: protein-coupled receptor 55
GSK-3: glycogen synthase kinase 3
GTF2I: general transcription factor II-I
GTF2IRD1: GTF2I repeat domain-containing protein I
GTF2IRD2: GTF2I repeat domain containing protein II
HSA21: human chromosome 21
i.p.: intraperitoneal
I-LTD: endocannabinoid-mediated long-term depression of inhibition
IQ: intelligence quotient
KO: knock-out
L-LTP: late phase long-term potentiation
LTD: long-term depression
LTP: long-term potentiation
MAGL: monoacylglycerol lipase
MAPK: mitogen-activated protein kinase
mGluR: metabotropic glutamate receptors
miRNA: micro-RNA
Mmu: *Mus musculus*
mTOR: mammalian target of rapamycin
MWM: Morris Water maze
NAPE: N-acylphosphatidylethanolamine
NAPE-PLD: NAPE-hydrolyzing phospholipase D
NMDA: N-methyl-D-aspartate
NORT: Novel object recognition test
NPRT: Novel place recognition test
PD: proximal deletion
PKA: protein kinase A
PKC: protein kinase C
PKC: protein kinase C
PLC: phospholipase C

PLD: phospholipase D
PPAR: peroxisome proliferator-activated receptor
PTPN22: protein tyrosine phosphatase N22
s.c.: subcutaneous
shRNA: short hairpin RNA
TBS: theta burst stimulation
TFII-I: transcription factor II-I
TgDyrk1A: transgenic DYRK1A
TRPV1: transient receptor potential vanilloid receptor 1
TTS: triple trisomic model
VGCC: voltage-gated Ca^{2+} channels
WBS: Williams-Beuren syndrome
WBS-CD: Williams-Beuren syndrome complete deletion
WBSCR: Williams-Beuren syndrome critical region
WT: wild-type
Δ^9-THC: Δ^9-tetrahydrocannabinol

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INTRODUCTION

INTRODUCTION

1. Intellectual disability

Intellectual disability is the term used to define neurodevelopmental disorders with onset during developmental period characterized by impairments in cognitive and adaptive functioning in conceptual, social and practical domains of living (American Psychiatric Association, 2013). Conceptual domain refers to skills in language, reading, writing, math, reasoning, knowledge and memory. Social domain encompasses empathy, social judgment, communication skills and making and retaining friends. Practical domain refers to self-management including personal care, school and job responsibilities and money management.

The prevalence of intellectual disability in general population is between 0.87 to 3.68% (Boat and Wu, 2015). Intellectual disability severely compromises the quality of life of affected individuals and their families and it has high medical, educational, social and economic burdens. It is estimated that the extra cost of an affected person throughout life is more than $1 million (Centers for Disease Control and Prevention, 2004).

The severity of intellectual disability is variable among individuals and it has been classically classified according to the intelligence quotient (IQ) score: mild (70-55), moderate (55-40), severe (40-25) and profound (<25). Nowadays, this classification is more based on daily skills than on a specific IQ range (Boat and Wu, 2015). Patients with intellectual disability usually present also other clinical manifestations which is known as syndromic intellectual disability.

Different etiologies are responsible of intellectual disability including genetic and environmental factors. Moreover, in some of the cases the cause is unknown. Environmental factors comprise prenatal exposure to teratogens (alcohols and drugs, chemicals or radiation), intrauterine infections, maternal malnutrition, premature birth, perinatal trauma or asphyxia, neonatal hypothyroidism and socio-economic and cultural factors (Kaufman *et al.*, 2010).

Genetic causes include chromosomal abnormalities (aneuploidies, deletions, translocations and duplications) and single gene mutations (Dierssen and Ramakers, 2006). The most common genetic cause of intellectual disability is **Down syndrome (DS)** whereas the most common inherited cause of intellectual disability is fragile X syndrome (FXS). Other genetic disorders include **Williams-Beuren syndrome (WBS),** Rett syndrome or tuberous sclerosis complex.

Several evidences suggest that intellectual disability is associated with abnormalities in brain development and brain plasticity caused directly or indirectly by the primary causative factors (Dierssen and Ramakers, 2006). For the proper establishment of synaptic connections and synaptic plasticity an accurate progression of multiple events during brain development is essential including neuronal and glia proliferation, migration, differentiation, maturation and synaptogenesis. Defects in some of these processes along with structural brain alterations have been found in fetuses with intellectual disability (Castren *et al.*, 2005; Contestabile *et al.*, 2007; Guidi *et al.*, 2007; Moon and Wynshaw-Boris, 2013). In addition, post-mortem brains of children and adults with intellectual disability have revealed that abnormalities in the volume and neuroarchitecture of several brain regions are frequent. Particularly, alterations in neuronal density and structural abnormalities at the level of

dendritic spines have been widely described (Kaufmann and Moser, 2000; Reiss *et al.*, 2000; Dierssen and Ramakers, 2006; Chailangkarn *et al.*, 2016). The current treatment for intellectual disability is focused on environmental optimization which includes early intervention programs, individualized education programs and the management of co-morbidities (Picker and Walsh, 2013). However, these therapeutic options have a limited efficacy (Bonnier, 2008; Couzens *et al.*, 2012). More recently, the development of mouse models of intellectual disability disorders has helped to understand the mechanisms behind cognitive deficits and to provide an avenue for the development of specific pharmacological approaches. Several preclinical studies have indicated that cognitive deficits may be improved by pharmacological interventions, which was unimaginable a decade ago.

Among the different cognitive functions affected in intellectual disability, this thesis is focused on memory and social behavior.

1.1. Cognitive function

1.1.1. Memory domains

Among the different cognitive functions impaired in intellectual disability, memory is one of the most important aspects (Vicari *et al.*, 2016). Memory consists in the faculty to storage past and present information in the brain. It is a crucial mechanism for adaptive behavior in animals since past experiences are used for future behavioral responses. Memory function consists of several domains with distinct characteristics that depend on the interaction between different brain regions (Squire, 2004). Memory can be classified based in its temporal or its content dimension (Figure 1). Regarding temporal dimension, it can be divided in four types. Sensory memory is the ability to retain impressions of sensory information once

the stimuli have ended. It is the shortest expression of memory and lasts a few 100 milliseconds (Sperling, 1960). Working memory refers to a mechanism that temporarily (from milliseconds to minutes) retains limited amounts of information allowing to remember the current state of an action that the individual is executing. It is necessary for higher cognitive functions including language comprehension, learning, reasoning and problem-solving (Miller *et al.*, 1986; Baddeley, 1992). The term of working memory has also been used in animal studies and it can be understood as the information that is only necessary to be retained during a session of a behavioral task (Funahashi, 2017). The prefrontal cortex is the main structure responsible for this type of memory (Goldman-Rakic, 2011; Funahashi, 2017). Short-term memory is the capacity to temporarily retain information from the immediate past. It lasts from minutes to days in humans and from minutes to few hours (3-4 hours) in rodents. It is susceptible to perturbations and the hippocampus is the main area involved (Kumaran, 2008).

Long-term memory lasts from days to years (even entire lifetime) in humans and from hours to days in mice. This type of memory requires synaptic and plastic changes, which involves protein synthesis (Barondes and Cohen, 1967) and the contribution of different brain regions. According to the type of content, long-term memory can be classified in explicit and implicit memory (also known as declarative and non-declarative memory respectively). Explicit or declarative memory is defined as the conscious recollection of facts and events. It is the one impaired in amnesic patients and it is dependent on medial temporal lobe structures (see section 1.1.2) (Cohen and Squire, 1980; Squire, 2004). Explicit memory can be subdivided in semantic memory, which comprises general facts about the world, and episodic memory, related to episodes

of personal life. The concept of explicit memory in nonhuman animals can be understood as the processing of spatial, contextual, configural and relational information (Richter-Levin, 2004). Animals seem to remember specific episodes from their past based on what happened, where did it occur and when (Morris, 2001; Crystal, 2010).

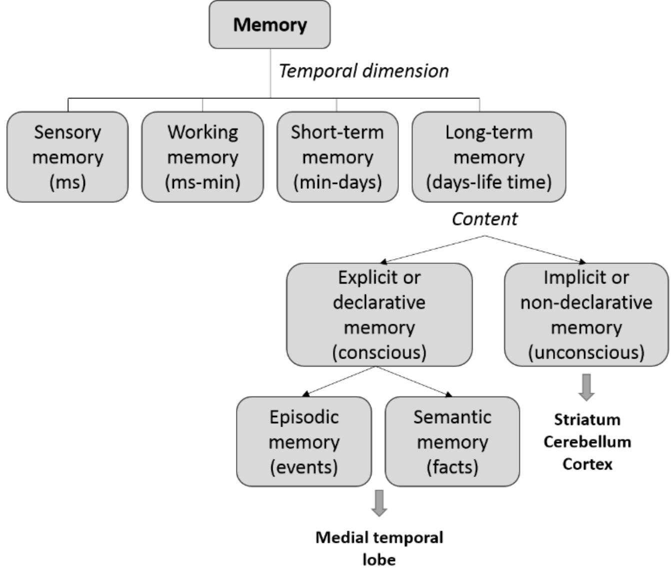


Figure 1. Schematic representation of the different memory types depending on the time that the information is available for the subject and its content.

Implicit or non-declarative memory is the non-conscious recollection of learning capacities that facilitates behavioral performance due to previous exposure (skills and habits, simple conditioning and priming). It requires longer acquisition times than declarative memory and it is thought to depend mostly on striatum, cerebellum and cortical association areas (Cohen and Squire, 1980; Squire, 2004).

Among the different etiological groups of intellectual disability, there are specific patterns of cognitive profiles in which some memory domains are disproportionately affected and others relatively preserved (Vicari *et al.*, 2016).

1.1.2. Neuroanatomical substrates of memory

First studies looking for the neuroanatomical substrate of memory pointed the medial temporal lobe as surgical removal of this region in patients with severe epilepsy caused amnesia (Scoville and Milner, 1957; Squire *et al.*, 2004; Eichenbaum *et al.*, 2007). The medial temporal lobe is a large region that includes the hippocampal formation and the parahippocampal region and seems to be critical only for explicit memory (Gaffan, 1974). The hippocampal formation is the center of the network that supports memory function (Scoville and Milner, 1957; Rosenbaum *et al.*, 2000; Cipolotti *et al.*, 2001). Alterations of this region have been extensively described in intellectual disability patients (Sylvester, 1983; Meyer-Lindenberg *et al.*, 2005; Bostrom *et al.*, 2016).

Structure and connections of hippocampal formation have been studied in detail in humans and in animal models (monkeys, rats and rodents). Unlike other brain areas such as the cerebral cortex, the hippocampal formation is highly conserved across these species (Figure 2) (Allen and Fortin, 2013).

Since in this thesis we have used mouse models, we will focus in the hippocampal formation of rodents. The rodent hippocampal formation is an elongated structure making a C shape with the long axis from the septal nuclei rostrally to the temporal cortex ventrocaudally. It includes three regions: the dentate gyrus, the hippocampus (*cornu ammonis* (CA)1, CA2 and CA3), and the *subiculum* (Amaral and Witter, 1989; van Strien *et al.*, 2009). The hippocampal formation has three layers. The deeper layer contains afferent and efferent fibers and interneurons (dentate gyrus: hilus; CA: *stratum oriens*), the contiguous layer is the cell layer composed by principal neurons and interneurons (dentate gyrus: granule layer; CA: pyramidal layer), and the superficial layer is the molecular layer. The

molecular layer in CA area is subdivided in sublayers including the *stratum lucidum* (only in CA3), the *stratum radiatum* and the *stratum lacunosum-moleculare*. The *stratum radiatum* contains the apical dendrites of pyramidal cells and the *stratum lacunosum-moleculare* the apical thufts (van Strien *et al.*, 2009). The parahippocampal region is contiguous to the subcubiculum and is comprised by the presubiculum, the parasubiculum, the entorhinal cortex, the perirhinal cortex and the postrhinal cortex. Unlike hippocampal formation, parahippocampal region has six layers (van Strien *et al.*, 2009).

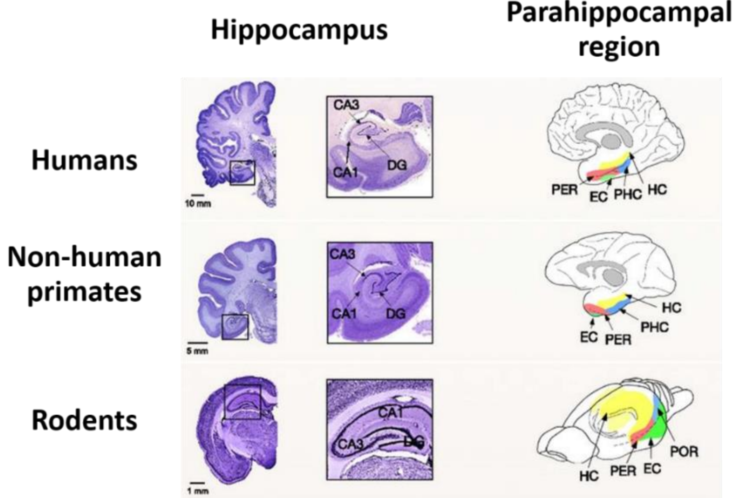


Figure 2. Anatomical comparison of the hippocampus and parahippocampal region of human, nonhuman primate (*Macaca mulatta*) and rodent (*Rattus norvegicus*). The hippocampus shows distinct subregions conserved among the distinct species. The parahippocampal region also conserves relative spatial locations among species. CA1, CA2 and CA3: cornu ammonis; DG: dentate gyrus; EC: entorhinal cortex; HC: hippocampus; PER: perirhinal cortex; PHC: parahippocampal region; POR: postrhinal cortex. Adapted from (Allen and Fortin, 2013).

A peculiarity of the hippocampal formation is that the fields are linked by unidirectional excitatory projections in a trisynaptic loop: entorhinal cortex→dentate gyrus→CA3→CA1 (Figure 3A).

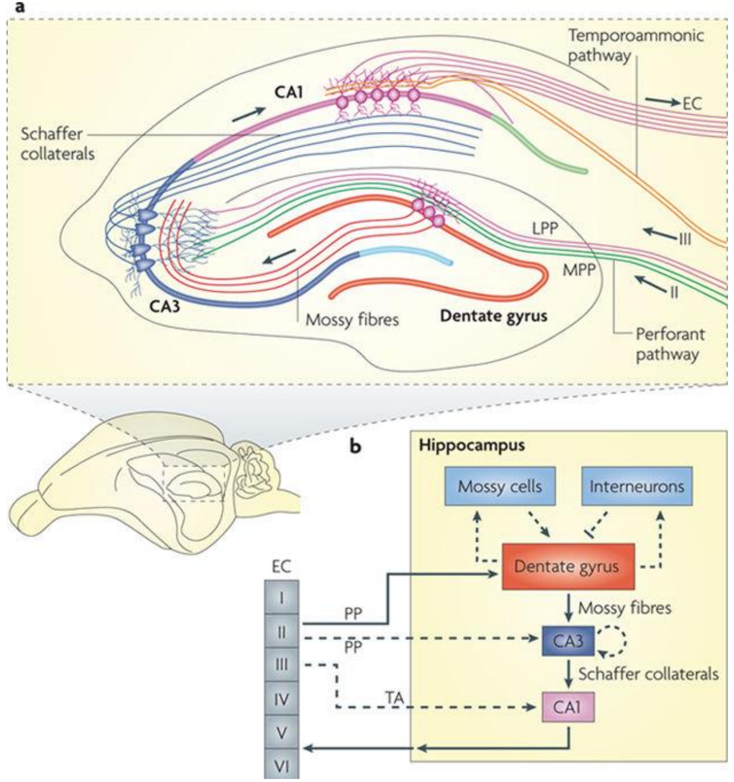


Figure 3. The hippocampal rodent network. (A) An illustration of the hippocampal circuitry. (B) Diagram of the hippocampal network. Solid arrows depicted the excitatory trisynaptic loop and dashed arrows other projections. CA1, CA2 and CA3: cornu ammonis; EC: entorhinal cortex; LPP: lateral perforant pathway; MPP: medial perforant pathway; TA: temporoammonic pathway (Deng *et al.*, 2010).

Entorhinal cortex layer II stellate cells project to the dentate gyrus through the medial and lateral perforant pathways. Dentate gyrus granule cells send excitatory inputs through the mossy fibers to the CA3 pyramidal cells. Axons from CA3 pyramidal neurons project to dendrites of CA1 pyramidal neurons in *stratum radiatum* through Schaffer collaterals. In turn, CA1 pyramidal neurons project back to the entorhinal cortex, specifically into the deep-layer neurons, to close the entorhinal cortex-hippocampal loop. Other projections besides the trisynaptic loop have been described (Figure 3B). CA1 and CA3 regions also receive direct inputs from the entorhinal cortex (layer III through temporoammonic pathway

and layer II through the perforant pathway respectively). In addition, CA3 axons also send their projections to other CA3 neurons. Furthermore, the granule cells of the dentate gyrus also have a direct input with mossy cells and hilar interneurons, which send excitatory and inhibitory projections respectively back to the granule cells (van Strien *et al.*, 2009; Deng *et al.*, 2010).

The hippocampal formation receives information from cerebral cortex regions through the parahippocampal region where two pathways are distinguished. The postrhinal cortex sends spatial/temporal information (context) to the medial entorhinal cortex and the perirhinal cortex sends non-spatial information (content) to the lateral entorhinal cortex. This second pathway is critical for object recognition memory (Brown and Aggleton, 2001; Feinberg *et al.*, 2012). It has been postulated that the hippocampus supports episodic memory combining the information received from the medial and lateral entorhinal cortex to form a representation of an experience within its spatial/temporal context (Knierim, 2015).

Besides hippocampal formation and parahippocampal region, other brain regions are also involved in memory processes. In fact, patients with lesions in the medial temporal lobe lose recent memories but retain older ones (i.e. from childhood) suggesting that over time, memories might be stored elsewhere (Squire and Alvarez, 1995). This hypothesis is supported by animal studies in which the disruption of hippocampal function affects more recent than remote memories (Frankland and Bontempi, 2005). It has been postulated that memories are initially stored in the hippocampal formation and over time, information is transferred to the neocortex where it is permanently retained. The process involves shifting the center of the network from

the hippocampus to the medial prefrontal/anterior cingulate cortex (Figure 4) (Takehara-Nishiuchi, 2014). Interaction between hippocampus and cortex networks during and after the experience is thought to be critical (Preston and Eichenbaum, 2013). Other reports have reformulated this model and postulate that information is encoded from the beginning in hippocampal-cortical networks and that implication of hippocampus is still present during remote contextual memories (Nadel and Moscovitch, 1997; Frankland and Bontempi, 2005).

The amygdala, and specifically, the basolateral amygdala, also plays an important role in memory processes when an emotional component is present (Figure 4) (LaBar and Cabeza, 2006).

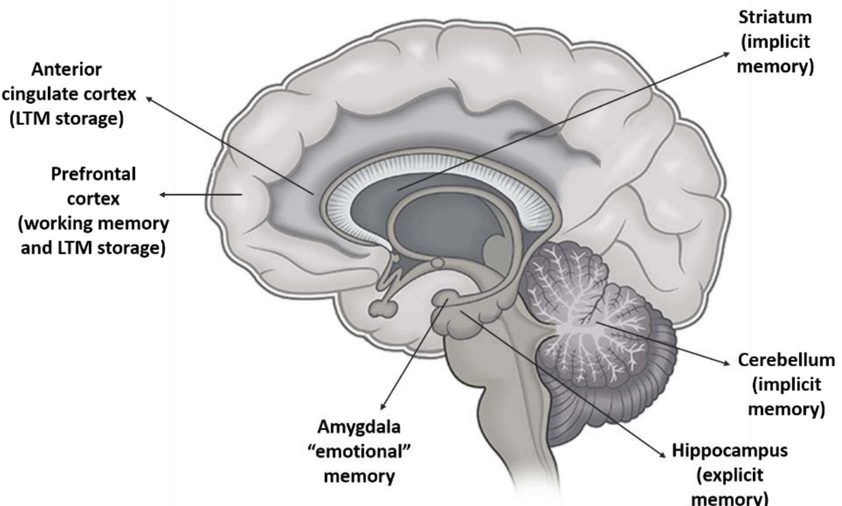


Figure 4. Schematic cartoon that summarizes the main brain areas involved in the different memory domains. LTM: long-term memory.

1.1.3. Behavioral mouse models to study memory

Several behavioral tests have been developed to study memory and learning in rodent models and they are of special interest to study models of intellectual disability. Tests are assessed in mazes or boxes where

animals perform a specific task. Usually, they take advantage of instinctive behaviors such as exploratory behavior which push animals to explore new environments allowing them to acquire information about novel places and things (Paul *et al.*, 2009). Sometimes, positive positive (e.g. food or water) and negative reinforcers (e.g. electric shock or loud noise) are used. Tests can be classified into operant and non-operant. In operant tests, mice perform an active behavior that produces an immediate or negative consequence. This consequence causes the strengthen or weaken of the active behavior. This thesis is focused on non-operant tests. Among several tests we have used two tests to assess explicit memory:

Novel object recognition test (NORT)

The NORT (Ennaceur and Delacour, 1988) assesses the ability to judge a previously encountered object. Object recognition is a subtype of declarative memory that is usually impaired in some human disorders (Winters *et al.*, 2008). It is normally assessed in humans by visual paired comparisons tasks which are really similar to NORT. In NORT, animals are exposed to two identical objects in a familiar maze and after a retention period, one of the objects is changed by a new one. Object recognition memory is inferred from the preference of rodents to explore the novel object over the familiar one, since they have tendency to approach and explore more the novelty (Berlyne, 1950). The preference over the novel object requires the encoding, consolidation and retrieval of the memory for the familiar object (Cohen and Stackman, 2015). The advantages of this test are that it does not require the use of positive or negative reinforcers, it does not generate stressful conditions, training can be performed in one session and it has been replicated in many laboratories using different maze designs, objects and strains (Ennaceur and Delacour, 1988; Sik *et al.*, 2003; Bevins and Besheer, 2006). All variables need to be

counterbalanced including which object serves as a familiar or new one, or where is placed the new object. Furthermore, the objects should be tested to check that each of them is explored the same time when both are new to mice.

There are different protocols of this test. Classically, it is performed in an open-field arena, although several studies have been performed in Y-shaped or V-shaped mazes to reduce contextual and spatial information (Busquets-Garcia *et al.*, 2013; Vallée *et al.*, 2014; Gomis-González *et al.*, 2016). Usually, it is performed in three phases. The first phase is habituation in which mice get used to the arena. The following phase is the training in which mice explore two identical objects. In the V-shaped version, each of the objects is placed at the end of the corridors. The final phase is the test and it is performed after a retention time (10 minutes-3 hours for short-term memory and 24 hours for long-term memory). During the test, one of the familiar objects is replaced by a new one. An illustration of the basic procedure is shown in Figure 5. The time exploring each of the objects is recorded and then a discrimination index can be measured (Bevins and Besheer, 2006). Discrimination index (DI) can be calculated as follows:

$$\text{DI} = \frac{\text{Exploration time novel object} - \text{exploration time familiar object}}{\text{Total exploration time}}$$

There are two principal areas required for object recognition memory, the hippocampus and perirhinal cortex. Although well established in humans, the involvement of hippocampus in rodents has been controversial (Squire *et al.*, 2007). Some studies have assessed this question showing a wide variety of results. These studies have been performed through lesions or pharmacological inactivation of the hippocampus and, whereas some authors have found object recognition spared others have found it

impaired (Clark *et al.*, 2000; Baker and Kim, 2002; Winters *et al.*, 2004; Ainge *et al.*, 2006; Cohen *et al.*, 2013). Differences may be attributed to the lesion size, the type of drug, dose, administration time and diffusion of the drug, or the retention time between the training and the test. In general, it seems that object recognition requires hippocampal integrity with long but not short (minutes) retention times (Cohen and Stackman, 2015). Several studies also support the role of perirhinal cortex in object recognition memory (Barker *et al.*, 2007; Olarte-Sánchez *et al.*, 2015). Importantly, both hippocampus and perirhinal cortex seem to be required during encoding, consolidation and retrieval stages (Winters and Bussey, 2005; Cohen *et al.*, 2013).

Novel place recognition test (NPRT)

The NPRT (Save *et al.*, 1992) assesses the ability of rodents to detect the displacement of a familiar object to a novel location. It is similar to the NORT but in this test spatial-object-location memory is studied (Ameen-Ali *et al.*, 2015). It is performed in an open-field arena and it also consists in three phases: habituation, training and test. The difference with the NORT is that during the test phase, one of the familiar objects is moved to a new location. Spatial-location memory is inferred from the preference of the rodent to explore the object placed in the new location over the object placed in the familiar one. An illustration of the basic procedure is shown in Figure 5.

Spatial-object-location seems to be hippocampal-dependent but perirhinal cortex not dependent (Save *et al.*, 1992; Barker *et al.*, 2007; Barker and Warburton, 2011).

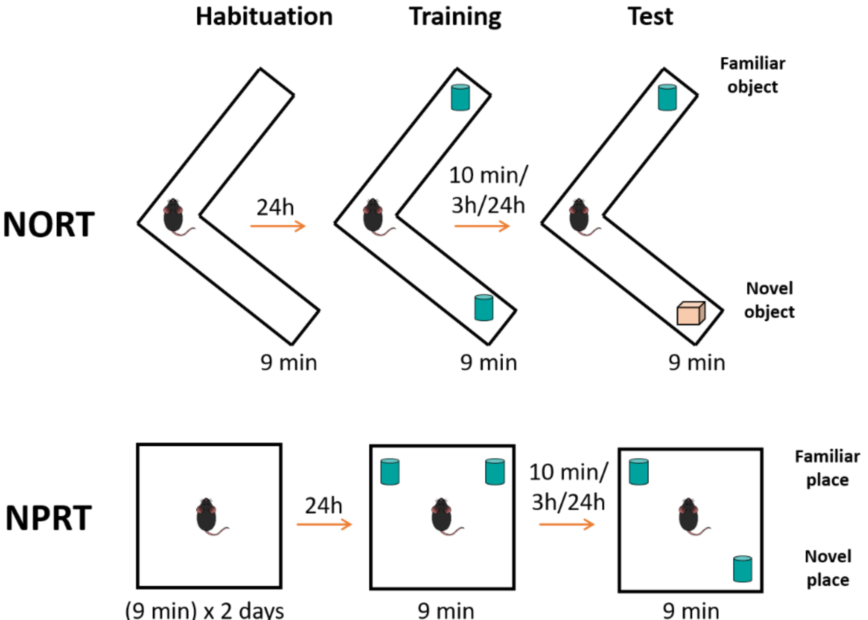


Figure 5. Schematic representation of the protocol and maze used to perform novel object recognition test (NORT) and novel place recognition test (NPRT).

Other tests commonly used to assess cognitive function in rodents are shown in Table 1.

Test	Description	Main brain regions involved	Scheme
Morris water maze (MWM)	Spatial learning memory task in which rodents have to learn the location of a submerged hidden platform using external cues during several training sessions. The day of the test, the time spent in the quadrant where the platform was located during training is recorded and can be used as an indicator of spatial memory. There are multiple versions of this test to assess for instance cognitive flexibility and working memory.	Hippocampus	
Radial arm maze	Spatial learning task with multiple variants. The maze has commonly eight arms that may or may not contain food pellets at the end. Food deprived rodents freely choose among the different arms and retrieve the baits. Animals can be trained to recognize which of the arms contains pellets and spatial reference memory can be assessed. Working memory can also be analyzed using a variant of the procedure.	Hippocampus Prefrontal cortex	
Spontaneous alternation task (Y-maze or T-maze)	Working memory task in which rodents can explore freely three arms in a Y-shaped or T-shaped maze. Animals will prefer to visit a new arm rather than returning to an arm that has been previously visited. Spontaneous alternation is used as indicator of working spatial memory.	Hippocampus Prefrontal cortex	
Fear conditioning	Pavlovian associative learning task where rodents associate a neutral conditional stimulus, that can be a cue (light or sound) or a context, with an aversive unconditioned stimulus (i.e. electrical foot shock). Different conditioned responses can be monitored as indicators of memory including active (e.g. rearings) or passive (e.g. freezing) responses.	Amygdala Hippocampus Prefrontal cortex	
Passive avoidance or Inhibitory avoidance	Avoidance task in which animals learn to inhibit a natural behavior (scape to a dark compartment) that has been punished before, usually with an electrical foot shock.	Amygdala Hippocampus Prefrontal cortex,	
Active avoidance	Avoidance task in which animals learn to avoid by a locomotor response an environment in which an aversive stimulus has been delivered (i.e. electrical foot shock).	Amygdala Prefrontal cortex Striatum	

Table 1. Non-operant behavioral tests to study learning and memory in rodents. The table includes a description, scheme and the main brain regions involved in each of the tests (Izquierdo *et al.*, 2006; Lee and Silva, 2009; Darvas *et al.*, 2011; Maren *et al.*, 2013; Martinez *et al.*, 2013; Ganella and Kim, 2014; Vorhees and Williams, 2014).

1.1.4. Neuronal plasticity

Nowadays, it is well established that mammalian brain displays persistent plasticity across the lifespan. Neuronal plasticity refers to the capacity of neural circuits to change in response to an experience (Sharma *et al.*, 2013). Neural circuits undergo continuous functional and structural rearrangements that include neurogenesis, synaptogenesis and changes in neuronal morphology. Neuronal plasticity is crucial for brain development and for learning and memory processes. Therefore, plasticity defects may play an important role in the mechanisms underlying intellectual disability. Of special interest for this thesis are synaptic plasticity and adult neurogenesis processes, both disrupted in intellectual disability (Dierssen and Ramakers, 2006; Pons-Espinal *et al.*, 2013a).

1.1.4.1. Synaptic plasticity

Synaptic plasticity is the process by which activity-dependent changes in the strength or the efficacy of synaptic transmission are thought to support learning and memory processes (Martin *et al.*, 2000). The concept of synaptic plasticity was already proposed by Cajal (Ramón y Cajal, 1911) more than a century ago, but was not demonstrated until the discovery of long-term potentiation (LTP) (Bliss and Gardner-Medwin, 1973; Bliss and Lømo, 1973). LTP is an experimental model of synaptic plasticity in which synaptic stimulation, such as prolonged high-frequency stimulation, produces a long-lasting increase in the strength of synaptic transmission. LTP can be induced by different stimulation protocols. The original protocol was high-frequency stimulation that consists in continuous 100 Hz tetanization for 1 second. However, this protocol is far from physiological conditions since pyramidal neurons fire action potentials at

around 5 Hz when a mouse is exploring (theta rhythm) (Bland, 1986). Other protocols more physiological were developed later including theta burst stimulation (TBS) which consists of episodes of 10 train stimuli at 5 Hz each 20 seconds, and each train includes 4 pulses at 100 Hz (Larson and Lynch, 1986). LTP can be divided in two main phases, the early phase (E-LTP) and the late-phase (L-LTP). E-LTP starts immediately after the induction and lasts about 1 to 3 hours. L-LTP lasts at least 10 hours and requires protein synthesis (Frey *et al.*, 1993).

LTP has been described extensively in different brain areas of animal models including hippocampus (Bliss and Gardner-Medwin, 1973; Bliss and Lømo, 1973), amygdala (Clugnet and LeDoux, 1990), cortex (Artola and Singer, 1987), striatum (Calabresi *et al.*, 1992) and cerebellum (Salin *et al.*, 1996). Furthermore, LTP has been described both *in vivo* and *in vitro* conditions (Kumar, 2011). The most studied and robust form is the one that occurs at CA3-CA1 synapses of the hippocampus (Nicoll, 2017). LTP at this area is dependent on N-methyl-D-aspartate (NMDA) receptors and involves postsynaptic modifications. Briefly, the process consists in the following: trains of high frequency cause the release of glutamate and as consequence, the depolarization of the postsynaptic membrane via the influx of charged sodium ions through 3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. The depolarization will allow the influx of Ca^{2+} into the cell if it is strong enough to remove the Mg^{2+} block from NMDA receptors. The increase of Ca^{2+} concentrations inside the cell activates different enzymes that are involved in the induction of LTP. The primary enzyme activated in dendritic spines is the Ca^{2+}-calmodulin-dependent kinase II (CaMKII). Other pathways activated are the mitogen-activated protein kinase (MAPK), protein kinase A (PKA) and protein kinase C (PKC) (Sweatt, 1999; Kumar, 2011).

LTP at CA3-CA1 synapses has some features that fit as candidate for the storage of memory: simultaneous weak stimulation of multiple pathways can induce LTP (associativity), presynaptic stimulation has to be paired with postsynaptic depolarization for its induction (cooperativity), the potentiation is restricted for the activated synapses (specificity) and it lasts beyond the initial stimulation (persistence). Furthermore, experimental mouse models have provided evidences about the functional relation of LTP and memory. These studies have mainly focused in the hippocampus, but also in other brain regions. Pharmacological or genetic blockade of key molecular targets of LTP not only disrupts LTP, but also memory and learning processes (Morris *et al.*, 1986; Grant *et al.*, 1992; Tsien *et al.*, 1996; Giese *et al.*, 1998; Shimizu *et al.*, 2000). In addition, saturation of LTP by repeated stimulation interferes with spatial memory (Moser *et al.*, 1998). LTP-like processes during learning have also been described in different tasks including fear-conditioning, inhibitory avoidance and novel-object recognition (Rogan *et al.*, 1997; Whitlock *et al.*, 2006; Clarke *et al.*, 2010). Furthermore, a report has demonstrated that an associative learning in the amygdala can be inactivated and reactivated with long-term depression (LTD) and LTP, respectively (Nabavi *et al.*, 2014). Notably, LTP is altered in different mouse models of intellectual disability disorders including DS and WBS (Costa and Grybko, 2005; Borralleras *et al.*, 2016), supporting the importance of this type of plasticity in memory function. Although there are several observations pointing a functional role of LTP on memory, some studies have found a dissociation of both phenomena (Nosten-Bertrand *et al.*, 1996; Bannerman *et al.*, 1997; Montkowski and Holsboer, 1997; Meiri *et al.*, 1998).

Other forms of activity-dependent plasticity have been described including LTD (Lynch *et al.*, 1977), excitatory postsynaptic potential (EPSP)-

spike potentiation (Abraham *et al.*, 1985) or spike-timing-dependent plasticity (Dan and Poo, 2004).

1.1.4.2. Adult neurogenesis

In the adult mammalian brain, adult neurogenesis is mainly restricted to two brain regions: the subgranular zone of the hippocampal dentate gyrus and the subventricular zone of the lateral ventricles (Gould, 2007). In humans, the extent of adult neurogenesis has been questioned by contradictory results depending on the tissues analyzed and the experimental approaches employed (Spalding *et al.*, 2013; Dennis *et al.*, 2016; Boldrini *et al.*, 2018; Sorrells *et al.*, 2018). In animals, adult neurogenesis has been widely studied, particularly, in the subgranular zone of the hippocampal dentate gyrus. This region is a thin layer between the granule cell layer and the hilus containing adult neural stem cell niches. There, newborn cells arise from neural stem cells, proliferate, differentiate into neurons or glial cells, and migrate towards the granular layer. New neurons can be integrated into the local network as glutamatergic granule cells (Figure 6) (Duan *et al.*, 2008). The role that new cells play is not fully understood (Deng *et al.*, 2010). It has been proposed that these new neurons replace the existing ones or that they are simply added to the network and modulate plasticity (Deng *et al.*, 2010). In fact, immature granule cells have a lower threshold for LTP induction indicating that newly generated neurons have unique mechanisms to facilitate synaptic plasticity (Schmidt-Hieber *et al.*, 2004). Both new mature and immature neurons seem to contribute to memory processes (Deng *et al.*, 2009).

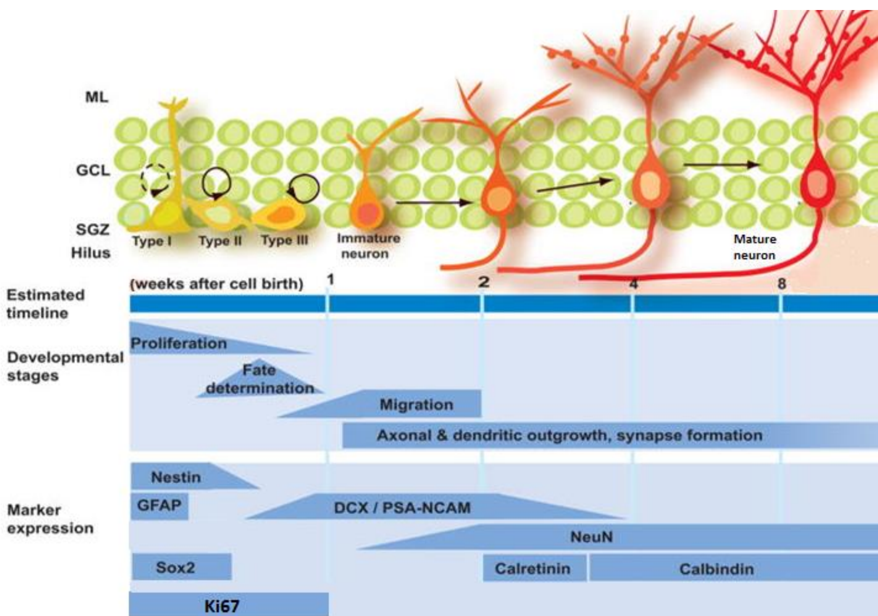


Figure 6. Adult neurogenesis in hippocampal dentate gyrus. Summary of the developmental stages during adult neurogenesis and the expression of stage-specific markers. Ki67 has been used in this thesis as a marker of cell proliferation. MCL: molecular layer; GCL: granule cell layer; SGZ: subgranular zone; GFAP: glial fibrillary acidic protein; DCX: doublecortin; NeuN: neuronal nuclei; PSA-NCAM: the polysialylated form of the neural cell adhesion molecule NCAM. Adapted from (Duan *et al.*, 2008).

Several preclinical studies have addressed the effect of neurogenesis over cognitive performance. After neurogenesis ablation, impairments were detected in hippocampal-dependent memory tests such as the NORT (Jessberger *et al.*, 2009), trace fear conditioning (Shors *et al.*, 2001, 2002), contextual fear conditioning (Saxe *et al.*, 2006; Winocur *et al.*, 2006; Farioli-Vecchioli *et al.*, 2008) and acquisition (Dupret *et al.*, 2008; Zhang *et al.*, 2008) and retention (Snyder *et al.*, 2005; Jessberger *et al.*, 2009) of the Morris water maze (MWM) test. However, some studies have found no effects of neurogenesis ablation in some of these tests, which may be attributable to differences in species, strains, age, ablation methods or the parameters evaluated in the specific tests (Deng *et al.*, 2010). The link between adult neurogenesis and learning is also supported by the fact that

the rate of neurogenesis is modulated by hippocampal-dependent tasks (Gould *et al.*, 1999; Leuner *et al.*, 2004; Epp *et al.*, 2007) and by LTP induction in dentate gyrus (Bruel-Jungerman *et al.*, 2006). Importantly, adult neurogenesis is impaired in some mouse models of intellectual disability disorders including DS, FXS and Rett syndrome (Pons-Espinal *et al.*, 2013a). Indeed, therapies targeting adult neurogenesis have demonstrated to improve memory deficits in DS and FXS mouse models (Pons-Espinal *et al.*, 2013a).

Overall, the weight of evidence indicates that hippocampal adult neurogenesis constitutes a functional mechanism of brain plasticity contributing to proper memory function. Deficits in this process may contribute to intellectual disability.

1.1.5. Social behavior

Impairments in social behavior are frequently core features of several neurodevelopmental disorders including autism spectrum disorders, in which patients display lack of interest in social interactions, or DS and WBS, in which patients display increased social interactions (Barak and Feng, 2016; Moss *et al.*, 2016).

Social behavior is a complex behavior that occurs between conspecifics. It includes multiple forms of interaction including parental care, pair bonding, mating, aggression, social affiliation or social communication (Chen and Hong, 2018). These interactions are essential for the survival of species and are modulated by the environment (Haller *et al.*, 2004). Social behavior requires cognitive function to detect and interpret social cues and guide exchange during social interactions which is known as social cognition (Millan and Bales, 2013). Social cognition is impaired in children

with autism spectrum disorders (Colle *et al.*, 2007) and with WBS (Plesa-Skwerer *et al.*, 2006, 2011; Riby and Hancock, 2009).

One of the brain regions that is a crucial component of social behavior is the prefrontal cortex, the most rostral part of the frontal lobe. Indeed, lesions in this area have been associated with impairments in social behavior (Eslinger and Damasio, 1985; Anderson *et al.*, 1999). Prefrontal cortex in humans can be subdivided into dorsolateral, dorsomedial, ventromedial and orbital prefrontal cortex (Carlén, 2017). Each of these subdivisions is associated with distinct aspects of social behavior. Medial prefrontal cortex is involved in interpreting information from past experiences and generating appropriate social responses within a context by regulating attention, emotion, behavioral flexibility and response inhibition (Arnsten, 2009; Bicks *et al.*, 2015). Orbitofrontal cortex is involved in social adjustment modulating emotion and reward processing (Rudebeck *et al.*, 2008). In rodents, several evidences have demonstrated that a functional comparable prefrontal cortex exists (Uylings *et al.*, 2003) and that it plays a role in social behavior (Ko, 2017).

The amygdala is also an essential substrate of social behavior. It receives and integrates social sensory information (mainly visual in humans and olfactory in rodents) from thalamus and sensory cortices and translates into behavioral outputs. Evidences from individuals with amygdala lesions have revealed a role of this brain region in eye contact, facial emotion recognition and social judgment (Adolphs *et al.*, 1994, 1998; Young *et al.*, 1995; Spezio *et al.*, 2007).

Other brain regions involved in social behavior are the anterior cingulate cortex, anterior insula, fusiform gyrus, inferior frontal gyrus, superior temporal sulcus and ventral hippocampus (Figure 7). Furthermore, subcortical areas such as the nucleus accumbens and the ventral

tegmental area mediate rewarding properties of social interactions (Frith, 2007; Felix-Ortiz and Tye, 2014; Bicks *et al.*, 2015; Barak and Feng, 2016).

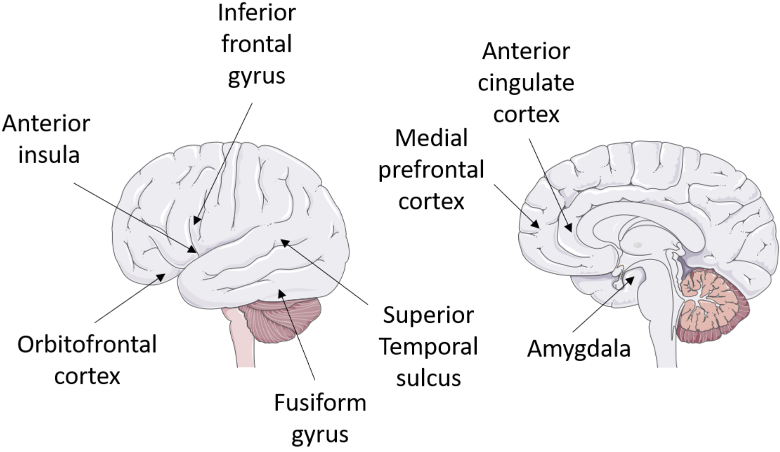


Figure 7. Brain regions of high relevance for social behavior.

Although social behavior is more complex in humans than in rodents, rodents are also social animals that display a great repertoire of social behaviors (Silverman *et al.*, 2010). These behaviors can be monitored by different behavioral tasks. One of the most interesting is the three-chamber paradigm (Moy *et al.*, 2004). The test consists in three phases and it is usually carried out in an open-field arena with three compartments. The first phase is the habituation period to the maze. The second phase, also known as sociability phase, assesses the degree of social motivation by comparing the time spent exploring an unfamiliar conspecific mouse (stranger 1) inside a compartment and the time spent exploring an object or an empty compartment. The third phase assesses preference for social novelty by analyzing the time spent exploring the stranger 1 and the time spent exploring a new unfamiliar mouse, stranger 2. Both strangers are inside a compartment with bars which allows sensory interactions (visual, tactile, auditory and olfactory), but prevents direct

physical contact avoiding potential confounds resulting from fighting and aggressive behavior (Moy *et al.*, 2004).

As a part of this thesis, we have set up, characterized and validated a novel approach to study social behavior with different strains and mouse models (see chapter 2). It is an adaptation of the three-chamber paradigm. In this case, the maze has a V-shape and at the end of each corridor there is a small chamber with plastic bars allowing sensory interactions. Each of the phases lasts 5 minutes and there is no inter-trial time among them (Figure 8).

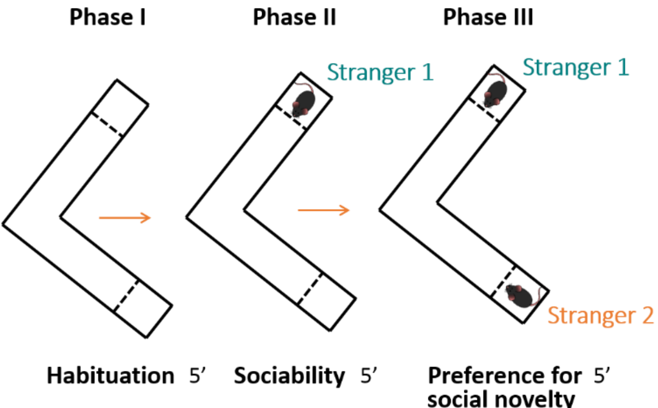


Figure 8. Protocol of the social test that has been designed and validated during this thesis.

2. Down syndrome

DS is the most common genetic form of intellectual disability and it affects around 1 in 700-1,000 live births (Dierssen, 2012; de Graaf *et al.*, 2015). The cause is an extra copy, total or partial, of the human chromosome 21 (HSA21). In 90-95% of DS patients, trisomy results from a non-disjunction during meiosis (Patterson, 1987). In the remaining cases, some patients present Robertsonian translocation anomalies (2-4%) whereas others present mosaicism having both trisomic and euploid cell populations (1.3-5%) (Papavassiliou *et al.*, 2015; Coppedè, 2016). Non-disjunction events have maternal origin in the majority of cases (>90%) and their incidence increases with maternal age (Lamb *et al.*, 2005; Freeman *et al.*, 2007).

DS individuals display a complex phenotype including the affectation of multiple organs and systems with variable penetrance. All DS patients present intellectual disability, muscle hypotonia and early onset of Alzheimer's disease neuropathology. However, other phenotypes such as cardiovascular, skeletal and motor alterations appear only in a fraction of patients (Antonarakis *et al.*, 2004) (Table 2).

Intellectual disability is the most limiting phenotype of DS, which really compromises patients' quality of life. Moreover, DS individuals show increased frequency of other neurological and psychiatric manifestations that also impact on their quality of life such as anxiety (Vicari *et al.*, 2013), sleep disturbance (Angriman *et al.*, 2015), hyperactivity (Pueschel *et al.*, 1991) or epilepsy (Robertson *et al.*, 2015).

Phenotype	% in DS/fold increase
Intellectual Disability	100%
Alzheimer’s disease neuropathology	100%*
Muscle hypotonia	100%
Characteristic dermatoglyphics	85%
Brachycephaly	75%
Short stature	70%
Short, broad hands	65%
Epicanthic folds	60%
Folded/dysplastic ear	60%
Short fifth finger	60%
Iris Brushfield spots	55%
Protruding tongue	45%
Congenital heart defect	40%
Atrioventricular canal	16%
Duodeneal stenosis/atresia	250x
Actue megakaryocytic leukemia	200-400x
Imperforate anus	50x
Hirschspung disease	30x
Acute lymphoblastic leukemia and acute megakaryocytic leukemia	10-20x

Table 2. Frequency of DS phenotypes. Frequency is shown as the percentage of DS patients with the phenotype or as the fold-increase having DS, (*= onset during adulthood). Adapted from (Antonarakis *et al.*, 2004).

Despite the availability of prenatal screening and termination of pregnancy in high-income countries, live birth prevalence of DS has remained stable in recent years due to a general increase in maternal age (Loane *et al.*, 2013). In addition, life expectancy of DS patients has dramatically augmented due to medical intervention in the last century (i.e. from 9 years in 1929 to 60 years nowadays in developed countries), increasing the population prevalence and making the improvement of cognitive abilities of DS patients a priority (Penrose, 1949; Glasson *et al.*, 2003; Bittles and Glasson, 2004; de Graaf *et al.*, 2017).

2.1. Neuropathology and neuropsychological aspects in Down syndrome

Intellectual disability in DS is characterized by deficits in adaptive functioning and specific domains such as learning and memory processes, executive function and language that lead to a general cognitive impairment (Grieco *et al.*, 2015). However, the severity of cognitive impairment and the domains affected are diverse among DS patients. This variability is probably multifactorial as a result of genetic, epigenetic variations and environmental factors plus stochastic events (Gardiner *et al.*, 2010). Moreover, cognitive deficits can change across lifespan, becoming more evident over the years (Carr, 1988) and may be influenced by several comorbid factors such as sensory impairments, sleep disruptions or other psychiatric conditions (Gasquoine, 2011).

DS patients typically display an IQ score in a mild to severe range (IQ score= 30-70) (Grieco *et al.*, 2015). Few patients have an IQ score higher than 70, which has been linked to partial trisomies (Papoulidis *et al.*, 2014). DS patients have weaknesses in explicit long-term memory, working memory and verbal short-term memory along with language deficits in articulation and syntax. Conversely, they have relative preserved visuospatial short-term memory and implicit memory (Carlesimo *et al.*, 1997; Jarrold and Baddeley, 2001; Vicari, 2001; Lanfranchi *et al.*, 2010; Grieco *et al.*, 2015; Godfrey and Lee, 2018). Importantly, everyday episodic memories are compromised limiting independent functioning of DS patients (Pennington *et al.*, 2003). DS subjects present deficits in different cognitive tests that are dependent on the medial temporal lobe, including tasks of pattern recognition memory, spatial recognition and association between objects and locations (Pennington *et al.*, 2003; Vicari *et al.*, 2005; Visu-Petra *et al.*, 2007; Edgin *et al.*, 2010). However, not all of the studies have

shown differences in performance of spatial memory tasks (Vicari *et al.*, 2005; Edgin *et al.*, 2010).

In addition to the cognitive impairment appeared during infancy and maintained during childhood and adulthood, DS patients present an early onset of Alzheimer's disease-like cognitive decline and dementia. Although by age 40-50 virtually all adults with DS have an Alzheimer's disease neuropathology, not all present dementia (Wisniewski *et al.*, 1985). Nevertheless, the incidence of dementia in DS is much higher than in general population reaching around 70% by the age of 55-60 years (Hartley *et al.*, 2015).

Several pathophysiological alterations have been described in DS brains that appear during brain's development and persist throughout life (Table 3). Post-mortem and neuroimaging studies indicate that DS individuals have brachycephaly and reduced overall brain volumes with disproportionately smaller volumes of some areas (e.g. hippocampus, entorhinal, prefrontal and temporal cortices, amygdala and cerebellum) and a larger volume of the parahippocampal gyrus (Raz *et al.*, 1995; Pinter *et al.*, 2001; Śmigielska-Kuzia *et al.*, 2011). Moreover, DS brain is also characterized by a decreased density of neurons in different regions including the cerebral cortex, hippocampus and cerebellum (Sylvester, 1983; Wisniewski *et al.*, 1984; Mann *et al.*, 1987; Wisniewski, 1990).

Both, reduction in brain size and in neuronal density may arise from defects of neurogenesis during brain development since DS fetuses present reduced proliferation of neural precursor cells and increased apoptosis (Contestabile *et al.*, 2007; Guidi *et al.*, 2007; Lu *et al.*, 2011).

Other alterations include reduced dendritic arborization and number of dendritic spines and changes in dendrite morphology which appear during childhood and continue throughout the lifespan in different brain regions

(e.g. visual motor and parietal cortex and hippocampus) (Marin-Padilla, 1976; Suetsugu and Mehraein, 1980; Takashima *et al.*, 1981; Becker *et al.*, 1986; Ferrer and Gullotta, 1990; Schulz and Scholz, 1992). Studies of fMRI have also revealed deficits in brain functional connectivity (Anderson *et al.*, 2013; Pujol *et al.*, 2015).

Brain region	Newborns	Adults (20–50 years of age)*	Elderly individuals (>50 years of age)*
Whole brain	Almost normal weight	Reduction in weight, brachycephalic	Smaller overall cerebral volumes
Prefrontal cortex	Reduction in volume	Reduction in volume	Reduction in volume
Parietal cortex	Normal or reduction in volume	Reduction in volume	Unknown
Temporal cortex	Narrow superior temporal gyrus	Reduction in volume of right middle or superior temporal gyrus	Decreased grey matter volume in posterior cingulate and entorhinal cortex
Hippocampus	Unknown	Reduction in volume	Unknown
Parahippocampal region	Unknown	Increase in size of the parahippocampal gyrus	Reduction in volume
Amygdala	Reduction in volume	Reduction in volume	Reduction in volume
Cerebellum	Reduction in volume	Reduction in volume	Reduction in volume
Brain stem	Reduction in volume	Increase in grey matter volume	Degeneration of locus coeruleus
Basal prosencephalon	Almost normal size	Normal	Degeneration of basal prosencephalon cholinergic nuclei (nucleus of Meynert)

Table 3. Brain alterations in DS patients. At the time of birth, gross neuroanatomical aspects are relatively similar between DS and euploid brains. However, by 6 months of age differences are already noticeable and are maintained across the whole life span (Nadel, 2003). Elderly individuals present Alzheimer's disease neuropathology which main hallmarks are degeneration of locus coeruleus, basal forebrain cholinergic neuron loss and the presence of amyloid plaques and neurofibrillary tangles (Head *et al.*, 2016). (Dierssen, 2012).

Alterations in various neurotransmitter systems have also been reported in DS individuals including decreased concentrations of glutamate, aspartate and noradrenaline in different adult brain regions, decreased levels of serotonin in both adult and fetal brain, and decreased levels of GABA in fetal frontal cortex (Reynolds and Warner, 1988; Risser *et al.*, 1997; Smigielska-Kuzia and Sobaniec, 2007; Śmigielska-Kuzia *et al.*, 2010). Interestingly, some associations between neuropsychological deficits and structural abnormalities have been reported in DS. The volume of hippocampus correlates positively with general intelligence whereas the

volume of the parahippocampal region correlates negatively (Raz *et al.*, 1995; Śmigielska-Kuzia *et al.*, 2011). Furthermore, grey matter density in the lateral and medial temporal lobe regions and the anterior cingulum positively correlated with spatial long-term memory abilities (Menghini *et al.*, 2011). These correlations and the fact that medial temporal lobe-dependent cognitive tasks are impaired in DS patients, indicate that alterations of this region have a key role in the cognitive deficits of DS.

2.2. Genetic cause of Down syndrome

HSA21 is the smallest human chromosome and carries 222 protein-coding genes and 325 non-protein-coding genes (Gupta *et al.*, 2016). The extra copy of the HSA21 in DS is expected to cause an increase of 50% in the transcripts of all genes mapping to this chromosome. However, although the average of all transcripts is close to 1.5 fold relative to euploid cells, not all transcripts are upregulated indicating a complex regulation of gene expression that is tissue-specific (Mao *et al.*, 2003; Prandini *et al.*, 2007).

Different hypotheses have been postulated to explain the link between the trisomy of HSA21 and the appearance of DS phenotypes. The "gene dosage effect" hypothesis states that the pathological features of DS are a direct consequence of the cumulative effects of the dosage imbalance of a subset of genes located at the HSA21. Consistent to this hypothesis, the analysis of genotype-phenotype correlation in DS patients with partial trisomy leaded to define a ~5.4 Mb region on HSA21q22 known as the DS critical region (DSCR). It was postulated that this region, that contains around 50 genes, was sufficient to underlie most of the phenotypic features of DS (Delabar *et al.*, 1993). However, more recent studies of patients with major features of DS carrying rare segmental trisomies that do not include DSCR have demonstrated that other regions are also

relevant to the different DS phenotypes (Korenberg *et al.*, 1994; Korbel *et al.*, 2009; Lyle *et al.*, 2009).

Another hypothesis is the "amplified developmental instability" which states that trisomy 21 causes generalized perturbations in genomic regulation and expression leading to a non-specific disturbance of cellular homeostasis (Pritchard and Kola, 1999). This second hypothesis tries to explain the fact that different aneuploid states share phenotypic traits and that all of these traits are also seen in euploid conditions, although at lower frequency, lower severity and usually presented as a single trait (Pritchard and Kola, 1999; Patterson and Costa, 2005). According to this hypothesis, the size of the triplicated region would correlate with the degree of cognitive impairment which has been argued since some patients with the full trisomy show mild intellectual disability (Korbel *et al.*, 2009). Moreover, several studies with mouse models have provided strong evidences of the contribution of the extra copy of specific individual genes, known as candidate dosage-sensitive genes, to the DS-associated phenotypes (Table 4) (Lana-Elola *et al.*, 2011). Of special interest are the candidate dosage-sensitive genes underlying DS-associated brain phenotypes: *DYRK1A* (dual specificity tyrosine-phosphorylation-regulated kinase 1A); *OLIG1* and *OLIG2*, which encode transcription factors involved in neurogenesis and oligodendrogenesis (Takebayashi *et al.*, 2000); *SIM2* (single-minded homologue 2), which encodes a master regulator in central nervous system (CNS) development (Nambu *et al.*, 1991); *DSCAM* (Down syndrome cell adhesion molecule), which encodes for a molecule involved in dendrite morphology and neuronal wiring (Fuerst *et al.*, 2008); *KCNJ6,* which encodes G protein-activated inwardly rectifying K^+ channel 2 (GIRK2); *SYNJ1* (Synaptojanin 1), which encodes a protein that regulates the metabolism of the lipid PtdIns(4,5)P_2 required for proper

neurotransmission (Di Paolo *et al.*, 2004); and *APP* (amyloid precursor protein).

Phenotype	Candidate dosage-sensitive genes	References
Learning, memory and brain development	*DYRK1A*	(García-Cerro et al., 2014)
	OLIG1, OLIG2	(Chakrabarti et al., 2010)
	SIM2	(Ema et al., 1999)
	DSCAM	(Alves-Sampaio et al., 2010)
	SYNJ1	(Voronov et al., 2008)
	APP	(Netzer et al., 2010)
	KCNJ6	(Kleschevnikov et al., 2017)
Neurodegeneration	*APP*	(Salehi et al., 2006)
	DYRK1A	(García-Cerro et al., 2017)
Motor control	*APP*	(Trazzi et al., 2011)
	DYRK1A	(Altafaj et al., 2001)
	ITSN1, SYNJ1, DSCR1	(Chang and Min, 2009)
Craniofacial alterations	*ETS2*	(Sumarsono et al., 1996)
Cardiac defects	*DSCAM*	(Korbel et al., 2009)
	SLC19A1	(Locke et al., 2010)
	COL6A1	(Davies et al., 1995)
Leukaemia	*ETS2, ERG*	(Stankiewicz and Crispino, 2009)
Reduction in solid tumors	*ETS2*	(Sussan et al., 2008)
	DSCR1	(Baek et al., 2009)
	ADAMTS1, ERG, JAM2, PTTG1IP	(Reynolds et al., 2010)

Table 4. Candidate dosage-sensitive genes causing DS phenotypes. Only genes for which there is direct evidence that their overexpression or allelic variation (SLC19A1 and COL6A1) induces a phenotype are included. Adapted from (Lana-Elola *et al.*, 2011).

Importantly, some of these dosage-sensitive genes encode proteins with master regulatory functions that may interfere in the transcription or the splicing of other genes outside the HSA21. In fact, 324 genes with genome-wide dosage effects were identified in a meta-analysis of 45 different data

sets of DS and the majority were located on chromosomes other than the HSA21 (Vilardell *et al.*, 2011).

Regulatory non-traditional genomic elements such as non-coding RNAs encoded by genes in the HSA21 may also contribute to DS pathology. HSA21 contains at least five micro-RNA genes (miRNA) (miR-99a, let-7c, miR-125b-2, miR-155 and miR-802) that are overexpressed in fetal brain and heart and their triplication may decrease expression of specific proteins (Elton *et al.*, 2010). Epigenetic mechanisms should also be taken into account since DS brains, leukocytes and lymphocytes have specific DNA methylation patterns (Kerkel *et al.*, 2010; Mendioroz *et al.*, 2015). Furthermore, the presence of the extra chromosome *per se* has an effect over chromatin regulation that may affect overall genomic expression (Hervé *et al.*, 2016).

To encompass all these data, the "genome instability" hypothesis has been postulated (Dierssen, 2012). This hypothesis suggests that DS associated phenotypes are caused by additive effects of multiple HSA21 and non-HSA21 genes that are directly and indirectly affected by dosage imbalance together with changes in functional regulation of mRNA and other non-coding and epigenetic elements.

2.3. Down syndrome mouse models

An animal model of a human disorder must fulfil three criteria: construct validity (similarity to the disorder's etiology), face validity (similarity to the disorder's phenotypes) and predictive validity (results in the model are analogous to results in humans) (Crawley, 2004). In this regard, several DS mouse models have been developed that partially satisfy these criteria. These mouse models have allowed to understand

better the mechanisms underlying the developmental changes in DS and to design and test multiple potential therapeutic approaches.

DS mouse models have been developed using two strategies: trisomic mice, which mimic a trisomic environment, and transgenic mice, in which single genes are overexpressed in a disomic environment. The HSA21 has three orthologous regions on mouse chromosomes 10, 16 and 17 (*Mus musculus* (Mmu) 10, 16, 17) in which gene order and orientation are conserved. There are 158 mouse genes that are homologous to HSA21 protein-coding genes. A total of 102 genes lie on the telomere proximal region of the Mmu16, and 19 and 37 in the internal region of the Mmu17 and Mmu10 respectively (Figure 9) (Davisson *et al.*, 2001).

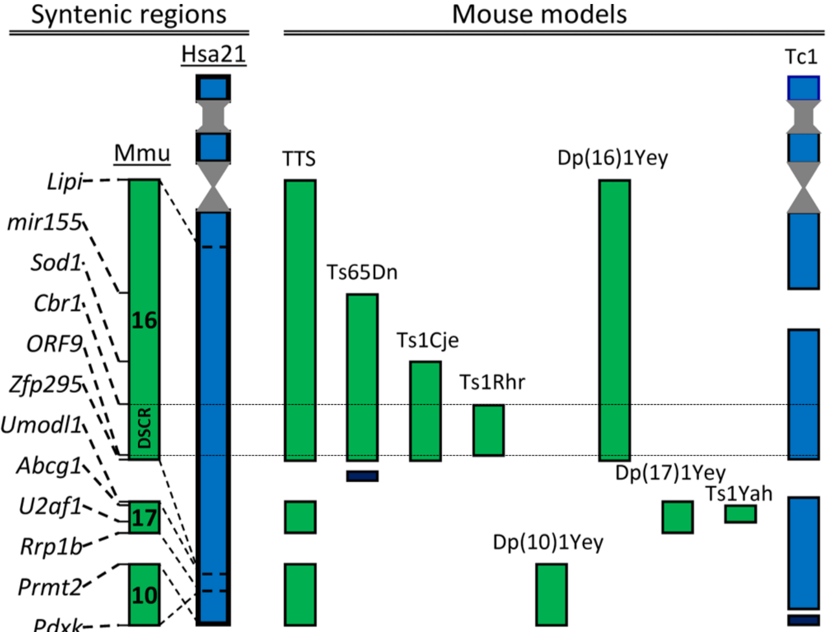


Figure 9. Trisomic mouse models of DS. Left: Correspondence of syntenic genomic regions on human chromosome 21 (HSA21) to mouse chromosomes (Mmu) 10, 16, and 17. Right: Representation of the triplicated genomic regions in trisomic mouse models of DS. Human and mouse genomic regions are shown in blue and green respectively. Mouse model genomic regions absent on HSA21 are shown in black. DSCR: Down syndrome critical region. Adapted from (Belichenko *et al.*, 2015).

The non-coding genes, such as miRNAs or long-non coding RNAs, are also well conserved and are located across all the three mouse chromosomes (Gupta *et al.*, 2016). Based on these homologies, multiple segmental trisomic mouse models have been developed. However, it is worthy to mention that there are some HSA21 genes that are not conserved in mice and other mouse genes that have no human homologues (Gupta *et al.*, 2016).

2.3.1. Ts65Dn mouse model

The Ts(17^{16})65Dn line (Ts65Dn) was the first viable trisomic mouse model of DS and it has been the most used model for preclinical studies. The Ts65Dn model was induced by cesium irradiation causing a reciprocal translocation between the distal region of Mmu16 (~13.4 Mb in total; from Mrp139 to Znf295) and the pericentromeric region of Mmu17 resulting in a small marker chromosome (Davisson *et al.*, 1993; Reeves *et al.*, 1995). This small chromosome contains a region of Mmu16 with 90 conserved protein-coding Hsa21 genes. However, it also contains an extra segment of Mmu17 with non-DS-related genes, including ∼35 protein-coding genes, 15 non-protein-coding genes and 10 pseudogenes (Duchon *et al.*, 2011). Despite this model does not have a perfect construct validity, it has a really good face validity recapitulating most of the phenotypes seen in DS patients at different ages including neuroanatomical and behavioral alterations (Rueda *et al.*, 2012; Aziz *et al.*, 2018).

Phenotypic features in the Ts65Dn model include reduced birth weight, postnatal developmental delay, craniofacial dysmorphogenesis and male sterility (Reeves *et al.*, 1995; Richtsmeier *et al.*, 2002; Aziz *et al.*, 2018). Since fertility is generally compromised in Ts65Dn males, the transmission is maintained through the maternal germline, which might affect the

phenotype of the trisomic and disomic progeny because mothers are trisomic unlike the human situation (Herault *et al.*, 2017).

Similarly to DS patients, Ts65Dn mice present impairments in hippocampal-dependent tasks including NORT (long-term but not short-term) (Fernandez *et al.*, 2007; Kleschevnikov *et al.*, 2012b; Contestabile *et al.*, 2013; Deidda *et al.*, 2015), NPRT (long-term and short-term) (Kleschevnikov *et al.*, 2012b; Contestabile *et al.*, 2013; Deidda *et al.*, 2015), MWM test (hidden platform during training and test phase and reversal test) (Reeves *et al.*, 1995; Sago *et al.*, 1998; García-Cerro *et al.*, 2014), radial arm maze (spatial reference and working memory) (Demas *et al.*, 1996), spontaneous alternation test (Fernandez *et al.*, 2007; Contestabile *et al.*, 2013) and contextual fear-conditioning (Contestabile *et al.*, 2013; García-Cerro *et al.*, 2014; Deidda *et al.*, 2015). In addition, this mouse model also exhibits other behaviors frequently observed in DS patients, such as sleep abnormalities (Colas *et al.*, 2008), locomotor hyperactivity (Escorihuela *et al.*, 1995) and increased seizures in some experimental epilepsy paradigms (Cortez *et al.*, 2009; Westmark *et al.*, 2010; Joshi *et al.*, 2016).

The overall brain volume of Ts65Dn mice is reduced during embryonic development. However after birth and during adulthood is similar to euploid brains (Aldridge *et al.*, 2007; Chakrabarti *et al.*, 2007). The overall hippocampal volume is similar to euploid animals but the volume of the hilus, dentate gyrus granule cell layer and cerebellum is decreased (Insausti *et al.*, 1998; Baxter *et al.*, 2000; Lorenzi and Reeves, 2006). Hypocellularity is present in hippocampus, perirhinal cortex, cerebellum and neocortex across all postnatal life stages (Baxter *et al.*, 2000; Olson *et al.*, 2004b; Lorenzi and Reeves, 2006; Chakrabarti *et al.*, 2007; Roncacé *et al.*, 2017). As in DS patients, it has been hypothesized that this

hypocellularity is secondary to deficits in neurogenesis. In fact, neurogenesis in Ts65Dn displays severe proliferative deficits during prenatal stages and from early postnatal stages until adulthood (Lorenzi and Reeves, 2006; Chakrabarti *et al.*, 2007; Belichenko and Kleschevnikov, 2011).

Other abnormalities include a reduction in spine density and enlarged spine head volumes in cortex and hippocampus (Dierssen *et al.*, 2003; Belichenko *et al.*, 2004; Kurt *et al.*, 2004). Electrophysiological studies also have shown altered synaptic function. LTP is decreased whereas LTD is increased in CA1 and dentate gyrus hippocampal regions (Siarey *et al.*, 1997, 1999; Kleschevnikov *et al.*, 2004; Costa and Grybko, 2005) (see Table 5 for a comparison between brain phenotypes of DS individuals and Ts65Dn mice).

Brain phenotypes	DS patients	Ts65Dn
Overall brain volume	Reduced brain volume from early developmental stages until adulthood	Reduced brain volume only during embryonic period
Hippocampal volume	Reduced around 27%	Reduced hilus and dentate gyrus granule cell layer volumes
Neuronal density	Hippocellularity appears during prenatal stages and persists after birth	Hypocellularity appears during prenatal stages and persists after birth
Neurogenesis	Deficits in embryonic neurogenesis	Deficits in embryonic and adult neurogenesis
Dendritic spines	Reduced spine density	Reduced spine density and enlarged spines
Synaptic plasticity	---	Reduced hippocampal LTP and increased LTD

Table 5. Comparison of the main brain phenotypes between DS patients and Ts65Dn mice.

Alterations in neurotransmitter systems have also been reported in Ts65Dn mice. In fact, an imbalance between excitation and inhibition, and specifically over-inhibition, has been postulated as one of the underlying causes of cognitive deficits in DS and has been linked with the reduction of hippocampal LTP (Siarey *et al.*, 1997; Kleschevnikov *et al.*, 2004; Costa and Grybko, 2005; Martínez-Cué *et al.*, 2014). Several reports have shown an increase of GABAergic interneurons and particularly, parvalbumin and somatostatin-positive cells in the cortex and hippocampus (Belichenko *et al.*, 2009b; Chakrabarti *et al.*, 2010; Pérez-Cremades *et al.*, 2010). This increase correlates at the synaptic level with an increase in inhibitory terminals in the primary somatosensory cortex and dentate gyrus (Pérez-Cremades *et al.*, 2010; Martínez-Cué *et al.*, 2013; García-Cerro *et al.*, 2014). However, in most of the brain areas the concentration of inhibitory terminals remains unaltered (Kurt *et al.*, 2000, 2004; Belichenko *et al.*, 2009b; Kleschevnikov *et al.*, 2012a; Parrini *et al.*, 2017). In dentate granule cells, there is also a shift in inhibitory synaptic connections away from the dendritic shafts and spine heads and onto the spine necks (Belichenko *et al.*, 2004). Alterations in $GABA_A$ and $GABA_B$ mediated inhibition have also been reported in hippocampus (Best *et al.*, 2012; Kleschevnikov *et al.*, 2012a; Deidda *et al.*, 2015), although the levels of GABA remain unaltered (Santin *et al.*, 2014).

Regarding excitatory neurotransmission, reductions in the number of excitatory synapses have been described, which may contribute to the imbalance between excitatory and inhibitory synaptic. A decrease of excitatory synapse density is found in CA1, CA3 and dentate gyrus regions (Kurt *et al.*, 2004; Stagni *et al.*, 2013; García-Cerro *et al.*, 2014; Parrini *et al.*, 2017). The decrease of dentate gyrus granule cells has been reported across all postnatal life stages (Lorenzi and Reeves, 2006; Contestabile *et*

al., 2007; Bianchi *et al.*, 2010; García-Cerro *et al.*, 2014), whereas the number of hippocampal pyramidal cells does not seem to be reduced during adulthood (Lorenzi and Reeves, 2006). In addition, Ts65Dn mice also exhibit alterations in excitatory glutamatergic transmission and in signaling mechanisms downstream glutamatergic receptors (Siarey *et al.*, 2006; Costa *et al.*, 2008). Instead, no changes have been found in glutamate levels in hippocampus of Ts65Dn mice although glutamine levels are reduced (Santin *et al.*, 2014).

Other alterations in the Ts65Dn model include decreased expression of the serotonin receptor 1A (Bianchi *et al.*, 2010) and a decrease of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) (Bimonte-Nelson *et al.*, 2003; Bianchi *et al.*, 2010) and nerve growth factor (NGF) (Salehi *et al.*, 2006).

As DS patients, Ts65Dn mice also show an Alzheimer's disease neuropathology including increased production of APP and amyloid-β peptide, and tau hyperphosporylation, progressive memory decline and progressive degeneration of basal forebrain cholinergic neurons and locus coeruleus noradrenergic neurons. However, these mice do not exhibit accumulation of amyloid plaques or neurofibrillary tangles (Hamlett *et al.*, 2016).

2.3.2. Other trisomic mouse models

Other segmentally trisomic models carrying different segments of Mmu10, 16, and 17 have been created. Another model of segmental trisomy of Mmu16 is the Ts1Cje (Sago *et al.*, 1998). The Ts1Cje model presents a trisomic segment shorter than the one present in Ts65Dn mice but does not present non-orthologous triplicated genes. These mice show slight phenotypic differences in comparison to Ts65Dn animals, although

learning deficits are less severe (Sago *et al.*, 1998, 2000; Aziz *et al.*, 2018). Importantly, *App* gene is absent in the trisomic segment of this model and, as a consequence, these mice do not present Alzheimer's disease neuropathology (Sago *et al.*, 1998).

Another interesting model is the Ts1Rhr mouse, which is a trisomic for the DSCR region (Olson *et al.*, 2004a). This mouse model presents deficits in NORT and spontaneous alternation task (Belichenko *et al.*, 2009a). However, it shows normal performance of the MWM test (Olson *et al.*, 2007) and it does not present craniofacial abnormalities supporting the idea that DSCR is not the only region responsible for DS phenotypes but it is sufficient to produce some structural and functional brain effects.

A mouse model trisomic for all three HSA21 syntenic mouse chromosomal regions has also been developed, the triple trisomic model (TTS) (Yu *et al.*, 2010a). This mouse model was generated by crossing three partial trisomy lines: *Dp(10)1Yey*/+, *Dp(16)1Yey*/+ and *Dp(17)1Yey*/+ (also known as Dp10, Dp16 and Dp17). Despite the construct validity is better in this model than in the Ts65Dn, both show similar phenotypes including cognitive deficits and reduced LTP (Yu *et al.*, 2010b; Belichenko *et al.*, 2015). In fact, the TTS model presents milder expression of some of the phenotypes in comparison to the Ts65Dn model. This may be explained because the additional genetic material is contained within the corresponding mouse chromosomes and not in a freely-segregating chromosome as in the Ts65Dn mouse model (Belichenko *et al.*, 2015). In addition, the use of the TTS mouse model is limited because of its poor viability and breading (Belichenko *et al.*, 2015). The comparison between the three partial trisomy lines and the TTS has emphasized the importance of the Mmu16 region in the brain phenotype since TTS and Dp16 but not

Dp17 nor Dp10, present cognitive and synaptic plasticity deficits (Yu *et al.*, 2010b).

Other models resulted from the insertion of the HSA21 in the mouse genome. The first transchromosomic mouse model was Tc(Hsa21)1TybEmcf (Tc1). It contains a freely segregating copy of the HSA21 (O'Doherty *et al.*, 2005). This model presents deficits in learning and memory processes and neuroanatomical alterations, such as deficits in hippocampal LTP. However, phenotypes are milder than the ones in Ts65Dn mice probably due to mosaicism and rearrangement processes (Gribble *et al.*, 2013).

2.3.3. Transgenic mouse models overexpressing Down syndrome candidate genes

Transgenic models overexpressing a single gene are very useful to dissect the effects of candidate dosage-sensitive genes. Among the different candidate dosage-sensitive genes underlying DS-associated brain phenotypes (Table 4), *DYRK1A* has been demonstrated to play a crucial role in the pathogenesis of DS disorder.

DYRK1A gene is located at the HSA21 in the DSCR and it encodes for a protein kinase that is overexpressed both in DS patients and in TS65Dn mice (Dowjat *et al.*, 2007). DYRK1A protein catalyzes both its autophosphorylation on a tyrosine residue in the activation loop and the phosphorylation of serine and threonine residues in its multiple substrates (Becker *et al.*, 1998; Himpel *et al.*, 2001). Through the phosphorylation of different proteins, DYRK1A modulates multiple processes including embryonic neuronal development, synaptic plasticity, neuronal progenitor proliferation and differentiation and synaptogenesis (Becker *et al.*, 2014). Examples of protein substrates for DYRK1A are the transcription factors cyclic AMP response element binding protein (CREB), nuclear

factor of activated T-cells (NFAT) and signal transducer and activator of transcription 3 (STAT3), the splicing factors cyclin L2, SF2 and SF3, the translation factor eIF2B, the cytoskeletal proteins TAU and microtubule-associated protein 1B (MAP1B), the synaptic proteins dynamin I, amphiphysin I and synaptojanin I or other proteins like glycogen synthase, caspase-9 and notch (Duchon and Herault, 2016).

Different strategies have been used to overexpress *Dyrk1A* in mice: yeast artificial chromosomes (YAC), bacterial artificial chromosomes (BAC) and expression vectors driven by exogenous promoters (Altafaj *et al.*, 2001; Branchi *et al.*, 2004; Ahn *et al.*, 2006; Guedj *et al.*, 2012). In this thesis, we have used the transgenic *Dyrk1A* (TgDyrk1A) developed by Dr.Cristina Fillat and colleagues (Altafaj *et al.*, 2001). This mouse model overexpresses the full-length cDNA of rat *Dyrk1A* under the control of the inducible sheep metallothionein-Ia promoter. Previous reports demonstrated that DYRK1A protein levels were similar to those observed in DS fetal human tissue when the transgene was not induced (Toiber *et al.*, 2010).

At the behavioral level, TgDyrk1A mice present hyperactivity, deficits in spatial learning and cognitive flexibility in MWM and in NORT (Altafaj *et al.*, 2001; de la Torre *et al.*, 2014). Concomitant to these cognitive deficits, adult TgDyrk1A mice also present neurological alterations. They show decreased cell proliferation rate and a reduced cell cycle exit in the subgranular zone of the dentate gyrus (Pons-Espinal *et al.*, 2013b). They also exhibit reduced spine density and dendritic branching in pyramidal cells of the motor cortex (Martinez de Lagran *et al.*, 2012). In addition, TgDyrk1A mice show a decreased firing rate and γ-frequency power in the prefrontal cortex of anesthetized and awake mice (Ruiz-Mejias *et al.*, 2016).

Other transgenic models overexpressing *Dyrk1A* also exhibit learning and memory deficits (Smith *et al.*, 1997; Ahn *et al.*, 2006; Souchet *et al.*, 2014), alterations in dendritic spines and in synaptic plasticity and associated pathways (Ahn *et al.*, 2006; Souchet *et al.*, 2014; Thomazeau *et al.*, 2014). Changes in GABAergic and glutamatergic related proteins also suggest that *Dyrk1A* is involved in the imbalance between excitatory and inhibitory synaptic inputs (Souchet *et al.*, 2014).

Interestingly, normalization of *Dyrk1A* overexpression in Ts65Dn mice improved hippocampal-dependent learning deficits. Consistently with these cognitive improvements, this strategy also normalized hippocampal synaptic plasticity, proliferation and differentiation in dentate gyrus and the balance between inhibitory and excitatory synaptic markers (Altafaj *et al.*, 2013; García-Cerro *et al.*, 2014). Moreover, it also normalized several of the Alzheimer's disease-like phenotypes found in the Ts65Dn mice (García-Cerro *et al.*, 2017).

All these results, provide compelling evidence of the role of *DYRK1A* overexpression in several neurological alterations of DS pathology.

2.4. Therapeutic interventions for Down syndrome

Nowadays, early intervention programs are the unique approach available to treat cognitive deficits in DS. These programs are focused on specific interventions to infants and young individuals to provide cognitive stimulation and emphasize training in cognitive domains especially affected in DS (Odom and Diamond, 1998; Bonnier, 2008). Several reports have demonstrated the beneficial effects of these programs in improving cognitive abilities, fine motor skills and self-sufficiency. However, their efficacy is limited and DS patients still face high limitations in their daily life (Hines and Bennett, 1996).

In recent years, a great effort has been focused to identify pharmacological interventions in an attempt to improve cognitive deficits of DS patients (Stagni *et al.*, 2015). With this objective, multiple studies have been performed in DS mouse models using different strategies.

To counterbalance increased GABAergic inhibition, $GABA_A$ antagonists were assessed (pentylenetetrazol and picrotoxin) in young and adult Ts65Dn mice. These compounds rescued hippocampal-dependent memory and hippocampal LTP (Fernandez *et al.*, 2007; Rueda *et al.*, 2008a). However, they also were anxiogenic and had proconvulsant properties (Dorow *et al.*, 1983; Little *et al.*, 1984). In order to avoid these negative effects, $GABA_AR$ α-5 negative allosteric modulators were explored demonstrating similar results with improvements in recognition and spatial memory, hippocampal synaptic plasticity and adult neurogenesis (Braudeau *et al.*, 2011; Martínez-Cué *et al.*, 2013). The concept of excessive inhibition was questioned by a report suggesting that $GABA_AR$ might mediate excitatory rather than inhibitory transmission in hippocampus of Ts65Dn mice. In that scenario, administration of bumetanide, an inhibitor of the Na^+-K^+-Cl^- cotransporter 1 (NKCC1), also restored synaptic plasticity and hippocampus-dependent memory in trisomic mice (Deidda *et al.*, 2015).

Targeting the glutamatergic system was also attempted. The NMDA receptor uncompetitive antagonist memantine had beneficial effects over cognitive impairment in adult Ts65Dn mice. Its mechanism of action may underlie the normalization of NMDA receptor functioning caused by the inhibition of the calcineurin protein or by other proteins encoded by HSA21 genes, such as the transient invasion and metastasis protein 1 (TIAM 1), intersectin 1 (ITSN1) or APP. An increase in BDNF expression after memantine administration may also contribute to the normalization

of behavior (Costa *et al.*, 2008; Rueda *et al.*, 2010; Lockrow *et al.*, 2011; Costa, 2014).

Administration of the antidepressant drug fluoxetine, which inhibits serotonin reuptake and increases cell proliferation in hippocampus, was also explored. Ts65Dn treated with fluoxetine at early postnatal stages showed improvements in explicit memory and adult neurogenesis, normalized hippocampal expression of serotonin receptor 1A and BDNF and restored functional connectivity between dentate gyrus and CA3 (Bianchi *et al.*, 2010; Stagni *et al.*, 2013). Interestingly, prenatal treatment with fluoxetine had positive effects on behavior that were maintained at postnatal day 45 along with the normalization of normalization of neuronal precursor proliferation, hypocellularity in dentate gyrus and neocortex, dendritic development, cortical and hippocampal synapse development and brain volume (Guidi *et al.*, 2014). The administration of the mood stabilizer lithium in adult Ts65Dn mice also restored hippocampal-dependent memory and dentate gyrus LTP, probably increasing neurogenesis by stimulating proliferation of neuronal progenitor cells via the Wnt/β-catenin pathway in the dentate gyrus (Contestabile *et al.*, 2013).

Targeting individual candidate dosage-sensitive genes was also investigated. The use of ethosuximide, a KCNJ6 inhibitor, failed to rescue cognitive performance in MWM and fear conditioning (Vidal *et al.*, 2012). By contrary, administration of the polyphenol epigallocatechin-3-gallate, an inhibitor of DYRK1A, restored deficits in cognition and synaptic plasticity in Ts65Dn mice (Xie *et al.*, 2008; de la Torre *et al.*, 2014), and neurogenesis and granule cell dendritic architecture in TgDyrk1A mice (Pons-Espinal *et al.*, 2013b). Other DYRK1A inhibitors also provided good results (Neumann *et al.*, 2018; Nguyen *et al.*, 2018).

Some studies focused specifically on targeting the Alzheimer's disease neuropathology present in aging Ts65Dn mice modulating APP (Netzer *et al.*, 2010; Belichenko *et al.*, 2016) and cholinergic and noradrenergic pathways (Granholm *et al.*, 2000; Chang and Gold, 2008; Rueda *et al.*, 2008b; Salehi *et al.*, 2009; Ash *et al.*, 2014). Interestingly, the endocannabinoid system was also studied as a potential target. Administration in aging mice (11-12 months of age) of JZL 184, a selective inhibitor of the enzyme monoacylglycerol lipase (see section 4.1.3), rescued deficits in long-term memory and hippocampal LTP (Lysenko *et al.*, 2014).

Other targets investigated were the Sonic hedgehog (Das *et al.*, 2013; Gutierrez-Castellanos *et al.*, 2013) and the mammalian target of rapamycin (mTOR) pathways (Andrade-Talavera *et al.*, 2015), oxidative stress (Lockrow *et al.*, 2009; Shichiri *et al.*, 2011) or neurotrophic factors (Blanchard *et al.*, 2011; Parrini *et al.*, 2017; Stagni *et al.*, 2017).

The lack of common experimental procedures across the studies makes difficult to compare the efficacy among the different therapies (Stagni *et al.*, 2015). Several experimental conditions differ in these studies including doses and duration of the treatments, age and sex of mice or the phenotypes assessed.

The studies above provide proof of concept observations that therapies can be attempted in DS individuals to improve learning and memory. In fact, some of these preclinical studies have prompted clinical trials in DS patients. Several clinical trials have been carried out in DS population (Table 6), although most of them failed to demonstrate significant beneficial effects over cognition. The most promising result was obtained with the epigallocatechin-3-gallate compound. In a phase II study, the combination of a green tea extract supplement containing 45%

epigallocatechin-3-gallate and cognitive training for 12 months improved significantly visual recognition memory, inhibitory control and adaptive behavior in young adult DS patients (16-34 years old) (de la Torre *et al.*, 2016). Phase III trials with a larger population should confirm the long-term efficacy of this strategy. At present, a phase II is being performed in pediatric population (6-12 years old).

Drug	Target	Reference
Vitamins and supplements	---	(Pueschel *et al.*, 1980; Weathers, 1983; Lonsdale and Kissling, 1986)
Donepezil	Cholinergic system	(Kishnani *et al.*, 1999, 2010; Kondoh *et al.*, 2011)
Thyroxine	Thyroid gland	(van Trotsenburg *et al.*, 2005)
Rivastigmine	Cholinergic system	(Heller *et al.*, 2006, 2010; Spiridigliozzi *et al.*, 2016)
Folinic acid	Folate metabolism	(Blehaut *et al.*, 2010)
Memantine	NMDA receptor	(Boada *et al.*, 2012) Ongoing (NCT02304302; University Hospitals Cleveland Medical Center)
Epigallocatechin-3-gallate	DYRK1A	(de la Torre *et al.*, 2014, 2016)
Basmisanil	$GABA_AR$ α-5	(NTC01436955, NTC02024789 NTC02484789; Hoffmann-La Roche)
ELND005	Amyloid pathway	(Rafii *et al.*, 2017)
Pentylenetetrazol	$GABA_A$	Ongoing (COMPOSE study)
Insuline glulisine	Glucose metabolism	Ongoing (NCT02432716; HealthPartners Institute)
ACI-24	Amyloid pathway	Ongoing (NCT02738450; AC Immune)

Table 6. Chronology of the main clinical trials carried out in DS population.

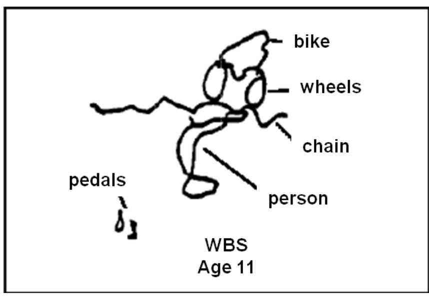
3. William's Beuren syndrome

WBS, also known as Williams' syndrome, is a rare neurodevelopmental disorder caused by the heterozygous deletion of a region containing 26 to 28 genes at 7q11.23. The estimated prevalence of this disorder is 1 in 7,500 individuals (Strømme *et al.*, 2002). Apart from few rare cases of autosomal dominant inheritance (Sadler *et al.*, 1993), the deletion occurs *de novo* during gamete formation. Patients present manifestations affecting mainly the vascular connective tissue, the CNS and the endocrine system (Pérez Jurado, 2003). The presence and severity of the different symptoms vary across individuals, which may be explained by environmental and genetic factors (Pérez Jurado, 2003). The cardiovascular phenotype is the most life-threatening complication for WBS patients. Moreover, intellectual disability has a major impact in their quality of life where independent living is infrequent (Howlin and Udwin, 2006).

3.1. Neuropathology and neuropsychological aspects in Williams-Beuren syndrome

The cognitive profile of WBS is characterized by weaknesses in visuospatial and visuomotor skills and relative strengths in language and facial processing abilities (Bellugi *et al.*, 2000; Schmitt, 2001). Patients with WBS show mild-to-moderate intellectual disability with an IQ score from 40 to 90, averaging around 60 (Bellugi *et al.*, 2000; Mervis and Klein-Tasman, 2000). WBS individuals show deficits in working memory and executive function abilities (Vicari *et al.*, 2003; O'Hearn *et al.*, 2009; Menghini *et al.*, 2011). Unlike DS patients, WBS patients have stronger alterations in implicit rather than in explicit memory (Vicari, 2001). However, deficits in explicit memory have also been described, especially in spatial memory

more than in object and face recognition (Vicari *et al.*, 2005). Visuospatial construction, understood as the ability to see an object/image as a set of parts and then construct a replica from these parts, is extremely impaired in WBS individuals (Mervis and Klein-Tasman, 2000) (Figure 10). These defects in visuospatial construction may be explained by deficits in spatial working memory (Vicari *et al.*, 2003; O'Hearn *et al.*, 2009).



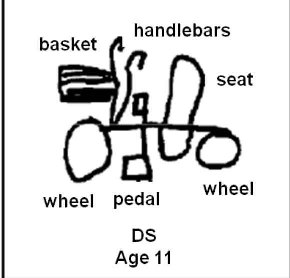


Figure 10. Visuospatial construction assessed by drawing in a child with WBS in the left and a patient with DS in the right. The WBS patient draws the parts of a bicycle but they are not organized coherently. Conversely, DS patient draws a simplified representation but with the correct organization (Bellugi *et al.*, 2000).

The personality of individuals with WBS has been described as hypersociable, including overfriendliness, high motivation to interact with strangers and increased empathy (Gosch and Pankau, 1994, 1997). This phenotype may be consequence of an increased interest for social stimuli, especially for human faces (Riby and Hancock, 2009). In addition, WBS individuals show difficulties to detect fear signs and reduced responses to negative social images indicating a decreased responsiveness to social threat (Plesa-Skwerer *et al.*, 2006, 2011). Despite their increased social behavior, they also exhibit problems in social adjustment, poor social judgement and difficulties in forming and maintaining friendships leading in several cases to social isolation (Gosch and Pankau, 1997; Stinton *et al.*, 2010). They also show psychiatric comorbidities such as anxiety, specific phobias or sleep problems (Dykens, 2003; Ashworth *et al.*, 2013).

Interestingly, musical skills of WBS patients exceed their cognitive level, being musical creativity and emotional reactions to music greater than normal controls (Don *et al.*, 1999). By contrary, they also exhibit hyperacusis (hypersensitivity to sounds), auditory allodynia (fearfulness to specific sounds) and a progressive hearing loss (Zarchi *et al.*, 2010).

Consistent with the neurological features above, WBS patients also present structural brain abnormalities. Brain volume is reduced by 10-15% in WBS individuals compared to age-matched controls, with greater affectations in white matter (Reiss *et al.*, 2000). Brain regions are not homogeneously affected. Parieto-occipital regions, *corpus callosum* and basal ganglia are reduced, whereas the frontal lobe and cerebellum seems to be preserved (Reiss *et al.*, 2000; Schmitt *et al.*, 2001).

A relative increase in amygdala volume has been reported in WBS individuals along with functional alterations of this region (Capitão *et al.*, 2011). Amygdala activation is attenuated by threatening faces and is increased in non-social and threatening scenes (Meyer-Lindenberg *et al.*, 2005b; Muñoz *et al.*, 2010). This may explain the social disinhibition and the non-social anxiety shown in WBS individuals. An increase in grey matter volume was found in two regions of the prefrontal cortex, the orbitofrontal cortex and the medial prefrontal cortex (Reiss *et al.*, 2004), although others found a decrease in the orbitofrontal cortex (Meyer-Lindenberg *et al.*, 2004). Both, the activation of the orbitofrontal cortex and its interaction with amygdala also display abnormalities in response to a specific task (Meyer-Lindenberg *et al.*, 2005a). Social interactions of patients with lesions in the frontal cortex share similarities with those of WBS individuals, supporting that the abnormal activity of the frontal cortex may have a key role in the social phenotype of WBS (Porter *et al.*, 2007).

Hippocampal volume is preserved in WBS individuals. However, they present a decrease in resting blood flow extending into the entorhinal cortex and a reduction N-acetyl aspartate, a marker of neuronal and synaptic integrity (Meyer-Lindenberg *et al.*, 2005b), indicating an overall depression of hippocampal metabolism and activity.

Cytoarchitecture abnormalities have also been described in WBS post-mortem brains including a decrease of neuronal density at the prefrontal cortex and an increase in other regions (e.g. visual area, primary somatosensory cortex and primary visual cortex) (Galaburda *et al.*, 2002; Lew *et al.*, 2017). In addition, pyramidal cortical neurons of layers V/VI exhibit an increase in total dendritic length and in number of dendritic spines (Chailangkarn *et al.*, 2016).

3.2. Cardiovascular phenotype

Cardiovascular disease affects the majority of WBS patients and it is characterized by stenosis of medium and large arteries and hypertrophy of smooth muscle cells. Stenosis is most frequently located above the aortic valve at the sinotubular junction, where it is known as supravalvular aortic stenosis. Supravalvular aortic stenosis occurs with an incidence of 70% whereas pulmonary aortic stenosis affects the 34% of patients (Pober *et al.*, 2008). Around 50% of individuals require surgical or catheter interventions (Bruno *et al.*, 2003).

Systemic hypertension is also frequent in WBS individuals (40-70%) beginning normally during adulthood, but sometimes found during childhood (Pober and Morris, 2007). The cause of hypertension remains unknown, although it may be a compensatory mechanism to abnormal vasculature (Faury *et al.*, 2003). There is a lack of specific treatments to ameliorate the WBS cardiovascular phenotype.

3.3. Genetic condition of Williams-Beuren syndrome

The deleted region in WBS is known as WBS critical region (WBSCR). WBSCR is flanked by three groups of low copy repeat sequences, also known as segmental duplications, which are fragments of 10-400 kb of high sequence homology. This high homology can produce a misalignment and an unequal crossing of these sequences during meiosis and as consequence, the deletion of the flanked region. Around 90-95% of patients present a 1.55 Mb deletion, whereas a larger deletion of 1.83 Mb occurs in the 5% of cases and smaller atypical deletions are present in few cases (Figure 11) (Bayés *et al.*, 2003).

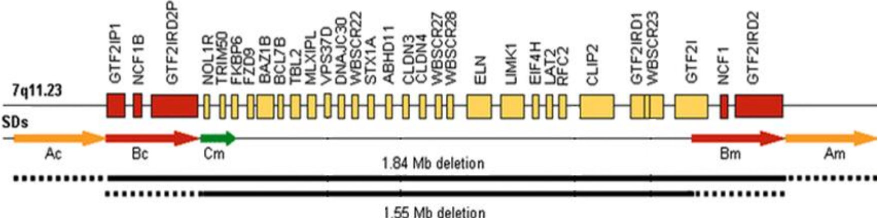


Figure 11. Schematic representation of the WBS *locus* at the 7q11.23 chromosomal region containing single copy genes (*yellow boxes*) and segmental duplications (SDs; *arrows*). The *black lines* below span the common 1.55 and 1.84 Mb WBS deletions. Adapted from (Antonell *et al.*, 2010b).

The absence of one copy of each of the genes of the deleted region is expected to reduce the expression of these genes by half. This has been confirmed with few tissue-specific exceptions (Merla *et al.*, 2006). Furthermore, transcriptomic profile of lymphoblastoid cell lines and primary skin fibroblasts showed a high number of differentially expressed genes, 151 and 868, respectively, in comparison to control cells (Antonell *et al.*, 2010b; Henrichsen *et al.*, 2011).

It is though that the hemizygous deletion of some of the genes of the WBSCR contributes to the phenotypes of WBS. Elastin gene was the first dosage-sensitive gene identified in WBS and its loss produces the

cardiovascular phenotype observed in patients (Ewart *et al.*, 1993). This gene encodes for elastin that is the main component of elastic fibers from the extracellular matrix of connective tissue. Deletions or point mutations of elastin gene were found in patients with isolated supravalvular aortic stenosis (Curran *et al.*, 1993). Moreover, heterozygous knock-out (KO) mice for this gene exhibit mild arterial stenosis like WBS patients (Li *et al.*, 1998). The effects of hemizygosity of other genes are less clear although some possible associations have been described (Table 7). Several genes have been linked to the neurocognitive phenotype of WBS patients including the transcription factor II-I (TFII-I) family, LIM domain kinase 1 (LIMK1), FRIZZLED 9 (FZD9), CAP-GLY domain containing linker protein 2 gene (CLIP2); bromodomain adjacent to a zinc-finger domain protein 1B gene (BAZ1B, which is also called Williams syndrome transcription factor gene [WSTF]), syntaxin 1A gene (STX1A) and eukaryotic translation initiation factor 4H (EIF4H) (Pérez Jurado, 2003).

Hemizygous Gene and Putative Effect	Likelihood of Effect	Data Sources†
FZD9		
Osteopenia	Possible	Mouse models
BAZ1B		
Hypercalcemia, intracardiac malformations	Possible	Mouse models
STX1A		
Impaired glucose tolerance	Possible	Mouse models, other human populations
ELN		
Arteriopathy with vascular stenoses, hypertension, vascular smooth-muscle-cell overgrowth	Definite	Mouse models, other human populations, and atypical deletions
Soft skin with premature aging, hoarse voice, inguinal hernias	Probable	Other human populations
Facial dysmorphology	Possible	Other human populations
LIMK1		
Impaired visuospatial abilities	Possible	Mouse models, atypical deletions
CLIP2		
Impaired visuospatial and motor abilities	Possible	Mouse models, atypical deletions
GTF2I* family, including *GTF2IRD1		
Craniofacial abnormalities, dental abnormalities, growth retardation, behavioral abnormalities, intellectual disability, WBS cognitive profile, decreased retinal thickness, impaired visual responses	Possible	Mouse models, atypical deletions
NCF1		
Reduced risk of hypertension	Possible	Fine mapping of WBSCR

Table 7. Candidate gene-dosage sensitive genes for WBS phenotypes (Pober, 2010).

3.4. Mouse models of Williams-Beuren syndrome

In mouse, the entire WBSCR is conserved on chromosome band 5G2, although it is inverted with respect to the centromere and the flanking genes (Valero *et al.*, 2000). WBS mouse models with partial and complete deletions of the WBSCR have been developed. The partial models have deletions spanning the proximal (proximal deletion, PD) or the distal part (distal deletion, DD) of the WBSCR (Figure 12). The study of the phenotypic features of these mouse models show that the distal region is associated with cognitive and brain abnormalities, whereas the proximal region is linked with social behavior and acoustic startle response alterations along with an increase in neuronal density in somatosensory cortex (Li *et al.*, 2009). The result of crossing PD and DD was the double heterozygous (D/P) mouse model, which carries the complete region deleted although *Limk1A* is lost on homozygosis and the two half deletions are *in trans* (Figure 12). D/P mice have a good face validity exhibiting many classical symptoms of WBS such as hypersociability, reduced brain size and increased neuronal density in primary somatosensory cortex (Li *et al.*, 2009).

A mouse model with higher construct validity was developed by Campuzano and colleagues, the WBS complete deletion (WBS-CD) mouse model. This mouse model carries an heterozygous deletion of the entire region of interest (Gtf2i-Fkbp6) in *cis* (Figure 12) (Segura-Puimedon *et al.*, 2014) and displays several WBS phenotypic traits. WBS-CD mice show an hypersociable phenotype, cognitive deficits in short-term NORT and spatial working memory, an increased startle response to acoustic stimuli and anxiety alterations (Segura-Puimedon *et al.*, 2014; Borralleras *et al.*, 2016; Ortiz-Romero *et al.*, 2018). WBS-CD mice also exhibit an overall reduction of the brain weight and alterations in neuronal density, dendritic spines and synaptic plasticity. Specifically, in hippocampus WBS-CD mice

display a volume reduction of the CA3 *stratum oriens* and pyramidal layer, a reduction of dendritic length and spine density in CA1 and a decrease in LTP (Segura-Puimedon *et al.*, 2014; Borralleras *et al.*, 2015, 2016). They also show a decrease in neuronal density in basolateral amygdala (Segura-Puimedon *et al.*, 2014). In addition, these mice display a mild cardiovascular phenotype with a borderline hypertension, mildly increased arterial wall thickness and cardiac hypertrophy (Segura-Puimedon *et al.*, 2014).

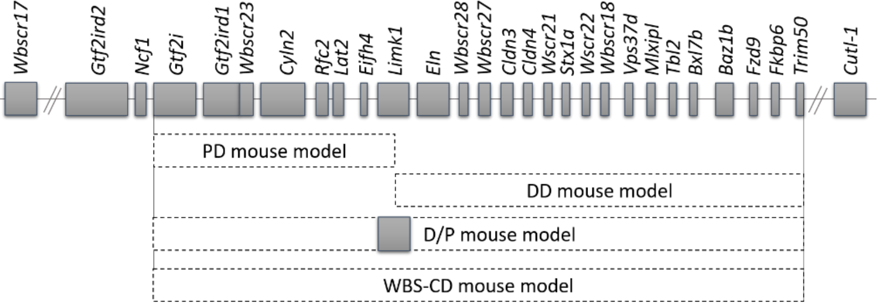


Figure 12. WBS mouse models with partial and complete deletions of the WBS critical region. Dashed lines indicate the deleted region of each mouse model.

Several KO mice of single genes from the WBSCR have been developed helping to elucidate the possible role of candidate dosage-sensitive genes. The members of the TFII-I family, the general transcription factor II-I (*GTF2I*), the GTF2I repeat domain-containing protein I (*GTF2IRD1*) and the GTF2I repeat domain containing protein II (*GTF2IRD2*) are strong candidates for the neurological and cognitive WBS-associated phenotypes. *GTF2I* and *GTF2IRD1* genes are invariably deleted in WBS individuals, whereas *GTF2IRD2* is only deleted in patients with the 1.83Mb deletion (Bayés *et al.*, 2003). These genes encode for transcription factors important for multiple biological processes, such as cell cycle and proliferation and Ca^{2+} and immune signaling (Roy, 2012). Heterozygous KO mice with a frame deletion of exon 2 of *Gtf2i* ($\Delta Gtf2i^{+/-}$) exhibit increased

sociability, abnormal anxiety behavior and enhanced acoustic sensitivity (Lucena *et al.*, 2010; Borralleras *et al.*, 2015). Another mutant model for *Gtf2i* also displays increased sociability and deficits in object recognition (Sakurai *et al.*, 2011). Interestingly, the normalization of Gtf2i expression in the WBS-CD mouse model normalized its social behavior and anxiety alterations (Borralleras *et al.*, 2015). All of these data indicate that the *GTF2I* deletion plays an important role in cognitive phenotypes of WBS and especially, in social behavior. This is supported by the phenotypic traits of patients with atypical deletions (Dai *et al.*, 2009; Antonell *et al.*, 2010a).

3.5. Therapeutic intervention for Williams-Beuren syndrome

WBS therapies are scarce. As in DS, early intervention programs are the only available treatment for the cognitive phenotype of WBS patients (Morris, 1999). Unfortunately, few studies have been performed in WBS mouse models to find potential therapeutic strategies for the WBS cognitive phenotypes. Normalization of *Gtf2i* expression by intracisternal gene therapy has beneficial effects in sociability, anxiety and motor coordination of WBS-CD mice (Borralleras *et al.*, 2015). However, the translation of this strategy to the clinics is very limited at the present. Recently, it was described that the epigallocatechin-3-gallate has beneficial effects over short-term memory and the cardiovascular phenotype of WBS-CD mice. However, this compound does not have effects over other phenotypes including sociability, working memory or anxiety-related behavior (Ortiz-Romero *et al.*, 2018). Therefore, novel approaches tackling this syndrome are needed.

4. The endocannabinoid system

The *Cannabis sativa* plant and its preparations such as marijuana, have been used for recreational and therapeutic purposes for thousands of years. During the 19th century, numerous attempts were made to isolate the active compounds of marijuana and to elucidate their structures, but they were unsuccessful. The reason is that *Cannabis sativa* contains over 120 compounds (Morales *et al.*, 2017), known as phytocannabinoids, with closely related structures and physical properties, making difficult their separation (Mechoulam and Parker, 2013). Among them, Δ^9-tetrahydrocannabinol (Δ^9-THC) is the primary psychoactive component of *Cannabis sativa* plant and it was finally isolated in 1964 (Gaoni and Mechoulam, 1964). Few years later, the identification and cloning of specific receptors from animal tissues (Matsuda *et al.*, 1990) brought the discovery of an endogenous modulatory system, which was entitled endocannabinoid system (ECS).

4.1. Components of the endocannabinoid system

The ECS is an endogenous lipidic signaling system involved in many physiological functions. It is composed by the cannabinoid receptors, their endogenous ligands known as endocannabinoids and the enzymes involved in their synthesis and inactivation.

4.1.1. Cannabinoid receptors

Originally, it was though that cannabinoid compounds exerted their pharmacological effects through nonspecific interactions with membrane lipids. It was not until the late 1980s when it was postulated that they would act through the activation of specific endogenous receptors (Howlett *et al.*, 1986; Devane *et al.*, 1988; Herkenham *et al.*, 1990).

Endogenous and exogenous cannabinoids act through the activation of at least two cannabinoid receptors, the cannabinoid type-1 receptor (CB1R) and the cannabinoid type-2 receptor (CB2R). They are both G-protein-coupled receptors (GPCRs) mainly coupled to the inhibitory Gi/o protein (Childers and Deadwyler, 1996) and they contain seven hydrophobic transmembrane domains connected by alternating extracellular and intracellular loops. However, they differ in their distribution. Indeed, CB1R is highly expressed on the CNS whereas CB2R is mainly expressed on the periphery (Svíženská *et al.*, 2008). Growing evidences indicate that cannabinoids may also bind to other receptors including the transient receptor potential vanilloid receptor 1 (TRPV1) (Di Marzo and De Petrocellis, 2010), G protein-coupled receptor 55 (GPR55), G protein-coupled receptor 18 (GPR18), the sphingosine-1-phosphate lipid receptors GPR3, GPR6 and GPR12 (Morales and Reggio, 2017) and the peroxisome proliferator-activated receptors (PPAR) (O'Sullivan, 2007).

4.1.1.1. Cannabinoid type-1 receptor

CB1R was cloned in 1990 from a rat brain cDNA library (Matsuda *et al.*, 1990). This discovery was followed by the cloning of its human (Gérard *et al.*, 1990) and mouse (Chakrabarti *et al.*, 1995) homologs, which share between 97 to 99% of amino acid sequence. CB1R is the most abundant GPCR in the CNS (Kano *et al.*, 2009), where it controls a wide spectrum of physiological processes including learning and memory, motor coordination, pain perception, regulation of appetite, body temperature and brain development (Busquets-Garcia *et al.*, 2016). Its distribution has been well characterized both in rodent (Herkenham *et al.*, 1991; Tsou *et al.*, 1998) and humans (Westlake *et al.*, 1994). The CNS areas with the highest density of CB1R are the hippocampus, amygdala, cerebellum,

periaqueductal gray, substantia nigra pars reticulata and some cortical areas including the somatosensory, cingulate and entorhinal cortex. Other regions exhibit moderate levels of CB1R such as the medial hypothalamus, basal forebrain, solitary nucleus or spinal cord. Low levels are displayed in other areas like the thalamus or brainstem (Figure 13) (Svíženská *et al.*, 2008). Furthermore, CB1R is also expressed in peripheral tissues including the cardiovascular system (Sierra *et al.*, 2018), fat tissue, liver, pancreas (Cota *et al.*, 2003), gastrointestinal tract (Izzo and Sharkey, 2010), immune system (Jean-Gilles *et al.*, 2015), retina (Porcella *et al.*, 2000), bone (Idris *et al.*, 2005) and skeletal muscle (Cavuoto *et al.*, 2007).

The expression of CB1R at mRNA and protein levels differs at distinct developmental stages and may be dysregulated in different pathological situations (Laprairie *et al.*, 2012).

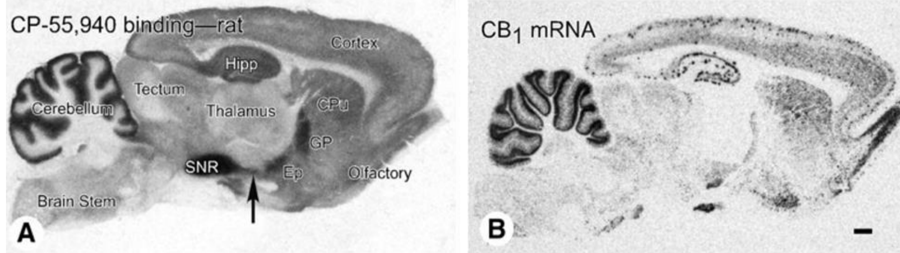


Figure 13. Autoradiographic film images showing CB1R protein and mRNA localization in rat brain. (A) Binding assay with the tritiated ligand CP-55,940 in a sagittal section of rat brain. (B) Hybridization of a CB1R-oligonucleotide probe showing expression of CB1R at mRNA level (Freund *et al.*, 2003)**.**

At the cellular level, CB1R is mainly expressed at the membrane of neuronal presynaptic terminals where it controls neurotransmitter release of GABA and glutamate. There are evidences that CB1R may also control the release of other neurotransmitters such as acetylcholine, noradrenaline, dopamine, D-aspartate and cholecystokinin (Pertwee and Ross, 2002). Furthermore, in the last decade, several reports have described the presence of CB1R in other locations rather than presynaptic

terminals (Figure 14). CB1R seems to be also expressed at postsynaptic terminals, at least in cortex, where it may regulate self-inhibition processes (Bacci *et al.*, 2004; Marinelli *et al.*, 2009) and, in hippocampus, where it may regulate synaptic plasticity through the hyperpolarization-activated cyclic nucleotide-gated channels (Maroso *et al.*, 2016). Other studies have demonstrated the presence of CB1R in astrocytes, where it contributes to synaptic plasticity processes, and in microglia (Cabral and Marciano-Cabral, 2005; Navarrete and Araque, 2008, 2010). In addition, new evidences reveal the presence of CB1R in a particular intracellular organelle, the mitochondria, where, by regulating cellular metabolism, may impact on synaptic function and memory formation (Bénard *et al.*, 2012; Hebert-Chatelain *et al.*, 2016). The presence of CB1R in mitochondria is not only restricted to neurons, but it seems to be also present in astrocytes (Gutiérrez-Rodríguez *et al.*, 2018). Furthermore, CB1R is also present in endosomal and lysosomal compartments where its function is not clear (Leterrier *et al.*, 2004; Thibault *et al.*, 2013).

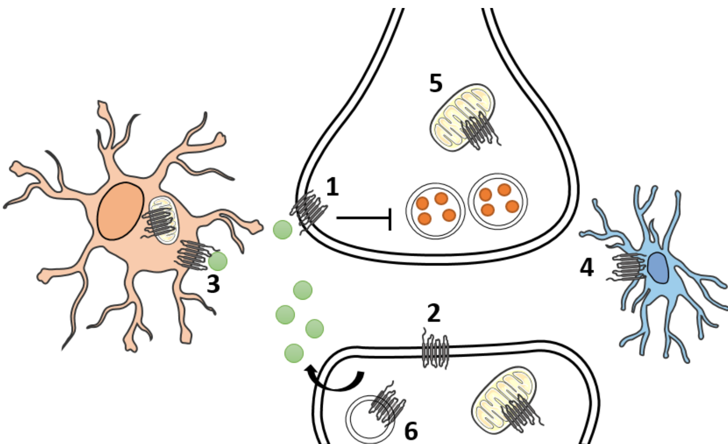


Figure 14. Schematic view of the cellular distribution of CB1R over a synapse. CB1R is expressed in neurons at presynaptic level (1), but also at postsynaptic level (2). Moreover, its presence has been described in other cell types including astrocytes (3) and microglia (4). It has also been detected in intracellular organelle, such as mitochondria (5) and endosomes (6).

The expression of CB1R is heterogeneous among different brain areas but also among different cell types. Interestingly, the levels of expression do not correlate with their functional relevance. For instance, in the hippocampus, CB1R is present at high levels on cholecystokinin inhibitory terminals and at low levels on glutamatergic terminals and astrocytes (Kano *et al.*, 2009). Although CB1R expression is lower on glutamatergic terminals than on GABAergic terminals, glutamatergic CB1R is more effectively coupled to downstream signal transduction (Steindel *et al.*, 2013). In fact, it has been demonstrated that CB1R on glutamatergic terminals has an important role in the regulation of hippocampal excitability (Marsicano *et al.*, 2003; Monory *et al.*, 2006). Differences in CB1R expression also exist among different synapses as it has been shown that the control of GABA release by CB1R of hippocampal interneurons differs between somatic and dendritic synapses, being more significant the control over the somatic ones (Lee *et al.*, 2010).

4.1.1.2. Cannabinoid type-2 receptor

CB2R was cloned in 1993 and has 44% overall homology to CB1R (Munro *et al.*, 1993). It is widely expressed in the immune system where it mediates immune response and the anti-inflammatory properties of cannabis (Buckley *et al.*, 2000). The presence of CB2R on healthy brains has been controversial probably due to its low expression levels and a lack of tools for its study. However, electrophysiological, anatomical and behavioral evidences have indicated that CB2R is present in healthy brains (Van Sickle *et al.*, 2005; Gong *et al.*, 2006; Onaivi, 2006; den Boon *et al.*, 2012; Stempel *et al.*, 2016). Interestingly, CB2R expression is inducible; it is very low under physiological conditions whereas it dramatically enhances under pathological conditions, such as neuropathic pain

(Svíženská *et al.*, 2013), neurodegenerative diseases (Palazuelos *et al.*, 2009; Concannon *et al.*, 2015; Aso and Ferrer, 2016; López *et al.*, 2018) or stroke (Yu *et al.*, 2015). It is though that this increase is a protective compensatory mechanism since activation of CB2R reduces neuroinflammation (Palazuelos *et al.*, 2008, 2009).

Among brain cell populations, CB2R is expressed in a subpopulation of neurons, but also in astrocytes and perivascular microglia (Núñez *et al.*, 2004; Sheng *et al.*, 2005; Gong *et al.*, 2006; Stempel *et al.*, 2016). Unlike CB1R, CB2R would be mainly expressed at postsynaptic terminals where it would regulate neuronal excitability (Zhang *et al.*, 2014; Stempel *et al.*, 2016). CB2R mediates a self-inhibitory type of plasticity in pyramidal cells of the hippocampal regions CA2 and CA3 (Stempel *et al.*, 2016). However, further studies should elucidate the physiological role of CB2R on CNS of healthy subjects.

4.1.2. Cannabinoids

Cannabinoid receptors are modulated, whether activated or inhibited, by the so-called cannabinoid compounds. These may be endogenous or exogenous ligands. Endogenous compounds are called endocannabinoids. Exogenous compounds can be classified with regards to their origin as phytocannabinoids and synthetic cannabinoids.

4.1.2.1. Endocannabinoids

The discovery of the cannabinoid receptors suggested the presence of possible endogenous ligands. The first endocannabinoid, N-arachidonoylethanolamine (AEA, also called anandamide), was discovered in 1992 (Devane *et al.*, 1992). Three years later, another endocannabinoid was described, the 2-arachidonoylglycerol (2-AG) (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). AEA and 2-AG are the most studied

endocannabinoids and they belong to the N-acylethanolamin and monoacylglycerol groups respectively (Figure 15). Both are lipidic molecules and unlike classical neurotransmitters they are not prestored in secretory vesicles. Instead, they are synthesized "on demand" in an activity-dependent manner. This classical view has been challenged since recent data indicate that at least AEA may also be stored inside the cell (Oddi *et al.*, 2008).

AEA is an endogenous eicosanoid derivative and acts as a partial agonist to the CB1R and CB2R. Moreover, it is also an endogenous ligand for other receptors, the TRPV1 (Zygmunt *et al.*, 1999) and the PPAR (O'Sullivan, 2007). 2-AG is a full agonist to CB1R and CB2R and its concentration in the brain is about 170 times higher than AEA (Stella *et al.*, 1997). 2-AG also activates PPAR, but does not activate TRPV1 (Du *et al.*, 2011). Both ligands, AEA and 2-AG, have slightly lower affinity for CB2R than for CB1R.

AEA

2-AG

Figure 15. Chemical structure of the main endocannabinoids, AEA and 2-AG. (Adapted from Mechoulam et al., 2014).

Other putative endocannabinoids have been identified including 2-arachydonoylglycerol ether (2-AGE, noladin ether) (Hanus *et al.*, 2001), N-arachidonoyldopamine (NADA) (Huang *et al.*, 2002), O-arachidonoylethanolamine (virodhamine) (Porter *et al.*, 2002), N-arachidonoylglycine (NAGly) (Huang *et al.*, 2001) and Cis-9,10-octadecanoamide (oleamide or ODA) (Leggett *et al.*, 2004). It has been demonstrated that these compounds activate CB1R or CB2R. However, some issues remain to be elucidated such as their affinity to the receptors,

their physiological role or their biosynthesis and inactivation pathways (Fonseca *et al.*, 2013).

Several lipidic molecules have structural similarities with endocannabinoids but are not able to activate cannabinoid receptors. These compounds, known as "endocannabinoid-like compounds", share some metabolic enzymes with endocannabinoids and may indirectly interfere with the ECS (Fonseca *et al.*, 2013; Kleberg *et al.*, 2014). Other N-acylethanolamines (palmitoylethanolamide, stearoylethanolamide, and oleoylethanolamide) and monoacylglycerols (2-linoleoylglycerol (2-LG), 2-oleoylglycerol (2-OG) and 2-palmitoylglycerol (2-PG)) are included in this group. Interestingly, some of these compounds (i.e. 2-LG, 2-PG and palmitoylethanolamide) potentiate endocannabinoid activity through the so-called "entourage effect" locally modifying endocannabinoids metabolism and probably by other unknown mechanisms (Ben-Shabat *et al.*, 1998; Jonsson *et al.*, 2001).

4.1.2.2. Phytocannabinoids

Phytocannabinoids are a group of C_{21} terpenophenolic constituents of the *Cannabis sativa* plant and its derivatives. The most abundant phytocannabinoids are Δ^9-THC, cannabidiol, cannabinol, cannabigerol, cannabichromene, Δ^9-tetrahydrocannabivarin and cannabidivarin (Turner *et al.*, 2017). Δ^9-THC is a partial agonist of CB1R and CB2R and it is the main responsible for the psychoactive effects of the marijuana (Figure 16) (Morales *et al.*, 2017). Cannabidiol is the second compound most abundant of the plant, and, unlike THC, does not present psychoactivity (Figure 16). Its affinity for CB1R and CB2R is very low (Table 8), however, *in vitro* studies suggest that cannabidiol may act as antagonist at CB1R and as inverse agonist at CB2R (Thomas *et al.*, 2009). Additionally, *in vivo*

evidences indicate that cannabidiol may also modulate GPR55 activity (Morales *et al.*, 2017).

Figure 16. Chemical structure of the phytocannabinoids Δ^9-THC and cannabidiol.

4.1.2.3. Synthetic cannabinoids

Beside natural compounds, several synthetic cannabinoids have been designed displaying different selectivity profiles for cannabinoid receptors. Most of them bind to the same site of cannabinoid receptors than the endogenous ligands, the orthosteric site. We can distinguish between agonist and antagonists and they may possess different affinities and intrinsic activities for CB1 and/or CB2R. The most used agonists are HU-210, CP55,940 and WIN55,212-2, and they possess similar activity for CB1R and CB2R (Table 8).

The most used CB1R-selective competitive antagonists are rimonabant (SR141716A), AM281, LY320135 and taranabant (MK-0364) (Figure 17) (Table 8). CB1R-selective competitive antagonists block activation of CB1R by either exogenous or endogenous cannabinoids in a competitive manner and bind with significant higher affinity to CB1R than CB2R (Pertwee *et al.*, 2010). However, most of these compounds (including rimonabant, AM251, AM281, LY320135 and taranabant) also behave as inverse agonists producing inverse cannabimimetic effects in the absence of agonists, suppressing tonic endocannabinoid signaling (Bouaboula *et al.*, 1997; Pan *et al.*, 1998; Mato *et al.*, 2002; Meye *et al.*, 2013). It has been postulated that these compounds may act as neutral CB1R antagonists at

low concentrations (nanomolar) and as inverse agonists at higher concentrations (micromolar) (Pertwee, 2005). However, more recently it has been described that rimonabant may act inhibiting Gαi/o subunit of heterotrimeric G proteins instead of acting as an inverse agonist at micromolar concentrations (Porcu *et al.*, 2018).

Figure 17. Chemical structure of the most used CB1R selective competitive antagonists. Adapted from (Pertwee *et al.*, 2010).

Several ligands behaving solely as neutral CB1R antagonists have also been developed such as NESS 0327 (Ruiu *et al.*, 2003) and O-2654 and O-2050 (Thomas *et al.*, 2004; Wiley *et al.*, 2011).

Moreover, in recent years, research has focused on compounds that can bind to allosteric sites, different from the orthosteric binding site. These ligands seem to act modifying the receptor conformation and altering the affinity and/or efficacy of orthosteric ligands in a positive or negative manner (Wootten *et al.*, 2013). Examples of CB1R allosteric modulators include ORG27569, ORG27759, ORG29647 (Price *et al.*, 2005), PSNCBAM-1 (Horswill *et al.*, 2009) or the endogenous molecules lipoxin A4 (Pamplona *et al.*, 2012), pepcans (Bauer *et al.*, 2012), cannabidiol (Laprairie *et al.*, 2015) and pregnenolone (Vallée *et al.*, 2014).

CANNABINOID RECEPTOR LIGANDS	Ki (nM)	
	CB1R	**CB2R**
Agonists with similar affinity for CB1R and CB2R		
(−)-Δ^9-THC	5.05–80.3	3.13–75.3
HU-210	0.06–0.73	0.17–0.52
CP55,940	0.5–5.0	0.69–2.8
R-(+)-WIN55,212-2	1.89–123	0.28–16.2
AEA	61–543	279–1940
2-AG	58.3, 472	145, 1,400
Agonists with higher affinity for CB1R		
ACEA	1.4, 5.29	195, >2,000
Arachidonylcyclopropylamide	2.2	715
R-(+)-methAEA	17.9–28.3	815–868
Noladin ether	21.2	>3,000
Agonists with higher affinity for CB2R		
JWH-133	677	3.4
HU-308	>10000	22.7
JWH-015	383	13.8
AM1241	280	3.4
CB1R-Selective Competitive Antagonists		
Rimonabant (SR141716A)	1.8–12.3	514–13,200
AM251	7.49	2,290
AM281	12	4,200
LY320135	141	14,900
Taranabant	0.13, 0.27	170, 310
NESS 0327	0.00035	21
O-2050	2.5, 1.7	1.5
CB2R-Selective Competitive Antagonists		
SR144528	50.3–>10,000	0.28–5.6
AM630	5152	31.2
JTE-907	2370	35.9
Others		
Cannabidiol	4350->10,000	2399->10,000
Cannabinol	120-1130	96-301

Table 8. Cannabinoid receptor ligands and their Ki values for the *in vitro* displacement of a tritiated compound (i.e [^{3}H] CP55,940, [^{3}H]SR141716A, [^{3}H]WIN55,212-2) from specific binding sites on rat, mouse or human CB1R and CB2R. Adapted from (Pertwee *et al.*, 2010).

4.1.3. Enzymes involved in the biosynthesis and degradation of endocannabinoids

There are different metabolic enzymes that regulate the bioavailability of the endocannabinoids by regulating their synthesis and degradation. Both AEA and 2-AG are arachidonic acid derivatives synthesized from precursors derived from membrane phospholipids. The enzymes involved in their synthesis are located mainly at synapses (Figure 18). However, their presence has also been observed in oligodendrocytes, astrocytes and microglial cells (Gomez *et al.*, 2010; Hegyi *et al.*, 2012).

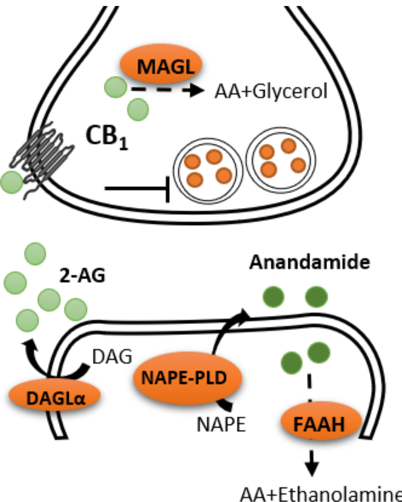


Figure 18. Location of the main biosynthetic and inactivating enzymes of endocannabinoids at synapse. DAG: 1,2-diacylglycerol; DAGLα: diacylglycerol lipase α; NAPE: N-acylphosphatidylethanolamine; NAPE-PLD: NAPE-hydrolyzing phospholipase D; FAAH: fatty acid amide hydrolase; AA: arachidonic acid; $EtNH_2$: ethanolamide. DAGLα is responsible for the synthesis of 2-AG and it is located at the plasma membrane of postsynaptic neurons (Dinh *et al.*, 2002) whereas MAGL degrades 2-AG and it is located presynaptically (Bisogno *et al.*, 2003). This distribution supports a role as retrograde messenger of 2-AG. NAPE-PLD is expressed at both presynaptic (Egertová *et al.*, 2008; Nyilas *et al.*, 2008) and postsynaptic level (Cristino *et al.*, 2008) and FAAH is located on neurons postsynaptic to CB1R (Egertová *et al.*, 2003). Solid arrows indicate transformation into active metabolites; dashed arrows indicate transformation into inactive metabolites.

AEA is synthesized in two main steps (Figure 19). First, an acyl group from the sn-1 position of glycerophospholipids is transferred to the amino group of phosphatidylethanolamine by the N-acyltransferase obtaining N-acylphosphatidylethanolamine (NAPE). Second, NAPE is hydrolyzed by the action of NAPE-hydrolyzing phospholipase D (NAPE-PLD) generating AEA and phosphatidic acid (Di Marzo *et al.*, 1994). Some studies have suggested that AEA can also be synthesized by alternative mechanisms dependent on phospholipase C (PLC) and protein tyrosine phosphatase N22 (PTPN22) (Liu *et al.*, 2006), and on the α/β-hydrolase domain type-4 (ABHD4) (Simon and Cravatt, 2006). This is supported by the fact that no significant changes on AEA levels were found in the brain of NAPE-PLD-KO mice (Leung *et al.*, 2006).

2-AG is also obtained from membrane phospholipids by two enzymatic reactions (Figure 19). In the first step, PLC degrades arachidonic acid-containing membrane phospholipids producing 1,2-diacylglycerol (DAG). Then, DAG is hydrolyzed by either of two selective diacylglycerol lipases, DAGL-α and DAGL-β, producing 2-AG (Prescott and Majerus, 1983). Among them, DAGL-α is the main enzyme responsible for the synthesis of 2-AG in the CNS (Gao *et al.*, 2010; Tanimura *et al.*, 2010).

Once the endocannabinoids released from the cell have activated their targets, they are rapidly transported into the intracellular space where they are degraded by hydrolysis or oxidation. Several mechanisms have been described to explain the endocannabinoid cellular uptake. Among them, the diffusion through the plasma membrane depending on the concentration gradient (Glaser *et al.*, 2003) has been proposed. Other hypothesis include the existence of a carrier protein, the "endocannabinoid membrane transporter" (Fegley *et al.*, 2004; Ligresti *et*

al., 2004) or that the uptake occurs via endocytosis (McFarland *et al.*, 2004).

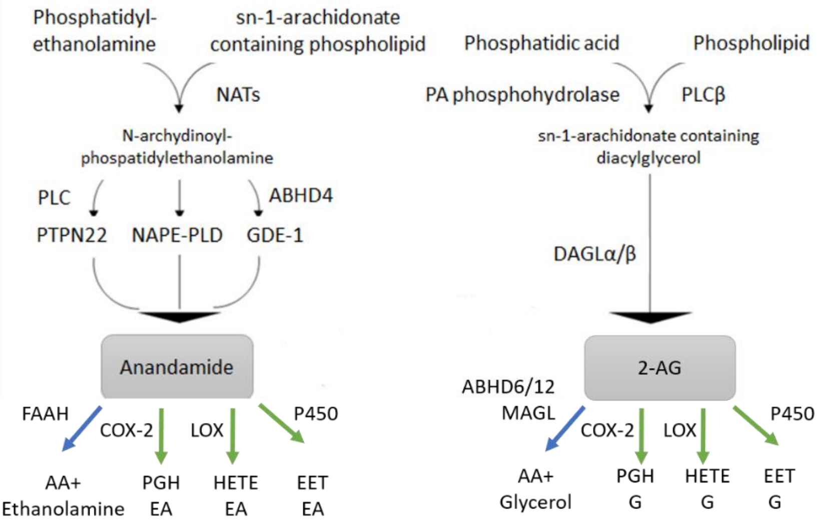


Figure 19. Main pathways of synthesis and inactivation of AEA and 2-AG. Blue arrows indicate inactivation and green arrows indicate oxidation. ABHD: α/β-hydrolase domain; COX2: cyclooxygenase 2; DAGL: diacylglycerol lipase; EA: ethanolamide; EET: epoxyeicosatrienoic acid; FAAH: fatty acid amide hydrolase; G: glycerol; GDE1: glycerophosphodiester phosphodiesterase 1; HETE: hydroxyepoxyeicosatrienoic acid; LOX: 15-lipoxygenase; MAGL: monoacylglycerol lipase; NAPE-PLD: N-acyl-phosphatidylethanolamine-specific phospholipase D; NATs: N-acyltransferases; PA: phosphatidic acid; PLC: phospholipase C; PTPN22: protein tyrosine phosphatase non-receptor type 22; PG: prostaglandins. Adapted from (Iannotti *et al.*, 2016).

AEA is mainly degraded by the fatty acid amide hydrolase (FAAH) to arachidonic acid and ethanolamine (Figure 19) (Cravatt *et al.*, 1996). Reports using FAAH KO mice have confirmed this fact as these mice show an increase of 15-fold in AEA levels (Cravatt *et al.*, 2001). Monoacylglycerol lipase (MAGL) seems to be responsible for 85% of 2-AG hydrolysis resulting in arachidonic acid and glycerol. The remaining is catalyzed by the α/β-hydrolase domain containing 6 and 12 (ABHD6 and ABHD12) (Blankman *et al.*, 2007). Both 2-AG and AEA can also be oxygenated by cyclooxygenase-

2, lipoxygenases and cytochrome P450 monooxygenases (Figure 19) (Kozak *et al.*, 2002; Snider *et al.*, 2010).

It is worthy to mention that all of these enzymes lack selectivity for 2-AG/AEA and also act over other members of N-acylethanolamines/monoacylglycerols families (Fonseca *et al.*, 2013).

4.2. Cannabinoid receptor signaling: intracellular signaling pathways

The stimulation of cannabinoid receptors produces several effects through the activation of multiple signaling pathways. As GPCR, CB1R and CB2R exert their biological functions by G-protein activation, mainly by the Gi/o family (Gα, Gβ and Gγ). The activation of Gαi/o causes the inhibition of adenylyl cyclase and a subsequent reduction of cyclic adenosine monophosphate (cAMP) levels and PKA activity (Howlett and Fleming, 1984; Howlett *et al.*, 1986). Furthermore, activation of Gβγi/o regulates phosphorylation and activation of different members of MAPK family including extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinase (JNK) (Figure 20) (Bouaboula *et al.*, 1995, 1996; Bosier *et al.*, 2010). Stimulation of CB1R also produces an intracellular transient increase of Ca^{2+} in a PLC-dependent manner that can be mediated by either Gi/o or Gq proteins (Sugiura *et al.*, 1997; Lauckner *et al.*, 2005). Under certain circumstances, CB1R can also couple Gs proteins causing adenylyl cyclase activation (Glass and Felder, 1997).

There are evidences that CB1R stimulation can modulate other signaling pathways including the phosphoinositide-3 kinase (PI3K)/Akt (Gómez del Pulgar *et al.*, 2000), glycogen synthase kinase 3 (GSK-3) (Ozaita *et al.*, 2007), mTOR (Puighermanal *et al.*, 2009) and PKC (Hillard and Auchampach, 1994; Busquets-Garcia *et al.*, 2018).

Receptor activation through the coupling to G proteins also modifies conductance of ions inhibiting voltage-gated Ca^{2+} channels (VGCCs) (N- and P/Q-type) (Mackie and Hille, 1992; Twitchell *et al.*, 1997) and activating A-type and inwardly rectifying K^+ channels (Deadwyler *et al.*, 1995; Mackie *et al.*, 1995). These effects lead to the repolarization of the plasmatic membrane and a decrease on neurotransmitter release in the presynaptic terminal (Bosier *et al.*, 2010).

Besides the effects mediated by G protein signaling, CB1R stimulation also triggers a later response recruiting the scaffold proteins β-arrestins at the plasma membrane, which mediates desensitization and internalization of CB1R and also activates intracellular pathways including the MAPK (Turu and Hunyady, 2010; Nogueras-Ortiz and Yudowski, 2016). CB1R activation also triggers sphingomyelin breakdown and ceramide accumulation through coupling with the adaptor protein FAN (factor associated with neutral sphingomyelinase activation) (Sánchez *et al.*, 2001).

Altogether, the response triggered by CB1R stimulation is complex not only because the immense diversity of effectors, but also due to the crosstalk among the different activated signaling pathways (Figure 20). Factors such as the lipid composition in the surrounding of the receptor, particularly the cholesterol content, could also be critical for the regulation of the signal transduction (Dainese *et al.*, 2010). Furthermore, a number of studies indicate that CB1R, as other GPCRs, may form homodimers (Wager-Miller *et al.*, 2002; Mackie, 2005) and heterodimers with other GPCRs including CB2R (Callén *et al.*, 2012), dopamine D2 (Kearn *et al.*, 2005; Khan and Lee, 2014), opioid (Rios *et al.*, 2006; Hojo *et al.*, 2008), orexin (Ellis *et al.*, 2006), adenosine (Carriba *et al.*, 2007) or serotonin receptors (Viñals *et al.*, 2015), further contributing to the diversity of signaling pathways and cellular functions of CB1R.

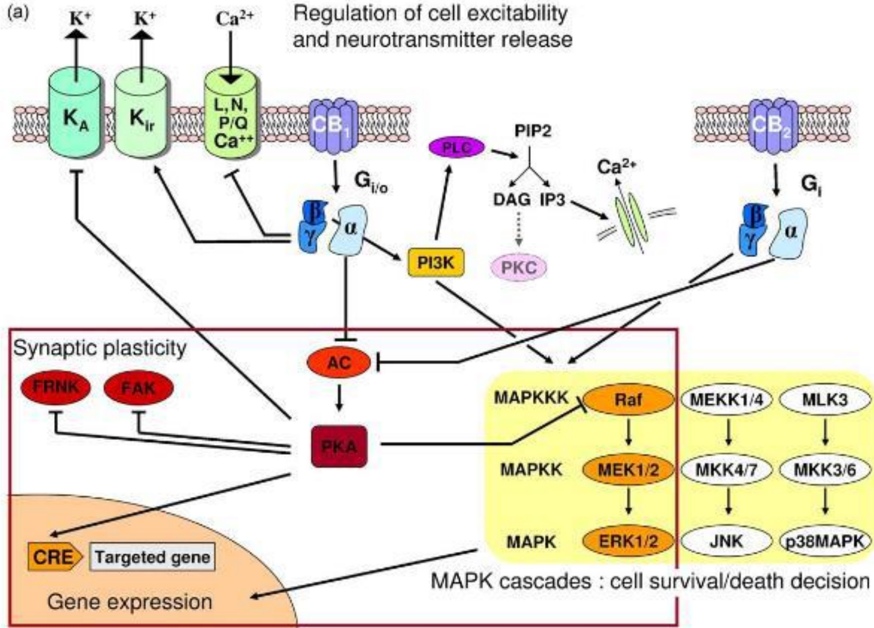


Figure 20. Complexity of the cannabinoid signaling. Crosstalk among the different pathways activated by the CB1R is illustrated by the variety of responses dependent on protein kinase A (PKA) inhibition. AC: adenylate cyclase; DAG: diacylglycerol; ERK1/2: extracellular signal-regulated kinase 1 and 2; FAK: focal-adhesion kinase; IP3: Inositol triphosphate; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; PI3K: phosphoinositide-3 kinase; PKC: protein kinase C; PLC: phospholipase C. (Bosier *et al.*, 2010)

4.3. Synaptic plasticity mediated by the endocannabinoid system

The ECS contributes to multiple forms of synaptic plasticity at different synapses. The main mechanism of action is the retrograde signaling, in which endocannabinoids are released from the postsynaptic cell and travel retrogradely to modulate pre-synaptic CB1R from the same or a neighboring synapse. Upon presynaptic activation of CB1R, neurotransmitter release is inhibited. This suppression can be transient (tens of seconds) leading to endocannabinoid-mediated short-term depression (eCB-STD), or long-lasting (minutes to hours) leading to endocannabinoid-mediated LTD (eCB-LTD) (Kano *et al.*, 2009; Kano, 2014). Different forms of eCB-STD/LTD have been described to occur at inhibitory

and excitatory synapses in various brain regions. Of special interest for this thesis is the hippocampus region. As mentioned above, in hippocampus CB1R is mainly expressed at presynaptic terminals of cholecystokinin-containing GABAergic interneurons but also of glutamatergic neurons. There, CB1R mediates two main forms of eCB-STD; the depolarization-induced suppression of inhibition (DSI) (Ohno-Shosaku *et al.*, 2001; Wilson and Nicoll, 2001) and depolarization-induced suppression of excitation (DSE) (Kreitzer and Regehr, 2001) in GABAergic and glutamatergic synapses, respectively. Both DSI and DSE are induced by postsynaptic depolarization leading to a Ca^{2+} influx through postsynaptic VGCC and to the subsequent release of endocannabinoids (Ohno-Shosaku *et al.*, 2012). However, there are also other forms of eCB-STD, in which the release of endocannabinoids is induced by strong activation of Gq/11-coupled receptors like metabotropic glutamate receptors (mGluRs) (Varma *et al.*, 2001) or M1/M3 muscarinic acetylcholine receptors (mAChRs) (Kim *et al.*, 2002) independent to postsynaptic Ca^{2+} elevation. eCB-STD mediates inhibition of neurotransmitter release through the inhibition of presynaptic VGCC likely through βγ subunits (Figure 21) (Kano *et al.*, 2009). The functional relevance of these forms of short-term plasticity mediated by endocannabinoids *in vivo* remains to be elucidated (Augustin and Lovinger, 2018).

Regarding eCB-LTD, LTD in hippocampus is mainly induced at inhibitory synapses of cholecystokinin-containing GABAergic interneurons where it is known as eCB-LTD of inhibition (I-LTD) (Heifets and Castillo, 2009). I-LTD involves more sustained CB1R activation than DSI. However once established, I-LTD maintenance does not require continued CB1R activation (Chevaleyre and Castillo, 2003). I-LTD is initiated by repetitive activity of neighboring excitatory synapse inputs leading to the activation

of mGluRs coupled to PLCβ and DGLα and to the subsequent mobilization of endocannabinoids (Figure 21) (Chevaleyre and Castillo, 2003). The mechanisms by which activation of CB1R causes long-lasting suppression on transmitter release are poorly understood (Araque *et al.*, 2017). Some downstream factors involved are PKA signaling (reduced by the inhibition of adenylyl cyclase), the active zone protein RIM1α, vesicle-associated protein Rab3B and VGCC (Chevaleyre *et al.*, 2007). Moreover, protein synthesis via mTOR signaling pathway may also be required (Younts *et al.*, 2016).

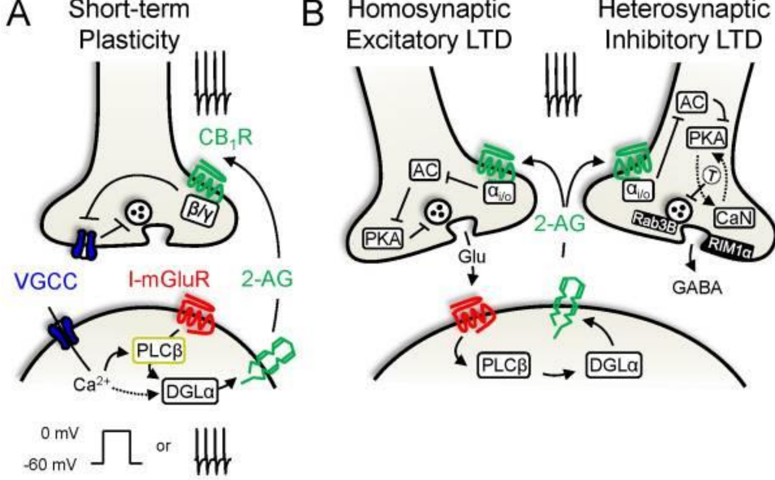


Figure 21. Endocannabinoid-mediated short- and long-term synaptic plasticity. (A) eCB-short term depression. (B) eCB-mediated excitatory long-term depression (E-LTD) and inhibitory LTD (iLTD). In hippocampus, E-LTD has only been described in neonatal hippocampus (Yasuda *et al.*, 2008). Dashed lines indicate putative pathways. AC: adenylyl cyclase; CaN: Ca^{2+}-sensitive phosphatase calcineurin; DGLα: diacylglycerol lipase; Glu: glutamate; I mGluRs: I metabotropic glutamate receptors (I mGluRs); PKA: protein kinase A; PLCβ: Phospholipase-Cβ; VGCC: voltage-gated Ca^{2+} channels; active zone protein RIM1α; vesicle-associated protein Rab3B (Castillo *et al.*, 2012).

Reducing inhibition during I-LTD but also during DSI, facilitates the induction of excitatory LTP at CA1 hippocampal region (Carlson *et al.*, 2002; Chevaleyre and Castillo, 2004). This facilitation may contribute to memory formation given the tight association between LTP and learning

and memory processes. In fact, ablation of I-LTD in hippocampus of mGluR5 CA1-KO mice also provokes the suppression of LTP facilitation and an impairment of an associative task with a temporal component, the trace-fear conditioning (Xu *et al.*, 2014).

Beyond the canonical retrograde endocannabinoid signaling, endocannabinoids may also exert their functions in a non-retrograde manner through the activation of postsynaptic CB1R, astrocytic CB1R or TRPV1. CB1R expressed postsynaptically in glutamatergic cells plays a role in hippocampal synaptic plasticity and spatial memory (Maroso *et al.*, 2016). This occurs through the modulation of hyperpolarization-activated cyclic nucleotide-gated channels that underlie the h-current, an important controller of dendritic excitability (Maroso *et al.*, 2016). Astrocytic CB1R expressed in hippocampus also plays a relevant role in synaptic plasticity and transmission. In fact, it is involved in different phenomena including eCB-LTD (Han *et al.*, 2012), spike timing-dependent LTD (t-LTD) (Andrade-Talavera *et al.*, 2016) and lateral synaptic regulation (Navarrete and Araque, 2008, 2010). The contribution to behavior of CB1R in this cell type is far from being understood. However, Han *et al.,* demonstrated that astrocytic CB1R mediates impairment of working memory caused by cannabinoids (Han *et al.*, 2012). In addition, a recent report showed that astrocytic CB1R may also contribute to long-term object recognition memory and LTP (Robin *et al.*, 2018). Activation of TRPV1 by AEA release mediates a postsynaptic form of LTD in dentate granule cells, which is dependent on AMPA receptor endocytosis (Chávez *et al.*, 2010).

In addition to activity-dependent release of endocannabinoids, tonic endocannabinoid signaling has also been suggested. Some studies suggest that CB1R may present constitutive activity even in the absence of ligands (Bouaboula *et al.*, 1997; Pan *et al.*, 1998; Mato *et al.*, 2002; Meye *et al.*,

2013). However, others suggest that tonic signaling relies in tonic endocannabinoid mobilization (Hentges *et al.*, 2005; Neu *et al.*, 2007). In any case, tonic signaling seems to mediate basal neurotransmitter release in specific conditions (Castillo *et al.*, 2012).

Finally, it is worthy to mention that the ECS itself also goes through plastic modulations triggered by neural activity or activation of CB1R by agonists. Thus, repeated stimulation of Schaffer collaterals potentiates DSI in a long-lasting form (Chen *et al.*, 2007; Zhu and Lovinger, 2007). The ECS also undergoes variations during development and aging (Castillo *et al.*, 2012). Interestingly, DSI is more robust in rats older than two weeks in comparison to rats at earlier postnatal days (Zhu and Lovinger, 2010). Moreover, I-LTD and suppressed inhibitory transmission mediated by CB1R agonist are higher in juvenile than in adolescent rats (Kang-Park *et al.*, 2007). In contrast, tonic endocannabinoid release has greater effects in GABAergic transmission in adult rather than in juvenile rats (Kang-Park *et al.*, 2007). Variations in the expression and function of CB1R or in endocannabinoids release may underlie these plastic changes (Castillo *et al.*, 2012). In fact, a reduction of the ECS signaling during aging has been reported. CB1R expression and functionality may be reduced in different brain areas including the hippocampus of old rodents in comparison with younger rodents (Berrendero *et al.*, 1998; Canas *et al.*, 2009). 2-AG levels seem to be also decreased through aging (Piyanova *et al.*, 2015). Importantly, changes in the components of the ECS have been described in several pathological conditions including neurological disorders like Alzheimer’s disease, Parkinson’s disease, Huntington's disease, multiple sclerosis or epilepsy (Iannotti *et al.*, 2016).

4.4. Physiological functions involving the ECS

The effects of *Cannabis sativa* in humans and animals plus the development of pharmacological (agonists and antagonists of the CB1R and CB2R) and genetic tools (KO mice) have allowed to elucidate the physiological functions controlled by the ECS.

The ECS regulates a plethora of functions both at central and peripheral level, which correlates with its ubiquitous distribution. At the central level, the ECS is crucial for synaptic homeostasis and the correct development of brain function. The different brain regions in which the ECS components are expressed, and particularly CB1R, have been associated with multiple brain functions. Expression of CB1R is found in areas involved in the control of learning and memory processes (hippocampus and cortex) (Kano *et al.*, 2009), fine control of movement and cerebellar learning performance (cerebellum and basal ganglia) (Fernández-Ruiz and Gonzáles, 2005; Kishimoto and Kano, 2006), anxiety, fear and stress (prefrontal cortex, various hypothalamic nuclei, the basolateral and the central amygdala) (Lutz *et al.*, 2015), food intake and energy balance (hypothalamus) (Di Marzo and Matias, 2005), reward processes (striatum and ventral tegmental area) (Solinas *et al.*, 2008) or pain modulation (spinal cord) (Guindon and Hohmann, 2009). At the peripheral level, the ECS plays a role in immune, reproductive, digestive and cardiovascular systems regulating processes such as inflammation, platelet aggregation, oocyte maturation, spermatogenesis progression, gastrointestinal motility and metabolism, energy balance via lipid and glucose homeostasis, blood pressure and heart rate (Maccarrone *et al.*, 2015; Chianese and Meccariello, 2016).

At the cellular level, different processes are mediated by the ECS including cell proliferation, differentiation, migration, survival and synapse

formation (Galve-Roperh *et al.*, 2013). The regulation of these processes may change depending on the cellular context (Galve-Roperh *et al.*, 2013). We will focus on the role of the ECS system in memory and social behavior.

4.4.1. Role of the endocannabinoid system in memory

Several behavioral evidences support that the ECS system plays a central role in the regulation of learning and memory processes (Davies *et al.*, 2002; Zanettini *et al.*, 2011; Puighermanal *et al.*, 2012). These evidences include the well described effects of cannabis consumption over memory in humans, but also the effects caused by targeting the ECS in mice or the memory status of CB1R KO mice. Furthermore, the distribution of the components of the ECS in the hippocampus, a key region for memory processes, is consistent with a role in memory function (Di Marzo *et al.*, 2000).

Consumption of marijuana in humans produces an impairment in episodic and working memory, but does not affect retrieval of previously learned memory (Ranganathan and D'Souza, 2006). Consistently, administration of CB1R agonists to rodents also produces deficits in several memory tasks such as the NORT (Schneider and Koch, 2002; Barna *et al.*, 2007; Clarke *et al.*, 2008), 8-arm radial arm (Lichtman *et al.*, 1995), spatial alternation in a T-maze (Jentsch *et al.*, 1997; Suenaga *et al.*, 2008), MWM (Ferrari *et al.*, 1999; Varvel *et al.*, 2001), contextual fear-conditioning (Pamplona and Takahashi, 2006), passive avoidance (Kruk-Slomka and Biala, 2016) or delayed matching/non-matching to position task with lever presentation (Heyser *et al.*, 1993; Hampson and Deadwyler, 2000). These effects seem to be dependent on CB1R as pretreatment with CB1R antagonists blocked some of these memory deficits (Pamplona and Takahashi, 2006; Barna *et al.*, 2007). Most of the results have been obtained by systemic

administration of the CB1R agonists. However, intrahippocampal infusion of the compounds has also been performed highlighting the importance of hippocampal CB1R (Lichtman *et al.*, 1995; Clarke *et al.*, 2008; Suenaga *et al.*, 2008).

The enhancement of the endocannabinoid tone by blocking the metabolism of endocannabinoids has shown a variety of effects. Increasing of AEA levels by FAAH inhibitors impairs object recognition memory (Busquets-Garcia *et al.*, 2011; Basavarajappa *et al.*, 2014) and working and spatial memory in the Y-maze test (Basavarajappa *et al.*, 2014). In contrast, it also facilitates spatial memory in the MWM (Varvel *et al.*, 2007) and passive avoidance learning (Mazzola *et al.*, 2009). These discrepancies may occur through a CB1R-independent mechanism since AEA and other fatty acids such as oleoylethanolamine and palmitoylethanolamine, that are also enhanced after FAAH inhibition, can also bind to PPAR-α (Mazzola *et al.*, 2009).

Contrary to CB1R agonists, the blockade of CB1R commonly produces memory improvements. Administration of the CB1R antagonist/inverse agonist rimonabant produces memory improvements in an olfactory recognition task (Terranova *et al.*, 1996), the radial-arm maze (Lichtman, 2000; Wolff and Leander, 2003) and elevated T-maze (Takahashi *et al.*, 2005). However, other paradigms such as the spatial delayed-non-match-to-sample are not modified after CB1R antagonist administration (Mallet and Beninger, 1996). Besides pharmacological studies, CB1R KO mice presents enhanced cognitive performance in different tasks like the NORT (Reibaud *et al.*, 1999; Maccarrone *et al.*, 2002), shock-probe burying test (Degroot and Nomikos, 2004) or contextual fear conditioning under highly aversive conditions (Jacob *et al.*, 2012). By contrary, these mice exhibit deficits in reversal learning in the MWM (Varvel and Lichtman, 2002).

Therefore, most of the results indicate that cannabinoid agonists impair working and long-term memory, while cannabinoid antagonists/inverse agonists or genetic deletion of cannabinoid receptors improve memory performance (Zanettini *et al.*, 2011). Discrepant results may be attributable to diverse experimental conditions (task, dose, route of administration, timing of administration, specie and strain among others). Different behavioral tests may be dependent on CB1R expressed in different brain regions or in different cell types. It is also worth mentioning that cannabinoids have effects over other behaviors including anxiety, locomotion, feeding, motivation or nociception, which may also influence the results.

Electrophysiological studies measuring synaptic plasticity also support the role of the ECS in learning and memory processes. Endocannabinoids mediate multiple forms of synaptic plasticity (see section 4.3) and several reports have shown that the ECS is involved in hippocampal LTP, whose association with memory and learning processes has been discussed above (section 1.1.4). Cannabinoid administration prevents LTP acting on CB1R (Stella *et al.*, 1997; Hoffman *et al.*, 2007; Abush and Akirav, 2009), whereas CB1R KO mice show an enhanced LTP (Bohme *et al.*, 2000; Jacob *et al.*, 2012) indicating that cannabinoid activation restricts LTP. Administration of CB1R antagonists cause both facilitation and impairment of LTP, which may be explained by different methodological conditions (Slanina *et al.*, 2005; de Oliveira Alvares *et al.*, 2006). In addition, whereas deletion of GABAergic CB1R leads to a decrease in hippocampal LTP, deletion of glutamatergic CB1R leads to an increase in hippocampal LTP revealing that the contribution to CB1R to LTP may depend on cell type population (Monory *et al.*, 2015).

Some evidences indicate that the ECS may also regulate adult neurogenesis. Neuronal progenitor cells express a functional ECS and can produce endocannabinoids (Aguado *et al.*, 2005; Butti *et al.*, 2012; Compagnucci *et al.*, 2013). Moreover, endocannabinoid signaling regulates multiple steps of adult neurogenesis including cell proliferation, cell differentiation or cell survival (Galve-Roperh *et al.*, 2013). In addition, chronic administration of cannabinoids have an effect over adult neurogenesis (Prenderville *et al.*, 2015). Although it seems clear that the ECS makes contributions to adult neurogenesis, how modulates neurogenesis remains to be elucidated. In fact, contradictory data have been found regarding cannabinoid effects. Cannabinoid agonists can enhance (Jiang *et al.*, 2005), decrease (Marchalant *et al.*, 2009; Abboussi *et al.*, 2014) or have no effect (Wolf *et al.*, 2010; Abboussi *et al.*, 2014) over cell proliferation depending in the conditions assessed. For example, chronic administration of HU-210 in rats enhanced cell proliferation in the dorsal hippocampus of adult rats (Jiang *et al.*, 2005). Conversely, administration of another CB1R agonist, WIN55,212-2, to adolescent but not to adult rats, caused a decrease in the number of new neurons in hippocampus (Abboussi *et al.*, 2014). Adult CB1R KO mice exhibited a reduction in astrogliogenesis, but an increase in neurogenesis (Aguado *et al.*, 2006). These data indicate that regulation of adult neurogenesis by the ECS is highly complex, may act at multiple steps and may be dependent on environmental conditions.

Altogether, these results suggest that the ECS contributes to memory and learning processes probably through the modulation of synaptic plasticity and adult neurogenesis processes.

4.4.2. Role of the endocannabinoid system in social behavior

The distribution of the ECS fits with a role in social behavior since CB1R expression is abundant to moderate in brain regions involved in social behavior like amygdala, cingulate cortex and frontal cortex (Tsou *et al.*, 1998; Svíženská *et al.*, 2008). First evidences of the role of the ECS in human social behavior come from marijuana smokers who were more communicative, interactive (Georgotas and Zeidenberg, 1979) and cooperative (Salzman *et al.*, 1976) in comparison with placebo groups. Nowadays, there are other evidences supporting this hypothesis. Thus, single nucleotide polymorphisms in the *CNR1* gene have been associated to striatal activity and duration of social gaze in response to happy faces (Chakrabarti *et al.*, 2006; Chakrabarti and Baron-Cohen, 2011). Moreover, a downregulation of CB1R has been reported in postmortem brains of individuals with autism, a condition where sociability is compromised (Purcell *et al.*, 2001).

Administration of CB1R exogenous agonists in rodents also produced effects over social phenotypes reducing social interactions and aggressive behaviors (Cutler and Mackintosh, 1984; van Ree *et al.*, 1984; Long *et al.*, 2010). However, increasing the levels of endogenous cannabinoids increased social play and social interactions and this effect was prevented by CB1R blockade (Trezza and Vanderschuren, 2008; Trezza *et al.*, 2012; Manduca *et al.*, 2015, 2016). In concordance with these results, social play increases levels of AEA in the amygdala and nucleus accumbens (Trezza *et al.*, 2012). Furthermore, a recent report has demonstrated that oxytocin release after social interaction triggers AEA mobilization in nucleus accumbens modulating social reward (Wei *et al.*, 2015). Release of 2-AG also occurs after social stimulation in nucleus accumbens and ventral-to-mid hippocampus (Wei *et al.*, 2016b). Altogether, these results suggest

that endocannabinoids are released during social interactions leading to the activation of CB1R in specific brain areas regulating social behavior (Trezza and Vanderschuren, 2008). Importantly, the regulation of social behavior by the ECS seems to be context-dependent (Haller *et al.*, 2004; Manduca *et al.*, 2014).

4.5. The endocannabinoid system as a therapeutic target

Alterations in the ECS signaling have been described in multiple pathophysiological states. Upregulation of ECS components have been reported in several disorders including increased expression of cannabinoid receptor, coupling receptor efficiency, endocannabinoid metabolizing enzymes expression or endocannabinoid levels (Pertwee, 2009). These alterations are thought to be a protective mechanism to decrease symptoms and the progression of disorders. In others cases, these changes may be maladaptive contributing or exacerbating symptoms (Pertwee, 2009; Miller and Devi, 2011). In this scenario, the modulation of the ECS is presented as an interesting therapeutic target for multiple disorders.

Activation of the ECS induced several pharmacological effects that may be beneficial for many conditions: analgesia, muscle relaxation, stimulation of appetite, antiemesis, bronchodilatation, anti-inflammatory and antineoplastic (Pertwee, 2009). However, it also produces other effects that may be detrimental, such as cognitive deficits, alterations in motor function and coordination or sedation (Pertwee, 2009, 2012). CB1R agonists, specifically Δ^9-THC (dronabinol; Marinol®) and its synthetic analogue nabilone (Cesamet®), were approved many years ago for suppressing nausea and vomiting produced by chemotherapy. Nowadays, Sativex® which contains Δ^9-THC and cannabidiol (proportion 1:1) has also

been approved for spasticity in multiple sclerosis patients and for chronic pain (Leocani *et al.*, 2015). Furthermore, the use of Epidiolex®, a purified form of cannabidiol, has been recently approved for treatment-resistant epilepsies associated with Lennox-Gastaut syndrome and Dravet syndrome (Kaufman, 2018).

Another strategy to increase the endocannabinoid tone is the inhibition the endocannabinoid-degrading enzymes. Inhibitors of FAAH and MAGL have been developed to increase the levels of AEA and 2-AG respectively (Toczek *et al.*, 2015). The most used are summarized in Table 9. Unlike cannabinoid agonists, these compounds may only increase endocannabinoid signaling at active sites of endocannabinoid production (Mechoulam and Parker, 2013).

Enzymes	Inhibitors	References
FAAH	URB597 OL-135 PF-3845 PF-04457845	(Mor *et al.*, 2004) (Lichtman *et al.*, 2004) (Ahn *et al.*, 2009) (Ahn *et al.*, 2011)
MAGL	JZL184 URB602 CAY10499 OMDM169	(Long *et al.*, 2009a) (King *et al.*, 2007) (Muccioli *et al.*, 2008) (Bisogno *et al.*, 2009)
FAAH/MAGL	JZL195	(Long *et al.*, 2009b)

Table 9. Main inhibitors of the endocannabinoid-degrading enzymes.

Interestingly, some of these drugs at effective therapeutic doses are devoided of most of the unwanted side effects associated to cannabinoid receptor agonists (Pertwee, 2014). Nowadays, none of these compounds modulating endocannabinoids have been approved for therapeutic use although there are several under clinical trials for the treatment of multiple disorders. One of these compounds, a FAAH inhibitor (BIA102474), caused the death of one participant and irreversible brain

damage of four participants in phase I clinical trial. However, these devastating adverse side effects were likely due to off-target effects not related to the direct stimulation of the ECS via FAAH (Kaur *et al.*, 2016; van Esbroeck *et al.*, 2017).

Blocking CB1R activity may also be useful for other pathophysiological conditions. Rimonabant (Acomplia®) was the first compound of this type approved in 2006 for body-weight reduction in obesity and related metabolic disorders. The drug successfully decreased weight in obese patients, improved their lipid profile and glucose control (Patel and Pathak, 2007). However, in 2008 the drug was withdrawn due to the appearance of psychiatric side effects including depression, anxiety and suicidal ideation (Christensen *et al.*, 2007). It was suggested that these unwanted effects were due to inverse agonist properties of rimonabant acting on CB1R (Meye *et al.*, 2013). Alternative drugs such as neutral antagonists or negative allosteric modulators of CB1R would be safer therapeutic options.

Other disorders in which the modulation of the ECS have demonstrated promising therapeutic applications are cancer (Abrams and Guzman, 2015), neuropathic, inflammatory and osteoarthritis pain (Jonsson *et al.*, 2006; La Porta *et al.*, 2014), intestinal disorders (Pesce *et al.*, 2017), post-traumatic stress disorder (Mizrachi Zer-Aviv *et al.*, 2016), neurodegenerative disorders (Alzheimer's disease, Parkinson's disease and Huntington's disease) (Kendall and Yudowski, 2017), epilepsy syndromes (Dravet syndrome, Lennox-Gastaut syndrome, tuberous sclerosis complex, and infantile spams) (De Caro *et al.*, 2017) and intellectual disability disorders (FXS) (Busquets-Garcia *et al.*, 2013). This thesis is focused on the modulation of the ECS in intellectual disability disorders.

4.5.1. Therapeutic potential of the endocannabinoid system in memory deficits of neurodevelopmental disorders

Given the role of the ECS in cognition, alterations of the ECS function may contribute to memory deficits observed in some neurodevelopmental disorders. The involvement of the ECS in the pathogenesis of FXS syndrome has been previously studied. In this rare genetic disorder, moderate intellectual disability is one of the most consistent phenotypes (Penagarikano *et al.*, 2007). In concordance with the cognitive impairment of FXS patients, mouse models also have deficits in memory tasks including NORT (Ventura *et al.*, 2004; Bhattacharya *et al.*, 2012; Busquets-Garcia *et al.*, 2013), MWM (D'Hooge *et al.*, 1997) and passive avoidance tests (Qin *et al.*, 2015). First evidences of altered ECS function in a mouse model of the disorder, the Fmr1 KO mice, showed that eCB-STD and eCB-LTD on GABAergic synapses of hippocampus and dorsal striatum were enhanced after mGluR1 activation (Maccarrone *et al.*, 2010; Zhang and Alger, 2010). Conversely, eCB-LTD was absent in prefrontal cortex and ventral striatum of Fmr1 KO mice (Jung *et al.*, 2012). Therefore, synaptic plasticity processes mediated by the ECS are altered in FXS mouse models. These alterations are different across several brain regions and may contribute to the cognitive deficits observed in the Fmr1 KO mice. Indeed, targeting the ECS has been explored as a therapeutic strategy to normalize cognitive deficits in this mouse model. Previous research of our lab demonstrated that blocking CB1R normalizes memory deficits of Fmr1 KO mice in the NORT (Busquets-Garcia *et al.*, 2013). This memory improvement is consistent with normalization of abnormal mTOR signaling, dendritic spine morphology and mGluR-LTD (Busquets-Garcia *et al.*, 2013; Gomis-González *et al.*, 2016). Other strategies used include the increase of 2-AG or AEA levels with specific inhibitors of MAGL and FAAH, respectively.

Increasing 2-AG levels in Fmr1 KO mice normalized synaptic plasticity of prefrontal cortex and ventral striatum, however, cognitive tasks were not assessed (Jung *et al.*, 2012). Conversely, increasing levels of AEA improved aversive memory in passive avoidance task (Qin *et al.*, 2015). Overall, several studies have demonstrated the involvement of the ECS in the pathophysiology of FXS. In addition, targeting the ECS seems to be a good approach to normalize memory deficits in this disorder. Taking into account this previous research in FXS and given the central role of the ECS in memory, one of the objectives of this thesis is to study of the role of the ECS and its therapeutic potential in cognitive deficits of DS.

4.5.2. Therapeutic potential of the endocannabinoid system in social deficits of neurodevelopmental disorders

Given that the ECS has a role in social behavior, dysregulation of this system may be involved in social deficits of different disorders. In fact, there are several evidences of different rodent models of autism-spectrum disorders which display social deficits. The prenatal exposure to valproic acid is a common preclinical model of autism-spectrum disorder. Mice exposed to valproic acid showed alterations in ECS components including increasing levels after social interaction of AEA and related compounds, increased expression of MAGL in hippocampus and changes in the expression of phosphorylated CB1 receptor in the amygdala, hippocampus and dorsal striatum (Kerr *et al.*, 2013). In addition, systemic administration of FAAH inhibitors in this model, normalized impairments in social interactions and communications (Kerr *et al.*, 2016; Servadio *et al.*, 2016). Another mouse model of autism-spectrum disorder is the prenatal administration of LPS, which provokes maternal immune activation and, as consequence, the affectation of the offspring with core symptoms of autism (Patterson, 2011). Post-natal LPS administration is not considered

a model of autism but also causes a decrease in social behavior in adolescent mice (Zamberletti *et al.*, 2017). Interestingly, these mice exhibited alterations in the ECS including decreased CB1R binding, increased AEA levels and increased FAAH activity in amygdala. Systemic administration but also local infusion in basolateral amygdala of the FAAH inhibitor PF-04457845 normalized social behavior of these mice (Doenni *et al.*, 2016). Furthermore, the BTBR mouse strain, which is also considered a model of autism, showed an increase of AEA cortical levels after social interaction (Gould *et al.*, 2012). Administration of a FAAH inhibitor also normalized the social impairment of this mouse model (Wei *et al.*, 2016a). In a similar way, administration of URB597 normalized social deficits in the Fmr1 KO mice (Wei *et al.*, 2016a). However, another report using the same mouse model in a different genetic background did not show social improvements (Qin *et al.*, 2015). Finally, mice with rare mutations in neuroligins, postsynaptic cell adhesion molecules which predispose to autism disorders, present endocannabinoid signaling defects in hippocampus and cortex (Földy *et al.*, 2013; Speed *et al.*, 2015). Further experiments should elucidate if these defects have a direct impact on the social phenotype of these mice.

Altogether, alterations of components of the ECS are recurrent in several mouse models of autism-spectrum disorders with different etiologies. One of the most frequent alteration among the different models are the increased levels of AEA. Decreasing AEA by FAAH inhibitors reverses social deficits in these mouse models, supporting that correct AEA signaling is essential to display normal social behavior and pointing this strategy as a good therapeutic approach to treat social deficits in autism-spectrum disorders. The role and therapeutic potential of the ECS in disorders with

enhanced sociability such as WBS has not been explored before and it is one of the main objectives of this thesis.

OBJECTIVES

OBJECTIVES

Objective 1

To study the role of the endocannabinoid system and its therapeutic potential in hippocampal-memory deficits of two mouse models of Down syndrome, the Ts65Dn model and the TgDyrk1A model.

Chapter 1: Cannabinoid type-1 receptor blockade restores cognitive impairment in mouse models of Down syndrome

Objective 2

To study the involvement of the endocannabinoid system and its therapeutic potential in social abnormalities of two mouse models of Williams-Beuren syndrome, the WBS-CD model and the $\Delta Gtf2i^{+/-}$ model.

Chapter 2: Monoacylglycerol lipase inhibition restores social alterations in mouse models of Williams-Beuren syndrome

CHAPTER 1

CANNABINOID TYPE-1 RECEPTOR BLOCKADE RESTORES COGNITIVE IMPAIRMENT IN MOUSE MODELS OF DOWN SYNDROME

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Submitted

CHAPTER 1

Cannabinoid type-1 receptor blockade restores cognitive impairment in mouse models of Down syndrome

Several mechanisms have been proposed to underlie cognitive deficits in DS, including alterations in hippocampal synaptic plasticity, adult neurogenesis in dentate gyrus and excitatory/inhibitory balance of neuronal circuits (Kleschevnikov *et al.*, 2004; Clark *et al.*, 2006; Contestabile *et al.*, 2013; Pons-Espinal *et al.*, 2013b). Interestingly, the ECS, a neuromodulatory system involved in synaptic homeostasis and plasticity fine-tunes all of these processes (Alger, 2002; Monory *et al.*, 2015; Prenderville *et al.*, 2015; Augustin and Lovinger, 2018). However, its possible role in the cognitive deficits of DS has not yet been explored. The ECS has been closely associated to learning and memory processes (Marsicano and Lafenêtre, 2009; Puighermanal *et al.*, 2012). Particularly, CB1R seems to be key regulator on memory processes since the activation or blocking of this receptor has a direct impact on cognitive function (Akirav, 2011; Zanettini *et al.*, 2011; Busquets-Garcia *et al.*, 2015). In this chapter, we have evaluated the involvement of the ECS in the pathophysiology of DS cognitive phenotypes using two well-characterized mouse models of the disorder and we have explored its pharmacological modulation as a possible pro-cognitive treatment.

1.1. Materials and Methods

Animals

All animal procedures were conducted following ARRIVE (Animals in Research: Reporting In Vivo Experiments) (Kilkenny *et al.*, 2010) guidelines and standard ethical guidelines (European Communities Directive 2010/63/EU) and approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB).

Ts65Dn experimental mice were obtained by repeated backcrossing Ts65Dn females to C57BL/6JEiJ x C3Sn.BLiA-Pde6b+/DnJ F1 hybrid males. The parental generation was obtained from The Jackson Laboratory. Euploid littermates of Ts65Dn mice served as wild-type (WT) controls. TgDyrk1A and WT littermates were obtained as described previously (Altafaj et al., 2001). To visualize hippocampal pyramidal neurons, double transgenic mice (Thy-YFP/TgDyrk1A) were obtained by backcrossing TgDyrk1A males to B6.Cg-Tg(Thy1-YFPH)2Jrs/J females from The Jackson Laboratory. Ts65Dn and TgDyrk1A mice were genotyped by PCR as previously described (Altafaj *et al.*, 2001; Duchon *et al.*, 2011).

Animals aged between 8 and 16 weeks were used for experiments. Both males and females were used for Ts65Dn experiments whereas only males were used for TgDyrk1A experiments. Mice were housed in Plexiglas cages with a maximum of four mice per cage and maintained in a temperature-controlled (21°C ± 1°C) and humidity-controlled (55 ± 10%) environment. Food and water were available *ad libitum*. All the experiments were performed during the light phase of a 12 hours light/dark cycle (light on at 8 am; light off at 8 pm). Mice were habituated to the experimental room and handled for 1 week before starting the experiments. All behavioral

experiments were conducted by an observer blind to the experimental conditions.

Drug treatment

Rimonabant (Sanofi-Aventis) and NESS 0327 (Cayman Chemical, Ann Arbor) were diluted in 5% ethanol, 5% Cremophor EL (Sigma) and 90% saline. They were injected in a volume of 10 ml/kg of body weight. Mice were randomly assigned to experimental groups and drugs or vehicle were administered daily by intraperitoneal (i.p.) injection for 7 days. The test phase of the cognitive tasks, LTP recordings and neurogenesis analysis were performed 24 hours after last injection of the treatment (Figure 22).

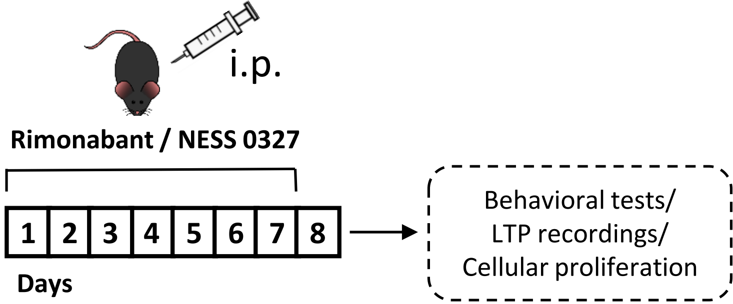

Figure 22. Schematic cartoon of drug treatment

For the surgery procedure, ketamine hydrochloride (Imalgène; Merial Laboratorios S.A.) and medetomidine hydrochloride (Domtor; Esteve) were mixed and dissolved in sterile 0.9 % physiological saline and administered i.p. in a volume of 10 ml/kg of body weight. Atipamezole hydrochloride (Revertor; Virbac) and meloxicam (Metacam; Boehringer Ingelheim, Rhein) were dissolved in sterile 0.9 % physiological saline and administered subcutaneously in an injection volume of 10 ml/kg of body weight. Gentamicine (Genta-Gobens; Laboratorios Normon) was dissolved in sterile 0.9 % physiological saline and administered i.p. in an injection volume of 10 ml/kg of body weight.

For perfusion, ketamine hydrochloride and xylazine hydrochloride (Sigma) were mixed and dissolved in ethanol (5 %) and distilled water (95 %).

Behavioral tests

Behavioral tests were performed in a sound-attenuated room at the animal facility with dim illumination 5-10 lux. A digital camera on top of the mazes was used to record the sessions.

Novel object recognition test

This test was performed as previously (Puighermanal *et al.*, 2009; Busquets-Garcia *et al.*, 2013; Gomis-González *et al.*, 2016) The tests consisted in three phases performed three consecutive days. On day 1, mice were habituated to a V-shaped maze for 9 minutes. On day 2, two identical objects (familiar objects) were located at the end of each corridor for 9 minutes and the time that the mice spent exploring each object was computed. Twenty-four hours later, one of the familiar objects was replaced by a new object (novel object). The time spent exploring each of the objects was computed to calculate a discrimination index. Discrimination index was calculated as the difference between the time spent exploring the novel object minus the time exploring the familiar object divided by the total exploration time (addition of the time exploring both objects). A higher discrimination index was considered to reflect greater memory retention for the familiar object. Object exploration was defined as the orientation of the nose towards the object at a distance of less than two cm. Objects used were chess pieces. Total exploration time was considered as a measure of general activity. Mice with total exploration times below 10 seconds were excluded from the analysis. Drug administration was performed immediately after habituation and training phases the 6th and 7th respective days of

treatment. Test was performed 24 hours after the last administration (see introduction 1.1.3).

Novel place recognition test

Mice were first habituated for 9 minutes to an empty open field during two consecutive days. On day 3, mice were trained for 9 minutes with two identical objects (training phase) located at two corners of the open field. Twenty-four hours later, on the test phase, one of the objects was located at a different corner (novel location), and the time spent exploring both objects in the novel and familiar locations was computed to calculate the discrimination index similar to above. Mice that explored less than 5 seconds both objects were excluded from the analysis. Total exploration time was considered as a measure of general activity during the test and did not show significant differences between different genotypes or treatments in the present study. Drug administration was performed immediately after habituation and training phases the 5th, 6th and 7th days of treatment. Test was performed 24 hours after the last administration (see introduction 1.1.3).

Immunoblots

Hippocampal tissue was dissected immediately after cognitive tests, frozen on dry ice and stored at −80 °C until used. Tissue was dounce-homogenized in 30 volumes of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 2 µg/mL aprotinin, 10 µg/mL leupeptine, 1 µg/mL pepstatin, 10 µg/mL phenylmethylsulfonyl fluoride, 1 mM Na^+ orthovanadate, 100 mM Na^+ fluoride, 5 mM Na^+ pyrophosphate, and 40 mM betaglycerolphosphate) plus 1% Triton X-100. Samples were vortexed for 10 minutes at 4ºC and then were centrifuged at 16,000 g for 30 minutes to eliminate insoluble debris. Protein content in supernatants

was determined using DC-micro plate assay (Bio-Rad) following manufacturer's instructions. Equal amounts of protein (15ug/well) were separated in 10% polyacrylamide gels and electrophoretic transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked for 1 hour at room temperature in tris-buffered saline (100 mmol/L NaCl, 10 mmol/L Tris, pH 7.4) with 0.1% Tween-20-tris-buffered saline and 3% of Bovine Serum Albumin (Ibian Technologies). Then, membranes were incubated for 2 hours with the following primary antibodies: anti-CB1R (rabbit and guinea pig, 1:500, CB1-Rb-Af380 and CB1-GP-Af530 Frontier Institute Co.Ltd), anti-FAAH (mouse, 1:1,200, ab54615, Abcam), anti-NAPE-PLD (guinea pig, 1:1000, NAPE-PLD-GP-Af720-1, Frontier Institute Co.Ltd), anti-MAGL (rabbit, 1:300, ab24701, Abcam), anti-DAGLα (guinea pig 1:300, Frontier Institute Co.Ltd, DGLa-GP-Af380-1), anti-actin (mouse, 1:50,000, MAB1501, MerckMillipore). Primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit, anti-mouse antibodies or anti-guinea pig and visualized by enhanced chemiluminescence detection (Luminata Forte Western HRP substrate, MerckMillipore). Digital images were acquired on a ChemiDoc XRS System (Bio-Rad) and quantified by The Quantity One software v4.6.3 (Bio-Rad). Optical density values for target proteins were normalized to actin as loading control in the same sample and expressed as a percentage of control group (WT).

Electrophysiological recordings

Mice were anesthetized with isoflurane (2 %) and decapitated for slice preparation. After decapitation, the whole brain, containing the 2 hippocampi, was removed into ice-cold solution (I) consisting of (in mM): 126 NaCl, 3 KCl, 1.25 KH_2PO4, 2 MgSO4, 2 $CaCl_2$, 26 $NaHCO_3$, and 10

glucose (pH 7.2, 300 mOs/ml), and positioned on the stage of a vibratome slicer and cut to obtain transverse hippocampal slices (350 μm), which were maintained continuously oxygenated for at least 1 hour before use. All experiments were carried out at room temperature (22–25ºC). For experiments, slices were continuously perfused with the solution described above.

To study evoked excitatory (eEPSCs) and inhibitory (eIPSCs) postsynaptic currents, whole-cell patch-clamp recording of pyramidal cells located in the CA1 field of the hippocampus were obtained under visual guidance by infrared differential interference contrast (DIC) microscopy. The neurons were verified as pyramidal cells through their characteristic voltage response to a current step protocol. The neurons were recorded in voltage-clamp configuration with a patch clamp amplifier (Multiclamp 700B) and the data were acquired using pCLAMP 10.2 software (Molecular Devices). To evoke eEPSCs, electrical pulses were delivered to Schaffer collaterals axons and to evoke eIPSCs electrical pulses were delivered to interneurons situated in the *stratum oriens*. Patch electrodes were pulled from borosilicate glass tubes and they had a resistance of 4-7 MΩ when filled with (in mM): 120 CsCl, 8 NaCl, 1 $MgCl_2$, 0.2 $CaCl_2$, 10 HEPES, 2 EGTA and 20 QX-314 (pH 7.2, 290 mOsm). Experiments were performed at -70 mV. Cell recordings were excluded from the analysis if the series resistance changed by more than 15% during the recording. All recordings were low-pass filtered at 3 kHz and acquired at 10 kHz. eEPSC were isolated by adding to the perfusion solution bicuculline (20 μM) to block $GABA_A$ receptors. Inhibitory postsynaptic currents (eIPSCs) were isolated adding to the perfusion solution D-AP5 and NBQX to block NMDA and AMPA/Kainate receptors, respectively.

Field EPSP (fEPSPs) were recorded in the CA1 region of the hippocampus and were evoked by stimulation with a stimulating electrode placed on the Schaffer collateral (0.2 Hz) (Figure 23). Extracellular recording electrodes were filled with the solution I. A stimulus-response curve (1–160 µA, mean of 5 fEPSPs at each stimulation strength) was compiled for the different mice used.

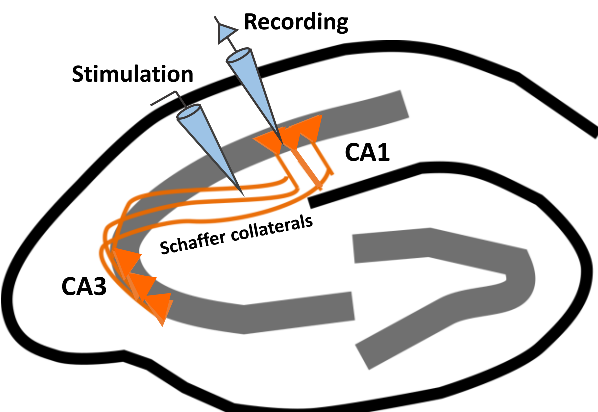

Figure 23. Schematic representation of electrode placement for stimulating Schaffer collaterals and recording field excitatory postsynaptic potentials (fEPSP) in CA1 region of hippocampal mice.

For plasticity experiments, fEPSPs were evoked at 0.2 Hz by a monopolar stimulation electrode placed in the *stratum radiatum* using brief current pulses (200 µs, 0.1–0.2 mA). Stimulation was adjusted to obtain a fEPSP peak amplitude of approximately 1 mV during control conditions. After a stable fEPSP baseline period of 10 minutes. Long-term potentiation was induced by a TBS protocol consisting in five episodes of 10 train stimulus at 5 Hz, each one with four pulses at 100 Hz. Recordings lasted 60 and 120 minutes after LTP induction.

Data were analyzed using the Clampfit 10.2 software (Molecular Devices). The last 5 minutes of recording were used to estimate changes in synaptic efficacy compared to baseline. LTP was quantified by comparing the mean

fEPSP slope over the 60 and 120 minutes post-tetanus period with the mean fEPSP slope during the baseline period and calculating the percentage change from 5 last minutes.

Endocannabinoid quantification by liquid chromatography–tandem mass spectrometry

The quantification of endocannabinoids and related compounds was based on the methodology previously described in plasma (Pastor *et al.*, 2014), adapted for the extraction of endocannabinoids from brain tissue. The following N-acylethanolamines and 2-monoacylglycerols were quantified: AEA, N-docosatetraenoylethanolamine (DEA), N-docosahexa-enoylethanolamine (DHEA), 2-AG, 2-LG and 2-OG. Half-right hippocampus (17.5 ± 1.7 mg) or half whole brain (226.3 ± 14.05 mg) of mice were placed in a 1 ml Wheaton glass homogenizer and spiked with 25 µl of a mix of deuterated internal standards dissolved in acetonitrile (mean ± S.D.). The mix contained 5 ng/ml AEA-d4, 5 ng/ml DHEA-d4, 5 µg/ml 2-AG-d5, and 10 µg/ml 2-OG-d5. All internal standards were purchased from Cayman Chemical (Ann Harbor), except for 2-OG-d5 (Toronto Research Chemicals). Tissues were homogenized on ice with 700 µl a mixture of 50 mM Tris-HCl buffer (pH 7.4): methanol (1:1) and the homogenates were transferred to 12 ml glass tubes. The homogenizer was washed twice with 0.9 ml of the same mixture and the contents were combined into the tube giving an approximate volume of 2.5 ml of homogenate. The homogenization process took less than 5 minutes per sample and homogenates were kept on ice until organic extraction to minimize the *ex-vivo* generation of endocannabinoids. Next, homogenates were extracted with 5 ml chloroform over 20 minutes by placing the tubes in a rocking mixer. Tubes were centrifuged at 1,700 g over 5 minutes at room temperature. The

lower organic phase was transferred to clean glass tubes, evaporated under a stream of nitrogen in a 39ºC water bath and extracts were reconstituted in 100 µl of a mixture water:acetonitrile (10:90, v/v) with 0.1 % formic acid (v/v) and transferred to high performance liquid chromatography vials with glass microvials. Endocannabinoids were separated using an Agilent 6410 triple quadrupole Liquid-Chromatograph equipped with a 1200 series binary pump, a column oven and a cooled autosampler (4 ºC). Chromatographic separation was carried out with a Waters C18-CSH column (3.1 x 100 mm, 1.8 µm particle size) maintained at 40 ºC with a mobile phase flow rate of 0.4 ml/minute. The composition of the mobile phase was: A: 0.1 % (v/v) formic acid in water; B: 0.1 % (v/v) formic acid in acetonitrile. Endocannabinoids and related compounds were separated by gradient chromatography. The ion source was operated in the positive electrospray mode. The selective reaction monitoring mode was used for the analysis. Quantification was done by isotope dilution with the response of the deuterated internal standards.

Stereotaxic surgery and AAV9 vector injection

To knockdown CB1R expression we used an adeno-associated viral serotype 9 (AAV9) vector-mediated short hairpin RNA (shRNA) approach previously described (Guegan *et al.*, 2013). The AAV9 vector was selected for its good transduction efficiency into the hippocampus (Aschauer *et al.*, 2013).

Mice were anaesthetized with a ketamine (75 mg/kg)/medetomidine (1 mg/kg) mixture before they underwent stereotaxic surgery and received a bilateral hippocampal injection. The intracranially injection of AAV9 was performed as previously described with slight modifications (Busquets-Garcia *et al.*, 2018) in the following coordinates: anteroposterior, − 1.82

mm; mediolateral, ± 1.00 mm; dorsoventral, 2.00 mm (Figure 24) (Paxinos and Franklin, 2004). The injection of 0.5 μl of AAV9-shSC (control, 5.03 x10^{13} vector genomes/ml) or AAV9-shCB1R (AAV9-shRNACB1A: 6.48 x10^{13} vector genomes/ml plus AAV9-shCB1B: 1.1 x10^{13} vector genomes/ml) was made through a bilateral injection cannula (33-gauge internal cannula, Plastics One) connected to a polyethylene tubing (PE-20, Plastics One) attached to a 10 µl Hamilton microsyringe (Sigma).

Figure 24. Schematic cartoon of target injection site of AAV9-shSC or AAV9-shCB1R.

The displacement of an air bubble inside the length of the polyethylene tubing that connected the syringe to the injection needle was used to monitor the microinjections. The volume was injected at a constant rate of 0.25 µl/minute by using a microinfusion pump (Harvard Apparatus, Holliston) for 2 minutes. After infusion, the injection cannula was left in place for an additional period of 10 minutes to allow the fluid to diffuse and to prevent reflux, then it was slowly withdrawn during 10 additional minutes. After surgery, anesthesia was reversed by a subcutaneous (s.c.) injection of atipamezole (2.5 mg/kg). In addition, mice received an i.p. injection of gentamicine (1 mg/kg) and a s.c. injection of the analgesic meloxicam (2 mg/kg). For tropism studies, we used an AAV9 vector expressing GFP cDNA (4.3 x10^{13} vector genomes/ml) under the control of the cytomegalovirus early enhancer/chicken β-actin (CAG) constitutive promoter and the woodchuck hepatitis post-transcriptional regulatory

element (WPRE). Behavioral tests were performed three weeks after the injection.

Immunofluorescence and cell quantification

Four hours after the last administration of rimonabant, bromodeoxyuridine (BrdU) (50 mg/kg, i.p.) was also injected. Twenty-four hours later, mice were deeply anesthetized by i.p. injection (0.2 ml/10 g of body weight) of a mixture of ketamine (100 mg/kg)/xylazine (20 mg/kg) prior to intracardiac perfusion of cold 4 % paraformaldehyde. Brains were removed and post-fixed overnight at 4 °C in the same fixative solution. The next day, brains were moved to phosphate buffer 0.1 M at 4 ºC. Coronal brains sections (50µm) for immunofluorescence staining were made on vibratome Leica VT1000 S (Leica Biosystems) and stored in a cryoprotectant solution containing 30 % ethylene-glycol (vol/vol), 30 % glycerol (vol/vol) at –20 ºC until they were used for immunodetections. Systematic series of coronal sections (1:6) per animal were selected, covering the rostral to caudal extension of the hippocampus (from 1.3 and 2.5 mm posterior to Bregma). Four brain sections per animal were used for performing immunofluorescence of Ki67 and BrdU.

For BrdU detection, DNA denaturalization was required. For this purpose, slices were pretreated with 2 N HCl at 37 ºC for 30 minutes. Then, free-floating sections were incubated with 0.1 M borate buffer pH=8.5 for 15 minutes to neutralize the pH. Afterwards, slices were rinsed in phosphate buffered saline, blocked in a solution containing 3% normal donkey serum and 0.3 % Triton X-100 in 0.1 M phosphate buffered saline at room temperature for 2 hours and incubated in the same solution with primary antibodies at 4 ºC. For detections we used the following primary antibodies: anti-BrdU (mouse, 1:150, B8434, Sigma-Aldrich) and anti-Ki67

(rabbit, ab15580, 1:150, Abcam). Forty-eight hours later, slices were rinsed with 0.1 M phosphate buffered saline and incubated with secondary antibodies in the blocking solution for 2 hours at room temperature. We employed the following secondary antibodies: donkey anti-mouse (1:700, Alexa Fluor-647, A31571, Invitrogen) and donkey anti-rabbit (1:600, Alexa Fluor-488, A21206, Life Technologies). Then, sections were rinsed and mounted onto gelatin-coated slides with Mowiol mounting medium. Images of stained sections were obtained with a confocal microscope TCS SP8 LEICA (Leica Biosystems) using a dry objective (20x) with a sequential line scan at 1024 × 1024 pixel resolution. The images were obtained choosing a representative 10 µm Z-stack from the slice. The density of positive cells (Ki67 or BrdU) was quantified manually over the projection visualized after the application of an optimal automatic threshold (MaxEntropy) from Fiji software (ImageJ). To avoid counting twice overlapped cells, all pictures of the z-stack were individually checked. The number of positive cells was calculated as the mean of total number of cells counted referred to the volume of the SGZ (μm^3). Positive cells density was referred to that calculated for the control group.

Dendritic spine analysis

Secondary and tertiary apical dendrites of pyramidal neurons from the stratum radiatum of CA1 region of the dorsal hippocampus were analyzed in Thy-YFP/TgDyrk1A mice. Brains were perfused as described in the previous section and were stored in a solution of 30 % sucrose at 4 °C. Coronal frozen sections were made at 60 µm on a freezing microtome (Leica) and stored in a 5 % sucrose solution. Images were acquired with a confocal microscope (TCS SP5 STED Leica) using a glycerol immersion lens

plus 3 times magnification (63X/3) with a sequential line scan at 1024 × 1024 pixel resolution. Serial optical sections were acquired with a 0.13 µm step size. A minimum of 8 dendrites per animal were selected from four different slices. A maximum of 2 dendrites were selected from the same neuron. Confocal images were deconvolved using Huygens Essential software and dendritic spines were analyzed using the semi-automated software NeuronStudio. Spine density was calculated by expressing the average number of spines in a 10 µm portion of the dendrite. NeuronStudio calculated for each spine the following parameters based on its morphology: head/neck ratio (threshold=1.100 pixel), length/head ratio (threshold=2.5 pixel) and head size (threshold=0.350 µm). Then, software classified spines into three major morphologic types: mushroom (if the head/neck ratio and the mushroom head size were above the threshold), stubby (if the head/neck and the length/head ratios were below the threshold) and thin (in the remaining cases) (Figure 25). Each spine was checked manually by an observer blind to conditions to accurate classification.

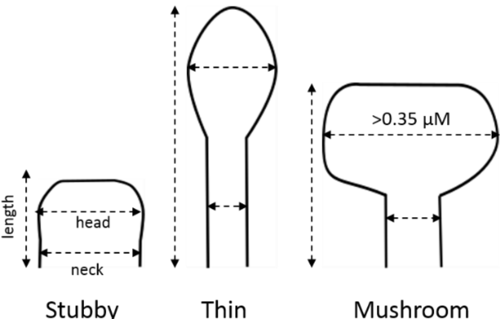

Figure 25. Classification of dendritic spines according their morphology.

Statistical analysis

Data were analyzed with Statistica Software using unpaired Student's t-test or two-way ANOVA for multiple group comparisons. Subsequent *post*

hoc analysis (Newman-Keuls) was used when required (significant interaction between factors). Comparisons were considered statistically significant when $p < 0.05$. Outliers (± 2 s.d. from the mean) were excluded.

1.2. Results

1.2.1. CB1R is overexpressed in the hippocampus of Ts65Dn mice

In order to study the possible role of the ECS in cognitive deficits of Down syndrome, we first analyzed the expression of the main components of the ECS in the hippocampus of young-adult Ts65Dn male mice. The expression of the most abundant cannabinoid receptor in the brain, CB1R, was increased in Ts65Dn in comparison to WT mice (Figure 26). Conversely, there were no changes in the expression of the enzymes involved in the synthesis (NAPE-PLD and DAGLα) and degradation (FAAH and MAGL) of the main endocannabinoids (Figure 26).

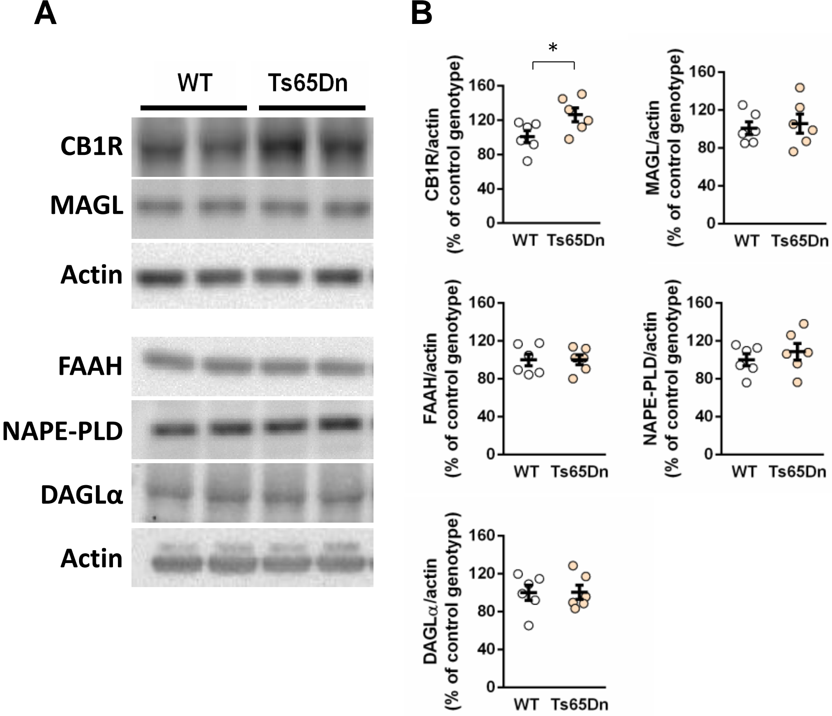

Figure 26. CB1R is overexpressed in hippocampus of Ts65Dn mice. (A-B) Representative immunoblots (A) and quantification (B) of the main components of the ECS in hippocampus of WT and Ts65Dn mice (WT, n=6; Ts65Dn, n=6). Actin immunodetection was used as housekeeping control. Distribution of individual data with mean ± s.e.m. * $p<0.05$ (genotype effect) by Student's t-test.

To assess the functional consequences of CB1R overexpression on synaptic transmission, we performed whole-cell patch-clamp recordings in CA1 pyramidal neurons from acute slices of WT and Ts65Dn mice. We found that the CB1R agonist WIN55,212-2 (300 nM) produced an enhanced inhibitory effect on the amplitude of eEPSCs, but had no effect on eIPSCs in trisomic mice compared to controls (Figure 27, A-D).

To determine whether the enhanced inhibitory effect on the amplitude of eEPSCs produced by WIN55,212-2 had a presynaptic or postsynaptic component, we estimated the noise-free coefficient of variation (CV) of the synaptic responses, in control conditions and in the presence of WIN55,212-2 (300 nM). We calculated the ratio of both CVs and plotted the observed change in the mean EPSC amplitude versus the change in the statistic $1/CV^2$, which denotes the variance of the evoked response (Malinow and Tsien, 1990; Rodríguez-Moreno *et al.*, 1997; Rodríguez-Moreno and Paulsen, 2008). This approach uncovered that, in control and Ts65Dn mice, the reduction in eEPSC amplitude by WIN55,212-2 closely follows the predicted relation for a presynaptic (diagonal dashed line) rather than a postsynaptic action (horizontal dashed line) (Figure 27E).

Together, these data revealed an overall increased function of CB1R at excitatory terminals of CA1 hippocampal region in Ts65Dn mice.

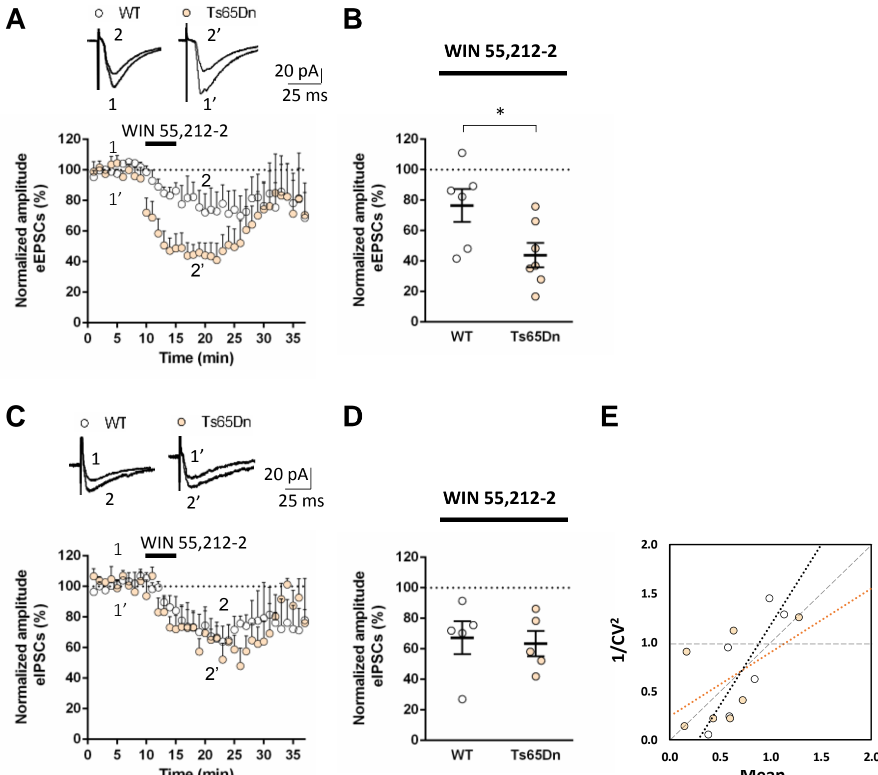

Figure 27. CB1R has an enhanced function in hippocampal CA1 excitatory terminals of Ts65Dn mice. (A) Average time course of evoked EPSCs amplitude in WT and Ts65Dn slices during baseline and after bath application of WIN 55,212-2 (300 nM). Inset, traces show EPSCs during baseline (1, 1') and after (2, 2´) bath application of WIN 55,212-2. (B) Summary of the results showed in (A) (WT, n=6; Ts65Dn, n=7). (C) Average time course of evoked IPSCs amplitude in WT and Ts65Dn slices during baseline (1, 1') and after (2,2') bath application of WIN 55,212-2 (300 nM). (D) Summary of the results showed in (D) (WT, n=5; Ts65Dn, n=5). (E) Normalized plot of CV^{-2} versus mean EPSCs yielded points closer to the diagonal after WIN 55,212-2 treatment. Distribution of individual data with mean ± s.e.m. * $p<0.05$ (genotype effect) by Student's t-test. CV: coefficient of variation.

We also determined the levels of endocannabinoids AEA and 2-AG, and related N-acylethanolamines (DEA and DHEA) and 2-monoacylglycerols (2-LG and 2-OG) compounds in hippocampus and whole brain homogenates. AEA, DEA and DHEA were decreased in hippocampus and DHEA was also decreased in whole brain of Ts65Dn mice (Table 10).

	Hippocampus		Whole brain	
	WT	Ts65Dn	WT	Ts65Dn
AEA	100 ± 4.3	82.3 ± 4.5 **	100 ± 4.3	86.9 ± 8.5
DEA	100 ± 3.3	87.9 ± 3.3 *	100 ± 3.6	97.5 ± 6.3
DHEA	100 ± 3.4	90.0 ± 3.4 *	100 ± 4.0	84.0 ± 3.4 **
2-AG	100 ± 5.6	107.5 ± 6.3	100 ± 2.9	105.0 ± 6.0
2-LG	100 ± 3.5	99.2 ± 5.7	100 ± 4.9	94.7 ± 12.3
2-OG	100 ± 2.5	95.4 ± 5.6	100 ± 9.0	94.2 ± 9.7

Table 10. Relative levels of endocannabinoids and related compounds in hippocampal and whole brain homogenates of Ts65Dn and WT controls. Data is expressed as mean ± s.e.m. * $p<0.05$, ** $p<0.01$ (genotype effect) by Student's t-test.

1.2.2. Rescue of hippocampal-dependent memory deficits by CB1R knockdown in Ts65Dn mice

As CB1R signaling has a major role in regulating memory processes (Akirav, 2011; Zanettini *et al.*, 2011; Busquets-Garcia *et al.*, 2015), we next evaluated the role of hippocampal CB1R overexpression in the cognitive impairment of Ts65Dn mice. To this aim, we used a set of AAV9 vectors, as previously described (Guegan *et al.*, 2013). To confirm AAV9 tropism and hippocampal diffusion, we bilaterally injected into the hippocampus AAV9 vector expressing GFP cDNA (Figure 28). Then, AAV9-shRNAs against CB1R (shCB1R) or AAV9-scramble shRNA (control, shSC) were bilaterally injected into the hippocampus in the same stereotaxic coordinates. Three weeks after the infusion, we assessed hippocampal-dependent memory using the NORT. Then, we obtained brain samples to analyze CB1R protein levels from hippocampal homogenates.

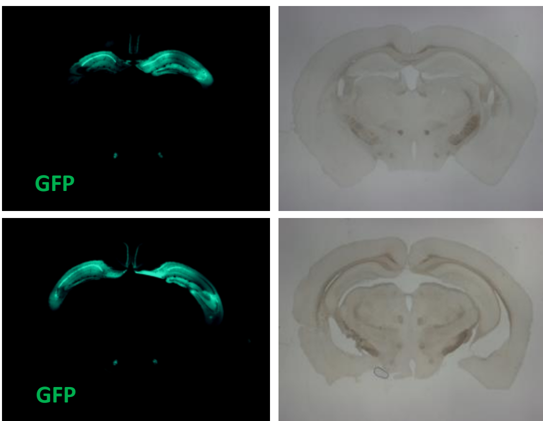

Figure 28. Representative images of localization and diffusion of AAV9 expressing GFP cDNA with the same stereotaxic coordinates than AAV9- shRNASC/CB1R.

Intra-hippocampal infusion of shCB1R, but not shSC, reduced expression of CB1R in hippocampus of Ts65Dn mice to the level observed in WT mice (Figure 29, A and B) and significantly reduced cognitive impairment in the NORT (Figure 29D). These improvements were not related to modifications in the exploratory behavior since total object exploration times did not change significantly in any of the tests (Figure 29E). shCB1R infusion, but not shSC, also reduced CB1R expression in WT mice without effects on NORT performance (Figure 29, C and D). Thus, the normalization of CB1R expression in hippocampus was sufficient to rescue hippocampal memory deficits in the Ts65Dn mice.

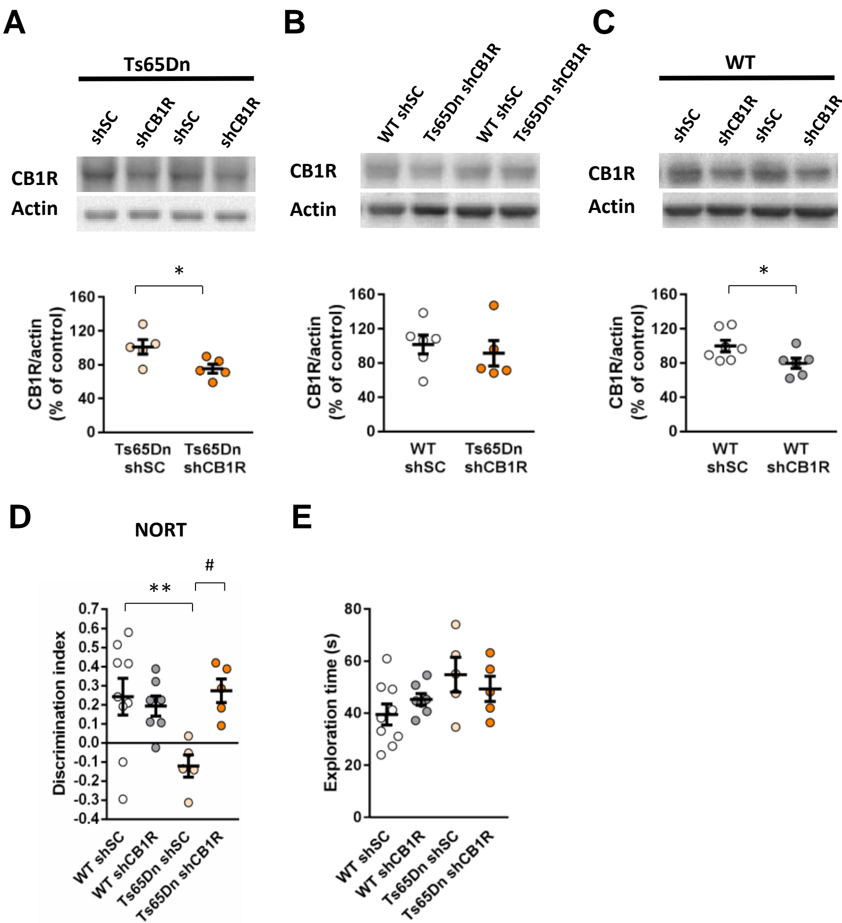

Figure 29. Injection of AAV9-shRNA against CB1R knocks down its expression and normalizes memory deficits of Ts65Dn mice. (A) Representative immunoblots and quantification of CB1R in hippocampus from Ts65Dn mice injected with with shSC and shCB1R-containing adenoassociated viral vectors (Ts65Dn shSC and Ts65Dn shSCB1R, n=5). (B) Representative immunoblots and quantification of CB1R in hippocampal samples comparing control mice (WT injected with shSC) versus Ts65Dn mice injected with shCB1R (WT shSC, n=6; Ts65Dn shCB1R, n=5). (C) Representative immunoblots and quantification of CB1R in hippocampal samples from WT mice injected with shSC or shCB1R (WT shSC, n=7; WT shCB1R, n=6). Actin was used as housekeeping control. (D) Discrimination index in NORT from WT and Ts65Dn mice injected with shSC or shCB1R in hippocampus (WT shSC, n=9; WT shCB1R, n=7; Ts65Dn shSC, n=5; Ts65Dn shCB1R, n=5). (E) Total object exploration time during test phase of NORT of mice injected with shSC and shCB1R. Distribution of individual data with mean ± s.e.m. * p<0.05, ** p<0.01 (genotype effect); # p<0.05 (treatment effect) by Student's t-test (A,B,C) and by Newman-Keuls post hoc test following two-way ANOVA (D,E).

1.2.3. CB1R pharmacological targeting rescues hippocampal-dependent memory deficits in Ts65Dn mice

We then tested whether a pharmacological intervention is suitable to treat memory deficits on DS using systemic administration of the CB1R selective antagonist/inverse agonist rimonabant in Ts65Dn mice. We administered 1 mg/kg of rimonabant for 7 days (i.p., last administration 24 hours before performing the test phase of the NORT). Rimonabant improved the memory performance of male and female of Ts65Dn mice (Figure 31, A and B) in the NORT.

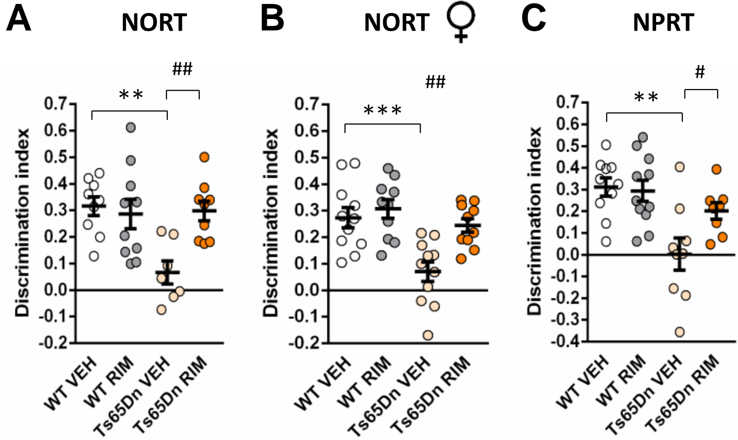

Figure 30. Pharmacological targeting of CB1R improves hippocampal-dependent memory in Ts65Dn mice. (A-B) Discrimination index in NORT after a sub-chronic treatment with vehicle (VEH) or rimonabant (RIM) (1 mg/kg) of male (A) and female (B) mice (males; WT VEH, n=9; WT RIM, n=11; Ts65Dn VEH, n=7; Ts65Dn RIM, n=8-11; females; WT VEH, n=11; WT RIM, n=10; Ts65Dn VEH, n=11; Ts65Dn RIM, n=10). (C) Discrimination index in NPRT after a sub-chronic treatment with vehicle or rimonabant (1 mg/kg) (WT VEH, n=10; WT RIM, n=11; Ts65Dn VEH, n=9; Ts65Dn RIM, n=8). Distribution of individual data with mean ± s.e.m. ** $p<0.01$, *** $p<0.001$ (genotype effect); # $p<0.05$, ## $p<0.01$ (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA.

Given this positive result, we also assessed place-recognition which is also dependent on the hippocampus but with less influence of cortico-hippocampal connections (i.e. perirhinal and prefrontal cortex) (Barker and Warburton, 2011; Warburton and Brown, 2015). Notably, rimonabant

administration also rescued the phenotype of Ts65Dn mice in the NPRT (Figure 31C). None of the memory tests revealed an effect of the treatment in WT mice (Figure 30, A-C). Neither differences in total exploration time were detected in any genotype (Figure 31, A-C).

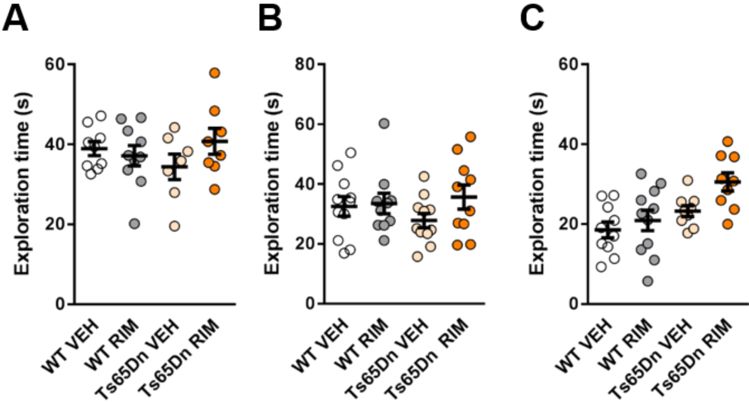

Figure 31. Pharmacological targeting of CB1R does not have an effect over total exploration times. (A-B) Total object exploration time during test phase of NORT of male (A) and female (B) mice treated with vehicle (VEH) or rimonabant (RIM) (1 mg/kg). (C) Total object exploration time during test phase of NPRT of male mice treated with vehicle or rimonabant (1 mg/kg). Distribution of individual data with mean ± s.e.m. Statistical significance was calculated by two-way ANOVA.

In addition, a comparable dose of the CB1R neutral antagonist NESS 0327 (0.1 mg/kg, i.p., 7 days) (Ruiu *et al.*, 2003), also improved NORT memory performance in Ts65Dn mice (Figure 32A) suggesting that the effect of rimonabant is associated to its antagonist and not to its inverse agonist profile. Again, no significant differences were detected in exploration times among the different experimental groups (Figure 32B). Therefore, the pharmacological intervention targeting CB1R improved hippocampal-dependent memory of Ts65Dn mice.

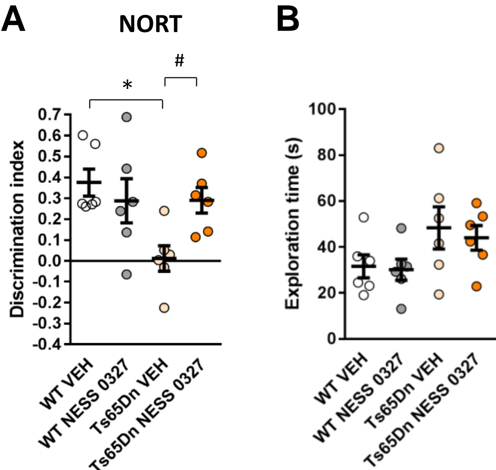

Figure 32. Pharmacological targeting of CB1R by a pure antagonist improves hippocampal-dependent memory in Ts65Dn mice. (A) Discrimination index in NORT after a sub-chronic treatment with vehicle (VEH) or NESS 0327 (0.1 mg/kg) WT VEH, n=6; WT NESS 0327, n=6; Ts65Dn VEH, n=6; Ts65Dn NESS 0327, n=6). (B) Total object exploration times during test phase of NORT of male mice treated with vehicle or NESS 0327. Distribution of individual data with mean ± s.e.m. * p<0.05 (genotype effect); # p<0.05 (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA.

1.2.4. CB1R pharmacological targeting improves hippocampal synaptic plasticity and cell proliferation in Ts65Dn mice

Since alterations in hippocampal synaptic plasticity and adult neurogenesis play a role in DS cognitive impairment (Kleschevnikov *et al.*, 2004; Clark *et al.*, 2006), we assessed whether pharmacological CB1R targeting could also rescue those phenotypes. We studied LTP elicited by TBS which is reduced in hippocampal CA3-CA1 synapses of Ts65Dn mice (Costa and Grybko, 2005). We analyzed E-LTP and L-LTP (60 and 120 minutes post-tetanus period, respectively) of hippocampal slices from WT and Ts65Dn mice treated for 7 days with vehicle or rimonabant (1 mg/kg, i.p., last administration 24 hours before slice collection). As expected, overall LTP was decreased in Ts65Dn mice compared to WT mice treated with vehicle with a significant reduction of L-LTP. Interestingly,

rimonabant administration normalized the Ts65Dn deficit in L-LTP (Figure 33 A and B; see Figure 33C for control groups) again with no effect of the treatment in WT mice. Changes in LTP were not due to differences in basal synaptic transmission as similar input/output curves were observed between genotypes (Figure 33D).

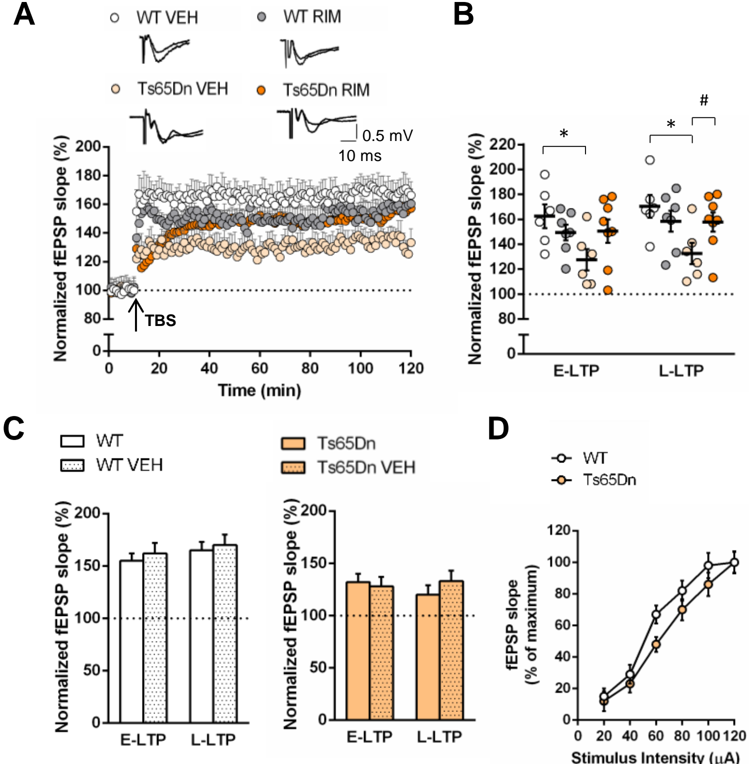

Figure 33. Pharmacological targeting of CB1R improves hippocampal synaptic plasticity of Ts65Dn mice. (A) Average time courses of the change in the slope of field excitatory postsynaptic potential (fEPSP) in hippocampal slices from mice treated for 7 days with vehicle (VEH) or rimonabant (RIM) (1 mg/kg). Traces represent samples of fEPSPs recorded for each experimental group before and after TBS. (B) Average LTP of the last 5 minutes of recordings in E-LTP and L-LTP (60 and 120 minutes post-tetanus period respectively) (WT VEH, n=6; WT RIM, n=7; Ts65Dn VEH, n=6; Ts65Dn RIM, n=8). (C) Average LTP of the last 5 minutes of recordings in E-LTP and L-LTP (60 and 120 minutes post-tetanus period, respectively) in hippocampal slices from naïve mice and mice treated with VEH (n=6-8). (D) Stimulation input/output curves for WT and Ts65Dn mice. Distribution of individual data with mean ± s.e.m. * p<0.05 (genotype effect); # p<0.05 (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA.

In addition, we studied adult neurogenesis, since Ts65Dn mice show a reduction in cell proliferation in the dentate gyrus (Clark *et al.*, 2006; Belichenko and Kleschevnikov, 2011). This phenotype can be quantified by the number of cells expressing Ki67, an endogenous marker of cell proliferation. Consistent with previous reports, Ts65Dn male mice treated with vehicle for 7 days (last administration 24 hours before brain perfusion) showed a decreased number of Ki67+ cells in the subgranular zone of the dentate gyrus (Figure 34). Treatment with rimonabant (1 mg/kg, i.p.) rescued this phenotype, with Ki67+ cell counts similar to WT controls, without modifying Ki67+ cell counts in WT mice (Figure 34). Thus, sub-chronic administration of rimonabant normalized defective synaptic plasticity and cellular proliferation in the hippocampus of Ts65Dn mice.

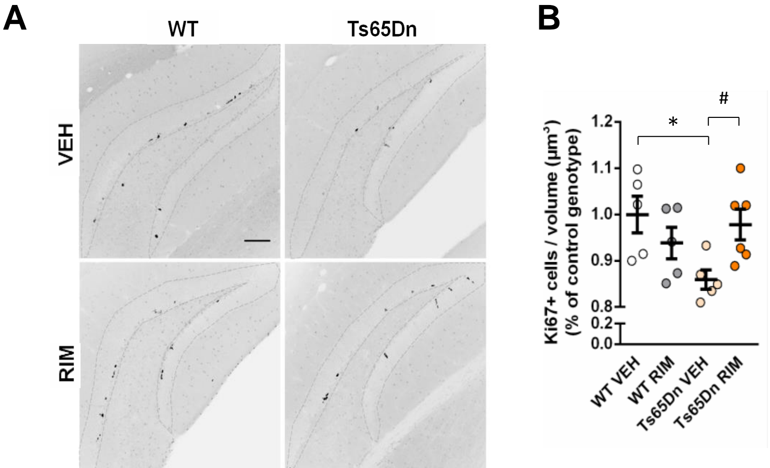

Figure 34. Pharmacological targeting of CB1R rescues the number of proliferating cells in the dentate gyrus of Ts65Dn mice. (A-B) Representative grey scale confocal images (A) and average density (B) of Ki67+ cells in the subgranular zone of the dentate gyrus of mice treated for 7 days with vehicle (VEH) or 1 mg/kg of rimonabant (RIM) (WT VEH, n=5; WT RIM, n=5; Ts65Dn VEH, n=5; Ts65Dn RIM, n=6) (scale bar=100µm). Distribution of individual data with mean ± s.e.m. * $p<0.05$ (genotype effect); # $p<0.05$ (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA.

1.2.5. CB1R pharmacological targeting is effective in a transgenic model overexpressing Dyrk1A

Dyrk1A overexpression in mice recapitulates cognitive deficits and brain alterations of DS (Altafaj *et al.*, 2001; Pons-Espinal *et al.*, 2013b). In addition, normalization of the dosage of this gene in Ts65Dn mice rescues cognitive, LTP and neuromorphological alterations (García-Cerro *et al.*, 2014) indicating that *Dyrk1A* overexpression is involved in these phenotypes. Therefore, we investigated whether rimonabant also improves memory deficits in a transgenic model overexpressing only this kinase, the TgDyrk1A mice (Altafaj *et al.*, 2001).

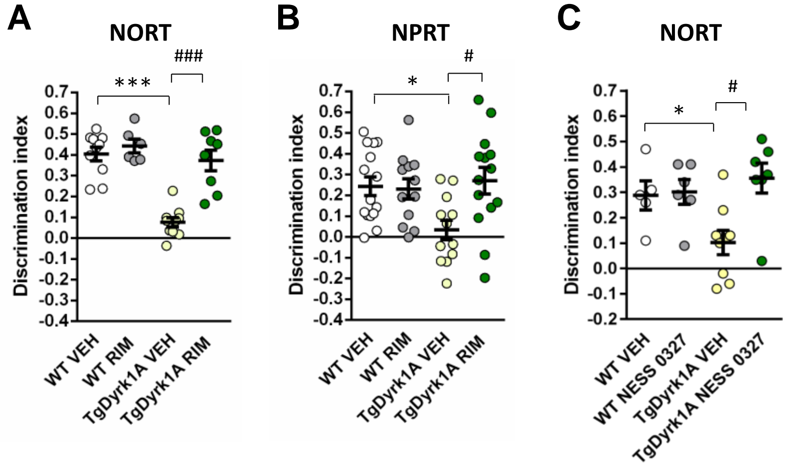

Figure 35. Pharmacological targeting of CB1R improves hippocampal-dependent memory in TgDyrk1A mice. (A-B) Discrimination index in NORT (A) and NPRT (B) after a sub-chronic treatment with vehicle (VEH) or 1 mg/kg of rimonabant (RIM) (NORT; WT VEH, n=10; WT RIM, n=6; TG VEH, n=10; TG RIM, n=8; NPRT; WT VEH, n=15; WT RIM, n=12; TG VEH, n=12; TG RIM, n=14). (C) Discrimination index of mice treated with vehicle or NESS 0327 (WT VEH, n=5; WT NESS 0327, n=6; TgDyrk1A VEH, n=8; TgDyrk1A NESS 0327, n=7). Distribution of individual data with mean ± s.e.m. * $p<0.05$, *** $p<0.001$ (genotype effect); # $p<0.05$, ### $p<0.001$ (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA.

TgDyrk1A mice showed a marked deficit in both NORT and NPRT. Interestingly, rimonabant treatment (1 mg/kg, i.p., 7 days) rescued cognitive performance in both tests also in this mouse model (Figure 35, A and B) without affecting total object exploration times (Figure 36, A and B). Again, NESS 0327 treatment (0.1 mg/kg, i.p., 7 days) also improved memory performance in TgDyrk1A mice (Figure 35C and 36C) further supporting CB1R as a relevant target in the cognitive improvement of this mouse line.

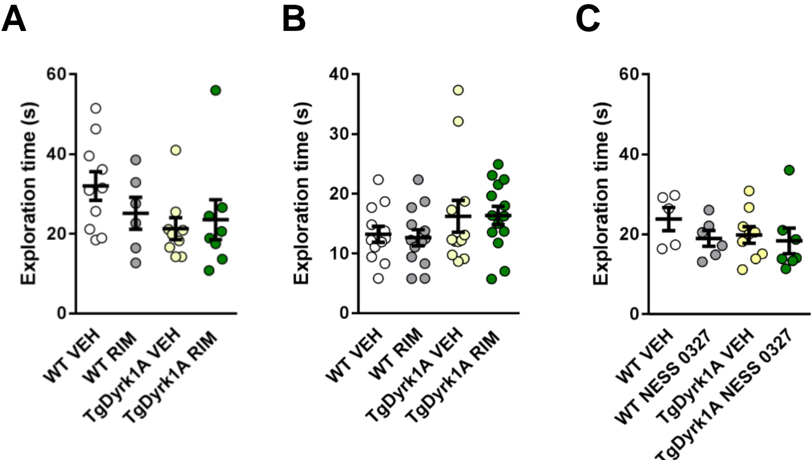

Figure 36. Pharmacological targeting of CB1R does not have an effect over total exploration times in TgDyrk1A mice. (A-B) Total object exploration times during test phase of NORT (A) and NPRT (B) treated with vehicle (VEH) or rimonabant (RIM). (C) Total object exploration times of mice treated with vehicle or NESS 0327. Distribution of individual data with mean ± s.e.m. Statistical significance was calculated by two-way ANOVA.

We also examined whether rimonabant treatment had an impact over hippocampal synaptic plasticity and cell proliferation in this model. We found a decrease in hippocampal L-LTP in slices of TgDyrk1A in comparison to WT. Sub-chronic rimonabant administration (7 days, i.p., 1 mg/kg) normalized this deficit in TgDyrk1A mice and did not have any effect on WT mice (Figure 37, A and B; see Figure 37 C for control groups). No

differences in basal synaptic transmission were observed between genotypes, as input/output curves were similar (Figure 37 D).

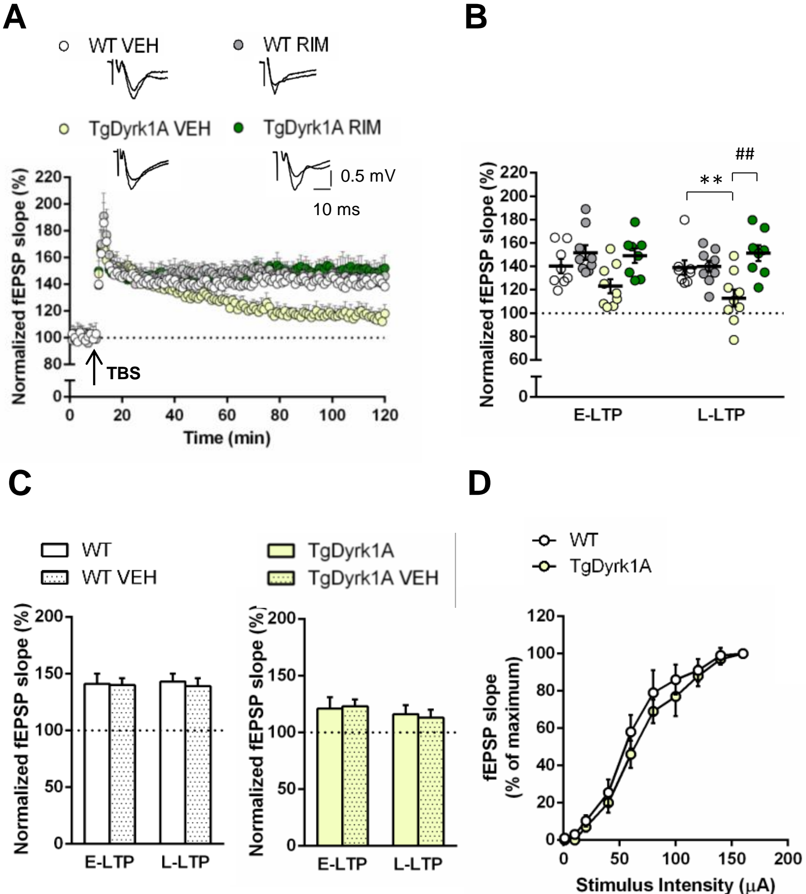

Figure 37. CB1R targeting improves hippocampal synaptic plasticity in TgDyrk1A mice. (A) Average time courses of the change in the slope of the fEPSP in hippocampal slices from mice treated for 7 days with vehicle (VEH) or rimonabant (RIM) (1 mg/kg). Traces represent samples of fEPSPs recorded for each experimental group before and after TBS. (B) Average LTP of the last 5 minutes of recordings in E-LTP and L-LTP (60 and 120 minutes post-tetanus period respectively) (WT VEH, n=8; WT RIM, n=9; TG VEH, n=9; TG RIM, n=8). (C) Average LTP of the last 5 minutes of recordings in E-LTP and L-LTP (60 and 120 minutes post-tetanus period respectively) in hippocampal slices from naïve mice and mice treated with vehicle, WT and TgDyrk1A (n=8-12). (C) Stimulation input/output curves for WT and TgDyrk1A mice. Distribution of individual data with mean ± s.e.m. ** p<0.01 (genotype effect); ## p<0.01 (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA.

TgDyrk1A mice also showed defects in adult neurogenesis. Conversely to what occurs in Ts65Dn mice, we observed a trend toward an increase of Ki67+ cells in TgDyrk1A mice, though this modification did not reach significance (Figure 38). We also found a trend to normalize this increase after rimonabant administration.

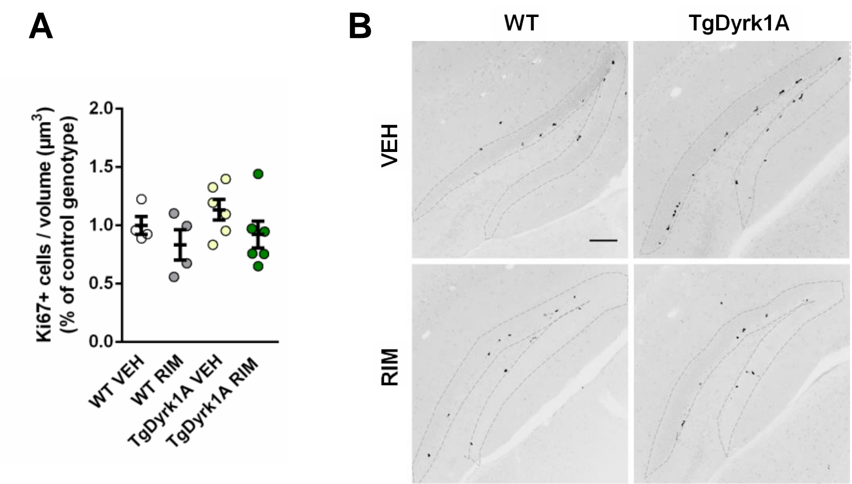

Figure 38. Pharmacological targeting of CB1R slightly decreases the number of proliferating cells in the dentate gyrus of TgDyrk1A mice. (A-B) Average density (A) and representative grey scale confocal images (B) of Ki67+ cells in the subgranular zone of the dentate gyrus of mice treated for 7 days with vehicle (VEH) or 1 mg/kg of rimonabant (RIM) (WT VEH, n=4; WT RIM, n=4; TG VEH, n=6; TG RIM, n=6) (scale bar=100μm). Distribution of individual data with mean ± s.e.m. Statistical significance was calculated by two-way ANOVA.

Since the increase of Ki67+ cells in TgDyrk1A mice seemed to be secondary to a cell cycle arrest rather than an increase in cell proliferation rate (Pons-Espinal *et al.*, 2013b), we analyzed the progenitors exiting the cell cycle. To address this specific issue, we injected a set of rimonabant/vehicle treated (1 mg/kg, 7 days) TgDyrk1A mice with a single dose of the DNA intercalating agent BrdU 24 hours before brain perfusion. BrdU labels *in vivo* those cells actively proliferating. We later quantified cells that exited the cell cycle by counting cells that had incorporated BrdU, but that did not express Ki67 (BrdU+/Ki67– cells). Interestingly, while rimonabant

administration slightly reduced the number of Ki67+ cells, it significantly increased BrdU+/Ki67– cells (Figure 39). This indicates that rimonabant rescued the decrease in cell cycle exit in dentate gyrus of TgDyrk1A mice. Therefore, repeated CB1R inhibition normalized long-term plasticity and cell cycle exit of hippocampus derived from *Dyrk1A* overexpression.

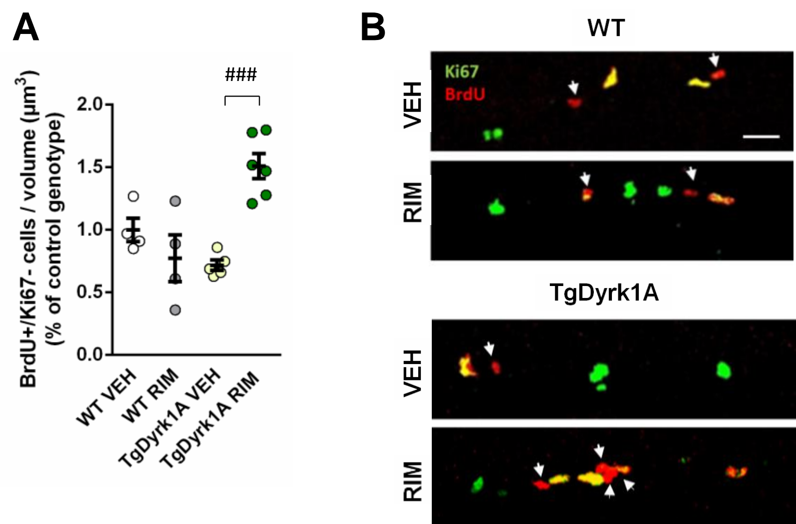

Figure 39. Pharmacological targeting of CB1R increases cell cycle exit in dentate gyrus of TgDyrk1A mice. (A-B) Average density (A) and representative confocal images (B) of BrdU+/Ki67– cells in the subgranular zone of the dentate gyrus of mice treated for 7 days with vehicle (VEH) or 1 mg/kg of rimonabant (RIM) (WT VEH, n=4; WT RIM, n=4; TG VEH, n=5; TG RIM, n=6) (scale bar=20µm). Distribution of individual data with mean ± s.e.m. ### p<0.001 (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA.

Abnormalities at the level of dendritic spines have been described in the hippocampus of several neurodevelopmental disorders associated with intellectual disability including DS (Ferrer and Gullotta, 1990). Taking advantage of a double transgenic line that expressed the yellow fluorescent protein (YFP) under the Thy1 promoter in the TgDyrk1A background, we analyzed spine density of apical dendrites from CA1 pyramidal neurons at the *stratum radiatum*. We found an enhanced spine density in TgDyrk1A in comparison to WT mice that was normalized after

rimonabant treatment (Figure 40A). We also classified dendritic spines depending on their morphology in stubby, thin and mushroom. No major changes were observed regarding spine morphology although a trend towards an increase in the number of stubby spines was found in TgDyrk1A mice (Figure 40, B and C). This result suggested that the enhancement in spine density of TgDyrk1A mice was secondary to an increase in immature spines. Rimonabant treatment resulted in an overall normalization of spine density with a non-significant reduction of stubby and thin spines (Figure 40).

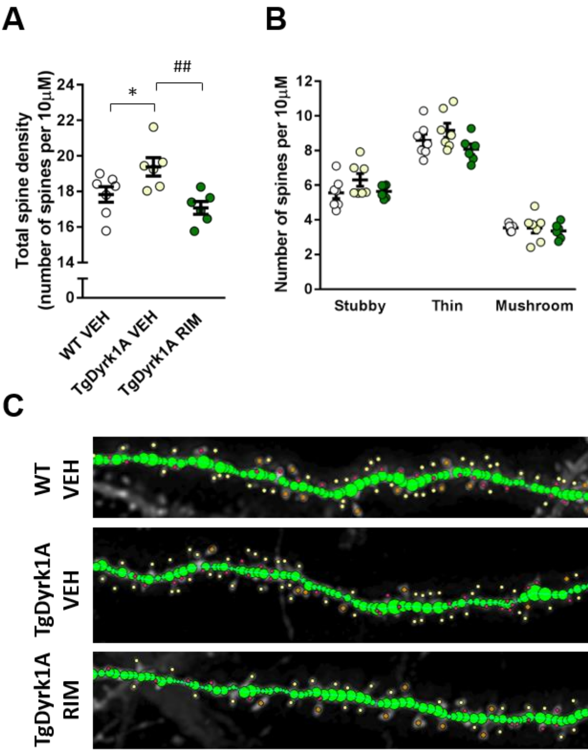

Figure 40. Pharmacological targeting of CB1R normalizes spine density of CA1 pyramidal dendritic spines of TgDyrk1A mice. (A) Quantification of the total number of spines in 10µm of CA1 pyramidal dendrites of mice treated for 7 days with vehicle (VEH) or 1 mg/kg of rimonabant. (B-C) Analysis (B) and representative images (C) of spine morphology (WT VEH, n=7; TG VEH, n=7; TG RIM, n=6). Distribution of individual data with mean ± s.e.m. * $p<0.05$ (genotype effect); ## $p<0.01$ (treatment effect) by Student's t-test.

CHAPTER 2

MONOACYLGLYCEROL LIPASE INHIBITION RESTORES SOCIAL ALTERATIONS IN MOUSE MODELS OF WILLIAMS-BEUREN SYNDROME

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Submitted

CHAPTER 2

Monoacylglycerol lipase inhibition restores social alterations in mouse models of Williams-Beuren syndrome

One of the most prominent features of the cognitive profile of WBS is an hypersociable phenotype characterized by uninhibited social interactions and a reduced response to social threat (Gosch and Pankau, 1994; Plesa-Skwerer *et al.*, 2006). This phenotype is opposite to the typical social phenotype of autism spectrum disorders characterized by lack of social interest and deficits in social communication (Barak and Feng, 2016). The neurobiological mechanisms that modulate these social abnormalities are still unknown.

The ECS plays a role in social behavior and, alterations of this system have been described in several mouse models of autism spectrum disorders. In addition, the pharmacological modulation of the ECS restores social abnormalities in some of these models (Wei *et al.*, 2017; Zamberletti *et al.*, 2017). The possible implication of the ECS and its therapeutic potential in the hypersociable phenotype of WBS had not been addressed before. In this chapter, we have assessed the involvement of the ECS and its therapeutic potential in the social abnormalities of WBS. For achieving these aims, we have used a mouse model that resembles the genetic condition of WBS subjects and another mouse model with a deletion of a single gene, the *Gtf2i*, with a central function in social behavior.

2.1. Materials and Methods

Animals

All animal procedures were conducted following ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines (Kilkenny *et al.*, 2010) and standard ethical guidelines (European Communities Directive 2010/63/EU) and approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB). C57BL/6J and CD1 mice were obtained from Charles River Laboratory. WBS-CD and Δ*Gtf2i*$^{+/-}$ mice were obtained as previously described (Segura-Puimedon *et al.*, 2014) and maintained on C57BL/6J background (backcrossed for nine generations). WT littermates were used as controls. Male mice aged between 8 and 16 weeks were used for experiments. For social behavior tests juvenile (4 weeks old) male C57BL/6J mice were used as stranger mice.

Mice were housed and maintained in the same conditions described in the previous chapter. Mice were habituated to the experimental room and handled for 1 week before starting the experiments. All behavioral experiments were conducted by an observer blind to the experimental conditions.

Drug treatment

JZL184 (Abcam) was diluted in 15% dimethyl sulfoxide (DMSO; Scharlau Chemie), 4.25% polyethylene glycol 400 (Sigma-Aldrich), 4.25% Tween-80 (Sigma-Aldrich) and 76.5% saline. Rimonabant (Sanofi-Aventis) was diluted in 5% ethanol, 5% Cremophor-EL and 90% saline. JZL184 and rimonabant were injected in a volume of 5 ml/kg and 10 ml/kg of body weight respectively. Drugs were administered daily by i.p. injection 2 hours prior behavioral testing. Mice were randomly assigned to experimental groups.

Behavioral tests

Sociability and preference for social novelty

V-maze test

We designed a modified version of the V-shaped maze to evaluate sociability (V-maze, Busquets-Garcia et al., 2011). It consists of two structures: the maze wall (150 mm high), made of black Plexiglas and the maze lead, made of transparent Plexiglas (Figure 41). Corridors in the V-maze are 300 mm long and 45 mm wide (internal measures). Two small chambers (65 mm long) were created at the end of the corridors when the lead was inserted into the V-maze. These chambers were used to allocate stranger mice (**Figure 41**).

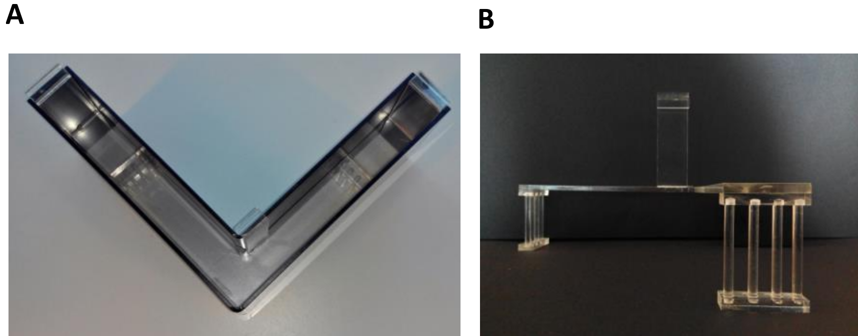

Figure 41. Photographs of the top view of the V-maze with the transparent lead and a detail of the lead which includes the Plexiglas bars for mice to interact.

Three-chamber maze test

It consisted in a rectangular box made of Plexiglas divided in three-identical-chambers by two Plexiglas walls containing small openings to allow mouse access between chambers. The stranger mice were enclosed in a round wire cage in the side chambers. The wire cage had vertical bars spaced 10 mm, which allow sniffing and exploration. There was a weighted cup on top of the wire cage to prevent the experimental animal from climbing (Moy *et al.*, 2004).

Experimental procedure

Social behavior was performed in both mazes to validate the V-maze approach (see Figure 42 for a comparison between mazes). The procedure was performed in a sound-attenuated room with dim illumination 5-10 lux. A digital camera on top of the maze was used to record the sessions. Social exploration was considered when the experimental mouse directed the nose in close proximity (1 cm) to the vertical bars of the chambers (V-maze) or the wire cups (three-chamber maze).

The protocol consists of the following phases (see also introduction 1.2).

Habituation (Phase I). Experimental mice were introduced into the central part of the V-maze or three-chamber maze for 5 minutes, where they freely explored the empty chambers/wire cups. The experimenter recorded the exploration time for each chamber/ cup analyzing the image obtained by a closed-circuit camera. This measurement is important to discard a possible bias for one particular chamber and it informs about the baseline activity of the mouse in the maze.

Sociability (Phase II). The sociability session was performed just after the habituation session. In this phase, an unfamiliar juvenile mouse assigned as stranger 1, was placed in one of the chambers/wire cups (both sides were alternated during the experiments). The experimental mouse was allowed to explore both compartments for 5 minutes. The experimenter recorded the time that the experimental mouse spent exploring the empty chamber/wire cup or the stranger 1. At the end of the sociability session the subject and stranger 1 were maintained in the maze to start the last phase of the test**.**

Preference for social novelty (Phase III). The preference for social novelty phase was performed just after the sociability session. A second novel juvenile mouse, assigned as stranger 2, was placed inside the previously

empty chamber/wire cup, while the stranger 1 remained inside the same chamber/wire cup as in phase II. For 5 minutes, the experimental animal was allowed to explore the two strangers and the time spent exploring each stranger was recorded.

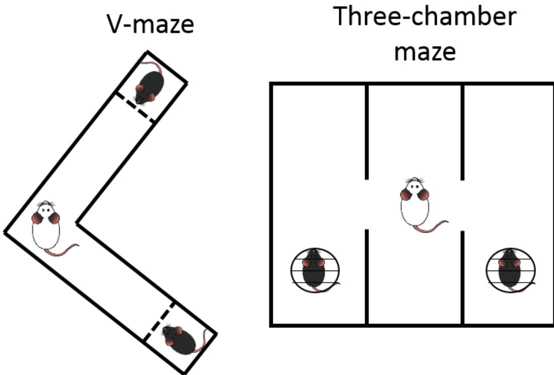

Figure 42. Schematic representation of the V-maze and the three-chamber maze.

Using the V-maze, a similar procedure was also performed with WBS-CD mice but with objects instead of juvenile stranger mice.

Locomotor activity

Locomotor activity was assessed for 30 minutes by using individual locomotor activity boxes (9 × 20 × 11 cm, Imetronic) in a low luminosity environment (5 lux). The number of horizontal movements was detected by a line of photocells located 2 cm above the floor.

Novel object recognition test

The NORT was performed as described in the previous chapter. However, short-term instead of long-term memory was studied. The time between the training and the test session was 10 minutes.

Oxcytocin analysis

Blood samples were collected by decapitation in tubes containing EDTA (1 mg/mL blood) and aprotinin (500 KIU/mL of blood) and centrifuged at

2000x g for 10 minutes at 4ºC. Plasma was transferred to new tubes and stored at -80ºC until used. Plasma oxytocin levels were measured using an ELISA kit (Enzo Life Sciences, ADI-900-153) according to manufacturers' protocol. For each animal 45 µl of plasma was used.

Immunoblot

Hippocampal, frontal cortex and amygdala tissue was dissected immediately after cognitive tests, frozen on dry ice, and stored at −80 °C until used. The preparation of the samples and the immunoblot was performed as in the previous chapter. The following primary antibodies were used: anti-CB1R (rabbit and guinea pig, 1:500, CB1-Rb-Af380 and CB1-GP-Af530 Frontier Institute Co.Ltd), anti-FAAH (mouse, 1:1,200, ab54615, Abcam), anti-NAPE-PLD (guinea pig, 1:1000, NAPE-PLD-GP-Af720-1, Frontier Institute Co.Ltd), anti-MAGL (rabbit, 1:300, ab24701, Abcam), anti-DAGLα (guinea pig 1:300, Frontier Institute Co.Ltd, DGLa-GP-Af380-1), anti-actin (mouse, 1:50,000, MAB1501, MerckMillipore) and anti-TFII-I (rabbit, 1:400, (Lucena *et al.*, 2010)). Primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-guinea pig antibodies.

Endocannabinoid quantification by liquid chromatography–tandem mass spectrometry

The quantification of endocannabinoids was performed as described in Chapter 1. In this case, the tissue analyzed was frontal cortex (20.24 ± 3.21 mg) and half whole brain (231.4 ± 20.38 mg) (Mean ± S.D.).

Statistical analysis

Data were analyzed with Statistica Software using unpaired Student's t-test and two-way ANOVA for multiple comparisons. Social interaction was

analyzed by repeated-measures ANOVA with maze/genotype/treatment as between-subject factor and compartment as within-subject factor. Subsequent *post hoc* analysis (Newman-Keuls) was used when required (significant interaction between factors). Comparisons were considered statistically significant when $p < 0.05$. Outliers (± 2 s.d. from the mean) were excluded.

2.2. Results

2.2.1. The V-maze as a new setting to characterize social behavior in mice

The V-shaped maze has been successfully used to measure cognitive responses with the NORT (Puighermanal *et al.*, 2009; Busquets-Garcia *et al.*, 2013; Gomis-González *et al.*, 2016). One of the standard approaches to assess social behavior is the three-chamber maze test, which is performed in a three-chamber box and can be used to measure sociability and preference for social novelty (Moy *et al.*, 2004). This test is time-consuming since it lasts around 35 minutes per mouse (10 minutes per phase plus the time of cleaning the apparatus). We hypothesized that the V-maze setting could enhance exploratory activity of the experimental mice by reducing spatial and contextual information and could allow to reduce the duration of the test and enhance reproducibility. For achieving this purpose, we compared social behavior of an inbreed and an outbreed mouse strain, C57BL/6J and CD1 mice respectively, in both, the V-maze and the three-chamber test. We performed both tests with 5 minutes per phase since it was reported that the majority of social interaction behaviors occurred within the first five minutes of each session (Nadler *et al.*, 2004). We also performed the three-chamber test with 10 minutes for each of the phases as a control. In the three-chamber test, the time spent in each chamber or the time spent sniffing the social target can be measured. We used the time spent sniffing for comparing the results with the V-maze.

The V-maze setting provided quite similar results in the habituation session (phase I) for both strains, although higher variability was shown in CD1 mice assessed with the three-chamber test (Figure 43, A and B). In the sociability session (phase II), all mice demonstrated a significant

preference for the chamber containing the juvenile stranger 1 compared to the empty chamber independently of the strain or the maze employed (Figure 43, C and D). Unexpectedly, we observed an increase in exploration times in the three-chamber maze in comparison to the V-maze in all mice. During preference for social novelty session (phase III), mice analyzed in the V-maze independently of their strain, showed a significant predilection for exploring stranger 2 compared to stranger 1. In the three-chamber maze, CD1 mice but not C57BL/6J, showed a clear preference for social novelty (Figure 43, E and F).

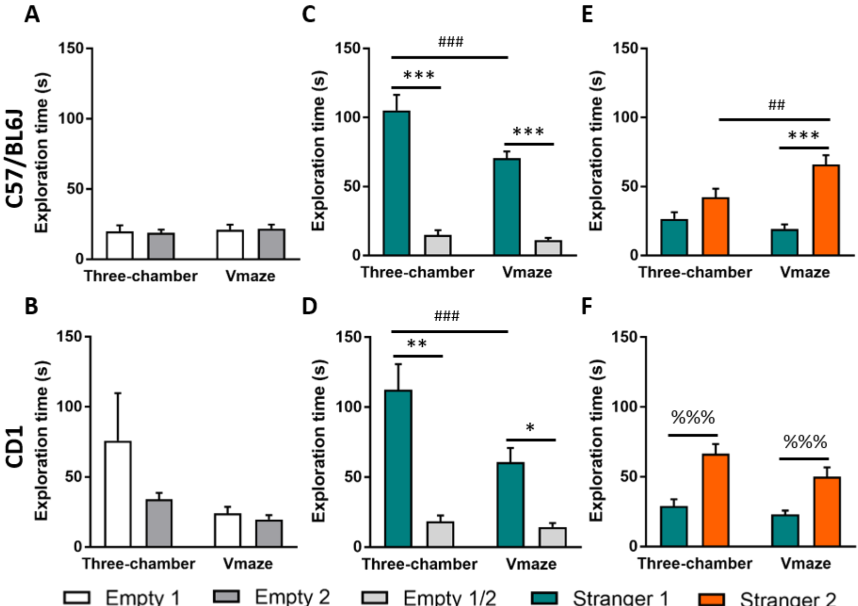

Figure 43. Comparison of social behavior assessed in the three-chamber maze or the V-maze using 5 minute-sessions. (A-B) Time spent by C57BL/6J (A) and CD1 mice (B) exploring the empty compartments during phase I. (C-D) Time spent by mice exploring either stranger 1 or the empty compartment during phase II. (E-F) Time spent by mice exploring either stranger 1 or stranger 2 during phase III (three-chamber C57BL/6J, n=6; CD1, n=11; V-maze C57BL/6J, n=8; CD1, n=10). Data are expressed as mean ± s.e.m. Statistical significance was calculated by repeated measures ANOVA comparison followed by Newman's Keuls *post hoc* test * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (compartment comparison); ## $p<0.01$; ### $p<0.001$ (maze comparison). Compartment x maze interaction was not significant in CD1 mice in phase III although a main effect of compartment was observed. %%% $p<0.001$ (main effect of compartment).

When 10 minutes were assessed in the three-chamber test, the preference for social novelty was apparent in both strains (Figure 44). These data revealed that the V-maze setting performed with 5 minutes for each of the phases was a new reliable and sensitive system to study social behavior in C57BL/6J and CD1 mice.

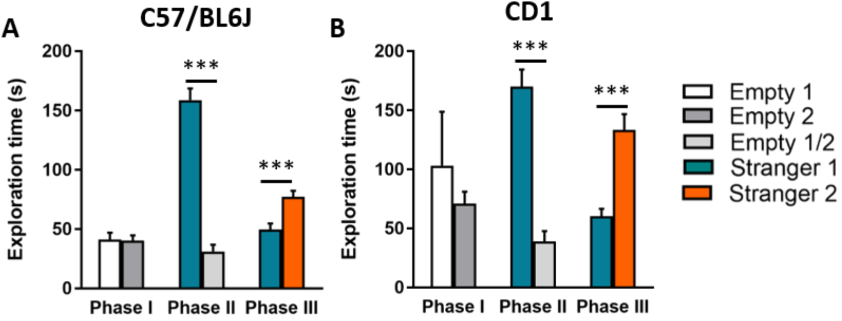

Figure 44. Sociability and preference for social novelty using the three-chamber maze for 10 minutes in C57BL/6J and CD1 mice. (A) Time spent by C57BL/6J mice exploring each of the compartments. (B) Time spent by CD1 mice exploring each of the compartments (C57BL/6J, n=8; CD1, n=7). Data are expressed as mean ± s.e.m. *** $p<0.001$ (compartment comparison). Statistical significance was calculated by Student's t-test.

2.2.2. WBS-CD present an hypersociable phenotype and no preference for social novelty

Alterations in social behavior have been described in both WBS individuals and mouse models of WBS (Gosch and Pankau, 1994, 1997; Li *et al.*, 2009; Segura-Puimedon *et al.*, 2014). Considering previous results, we assessed social behavior of WBS-CD mice (C57BL/6J background) using the V-maze setting. No changes between genotypes were observed in exploration times of the empty compartments during the habituation session. During the sociability phase, both WT and WBS-CD displayed a preference for exploring the compartment with a juvenile stranger mouse rather than the empty compartment. Notably, WBS-CD mice spent significantly more time exploring stranger 1 than WT mice (Figure 45B). During the preference for

social novelty phase, WBS-CD mice explored similarly stranger 1 and stranger 2 in contrast to WT animals (Figure 45C). These data indicated that WBS-CD mice presented an hypersociable phenotype and a lack of preference for social novelty.

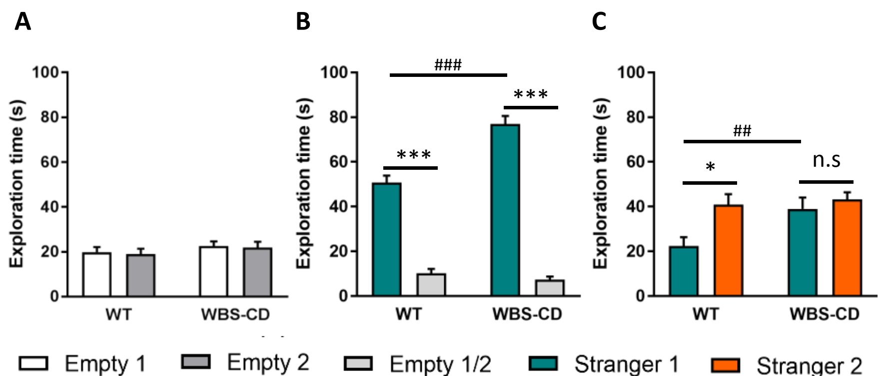

Figure 45. WBS-CD mice show an hypersociable phenotype and no preference for social novelty. (A) Time spent exploring the empty compartments. (B) Time spent exploring either stranger 1 or the empty chamber during sociability phase. (C). Time spent exploring either stranger 1 or stranger 2 during preference for social novelty. Data are expressed as mean ± s.e.m. (WT, n=11; WBS-CD, n=11). *p<0.05; *** p<0.001 (compartment comparison). ## p<0.01; ### p<0.001 (genotype comparison). Statistical significance was calculated by repeated measures ANOVA comparison followed by Newman's Keuls *post hoc* test.

To confirm that both phenotypes were dependent on social stimuli, we repeated the same procedure using objects instead of unfamiliar mice (Figure 46A).. Both WT and WBS-CD mice displayed a preference for the compartment with an object (object 1) instead the empty compartment. In contrast to social behavior, WT and WBS-CD mice spent similar times exploring the object 1 (Figure 46C). When the object 2 was introduced, both WT and WBS-CD mice spent more time exploring the object 2 than object 1 (Figure 46D). Therefore, WBS-CD mice showed no preference for social novelty, but preference for object novelty.

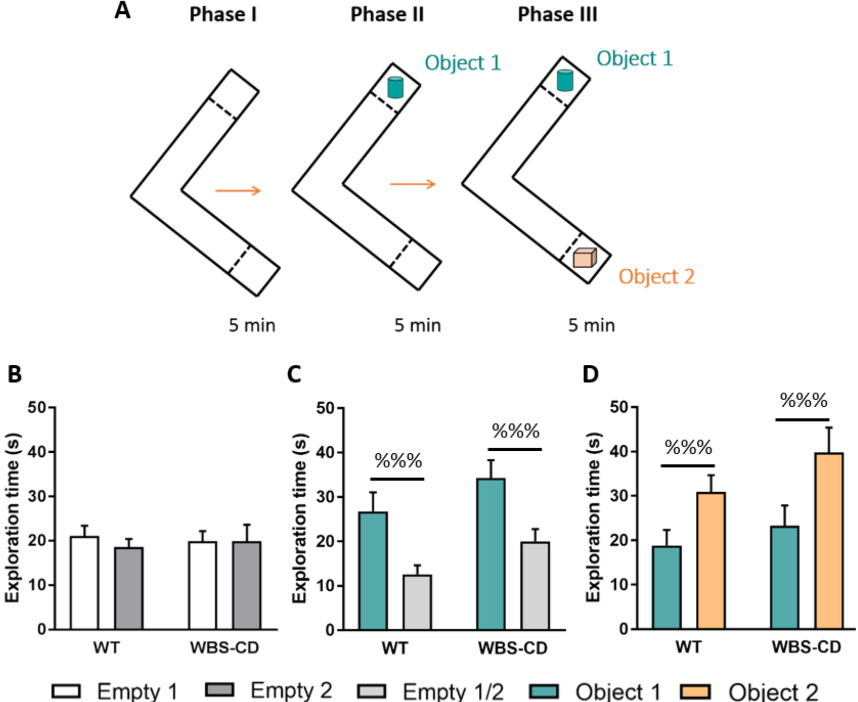

Figure 46. WBS-CD mice show preference for object novelty. (A) Schematic cartoon of the procedure. (B) Time spent exploring the empty compartments. (C) Time spent exploring either the object 1 or the empty chamber. (D) Time spent exploring either the object 1 or the object 2 (WT, n=12; WBS-CD, n=8). Data are expressed as mean ± s.e.m. Compartment x genotype interaction was not observed by repeated measures ANOVA comparison although a main effect of compartment was observed in phases II and III. %%% $p<0.001$ (main effect of compartment).

2.2.3. WBS-CD mice do not show major alterations of the endocannabinoid system

Alterations in the components of the ECS have been associated with social abnormalities in mouse models of autism-spectrum disorders (Zamberletti *et al.*, 2017). Given the social abnormalities of the WBS-CD mice, we studied possible alterations of the ECS in this mouse model. We analyzed by immunoblot the main components of the ECS in brain areas affected in WBS patients: the frontal cortex, amygdala and hippocampus (Meyer-Lindenberg *et al.*, 2005b, a; Capitão *et al.*, 2011). A slight trend toward a

decrease in CB1R was found in frontal cortex of WBS-CD mice, although it did not reach significance (Figure 47). No major changes were found in the main enzymes responsible for the synthesis and inactivation of 2-AG and AEA in the three areas analyzed (Figure 47-48).

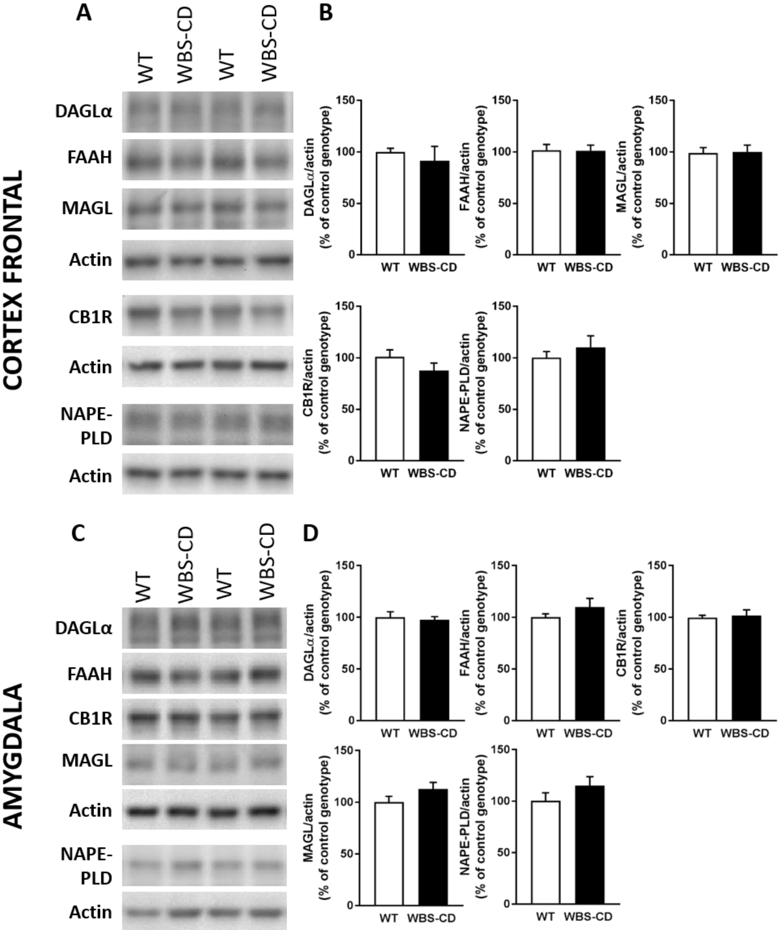

Figure 47. WBS-CD mice do not present major alterations in the main components of the endocannabinoid system in frontal cortex and amygdala. (A-B) Representative immunoblots (A-C) and quantification (B-D) of the main components of the endocannabinoid system in amygdala and frontal cortex from WT and WBS-CD mice (WT, n=6; WBS-CD, n=6). Data are expressed as mean ± s.e.m. Actin immunodetection was used as housekeeping control. Statistical significance was calculated by Student's t-test.

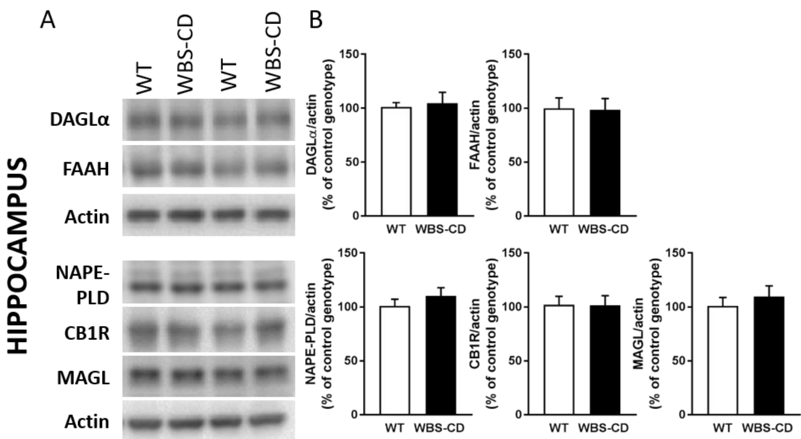

Figure 48. WBS-CD mice do not present major alterations in the main components of the endocannabinoid system in hippocampus. (A-B) Representative immunoblots (A) and quantification (B) of the main components of the endocannabinoid system in hippocampus from WT and WBS-CD mice (WT, n=7; WBS-CD, n=7). Actin immunodetection was used as housekeeping control. Statistical significance was calculated by Student's t-test.

We also determined the levels of endocannabinoids AEA and 2-AG, and related N-acylethanolamine (DEA and DHEA) and 2-monoacylglycerol (2-LG and 2-OG) compounds in the frontal cortex and whole brain homogenates of WBS-CD mice. Among the brain areas most affected in WBS individuals, we selected the frontal cortex since it has been reported that balance between excitatory and inhibitory synaptic inputs of this area is essential for normal social behavior (Yizhar *et al.*, 2011). Therefore, changes in the levels of endocannabinoids there may have an impact on endocannabinoid signaling and, as consequence, in the balance between synaptic inputs. No significant changes were revealed in the different experimental conditions, although a trend toward an increase in 2-AG levels was found in whole brain homogenates of WBS-CD mice (Table 10).

	Frontal Cortex		Whole brain	
	WT	WBS-CD	WT	WBS-CD
AEA	100 ± 5.6	89.75 ± 7.6	100 ± 1.73	101.54 ± 5.8
DEA	100 ± 6.3	83.48 ± 5.5	100 ± 6.6	97.60 ± 3.0
DHEA	100 ± 5.4	98.61 ± 7.4	100 ± 4.5	93.95 ± 4.9
2-AG	100 ± 8.4	93.1 ± 11.2	100 ± 3.5	111.1 ± 5.9
2-LG	100 ± 5.8	109.7 ± 5.1	100 ± 3.7	109.23 ± 6.1
2-OG	100 ± 7.6	98.06 ± 4.9	100 ± 2.9	100.05 ± 5.1

Table 11. Relative levels of endocannabinoids and related compounds in frontal cortex and whole brain homogenates of WBS-CD and WT. Data is expressed as mean ± s.e.m. Statistical significance was calculated by Student's t-test.

2.2.4. JZL184 administration corrects social impairment in WBS-CD mice

Limited literature has addressed the role of 2-AG on social behavior and the studies have reported contradictory results. The increase of 2-AG signaling by blocking MAGL decreases aggressive behavior in adult mice (Aliczki *et al.*, 2015), whereas increases social interactions in adolescent mice (Manduca *et al.*, 2016). Administration of the dual inhibitor of MAGL and FAAH, JZL195, increases social interactions in adolescent and adult rats, although only at low doses (Manduca *et al.*, 2015). Therefore, the inhibition of MAGL has an impact on social behavior, but its effects are probably context-dependent.

We used JZL184 (8 mg/kg, i.p.), an irreversible inhibitor of the MAGL, to increase the 2-AG tone in young-adult WBS-CD mice. After a single administration of the drug (2 hours before starting the V-maze test), no significant effect of the treatment was observed in sociability and preference for social novelty in WBS-CD mice neither in WT mice (Figure 49, A-C), although a slight effect was observed during phase III.

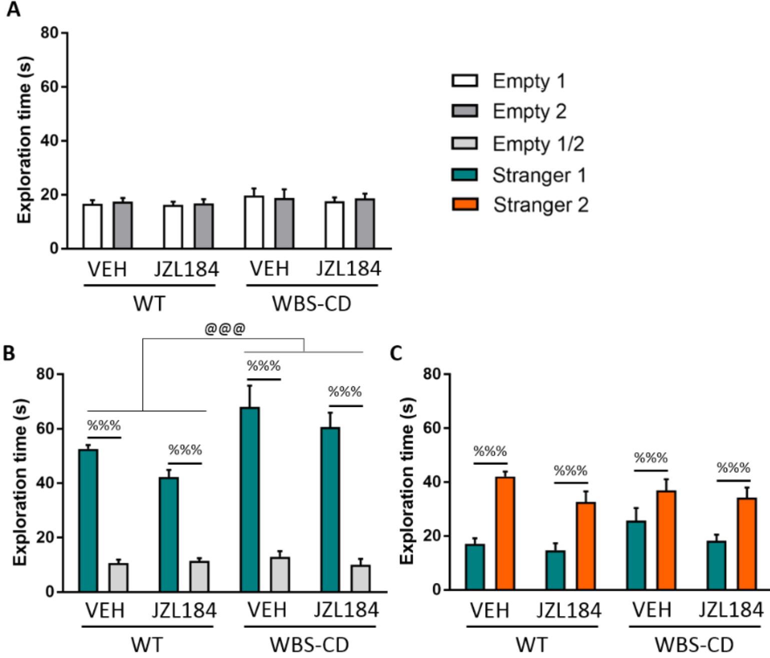

Figure 49. A single dose of the MAGL inhibitor JZL184 does not have a major effect over the social phenotype of WBS-CD mice. (A) Time spent exploring either the empty chamber 1 or the empty chamber 2 after a single dose of vehicle (VEH) or JZL184 (8 mg/kg) (WT VEH, n=8; WT JZL184, n=7; WBS-CD VEH, n=5; WBS-CD JZL184, n=6). (B) Time spent exploring either the stranger 1 or the empty chamber (WT VEH, n=9; WT JZL184, n=8; WBS-CD VEH, n=6; WBS-CD JZL184, n=6). (C) Time spent exploring either the stranger 1 or the stranger 2 (WT VEH, n=9; WT JZL184, n=8; WBS-CD VEH, n=6; WBS-CD JZL184, n=6). Data are expressed as mean ± s.e.m. Compartment x genotype x treatment interaction was not observed by repeated measures ANOVA comparison although a main effect of the compartment was found in phases II and III, and a main effect of the genotype was found in phase II. %%% p<0.001 (main effect of compartment); @@@ p<0.001 (main effect of genotype).

Administration of JZL184 (8 mg/kg, i.p.) for 10 days during the sociability phase significantly decreased the time that WBS-CD mice spent exploring stranger 1 reaching levels comparable to those displayed by WT mice (Figure 50 B). In addition, during phase III, WBS-CD mice treated with JZL184 showed a preference for social novelty similar to WT animals (Figure 50 C). Notably, administration of JZL184 did not alter the exploration times of WT mice neither the exploration times during phase

I (Figure 50, A-C). Pretreatment with rimonabant (2 mg/kg, i.p.; 15 minutes before the injection of JZL184) blocked the effect of JZL184 on WBS-CD mice in both phases, II and III (Figure 50, B-C). Therefore, effects of monoacylglycerol inhibition over social phenotypes in WBS-CD mice were dependent on CB1R, and thus, on 2-AG accumulation.

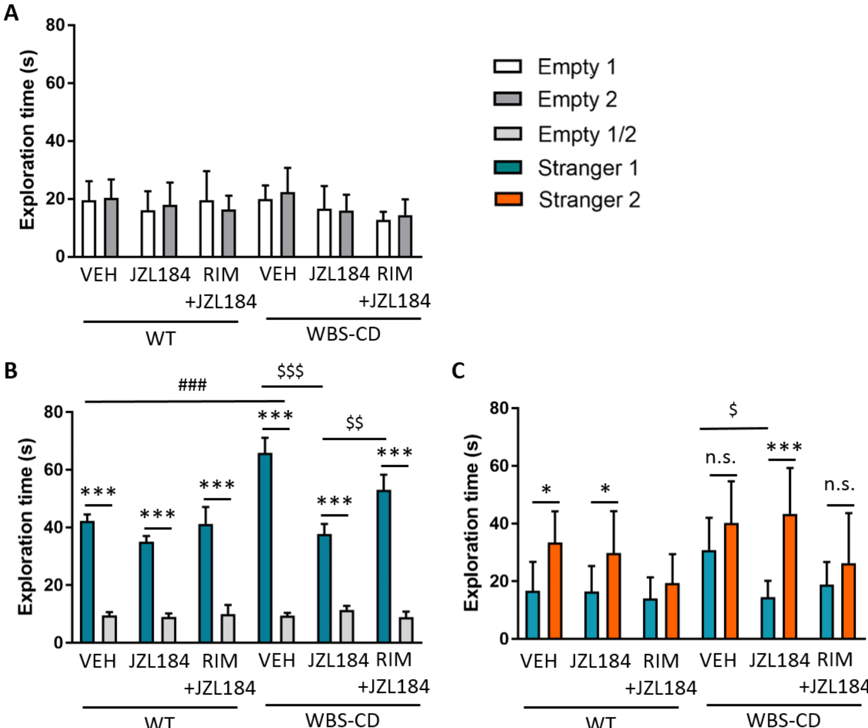

Figure 50. Inhibition of MAGL by JZL184 normalizes the hypersociable phenotype and preference for social novelty of WBS-CD mice by a CB1R-dependent mechanism. (A) Time spent exploring empty chambers after 10 days of treatment of vehicle (VEH), JZL184 (8 mg/kg) or rimonabant (RIM) (2 mg/kg) + JZL184 (8 mg/kg (WT VEH, n=17; WT JZL184, n=16; WT RIM JZL184, n=5; WBS-CD VEH, n=14; WBS-CD JZL184, n=13; WBS-CD RIM JZL184, n=8). (B) Time spent exploring either the stranger 1 or the empty chamber (WT VEH, n=17; WT JZL184, n=15; WT RIM JZL184, n=5; WBS-CD VEH, n=15; WBS-CD JZL184, n=13; WBS-CD RIM JZL184, n=7). (C) Time spent exploring either the stranger 1 or the stranger 2 (WT VEH, n=16; WT JZL184, n=15; WT RIM JZL184, n=5; WBS-CD VEH, n=14; WBS-CD JZL184, n=13; WBS-CD RIM JZL184, n=8). Data are expressed as mean ± s.e.m. Statistical significance was calculated by repeated measures ANOVA comparison. * $p<0.05$; *** $p<0.001$ (compartment comparison); ### $p<0.001$ (genotype comparison); $ $p<0.05$; $$ $p<0.01$; $$$ $p<0.001$ (treatment comparison).

To discard that the effects of JZL184 (8 mg/kg, i.p.) were dependent on changes in locomotion we assessed locomotor activity. No changes were observed after JZL184 administration neither in WT nor in WBS-CD mice (Figure 51).

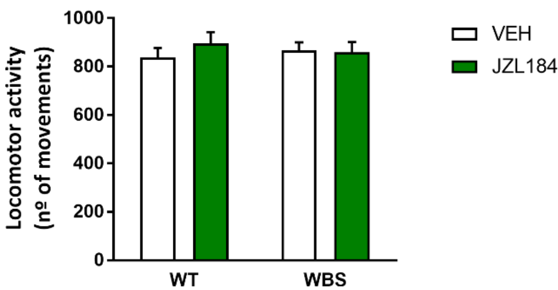

Figure 51. JZL184 treatment does not modify locomotor activity in WT and WBS-CD mice. Horizontal movements performed in locomotor activity boxes for 30 minutes by mice treated with vehicle (VEH) or JZL184 (8 mg/kg) (WT VEH, n=8; WT JZL184, n=8; WBS-CD VEH, n=8; WBS-CD JZL184, n=8). Data are expressed as mean ± s.e.m. Statistical significance was calculated by two-way ANOVA.

2.2.5. ΔG*tf2i* heterozygous knock-out mice present an hypersociable phenotype also corrected by JZL184

The deletion of *Gtf2i* gene has a major contribution in social abnormalities of WBS (Lucena *et al.*, 2010; Sakurai *et al.*, 2011; Borralleras *et al.*, 2015). Previous studies of the Δ*Gtf2i*$^{+/-}$ mice showed that they had an hypersociable phenotype (Borralleras *et al.*, 2015). We analyzed social behavior of the Δ*Gtf2i*$^{+/-}$ mice in the V-maze paradigm. Both WT and Δ*Gtf2i*$^{+/-}$ mice showed a significant increase in the time spent exploring the stranger 1 in comparison to the empty chamber in phase II. Interestingly, Δ*Gtf2i*$^{+/-}$ mice spent more time exploring the stranger 1 than WT mice (Figure 52 B). Unlike WBS-CD mice, Δ*Gtf2i*$^{+/-}$ mice presented preference for social novelty similar to control animals (Figure 52 C). A single dose of JZL184 (8 mg/kg, i.p., 2 hours before starting the V-maze test) normalized the hypersociable phenotype of Δ*Gtf2i*$^{+/-}$ during phase II (Figure 52 B).

Notably, JZL184 did not modify the behavioral responses of WT mice in any of the phases (Figure 52, A-C).

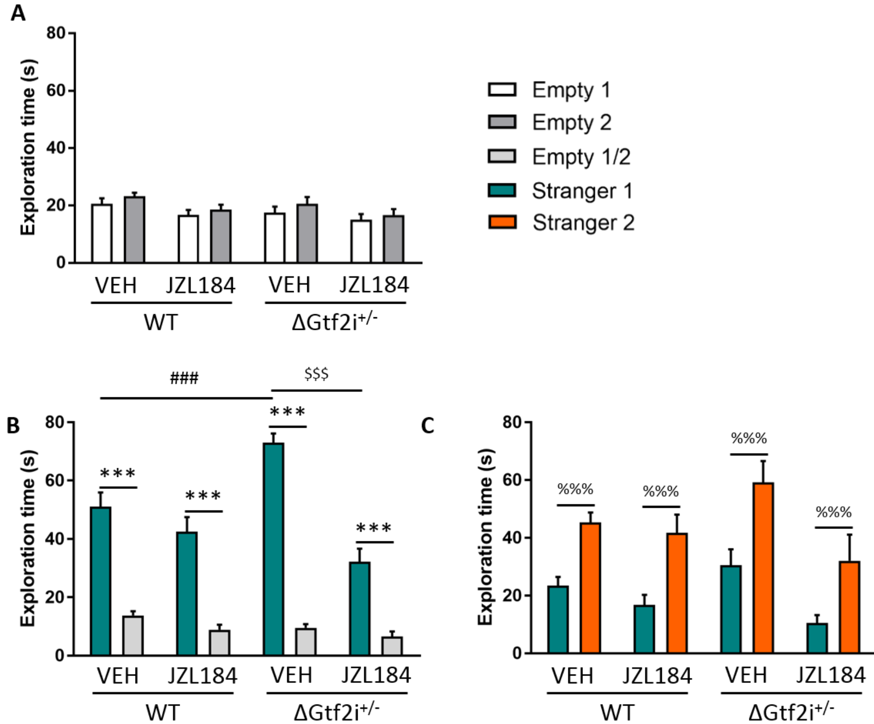

Figure 52. A single dose of the MAGL inhibitor JZL184 rescues the hypersociable phenotype of Δ*Gtf2i*$^{+/-}$. (A) Time spent exploring either the empty chamber 1 or the empty chamber 2 after a single dose of vehicle (VEH) or JZL184 (8 mg/kg). (B) Time spent exploring either the stranger 1 or the empty chamber after a single dose of vehicle or JZL184. (C) Time spent exploring either the stranger 1 or the stranger 2 after a single dose of VEH or JZL184. (WT VEH, n=7; WT JZL184, n=8; ΔGtf2i+/- VEH, n=6; ΔGtf2i+/- JZL184, n=6). Data are expressed as mean ± s.e.m. Statistical significance was calculated by repeated measures ANOVA comparison. *** p<0.001 (compartment comparison); ### p<0.001 (genotype comparison); $$$ p<0.001 (treatment comparison). In phase III, only a main effect of compartment was observed. %%% p<0.001 (main effect of compartment).

2.2.6. JZL184 treatment does not alter protein expression of TFII-I in WBS-CD mice

Immunoblot from frontal cortex of WBS-CD mice were performed to elucidate whether JZL184 treatment was directly acting over the expression of the encoding transcription factor of *Gtf2i,* TFII-I. As

expected, TFII-I protein levels were decreased by a half in WBS in comparison to WT mice. However, no changes in protein expression of TFII-I were found after 10 days of JZL184 treatment (8 mg/kg, i.p.) (Figure 53).

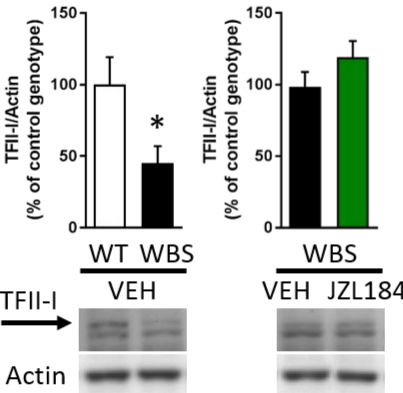

Figure 53. Inhibition of MAGL does not change expression of TFII-I in WBS-CD mice. (A-B) Representative immunoblots and quantification of TFII-I in frontal cortex of mice treated for 10 days with vehicle (VEH) or JZL184 (8 mg/kg) (WT VEH, n=6; WBS-CD VEH, n=6; WBS JZL184, n=6). Actin immunodetection was used as housekeeping control. Distribution of individual data with mean ± s.e.m. * $p<0.05$ (genotype effect) by Student's t-test.

2.2.7. Plasma oxytocin levels are unchanged after JZL184 administration in WBS-CD mice

Alterations in oxytocin biology have been linked with modifications of social behavior (Jacob *et al.*, 2007; Parker *et al.*, 2014). Interestingly, an increase in oxytocin plasma levels was described in WBS patients which correlates with approach to strangers (Dai *et al.*, 2012). We assessed plasma oxytocin levels in WBS-CD mice treated with vehicle or JZL184. We found a trend toward a reduction in oxytocin plasma concentration in WBS-CD mice treated with vehicle in comparison to WT mice (Figure 54). JZL184 administration (8 mg/kg, i.p.) for 10 days did not change oxytocin plasma levels, neither pretreatment with rimonabant (2 mg/kg, i.p.).

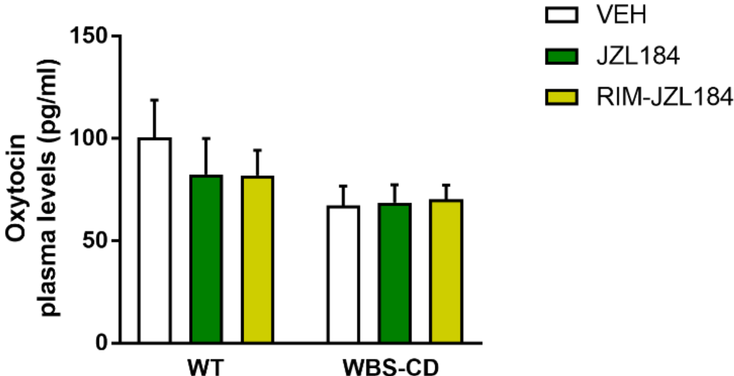

Figure 54. WBS-CD mice show a non-significant decrease in oxytocin plasma levels that is not modified after JZL184 administration. Levels of oxytocin in plasma of WT and WBS-CD mice treated with vehicle (VEH), JZL184 (8 mg/kg) and rimonabant (2 mg/kg) + JZL184 (8 mg/kg) (WT VEH, n=7; WT JZL184, n=4; WT RIM-JZL184, n=5; WBS-CD VEH, n=5; WBS-CD JZL184, n=5; WBS-CD RIM-JZL184, n=7). Data are expressed as mean ± s.e.m. Statistical significance was calculated by two-way ANOVA.

2.2.8. JZL184 also restored short-term memory deficits

WBS-CD mice display an impairment in short-term memory in novel-object recognition test (Ortiz-Romero *et al.*, 2018). Given the role of the ECS in learning and memory processes (Marsicano and Lafenêtre, 2009), we studied the effect of blocking MAGL over this phenotype.

Sub-chronic administration of JZL184 (8 mg/kg, i.p.) for 7 days (last administration 2 hours before starting the training phase of the NORT), restored memory impairment in WBS-CD mice (Figure 55).

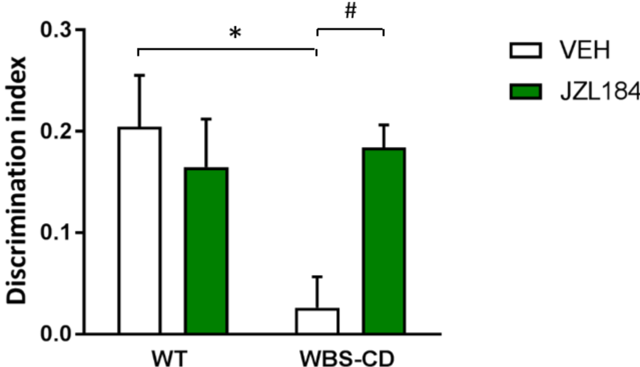

Figure 55. Inhibition of MAGL by JZL184 rescue short-term object recognition memory. Discrimination index of WT and WBS-CD mice treated for 7 days with vehicle (VEH) or JZL184 (8 mg/kg) (WT VEH, n=6; WT JZL184, n=6; WBS-CD VEH, n=6; WBS-CD JZL184, n=6). Data are expressed as mean ± s.e.m. Statistical significance was calculated by calculated by Newman-Keuls post hoc test following two-way ANOVA. * < 0.05 (genotype effect); # p< 0.05 (treatment effect).

2.2.9. JZL184 have an impact on the cardiovascular phenotype of WBS-CD mice

WBS-CD mice present a cardiovascular phenotype similar to WBS patients including a cardiac hypertrophy that correlates with an increase in the size of cardiomyocites, an increase in aortic wall thickness and a slight increase in arterial blood pressure (Segura-Puimedon *et al.*, 2014). The ECS plays a role in cardiovascular function, especially in pathological conditions. Notably, increasing the 2-AG tone has effects over blood pressure, cardiac contractility and vascular resistance (Járai *et al.*, 2000; Pulgar *et al.*, 2014; Szekeres *et al.*, 2015; Karpińska *et al.*, 2017). Therefore, a possible modification in the activity of the ECS may also have an impact over the cardiac hypertrophy in WBS-CD mice. As expected, we observed a cardiac hypertrophy in WBS-CD mice in comparison with WT mice. Administration of JZL184 for 10 days (8 mg/kg, i.p.) normalized this phenotype (Figure 56).

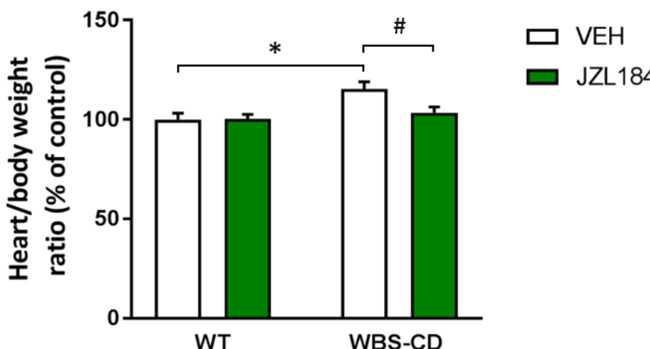

Figure 56. Inhibition of MAGL by JZL184 administration improves cardiac hypertrophy of WBS-CD mice. Heart/body weight ratios obtained from WT and WBS-CD mice treated for 10 days with vehicle (VEH) or JZL184 (8 mg/kg). (WT VEH, n=14; WT JZL 184, n=12; WBS-CD VEH, n=12; WBS-CD JZL 184, n=13). Data are expressed as mean ± s.e.m. Statistical significance was calculated by Newman-Keuls post hoc test following two-way ANOVA. * < 0.05 (genotype effect); $^{\#}$ p< 0.05 (treatment effect).

DISCUSSION

DISCUSSION

During this thesis we used animal models resembling the genetic conditions of DS and WBS to investigate the role of the ECS in those phenotypes associated to intellectual disability. In each disorder, we focused on the most striking phenotypes that are hippocampal-dependent memory deficits for DS and abnormalities in social functioning for WBS.

1.1. Involvement of the endocannabinoid system in hippocampal-dependent memory deficits of Ts65Dn mice

We focused our attention in DS, a disorder in which intellectual disability is the main limitation for patients' daily life. Currently, there are no available gold-standard treatments to alleviate cognitive impairment of DS individuals. Therefore, there is an urgent need to discover new targets that may alleviate such traits. We focused our attention in the ECS since this neuromodulatory system has an important role in memory and regulates several processes that seem to underlie deficits in DS (Alger, 2002; Monory *et al.*, 2015; Prenderville *et al.*, 2015; Augustin and Lovinger, 2018).

We studied the main components of the ECS in the hippocampus of young adult Ts65Dn mouse model, the most used and studied mouse model of DS. We focused our attention in the hippocampus because it is an area involved in memory processes and appears specially impaired in DS patients (Pennington *et al.*, 2003). In this brain area, we observed alterations in CB1R and endocannabinoid levels. We found an increase in CB1R expression, the most abundant GPCR in the CNS (Kano *et al.*, 2009). Our results are in agreement with a recent report where the proteome of subsynaptic compartments has been analyzed in Ts65Dn mouse hippocampus (Gómez de Salazar *et al.*, 2018). CB1R is increased in Ts65Dn

mice at the extrasynaptic fraction, where CB1R is predominantly located (Nyíri *et al.*, 2005; Mikasova *et al.*, 2008; Thibault *et al.*, 2013).

In hippocampus, at the cellular level, CB1R is mainly expressed on cholecystokinin-expressing inhibitory terminals and to a minor extent on glutamatergic terminals (Kano *et al.*, 2009). Moreover, CB1R is also expressed on other types of terminals and cell types including astrocytes and microglia (Pertwee and Ross, 2002; Cabral and Marciano-Cabral, 2005; Navarrete and Araque, 2008). According to our patch-clamp studies, the increase of CB1R in hippocampus of Ts65Dn mice seems to be functionally relevant in excitatory terminals and not in inhibitory terminals. This suggests that overexpression of CB1R may be restricted to excitatory terminals although overexpression of CB1R in other terminals or cell types cannot be disregarded. Importantly, the differential level of expression of CB1R does not correlate with their functional relevance and indeed, glutamatergic CB1R has an important role in the modulation of hippocampal excitability (Marsicano *et al.*, 2003; Monory *et al.*, 2006). This is likely because glutamatergic CB1R is more effectively coupled to G-protein signaling than GABAergic CB1R (Steindel *et al.*, 2013) and because the connectivity of pyramidal cells is higher than the connectivity of interneurons (Buhl and Whittington, 2007).

The increased inhibition of glutamatergic neurotransmission produced by the CB1R agonist WIN55,212-2 in the hippocampus of Ts65Dn mice indicates a decrease on excitatory transmission. This decrease on excitability may contribute to the imbalance between excitatory and inhibitory circuits that has been proposed to occur in Ts65Dn mice (Zorrilla de San Martin *et al.*, 2018). Reductions in glutamate have also been observed in adult hippocampal tissue from DS subjects (Reynolds and Warner, 1988), although such differences have not been observed in

hippocampus of Ts65Dn mice (Santin *et al.*, 2014). In this regard, decreased levels of glutamate release by CB1R activity may not affect total levels of glutamate. Therefore, quantification of extracellular pools of glutamate would be more accurate to reveal whether CB1R altered functioning decreases glutamate release in Ts65Dn mice (Figure 57).

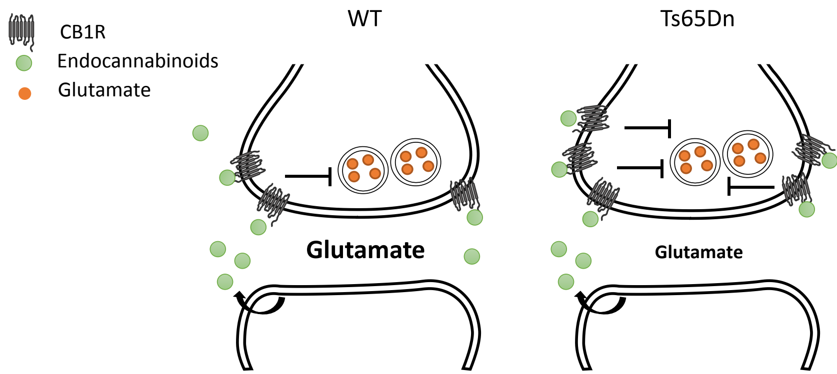

Figure 57. Model of the endocannabinoid signaling at hippocampal excitatory terminals of Ts65Dn mice according to the results obtained in this thesis.

The molecular mechanisms underlying CB1R overexpression remain to be elucidated. The *Cnr1* gene, which encodes for CB1R, is located at the Mmu 4, and therefore, it is not in trisomy in Ts65Dn mice. However, genes or non-coding elements in trisomy in Ts65Dn mice may interfere directly or indirectly in the expression of CB1R altering processes such as transcription, splicing, methylation or turnover of the receptor. Alternatively, CB1R expression may be conditioned by the availability of the endocannabinoids (Laprairie *et al.*, 2012).

Regarding endocannabinoid levels, we found a decrease of AEA and no changes on 2-AG in hippocampus of Ts65Dn mice. Since we did not find changes in the main enzymes responsible for the synthesis and degradation of AEA, such decrease may be secondary to alterations in the activity of these enzymes or alterations in secondary enzymes (ex. PTPN22

or PLC). Changes in neuronal activity may also have an effect over AEA levels (Kim and Alger, 2010), contributing to the observed differences.

Decreased levels of AEA in hippocampus could lead to a decrease in CB1R signaling which could compensate the overexpression of CB1R shown in Ts65Dn mice. However, this is unlikely since 2-AG levels are much higher than AEA ones (Stella *et al.*, 1997). Furthermore, 2-AG mediates major forms of hippocampal synaptic plasticity through the activation of CB1R including DSI, DSE and iLTD (Chevaleyre and Castillo, 2003; Straiker and Mackie, 2005; Hashimotodani *et al.*, 2013). Conversely, AEA mediates hippocampal synaptic plasticity mainly through the activation of the TRPV1 (Chávez *et al.*, 2010).

AEA alterations in Ts65Dn mice would be anatomically restricted since we did not observe alterations on AEA when whole brain homogenates of young-adult mice were analyzed. Our results of endocannabinoid content in whole brain extracts from young-adult Ts65Dn mice do not conform with a previous report that described enhanced levels of 2-AG in whole brain homogenates of 11-month-old Ts65Dn mice (Lysenko *et al.*, 2014). Such differences could be due to the fact that middle-aged Ts65Dn mice also present an AD neuropathology including age-dependent cognitive decline, cholinergic neurodegeneration in the basal forebrain, increased levels of APP, amyloid-β peptide and tau hyperphosphorylation (Hamlett *et al.*, 2016). In this sense, stereotaxic injection of amyloid-β peptide in WT rats drives an increase in 2-AG, but not in AEA (van der Stelt *et al.*, 2006). Therefore, the specific increase in 2-AG levels observed in middle-age Ts65Dn mice could be related to the associated AD neuropathology.

1.2. Cannabinoid type-1 receptor blockade in Ts65Dn mice

Taking into account the considerations explained in the previous sections and given the role of CB1R on memory and learning (Abush and Akirav, 2009; Zanettini *et al.*, 2011), we assessed a genetic approach to specifically target CB1R in hippocampus of Ts65Dn mice. The attenuation of CB1R expression by shRNA rescued hippocampal-dependent memory in Ts65Dn mice suggesting that the overexpression of CB1R is involved in cognitive deficits of Ts65Dn mice. This approach, not only revealed the involvement of CB1R in cognitive deficits of trisomic mice, but also identified a new druggable target to treat cognitive deficits in DS.

We used a pharmacological treatment to further confirm these results and to use an approach that could be translated to DS individuals. We administrated rimonabant, a selective CB1R antagonist/inverse agonist, for 7 days and we observed an improvement on hippocampal-memory deficits of Ts65Dn mice. Along with the improvement of memory-deficits, sub-chronic rimonabant treatment also restored LTP, a process that is closely related to hippocampal-memory deficits on DS (Kleschevnikov *et al.*, 2004, 2012b; García-Cerro *et al.*, 2014). Several reports have linked excessive GABAergic activity with abnormal LTP in Ts65Dn mice (Kleschevnikov *et al.*, 2004; Costa and Grybko, 2005; Martínez-Cué *et al.*, 2013). However, according to our results, CB1R may also play a putative role since CB1R blockade was able to restore impaired LTP. In concordance with this hypothesis, it has been reported that deletion of CB1R in GABAergic or glutamatergic terminals has opposed effects over LTP indicating that a change on expression of CB1R in either population would produce a deregulation over LTP (Monory *et al.*, 2015). LTP stimulation protocol is known to lead to the release of endocannabinoids postsynaptically at activated glutamatergic neurons, and then

endocannabinoids travel retrogradely to reach local presynaptic CB1R on both glutamatergic and GABAergic terminals (Stella *et al.*, 1997). An increase in functionality of CB1R on glutamatergic terminals in Ts65Dn mice may produce a reduction in glutamatergic transmission contributing to a reduction of LTP.

Interestingly, increasing evidences relate adult hippocampal neurogenesis of the dentate gyrus in the establishment of hippocampal-dependent memory and LTP (Saxe *et al.*, 2006; Dupret *et al.*, 2008; Jessberger *et al.*, 2009; Massa *et al.*, 2011). Indeed, several compounds that normalize adult neurogenesis in Ts65Dn mice, such as the antidepressant fluoxetine (Bianchi *et al.*, 2010) or the mood stabilizer lithium (Contestabile *et al.*, 2013) also normalize memory deficits. We assessed adult neurogenesis and we showed a decrease in cell proliferation in young Ts65Dn mice, which is consistent with previous results (Clark *et al.*, 2006; Belichenko and Kleschevnikov, 2011). Notably, rimonabant treatment normalized the number of proliferating cells in Ts65Dn without modifying that in WT mice. Several studies have addressed the role of CB1R over adult neurogenesis. However, there is no a general consensus as effects vary considerably according to the pathological and experimental conditions including the drug, dose and duration of administration (Prenderville *et al.*, 2015). In this regard, the sub-chronic CB1R blockade in Ts65Dn mice may have a direct effect on the number of proliferating cells. However, it is also plausible that CB1R blockade modulates adult neurogenesis indirectly, as a result of altering local network activity in the dentate gyrus of the hippocampus (Lehmann *et al.*, 2005).

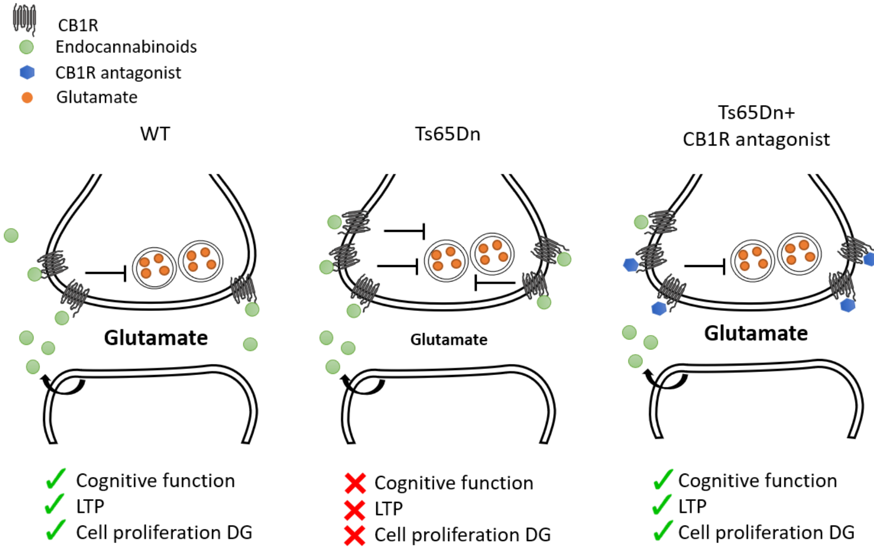

Figure 58. Schematic diagram of the effects of the CB1R blockade at hippocampal excitatory terminals of Ts65Dn mice. DG: dentate gyrus; LTP: long-term potentiation.

1.3. Cannabinoid type-1 receptor blockade in transgenic Dyrk1A mice

DYRK1A gene is in trisomy in DS patients and in the Ts65Dn mouse model. The encoding protein is expressed in hippocampus and its dosage is critical for hippocampal-dependent memory (Altafaj *et al.*, 2001; Fotaki *et al.*, 2002; Ahn *et al.*, 2006; Arqué *et al.*, 2008; de la Torre *et al.*, 2014). Furthermore, mouse models with changes over *Dyrk1A* gene dosage and normalization of the *Dyrk1A* gene dosage on Ts65Dn mice have revealed a key role of this protein in hippocampal synaptic plasticity, proliferation and differentiation in dentate gyrus, synaptogenesis, and neuronal circuit excitatory/inhibitory imbalance (Smith *et al.*, 1997; Ahn *et al.*, 2006; Martinez de Lagran *et al.*, 2012; García-Cerro *et al.*, 2014; Souchet *et al.*, 2014; Thomazeau *et al.*, 2014; Ruiz-Mejias *et al.*, 2016). We used a mouse model overexpressing *Dyrk1A*, the TgDyrk1A (Altafaj *et al.*, 2001), and we observed memory deficits in the NORT, as previously described (de la

Torre *et al.*, 2014), and in the NPRT. These deficits were prevented by the blockade of CB1R. In addition, we found for the first time a decrease in hippocampal LTP in this mouse model. These results are in contradiction with a previous publication revealing an increase in LTP in an analogous model of *Dyrk1A* overexpression (Ahn *et al.*, 2006). However, our results are in agreement with those in the Ts65Dn model and in other trisomic models , the Ts1Cje, Ts1Rhr and TTS (Costa and Grybko, 2005; Siarey *et al.*, 2005; Belichenko *et al.*, 2009a, 2015). In addition, normalizing *Dyrk1A* dosage on Ts65Dn mice increases LTP to control values (García-Cerro *et al.*, 2014), which further supports our findings of the decreased LTP in the TgDyrk1A model.

In agreement with previous reports (Pons-Espinal *et al.*, 2013b), we observed in the dentate gyrus of TgDyrk1A mice an increase on Ki67+ cells and a decrease on BrdU+/Ki67– cells, which may be explained by a reduction on cell cycle exit. This reduction on cell cycle exit seems to be secondary to an arrest on G2 phase during cell cycle (Pons-Espinal *et al.*, 2013b), which has also been described in Ts65Dn mice (Contestabile *et al.*, 2007). Therefore, both Ts65Dn and TgDyrk1A models show a decrease in cell proliferation and cell cycle exit, but probably at different extents, which may explain the different cell counts observed with the proliferative marker Ki67. Notably, CB1R sub-chronic blockade in the TgDyrk1A model normalized LTP and significantly facilitated cell cycle exit of neuronal precursors.

Changes in the density and morphology of dendritic spines is a common feature in several neurodevelopmental disorders (Levenga and Willemsen, 2012). Regarding DS, a decrease in the number of spines in the CA1 hippocampal region was found in DS subjects (Ferrer and Gullotta, 1990) and in Ts65Dn mice (Catuara-Solarz *et al.*, 2016). Conversely, we found an

increase in spine density of apical dendrites from CA1 pyramidal neurons of TgDyrk1A mice. This phenotype was likely secondary to an increase in stubby spines, which are small spines without a well-defined neck. Changes in the number of this type of spine may impact on synaptic plasticity since morphology and size of spines are determinants of synaptic strength (Hayashi and Majewska, 2005; Noguchi *et al.*, 2005). A previous report using the same mouse model, described a decrease in spine density in the motor cortex (Martinez de Lagran *et al.*, 2012) while an increase was found in the prefrontal cortex of a similar mouse model overexpressing *Dyrk1A* (Thomazeau *et al.*, 2014). These discrepancies indicate that Dyrk1A overexpression may result in changes in spine density in a region-dependent manner.

Pharmacological blockade of CB1R was able to normalize alterations in spine density of TgDyrk1A mice, probably due to a reduction in the number of stubby and thin spines. These results are in concordance with a previous study of our group where administration of rimonabant reduced spine density in Fmr1 KO mice to WT control levels (Busquets-Garcia *et al.*, 2013). The modulation of spine density by targeting CB1R may be secondary to changes in synaptic transmission (Lai and Ip, 2013).

Altogether, these findings strongly indicate that CB1R signaling is implicated in the deficits derived from the increase on *Dyrk1A* gene dosage. Hence, an interplay between DYRK1A expression and CB1R may exist. Whether the blockade of CB1R have a direct impact over DYRK1A expression, activity or downstream pathways remains to be elucidated.

1.4. Translational validity and future directions of the blockade of cannabinoid type-1 receptor in Ts65Dn mice

In the first chapter, we revealed CB1R as a relevant target to improve hippocampal-dependent memory, long-term synaptic plasticity and adult

neurogenesis in two relevant models for DS, the segmentally trisomic model Ts65Dn and the transgenic model TgDyrk1A (Figure 59). These preclinical evidences strongly suggest CB1R as a target worth exploring in the improvement intellectual disability in DS subjects.

Although several drugs have been successful in preclinical studies of DS, most of them have produced marginally positive results in clinical trials (Hart *et al.*, 2017). For this reason, we took several considerations to maximize the translational potential of our study:

- We used two mouse models whose predictive validity has been recently demonstrated for novel experimental approaches to treat intellectual disability in DS individuals (de la Torre *et al.*, 2014, 2016).
- Although most of the experiments were carried out in male mice due to limitations of costs, space and time, hippocampal-dependent memory after CB1R blockade was also assessed in female mice, showing comparable results between genders.
- We randomly assigned mice to experimental groups and we blinded the experimenter to experimental conditions.
- We assessed three different methods directed to the same target, CB1R, a genetic approach and two pharmacological approaches (rimonabant and NESS 0327).

Despite these strengths, our study also has some limitations that should be taken into consideration. The Ts65Dn mouse model is trisomic for 90 ortholog genes to those found in HSA21 but also for other 35 coding genes not in trisomy in DS individuals which may contribute to the results obtained in this work. Although the construct validity of this model is not the best one among the different DS mouse models, it recapitulates most

of the phenotypes observed in DS patients at different ages (Aziz *et al.*, 2018). Unfortunately, a perfect DS mouse model does not exist. There are other models with better construct validity, such as the Dp16, which contains three copies of 119 genes orthologues on Mmu16 without containing DS-not related genes, or the TTS, which is trisomic for all three syntenic regions homologous to HSA21 (Yu *et al.*, 2010b, a). Both mouse models show phenotypes milder than those found in the Ts65Dn model and they do not present some of the phenotypes observed in DS patients (Belichenko *et al.*, 2015; Aziz *et al.*, 2018). Probably, this is because the additional genetic material in these models is not contained in a freely segregating chromosome, as in the Ts65Dn mice (Hervé *et al.*, 2016).

The TgDyrk1A mouse model was constructed using an exogenous promoter, the inducible sheep metallothionein-Ia (Altafaj *et al.*, 2001). As consequence, spatial and temporal regulation of the transgene may not match with the endogenous *Dyrk1A* gene. However, it has been demonstrated that the expression in cerebral cortex of DYRK1A in this model is similar to DS fetal tissue (Toiber *et al.*, 2010).

Another limitation is that the CB1R antagonist that we used in most of the experiments, rimonabant, had been previously used for the treatment of obesity in the clinic, but was later discontinued due to dose-dependent adverse psychiatric side-effects (Christensen *et al.*, 2007). Two doses of rimonabant were assessed in clinical trials, 20 mg/day and 5 mg/day, and the highest dose was the one that presented a higher incidence of severe psychiatric side-effects (Scheen *et al.*, 2006; Christensen *et al.*, 2007; Topol *et al.*, 2010). In our study, we used 1 mg/kg equivalent to 4.86 mg/day for a 60 kg person according to the dose conversion previously described (Reagan-Shaw *et al.*, 2008). This lower dose may preclude the appearance of adverse psychiatric symptoms. Moreover, it has been reported that the

inverse agonist profile of rimonabant could be responsible for its adverse psychiatric side effects (Bergman *et al.*, 2008; Meye *et al.*, 2013). We observed that the sub-chronic administration of a neutral antagonist, NESS 0327, also restored hippocampal-memory deficits in Ts65Dn and TgDyrk1A mice. Therefore, other compounds directed to block CB1R with a safer profile than rimonabant including neutral antagonists or negative allosteric modulators (Vallée *et al.*, 2014) may become interesting approaches to treat cognitive deficits in DS individuals.

Another aspect to consider is that we assessed the treatment only for a brief period of time, 7 days. Further work is required to assess the effect of a longer treatment, which may be more realistic to what happens in clinics. It will be also interesting to assess whether therapy outlasts treatment cessation. This would be crucial to clarify whether continuous administration would be required which may be inconvenient for patients' day life.

Our study was directed to young adult mice ranging 2 to 4 months of age. Since during prenatal and early postnatal periods the bulk of neuron proliferation and maturation takes place (Semple *et al.*, 2013; Stagni *et al.*, 2015), further experiments are required to address whether starting the treatment at younger ages, such as the time of weaning, earlier or even during the prenatal period, could result in enhanced benefits. Given that brain alterations in DS are present since embryonic stages, prenatal treatment may have a major impact, probably affecting the development of the whole brain and having permanent effects. Some prenatal treatments have been assessed in DS mouse models including fluoxetine (Guidi *et al.*, 2014), choline (Moon *et al.*, 2010), neurotrophic factors (Toso *et al.*, 2008; Incerti *et al.*, 2012) and epigallocatechin-3-gallate (Guedj *et al.*, 2009). Interestingly, prenatal treatment with fluoxetine has enduring

effects and 1.5 months after cessation of the treatment Ts65Dn mice show a restored cognitive performance, along with normalization of neuronal precursor proliferation, cellularity in dentate gyrus and neocortex, dendritogenesis, cortical and hippocampal synapse development and brain volume (Guidi *et al.*, 2014). The early prenatal diagnosis of DS makes possible the prenatal treatment in humans. However, the potential treatment should be carefully assessed before starting clinical trials. Off-target side effects may appear given that this period is so critical for the development of individuals. For instance, several studies assessing the effects of fluoxetine administration in depressed pregnant women have described an increased risk for fetal growth restriction, low birth weight, increased neonatal abstinence syndrome or cardiac malformations (Millard *et al.*, 2017).

Another time window important to be considered in DS is aging. Middle-aged DS individuals develop an early-onset form of Alzheimer's disease related to the additional copy of the *APP* gene in trisomy. In fact, enhancement of 2-AG, through the MAGL inhibitor JZL184, improved cognitive performance and synaptic plasticity of 11-months old Ts65Dn mice (Lysenko *et al.*, 2014). Although the approach in our study with young adult mice and that followed on middle-aged Ts65Dn mice seems contradictory (CB1R blocker vs. 2-AG signaling enhancer, respectively), we cannot discard that JZL184 could be acting through a CB1R-independent mechanism. Indeed, administration of JZL184 in a mouse model of Alzheimer's disease demonstrated anti-inflammatory properties which were independent of CB1R or CB2R function (Chen *et al.*, 2012).

In Alzheimer's disease rodent models, different strategies to improve cognitive decline targeting the ECS have been assessed. These strategies include JZL184 (Chen *et al.*, 2012), but also others compounds, such as

cannabidiol and the CB1R agonists ACEA, WIN55,212-2, JWH-133 and THC (Ramirez *et al.*, 2005; Aso *et al.*, 2012, 2014, 2016; Martín-Moreno *et al.*, 2012). All these strategies are in the line of enhancing the signaling of the ECS. Future experiments should assess the effect of CB1R blockade over cognition in middle-aged Ts65Dn mice or whether long-term treatment have an impact over Alzheimer's disease phenotypes. As a first approach, it would be interesting to assess whether the overexpression of CB1R is also present in aged Ts65Dn mice, which may help to predict the effect of our treatment in these conditions.

It will be important that future research investigate the efficacy of our treatment in comparison with other compounds in preclinical models. Of special interest would be to compare rimonabant or other CB1R-dissected compounds with the green tea extract supplement containing 45% epigallocatechin-3-gallate, which has demonstrated promising results on clinical trials (de la Torre *et al.*, 2016). In addition, the combination of CB1R blockade with environmental enrichment or other compatible pharmacological approaches should also be explored.

Analysis of the expression of ECS components in post-mortem brains of DS individuals could also help to predict the effects of the CB1R blockade in humans. In fact, a previous report analyzed the expression of CB1R, CB2R and FAAH in cortices of DS individuals. Young DS individuals did not show changes as compared with control ones in the expression of none of these components (Núñez *et al.*, 2008). Examination of the expression of CB1R in hippocampus region of young DS subjects would be relevant.

1.5. Involvement of endocannabinoid system in cognitive deficits of neurodevelopmental disorders

The involvement of the ECS in the pathogenesis of cognitive deficits had been previously studied in FXS. In this thesis, we have revealed the

involvement of the ECS in the pathogenesis of another intellectual disability disorder, DS.

DS and FXS are both genetic developmental disorders. Although the genetic cause is different, DS and FXS patients show common phenotypic features including defects in hippocampal-dependent memory, neurogenesis and synaptic plasticity (Faundez *et al.*, 2018). Both disorders also present several differences and some of the alterations go in opposite directions including brain size, synaptic plasticity and excitatory/inhibitory synaptic balance. Children with DS have smaller brain volumes (Pinter *et al.*, 2001) than typically developing children while children with FXS have larger ones (Hazlett *et al.*, 2012). In addition, DS subjects present a reduced synaptic density (Takashima *et al.*, 1981) whereas FXS subjects present an increase of this parameter (Irwin *et al.*, 2000). multiple evidences point to an over-inhibition in the balance between excitatory and inhibitory activity of neuronal circuits in DS (Zorrilla de San Martin *et al.*, 2018), whereas an hyperexcitability seems to occur in FXS (Contractor *et al.*, 2015). These similar and opposite phenotypes may be linked to partial to shared mechanisms across both disorders (Faundez *et al.*, 2018). Therefore, targets in these common mechanisms could be equally valid for both disorders. Previous results of our laboratory (Busquets-Garcia *et al.*, 2013) together with the results presented in this thesis point to the ECS as one of these common mechanisms. The same strategy, the blockade of CB1R, rescues memory deficits in mouse models of DS and FXS. In DS, we have found an increase in the expression of CB1R, whereas, no changes of this receptor were found in Fmr1 KO mice (Busquets-Garcia *et al.*, 2013). The rationale of using a CB1R blockade in the Fmr1 KO mouse model may be explained by the alterations in synaptic plasticity found in these mice. Indeed, eCB-STD and eCB-LTD are enhanced on GABAergic synapses of

hippocampus of Fmr1 KO mice after mGluR1 activation (Zhang and Alger, 2010). The possible role of the ECS and its therapeutic potential in other neurodevelopmental disorders with intellectual disability should also be taken into consideration.

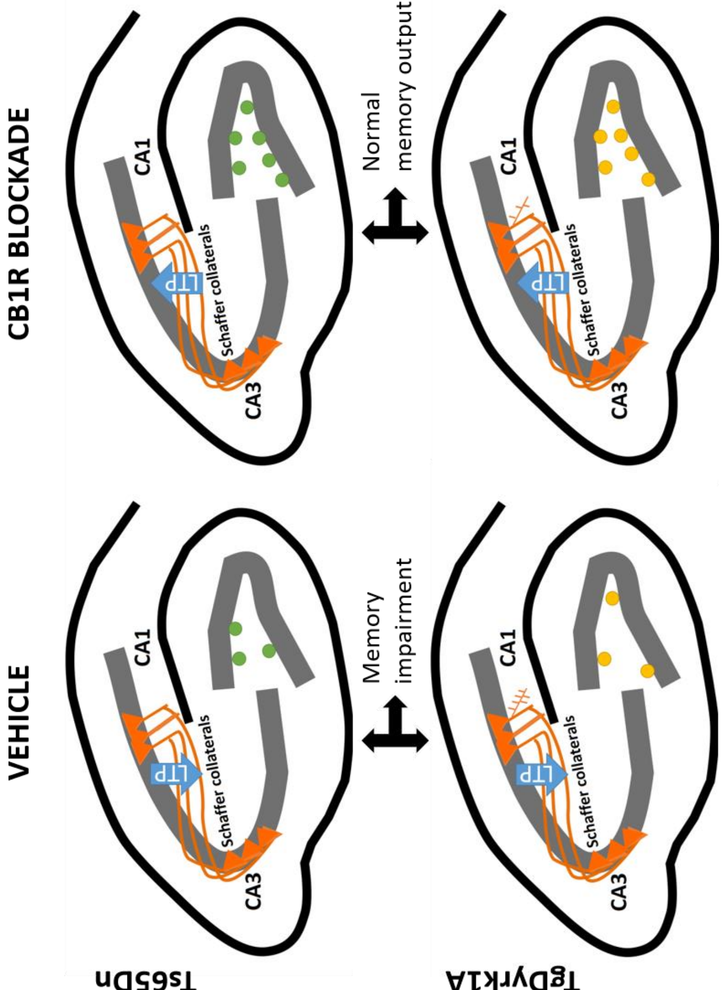

Figure 59. Summary of the results obtained in the first chapter of this thesis. Blockade of CB1R increased the number of proliferating cells (green dots) and long-term potentiation (LTP) in Ts65Dn mice to control levels. In TgDyrk1A mice, blockade of CB1R increased the number of cells exiting the cell cycle (yellow dots) and LTP to WT levels. In addition, CB1R blockade also decreased spine density of the apical dendrites of CA1 pyramidal neurons (orange).

2.1. Social behavior of WBS-CD mice assessed by V-maze setting

We validated a new setting to measure social behavior, the V-maze. For this purpose, we used an inbreed and an outbreed mouse strain and we compared the novel paradigm to the three-chamber maze, the standard approach to study sociability and preference for social novelty (Moy *et al.*, 2004). All tests were performed under the same experimental conditions in terms of lighting, room environment, experimental mouse strain and stranger strain, as well as overall experimental procedure.

As previously reported (Hsieh *et al.*, 2017), the exploration times spent in social interactions were similar for C57BL/6J and CD1 mice. As stranger mice, we used juvenile C57BL/6J mice for both strains. Therefore, a different strain in stranger mice does not seem to have an effect over social approach behaviors. This is consistent with the results found in a previous study (Nadler *et al.*, 2004).

The test in both experimental conditions consists in three phases. During phase I, similar results were obtained in both mazes. During phase II, the duration of 5 minutes was enough to detect in both mazes a robust phenotype of sociability in both strains revealed by the preference for exploring a stranger mouse rather than an empty compartment. Unexpectedly, exploration times towards the stranger mouse were higher in the three-chamber maze in comparison to the V-maze. Therefore, although spatial and contextual information was reduced in the V-maze, mice spent less time exploring the stranger mice. The surface area of social interaction was higher in the three-chamber test (round wire cage) in comparison to the V-maze (one-side plastic bars), which may explain this difference. During phase III, we obtained robust results of preference for social novelty in both strains using 5 minutes per phase with the V-maze setting, but not with the three-chamber maze. Therefore, we set up a new

experimental system that allows evaluating sociability and preference for social novelty in mice in a time-efficient and reproducible manner. Moreover, this system is more compact than the three-chamber maze test, cleaning between sessions and transport and storage. A pharmacological validation of the V-maze using for instance an oxytocin receptor antagonist, which decreases social interactions in WT mice (Lukas *et al.*, 2011), would reinforce the results obtained.

Using this new setting, we assessed social behavior in WBS-CD mice on a C57BL/6J background. In line with previous findings (Segura-Puimedon *et al.*, 2014), we observed a significant increase in the sociability of these mice compared to WT mice. This phenotype resembles the human condition in which WBS subjects show higher social motivation (Riby and Hancock, 2009; Riby *et al.*, 2013). In addition, we described for the first time that WBS-CD mice did not show preference for social novelty. This lack of preference is dependent on social stimuli and may be due to a lack of habituation to the previously encountered animal (stranger 1). In fact, WT but not WBS-CD mice exhibited an habituation effect after exploring for 5 minutes a stranger mouse (Segura-Puimedon *et al.*, 2014). This trait is reminiscent of the lack of habituation to faces observed through electrodermal measures in WBS individuals, which may cause that social stimuli appear continuously novel and interesting (Järvinen *et al.*, 2012).

These results not only improved the characterization of the social behavior in WBS-CD mice but also but also demonstrated that the V-maze setting is useful to detect social traits.

2.2. Involvement of the endocannabinoid system in the hypersociable phenotype of WBS-CD mice

We analyzed the expression of the main components of the ECS (CB1R, MAGL, NAPE-PLD, DAGL-α, FAAH, endocannabinoids) in the frontal cortex,

amygdala and hippocampus of WBS-CD mice and we did not find major alterations compared to WT animals. However, alterations in the activity of cannabinoid receptors or the activity of these enzymes or in secondary enzymes cannot be discarded.

Sub-chronic administration of the MAGL inhibitor JZL184 normalized the hypersociability and the lack of preference for social novelty of WBS-CD mice. Blockade of CB1R prevented this effect indicating that was dependent on CB1R activation and 2-AG accumulation. The blockade of MAGL did not have any effect over WT animals revealing that the effects of increasing 2-AG over social interactions were selective for the social phenotype of WBS-CD mice. These findings are in contradiction with a previous report which describes that the same dose of JZL184 increases social interactions in adolescent mice (Manduca *et al.*, 2016). Such a difference may be attributed to the age given that the ECS signaling undergoes age-related variations (Kang-Park *et al.*, 2007; Zhu and Lovinger, 2010). In addition, a low but not a high dose of JZL195 (dual inhibitor FAAH and MAGL), increases social interactions in adult WT mice, likely by increasing 2-AG and not AEA levels (Manduca *et al.*, 2015). Hence, although our results do not suggest a role of 2-AG in normal social interactions of adult WT mice we cannot rule out that other doses of JZL184 have a different effect.

Based on our results, we can speculate different possible scenarios that lead to normalize the social phenotype of WBS-CD mice by increasing 2-AG levels. A first simplified interpretation of the result may be the possible presence of decreased 2-AG activity in the brain of WBS-CD mice. However, we did not find changes in 2-AG levels in frontal cortex of WBS-CD mice, and a trend to increase 2-AG levels was found in whole brain homogenates. This increase may be a protective mechanism trying to

reestablish brain functions, and therefore, a further increment of 2-AG would facilitate this restoration. We cannot discard changes of 2-AG levels in other brain areas. Alternatively, the increase of 2-AG by blocking the MAGL may counteract another defect of the ECS.

Convergent lines of evidence from mouse models (Sakurai *et al.*, 2011; Borralleras *et al.*, 2015), WBS patients (Dai *et al.*, 2009; Antonell *et al.*, 2010a) and healthy population (Crespi and Hurd, 2014) have linked *GTF2I* gene with social behavior. In line with previous experiments (Borralleras *et al.*, 2015), we found that the Δ*Gtf2i*$^{+/-}$ mice have increased sociability in comparison with WT animals. Unlike WBS-CD mice, the Δ*Gtf2i*$^{+/-}$ mouse model shows a clear preference for social novelty. Such discrepancy among models suggests that this phenotype may be secondary to the hemizygous deletion of another gene in WBS-CD mice. One candidate may be the G*TF2IRD1* gene that is also involved in the social phenotype of WBS (Young *et al.*, 2008; vonHoldt *et al.*, 2017).

The acute treatment with the MAGL inhibitor restores the hypersociable phenotype of Δ*Gtf2i*$^{+/-}$ mice. These results suggest a scenario in which administration of JZL184 may normalize social behavior of WBS mouse models by impacting somehow in the expression/activity of the protein encoded by the *Gtf2i* gene, the TFII-I, or its downstream pathways. To explore this hypothesis, we analyzed TFII-I expression in WBS-CD mice after the administration of JZL184 and we did not find any change in frontal cortex.

How the hemizygous deletion of the *GTF2I* gene causes the social phenotype of WBS remains unanswered. A recent publication has demonstrated in healthy population a link between oxytocin reactivity and a polymorphism in the gene *GTF2I* (Procyshyn *et al.*, 2017). In this study, they propose that the reduced expression or activity of the TFII-I in WBS

may regulate sociability via oxytocin. Therefore, another possibility is that JZL184 administration mediates its effects modulating oxytocin, which may be a downstream effector of the TFII-I. Oxytocin is one of the most studied molecular mechanisms that modulate social behavior. This neuropeptide has a faciliatory role in different aspects of social behavior including social motivation, social memory and social recognition through central mechanisms (Popik and van Ree, 1991; Engelmann *et al.*, 1998; Ferguson *et al.*, 2000; Lukas *et al.*, 2011). In WBS individuals, a basal increase in oxytocin plasma levels has been described which positively correlates with social approach (Dai *et al.*, 2012). By contrary, a decrease is shown in autism-spectrum disorder patients (Modahl *et al.*, 1998). We analyzed oxytocin plasma levels of WBS-CD mice and we found a slight decrease (not significant) in comparison to WT mice in contrast to what happens in humans. In addition, we did not find differences in oxytocin plasma levels after JZL184 administration, neither with the pretreatment of rimonabant. Since correlation between peripheral and central oxytocin concentrations does not occur in all contexts (Landgraf and Neumann, 2004; Lefevre *et al.*, 2017), we cannot exclude that an increase in central oxytocin may contribute to the hypersociable phenotype of WBS-CD mice. Neither, we cannot discard that the increase of 2-AG by MAGL blockade in WBS-CD mice may have some effect over central oxytocin levels.

The balance between excitatory and inhibitory synaptic inputs in the prefrontal cortex seems to be essential for normal social behavior (Yizhar *et al.*, 2011). In autism-spectrum disorders, several evidences from human and rodent studies point to an increase in the ratio of excitation/inhibition (Rubenstein and Merzenich, 2003). Normalization of this balance in the prefrontal cortex of *CNTNAP2* KO mice, which exhibit autism-like phenotypes, rescues abnormal social behavior (Selimbeyoglu *et al.*, 2017).

Regarding WBS, increases in the number of glutamatergic synapses and spike frequency have been found in layer V/VI cortical neurons derived from induced pluripotent stem cells from patients (Chailangkarn *et al.*, 2016). These results suggest an imbalance between excitatory and inhibitory inputs in cortex of WBS patients. Future work should address whether an imbalance in excitatory/inhibitory inputs is present in the prefrontal cortex of WBS-CD mice and whether it is contributing to their social phenotype. In that scenario, an increase on 2-AG may normalize the balance between excitatory and inhibitory inputs regulating neurotransmitter release.

Altogether, we demonstrated that the pharmacological blockade of MAGL normalizes social abnormalities in WBS mouse models. However, the results do not allow determining whether alterations in the ECS contribute to the social phenotypes observed in WBS-CD mice.

2.3. Translational validity of the study and future directions of the inhibition of monoacylglycerol lipase in WBS-CD mice

The results of this chapter point that the inhibition of MAGL may be a suitable approach to normalize social abnormalities of WBS. Atypical social functioning of WBS subjects predisposes to social vulnerability (Jawaid *et al.*, 2012). In fact, WBS subjects have difficulties in peer interactions, maintaining friendships and around 73% have experienced social isolation (Davies *et al.*, 1998). In addition, they have an increased risk to suffer psychiatric conditions that are not associated to the IQ range neither language disability (Stinton *et al.*, 2010) and seem to be related to the social phenotype (Riby *et al.*, 2014; Ng-Cordell *et al.*, 2018). Therefore, improvements in social functioning may have a beneficial effect over the quality of life of WBS subjects.

The treatment with the MAGL inhibitor JZL184 also restores short-term hippocampal-memory deficits of WBS-CD mice. In line with previous findings, the same treatment does not have any effect over WT mice (Busquets-Garcia *et al.*, 2011) and therefore, it is specific for the disorder. These results indicate that the inhibition of MAGL may also be beneficial to restore cognitive deficits of WBS. However, it would be interesting to assess other phenotypes particularly impaired in WBS individuals such as working or spatial memory (Vicari *et al.*, 2005; O'Hearn *et al.*, 2009).

Notably, the treatment with JZL184 may have additional benefits in WBS since it has a positive effect over the cardiac hypertrophy of WBS-CD mice. This result may be explained because the effects of cannabinoids over the cardiovascular system are dominated by a decrease in arterial blood pressure, cardiac contractility, and heart rate (Pacher and Kunos, 2013). Beneficial effects over the cardiovascular phenotype of WBS are very relevant given that it is the most life-threatening complication of the disorder. Further experiments assessing other parameters, such as the size of the cardiomyocytes, the cardiac function or the blood pressure should be performed to confirm these results.

Taken together, MAGL inhibition may be a good strategy to treat not only social function but also memory and cardiovascular deficits in WBS. These results are of great importance given that few preclinical studies have addressed potential treatments for WBS.

Unlike CB1R antagonists, clinical research in MAGL inhibitors is still at initial stages. To date, the JZL184 compound has not been assessed in clinical trials. However, another MAGL inhibitor, ABX-1431, has demonstrated its safety in a placebo-controlled phase Ia study (Gil-Ordóñez *et al.*, 2018). More research on the safety and tolerability of this type of compounds is required.

CONCLUSIONS

CONCLUSIONS

The findings revealed in the present thesis allow to draw the following conclusions:

1) CB1R is overexpressed in hippocampus and its functionality is increased at CA1 pyramidal neurons of Ts65Dn mice.
2) Knockdown of CB1R in hippocampus restores hippocampal-dependent memory deficits of Ts65Dn mice.
3) Repeated administration of the CB1R antagonist/inverse agonist rimonabant (1 mg/kg, 7 days) or the CB1R neutral antagonist NESS 0327 (0.1 mg/kg, 7 days) normalizes hippocampal-dependent memory deficits of Ts65Dn mice in the novel object recognition and novel place recognition tests.
4) The same treatment schedule of rimonabant restores long-term potentiation in CA3-CA1 synapses and the number of proliferating cells in dentate gyrus of the Ts65Dn mouse model.
5) Administration of rimonabant or NESS 0327 normalizes hippocampal-dependent memory deficits of TgDyrk1A mice.
6) Rimonabant treatment also normalizes long-term potentiation in CA3-CA1 synapses, the number of cells exiting the cell cycle in the dentate gyrus, and dendritic spine density of CA1 pyramidal neurons in TgDyrk1A mice.
7) The V-maze test is a novel paradigm to study sociability and preference for social novelty in the CD1 outbreed strain and the C57BL/6J inbreed strain in a reproducible and time-efficient manner.
8) WBS-CD mice show an hypersociable phenotype and no preference for social novelty in the V-maze approach.

9) Social abnormalities of WBS-CD mice are restored after the sub-chronic administration of the MAGL inhibitor JZL184 (8 mg/kg, 10 days) in a CB1R-dependent manner.
10) Acute administration of JZL184 restores the hypersociable phenotype of *Gtf2i* heterozygous knock-out mice.
11) Object recognition short-term memory and cardiac hypertrophy of WBS-CD mice are also normalized after a sub-chronic administration of JZL184.

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