Novel pharmacological and electroceutical approaches for memory improvement in animal models of intellectual disability

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A totes les persones que m'he trobat en el camí,

Abstract

Intellectual disability is a term used to describe a group of neurodevelopmental disorders defined by deficits in cognitive and adaptive functioning with onset during the developmental period. Preclinical research suggest that pharmaceutical approached might benefit people with intellectual disabilities since mouse models bearing similar genetic modifications to those in human syndromes improve cognitive performance upon treatment. This thesis focused on the therapeutic potential of two different approaches, pharmacological and electroceutical, in two mouse models of intellectual disability. First, we studied therapeutic potential of long-term treatments targeting the endocannabinoid system for memory improvement in a well-characterized mouse model of Down syndrome. Secondly, we explored a new electroceutical approach for memory enhancement in a mouse model of fragile X syndrome based on vagus nerve stimulation. Together, using behavioral, pharmacological, electrostimulation, histological and biochemical approaches, we have described two new therapeutical interventions for memory enhancement in mouse models of intellectual disability.

Resumen

La discapacidad intelectual es un término que se utiliza para describir un grupo de trastornos del neurodesarrollo definidos por déficits en el funcionamiento cognitivo que empiezan a en el periodo de desarrollo. Esta tesis se centró en el potencial terapéutico de dos aproximaciones diferentes, farmacológica y electrocéutica, en dos modelos de ratón de discapacidad intelectual. En primer lugar, hemos estudiado el potencial terapéutico de los tratamientos a largo plazo dirigidos al sistema endocannabinoide para mejorar la memoria en un modelo de ratón de síndrome de Down. En segundo lugar, hemos explorado un nuevo enfoque electrocéutico para la mejora de la memoria en un modelo de ratón del síndrome X frágil basado en la estimulación del nervio vago. Conjuntamente, utilizando técnicas conductuales, farmacológicos, de electroestimulación, histológicos y bioquímicos, hemos descrito dos nuevas aplicaciones terapéuticas para la mejora de la memoria en modelos de ratón de discapacidad intelectual.

Resum

La discapacitat intel·lectual és un terme que s'utilitza per a descriure un grup de trastorns del neurodesenvolupament definits per dèficits en el funcionament cognitiu que comencen al llarg del període de desenvolupament. Aquesta tesi es va centrar en el potencial terapèutic de dues aproximacions diferents, farmacològica i electrocèutica, en dos models de ratolí de discapacitat intel·lectual. En primer lloc, hem estudiat el potencial terapèutic dels tractaments a llarg termini dirigits al sistema endocannabinoide per a millorar la memòria en un model de ratolí de síndrome de Down. En segon lloc, hem explorat un nou enfocament electrocèutic per a la millora de la memòria en un model de ratolí de la síndrome X fràgil basat en l'estimulació del nervi vague. Conjuntament, utilitzant tècniques conductuals, farmacològics, d'electroestimulació, histològics i bioquímics, hem descrit dues noves aplicacions terapèutiques per a la millora de la memòria en models de ratolí de discapacitat intel·lectual.

Abbreviations

- 2-AG: 2-arachidonoylglycerol
- Aβ: β-amyloid
- AA: arachidonic acid
- ABVN: auricular branch of the vagus nerve
- AC: adenylate cyclase
- AD: Alzheimer's disease
- ADHD:attention-deficit/hyperactivity disorder
- AEA: N-arachidonoylethanolamine
- APP: amyloid precursor protein
- ASD: autism spectrum disorder
- atVNS: auricular transcutaneous vagus nerve stimulation
- BDNF: Brain-derived neurotrophic factor
- BFCN: Basal forebrain cholineric neurons
- CA: Cornu ammonis
- Cal: calbindin-D28K
- CB1R: Cannabinoid type-1 receptor
- CB2R: Cannabinoid type-2 receptor
- ChAT: choline acetyltransferase
- CNS: central nervous system
- COX2: cyclooxygenase-2
- DAGL: Diacylglycerol lipase
- DG: dentate gyrus
- DI: Discrimination index
- Dp10: Dp(10)1Yey/+

- Dp16: Dp(16)1Yey/+
- Dp17: Dp(17)1Yey/+
- DS: Down syndrome
- DSCR: Down syndrome critical region
- DYRK1A: dual specificity tyrosine-phosphorylation-regulated kinase 1A
- DYRK1A: dual specificity tyrosine-phosphorylation-regulated kinase 1A
- EC: entorhinal cortex
- ECS: Endocannabinoid system
- ERK1/2: Extracellular signal-regulated kinase 1 and 2
- FAAH: Fatty acid amide hydrolase
- Fmr1: fragile X mental retardation 1
- FMRP: fragile X mental retardation protein
- FXS: fragile X syndrome
- GABA: Γ-aminobutyric acid
- GPCR: G-protein coupled receptors
- GSK-3: glycogen synthase kinase 3
- HC: hippocampus
- HSA21: human chromosome 21
- HSA21: human chromosome 21
- ID: intellectual disability
- IL: interleukin
- IP3: Inositol triphosphate
- IQ: intelligence quotient
- JNK: Jun N-terminal kinase

- KO: Knock-out
- LC: *locus coeruleus*
- LC-NE: locus coeruleus norepinephrinergic
- LTP: long-term potentiation
- MAGL: Monoacylglycerol lipase
- MAPK: mitogen-activated protein kinase
- mGluR: metabotropic glutamate receptors
- MMU: *Mus musculus*
- mTOR: mammalian target of rapamycin
- MWM: Morris water maze
- NAPE: N-arachidonoyl phosphatidyl ethanol
- NE: norepinephrine
- NGF: nerve growth factor
- NMDA: N-methyl-D-aspartate
- NMDA: N-methyl-D-aspartate
- NOR: novel-object recognition
- NPRT: novel-place recognition memory test
- NTS: nucleus of the solitary tract
- p75NTR: p75 neurotrophin receptor
- PER: perirhinal cortex
- PFC: prefrontal cortex
- PHC: parahippocampal region
- PKA: protein kinase A
- PKC: protein kinase C
- PLC: phospholipase C
- POR: postrhinal cortex

- PPAR: Peroxisome proliferator-activated receptors
- RCAN1: regulator of calcineurin 1
- Rimonabant: SR14176A
- STM: short-term memory
- Tc1: Tc(Hsa21)1TybEmfc
- TgDyrk1A: transgenic DYRK1A
- TRPV1: transient receptor potential vanilloid 1
- Ts65Dn: Ts(17^{16})65Dn   superindex
- TTS: triple trisomic model
- tVNS: transcutaneous vagus nerve stimulation
- VN: vagus nerve
- VNS: vagus nerve stimulation
- WM: Working memory
- WT: wild type
- Δ^9-THC: Δ^9-tetrahydrocannabinol

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Introduction

1. Cognitive function and dysfunction

1.1. Memory domains

Memory is among the most significant element of cognition impaired in ID (Vicari *et al.*, 2016). It consists in the ability to store knowledge from the past and present in the brain. Because previous experiences are utilized to predict future behavioral reactions, it is a critical process for adaptive behavior. Memory function is divided into numerous domains, each with its own set of properties that are dependent on the interplay of various brain areas (Squire, 2004). Memory may be divided based on two characteristics: its temporal course and its content (Figure 1). There are four different sorts based on their temporal dimension (Harvey, 2019):

1) Sensory memory is the shortest-lived memory and consists in the ability to remember sensory experiences after the stimulus has stopped. It lasts only a few hundred milliseconds but allows the brain to create a continuum out of stimuli (Sperling, 1960).
2) Working memory temporarily stores limited quantities of information (from milliseconds to minutes) that is recalled continuously to allow performing specific actions that require previously acquired information (Funahashi, 2017). Higher cognitive processes such as language comprehension, learning, thinking, and problem-solving require working memory (Baddeley, 2012). The major brain region responsible for this sort of memory is the prefrontal cortex

(Funahashi, 2017), although the hippocampus has been also related to WM (Crouch *et al.*, 2018).

3) Short-term memory refers to the ability to remember knowledge from the recent past for a short period of time. In humans, it can last anywhere from minutes to days, whereas in rodents, it can last anywhere from minutes to a few hours (3-4 hours). It is vulnerable to disturbances, with the hippocampus being the most relevant region (Kumaran, 2008).
4) In humans, long-term memory lasts from days to years (even a lifetime), but in mice, it lasts from hours to days. Synaptic and morphological modifications are required for this form of memory, which includes protein production and the involvement of several brain areas (Costa-Mattioli *et al.*, 2009; Xu *et al.*, 2009).

Long-term memory is divided into explicit and implicit memory categories based on the content dimension (also known as declarative and non-declarative memory respectively). The conscious remembering of facts and experiences is known as explicit or declarative memory. It is the one that is affected in amnesic individuals and is dependent on structures in the medial temporal lobe (Cohen and Squire, 1980; Squire, 2004). Semantic memory, which includes basic information about the environment, and episodic memory, which is concerned with personal events, are two types of explicit memory (Tulving and Markowitsch, 1998).

The processing of spatial, contextual, configural, and relational information in nonhuman animals is referred to as explicit memory (Richter-Levin, 2004). Animals appear to recall specific events from their history based on what occurred, where it occurred, and when it occurred (Crystal, 2010).

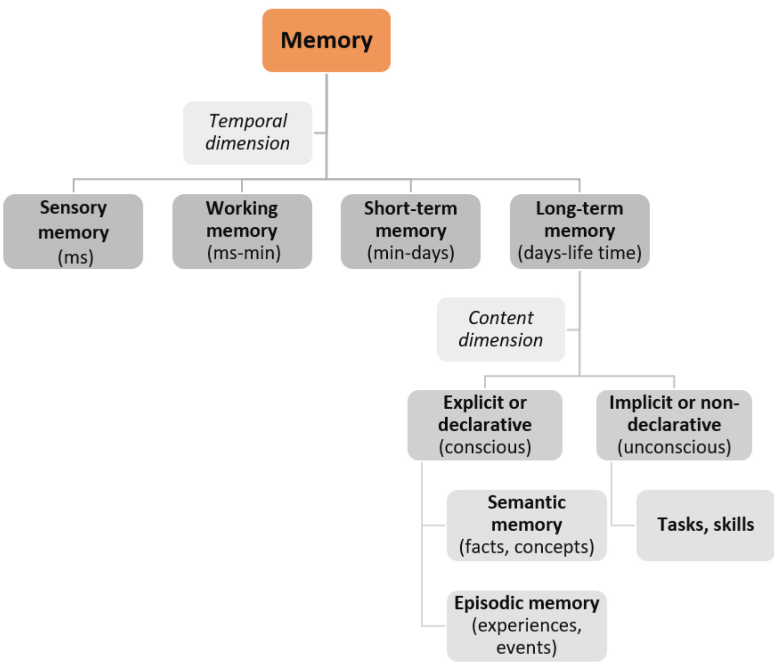

Figure 1. **Memory classification**. The multiple memory types are represented schematically based on the temporal and content dimensions.

The non-conscious recollection of learning capacity that enhances behavioral performance owing to past exposure is known as implicit or non-declarative memory (skills and habits, simple conditioning and priming). It takes longer to learn than declarative memory and

is primarily dependent on the striatum, cerebellum, and cortical association regions (Cohen and Squire, 1980; Squire, 2004).
There are distinct patterns of cognitive profiles among the various etiological groups of intellectual impairment, in which some memory areas are disproportionately damaged while others are largely unaffected (Vicari *et al.*, 2016).

1.2. Neuroanatomical substrates of memory

The medial temporal lobe was first identified as the neuroanatomical basis of memory, with surgical ablation of this area in individuals with severe epilepsy causing amnesia (Scoville and Milner, 1957; Squire, 2004). The hippocampal formation and the parahippocampal area make up the medial temporal lobe, which appears to be important solely for explicit memory (Gaffan, 1974). The hippocampus formation is at the core of the memory-supporting network (Scoville and Milner, 1957; Cipolotti *et al.*, 2001). In patients with intellectual disabilities, changes in this area have been well documented (Sylvester, 1983; Meyer-Lindenberg *et al.*, 2005; Bostrom *et al.*, 2016).
In humans and animal models, the structure and connectivity of the hippocampal formation have been explored in depth (monkeys, rats and mice). The hippocampal formation, unlike other brain regions such as the cerebral cortex, is substantially conserved throughout different species (Figure 2) (Allen and Fortin, 2013).

Because we used mouse models in this work, we will concentrate on rodent hippocampus formation. The hippocampal formation in rodents is an extended structure with a long axis that runs from the septal nuclei rostrally to the temporal cortex ventrocaudally, forming a C shape. The dentate gyrus, the hippocampus (cornu ammonis (CA)1, CA2, and CA3), and the subiculum are the three areas that make up the dentate gyrus (Schröder *et al.*, 2020). Afferent and efferent fibers and interneurons are found in the deeper layer (dentate gyrus: hilus; CA: *stratum oriens*), main neurons and interneurons are found in the adjacent layer (dentate gyrus: granule layer; CA: pyramidal layer), and the molecular layer is found in the superficial layer. The stratum lucidum (only in CA3), stratum radiatum, and stratum lacunosum-moleculare are sublayers of the molecular layer in the CA region. The apical dendrites of pyramidal cells are found in the stratum radiatum, whereas the apical thufts are found in the stratum lacunosum-moleculare (Schröder *et al.*, 2020). The presubiculum, parasubiculum, entorhinal cortex, perirhinal cortex, and postrhinal cortex make up the parahippocampal area, which is connected to the subiculum. The parahippocampal region, unlike the hippocampal formation, contains six layers (Schröder *et al.*, 2020).

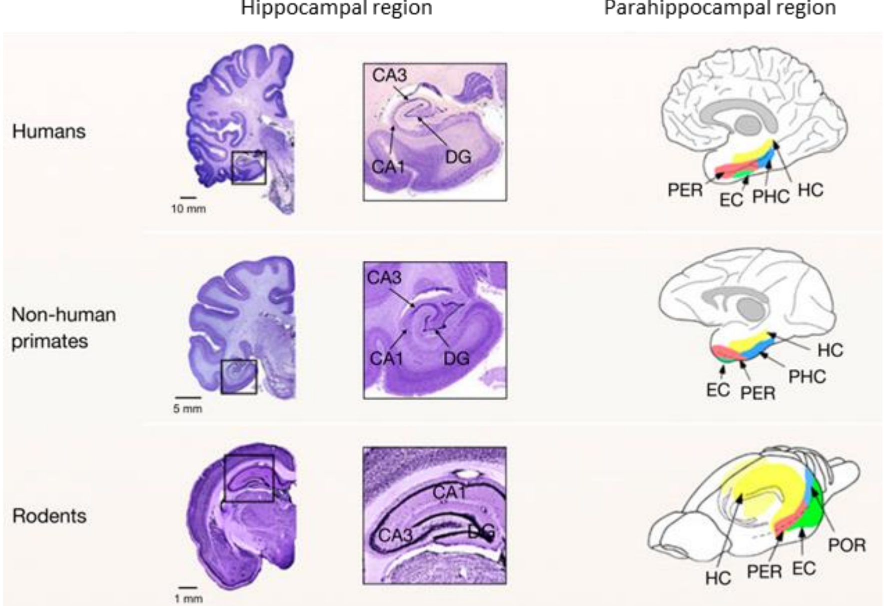

Figure 2. **Humans, nonhuman primates, and rodents' hippocampus and parahippocampal regions are compared anatomically. There are different subregions in each area that are well conserved among the species.** CA1, CA2 and CA3: *Cornu Ammonis*; DG: dentate gyrus; EC: entorhinal cortex; HC: hippocampus; PER: perirhinal cortex; PHC: parahippocampal region; POR: postrhinal cortex (Modified from (Allen and Fortin, 2013).

A distinctiveness of the hippocampal formation is that the fields are linked by unidirectional excitatory projections in a trisynaptic loop: entorhinal cortex → dentate gyrus → CA3 → CA1 → and back to entorhinal cortex (Figure 3A) (Schröder *et al.*, 2020).

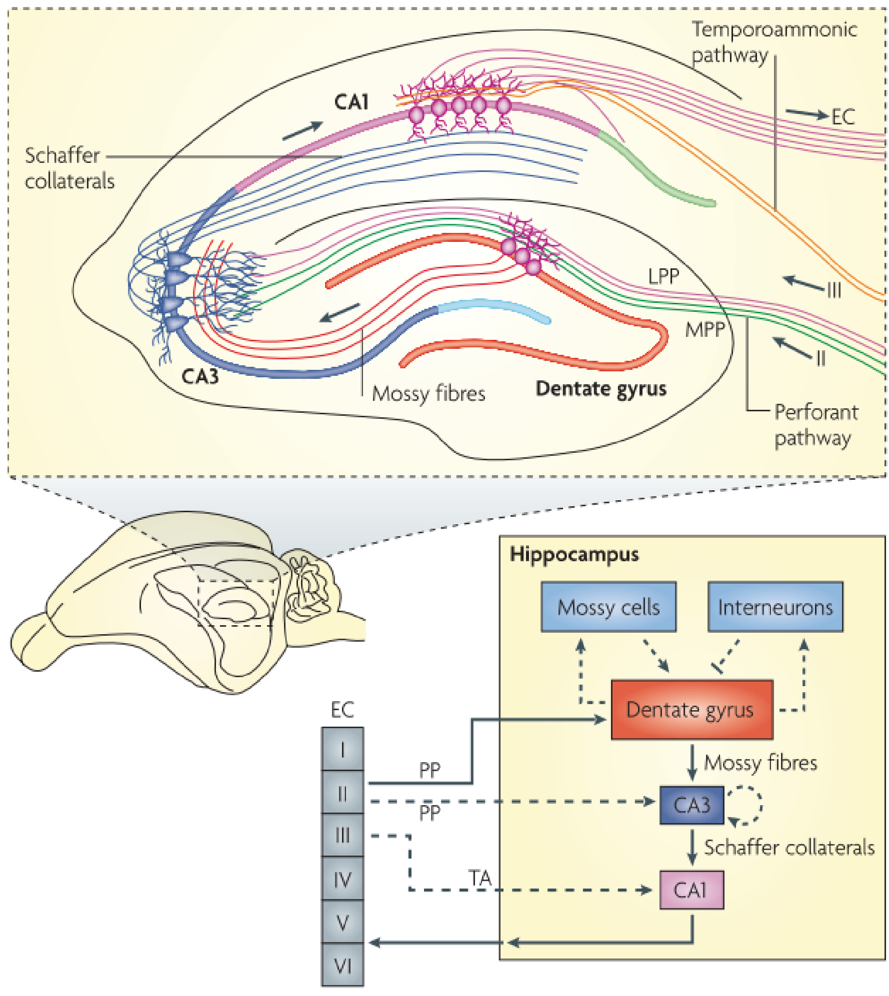

Figure 3. Illustration of the hippocampal rodent network. (A) Schematic representation of the hippocampal circuitry. (B) Diagram of the hippocampal network. Solid arrows show the excitatory trisynaptic loop and dashed arrows other projections. CA1, CA2 and CA3: cornu ammonis; EC: entorhinal cortex; LPP: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

The medial and lateral perforant routes connect the entorhinal cortex layer II stellate cells to the dentate gyrus. Dentate gyrus granule cells provide excitatory inputs to CA3 pyramidal cells via mossy fibers. Schaffer collaterals connect the axons of CA3

pyramidal neurons to the dendrites of CA1 pyramidal neurons in the stratum radiatum. To conclude on the entorhinal cortex-hippocampal loop, CA1 pyramidal neurons transmit back to the entorhinal cortex, especially into the deep-layer neurons. Aside from the trisynaptic loop, other projections have been reported (Figure 3B). The entorhinal cortex also sends direct signals to the CA1 and CA3 areas (layer III through temporoammonic pathway and layer II through the perforant pathway respectively). CA3 axons also communicate with other CA3 neurons through projections. Furthermore, the dentate gyrus' granule cells get direct input from mossy cells and hilar interneurons, which transmit excitatory and inhibitory projections back to the granule cells, respectively (Van Strien *et al.*, 2009; Deng *et al.*, 2010).

The hippocampal formation gets information from the cerebral cortex via the parahippocampal region, which is divided into two routes. The medial entorhinal cortex receives spatial/temporal information (context) from the postrhinal cortex, whereas the lateral entorhinal cortex receives non-spatial information (content) from the perirhinal cortex. This second route is crucial for memory of object recognition (Aggleton *et al.*, 2012). The hippocampus is thought to assist episodic memory by integrating information from the medial and lateral entorhinal cortex to create a representation of an experience in its spatial/temporal context (Knierim, 2015).

Other brain areas, in addition to the hippocampal formation and parahippocampal region, are involved in memory processing. Patients with lesions in the medial temporal lobe lose recent

memories but maintain older ones (from infancy), indicating that memories may be stored elsewhere over time (Squire and Alvarez, 1995). Animal studies show that disrupting hippocampal function impacts more current memories rather than distant memories, supporting this notion (Frankland *et al.*, 2019).

It has been proposed that memories are first stored in the hippocampus formation, and then transported to the neocortex, where they are permanently preserved. The network's core is moved from the hippocampus to the medial prefrontal/anterior cingulate cortex in this phase (Takehara-Nishiuchi, 2014). During and after the event, interaction between the hippocampus and cortical networks is crucial (Preston and Eichenbaum, 2013).

Other studies have recast this paradigm, claiming that information is recorded in hippocampal-cortical networks from the start, and that the hippocampus's implication is still present throughout distant contextual memories (Nadel and Moscovitch, 1997; Frankland and Bontempi, 2005).

1.3. Behavioral test to study memory in mice

Several behavioral tests have been devised to examine memory and learning in rodent's models, and they are particularly useful in studying intellectual impairment models. Animals are tested in mazes or boxes that require them to complete a certain task. Typically, these tasks rely on innate behavior such as exploration behavior, which encourages animals to explore new habitats to

learn about new places and objects; positive reinforcers, such as food or water; and negative reinforcers, such as electric shock or loud noise (Paul *et al.*, 2009).

A set of behavioral tests was designed using exploratory behavior: the Y-maze alternation task (Gerlai, 1998a), the novel object (Ennaceur and Delacour, 1988) and novel place recognition test (Save *et al.*, 1992). More complex learning tasks containing positive reinforcers like food, sweetened water, or the possibility to stay in a safe compartment or hole, such as the classical operant behavior acquisition models, were established (Baron and Meltzer, 2001). Other memory paradigms containing an aversive component or reinforcer, such as a foot shock or a loud noise, have also been developed, including the active avoidance test and the fear conditioning paradigm (Gerlai, 1998b). In this thesis, the novel object recognition (NOR) test was used to investigate non-emotional hippocampal-dependent memory (Ennaceur and Delacour, 1988).

The NOR test assesses the ability to remember an object seen before. Recognition memory is a form of declarative memory that is often impaired for instance in people that suffers neurodegenerative disease or brain injury (Winters *et al.*, 2008). Visual paired comparison tasks, which are rather comparable to NOR test, are commonly used to test recognition memory in humans (Pascalis *et al.*, 2004). In NOR test, animals are exposed to two similar items in a familiar maze, and one of the objects is replaced

with a new one after a retention time. Rodents prefer to investigate unfamiliar objects over familiar ones; thus object recognition memory is inferred from their inclination to approach and explore novel objects more (Berlyne, 1950). The encoding, consolidation, and retrieval of the memory for the familiar object is required for the preference for the novel object. (Cohen and Stackman, 2015). The benefits of this test include the fact that it does not involve the use of positive or negative reinforcers, does not create stressful conditions, can be completed in a single session, and has been duplicated in several laboratories using various labyrinth designs, items, and strains (Ennaceur and Delacour, 1988; Bevins and Besheer, 2006). All variables, including which item acts as a familiar or new one, and where the new object is located, must be balanced. The objects should also be checked to ensure that they are both investigated for the same time when both are new to mice.

This test has several distinct variations. Although numerous studies have been done in Y-shaped or V-shaped mazes to minimize contextual and spatial information (Busquets-Garcia *et al.*, 2013; Vallée *et al.*, 2014; Oliveira da Cruz *et al.*, 2020), it is traditionally performed in an open-field arena. In this thesis was performed in a V-shaped maze.

The test has three stages. Habituation is the initial step, during which mice become used to the arena. The training or familiarization phase follows, in which mice investigate two similar objects. Each object is located at the end of the corridors in the V-

maze. The memory test is the final phase, and it is carried out after a period of time has passed (10 min-3 h for short-term memory and 24 h for testing long-term memory). On this phase, one of the familiar items gets replaced with a new one. Figure 4 is an illustration of the fundamental method.

Figure 4. Procedure and maze used to execute the novel object recognition (NOR) test. LTM: long-term memory; STM: short-term memory.

After recording the amount of time mice spent exploring each object, a discrimination index (DI) can be calculated (Bevins and Besheer, 2006), using the following formula:

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

The hippocampus and perirhinal cortex are the two main regions involved in object recognition memory. The role of the hippocampus in rodents has been disputed, despite its well-established role in humans (Squire *et al.*, 2007). Some research has looked at this topic and come up with a broad range of answers. These investigations used hippocampal lesions or pharmacological inactivation, and although some found object recognition to be unaffected, others found it to be impaired (Baker and Kim, 2002; Ainge *et al.*, 2006; Winters *et al.*, 2008).

Table 1 lists some of the other tests commonly used to measure cognitive function in mice

Test	Description	Brain areas	Scheme
Novel Place Recognition	**Spatial memory:** During the familiarization phase, mice are exposed to two identical objects and one is relocated during the test phase. A discrimination index is calculated as in the NOR test.	Hippocampus and postrhinal cortex	
Morris Water Maze	**Spatial learning:** This task consists in finding the location of a hidden platform immersed in a pool of water surrounded by a variety of spatial stimuli as spatial reference. Mice are taught over several days and the time spent finding the platform and in the target quadrant is recorded as a learning index.	Hippocampus	
Spontaneous alternation task (Y-maze)	**Working memory**: The percentage of correct spontaneous alternation is assessed as a learning metric since animals prefer to visit a new arm over returning to a prior arm.	Hippocampus and prefrontal cortex	
Active Avoidance	**Fear conditioning learning:** This test asses emotional memory and it is performed in a shuttle box with two compartments. Mice must learn that if they do not move compartments following a cue light, they will receive an electrical foot shock. A learning index is calculated as a percentage of conditioned responses.	Amygdala, striatum and prefrontal cortex	
Radial Arm Maze	**Working memory:** Food-deprived mice are expected to recall which arms they have already visited in a maze with eight arms baited with a reward. The number of errors that occur when an animal returns to a previously visited arm is measured.	Hippocampus and prefrontal cortex	

Table 1. Summary of behavioral test used to investigate learning and memory in rodents. A description, a scheme, and the key brain areas involved for each behavioral test (Sunyer *et al.*, 2007; Lee and Silva, 2009; Ameen-Ali *et al.*, 2015)

1.4. Intellectual disability

Intellectual disability (ID) is a term used to describe a group of neurodevelopmental disorders that begin during childhood and are marked by deficits in cognitive and adaptive functioning in the conceptual, social, and practical domains of life (American Psychiatric Association, 2013). Language, reading, writing, reasoning, knowledge, and memory are all part of the conceptual domain. Empathy, social judgment, communication skills, and establishing and maintaining friends are all part of the social domain. Self-management, which includes personal care, school and employment obligations, and money management, is referred to as the practical domain.

In the general population, the frequency of intellectual impairment ranges from 0.87 to 3.68 % (Boat and Wu, 2015). Intellectual impairment has a significant impact on the quality of life of those afflicted and their families, as well as significant medical, educational, social, and economic costs. An affected person's lifetime additional cost is anticipated to be more than $1 million (Centers for Disease Control and Prevention (CDC), 2004).

The level of intellectual impairment varies from person to person, and it has traditionally been measured using the intelligence quotient (IQ) score: mild (70-55), moderate (55-40), severe (40-25), and profound impairment (<25). Nowadays, rather than a defined IQ range, this categorization is based on daily abilities (Boat and Wu,

2015). Syndromic intellectual impairment refers to patients with ID who also have other clinical symptoms.

The etiology of intellectual impairment is very heterogeneous, including hereditary and environmental factors. Furthermore, in certain situations, the causes are unknown. Prenatal teratogen exposure (alcohols and drugs, chemicals, or radiation), intrauterine infections, maternal malnutrition, preterm delivery, perinatal trauma or hypoxia, neonatal hypothyroidism, and socioeconomic and cultural variables are all examples of environmental influences (Kaufman *et al.*, 2010).

Chromosomal abnormalities (aneuploidies, deletions, translocations, and duplications) and single gene mutations are among the genetic reasons (Dierssen and Ramakers, 2006). Down syndrome (DS) is the most frequent genetic cause of ID, whereas fragile X syndrome (FXS) is the most common inherited cause of ID. Williams-Beuren syndrome, Rett syndrome, and tuberous sclerosis complex are examples of other genetic disorders.

Several studies have found that intellectual impairment is linked to anomalies in brain development and plasticity, which are produced whether environmental or genetic factors (Dierssen and Ramakers, 2006). Multiple processes throughout brain development, including neuronal and glial proliferation, migration, differentiation, maturation, and synaptogenesis, are required for the correct formation of synaptic connections and synaptic plasticity. In foetuses with ID, defects in several of these processes, as well as

structural brain abnormalities, have been discovered (Castrén *et al.*, 2005; Contestabile *et al.*, 2007; Guidi *et al.*, 2008). Furthermore, changes in the volume and neuroarchitecture of numerous brain areas have been found in *post-mortem* brains of children and people with ID. Changes in neuronal density and structural anomalies at the level of dendritic spines, in particular, have been well documented (Kaufmann and Moser, 2000; Dierssen and Ramakers, 2006) and are supposed to be at the core of the condition.

The current therapy for intellectual impairment focuses on optimizing the environment, which includes early intervention programs, personalized education programs, and co-morbidity management (Picker and Walsh, 2013). However, the effectiveness of these treatment alternatives is limited (Bonnier, 2008; Couzens *et al.*, 2012). More recently, the creation of mouse models of intellectual impairment disorders has aided in the understanding of the processes behind cognitive deficiencies as well as the development of particular pharmacological treatments. Several preclinical investigations have shown that pharmacological treatments can alleviate cognitive impairments, which was unthinkable some years ago.

This thesis focuses on the study of memory as the cognitive function most impacted by intellectual impairment.

1.5. Alzheimer's disease

Alzheimer's disease (AD) is the most prevalent form of dementia, affecting 50 million individuals in 2018, with the number expected to triple by 2050 (Livingston *et al.*, 2020). The term "dementia" refers to a decline in intellectual capacity or the emergence of numerous cognitive impairments that impact language, comprehension, memory, and everyday activities.

The most frequent symptom is a progressive memory loss, although it is also accompanied by executive dysfunction and visuospatial impairment, which impact cognition and behaviour (Tiwari *et al.*, 2019). AD is a progressive neurodegenerative illness characterized by increased extracellular amyloid plaques composed of β-amyloid (Aβ) peptides (neuritic plaques) and intracellular neurofibrillary tangles comprised of hyperphosphorylated tau protein in the cortical and limbic regions of the brain (Ulep *et al.*, 2018). Plaques are tiny lesions with a core of extracellular Aβ-peptide and increased axonal terminals that are spherical in shape. The Aβ peptide is generated from an amyloid precursor protein (APP) which is a transmembrane protein. Proteases called α, β, and γ-secretase break the Aβ peptide from the APP. APP is usually cleaved by α or β secretase, and the resulting small fragments are not harmful to neurons. Sequential cleavage by β and then γ-secretase, on the other hand, yields 42 amino acid peptides (Kumar *et al.*, 2021).

On the other hand, tau protein forms fibrillary intracytoplasmic aggregates in neurons called neurofibrillary tangles. The tau

protein's main task is to keep axonal microtubules stable. Hyperphosphorylation of tau occurs in AD because of extracellular Aβ peptide accumulation, resulting in tau aggregate development. Tau aggregates produce neurofibrillary tangles, which are twisted paired helical filaments. They start in the hippocampus and subsequently spread to the rest of the cerebral cortex (Kumar *et al.*, 2021).

In this cellular pathological environment, neuroinflammation, vascular changes, ageing, and lymphatic system dysfunction operate upstream or parallel to Aβ accumulation (Scheltens *et al.*, 2021). Indeed, neuroinflammation is considered a key component of AD pathogenesis, leading to disease development and neurodegeneration.

Cholinesterase inhibitors and the N-methyl-D-aspartate (NMDA) receptor antagonist memantine are the only approved therapies that are considered standard of care for many AD patients (Livingston *et al.*, 2020).

1.5.1. Neuroinflammation

The activation of the innate immune system in response to an inflammatory event in the CNS is known as neuroinflammation. Different cellular and molecular changes characterize it, and they play an essential role in both healthy and pathological situations. Neuroinflammation is a defining feature of a variety of neurologic

illnesses, including chronic pain, traumatic brain injury, neurodegenerative diseases, and stroke where microglia cells plays a key role (Di Vito *et al.*, 2017; Olah *et al.*, 2020).

Microglial cells are immunological effector cells in the CNS, accounting for 5-20% of total glial cells in mice depending on the brain region (Lawson *et al.*, 1990), and being more prevalent in the gray matter than the white matter (Kofler and Wiley, 2011). They arose from the CNS invasion of peripheral mesodermal primitive macrophages and have a hematopoietic origin (Alliot *et al.*, 1999). These cells migrate, develop, and proliferate into the CNS during embryogenesis, where they form a dense network along the parenchyma that contributes to brain homeostasis (Saijo and Glass, 2011).

Microglial cells are thought to be key actors in inflammation, helping to restore homeostasis after damage or infection (Kempermann and Neumann, 2003). Microglia can also govern the proliferation and differentiation of neurons, as well as the creation of new synapses, in a healthy CNS (Graber *et al.*, 2012). Microglia dysfunction has therefore been linked to brain development and aging, as well as the advancement of a number of neurodegenerative illnesses and neuropathologies (Colonna and Butovsky, 2017).

Microglial cells are highly dynamic, according to two-photon imaging investigations. Based on their morphology and the expression of activation markers, they are now divided into two groups: "resting state" and "active state" (Figure 5) (Colonna and

Butovsky, 2017). While transcriptomic studies have revealed multiples subtypes (Sankowski *et al.*, 2019; Ochocka and Kaminska, 2021).

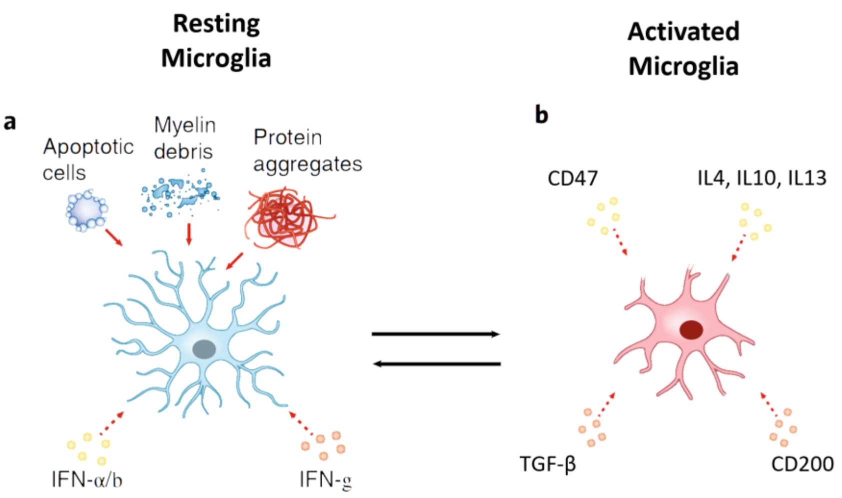

Figure 5. Classical stages of microglial cells activation from a morphological point of view. Surveillant/resting microglia cells are activated by several factors, which cause them to change shape from a small soma to a more amoeboid and less ramified state. Different variables allow activated microglia to return to a resting state. Adapted from (Song and Colonna, 2018).

Microglial cells morphology is ramified with numerous branches and processes that extend from the soma under normal conditions, without brain damage (Helmut *et al.*, 2011; Benarroch, 2013). Microglial ramifications contact neurons, astrocytes, and blood vessels in this resting surveillance state, monitoring synaptic function. Microglial cells are constantly scouring their surroundings, and their branches expand and retract at speeds of up to 3 m/min (Nimmerjahn *et al.*, 2005).

Microglial cells get activated in response to brain damage or inflammatory stimuli, and their morphology changes to that of an amoeboid. An expansion of the soma and a retraction of microglial branches define this morphology. Microglial cells follow chemotactic gradients to the location of the lesion or the invading pathogen during this phase. Depending on the type of stimulus or the environmental conditions that activate them, activated microglia may exhibit a variety of adaptive responses (Benarroch, 2013). Activated microglia can take on many morphologies, including amoeboid, rod, and multinucleated, and they can perform pro-inflammatory, cytotoxic, immunoregulatory, and repair activities (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009; Ransohoff and Cardona, 2010).

In summary, microglial cells respond to several types of transforming factors that mediate the exchange from a surveillance state to activated state involving cell morphology, gene expression and functional changes.

2. Down syndrome

DS is the most significant genetic cause of ID affecting around 1 in about 700-1,000 live births worldwide (de Graaf *et al.*, 2021; de Graaf *et al.*, 2020). This disorder is produced by an autosomal aneuploidy (defined as an abnormal number of copies of a genomic region), which consists in the trisomy of human chromosome 21 (HSA21) (Lejeune *et al.*, 1959).

The main risk factor for DS is advanced maternal age due to non-disjunction of homologous chromosomes during the meiotic division in the oocytes generation (Nagaoka *et al.*, 2012). The prevalence of DS in live births has been stable or slightly decreasing in the last decades, although important differences exist between countries (De Graaf *et al.*, 2017a). Although the number of foetuses with DS has been increasing during the last decades due to an increase in maternal age, this has been counterbalanced with an increase in spontaneous miscarriages and the number of pregnancies that are electively terminated (de Graaf *et al.*, 2015).

DS is defined by several heterogeneous developmental anomalies, which affect multiple organs with different penetrance and that can appear in different times of individual's life (Figure 6). The principal symptoms are ID, slow growth, hypotonia, heart defects, epilepsy, thyroid diseases, and neuropathological alterations typical of AD, among others (Antonarakis *et al.*, 2020). Furthermore, people with DS present also craniofacial changes like slanted eyes; short, wide neck and large tongue.

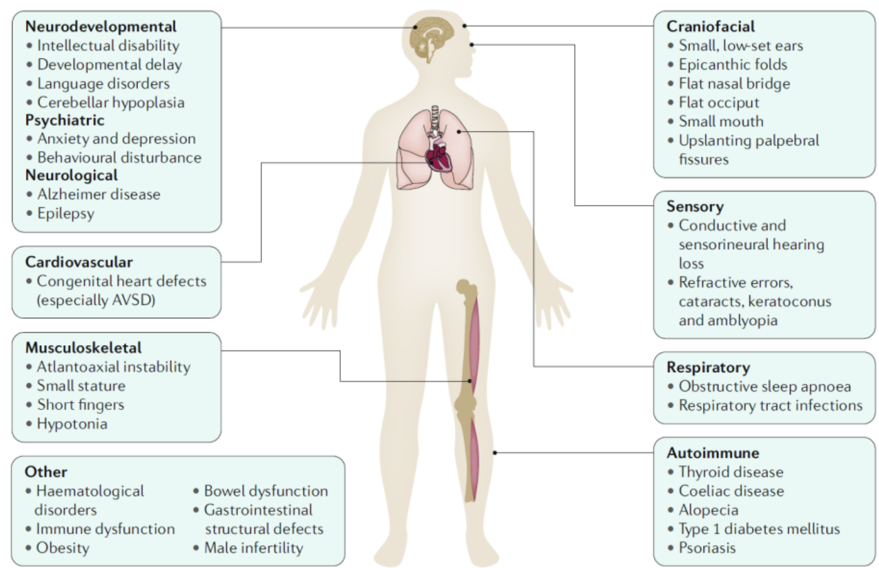

Figure 6. Signs and symptoms in Down syndrome. DS population present affectations in many tissues including the brain, both during development and later in adult life, leading to multiple and variable symptoms and manifestations. Overall, the severity of the symptoms is different for every individual. Subjects with DS present specific craniofacial and musculoskeletal signs such as small stature and hypotonia in addition to the presence of small ears and epicanthic folds among others. All DS individuals share neurodevelopmental symptoms such as ID in addition to psychiatric disorders and an early-onset AD. Furthermore, other comorbidities are frequent in the DS population including congenital heart defects, endocrine disorders (specifically hypothyroidism), higher risk for infections, diabetes, and obstructive sleep apnea. It also affects visual and audition systems coupled with speech production disabilities (Antonarakis *et al.*, 2020).

ID is the most prevalent and limiting phenotype of DS. This cognitive dysfunction is an important limitation for DS people independence, and negatively affects their quality of life. Therefore, understanding the pathophysiology of cognitive impairment in DS has been one of the main objectives in this field.

Individuals with DS also have an increased prevalence of neurological and psychiatric disorders in contrast with the general population (Capone *et al.*, 2006) such as anxiety, depression (Tassé *et al.*, 2016) or hyperactivity (Pueschel *et al.*, 1991).

The median life expectancy for people with DS has increased remarkably in the last decades, because of medical advances. For instance, life expectancy for DS subjects has increased from 26 years old in 1950 to 53 years old in 2010 in the USA (De Graaf *et al.*, 2017b). Therefore, nowadays DS population is more prone to suffer from age-related comorbidities throughout their lives, such as an early-onset AD which is present in all adults with DS by age 65 (Mccarron *et al.*, 2014). As a result, increased survival is not only associated with a longer period of necessary care, but it is also intermingled with the real demand of more specialized attention at an early age.

2.1. Genetic cause of Down syndrome

The genetic cause of DS is the partial or complete trisomy of the HSA21 (Lejeune *et al.*, 1959) which is the shortest human chromosome and is composed by 222 protein-coding genes and 325 non-protein-coding genes (Gupta et al., 2016).

A free trisomy 21 is phenotypically expressed in 95% DS individuals, originated from errors in cell divisions during the development of the oocyte, sperm, or after the formation of the zygote (Table 2).

The remaining cases are attributed to other chromosomal abnormalities including mosaicism, translocation (usually t(14;21) called the Robertsonian translocation) or partial trisomy which is a rare condition that derives to a diverse penetrance of the symptoms depending on the length of the partial triplication of the HSA21.

Type of HSA21 trisomy	Percentage of cases	References
Maternal meiosis I	~66%	(Antonarakis, 1991; Antonarakis *et al.*, 1993)
Maternal meiosis II	~21%	
Paternal meiosis I	~3%	
Paternal meiosis II	~5%	
Mitosis after the zygote formation	~5%	
Translocation	~5%	(Antonarakis, 1998; Morris *et al.*, 2012)
Mosaicism	~2%	(Barlow *et al.*, 2001; Lyle *et al.*, 2009)

Table 2. Genetic causes for trisomy 21 in DS.

Two main hypotheses have been postulated to understand the genetic aetiology of the increased presence of pathologic conditions in the DS population. The first one is the "*gene dosage effect*" hypothesis which proposes that the effects of the overexpressed HSA21 genes and their downstream outcome directly contribute to the phenotypes encountered in DS individuals. Then, each result of HSA21 trisomy is the direct consequence of an increased dose of a single HSA21 gene. Studies of genotype-phenotype correlation in DS subjects with partial trisomy determined a ~5.4 Mb region on HSA21

called the DS critical region (DSCR). This region contains in the order of 50 genes that are sufficient to produce the most relevant signs and manifestations of DS (Delabar *et al.*, 1993). But further studies on DS individuals with infrequent segmental trisomies that do not contain the DSCR demonstrated that other regions are also important (Korenberg *et al.*, 1994; Korbel *et al.*, 2009; Lyle *et al.*, 2009). Studies with DS mouse models have demonstrated that the extra copy of particular genes, called candidate dosage-sensitive genes, contribute to the phenotypes found in the DS population. Some of the genes particularly involved in neuropathological features are *DYRK1A* (dual specificity tyrosine-phosphorylation-regulated kinase 1A) and *APP* (amyloid precursor protein) among others. DYRK1A plays a role in proliferation, survival and development of neural progenitor cells (Tejedor *et al.*, 1995), and has been proposed as a candidate gene for ID in DS (Altafaj *et al.*, 2001). Besides, an increased dosage of APP enhances propensity to early-onset AD in DS population (Salehi *et al.*, 2006). A second hypothesis, called "amplified development instability" propose that trisomy 21 can also disrupt global transcription because the increased genetic material could change genome-wide gene expression (Pritchard and Kola, 1999). Recent studies have found that an extra HSA21 can change chromosome compactation and chromosome territories in the nucleus (Kemeny *et al.*, 2018). Alterations in methylation patterns are also found in different chromosomes and tissue types in DS and they can be observable from the developmental stage (Do *et al.*, 2017).

Then a third hypothesis that combines the previous two hypothesis, called the "genome instability" hypothesis, proposes that both mechanisms contribute to the phenotypes found in DS individuals (Dierssen, 2012). Then this hypothesis postulate that DS manifestations are due to the dosage imbalance of HSA21 and non-HSA21 genes, in addition to the global transcriptomic and epigenetic changes (Dierssen, 2012).

2.2. Neuropathology and neuropsychological features in Down syndrome

ID is the most prevalent and prominent feature in DS, being DS the main cause of genetic ID (Pulsifer, 1996). ID in DS is characterised by defects in adaptive functions in specific areas, such as learning and memory, executive functions, and language (Grieco *et al.*, 2015). This cognitive dysfunction is an important limitation for DS people independence, and negatively affects their quality of life being relevant to figure out potential remediation approaches. Therefore, understanding the pathophysiology of cognitive impairment in DS has been one of the main objectives in this field.

The severity of ID in DS population is in a mild to severe range (IQ score=30-70) (Vicari *et al.*, 2005; Contestabile *et al.*, 2010). This variability has been related to diverse factors, such as genetic and epigenetic variations, environmental causes and random events (Gardiner *et al.*, 2010). Then, the domains affected vary between

individuals with DS. Mainly verbal short-term memory, explicit long-term memory and working memory are globally impaired in DS people (Wang and Bellugi, 1994). Furthermore, language deficits in articulation and lexical and morphosyntactic abilities are also present in this population (Vicari *et al.*, 2004). In addition, daily episodic memory is impaired, limiting the independent function of DS individuals (Pennington *et al.*, 2003). In contrast, implicit long-term memory or associative learning are relatively conserved (Carlesimo *et al.*, 1997; Vicari *et al.*, 2006, 2007). Furthermore, deficits in spatial memory are not observed in all the studies (Vicari et al., 2005; Edgin et al., 2010).

Individuals with DS also have an increased prevalence of neurological and psychiatric disorders competed with the general population (Capone et al., 2006) such as hyperactivity and anxiety (Pueschel et al., 1991). Some studies determined that almost the half of DS children present hyperactivity but no significant correlation was found with the cognitive impairment severity (Ekstein et al., 2011). Furthermore, anxiety is more present in older adults with DS (Tassé et al., 2016).

The origin of the cognitive deficit lies in neurodevelopmental alterations, which produce several changes in the brain of DS adults (Table 3). First, people with DS display brachycephaly and microcephaly, especially affecting areas such as the hippocampus, prefrontal cortex, and cerebellum (Kesslak et al., 1994; Raz et al., 1995; Pinter et al., 2001; Śmigielska-Kuzia et al., 2011). Indeed, a

negative correlation between the parahippocampal volume and the cognitive performance is observed, evidencing the functional importance of such morphological alterations (Śmigielska-Kuzia et al., 2011). Furthermore, a reduced density of neurons can be observed in some regions such as the hippocampus, cortex, and cerebellum (Sylvester, 1983; Wisniewski et al., 1984; Wisniewski, 1990; Olmos-Serrano et al., 2016). Because the proliferation of neural progenitor cells in DS foetuses is decreased and apoptosis is increased, the decrease in brain size and neuronal density is likely due to defects in neurogenesis in brain development (Contestabile *et al.*, 2007; Guidi *et al.*, 2008; Lu *et al.*, 2011). In addition to volume reduction, some brain areas display alterations in their morphology, connectivity, and functionality. At the morphological level, neurons show reduced dendritic branches and reduced numbers of dendritic spines in multiple brain regions such as the hippocampus and motor and parietal cortex (Marin-Padilla, 1976; Suetsugu and Mehraein, 1980). Remarkably, these alterations appear during childhood and remain present until adulthood. Finally, at the functional level, fMRI studies have found brain functional connectivity defects contributing to poor adaptive behaviour in addition to immature development of connectivity in subjects with DS (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 changes in new-borns, adults and elderly DS individuals (Dierssen, 2012).

Therefore, the overall changes in the hippocampus as well as the other affected areas related with memory and learning, have been proposed to be an explanation for the cognitive deficit present in DS.

2.1.1. Alzheimer's disease in Down syndrome

As mentioned above, through medical care advances, life expectancy has been extended to more than 60 years for DS population (Bittles et al., 2007; Contestabile et al., 2010). All individuals with DS develop early-onset AD, mainly due to APP overproduction (Prasher et al., 1998; Wiseman et al., 2015a). Importantly, dementia is the leading cause of death in 70% of elderly people with DS (Hithersay et al., 2019). Clinical symptoms of the

cognitive decline begin after the age of 40, but affect all the individuals by the age of 70 (Ballard *et al.*, 2016a; Bayen *et al.*, 2018). A characteristic of AD which correlates with memory loss is the degeneration of cholinergic neurons (Ikonomovic et al., 2003). In fact, DS subjects also display a progressive loss of basal forebrain cholinergic neurons (BFCN) (Yates et al., 1983; Mann et al., 1985).

Post-mortem studies show that while most DS individuals have neuropathological changes related to AD, some people do not present strong symptoms of dementia (Devenny *et al.*, 1996; Krinsky-McHale and Silverman, 2013). It must be considered that, there are some difficulties to distinguish age-related decline from progressive cognitive decline related to dementia. One of the first symptoms associated with the emergence of dementia is the impairment in executive function, especially with planning ability and attention (Das *et al.*, 1995; Rowe *et al.*, 2006; Ball *et al.*, 2008). Other symptoms such as episodic memory impairment are related with weakening in selective attention (Krinsky-McHale *et al.*, 2008). Changes in the frontal lobes, evidence for the appearance of apathy or depression, can also indicate neurodegeneration secondary to AD (Lott and Head, 2001).

As mentioned above, the origin of AD in DS has been related with the overexpression of *APP* (Rumble et al., 1989). This overexpression results in an increased formation of Aβ plaques, and its deposition in DS individual's brain. Deposition of plaques has been found in young DS people, but it is not until 30 years of age that is systematically observed (Figure 7)(Leverenz and Raskind, 1998;

Stoltzner et al., 2000). With age, Aβ manifests itself as diffuse deposits that evolve to compact neuritic plaques. The increase of brain Aβ in people with DS is not linear but exponential after 40 years of age, implying that at this age, disease progression has an acceleration phase (Nistor et al., 2007).

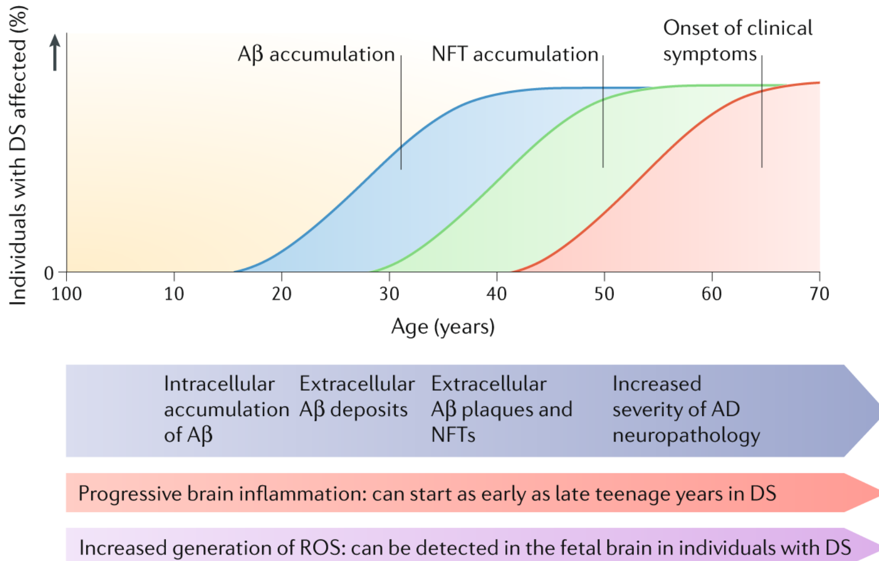

Figure 7. AD pathology timeline in people with DS from birth to over 60 years of age. Mitochondrial dysfunction and an increase in reactive oxygen species production can start as early as the prenatal brain. The existence of activated microglial cells, which are associated with Aβ plaques later in the disease, can start brain inflammation as early as late adolescence. Extracellular Aβ and neurofibrillary tangles (NFTs) are present in sufficient amounts for a neuropathological diagnosis of AD by the age of 40. When people with DS reach the age of 50, the severity of AD neuropathology increases, and clinical symptoms of dementia become more prominent (Lott and Head, 2019).

Furthermore, the presence of an Apo E ε4 allele increases the burden of cerebral Aβ plaques and shortens life duration in DS.

Some studies of rare cases of people with DS but disomic for *APP* show no neuropsychological signs of dementia and no evidence of AD at autopsy, reinforcing the role of *APP* overexpression in the origin of AD in DS (Prasher et al., 1998; Doran et al., 2017).

Other familial forms of AD consist in *APP* gene locus duplication (dup-*APP*). When compared to this familial form of AD, people with DS have a similar early age of onset of Aβ pathology (Wiseman et al., 2015b; Carmona-Iragui et al., 2017) and a similar AD neuropathology, as well as an increased prevalence of cerebral amyloid angiopathy (Wallon et al., 2012) . But some phenotypic differences occur between these two conditions, since in DS the variability in the prevalence of dementia is more striking that in dup-APP even most DS subjects have AD neuropathology (Hooli et al., 2012). These behavioural differences provide a foundation for learning more about the roles that additional genes on HSA21 play in AD etiology.

Tau phosphorylation and aggregation is manifested in DS individuals' brains. These can be found as dystrophic neurites around Aβ plaques or neurofibrillary tangles for instance in CA1 in the hippocampus and subiculum (Mann and Esiri, 1989; Hof et al., 1995). Numerous genes on HSA21 may also play a role in tau pathology in DS, including *DYRK1A* (facilitating GSK3β phosphorylation) and *RCAN1*.

Other factors can contribute to AD pathogenesis in DS (Figure 7). The most significant ones include oxidative stress (Cenini et al.,

2012; Barone et al., 2018) or neuroinflammation (Wilcock, 2012; Wilcock and Griffin, 2013).

2.1.2. Neuroinflammation in Down syndrome

Neuroinflammation is a major contributor to neurodegenerative disorders (Colonna and Butovsky, 2017; Yin *et al.*, 2017) including AD in DS. Microglial cells are the major mediators of the neuroinflammatory response in the brain. Microglial cells in persons with DS over the age of 40 display morphological and pathological alterations, including a decrease in the number of microglial cells with a resting state morphology and an increase in the frequency of dystrophic microglial cells (Xue and Streit, 2011). In addition, recent studies that assessed microglial morphology and activation markers, found activated microglia and neuroinflammation in *post-mortem* samples of young individuals with DS (Pinto *et al.*, 2020). Some genes are directly involved in the neuroinflammatory phenotype in DS such as S100β which is on HSA21. Indeed, increased S100β and IL-1 β expression in astrocytes was observed (Griffin *et al.*, 1989). Furthermore, C1q activation of the complement system that governs immunological activities seems to be prevalent in adults with DS (Head *et al.*, 2001). At the peripheral level, an increase of inflammatory cytokines in plasma samples of people with DS was found (Rodrigues *et al.*, 2014). Interestingly, these levels were even higher in DS subjects with dementia (Iulita *et al.*, 2016), further

supporting a link of neuroinflammation with the neurological dementia.

2.3. Down syndrome mouse models

Animal models that resemble genetic features of DS have been developed as a tool for studying the pathophysiology of this condition and as an instrument for drug testing. More precisely, mouse models have been used due to the difficulty to replicate DS genetic conditions in other animal species.

HSA21 is orthologous to three different regions that are located in the murine chromosomes 10, 16 and 17 (*Mus musculus* (MMU) 10, 16 and 17). In the telomere proximal of MMU16 there are 102 of the 158 mouse genes that are homologous to human protein-coding genes, 37 in the MMU10 and 19 in the internal region of the MMU17 (Figure 8) (Davisson et al., 1993).

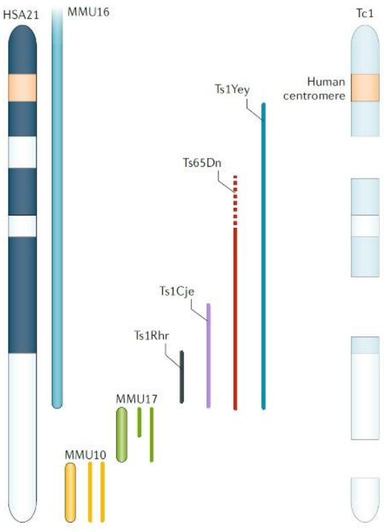

Figure 8. Representation of HSA21 with murine homologous parts and mouse models of DS. Left: correspondence of genomic areas of HSA21 to murine chromosomes 10, 16, and 17. Right: representation of the triplicated genomic regions in mouse models for DS. Adapted from (Antonarakis et al., 2020).

Producing animal models to study DS has been difficult due to this genetic different distribution (Antonarakis et al., 2004). Furthermore, non-coding genes, for example those encoding miRNAs are also found on these three murine chromosomes and are well conserved (Gupta et al., 2016). But some mouse genes do not have human homologues and some HSA21 genes are not conserved in the mouse, which makes creating DS models a more challenging task (Antonarakis et al., 2004). Due to the genetic complexity of DS, several mouse models have been generated. In this thesis we have used exclusively the Ts65Dn mouse model.

2.3.1. Ts65Dn mouse model

The first trisomic mouse model reaching adulthood was the $Ts(17^{16})65Dn$ (Ts65Dn) mouse, enabling postnatal studies (Davisson et al., 1993). This model has been the most-used mouse model for study of DS.

It consists in a partial trisomic model that contains the distal region of MMU16 (from *Mrp139* to *Znf95*) and the pericentromeric region of MMU17 resulting in a free segregating mini chromosome (Davisson et al., 1993; Reeves et al., 1995). Although this model possesses a region of MMU16 with 90 conserved protein-coding HSA21 genes, it also contains some genes that are not present in the human condition. For instance, in the extra segment of MMU17 there are ∼35 protein-coding genes, 15 non-protein-coding genes and 10 pseudogenes (Duchon et al., 2011). But although the Ts65Dn does not present a perfect construct validity, it is important to take into consideration the impact on cellular division and gene expression that the independent chromosome brings to the model. Furthermore, the fertility of Ts65Dn males is usually affected. Then the transmission is maintained through the maternal germline, which may alter the phenotype of trisomic and disomic offspring, because unlike the human condition, the mother is trisomic (Herault et al., 2017). Nevertheless, this model recapitulates relevant phenotypes observed in the human condition.

Ts65Dn mice present reduced birth weight (Reeves et al., 1995), similar DS-related cranofacial alterations (Richtsmeier et al., 2002;

Starbuck et al., 2014) in addition to hypotonia which is widely observed in DS new-borns (Vicari et al., 2006). This model also presents congenital cardiac defects that have been related with an increased postnatal lethality (Moore, 2006).

Importantly, Ts65Dn mice show several neurological and behavioural traits, making them a feasible model for pharmacological intervention in cognitive remediation. At the neuroanatomical level, a reduced brain volume is observed during the embryonic stage but not in the adult stage (Aldridge et al., 2007; Chakrabarti et al., 2007b). The global hippocampal volume is not altered, but the volume of the hilus and the dentate gyrus granule cell layer is reduced (Insausti et al., 1998; Lorenzi and Reeves, 2006). Hypocellularity can also be observed during adulthood in some brain regions such as the hippocampus, the perirhinal cortex, the neocortex and cerebellum (Baxter et al., 2000; Olson et al., 2004; Lorenzi and Reeves, 2006; Chakrabarti et al., 2007a; Roncacé et al., 2017).

As in individuals with DS, it has been hypothesized that this cellular deficiency is secondary to neurogenesis defects. Indeed, in Ts65Dn it has been described proliferation defects during the prenatal period that are maintained through life (Lorenzi and Reeves, 2006; Chakrabarti et al., 2007a).

The Ts65Dn mouse model also present synaptic plasticity deficits. This deficits include structural changes such as decreased spine density and enlarged spine head volumes in the hippocampal and cortical region (Dierssen et al., 2003; Ayberk Kurt et al., 2004;

Kleschevnicov et al., 2004). At the functional level, electrophysiological experiments found that LTP is decreased while LTD is increased in CA1 and the dentate gyrus of the hippocampus (Siarey et al., 1997, 1999; Kleschevnicov et al., 2004; Costa and Grybko, 2005).

Extensive behavioural testing of the Ts65Dn mouse revealed severe memory deficits like DS subjects. Indeed, several hippocampal-dependent tasks have been found to be impaired. Deficits in object-recognition memory are described using the NOR test (Fernandez *et al.*, 2007a; Contestabile *et al.*, 2013; Deidda *et al.*, 2015). Spatial memory deficits are also observed in NPRT (Kleschevnikov et al., 2012; Contestabile et al., 2013), MWM (Reeves et al., 1995; García-Cerro et al., 2014), Barnes maze (Kazim et al., 2017) and radial arm maze (Demas et al., 1996). Deficits in working memory can also be noted in the spontaneous alternation task (Contestabile et al., 2013) as well as in emotional memory in the fear-conditioning test (Fernandez *et al.*, 2007a; Contestabile *et al.*, 2013; García-Cerro *et al.*, 2014; Deidda *et al.*, 2015). In addition, Ts65Dn mice performance in some hippocampal-dependent tasks deteriorates from 6 months of age, that could be secondary to the neurodegenerative state developed from this age (Hyde and Crnic, 2001; Hunter et al., 2003). Furthermore, this mouse model displays additional DS-related characteristics such as hyperactivity (Whitney and Wenger, 2013), sleep disorders (Colas et al., 2008), and increased seizures (Cortez et al., 2009; Joshi et al., 2016). In addition, less anxiety-like behaviour has been described in Ts65Dn using the

plus-maze and open-field tests were used (Escorihuela et al., 1995, 1998; Coussons-Read and Crnic, 1996).

2.3.1.1. Neurodegenerative phenotype

The Ts65Dn mouse model has been the most extensive used for preclinical studies of AD in DS. Even other mouse models for DS have been developed, the Ts65Dn model is the only one where age-related memory deficits have been examined (Hamlett *et al.*, 2015). The first signs of neurodegeneration can be observed at 6 months of age when norepinephrinergic neurons in the *locus coeruleus* (LC-NE) degenerate (Salehi et al., 2009; Lockrow et al., 2011). The LC is the main source of norepinephrine inputs to the hippocampus (Ennis and Aston-Jones, 1988) and also exerts a direct influence on BFNCs (Wenk et al., 2003). The outcomes of LC-NE degeneration on brain function are transmitted directly through neurotransmission. In addition, LC-NE indirectly contribute to Aβ accumulation, inflammation, and oxidative stress pathways (Marien et al., 2004; Counts and Mufson, 2010).

The main cholinergic innervation to frontal cortical areas and the hippocampus is provided by BFCNs, which influences the processing of information required for attention and cognition in both animal models and humans (McGeer, 1984; Dunnett et al., 1991). The phenotypic loss of cholinergic neurons is a characteristic of AD and significantly corresponds with memory decline in this pathology (Ikonomovic et al., 2003). The Ts65Dn mouse model mirrors BFCN

neuropathology, with age-related degeneration beginning at 6–8 months of age, and severe phenotypic loss at 10 months of age (Holtzman *et al.*, 1996; Granholm *et al.*, 2000; Cooper *et al.*, 2001). Furthermore, at this age, BFCN show a reduction in immunohistochemistry for both choline acetyltransferase (ChAT) and low and high-affinity nerve growth factor (NGF) receptors such as p75 neurotrophin receptor (p75NTR) and TrkA (Granholm *et al.*, 2000; Hunter *et al.*, 2004b).

Other neuronal population that also degenerates is Calbindin-D28K neurons in the hippocampal CA1 region, which play and important function in AD neuropathology in this brain region (Sutherland et al., 1993; Pappas and Parnavelas, 1997) and it is also reduced in adult Ts65Dn (Hunter *et al.*, 2003; Hamlett *et al.*, 2020b).

Since mouse Aβ is not able to aggregate (Lomoio et al., 2009), its accumulation does not lead to Aβ plaque formation, representing that the Ts65Dn trisomic model undergoes neurodegeneration without the presence of Aβ plaques. Furthermore, neurodegenerative hallmark changes correlate with progressive memory decline and loss of cholinergic neurons, directly dependent on App overexpression (Salehi et al., 2006). These factors and others are the cause of the early neurodegenerative phenotype developed in trisomic mice, reminiscent of AD present in middle-aged individuals with DS. These shared characteristics indicate that Ts65Dn mice can be used to simulate the progression of neuropathology in DS and provide an opportunity to study DS and the potential therapeutic interventions for AD (Hamlett *et al.*, 2015).

AD and other neurodegenerative disorders are known to have a close relationship with microglial reactivity (Colonna and Butovsky, 2017; Yin et al., 2017). As in DS subjects, different studies have observed an increase in microglial reactivity in adult Ts65Dn mice (Hunter *et al.*, 2004b; Lomoio *et al.*, 2009; Illouz *et al.*, 2019; Hamlett *et al.*, 2020a). Furthermore, pharmacological intervention of inflammatory activity reduces microglia activation, prevents cholinergic cell loss, and enhances working and reference memory in Ts65Dn mice (Hunter et al., 2004b).

2.3.2. Other mouse models for Down syndrome

In addition to Ts65Dn other mouse models for DS have been developed. The Ts1Cje is another model of MMU16 segmental trisomy (Sago et al., 1998). This model has a trisomic segment shorter than in Ts65Dn mice but in contrast it does not present non-orthologous triplicated genes. Importantly, in comparison to Ts65Dn mice, Ts1Cje mice present less prominent memory deficits (Sago et al., 1998; Aziz et al., 2018). Furthermore, this mouse model do not present AD neuropathology since App gene is not in trisomy (Sago et al., 1998).

Another relevant model is the Ts1Rh mouse, which has the DSCR region in trisomy (Olson et al., 2004). Although Ts1Rh mice present deficits in certain memory tasks such as the NOR test and the spontaneous alternation task (Belichenko et al., 2009), they do not present deficits in the MWM test (Olson et al., 2007) and

craniofacial abnormalities are not observable. This evidence confirms the idea that DSCR is not the only region responsible for the phenotypes associated with DS, but this is sufficient to cause some structural and functional effects in the brain.

Some models with a better construct validity than Ts65Dn has been developed. For instance, the triple trisomic model (TTS) (Yu *et al.*, 2010a) carries the three HSA21 mouse chromosomal regions and is 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 these mice display memory deficits and reduced LTP (Yu *et al.*, 2010b; Belichenko *et al.*, 2015), they also exhibit milder phenotypes than the Ts65Dn model. Nonetheless, unlike the Ts65Dn model and human condition, this model lack a free segregating chromosome. The significance of this independent minichromosome on cellular division (proliferation) and nuclear chromatin structure (gene expression) should not be underestimated (Belichenko *et al.*, 2015). Comparative studies between TTS and Dp16, Dp17 and Dp10 have emphasized the importance of the MMU16 in the neurologic phenotye, since the last two do not shot cognitive impairment and synaptic plasitcy defects (Yu *et al.*, 2010b). Nonetheless, Dp16 animals do not display prenatal neurogenesis defects and abnormal brain growth (Aziz *et al.*, 2018). This remarkable result raises critical concerns regarding how prenatal and postnatal traits interact in DS animal models.

Other models such as the Tc(Hsa21)1TybEmfc (Tc1), were created as a result of the insertion of a freely segregating copy of the HSA21 into the mouse genome. However, this model also shows milder phenotypes than the Ts65Dn (Gribble *et al.*, 2013).

Transgenic animals that overexpress a single gene can be used to investigate the impact of potential dosage-sensitive genes. For instance, transgenic mouse models for *Dyrk1A* (Altafaj *et al.*, 2001; García-Cerro *et al.*, 2014; Navarro-Romero *et al.*, 2019) have shown that this have a critical role driving DS-associated brain abnormalities.

2.4. Therapeutic interventions for intellectual disability in Down syndrome

Early intervention with cognitive stimulation programs are now the only way to improve cognitive impairments in people with DS. Several studies have shown that these programs can improve cognitive ability, fine motor skills, and self-sufficiency (Table 4) (Bonnier, 2008). However, individuals with DS continue to experience significant constraints in their everyday lives (Hines and Bennett, 1996).

Although no pharmacotherapy for the cognitive and adaptive impairments associated with DS is currently licensed, there have been numerous pharmacological compounds capable of restoring

learning/memory abnormalities found in DS animal models (Potier and Reeves, 2016).

Currently, the most relevant results obtained from a clinical study are from using epigallocatechin-3-gallate. Researchers found that the combination of 45 % epigallocatechin-3-gallate green tea extract supplement and cognitive training for 12 months significantly enhanced visual recognition memory, inhibitory control, and adaptive behaviour in young adult DS subjects (16-34 years old) (de la Torre *et al.*, 2016) . The long-term effectiveness of this approach should be confirmed in phase III studies with a bigger sample size. Phase II trials are under underway in the paediatric population (6-12 years old) (NCT03624556).

The main therapies tested in Ts65Dn mice in the last decades targeted transmitter and receptor systems (Stagni *et al.*, 2015a). Several studies found an excessive GABA-mediated inhibitory tone in Ts65Dn, that reduce hippocampal-LTP (Kleschevnicov *et al.*, 2004; Costa and Grybko, 2005; Martínez-Cué *et al.*, 2014; Zorrilla de San Martin *et al.*, 2018). In this sense, pentylenetetrazol and picrotoxin which are $GABA_A$ antagonists have been tested in young and adult Ts65Dn mice. These drugs improved hippocampal-dependent memory and hippocampal LTP (Fernandez *et al.*, 2007a; Rueda *et al.*, 2008), but they also produced anxiety (Dorow *et al.*, 1983). For this reason, $GABA_AR$ α-5 negative allosteric modulators have been tested showing similar results without any of the previously observed negative side effects (Martínez-Cué *et al.*, 2013). These studies were the first to show that treatment of cognitive deficits in

DS is also possible in adulthood, despite widespread belief that pharmacological cognitive improvement can only occur during a short window of time in childhood (Stagni *et al.*, 2015b). Indeed, the $GABA_AR$ α-5 negative allosteric modulator basmisanil (RG1662) was assessed in the clinics, however, phase II was stopped prematurely because no efficacy was found (NCT01920633, NCT01436955, NCT02484703).

In addition to the gabaergic system, the glutamatergic system has been also targeted. Memantine, NMDA receptor uncompetitive antagonist, improves memory in Ts65Dn mice(Rueda *et al.*, 2010). Nowadays a phase II trial in young adults DS individuals (15-32 years old) is being carried out (NCT02304302).

Pro-neurogenic drugs have also been investigated. Fluoxetine, which is an inhibitor of serotonin reuptake, widely used as an antidepressant, enhances memory mainly by promoting adult neurogenesis (Bianchi et al., 2010; Stagni et al., 2013). Furthermore, prenatal treatment with fluoxetine maintained memory improvement at post-natal day 45 in addition to sustained improvement in brain structure such as cortical and hippocampal synapse development (Guidi *et al.*, 2014a). In addition to fluoxetine, the mood stabilizer lithium also improved improves hippocampal-dependent memory and hippocampal LTP by promoting adult neurogenesis in the dentate gyrus through Wnt/β-catenin pathway (Contestabile et al., 2013).

Several other targets have been tested in DS mouse models such as oxidative stress (Lockrow *et al.*, 2009), neurotrophic factors (Stagni *et al.*, 2017) and the mammalian target of rapamycin (mTOR) pathways (Andrade-Talavera *et al.*, 2015).

DRUG	MECHANISM	RESULTS IN Ts65Dn	REFERENCES	CLINICAL TRIAL
FOLINIC ACID	Folate metabolism	-	-	(Blehaut *et al.*, 2010; Mircher *et al.*, 2020)
EPIGALLOCATECHIN-3-GALLATE	DYRK1A inhibition	Rescued Neurogenesis, cellularity, LTP and long-term memory	(De la Torre *et al.*, 2014; Stagni *et al.*, 2016)	(De la Torre *et al.*, 2014; de la Torre *et al.*, 2016)
BASMISANIL	$GABA_AR$ α-5 Negative allosteric modulator	-	-	**Failed** - NTC02024789 (Hoffmann-La Roche)
PENTYLENETETRAZOL	Non-competitive $GABA_A$ antagonist	Rescued LTP and long-term memory	(Fernandez *et al.*, 2007a; Colas *et al.*, 2013)	**Ongoing** (COMPOSE study)
INSULINE GLULISINE	Glucose metabolism	-		(Rosenbloom *et al.*, 2020)

Table 4. **Summary of some of the pharmacological trials in Ts65Dn for cognitive enhancement and clinical trials performed with DS subjects**. Adapted from (Stagni *et al.*, 2015a).

The cholinergic theory of AD and anticholinesterase inhibitors have been key to pharmacological methods to treating AD in persons with DS. These early pharmacological therapies were shown to partially alleviate the symptoms of AD in DS, but had little effect on the disease's progression (Prasher *et al.*, 2002). Possible treatments targeting the amyloid pathway and trying to alter the AD progression are being studied, and they may be beneficial for treating AD in DS as well. Nevertheless, most of currently available treatments are just symptomatic (Table 5).
BACE inhibitors and secretase modulators decrease Aβ in Ts65Dn (Netzer *et al.*, 2010).At the preclinical level, active Aβ immunotherapy improved cognitive performance and reduced neuronal loss in animal models for DS (Belichenko *et al.*, 2016). Furthermore, anti-inflammatory drugs such as minocycline improved cholinergic cell loss and reduced microglial reactivity in Ts65Dn mice (Hunter *et al.*, 2004b).

DRUG	MECHANISM	RESULTS IN Ts65Dn	REFERENCES	CLINICAL TRIAL
ESTROGEN	Anti-oxidant, protects BFCNs	Protective and rescue memory	(Granholm *et al.*, 2002; Hunter *et al.*, 2004a)	NO
VITAMIN E	Anti-oxidant	Preventive	(Lockrow *et al.*, 2009)	(Lott *et al.*, 2011; Parisotto *et al.*, 2014)
DONEPEZIL	AChE inhibitor	No efficacious	(Rueda *et al.*, 2008)	FDA approved
MEMANTINE	NMDA reverse agonist	Rescue memory	(Costa *et al.*, 2008; Rueda *et al.*, 2010)	(Hanney *et al.*, 2012; Eady *et al.*, 2018)
L-DOPS	NE precursor	Rescue AD pathology and memory	(Salehi *et al.*, 2009; Fortress *et al.*, 2015)	NO

Table 5. Summary of pharmacological trials in Ts65Dn for AD and clinical trials performed with DS subjects. Adapted from (Hamlett *et al.*, 2015).

3. Fragile X syndrome

Fragile X syndrome (FXS) is the most common monogenic cause of inherited human ID and autism (De Vries *et al.*, 1998; Penagarikano *et al.*, 2007) and is caused by a lack of fragile X mental retardation 1 protein (FMRP; also known as synaptic functional regulator *FMR1*), an RNA binding protein that plays an important role in the regulation of several mRNAs in postsynaptic neurons. In the general population, the frequency of the FXS complete mutation is estimated to be 1 in 5,000 in males and 1 in 4,000 to 1 in 8,000 in females (Jin and Warren, 2003).

FXS subjects have a variety of physical features that might vary greatly between individuals. They have a long face, big and prominent ears, and a high broad forehead (Hagerman *et al.*, 2017). Other physical characteristics are macroorchidism in males, joint laxity, hypotonia, and mitral valve prolapse (Brady, 1984). Importantly, individuals with FXS present several neuropsychological manifestations such as ID, hyperactivity, hypersensitivity to sensorial stimuli, anxiety, attention deficit, epileptic seizures, and autistic traits (Penagarikano *et al.*, 2007). These manifestations produce an important limitation for FXS subjects' independence, and negatively affect their quality of life.

3.1. Genetic cause of fragile X syndrome

FXS is a X-linked dominant disease produced by a trinucleotide CGG expansion in the 5'-untranslated region of the fragile X mental retardation gene (*FMR1*), which encodes for the fragile X mental retardation protein (FMRP) (Verkerk *et al.*, 1991; Penagarikano *et al.*, 2007). The amount of CGG repeats in humans is highly variable. The *FMR1* gene has been categorized into four allelic variants based on these repeats: normal allele (5-55 repeats), premutation allele (55-200 repeats), and complete mutation allele (>200 repeats) (Dean *et al.*, 2016). The existence of the complete mutant allele causes the *FMR1* gene to be hypermethylated, resulting in transcriptional silence and the lack or deficiency of FMRP (Sutcliffe *et al.*, 1992; Coffee *et al.*, 1999). Even though premutation alleles do not induce FXS, RNA toxicity can be caused by overexpression of mRNA with the CGG expansion (Pretto *et al.*, 2014), with the risk of developing fragile X-associated tremor/ataxia syndrome, that courses with executive function impairment and slowly progressing neurodegenerative diseases, or developing premature ovarian insufficiency in females (Figure 9) (Hagerman and Hagerman, 2015).

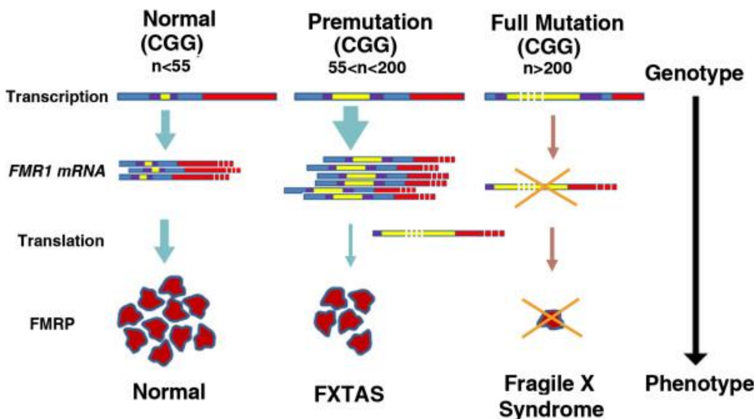

Figure 9. Pathogenic consequences of expanded CGG repeat in the *FMR1* gene. In the fragile X premutation range, repeat length is 55 to 200, leading to an increase in *FMR1* mRNA levels, a slight drop in FMRP, and an increased chance of developing fragile X-associated tremor/ataxia syndrome. The complete mutation has more than 200 repeats; *FMR1* transcription is suppressed owing to DNA hypermethylation; and the lack of FMRP leads in FXS (Berman *et al.*, 2014).

Importantly, FMRP is an RNA-binding protein that is abundant in the brain, particularly in synapses and its absence disrupts normal synaptic plasticity and appears to be the cause of intellectual impairment in FXS individuals (Penagarikano *et al.*, 2007).

3.2. Neuropathology and neuropsychological features in fragile X syndrome

One of the most limiting and prevalent features in FXS subjects is ID. Individuals with FXS present an IQ value between 20 and 70 (Penagarikano et al., 2007; de Esch et al., 2014). Interestingly, 15% of males and 70% of females have an IQ in the borderline to normal range but have cognitive and emotional difficulties (De Vries et al., 1996; Loesch et al., 2004). FXS individuals present affectations in

working memory, short-term memory, executive function, visuo-spatial abilities, and speech delay (Penagarikano et al., 2007; de Esch et al., 2014).

Many children with FXS develop anxiety and sensory hyperarousal (Talisa *et al.*, 2014). Indeed, 80% of males with FXS exhibit significant hyperactivity and are diagnosed with attention-deficit/hyperactivity disorder (ADHD), but only 40% of females with FXS are diagnosed with ADHD by school age (Cornish *et al.*, 2008). Furthermore, 50–60% of males and 20% of females with FXS also meet the criteria for autism spectrum disorder (ASD) (Harris *et al.*, 2008; Roberts *et al.*, 2009) in addition to a higher susceptibility to epilepsy (Figure 10) (Berry-Kravis, 2002).

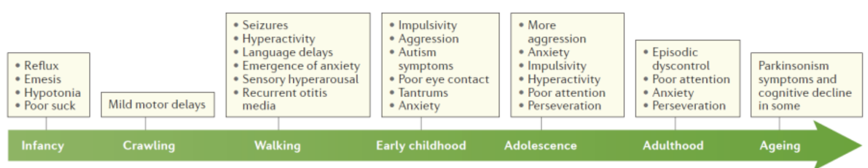

Figure 10. Main clinical manifestations of FXS subjects across the lifespan. (Hagerman *et al.*, 2017)**.**

Interestingly, there are no gross significant brain abnormalities in FXS subjects *post mortem* samples (Kooy *et al.*, 1999). Nevertheless, microanatomy abnormalities in individuals with FXS can be observeved such as changes in dendritic spine density and maturation, which are linked to synaptic plasticity impairments (Bakker *et al.*, 1994; He and Portera-Cailliau, 2013).

3.3. Fragile X syndrome mouse models

Over the years, animal models of FXS have been created to better understand the genetic and molecular mechanisms underlying this illness and, as a result, to develop effective treatments. The *Fmr1* gene is highly conserved between mice and humans (95 % homology) (Ashley *et al.*, 1993), and the identification of the genetic basis of FXS resulted in the creation of the first complete mutant mouse model, the *Fmr1* KO mouse.

There are now numerous mouse models of FXS available, including transgenic models that replicate some of the disorder's most significant characteristics (Bontekoe *et al.*, 1997, 2001; Lavedan *et al.*, 1998) and KO for *Fmr1* autosomal homolog 1 gene (Mikiko *et al.*, 1995). However, the most frequently studied mouse model of FXS is the *Fmr1* KO mouse, which was created by homologous recombination in which *Fmr1* was specifically knocked out (Bakker *et al.*, 1994).

The *Fmr1* KO mouse model replicates some of the FXS features, such as hyperactivity, macroorchidism, and heightened sensitivity to auditory stimuli, as well as a decreased acoustic startle response (Bakker *et al.*, 1994; Spencer *et al.*, 2005; Price *et al.*, 2007). Remarkably, the *Fmr1* KO mouse exhibits cognitive impairments in the NOR test (Busquets-Garcia *et al.*, 2013), Morris water maze task (D'Hooge *et al.*, 1997), radial arm maze task (Mineur *et al.*, 2002), avoidance task (Brennan *et al.*, 2006), and trace fear-conditioning test (Zhao *et al.*, 2005).

Defects in spine shape and density are reported in the hippocampus and neocortex of *Fmr1* KO mice (Nimchinsky *et al.*, 2001; Busquets-Garcia *et al.*, 2013). Furthermore, abnormal synaptic function and spine shape have been linked to abnormal signalling of excitatory group I mGluR (mGluR1 and mGluR5) (Levenga *et al.*, 2010). Notably, unregulated mGluR5 activity has been observed in FXS (Bear *et al.*, 2004; Michalon *et al.*, 2012), and genetic decrease of mGluR5 expression is sufficient to restore several characteristics of the *Fmr1* KO mice model (Dölen *et al.*, 2007). The lack of FMRP, which weakens the synapse, causes an exacerbated LTD in response to mGluR activation in FXS (Figure 11) (Bear *et al.*, 2004; Pop *et al.*, 2014).

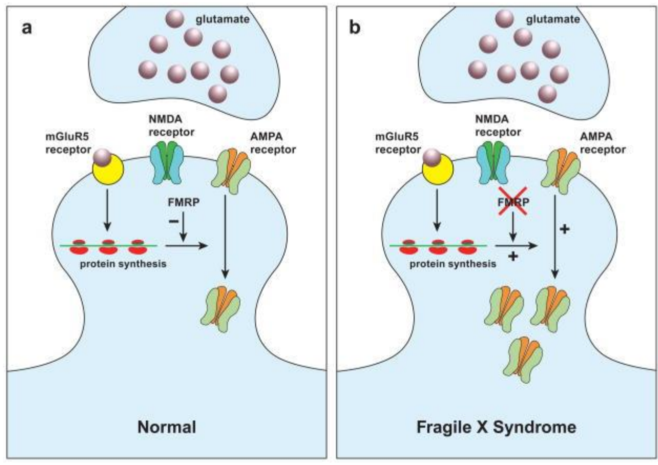

Figure 11. **The mGluR theory of fragile X syndrome**. (a) Glutamate stimulation of mGluR5 causes local mRNA translation at the synapse. Local protein synthesis promotes AMPA receptor internalization, which is required for long-term synaptic plasticity. FMRP suppresses transcription and decreases AMPA receptor internalization. (b) Neurons from FXS patients exhibit greater internalization of AMPA receptors in the absence of FMRP, which weakens the synapse, based on results in *Fmr1* KO mice (Levenga *et al.*, 2010).

In addition, several changes in the GABAergic system have been observed in the hippocampus of *Fmr1* KO mice, including a decrease in the expression of GABA receptor subunits resulting in a decreased GABAergic signalling (D'Hulst *et al.*, 2009; Paluszkiewicz *et al.*, 2011).

3.4. Therapeutic interventions for fragile X syndrome

The discovery of cellular and molecular changes in FXS spurred the introduction of therapies that operate on this syndrome. Several characteristics of the *Fmr1* KO mice were improved by either genetic decrease or pharmacological blockade of mGluR5, including brain structural changes, susceptibility to audiogenic seizures, and hyperactivity (Santoro *et al.*, 2012). Furthermore, mGluR5 inhibitors repaired certain phenotypic changes in FXS models (Krueger and Bear, 2011; Michalon *et al.*, 2012). An initial phase I/II study with the mGluR5 negative modulator AFQ056 demonstrated improvement in hyperactivity, stereotypic behavior, and improper speech in individuals with FXS, but successive phase IIb trials of AFQ056 and a comparable mGluR5 modulator RO4917523 did not show such an improvement (Hagerman *et al.*, 2018).

Treatment with certain $GABA_B$R agonists also decreased *Fmr1* KO mice's sensitivity to audiogenic seizures (Pacey *et al.*, 2009) and corrected *Fmr1* KO mice's increased protein synthesis in the hippocampus and social behaviour (Henderson *et al.*, 2012). However, treatment with $GABA_B$R arbaclofen did not demonstrate any improvement in a phase III study (Berry-Kravis *et al.*, 2018).

$GABA_AR$ agonists can also be used to enhance GABAergic tone and they have seen to improve anxiety, hyperactivity, rotarod performance, and the incidence of audiogenic seizures in *Fmr1* KO mice (Heulens *et al.*, 2012).

Other treatment options for FXS may include minocycline, an MMP9 inhibitor (Leigh *et al.*, 2013), temsirolimus, an mTOR inhibitor (Busquets-Garcia *et al.*, 2013) and rimonabant, a CB1R antagonist (Busquets-Garcia *et al.*, 2013; Gomis-González *et al.*, 2016).

4. The endocannabinoid system

For thousands of years, *Cannabis sativa* plant and its derivatives, such as marijuana, have been utilized for recreational and medicinal purposes. *Cannabis sativa* plant includes more than 120 phytocannabinoids (Morales *et al.*, 2017a) with closely similar structures and physical characteristics (Mechoulam and Parker, 2013). Among them, Δ^9-tetrahydrocannabinol (Δ^9-THC) was identified in 1964 (Gaoni and Mechoulam, 1964). Several years later, the identification and cloning of Δ^9-THC receptors from animal tissues (Matsuda *et al.*, 1990) resulted in the discovery of an endogenous modulatory system known as the endocannabinoid system (ECS).

4.1. Components of the endocannabinoid system

The ECS is widely distributed in the organism and plays a role in a variety of physiological processes. Cannabinoid receptors, their endogenous ligands known as endocannabinoids, and the enzymes involved in their production and degradation make up this system.

Cannabinoid compounds were once thought to produce their pharmacological effects via nonspecific interactions with membrane lipids due to their hydrophobic characteristics. It wasn't until the late 1980s that the hypothesis of the existence of dedicated receptors was considered and the first cannabinoid receptors were characterized (Devane *et al.*, 1988; Herkenham *et al.*, 1990).

4.1.1. Cannabinoid receptors

The cannabinoid type-1 receptor (CB1R) and the cannabinoid type-2 receptor (CB2R) are nowadays the two most well-known cannabinoid receptors. The first cannabinoid receptor to be cloned was CB1R (Matsuda *et al.*, 1990), followed by CB2R three years later (Munro *et al.*, 1993). Cannabinoid receptors are G-protein coupled receptors (GPCRs) with seven transmembrane domains that are mostly linked to Gi/o proteins (Childers and Deadwyler, 1996). Furthermore, these cannabinoid receptors are distributed differently across the body. CB1R is significantly expressed in the central nervous system (CNS), whereas CB2R is mostly expressed in the immune system (Svíženská *et al.*, 2008).

Furthermore, numerous recent investigations have suggested that additional receptors may bind cannabis ligands and modulate their actions, including some orphan GPCRs, such as GPR55, GPR18, and GPR110 (Kohno *et al.*, 2006; Pertwee, 2007; Lee *et al.*, 2016), transient receptor potential vanilloid 1 (TRPV1) (De Petrocellis and Di Marzo, 2010), sphingosine-1-phosphate lipid receptors GPR3, GPR6, and GPR12 (Morales and Reggio, 2017), and peroxisome proliferator-activated receptors (PPAR) (O'Sullivan, 2007).

4.1.1.1. Cannabinoid type-1 receptor

CB1R is the most common GPCR in the central nervous system and it is responsible for Δ^9-THC's psychotropic effects, including learning and memory impairments (Kano *et al.*, 2009; Puighermanal *et al.*,

2009). CB1R was discovered in a rat brain cDNA library in 1990 (Matsuda *et al.*, 1990). Subsequent investigations cloned CB1R homologs from human (Gerard *et al.*, 1991) and mice (Chakrabarti *et al.*, 1995), which share 97 to 99 percent of the amino acid sequence. CB1R regulates a wide range of physiological functions, including learning and memory, motor coordination, pain perception, hunger management, body temperature regulation, and brain development (Garcia *et al.*, 2016).

CB1R distribution in rodents (Herkenham *et al.*, 1991; Tsou *et al.*, 1998) and humans (Glass *et al.*, 1997) has been thoroughly studied. The hippocampus, amygdala, cerebellum, periaqueductal gray, substantia nigra pars reticulata, and certain cortical regions such as the somatosensory, cingulate, and entorhinal cortex have the greatest density of CB1R in the CNS. The medial hypothalamus, basal forebrain, solitary nucleus, and spinal cord all reveal moderate levels of CB1R. Low amounts can also be detected in other parts of the brain, such as the thalamus and the brainstem (Figure 12)(Svíženská *et al.*, 2008; Flores *et al.*, 2013). CB1R is also found in peripheral tissues such as the cardiovascular system (Sierra *et al.*, 2018), fat tissue, liver, and 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). Remarkably, CB1R mRNA and protein expression vary at different developmental stages and can be dysregulated in a variety of clinical conditions (Laprairie *et al.*, 2012).

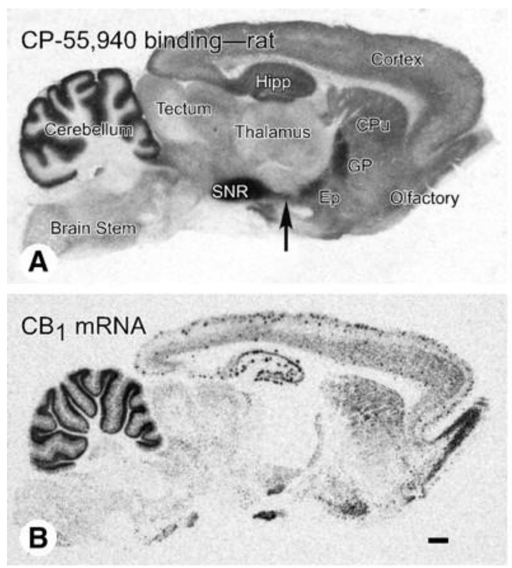

Figure 12. Autoradiographic film images displaying CB1R protein and mRNA localization in rat brain. (A) Binding assay with the tritiated ligand CP-55,940 in a sagittal section of rat brain. (B) Hybridization of a CB1R-oligonucleotide probe displaying expression of CB1R at mRNA level (Freund *et al.*, 2003).

CB1R is mostly expressed in the membrane of neuronal presynaptic terminals, where it regulates the release of neurotransmitters. CB1R may also regulate the release of other neurotransmitters such as acetylcholine, noradrenaline, dopamine, D-aspartate, and cholecystokinin (Pertwee and Ross, 2002; Lutz, 2020). Furthermore, other than presynaptic terminals, CB1R has been found in various places in the recent decade, according to many publications. CB1R appears to be present at postsynaptic terminals in at least two areas: the cortex, where it may control self-inhibition processes (Marinelli *et al.*, 2009), and the hippocampus, where it may regulate synaptic plasticity via hyperpolarization-activated cyclic nucleotide-

gated channels (Maroso *et al.*, 2016). CB1R has also been discovered in cells other than neurons, such as astrocytes, where it plays a role in synaptic plasticity, and microglia, where it plays a role in inflammatory processes (Cabral, 2005; Navarrete and Araque, 2008). In addition, astrocytic CB1R can control the release of gliotransmitters (Lutz, 2020). Furthermore, recent research has discovered the existence of CB1R in intracellular organelles. CB1R has been found in the mitochondria, where it regulates cellular metabolism and may have an influence on synaptic function and memory formation (Hebert-Chatelain *et al.*, 2016). Furthermore, the presence of CB1R in mitochondria has been described in neurones and astrocytes (Jimenez-Blasco *et al.*, 2020). There is additional evidence that CB1R is present in endosomal and lysosomal compartments, where its function is unknown (Thibault *et al.*, 2013). In addition, CB1R has been found in oligodendrocytes, oligodendrocyte precursor cells, and adult neural stem cells (Figure 13) (Lutz, 2020).

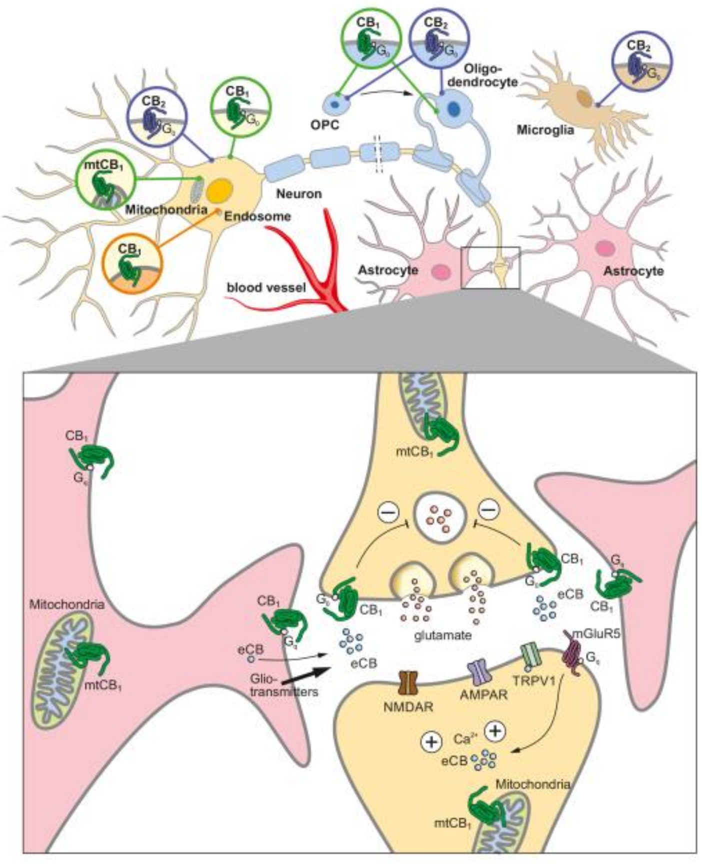

Figure 13. **Illustration of the cellular distribution of CB1R and CB2R in the CNS**. CB1R is present in neurons, astrocytes, oligodendrocytes and microglia. Functional CB1R are found on the plasma membrane, but also in mitochondria (mtCB1) of neurons and astrocytes. Presynaptic CB1 receptor reduces neurotransmitter release, at a glutamatergic synapse, among others. CB2R can be found in reactive microglia, oligodendrocytes, and to a lesser extent, in neurons (Lutz, 2020).

CB1R expression varies not just across different brain regions but also between various cell types. Surprisingly, the levels of expression are unrelated to their functional significance. CB1R is found at high levels on cholecystokinin inhibitory terminals in the hippocampus

and at low levels on glutamatergic terminals and astrocytes (Kano *et al.*, 2009). Even though glutamatergic CB1R expression is lower than GABAergic CB1R expression, glutamatergic CB1R is better linked to downstream signal transduction (Steindel *et al.*, 2013). CB1R on glutamatergic terminals play a significant role in the control of hippocampal excitability (Marsicano *et al.*, 2003; Monory *et al.*, 2006).

4.1.1.2. Cannabinoid type-2 receptor

CB2R was cloned in 1993 and shares 44% of homology with CB1R. (Munro *et al.*, 1993). It is extensively expressed in the immune system, where it regulates immunological response and mediates cannabis' anti-inflammatory effects (Buckley *et al.*, 2000). Because of its modest expression levels and a lack of accurate methods to investigate it, the existence of CB2R on healthy brains has been disputed. CB2R is present in healthy brains, according to electrophysiological, anatomical, and behavioral data (Van Sickle *et al.*, 2005; Gong *et al.*, 2006; Onaivi, 2007; Den Boon *et al.*, 2012; Stempel *et al.*, 2016). CB2R expression is inducible; it is modest in healthy situations but substantially increases in pathological conditions such neuropathic pain (Svíženská *et al.*, 2013), neurological disorders (Palazuelos *et al.*, 2009; Aso and Ferrer, 2016; Concannon and Dowd, 2016; López *et al.*, 2018), or stroke (Yu *et al.*, 2015). This rise is thought to be a protective compensating

mechanism, as CB2R activation decreases neuroinflammation (Palazuelos *et al.*, 2008, 2009).

CB2R is expressed in astrocytes, perivascular microglia, and a subpopulation of neurons in the brain (Sheng *et al.*, 2005; Gong *et al.*, 2006; Stempel *et al.*, 2016). CB2R, unlike CB1R, is mostly expressed in postsynaptic terminals, where it regulates neuronal excitability (H. Y. Zhang *et al.*, 2014; Stempel *et al.*, 2016). In pyramidal cells of the hippocampal regions CA2 and CA3, CB2R regulates a self-inhibitory form of plasticity (Stempel *et al.*, 2016). However, further research is needed to determine the physiological significance of CB2R in the CNS in healthy conditions.

4.1.2. Endocannabinoids

Endogenous cannabinoid ligands, also known as endocannabinoids, and exogenous cannabinoids are the two primary types of cannabinoids. In 1992, N-arachidonoyl ethanolamine (AEA, often known as anandamide) was identified as the first endocannabinoid (Devane *et al.*, 1992). Another endocannabinoid, 2-arachidonoyl glycerol (2-AG), was discovered three years later (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). Although the physiological importance of other endocannabinoids is still being debated (Fonseca *et al.*, 2013). AEA and 2-AG are the most studied endocannabinoids. They belong to the N-acylethanolamine and monoacylglycerol families, respectively (Figure 14). Both are lipidic compounds that are not pre-stored in secretory vesicles, unlike conventional

neurotransmitters. Most evidence suggest that, they are created "on demand" in response to activity, although other observations suggest that at least AEA may be kept inside the cell, challenging the traditional notion (Oddi *et al.*, 2008).

N-arachidonoyl ethanolamine (AEA) **2-arachidonoyl glycerol (2-AG)**

Figure 14. **Chemical structure of AEA and 2-AG, the two primary endocannabinoids**. Adapted from (Mechoulam *et al.*, 2014).

While AEA is a partial agonist for both CB1R and CB2R, 2-AG is a complete agonist for both receptors, with a concentration in the brain homogenate 170 times that of AEA (Stella *et al.*, 1997). As a result, 2-AG is the most significant endogenous ligand of CB1R and CB2R, as well as the principal endogenous cannabinoid agonist (Sugiura *et al.*, 2006). The affinity of both ligands, AEA and 2-AG, for CB2R is somewhat lower than that of CB1R (Pertwee *et al.*, 2010). Furthermore, both endocannabinoids bind to additional receptors: TRPV1 (Zygmunt *et al.*, 1999) and PPAR (O'Sullivan, 2007) are activated by AEA, but TRPV1 is not activated by 2-AG (Du *et al.*, 2011).

The "on demand" biosynthesis of endocannabinoids at the synapse is triggered by an increase in calcium intracellular concentration, according to the traditional understanding of endocannabinoid signaling (Matias and Di Marzo, 2007). Endocannabinoids are

released from the postsynaptic neuron and diffuse retrogradely to activate cannabinoid receptors. Then endocannabinoid build-up causes presynaptic cannabinoid receptor activation and a temporary reduction in neurotransmitter release in the neighboring synapses expressing cannabinoid receptors. In both excitatory and inhibitory synapses, endocannabinoids serve as retrograde synaptic messengers, preventing excessive neuronal activity and maintaining balance in healthy and pathological circumstances (Wilson and Nicoll, 2002; Ohno-Shosaku *et al.*, 2012).

Several lipidic compounds resemble endocannabinoids in structure but are unable to modulate cannabinoid receptors. Endocannabinoid-like compounds are similar to endocannabinoids in that they share certain metabolic enzymes. It has been suggested that certain chemicals may interfere with the ECS function in some way (Fonseca *et al.*, 2013). For example, they specifically modify endocannabinoid activity via the so-called "entourage effect," which affects endocannabinoid metabolism locally (Ben-Shabat *et al.*, 1998; Jonsson *et al.*, 2001; Ferber *et al.*, 2020).

4.1.3. Enzymes involved in the biosynthesis and degradation of endocannabinoids

Endocannabinoids levels are constantly regulated since they are not stored in vesicles and must be synthesized on demand. Their production and degradation are aided by a variety of enzymes. Both AEA and 2-AG are lipidic structures that are obtained from arachidonic

acid, produced by the hydrolysis of membrane phospholipid precursors (Iannotti *et al.*, 2016).

Two major enzymatic processes are required for AEA synthesis: first, phosphatidyl-ethanolamine is transacylated to generate N-acylphosphatidyl-ethanolamines (NAPEs) by a calcium-dependent N-acyltransferase (NAT). Second, a particular phospholipase, NAPE-PLD, hydrolyzes NAPE to produce AEA and phosphatidic acid (Di Marzo, 2006). Alternative routes to produce AEA exist, including the phospholipase C (PLC) (Liu *et al.*, 2006) and the α/β-hydrolase domain type-4 (ABHD4) (Simon and Cravatt, 2006).

Two enzymatic processes also contribute to the formation of 2-AG. PLC produces 1,2-diacylglycerol by hydrolyzing the membrane phospholipid sn-2-arachidonoyl-PIP2 (DAG). Second, DAG is degraded into 2-AG by one of two diacylglycerol lipases, DAGL-α or DAGL-β. The primary enzyme responsible to produce 2-AG in the CNS is DAGL- α (Tanimura *et al.*, 2010).

Once the cell's endocannabinoids have activated their targets, they are quickly carried into the intracellular region, where they are hydrolyzed or oxidized. Several methods for endocannabinoid cellular absorption have been proposed. Diffusion through the plasma membrane as a function of concentration gradient (Kiecolt-Glaser *et al.*, 2003) has been proposed as one of them. Other hypothesis suggests the presence of a carrier protein, the “endocannabinoid membrane transporter” (Fegley *et al.*, 2004; Ligresti *et al.*, 2004) or that the uptake happens via endocytosis (McFarland *et al.*, 2004).

Endocannabinoids will be degraded by particular enzymes once they have been reuptaken. The fatty acid amide hydrolase (FAAH) breaks down AEA into AA and ethanolamine (Di Marzo, 2006). Nevertheless, the inducible cyclooxygenase-2 (COX-2) and other lipoxygenases also degrade AEA to produce prostanglandin-ethanolamides and hydroxyeicosatetraenoic-ethanolamide, respectively (Deutsch and Chin, 1993; Cravatt *et al.*, 1996).

For 2-AG degradation, the enzyme monoacylglycerol lipase (MAGL) metabolizes roughly 85% of 2-AG, generating AA and glycerol as products (Di Marzo, 2006). The remaining 15% is broken down by the enzymes α/β-hydrolase 6 and 12 (ABHD6 and ABHD12) (Blankman *et al.*, 2007). COX-2 and other lipoxygenases may also oxygenate 2-AG, resulting in prostaglandin-glycerol esters and hydroxyeicosatetraenoic-glycerol esters (Figure 15) (Kozak *et al.*, 2002).

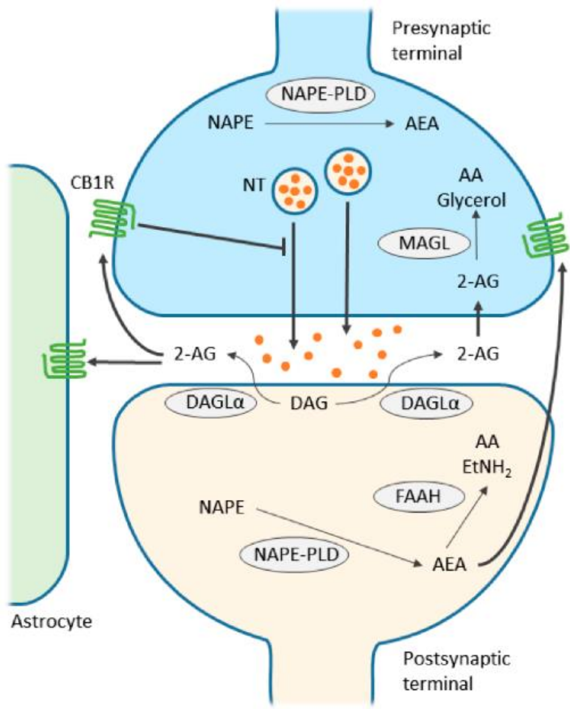

Figure 15. Graphic representation of the principal pathways of synthesis and degradation of endocannabinoids in the synapse. Arachidonic acid (AA); 2-arachidonolglycerol (2-AG); diacylglycerol (DAG); diacylglycerol lipase-α (DAGLα); ethanolamine ($EtNH_2$); fatty acid amide hydrolase (FAAH); monoacylglycerol lipase (MAGL); N-acyl-phosphatidylethanolamine (NAPE); NAPE-specific phospholipase D (NAPE-PLD); transient receptor potential cation channel subfamily V member 1 (TRPV1) (Zou and Kumar, 2018).

To conclude, the ECS is a neuromodulatory system found in virtually all mammals that is involved in fine-tuning synaptic homeostasis. The following diagrams show an overview of ECS regulation in synaptic content, which includes all ECS components as well as neurotransmitter release modulation (Figure 16).

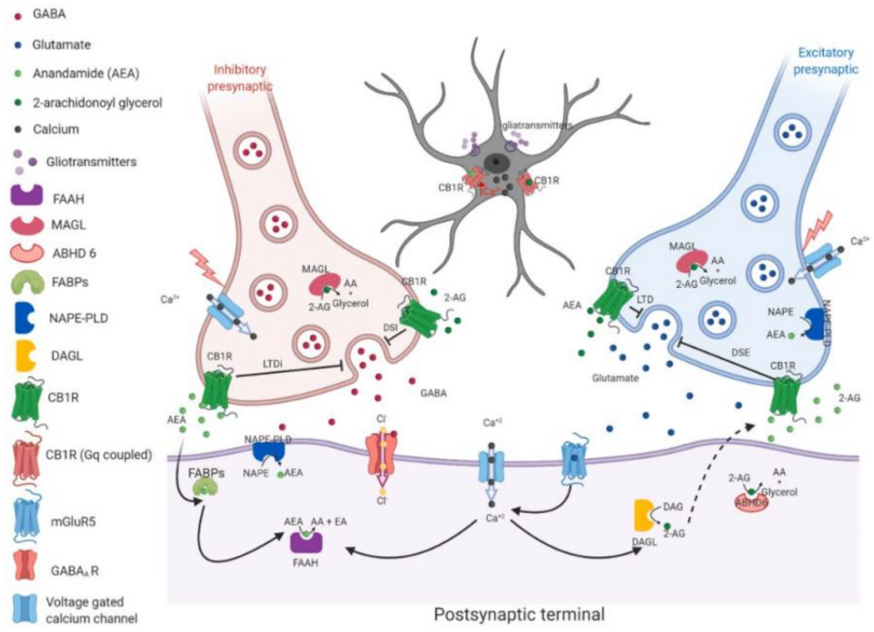

Figure 16. **The ECS in its synaptic environment**. Endocannabinoid synthesis is boosted by calcium influx. Endocannabinoids will bind to presynaptic CB1R after retrograde diffusion from the postsynaptic membrane. Synaptic plasticity is caused by CB1R signalling events that limit neurotransmitter release (DSE or DSI). The activation of astrocytic CB1Rs causes the release of gliatransmitters, which shifts the balance of excitement and inhibition (Gunduz-Cinar, 2021).

4.2. Cannabinoid intracellular signalling pathways in the brain.

The activation of various signaling pathways when cannabinoid receptors are stimulated generates a variety of consequences. CB1R works biologically as a GPCR by activating G-proteins, namely the Gi/o family (Gα, Gβ, and Gγ). When Gi/o is activated, adenylyl cyclase is inhibited, resulting in a decrease in cyclic adenosine monophosphate (cAMP) levels and PKA activity (Howlett, 1985; Howlett *et al.*, 1986). Gi/o activation also controls the phosphorylation and activation of MAPK family members such as

extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK) (Bouaboula *et al.*, 1995, 1996).

Stimulation of CB1R results in a PLC-dependent intracellular temporary rise in calcium, which can be mediated by either Gi/o or Gq proteins (Sugiura *et al.*, 1997; Lauckner *et al.*, 2005). CB1R can also link Gs proteins, triggering adenylyl cyclase activation, in certain situations (Glass and Felder, 1997).

There is evidence that CB1R activation can alter other signaling pathways such as 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.*, 2018b).

Aside from the effects mediated by G protein signalling, CB1R activation also causes the scaffold proteins β-arrestins to be recruited to the plasma membrane, which facilitates CB1R desensitization and internalization, as well as activating intracellular pathways such as the MAPK (Turu and Hunyady, 2010; Nogueras-Ortiz and Yudowski, 2016).

Overall, the response elicited by CB1R stimulation is complicated, not only because of the wide range of effectors, but also because of crosstalk across the many activated signalling pathways (Figure 17).

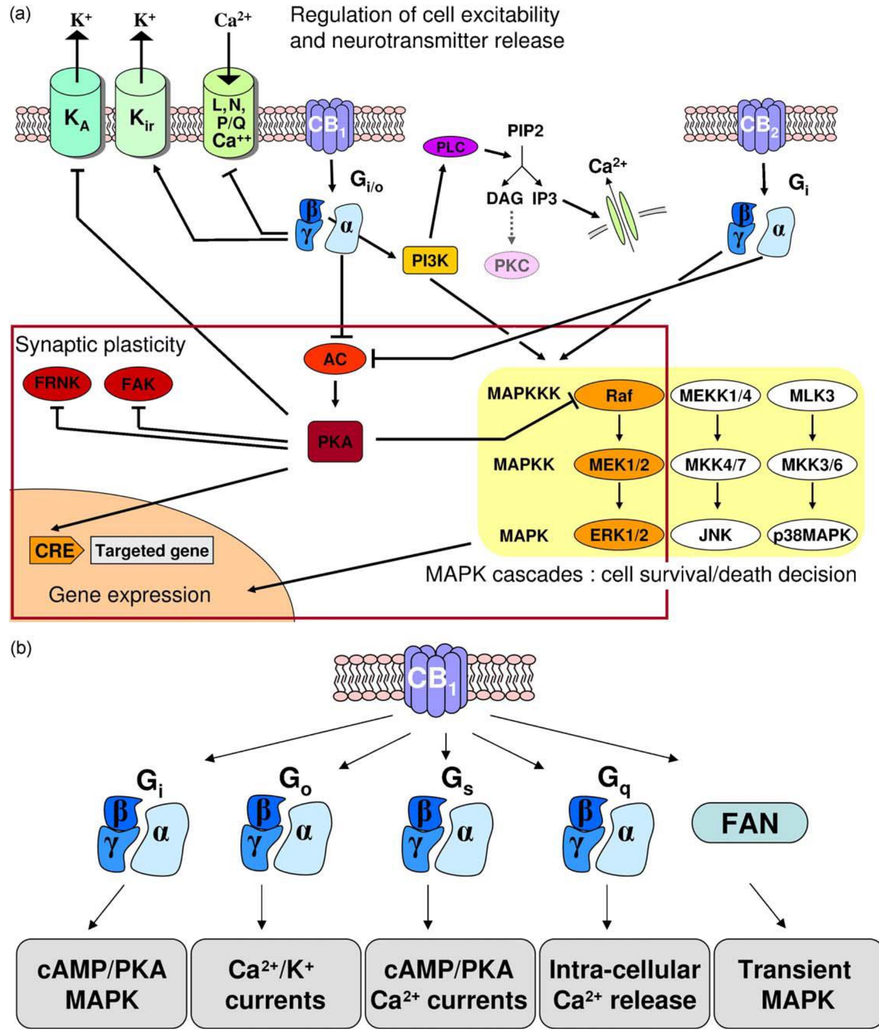

Figure 17. Cannabinoid signalling complexity. (A) The diversity of responses to protein kinase A (PKA) inhibition demonstrate crosstalk among the various pathways activated by the CB1R. (B) The CB1 cannabinoid receptor also interacts with non-G protein partners, including the adaptor protein FAN. The variety and selectivity of responses mediated by cannabinoid receptors is facilitated by each G protein's preferential activation of various intracellular effectors. 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. Physiological role of the endocannabinoid system

Because cannabinoid receptors are widely distributed across the CNS and peripheral organs, the ECS is engaged in a variety of physiological activities. Such physiological activities have been well described owing to research into the effects of *Cannabis sativa* derivates in people and animals, as well as the development of pharmacological (agonists and antagonists of the CB1R and CB2R) and genetic (KO mice) tools (Zou and Kumar, 2018).

In the nervous system the ECS is critical for synaptic homeostasis and the proper development of brain function. The numerous brain areas where ECS components are expressed, notably CB1R, have been linked to a range of brain processes, indicating that the ECS plays a variety of physiological roles (Table 6) (Shu-Jung Hu and Mackie, 2015). Particularly, relevant for the thesis CB1R is located in areas involved in the control of learning and memory processes (hippocampus and cortex) (Kano, 2009), fine control of movement and cerebellar learning performance (cerebellum and basal ganglia) (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, energy balance and temperature regulation (hypothalamus) (Di Marzo and Matias, 2005), reward processes (striatum and ventral tegmental area) (Solinas *et al.*, 2008) and pain modulation (spinal cord) (Guindon and Hohmann, 2012).

CNS STRUCTURE	PHYSIOLOGICAL ROLE	EXAMPLES OF PATHOLOGICAL ROLE
HIPPOCAMPUS	Learning and memory	Memory impairment
BASAL GANGLIA	Movement control	Slowed reaction time
CEREBELLUM	Motor coordination balance	Motor coordination impairment
NEOCORTEX	Higher cognitive functions	Altered cognitive functions (judgement, consciousness)
NUCLEUS ACCUMBENS	Motivation and reward	Drug addiction
HYPOTHALAMUS	Body housekeeping functions (body temperature regulation, reproductive function)	Neuroendocrine alterations (increase appetite)
BRAIN STEM	Sleep and arousal, motor control	Alterations on heart rate and blood pressure
AMYGDALA	Emotional response and fear	Anxiety and paranoia
SPINAL CORD	Nociception	Altered pain sensitivity

Table 6. ECS function in brain areas with the greatest CB1R expression, both physiological and pathological. CB1R is found throughout the brain, where it performs several physiological functions and contributes to a variety of pathological conditions. Adapted from (Kano, 2009; Shu-Jung Hu and Mackie, 2015).

The ECS has been shown to have a function in the immunological, reproductive, digestive, and cardiovascular systems, among other things, at the peripheral level. Inflammation, platelet aggregation, oocyte maturation, spermatogenesis development, gastrointestinal motility and metabolism, energy balance via lipid and glucose homeostasis, blood pressure, and heart rate are all regulated by this system (Zou and Kumar, 2018).

At the cellular level, the ECS regulates a variety of biological activities, including cell proliferation, differentiation, migration, survival, and synapse formation, and its regulation may vary depending on the cellular environment (Galve-Roperh *et al.*, 2013).

4.3.1. Role of the endocannabinoid system in learning and memory

Several behavioral evidences support the idea that the ECS system is important for learning and memory control (Davies *et al.*, 2002; Puighermanal *et al.*, 2012). Furthermore, the distribution of ECS components in the hippocampus, a critical memory-processing area, is compatible with a memory-functioning role (Di Marzo *et al.*, 2000).

In general, cannabinoid agonists impair learning and memory, whereas cannabinoid antagonists increase memory performance. The memory effects produced by cannabinoids, however, can vary depending on a variety of factors, including the type of compound tested (direct/indirect agonist or antagonist), the dosage, the route of administration, the memory task performed (Kruk-Slomka *et al.*,

2017), the age of the animals (Bilkei-Gorzo *et al.*, 2017), and whether the animals are naive or an animal model for a pathological condition (Calabrese and Rubio-Casillas, 2018; Escudero-Lara *et al.*, 2020). It is also worth noting that cannabinoids have an impact on other behaviors including anxiety, locomotion, eating, motivation, and nociception, all of which might affect the results in the cognitive tests.

Cannabis use in humans has been shown to cause deficiencies in various elements of learning and memory (Volkow *et al.*, 2016). Cannabis use affects episodic and working memory in adults but has no effect on recall of previously learned information (Ranganathan and D’Souza, 2006). As a result, administering CB1R agonists to rodents causes impairments in a variety of memory tasks, including the NOR test (Puighermanal *et al.*, 2009), 8-arm radial arm (Egashira *et al.*, 2002), spatial alternation in a T-maze, working-memory (Fadda *et al.*, 2004), Morris water maze (Da Silva and Takahashi, 2002), contextual fear-conditioning (Pamplona and Takahashi, 2006) and passive avoidance (Kruk-Slomka and Biala, 2016). These effects appear to be CB1R-dependent, since CB1R antagonists prevented some of the memory impairments (Pamplona and Takahashi, 2006; Barna *et al.*, 2007). The majority of the results were achieved by administering CB1R agonists systemically. The compounds have also been infused intrahippocampally, demonstrating the significance of hippocampus CB1R (Lichtman *et al.*, 1995; Clarke *et al.*, 2008; Suenaga *et al.*, 2008).

As mentioned, in contrast to CB1R agonists, CB1R blockage is frequently associated with improved memory. The CB1R antagonist/inverse agonist rimonabant improves memory 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). Nevertheless, other paradigms, such as the spatial delayed-non-match-to-sample, are unaffected by CB1R antagonist treatment (Mallet and Beninger, 1996).

In addition to pharmacological studies, CB1R KO mice show improved cognitive performance in a variety of tasks, including the NOR test (Reibaud *et al.*, 1999; Maccarrone and Finazzi-Agró, 2002) and contextual fear conditioning (Jacob *et al.*, 2012). In the MWM, however, these mice show impairments in reversal learning (Varvel and Lichtman, 2002).

The increase of endocannabinoid tone by inhibiting endocannabinoid metabolism has a wide range of mnemonic implications. Increasing AEA levels by FAAH inhibitors affects object recognition memory, working memory and spatial memory in the Y-maze (Busquets-Garcia *et al.*, 2011; Basavarajappa *et al.*, 2014). It also promotes spatial memory (Varvel *et al.*, 2007) and passive avoidance learning in the MWM (Mazzola *et al.*, 2009). Because AEA and other fatty acids like oleoylethanolamine and palmitoylethanolamine, which are likewise increased following FAAH inhibition, can also bind to PPAR-α, these differences may arise through a CB1R-independent process (Mazzola *et al.*, 2009).

In general, the findings show that cannabinoid agonists decrease working and long-term memory, whereas cannabinoid antagonists/inverse agonists or genetic deletion of cannabinoid receptors enhance memory (Zanettini *et al.*, 2011). Various experimental circumstances may be responsible for differing outcomes (task, dose, route of administration, timing of administration, specie, and strain among others). CB1R expression in various brain areas or cell types may be required for different behavioral tasks. It's also worth noting that cannabinoids have an impact on other behaviors including anxiety, locomotion, food intake, motivation, and nociception, all of which might affect the results.

The importance of the ECS in learning and memory is further supported by electrophysiological experiments that measure synaptic plasticity. Multiple kinds of synaptic plasticity are mediated by endocannabinoids, and various investigations have indicated that the ECS is involved in hippocampus LTP, which has previously been linked to memory and learning processes. Cannabis treatment inhibits LTP from acting on CB1R (Stella *et al.*, 1997), but CB1R KO mice have increased LTP (Bohme *et al.*, 1999; Jacob *et al.*, 2012), suggesting that cannabinoid activation limits LTP.

4.3.2. Role of the endocannabinoid system in neuroinflammation

Immunomodulation and inflammation are essential functions of the ECS (Mecha *et al.*, 2016). Several ECS components are increased

during inflammation to protect cells from harm and to counteract microglia's large production of toxic cytokines and inflammatory mediators (Sánchez and García-Merino, 2012). As previously stated, CB2R expression is low in surveillant/resting microglial cells, but it is increased in activated cells (Cabral *et al.*, 2008). CB2R activation decreases antigen presentation, suppresses cytokine production, and modifies Immune cell motility (Ehrhart *et al.*, 2005; Miller and Stella, 2008). Endocannabinoids have also been shown to cause the anti-inflammatory M2 microglial phenotype (Sánchez and García-Merino, 2012; Mecha *et al.*, 2016). As a result, CB2R activation has been suggested as a therapy for neuroinflammation in neurodegenerative disorders.

Inflammatory processes are also influenced by CB1R. Nitric oxide and several pro-inflammatory cytokines are inhibited when CB1R is stimulated (Waksman *et al.*, 1999). In this context, 2-AG treatment following brain damage in mice suppresses the production of pro-inflammatory cytokines IL-6, IL-1α, IL-1β, and TNF-α (Panikashvili *et al.*, 2006). Other cannabinoid-like receptors expressed in microglial cells include GPR55 (Kallendrusch *et al.*, 2013), PPARγ (Lee and Won, 2014), and TRPV1 (Raboune *et al.*, 2014), which respond to endogenous or synthetic cannabinoids to influence microglial activation, migration, and proliferation processes.

In conclusion, the ECS influence a variety of activities in microglial cells, including activation, proliferation, cytokine production,

migration, and phagocytosis, suggesting that the ECS system might be a significant target in the treatment of neuroinflammation

4.4. Pharmacological modulation and therapeutic potential of the ECS

Multiple pathophysiological conditions have been linked to changes in ECS signalling. Increased expression of cannabinoid receptors, coupling receptor effectiveness, endocannabinoid metabolizing enzymes expression, or endocannabinoid levels have all been linked to upregulation of ECS components in various diseases. These changes are a protective mechanism that slows the course of diseases and reduces symptoms. These alterations may also be maladaptive, leading to or worsening symptoms in certain circumstances (Pertwee, 2009; Miller and Devi, 2011). The manipulation of the ECS is offered in this scenario as a promising treatment target for a variety of diseases.

Phytocannabinoids and synthetic cannabinoids are two types of exogenous cannabinoids that are categorized based on their origin.

4.4.1. Phytocannabinoids

For centuries, cannabis medicines have been used for medicinal purposes. Cannabinoids are continuously being studied in the hopes of finding medicinal uses. Analgesia, appetite stimulation, antiemesis, immunosuppression, antineoplastic, and anti-

inflammatory effects are only a few of the advantages of using cannabis agonists (Pertwee, 2012).

Phytocannabinoids are a group of C_{21} terpenophenolic components 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 (Figure 18) (Turner *et al.*, 2017). Δ^9-THC is a partial agonist of CB1R and CB2R, and it is the primary cause of marijuana's psychoactive effects (Morales *et al.*, 2017b). Cannabidiol is the plant's second most prevalent component, yet unlike Δ^9-THC, it has no psychoactive properties. Although cannabidiol's affinity for CB1R and CB2R is modest, *in vitro* studies show that it may operate as an antagonist at CB1R and as an inverse agonist at CB2R (Thomas *et al.*, 2007). Furthermore, *in vivo* research suggests that cannabidiol may influence GPR55 activation (Morales and Reggio, 2017). Cannabidiol has recently been discovered to bind to adenosine A_{2A} and CB1R heteromers, reducing the cognitive impairment caused by Δ^9-THC (Aso *et al.*, 2019). Cannabidiol is currently gaining popularity due to its anti-inflammatory, analgesic, anti-anxiety, and anti-tumor properties, among other things. The mechanistic basis of cannabidiol effects, on the other hand, are yet to be understood (Morales and Reggio, 2017).

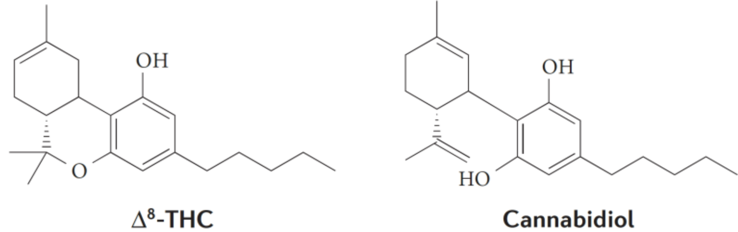

Figure 18. Chemical structure of the phytocannabinoids Δ⁹-THC and cannabidiol. Adapted from (Mechoulam *et al.*, 2014).

A variety of CB1R/CB2R agonists have previously been created (Table 7). For the control of nausea and vomiting caused by chemotherapy, Δ^9-THC (dronabinol; Marinol®) and its synthetic counterpart nabilone (Cesamet®) were first authorized. Following that, dronabinol was used as an appetite stimulant in individuals suffering from cachexia brought on by chemotherapy or AIDS (Pertwee, 2009, 2012). Sativex®, which contains about equal amounts of Δ^9-THC and cannabidiol, is now utilized to treat spasticity in multiple sclerosis patients as well as neuropathic pain (Urits *et al.*, 2019). Epidiolex®, a pure extract containing cannabidiol, is also used to treat Lennox-Gastaut syndrome and Dravet syndrome refractory epilepsies (Sekar and Pack, 2019).

4.4.2. Synthetic cannabinoids

Beyond natural substances, numerous synthetic compounds with distinct selectivity profiles for cannabinoid receptors have been developed. Most of them bind to the orthosteric location of cannabinoid receptors, which is the same site where endogenous

ligands, but there are also compounds with allosteric binding properties. Agonists, antagonists and allosteric modulators can be distinguished, and they may have distinct affinities and intrinsic activity for CB1R and/or CB2R.

4.4.2.1. Agonists

The most often utilized agonists on basic research are HU-210, CP55,940, and WIN55,212-2, which have comparable affinity for CB1R and CB2R. CB1R activation, has a variety of deleterious effects, including cognitive impairments, motor impairment, and sedation (Pertwee, 2009). Preclinical studies have shown that activation of CB1R may be advantageous neuropathic and inflammatory pain (Donvito *et al.*, 2018); neuropsychiatric disorders such as depression and anxiety (Hillard *et al.*, 2012); neurodegenerative diseases including multiple sclerosis, and AD (Aso and Ferrer, 2014); brain ischemia and inflammatory bowel disorders (Hasenoehrl *et al.*, 2017).

4.4.2.2. Antagonists

Rimonabant (SR141716A), AM281, LY320135, and taranabant (MK-0364) are the most often used CB1R-selective competitive orthosteric antagonists (Figure 19). CB1R-selective competitive antagonists bind to CB1R with significantly greater affinity than CB2R and inhibit CB1R activation by either exogenous or endogenous cannabinoids in a competitive manner (Pertwee *et al.*,

2010). However, the majority of these drugs (including rimonabant, AM251, AM281, LY320135, and taranabant) also act as inverse agonists, reducing tonic endocannabinoid signaling in the absence of agonists (Bouaboula *et al.*, 1997; Mato *et al.*, 2002; Meye *et al.*, 2013). At low doses (nanomolar), these drugs may function as neutral CB1R antagonists, whereas at higher concentrations (micromolar), they may behave as inverse agonists (Pertwee, 2005). Nevertheless, it has been recently described that rimonabant can act inhibiting Gαi/o subunit of heterotrimeric G proteins instead of acting as an inverse agonist at micromolar concentrations (Porcu *et al.*, 2018).

Figure 19. Chemical structure of the most used CB1R selective competitive antagonists. Adapted from (Pertwee *et al.*, 2010).

Several ligands that act exclusively as neutral CB1R antagonists, such as NESS 0327 (Ruiu *et al.*, 2003), O-2654, and O-2050, have also been produced (Wiley *et al.*, 2011).

CB1R inhibition has been proposed for disorders where CB1R activity contributes to disease development, such as obesity, type 2 diabetes (Richey and Woolcott, 2017), reproductive disorders (Battista *et al.,* 2015), schizophrenia (Saito *et al.*, 2013), and ID such as FXS (Busquets-Garcia *et al.*, 2013; Gomis-González *et al.*, 2016) and DS (Navarro-Romero *et al.*, 2019).
The CB1R antagonist/inverse agonist SR141716A (rimonabant, Acomplia®) was approved for use in Europe in 2006 to treat obesity and cardiometabolic disease. Rimonabant has been shown to help obese patients lose weight while also improving their lipid profile and glycaemic management (Patel and Pathak, 2007). Rimonabant was taken off the market in 2008 due to the emergence of psychiatric side effects such as depression, anxiety, and suicide ideation in the obese population (Christensen *et al.*, 2007). Rimonabant's adverse effects in the CNS have been associated to its CB1R inverse agonist characteristics (Meye *et al.*, 2013). In an effort to avoid such undesirable effects, alternatives such as allosteric CB1R modulators, neutral CB1R antagonists and peripherally restricted CB1R antagonists have been developed.

4.4.2.3. Allosteric modulators

Due to failure of rimonabant, alternative methods, such as peripherally acting CB1R antagonists, have been developed and proved to be effective without causing significant CNS adverse effects in recent years (Lu *et al.*, 2019). In addition, the ECS is distinguished by its temporal and topographical selectivity in signalling, as mentioned previously. Then, in comparison to direct receptor agonism/antagonism, both positive and negative allosteric receptor modulators are new potential alternatives.

Recent GPCR research has switched its attention to finding ligands that bind to a topographically different site(s) from the orthosteric site. These sites are called allosteric sites, while allosteric modulators are ligands that bind to these sites to control receptor function (Khurana *et al.*, 2017). Many GPCRs, including CB1R, present allosteric sites (Kenakin, 2012; Conn *et al.*, 2014). Targeting these locations has numerous advantages, including increased subtype selectivity (Conn *et al.*, 2009), preservation of spatial and temporal features of receptor activation, and reduced side effects (Conn *et al.*, 2009; Burford *et al.*, 2013).

Allosteric modulators are divided into four categories (Figure 20)(Gentry *et al.*, 2015; Khurana *et al.*, 2017):

- Positive allosteric modulators are ligands that improve the activity of receptors in the presence of an agonist. They may increase agonist affinity or effectiveness. Positive allosteric modulators can potentially prevent receptor desensitization.

- Negative allosteric modulators or allosteric antagonists are ligands that reduce receptor function by lowering agonist affinity or effectiveness.
- Allosteric agonists are allosteric drugs that exhibit positive modulation even when the orthosteric ligand is absent.
- Neutral allosteric ligand binds to the allosteric site but have no effect on receptor activity.

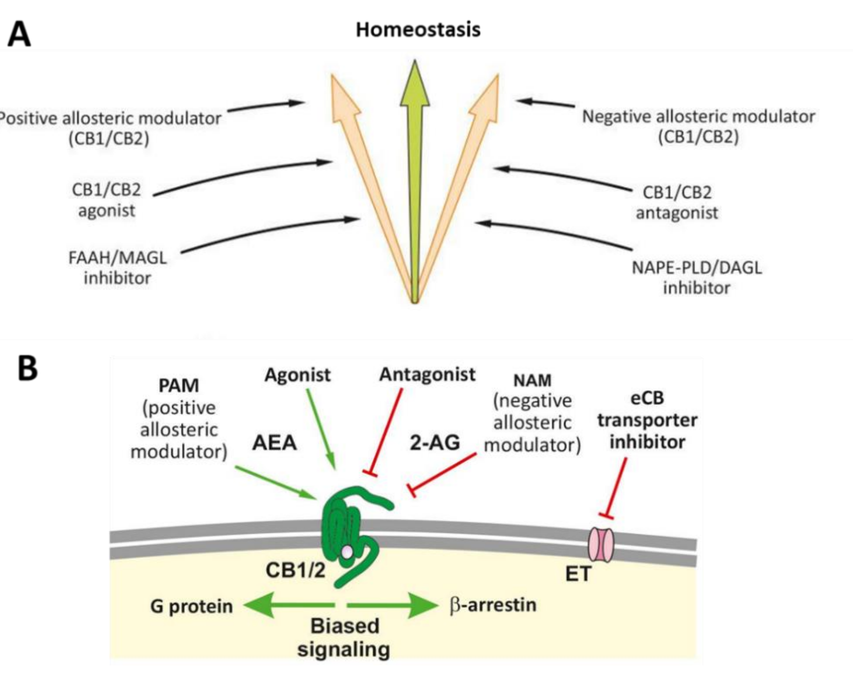

Figure 20. Pharmacological approaches to target cannabinoid receptors. (A) Pharmacological treatments aimed at restoring homeostasis by focusing on different ECS components and employing various mechanistic methods. (B) Pharmacological treatments directed at the various components of the ECS. Compounds that act on cannabinoid receptors and have signalling effects that are directed toward the G protein or β–arrestin pathways have a lot of potential. Adapted from (Lutz, 2020).

CB1R has several allosteric sites that have been targeted with different modulators (Figure 21). Many orthosteric ligands for CB1R also bind CB2R with high affinities. This led to the development of type-specific ligands. Interestingly, the steroid hormone precursor pregnenolone was found to binding specifically CB1R acting as a negative allosteric modulator (Vallée *et al.*, 2014). Pregnenolone is produced in steroidogenic tissues, the brain, and lymphocytes (Vallée *et al.*, 2001; Miller and Auchus, 2011). Pregnenolone, in addition to functioning as a precursor for other steroid hormones, has its own impact in the brain. Pregnenolone and its metabolic products, have been found to improve learning and memory in the brain, as well as alleviate depression and alter cognitive processes (Vallée *et al.*, 2001). Specifically, pregnenolone has shown potential in preventing psychotic-like symptoms such as decreased cognitive function caused by THC (Busquets-Garcia et al., 2017).

Other CB1R allosteric modulators are for instance, PSNCBAM-1, which has acute hypophagic effects (Horswill *et al.*, 2007) and antagonizes neural excitability (Wang *et al.*, 2011), both of which might lead to new therapies for obesity and CNS diseases. Lipoxin A4 has been demonstrated to protect neuronal cells against Aβ-induced neurotoxicity (Pamplona *et al.*, 2012), indicating that it might be used to treat AD. Furthermore, the positive allosteric modulator ZCZ011, reduces neuropathic pain in a mouse model with no cannabimimetic adverse effects (Ignatowska-Jankowska *et al.*, 2015).

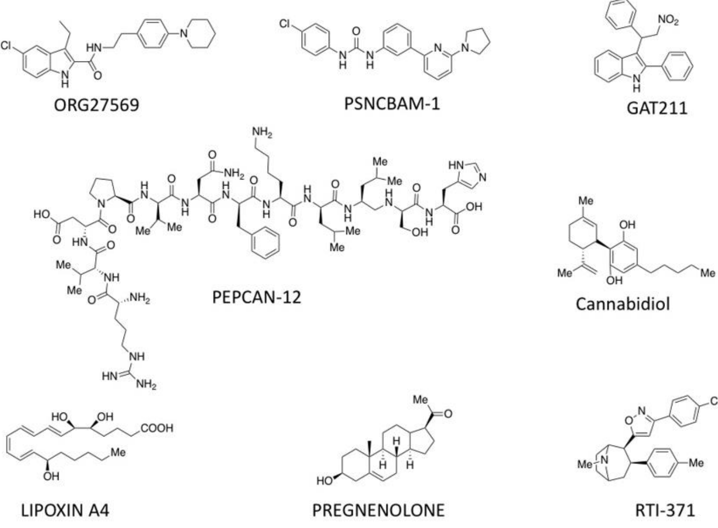

Figure 21. Allosteric modulators of the CB1R. The chemical structures of ORG27569, PSNCBAM-1, GAT211, Pepcan-12, Cannabidiol, Lipoxin A4, Pregnenolone and RTI-371 are shown in the diagram (Khurana *et al.*, 2017).

4.4.2.4. Modulators of endocannabinoid system metabolic enzymes.

The discovery of enzymes involved in the synthesis and breakdown of endocannabinoids paved the way for the development of inhibitory drugs that target these enzymes to boost endocannabinoid tone. FAAH enzyme inhibitors including URB532, URB597, OL-135, OL-92, and PF-3845 can boost AEA levels. Selective MAGL inhibitors such as JZL184, URB602, SAR127303, or OMDM169, on the other hand, enhance 2-AG levels (Tuo *et al.*, 2017). These compounds, unlike cannabinoid agonists, may only enhance endocannabinoid signalling at active endocannabinoid

synthesis sites (Mechoulam and Parker, 2013). Surprisingly, at effective therapeutic levels, several of these medications do not produce most of the negative side effects associated with cannabinoid receptor agonists (Pertwee, 2014). Currently, none of these endocannabinoid-modulating drugs have been authorized for therapeutic use, while several are under clinical studies for the treatment of a variety of illnesses. In a phase I clinical trial, one of these drugs, an FAAH inhibitor (BIA102474), caused the death of one participant and irreparable brain damage in four others. However, these devastating negative side effects were most likely caused by off-target effects unrelated to FAAH-mediated ECS stimulation (Kaur *et al.*, 2016; Van Esbroeck *et al.*, 2017).

4.4.2.5. CB2R modulators

Targeting CB2R, which is primarily expressed in immunological organs, is also potential strategy for treating inflammatory and autoimmune disorders. Various CB2R agonists have been utilized in pre-clinical investigations of pain, arthritis, cancer, Parkinson's, Huntington's, and AD, among others (Cassano *et al.*, 2017).

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 7. 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).

In addition to all the pathological situations mentioned in this section the manipulation of the ECS has also shown interesting therapeutic implications in the treatment of other conditions such as cancer (Abrams and Guzman, 2015), neuropathic, inflammatory and osteoarthritis pain (Jonsson *et al.*, 2006; La Porta *et al.*, 2014), intestinal disorders (Pesce *et al.*, 2018), post-traumatic stress disorder (Zer-Aviv *et al.*, 2016) and endometriosis (Escudero-Lara *et al.*, 2020). In this thesis we will focus on the therapeutic potential of the ECS in learning and memory, and more specifically in intellectual disability.

4.5. Therapeutic potential of the endocannabinoid system in cognitive deficits

Changes in ECS function may contribute to memory impairments seen in some neurodevelopmental disorders, given the ECS's role in cognition. Previous research focused on the role of the ECS in the etiology of FXS syndrome. In FXS mouse models, synaptic plasticity mechanisms mediated by the ECS are disrupted (MacCarrone *et al.*, 2010; Zhang and Alger, 2010). Indeed, targeting the ECS as a therapeutic method to restore memory impairments in Fmr1 KO mice has been investigated. Blocking CB1R normalizes memory impairments in Fmr1 KO mice in the NOR test, according to previous studies from our group (Busquets-Garcia et al., 2013). Normalization of aberrant mTOR signalling, dendritic spine shape, and mGluR-LTD is associated with improved memory (Busquets-Garcia et al., 2013;

Gomis-González et al., 2016). Other approaches involve increasing 2-AG or AEA levels by using MAGL and FAAH inhibitors, respectively. Increased 2-AG levels restored synaptic plasticity in the prefrontal cortex and ventral striatum in *Fmr1* KO mice, but no cognitive tasks were tested (Jung *et al.*, 2012). In a passive avoidance exercise, raising AEA levels improved unpleasant memory (Qin *et al.*, 2015).

In addition, CBD has been proposed as a therapeutic tool for anxiety, insomnia, and cognitive impairments, as well as for people who have endocannabinoid deficiencies, such as FXS subjects. CBD was found to reduce anxiety-like behaviour in *Fmr1* KO mice but do not modify cognitive performance (Zieba *et al.*, 2019). Case studies have also been reported, where they described that administration of oral CBD solutions, showed significant improvements in social avoidance and anxiety, as well as sleep, feeding, motor coordination, language abilities, anxiety, and sensory processing (Tartaglia *et al.*, 2019). Furthermore, a randomized, double-blind, placebo-controlled study showed the efficacy and safety of ZYN002, a clear CBD gel that can be applied to the skin for the treatment of behavioural symptoms of FXS (NCT03614663). Social avoidance, irritability, hyperactivity, and inappropriate speech were improved specifically in treated participants with full methylation of the *Fmr1* gene. The above findings, when combined with the existing preclinical evidence, point to CBD's and other ECS pharmacological approaches as a potential treatment for people with FXS.

The manipulation of the ECS might also be a potential pharmacological therapy for AD (Aso and Ferrer, 2014; Talarico *et al.*, 2018). On the one hand, manipulation should be aimed at inhibiting neuroinflammation by activating CB2R to prevent reactive oxygen species formation and cytokine release from microglia (Iuvone *et al.*, 2004; Benito *et al.*, 2008). On the other hand, at reducing neurotoxicity by activating CB1R to inhibit glutamate release (Marsicano *et al.*, 2003; Zhuang *et al.*, 2005) and enhance neurotrophin expression and neurogenesis (Esposito *et al.*, 2011). It has also been shown that Δ^9-THC inhibits acetylcholinesterase, resulting in improved cholinergic transmission and decreased amyloidogenesis (Eubanks *et al.*, 2006). However, because the ECS's potential impact on cognitive and behavioral functions is unknown, randomized controlled studies are needed to assess whether the ECS can slow or stop disease progression.

4.5.1. Therapeutic potential of the endocannabinoid system in Down syndrome

In the laboratory we have studied, the involvement of the ECS in the pathogenesis of DS (Navarro-Romero *et al.*, 2019). We found that CB1R expression was enhanced, and its function increased in hippocampal excitatory terminals of Ts65Dn mice. Thereupon, hippocampal CB1R knockdown reestablished cognitive execution in the NOR test. Concomitant with this result, pharmacological blockade of CB1R by a subchronic administration of the CB1R

antagonist rimonabant or NESS0327, also rescued specific hippocampal memory deficits in male and female Ts65Dn trisomic mice. Remarkably, CB1R inhibition also restored hippocampal long-term potentiation and adult neurogenesis in the dentate gyrus. We used TgDyrk1A mouse model to investigate the mechanism behind the cognitive improvement mediated by the blockade of CB1R. This model overexpresses Dyrk1A, which is proposed to be one of the main implicated genes in ID associated with DS. TgDyrk1A mice also present an enhanced CB1R expression in the hippocampal region. Moreover, pharmacological CB1R blockade similarly restored memory deficits, synaptic plasticity, and adult neurogenesis. Thus, our results pointed the CB1R as a novel druggable target potentially relevant for the improvement of cognitive performance in the context of DS. However, this study only utilizes young-adult mice and uses a short treatment. Further studies are necessary to determine the possible efficacy of long-term treatments with inhibitors of CB1R and its impact in other neurological phenotypes of the Ts65Dn.

5. Vagus nerve stimulation

5.1. Vagus nerve anatomy

The sympathetic and parasympathetic divisions of the autonomic nervous system regulate various organs, glands, and involuntary muscles in the body. The vagus nerve (VN), the tenth and longest of the cranial nerves, is a key component of the parasympathetic nervous system, functioning as a bidirectional conduit between the body and the brain (Roberts and Bullis, 2018). It is a mixed nerve with 20% efferent fibers and 80% afferent fibers which transmit sensory impulses from visceral organs to the Nucleus Tractus Solitarius (NTS), Spinal nucleus of Trigemial Nerve, and Area Postrema (Roberts and Bullis, 2018). From the NTS projections are sent to higher brain regions such as the *locus coeruleus* (LC), amygdala, hypothalamus, dorsal raphe and thalamus. It also possesses parasympathetic innervation which fibers originate in the medulla and converge in the jugular and nodose ganglia. Then the VN descends into the neck and splits into four branches, being one of them the auricular branch, which innervates the auricular concha meatus. It continues down until the junction of the cardiac, pulmonary, and aortic branches (Yuan and Silberstein, 2016). The left and right VNs, innervate distinct regions of the heart, with the right vagus innervating the sinoatrial node and the left vagus innervating the atrioventricular node (Ben-Menachem, 2002). Then it crosses the diaphragm and splits into different branches that

innervated the stomach, liver and kidney, among other abdominal organs (Figure 22) (Ruffoli *et al.*, 2011; Yuan and Silberstein, 2016).

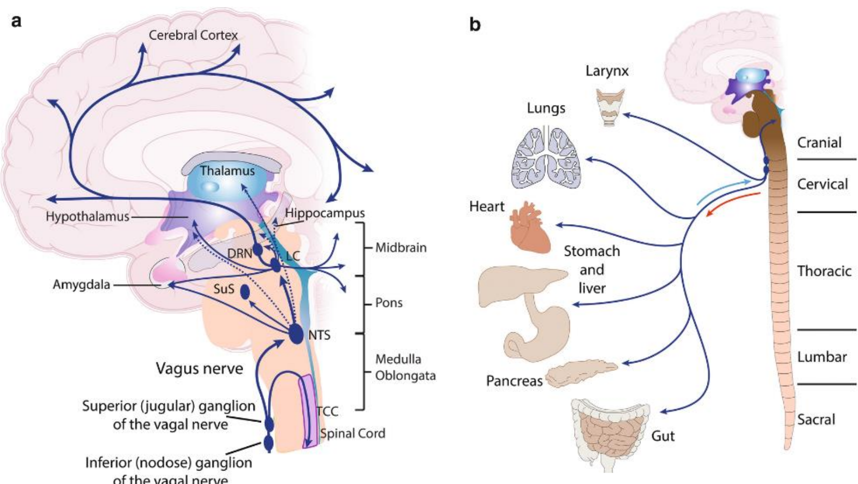

Figure 22. **VN anatomy and innervations**. Adapted from (Akerman and Romero-Reyes, 2020). DRN: dorsal raphe nucleus; LC: *locus coeruleus;* NTS: nucleus solitary tract; TTC: trigemino cervical cortex.

5.2. Physiological role of the vagus nerve

Since the VN innervates a variety of bodily parts and serves as a direct link to the brain, it is involved in an important number of physiological processes. Pressure, pain, temperature, chemical, osmotic pressure, and inflammation are all interoceptive stimuli that the vagal afferents detect. Sensory information converges in the vagal nuclei, which transfer data to numerous brain areas and communicate regulatory information via the descending vagal efferents (Yuan and Silberstein, 2016).

The VN integrates signaling from the body and regulate homeostasis in many organ (Craig, 2003). Heart rate, blood pressure, vascular resistance, airway diameter and breathing are all controlled by the VN. Eating is also regulated by the VN by the named "gut-brain signaling pathway" since luminal nutrients cause the production of enteroendocrine mediators which interact with the VN (Dockray, 2013). Furthermore, vago-vagal reflex initiates digestion and peristalsis.

On the one hand, the VN has three afferent types that terminates in different vagal nuclei such as the spinal nucleus of the trigeminal nerve and the NTS (Ruffoli *et al.*, 2011). For instance, the spinal nucleus of the trigeminal nerve receives sensory inputs from external auditory meatus, the larynx and esophagus (Yuan and Silberstein, 2016). In addition, the rostral NTS is responsible for the taste sensation of the epiglottis and pharynx. On the other hand, the VN has different visceral efferent types that originate in the dorsal motor nucleus of the VN and the nucleus ambiguous which respectively innervate most abdominal organs and produce cardiac inhibition and maintain airway caliber (Prechtl and Powley, 1990).

5.3. Therapeutic potential of vagus nerve stimulation

When it was discovered that electrical stimulation of the VN might halt chemically induced seizures in 1985, it became a possible therapeutic technique in epilepsy. In the 1990s, VN stimulation (VNS) devices were first implanted in people with refractory

epilepsy. By 2015, over 100,000 VNS devices have been implanted around the world after the FDA approved VNS therapy for pharmacoresistant depression and epilepsy. (Nemeroff *et al.*, 2006; Johnson and Wilson, 2018)

The device comprises of a battery-powered pulse generator implanted near the clavicle in the chest (Figure 23), which will supply the necessary current for stimulation. The generator is connected to a bipolar stimulating electrode that is wrapped around the left VN by a lead (Figure 23). The electrodes are not implanted in the right VN to prevent bradycardia, which is not observed after left VNS (Randall *et al.*, 1987). The surgical procedure is quick and easy to execute under local anesthetic. Despite being a minimally invasive technique, the surgery is inherently dangerous owing to the electrode placement, which necessitates dissection of the VN and carotid artery (Handforth *et al.*, 1998). Possible side effects of the surgical procedure are bradyarrhythmias during device insertion, peritracheal hematoma (due to surgical trauma), and other respiratory problems (Asconapé *et al.*, 1999; Santos, 2003; Fahy and Dickey, 2010; Yap *et al.*, 2020).

With the use of a computer and a magnetic programming wand, the physician may customize the stimulation parameters. Afferent projections of the VN can be one of three types of neurons: myelinated A and B fibres, or unmyelinated C fibres (Table 8). According to research performed on cats, the afferent C fibres are the most abundant, accounting for 65-80 % of the total (Woodbury

and Woodbury, 1990). Each one has a unique excitation threshold below which action potentials can be produced.

	FIBRE TYPE		
	A	B	C
Fibre diameter (mm)	5-20	<3	0.4-2
Gross	Large	Small	Small
Myelinated	Yes	Yes	No
Threshold (mA)	0.02 - 0.2	0.04 - 0.6	2.0+

Table 8. Summary of the characteristics of the different fibre types of VN. A-fibres have the lowest thresholds for conventional invasive VNS cuff electrodes, with some requiring as little as 0.02 mA currents to recruit, with thresholds ranging up to 0.2 mA. B-fibres require greater currents, ranging from 0.04 to 0.6 mA. C-fibres, which are not myelinated and hence have a lower conductance than A-fibres, require currents greater than 2 mA to be recruited (Erlanger and Gasser, 1930).

The amplitude of clinically effective stimulation is dependent on pulse width, whose decreasing amplitudes required the longer the pulse width (Koo *et al.*, 2001). This amplitude in adults is below the C-fibre excitation threshold, which ranges from 0.5 mA (for 500 ms pulses) to 1.5 mA (for 100 ms pulses) (Koo *et al.*, 2001). However, some studies suggest that activation of these fibres was not required for therapeutic benefits of VNS (Krahl *et al.*, 2001). As for frequency, the 20-30 Hz range have been authorized by the FDA for clinical usage, since axonal damage may occur at frequencies over 50 Hz (Agnew and McCreery, 1990).

5.3.1. Vagus nerve stimulation applications

VNS was initially authorized by the FDA as a treatment for individuals who had failed to react to traditional anti-seizure medications (Ben-Menachem, 2002). After 2–3 years of therapy, around 40% of patients who used VNS saw a 50% reduction in seizures (Morris and Mueller, 1999). The mechanisms by which VNS induces changes in neurochemistry and prevents epileptic seizures remain unknown. Some evidence shows the VN has a role in quenching kindling of seizures in areas prone to heightened excitability such as the thalamus, the limbic system and the thalamocortical projections (Bonaz *et al.*, 2013). VNS also enhances activity in the LC and raphe nuclei, as well as moderating the downstream release of norepinephrine and serotonin, both of which have antiepileptic effects (Figure 23) (Krahl and Clark, 2012).

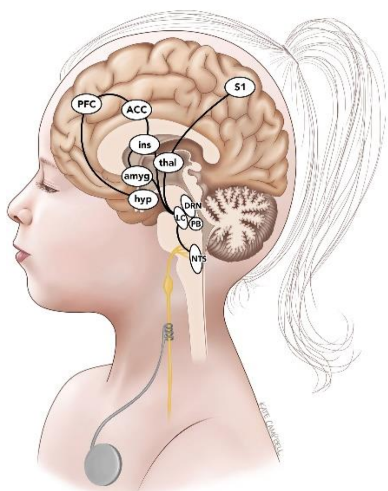

Figure 23. **The afferent network of the VN**. The major brainstem areas, as well as subcortical and cortical regions, that are thought to underpin VNS. ACC = anterior cingulate cortex; amyg = amygdala; hyp = hypothalamus; ins = insula; PB = parabrachial nucleus; PFC = prefrontal cortex; S1 = primary somatosensory cortex; thal = thalamus (Hachem *et al.*, 2018).

An overall mood improvement was observed in certain epileptic patients who were already receiving VNS treatment, regardless of whether seizure reduction improved or not (Harden, 2002). This raised the possibility that VNS may be useful as an adjuvant in the treatment of drug-resistant depression. The first trials revealed that 40% of patients experience a 50% reduction in baseline Hamilton Depression Rating Scale (HDRS) scores, a standard test for assessing depression, and 17% of patients experience complete remission of depression (Rush *et al.*, 2000). Based on these studies, in 2005 the FDA authorized VNS use for depression. Increased NE concentration in several of the regions normally associated with mood regulation,

such as the hippocampus, amygdala, and prefrontal cortex, is caused by an increase in firing rate in the LC caused by VNS (Ruffoli *et al.*, 2011). Because the LC contains excitatory projections to the Dorsal Raphe Nucleus, which is the brain's major source of serotonin (Henry, 2002), its activity can be regulated indirectly by VNS (Dorr and Debonnel, 2006).

VNS has also been found to enhance memory. First study described that VNS improved word-recognition memory performance (Clark *et al.*, 1999). Other studies also found that VNS improves working memory and memory retention (Ghacibeh *et al.*, 2006b; Sun *et al.*, 2017). Given this results, VNS emerged as a possible treatment for AD. Pilot studies of daily VNS treatment revealed that after six months, 70% of patients exhibited deterioration in cognitive status, but this dropped to 40% after a year (Sjögren *et al.*, 2002; Merrill *et al.*, 2006).

The mechanisms by which VNS induces improves memory performance is still unclear. The only source of NE for the hippocampus is the LC (Loy *et al.*, 1980). VNS induces a rise in NE in brain regions important for cognitive function such as hippocampus and cerebral cortex. Furthermore, this NE increase disappears when VNS is switched off, according to animal research (Roosevelt *et al.*, 2006).

5.3.2. Non-invasive vagus nerve stimulation

VNS is a promising technique for CNS modulation that has proven effective in a variety of illnesses and shows potential in others. However, there are significant disadvantages, such as the danger of surgical and technical problems, as well as the medical expense (Spuck *et al.*, 2010). This also restricts the research of its potential therapeutic effects, as most studies must be conducted on individuals who have already had the device implanted. To date, two techniques have been used to circumvent these issues: cervical and auricular transcutaneous vagus nerve stimulation (atVNS). Non-invasive stimulation of the cervical branch of the VN has gained popularity due to the technique's low cost, minimal side effects, and low morbidity (Goadsby *et al.*, 2014; Grazzi *et al.*, 2014). Nevertheless, in this thesis we will focus on atVNS.

According to anatomical studies of the ear, the auricular branch of the vagus nerve (ABVN) formed by the *tragus*, *concha*, and *cymba concha* are the locations on the human body where cutaneous afferent VN distributions exist (Figure 24) (Peuker and Filler, 2002), and stimulation of these afferent fibers produce therapeutic effects like regular VNS (Rong *et al.*, 2012; Stefan *et al.*, 2012; Hein *et al.*, 2013). Furthermore, at least in rats, these afferent projections of the VN terminate in the NTS (He et al. 2013).

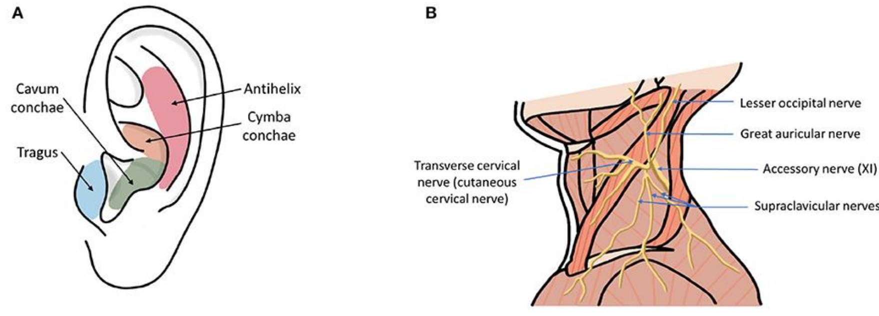

Figure 24. **Transcutaneous afferent VN regions for non-invasive VNS.** (A) The cutaneous ABVN innervates the ear. (B) Nerves in the neck, including the VN's cervical branch. (Yap *et al.*, 2020)

Because the therapeutic benefits of VNS are thought to be mediated via increasing NTS activity, the ABVN has attracted interest as a target for non-invasive VNS. The ABVN is stimulated electrically by implanting a pair of electrodes in the auricular concha, to which a pulse generator delivers a current. In animal models, there was an increase in NTS firing rate and antiepileptic effects when atVNS was performed using already established effective parameters for invasive VNS (He *et al.*, 2013). Since then, it has been demonstrated the efficacy of atVNS in a variety of illnesses, including heart failure (Afanasiev *et al.*, 2016), migraine (Straube *et al.*, 2015), and depression (Liu *et al.*, 2016). Importantly, some studies found that atVNS is also efficacious for the improvement of associative memory (Jacobs *et al.*, 2015), in visuospatial reasoning (Klaming *et al.*, 2020) and recognition tasks and the establishment of emotional memories (Ventura-Bort et al., 2021).

The most commonly used devices are gammaCore electroCore or Nemos Cerbomed, a commercial atVNS stimulators which has already received European comission approval for the European market, included in the third of the studies performed (Figure 25) (Yap et al., 2020). The gammaCore electroCore device is almost always utilized for neck stimulation, whereas the NEMOS Cerbomed device is mostly used for ear stimulation of the ABVN (Yap et al., 2020).

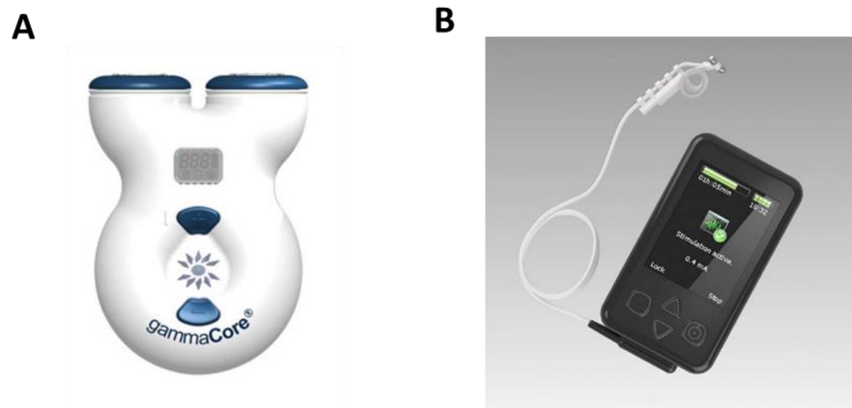

Figure 25. Commercial devices for VNS. (A) Electrocore gammaCore. Adapted from www.gammacore.com. (B) Cerbomed NEMOS. Adapted from www.cerbomed.com.

Objectives

Objective 1

To study the cognitive and neurologic effects of a long-term treatment with a low dose of the CB1R antagonist rimonabant in the Ts65Dn mouse model of Down syndrome.

Article #1

Long-term decreased cannabinoid type-1 receptor activity restores specific neurological phenotypes in the Ts65Dn mouse model of Down syndrome

Anna Vázquez-Oliver, Silvia Pérez-García, Nieves Pizarro, Laura Molina-Porcel, Rafael de la Torre, Rafael Maldonado*, Andrés Ozaita*

** equal contribution.*

bioRxiv 2021.11.22.469296;

doi: https://doi.org/10.1101/2021.11.22.469296

<u>Objective 2</u>

To assess the efficacy of a CB1R allosteric modulator in the Ts65Dn mouse model of Down syndrome after long-term treatment.

Article #2

Long-term cannabinoid type-1 receptor inhibition with a novel allosteric modulator improves memory in the Ts65Dn mouse model of Down syndrome

Anna Vázquez-Oliver, Gabriela Bordeanu, Pier-Vincenzo Piazza, Rafael Maldonado*, Andrés Ozaita*

** equal contribution.*

In preparation, 2021

Objective 3

To investigate auricular transcutaneous vagus nerve stimulation as a method for memory enhancement in naïve mice and in a mouse model of fragile X syndrome.

Article #3

Auricular transcutaneous vagus nerve stimulation improves memory persistence in naïve mice and in an intellectual disability mouse model

Anna Vázquez-Oliver*, Cecilia Brambilla-Pisoni*, Mikel Domingo-Gainza, Rafael Maldonado, Antoni Ivorra, Andrés Ozaita.

** equal contribution*

Brain stimulation. 2020; 13(2), 494–498. DOI: 10.1016/j.brs.2019.12.024

Results

Objective 1

To study the cognitive and neurologic effects of a long-term treatment with a low dose of the CB1R antagonist rimonabant in the Ts65Dn mouse model of Down syndrome.

Article #1

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Long-term decreased cannabinoid type-1 receptor activity restores specific neurological phenotypes in the Ts65Dn mouse model of Down syndrome

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Keywords: Down syndrome, CB1 cannabinoid receptor, rimonabant, Ts65Dn, memory

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Conflict of interest statement

R.M. and A.O. declare intellectual property of the patent PCT/EP2013/055728. The remaining authors declare no conflict of interest.

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Abstract

Intellectual disability is the most prevalent and limiting hallmark of Down syndrome (DS), without any pharmacological treatment available. Neurodegeneration and neuroinflammation are relevant neurological features of DS reaching to early development of Alzheimer's disease. Preclinical evidence suggests that the endocannabinoid system, an important neuromodulator on cognition and neuroinflammation, could act as beneficial target in DS. Indeed, cannabinoid type-1 receptor (CB1R) activity was enhanced in the hippocampus of young-adult trisomic Ts65Dn mice, a well-characterized surrogate model of DS. In previous studies, inhibition of CB1R, was able to restore key neurological deficits in this mouse model. To determine the possible clinical relevance of this target, it is mandatory to evaluate the long-term consequences of attenuated CB1R activity and to minimize the possible side-effects associated to this mechanism. We found that CB1R expression was significantly enhanced in the hippocampus brains of aged DS subjects. Similarly, middle-aged trisomic mice showed enhanced CB1R expression. Long-term oral administration of a low dose of the CB1R specific antagonist rimonabant was administered to male and female Ts65Dn trisomic and wild-type mice from the time of weaning to 10 months, an age when signs of neurodegeneration have been described in the model. CB1R inhibition resulted in significant cognitive improvement in novel object-recognition memory in trisomic male and female mice, reaching a similar performance to that of wild-type littermates. Interestingly, this long-term rimonabant treatment modify locomotor activity, anxiety-like behavior, body weight or survival rates. Brain analysis at 10 months of age revealed noradrenergic and cholinergic neurodegeneration signs in trisomic mice that were not modified by the treatment, although the alterations in hippocampal microglia morphology shown by vehicle-treated trisomic mice was normalized in trisomic mice exposed to rimonabant. Altogether, our results demonstrate a sustained pro-cognitive effect of CB1R inhibition at doses that do not produce major side effects that could be associated to an anti-inflammatory action, suggesting a potential interest in this target of to preserve cognitive functionality in DS.

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Introduction

Down syndrome (DS) is the most significant genetic cause of intellectual disability affecting around 1 over 700-1,000 live births worldwide (de Graaf *et al.*, 2021; de Graaf *et al.*, 2020) and is produced by trisomy of human chromosome 21 (HSA21) (Lejeune *et al.*, 1959). Intellectual disability is the most prevalent feature of DS an important limitation for people with DS independence especially in hippocampus-related cognitive domains (Dierssen, 2012; Pennington et al., 2003). The life expectancy for people with DS has markedly increased because of medical advances reaching to a median age at death of almost 60 years (De Graaf *et al.*, 2017; Bayen *et al.*, 2018). As a result, nowadays DS population is more prone to suffer from age-related comorbidities throughout their lives, such as an early-onset Alzheimer's disease which is present in almost all adults with DS by age 65 (Mccarron *et al.*, 2014). At the cellular level, aging subjects with DS show decreases in the density of noradrenergic neurons in the *locus coeruleus*, the main noradrenergic center in the brain (Mukhin *et al.*, 2017) and in cholinergic neurons in the basal forebrain (Yates *et al.*, 1983; Mann *et al.*, 1985) as well as neuroinflammation (Wilcock and Griffin, 2013), all associated to the development of the Alzheimer's disease pathology (Fortea *et al.*, 2021).

Several animal models for DS have been established as a tool for investigating this condition based on the fact that HSA21 is orthologous to three distinct regions in murine chromosomes 10, 16, and 17 (Herault *et al.*, 2017; Antonarakis *et al.*, 2020). The Ts65Dn mouse model is the most-used mouse model for DS and it consists in a partial trisomy of murine chromosome 16 (from *App* to *Mx1*), covering more than 50% of genes in the homologous region of HSA21 (Davisson *et al.*, 1993; Reeves *et al.*, 1995). Importantly, Ts65Dn mice at 10 months of age also display common features with Alzheimer's disease. Indeed, Ts65Dn mice show increased amyloid precursor protein (APP) expression, and degeneration of *locus coeruleus* noradrenergic neurons and basal forebrain cholinergic neurons, among others (Hamlett *et al.*, 2015).

The endocannabinoid system (ECS) is a neuromodulatory system involved in synaptic homeostasis relevant for neuroinflammation and memory functions (Lutz, 2020). The ECS is composed by the cannabinoid receptors, mainly the cannabinoid type-1 and type-

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2 receptors (CB1R and CB2R, respectively), their endogenous ligands known as endocannabinoids, and the enzymes involved in their synthesis and degradation (Zou and Kumar, 2018). In the Ts65Dn model, previous studies showed that CB1R expression and function was enhanced in the hippocampus of young-adult Ts65Dn mice. Moreover, pharmacological and genetic CB1R inhibition restored memory deficits, synaptic plasticity, and adult neurogenesis in this mouse model at a young age (Navarro-Romero *et al.*, 2019), although similar results have also been demonstrated after protecting 2-AG from enzymatic degradation (Lysenko *et al.*, 2014). However, the possible efficacy of long-term treatments with CB1R antagonists in behavioral and in specific neurological phenotypes characteristic of aged Ts65Dn trisomic mice.

In this study, we found that CB1R expression is enhanced in hippocampal *post-mortem* samples of human subjects with DS further confirming CB1R as a potential therapeutic target. In addition, long-term pharmacological inhibition of CB1R with a low dose of rimonabant, enhanced memory in Ts65Dn mice at an age when this mouse model has a noticeable neurodegenerative and neuroinflammatory phenotype, the latter being sensitive to CB1R inhibition.

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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). Procedures were approved by the local ethical committee (Comité Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB) and local authorities (Generalitat de Catalunya).
All experimental mice were bred at the Barcelona Biomedical Research Park (PRBB) Animal Facility. 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 purchased from The Jackson Laboratory. Euploid littermates of Ts65Dn mice served as wild-type (WT) controls.
Mice were housed in Plexiglas cages with a maximum of 4 males or 5 female mice per cage in a temperature-controlled (21°C ± 1°C) (mean ± range) and humidity-controlled (55% ± 10%) environment. Lighting was maintained at 12 h cycles (light on at 8 AM; light off at 8 PM). All the experiments were conducted during the light phase in an experimental room at the animal facility. Food and water were available *ad libitum*. All behavioral experiments were performed by an observer blind to the genotype and treatment.

Drug treatments

Rimonabant was purchased from Axon Medchem. Rimonabant was first prepared as a 40 mM stock solution in ethanol. Then, rimonabant stock solution was diluted to a final concentration of 10.8 µM in 0.3 % 2-hydroxypropyl-β-cyclodextrin in water. The compound was administered through the drinking bottle, and the same solution without rimonabant was used in littermate mice as control/placebo/vehicle condition. Animals with different treatment (vehicle or rimonabant) were maintained in different home-cages, while home-cages could hold both genotypes (Ts65Dn or WT). Mice were included in the study at the age of weaning (postnatal day 21, PND21) and randomly associated to one of the treatments. During the first 2.5 months of treatment mice

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received rimonabant 0.1 mg/kg/day. This dose was increased to the final concentration of 0.5 mg/kg/day by 3.5 months of age. The dosage was then maintained until mice were euthanized at 10 months of age.

Behavioral tests

All behavioral tests were performed in a sound-attenuated room with dim illumination. A digital camera on top of the maze was used to record the sessions.

The novel object-recognition memory test was performed following a previously described protocol (Puighermanal *et al.*, 2009). Briefly, novel object-recognition memory was assessed in a V-shape maze with dim illumination (3-5 lux). This task consists in 3 different phases (habituation, familiarization/training, and test) performed on 3 consecutive days for 9 min. On day 1, mice were habituated to the empty V-maze. Next day, mice were introduced in the V-maze where 2 identical objects were presented in the familiarization/training phase. Finally, the test was performed 24 h later, where 1 of the familiar objects was replaced for a novel object and the exploration time for both objects was recorded. Object exploration was defined as orientation of the nose toward the object at a distance < 2 cm. A discrimination index (DI) was calculated as the difference between the time spent exploring either the novel (Tn) or familiar (Tf) object divided by the total time spent exploring both objects: DI = (Tn − Tf) / (Tn + Tf).

Locomotor activity was assessed for 120 min. Individual locomotor activity boxes (9 × 20 × 11 cm) (Imetronic) were used in a low luminosity environment (5 lux). The total activity was detected by infrared sensors.

The elevated plus maze test was performed in a black Plexiglas apparatus with 4 arms (29 cm long x 5 cm wide), 2 open and 2 closed, set in cross from a neutral central square (5 cm x 5 cm) elevated 30 cm above the floor and indirectly illuminated from the top (40–50 lux in the open arms/4–6 lux in the close arms). Five-minute test sessions were performed, and total number of entries and the percentage of time spent in the open arms were used as a measure of anxiety-like behavior.

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Preparation of histological brain samples

Mice were deeply anesthetized by intraperitoneal injection (0.2 mL per 10 g body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg). Then, mice were perfused intracardially with 4 % paraformaldehyde (PFA) in a 0.1M Na_2HPO_4/NaH_2PO_4 (pH 7.5) phosphate buffer (PB) using a peristaltic pump. The brains were removed from the skull and postfixed overnight at 4 °C in the same fixative solution. The next day, brain sections were moved to 30 % sucrose in PB solution. Brain sections (30 µm) were obtained with a sliding microtome and kept in a 5 % sucrose in PB solution at 4 °C until they were used for immunodetection.

Immunofluorescence

Firstly, free-floating sections were washed three times (5 min each) in PB. Afterwards, the tissue was incubated in blocking buffer for 2 h which was made of 0.3 % Triton X-100 and 3 % normal donkey or goat serum diluted in 0.1 M PB, for neurodegeneration assessment and inflammation assessment respectively. Then, sections were incubated with one of the primary antibodies (mouse anti-TH (1:1,000, Sigma); rabbit anti-p75NTR (1:1,000, Millipore); mouse anti-Iba1 (1:500, Wako)) for 24 h at 4 °C. Then, sections were washed 3 times (10 min each) and subsequently incubated with the corresponding secondary fluorescent antibodies for 2 h at room temperature. As secondary antibodies were used goat anti-mouse Alexa Fluor 488 (1:1,000), donkey anti-rabbit Alexa Fluor 555 (1:500) and donkey anti-mouse Alexa Fluor 555 (1:500). Finally, the sections were washed 3 times (10 min each) and were mounted onto gelatin-coated slides with Fluoromont/DAPI mounting medium.

Image acquisition and analysis

For TH+ cell counting, 4 coronal sections of the *locus coeruleus* were selected (from 5.34 to 5.52 posterior to Bregma) (Gould *et al.*, 2012). Images of stained sections were obtained with a confocal microscope TCS SP8 LEICA (Leica Biosystems) using a dry objective (20× objective, 0.75 zoom) with a sequential line scan at 1024 × 1024-pixel resolution. The number of TH+ cells was manually quantified using Fiji software (Image

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J). The number of positive cells was calculated as the mean of total number of cells counted referred to the area of the *locus coeruleus* (µm^2).

For p75NTR+ cell counting, systematic series of coronal sections (1 every 6 sections) per animal were selected, covering the rostral to caudal extension of the medial septum (from 1.18 and 0.38mm posterior to Bregma) (Gould *et al.*, 2012).

The Leica DM6000B microscope was used (10x objective). To obtain the macro used for the quantification of p75NTR+ cells, firstly the Yen threshold was applied. Then an erosion and dilatation operation were applied with the command OPEN followed by remove outliers command (radius=2 and threshold=50). Finally, watershed command was executed and cells higher than 70µm^2 were counted as positive. The number of positive cells was calculated as the mean of total number of cells counted referred to the area of the medial septum (µm^2).

Iba1 staining was used to evaluate the microglial morphology. Confocal microscopy images of whole Z-stack from the slice were acquired in the *stratum radiatum*. Images of stained sections were obtained with a confocal microscope TCS SP5 STED LEICA (Leica Biosystems) using an immersion-oil objective (40× objective, 1.5 zoom) with a sequential line scan at 1024 × 1024-pixel resolution and with 0.3 µm depth intervals. The perimeter of the microglial soma was quantified in 20 cells per animal using Fiji software (Image J).

Human samples slice histology, immunofluorescence, image acquisition and analysis

Brain samples (n=5 from subjects with DS, median age 64 and n=5 from typically developing subjects median age 66, all male) were obtained from the Neurological Tissue Bank, Biobanc-Hospital Clínic-IDIBAPS, Barcelona, Spain (Supplementary Table 1). Neuropathologic examination was performed according to standardized protocols (Borrego-Écija *et al.*, 2021). Half-brain was fixed in formaldehyde solution for 3 weeks. Middle-posterior hippocampal region was embedded in paraffin and cut at 4 µm. The sections were placed in a BOND-MAX Automated Immunohistochemistry Stainer (Leica Biosystems Melbourne Pty Ltd, Melbourne, Australia). Tissues were deparaffinized and pre-treated with the Epitope Retrieval Solution 2 (EDTA-buffer pH8.8) at 98°C for 40 min. CB1R primary antibody (rabbit, 1:1,000, Immunogenes) was incubated for 60 min.

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Subsequently, tissues were incubated with polymer-HRP for 8 min and developed with DAB-Chromogen for 10 min. Slides were counterstained with hematoxylin.

Hippocampal subregions were defined using QuPath software (Bankhead *et al.*, 2017) and following Allen Brain Atlas guidelines. Mean gray value optical density and % of occupied area were analyzed for CB1R and Nissl staining respectively, using Fiji software (Image J). The % of Nissl occupied area was quantified after the application of an optimal automatic threshold (Triangle dark) from Fiji software (ImageJ). Then, an erosion and dilatation operation were applied with OPEN command to erase unspecific signal. Ratio between CB1R optical density values and % of Nissl occupied area were expressed as a percentage of control group.

Western Blotting

Mouse brain tissue was rapidly dissected, immediately frozen on dry ice and stored at −80 °C until used. Samples were processed following a protocol previously described (Ozaita *et al.*, 2007) to obtain the total solubilized fraction and separated on SDS-PAGE gels and transferred into nitrocellulose membranes as previously described (Ozaita et al., 2007). The primary antibodies used were rabbit anti-CB1R (1:1,000, Immunogenes) and mouse anti-actin (1:50,000, MAB1501, Merck Millipore). Primary antibodies were detected with horseradish peroxidase conjugated anti-rabbit and anti-mouse antibodies and visualized by enhanced chemiluminescence detection (Luminata Forte Western HRP substrate, Merck Millipore). Digital images were acquired on a ChemiDoc XRS System (Bio-Rad) and quantified using The Quantity One software v4.6.3 (Bio-Rad). Optical density values for CB1R were normalized to actin optical density values as loading control in the same sample and expressed as a percentage of control group (WT).

Rimonabant detection

For the analysis of mice brain content, tissues (40-80 mg) where weighted and homogenized with 1 mL grinder dounce (Wheaton, USA) in two steps: first by adding 400 µL of HCOOH 0.1 %, (thirty movements “loose”, followed by thirty movements “tight” were used for homogenization) followed by protein precipitation with 800 µL of ethanol. The mixture was centrifuged 10 min at 15,700 g, 4 °C, and supernatant was

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recovered and stored at -20 °C until use. Then, 100 µL of supernatant was mixed with 25 µL of the internal standard (rimonabant-d10, 0.004 µg/mL in ethanol, Bertin technologies, Montigny le Bretonneux, France) and 4 µL of the final mixture were used for the HPLC-MS/MS analysis.

Samples were analyzed in an Acquity UPLC System (Waters Associates, Milford, MA, USA) coupled to a mass spectrometer (Quattro Premier, Watters Associates). Chromatographic separation was carried out in an Acquity BEH C18 (100 mm x 2.1 mm i.d., 1.7 µm) (Waters Associates) at a flow rate of 0.4 mL/min. Ammonium formate (1 mM)-HCOOH 0.01% (A) and MeOH with ammonium formate (1 mM)-HCOOH 0.01%(B) were used as mobile phases. After keeping 40%B for 0.5 min, the gradient was increased to 95%B in 3 min and maintained at 95%B for 1 minute after going back to initial conditions. Detection of analytes was done by the selected reaction monitoring (SRM) method, being the transitions used for identification and quantification (in bold) for each compound as follows: 465→84, 99, 365 (rimonabant); 475→94, 365 (rimonabant-d10).

Experimental design and statistical analysis

Sample size choice was based on previous studies with similar experimental approached (Busquets-Garcia *et al.*, 2013, 2016) and it is indicated in figure legends for each experiment. Data were analyzed with GraphPad Software using unpaired Student's t-test or two-way ANOVA for multiple group comparisons. Subsequent *post-hoc* analysis (Bonferroni) was used when significance in interaction between factors was found. The Pearson correlation coefficient was used to analyze the relationship between discrimination index and the area of microglial soma. Comparisons were considered statistically significant when $p < .05$. Outliers (±2 s.d. from the mean) were excluded.

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Results

CB1R expression is enhanced in hippocampal tissue of subjects with DS and middle aged trisomic Ts65Dn.

Previous studies in our laboratory described that the expression of CB1R was increased in the hippocampus of young-adult Ts65Dn male mice (Navarro-Romero *et al.*, 2019). We hypothesized that hippocampal CB1R expression could be also enhanced in human subjects with DS. Therefore, we immunodetected and quantified the expression of CB1R in brain slices corresponding to the hippocampal formation of aged subjects with DS and sex, age, and *post-mortem* interval-matched controls (Supplementary Table 1). CB1R immunodetection was non-significantly increased when all subregions of the hippocampus were considered together (Figure 1A). Interestingly, the analysis of the CB1R immunoreactivity in the dentate gyrus showed a significant increase in samples from DS subjects (Figure 1B). Protein expression analysis of CB1R in hippocampus of trisomic Ts65Dn middle-aged mice also showed a significant increase in comparison to WT litter mates' mice (Figure 1C). Together, these data revealed that CB1R overexpression is shared in both human subjects with DS and middle-aged trisomic Ts65Dn mice.

Sustained oral administration of a low dose of rimonabant improves memory performance in young-adult and middle-aged Ts65Dn mice.

We then tested whether a long-term sustained pharmacological intervention with a low dose of the selective CB1R antagonist rimonabant (Rinaldi-Carmona *et al.*, 1994), would be suitable to improve memory performance in Ts65Dn trisomic mice. To this aim, we first established the conditions to perform an oral treatment to deliver rimonabant in the drinking water. Rimonabant was stable in solution at room temperature for at least 4 days (data not shown), so bottle content was changed every 4 days for the length of the treatment. Both male and female mice (Ts65Dn and WT littermates) received the treatment (rimonabant or vehicle) through the drinking water from PND21 until 10 months of age, at this time brain samples were collected. During treatment, mice were analyzed in their behavioral response to assess different aspects of general activity as

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that could be of relevance for the possible side effects associated to CB1R blockade as well as for the efficacy of the treatment at the cognitive level (Figure 2A). First, mice were tested at 4 months of age using the novel object recognition (NOR) task. We observed that Ts65Dn male mice treated with vehicle presented a deficit in this task, as expected (Reeves *et al.*, 1995; Fernandez *et al.*, 2007). Instead, Ts65Dn male mice treated with rimonabant presented a better cognitive performance, comparable to controls treated with vehicle (Figure 2B). This observation confirmed that sustained inhibition of CB1R does not show tolerance to the mnemonic effects observed in trisomic mice.

To assess any possible behavioral disruption that could be related to potential side effects associated to CB1R blockade, we first evaluated anxiety-like behavior using the elevated plus maze task (EPMT) in the same cohort of mice. Ts65Dn trisomic male mice showed a low anxiety-like phenotype and rimonabant treatment did not modify such responses (supplementary Figure 1A). In contrast, rimonabant treatment increased anxiety-like behavior in WT female mice at this time point (supplementary Figure 1B). Interestingly, we observed a significant increase in the total number of entries in Ts65Dn trisomic mice compared to WT that was not altered by rimonabant treatment (supplementary Figure 1C and 1D). Locomotor activity (LAT) assessment of the same cohorts revealed an hyperlocomotor phenotype in Ts65Dn trisomic mice that was not modified by rimonabant exposure (supplementary Figure 2A and 2B). Together, these findings show that chronic long-term rimonabant therapy is effective in restoring NOR memory in young-adult Ts65Dn trisomic mice without compromising other behavioral features in these trisomic animals.

Because Ts65Dn mice display a noticeable neurodegenerative phenotype that resembles that of middle-aged subjects with DS, NOR memory was evaluated again in the same cohort of mice at 10 months of age. Interestingly, no tolerance was developed over the months of treatment since memory improvement was detected in the same cohort of rimonabant-treated male and female Ts65Dn mice of this second time point (Figure 2C and 2D). Remarkably, NOR test did not reveal an effect of the treatment in WT mice (Figure 2C and 2D) demonstrating that blocking CB1R specifically enhances hippocampal-dependent memory in Ts65Dn mice. Furthermore, the presence of

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rimonabant was detected in brain samples of treated mice at that time (mean ± s.e.m., Rimonabant treated groups = 12. 6 ± 6.0 pg/mg).

Noradrenergic and cholinergic alterations are not prevented by long-term rimonabant treatment.

Noradrenergic neurons in the *locus coeruleus* (LC-NE neurons) suffer degeneration in DS (Mukhin *et al.*, 2017). We assessed whether long-term rimonabant treatment could alter LC-NE neurodegeneration in Ts65Dn trisomic mice where we revealed that this treatment improved memory performance. We used tyrosine hydroxylase (TH), the limiting enzyme in the synthesis of dopamine and norepinephrine (Vecchio *et al.*, 2021), as a specific marker of LC-NE neurons. Analysis of LC-NE neurons revealed a significant decrease in the density of TH+ neurons in this region of Ts65Dn male mice, replicating similar results of previous studies (Salehi *et al.*, 2009). Notably, LC-NE neuron loss was not prevented by long-term rimonabant treatment (Figure 3A).

Similarly, basal forebrain cholinergic neurons, the main source of acetylcholine to the hippocampus, undergo age-related degeneration in DS (Yates *et al.*, 1983; Mann *et al.*, 1985). Ts65Dn trisomic male mice show age-related atrophy and loss of cholinergic neurons particularly in the medial septum (Hamlett *et al.*, 2015) as measured using immunostaining for either p75 neurotrophin receptor (p75NTR) or choline acetyltransferase (Salehi *et al.*, 2006). We used p75NTR as a cholinergic marker. Under our experimental conditions, we found that Ts65Dn mice exhibit a non-significant tendency to decrease in the density of cholinergic cells in the medial septum of Ts65Dn compared to WT (Figure 3B), in line with previous studies (Granholm *et al.*, 2000; Hunter *et al.*, 2004b). Nevertheless, this trend to cholinergic degeneration was not modified by rimonabant treatment (Figure 3B). Together, these findings suggest that the effects of rimonabant treatment improving memory performance in aged Ts65Dn mice is independent from preventing the neurological changes revealed in noradrenergic and cholinergic neurons in these brain areas.

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Anomalous microglial morphology in Ts65Dn trisomic mice is normalized by long-term treatment with rimonabant.

Together with early neurodegeneration, brain inflammation is a relevant neurological feature in Alzheimer's disease in DS (Wilcock and Griffin, 2013; Wilcock *et al.*, 2015). Microglial cells are the major mediators of the neuroinflammatory response in the brain and different studies have observed an increase in microglial reactivity in adult Ts65Dn mice (Hunter *et al.*, 2004b; Lomoio *et al.*, 2009; Illouz *et al.*, 2019; Hamlett *et al.*, 2020a) and in subjects with DS (Pinto *et al.*, 2020). In the same previously studied cohort of mice, we observed an increase in microglial soma size, measured as cell body area, in the hippocampus of trisomic mice. This microglial activation was reversed to control values in long-term rimonabant-treated trisomic mice (Figure 4A). Moreover, microglia reactivity, negatively correlated with NOR memory performance (Figure 4B), pointing to a potential relationship between memory performance improvement and diminished microglial reactivity due to rimonabant treatment. Together, these results indicate that long-term CB1R pharmacological inhibition normalized microglia morphology in the hippocampus of trisomic mice.

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Discussion

Intellectual disability in DS is characterized by learning and memory impairments together with age-related comorbidities, such as Alzheimer's disease. Currently, there is no effective treatment to circumvent the genetic alterations in DS. Therefore, there is an urgent need to investigate novel therapeutic targets. In this study, we describe, for the first time to the best of our knowledge, a dysregulation in the expression of CB1R in the hippocampus of human subjects with DS and Ts65Dn middle-aged trisomic mice, revealing CB1R as a promising target for long-term treatments to enhance hippocampal-dependent memory in this disease.

We previously described that CB1R expression and function were upregulated in young-adult Ts65Dn trisomic mice (Navarro-Romero *et al.*, 2019), but whether this feature is observed in human subjects with DS had not been previously addressed. Therefore, we analyzed CB1R immunodetection in the hippocampus of aged subjects with DS compared to age-matched healthy controls. Interestingly, 4 of 5 subjects with DS involved in this study suffered from Alzheimer's disease. A significant enhancement of CB1R immunodetection was found in the dentate gyrus of the hippocampus, whereas a non-significant increased was observed in the whole hippocampus. These findings further underlie the potential interest of diminishing CB1R function as a suitable pharmacological approach for the treatment of memory impairment in DS, as we previously suggested (Navarro-Romero *et al.*, 2019). We also observed that hippocampal CB1R expression was enhanced in homogenates of middle-aged Ts65Dn trisomic mice. At this age, Ts65Dn trisomic mice show signs of a neurodegenerative phenotype (Hamlett *et al.*, 2015). Studies of CB1R expression in the brains of Alzheimer's disease patients have been inconsistent. Indeed, CB1R has been shown to be downregulated, upregulated, and unaltered in these patients (Westlake *et al.*, 1994; Lee *et al.*, 2010; Ahmad *et al.*, 2014; Manuel *et al.*, 2014). Therefore, the enhanced hippocampal CB1R expression could be an up-to-now undescribed novel feature in subjects with DS and supports the potential interest of therapeutical approaches to attenuate CB1R function. Further studies will be needed to determine whether CB1R overexpression appears in younger subjects with DS as a common feature of the DS condition or whether it is associated to the neurodegenerative status in humans.

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We then assessed whether a long-term CB1R pharmacological inhibition intervention would be efficacious for the improvement of memory performance in the mouse model. Rimonabant, a well stablished CB1R specific antagonist (Rinaldi-Carmona *et al.*, 1994), reached the market (Acomplia®, Sanofi Aventis) as a treatment for obesity since high doses of rimonabant were found to reduce food intake and food reinforcing properties (Pacher, 2006). However, Acomplia® was withdrawn from the market due to psychiatric side effects affecting a subpopulation of obese patients (Christensen *et al.*, 2007; Rucker *et al.*, 2007). At high dosages, rimonabant has inverse agonist properties on CB1R, which has been hypothesized to be the cause of some of the negative effects associated to the treatment with this CB1R antagonist (Landsman *et al.*, 1997; Silvestri and Di Marzo, 2012). Therefore, the use of low doses of rimonabant is mandatory to evaluate any possible therapeutic interest of this target in order to obtain results with potential translational value.

We observed that long-term rimonabant exposure, using at a dose 20 times lower that that used to reveal the anti-obesity properties in diet-induced obesity (Martín-García *et al.*, 2010), was well tolerated and did not modify the body weight in mice compared to vehicle-treated animal (data not shown). Notably, recognition memory performance was preserved in both male and female young-adult and middle-aged Ts65Dn trisomic treated mice discarding tolerance processes, which have been observed to be key adverse feature to reduce the effectiveness of other treatments envisioned to improve cognition in other intellectual disability disorders (Stoppel *et al.*, 2021). Our results of long-term exposure further support the relevance of CB1R to tackle cognitive impairment in DS as observed in sub-chronic treatments previously assessed (Navarro-Romero *et al.*, 2019), and open the possibility to evaluate this approach in other intellectual disability disorders where low doses of CB1R antagonists have also been found effective, such as in fragile X syndrome (Busquets-Garcia et al., 2013; Gomis-González et al., 2016).

Other studies have found that increasing the endocannabinoid 2-arachidonoil glycerol (2-AG), by inhibiting the metabolizing enzyme monoacylglycerol lipase (MAGL) through JZL184, has favorable benefits on cognitive function and synaptic plasticity in 11-month-old Ts65Dn trisomic mice (Lysenko *et al.*, 2014). Although the approach used in our study

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with middle-aged Ts65Dn mice would appear to be contradictory, both rimonabant and JZL184 treatments could reduce CB1R functionality. Indeed, JZL184 treatment in mice, or mice knockout for MAGL, has been shown to cause CB1R desensitization has been reported due to the extra 2-AG available (Schlosburg *et al.*, 2010; Bernal-Chico *et al.*, 2015). Therefore, both therapies, through different mechanisms, may eventually reduce CB1R functionality after long term exposure. Furthermore, the possibility that JZL184 is acting through a CB1R-independent mechanism cannot be discarded.

Middle-aged human adults with DS present neuropathological alterations common to Alzheimer's disease, involving the degeneration of noradrenergic neurons in the *locus coeruleus* and basal forebrain cholinergic neurons (Ballard *et al.*, 2016; Fortea *et al.*, 2021), features that are reproduced by middle-aged Ts65Dn mice (Granholm *et al.*, 2000; Salehi *et al.*, 2009; Hamlett *et al.*, 2015). Therefore, we have analyzed the neurodegenerative features of Ts65Dn mice after long-term rimonabant exposure. Interestingly, we observed that Ts65Dn treated with rimonabant presented evident noradrenergic cell loss and an emerging cholinergic impairment to a similar extent that Ts65Dn treated with vehicle. These results discard an effect of rimonabant in these particular neuronal populations to explain its positive effects on cognition in Ts65Dn mice.

Previous research has revealed that endocannabinoids are dysregulated in Alzheimer's disease and contribute to the disorder's progression (Cristino *et al.*, 2020). Because research shows conflicting results, it is difficult to determine if an increase or reduction in cannabinoid tone is associated to an improvement in pathology. For instance, CB1R and/or CB2R agonists improved memory and/or cognitive deficits in Tg2576 mice, APP/PS1 mice (Aso *et al.*, 2013), and in rodents receiving intracerebral injections of Aβ (Ramírez *et al.*, 2005; Martín-Moreno *et al.*, 2011). Conversely, CB1R antagonism protected against Aβ-induced memory impairment in mice (Mazzola *et al.*, 2003), suggesting that activation of CB1R by endocannabinoids inhibits neurotoxicity, but may worsen its long-term consequences (such as reduced acetylcholine signaling) that lead to cognitive impairment.

Given that long-term rimonabant treatment prevented memory deficits in the Ts65Dn mice, we explored other neurological parameters relevant for cognitive performance, such as neuroinflammation. Neuroinflammation is a major contributor to

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neurodegenerative disorders (Colonna and Butovsky, 2017; Yin *et al.*, 2017) including Alzheimer's disease in DS (Wilcock, 2012; Wilcock *et al.*, 2015; Flores-Aguilar *et al.*, 2020; Pinto *et al.*, 2020). Interestingly, recent studies also demonstrated that a reduction in microglial reactivity was associated to improvements in learning and memory in a mouse model of DS (Pinto *et al.*, 2020). We found that Ts65Dn trisomic mice displayed an increase in microglial soma size, which is associated to a reactive type of microglia (Helmut *et al.*, 2011), in agreement with previous studies in microglial populations in the Ts65Dn model (Hunter *et al.*, 2004b). Interestingly, rimonabant-treated Ts65Dn trisomic mice that showed regular recognition memory exhibited as well similar microglial body area that control mice. This anti-inflammatory effect of rimonabant could be directly impinged onto microglial cells or could be secondary to the effect of rimonabant on neuronal circuits that promote microglial reactivity. Notably, the microglial soma area correlated with memory performance in the novel-object recognition test. Previous studies have linked microglial activation and cholinergic cell loss (Hunter *et al.*, 2004b), although we did not find an association between these two brain alterations after long-term rimonabant treatment in our study. One possibility would be that the neuroinflammatory effect is limited to the hippocampal region, with no effect in degenerative regions like the basal forebrain. As a result, these findings suggest a link between improved memory function and reduced microglial reactivity after rimonabant treatment.

At the present moment, there are no approved treatments for intellectual disability in DS in spite of the large amounts of preclinical studies that have been performed (Hamlett *et al.*, 2015; Stagni *et al.*, 2015). In our current study, we maximized the potential translational value of our experimental approach by taking numerous variables into consideration. First, we studied the status of CB1R in DS human hippocampus, and we found interesting parallelisms with the preclinical models. Second, we performed our study in the most used preclinical model for DS, the Ts65Dn, which predictive validity was recently proven for novel experimental approaches to enhancing memory performance in subjects with DS (de la Torre *et al.*, 2014; de la Torre *et al.*, 2016). In addition, Ts65Dn mouse model is the only model which neurodegenerative phenotype is widely described, necessary for the assessment of long-term effects of our treatment (Herault *et al.*, 2017; Antonarakis *et al.*, 2020). Third, we assessed rimonabant efficacy

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in male and female mice, presenting comparable sex results. Altogether, our results reinforce the potential interest of decreasing CB1R activity to maintain cognitive function and prevent specific neurological deficits in DS and expands our understanding of the potential use of CB1R inhibition for long-term periods in the treatment of this disease.

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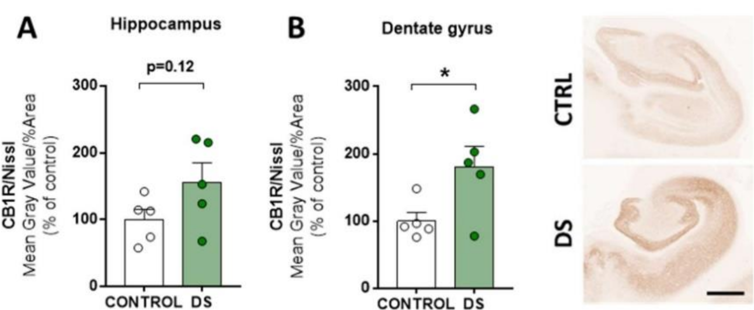

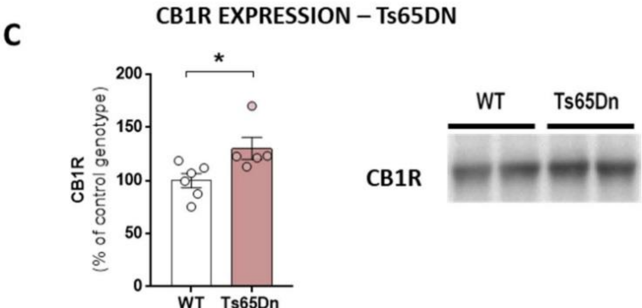

Figure 1. CB1R expression is enhanced in human *post-mortem* samples of aged Down syndrome subjects and in hippocampus of middle-aged Ts65Dn mice. (A-B) Representative images and average intensity of CB1R expression in the hippocampus (A) and dentate gyrus (B) of Control and subjects with DS (Control, n=5, DS=5) (scale bar = 2 mm). (C) Representative immunoblots and quantification of CB1R in hippocampus from WT and Ts65Dn mice of 10 months of age (WT, n=6, Ts65Dn, n=4). Distribution of individual data with mean ± s.e.m. * $p < .05$ (genotype effect) by Student's *t*-test.

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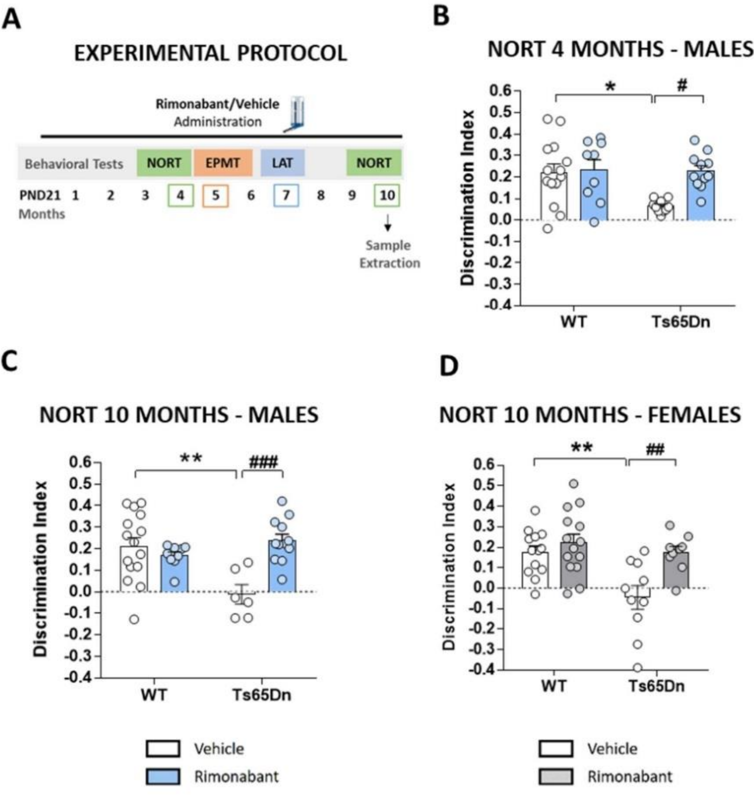

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Figure 2. Long-term CB1R inhibition improves memory performance in young and in middle-aged male and female Ts65Dn mice. (A) Schematic representation of the experimental protocol. EPMT = elevated plus maze test, LAT = locomotor activity test, NORT = novel object recognition test, PND21 = postnatal day 21. (B) Discrimination index in novel object-recognition test (NORT) of WT and Ts65Dn male of 4 months of age with VEH or RIM (0.5 mg/kg/day) (WT VEH, n=14; WT RIM, n=9; Ts65Dn VEH n=10; Ts65Dn RIM n=11). (C-D) Discrimination index in novel object-recognition test (NORT) of WT and Ts65Dn male (C) and female (D) mice treated of 10 months of age with VEH or RIM (0.5 mg/kg/day) (males: WT VEH, n=15; WT RIM, n=9; Ts65Dn VEH n=6; Ts65Dn RIM n=12; females: WT VEH, n=13; WT RIM, n=14; Ts65Dn VEH n=10; Ts65Dn RIM n=9). Distribution of individual data with mean ± s.e.m. * $p < .05$, ** $p < .01$ (genotype effect); # $p < .05$, ## $p < .01$, ### $p < .001$ (treatment effect) by Bonferroni *post-hoc* test following two-way ANOVA.

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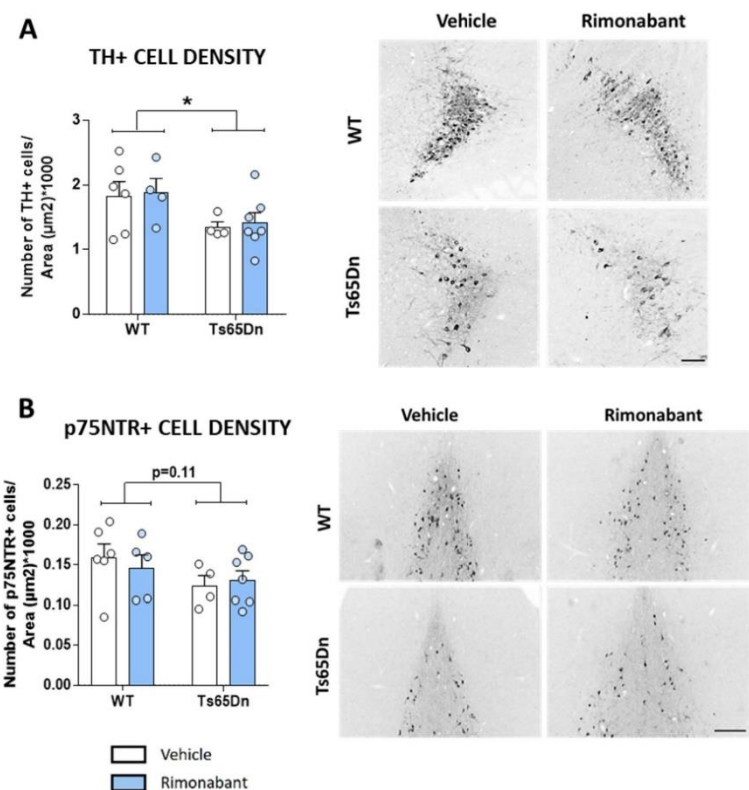

Figure 3. Long-term CB1R pharmacological inhibition did not modify the degeneration of cholinergic and adrenergic neurons in male Ts65Dn mice. (A) Representative grey scale confocal images and average density of TH+ cells in the *locus coeruleus* of WT and Ts65Dn mice of 10 months of age treated with VEH or RIM (0.5 mg/kg/day) (WT VEH, n=6; WT RIM, n=4; Ts65Dn VEH n=4; Ts65Dn RIM n=7) (Scale bar= 100 µm). (B) Representative grey scale images and average density of p75NTR+ cells in the medial septum of the basal forebrain of WT and Ts65Dn mice of 10 months of age with VEH or RIM (0.5 mg/kg/day) (WT VEH, n=6; WT RIM, n=5; Ts65Dn VEH n=4; Ts65Dn RIM n=7) (Scale bar= 200 µm). Distribution of individual data with mean ± s.e.m. * $p < .05$ (genotype effect) by two-way ANOVA.

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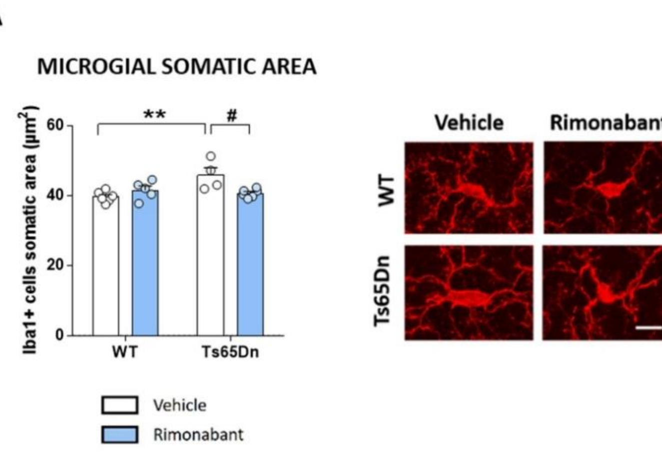


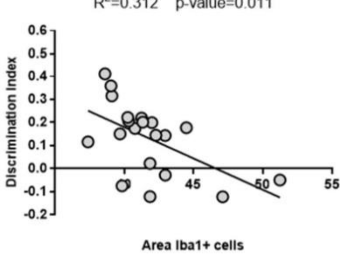
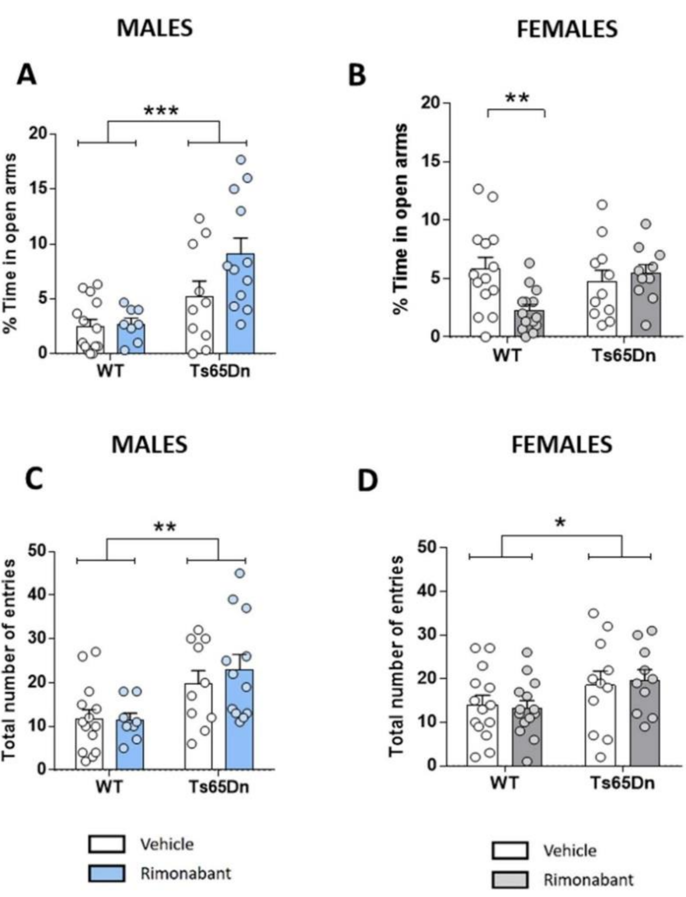


Figure 4. Long-term CB1R pharmacological inhibition reduced microglia reactivity in the hippocampus of male Ts65Dn mice. (A) Representative confocal images and average microglial somatic area of Iba1+ cells in the hippocampus of WT and Ts65Dn mice of 10 months of age with VEH or RIM (0.5 mg/kg/day) (WT VEH, n=6; WT RIM, n=5; Ts65Dn VEH n=4; Ts65Dn RIM n=6) (scale bar=10 µm). (B) Correlation between discrimination index in the NORT and microglial activation (area of the soma) in the hippocampus. Distribution of individual data with mean ± s.e.m. ** $p < .01$ (genotype effect); # $p < .05$ (treatment effect) by Bonferroni *post-hoc* test following two-way ANOVA.

Case Number	Diagnosis	Age (years)	Gender	Clinical diagnosis of dementia
1	Control	66	M	Absent
2	Control	70	M	Absent
3	Control	64	M	Absent
4	Control	58	M	Absent
5	Control	76	M	Absent
6	Down syndrome	62	M	Present
7	Down syndrome	59	M	Present
8	Down syndrome	64	M	Present
9	Down syndrome	61	M	Present
10	Down syndrome	71	M	Absent

Supplementary Table 1. Human samples used for immunohistochemistry for CB1R in Figures 1A-B.



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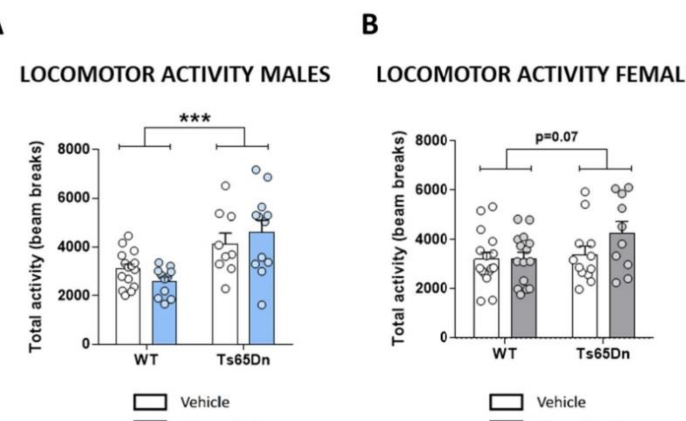
Supplementary Figure 1. Long-term CB1R pharmacological inhibition does not modify anxiety-like behavior in Ts65Dn. (A-B) Percentage of time in open arms and number of total entries in the elevated plus maze test of male (A) and female (B) WT and Ts65Dn mice of 7 months of age treated with VEH or RIM (0.5 mg/kg/day) (males: WT VEH, n=14; WT RIM, n=8; Ts65Dn VEH n=10; Ts65Dn RIM n=12; females: WT VEH, n=14; WT RIM, n=14; Ts65Dn VEH n=11; Ts65Dn RIM n=10). Distribution of individual data with mean ± s.e.m., *** $p < .001$, ** $p < .01$, * $p < .05$ (genotype effect) by Bonferroni *post-hoc* test following two-way ANOVA.



Supplementary Figure 2. Long-term CB1R pharmacological inhibition does not modify locomotor activity in Ts65Dn. (A-B) Total activity measured as number of beam breaks of male (A) and female (B) WT and Ts65Dn mice of 5 months of age treated with VEH or RIM (0.5 mg/kg/day) (males: WT VEH, n=15; WT RIM, n=11; Ts65Dn VEH n=9; Ts65Dn RIM n=12; females: WT VEH, n=16; WT RIM, n=15; Ts65Dn VEH n=12; Ts65Dn RIM n=10). Distribution of individual data with mean ± s.e.m. *** $p < .001$ (genotype effect) by two-way ANOVA.

Objective 2

To assess the efficacy of a CB1R allosteric modulator in the Ts65Dn mouse model of Down syndrome after long-term treatment

Article #2

Long-term cannabinoid type-1 receptor inhibition with a novel allosteric modulator improves memory in the Ts65Dn mouse model of Down syndrome

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** equal contribution.*

In preparation, 2021.

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Introduction

Down syndrome (DS) affects 1 in about 700-1,000 live births worldwide (de Graaf *et al.*, 2021; de Graaf *et al.*, 2020) and is produced by trisomy of human chromosome 21 (HSA21) (Lejeune *et al.*, 1959). Intellectual disability is the most limiting and prevalent hallmark. However, except for nutritional supplements, there is no approved clinical treatment to ameliorate this hampering condition. A high proportion of DS individuals develop Alzheimer's disease by the age of 65 (De Graaf *et al.*, 2017b; Bayen *et al.*, 2018). With the average lifespan of DS individuals increasing during the last decades due to healthcare improvement, dementia has become more prevalent in this population (Fortea *et al.*, 2021). Preclinical mouse models of DS have been used not only to study the physiopathological mechanisms derived from the trisomy but also to assess potential therapeutic approaches that can be later translated to the clinical setting (Herault *et al.*, 2017). One of the most studied mouse models of DS is the partially trisomic Ts65Dn mouse, which contains a majority of the HSA21 orthologous protein-coding genes that map to murine chromosome 16 (Davisson *et al.*, 1993). Ts65Dn mice exhibit several deficits common to individuals with DS, including progressive memory decline and hippocampal abnormalities (Reeves *et al.*, 1995). Importantly for this project, Ts65Dn mice also display common features with AD (Hamlett *et al.*, 2015). More specifically, at 10 months of age Ts65Dn mice show increased APP production, degeneration of *locus coeruleus* neurons

and basal forebrain cholinergic neuron loss, among others (Hamlett *et al.*, 2015).

Our group has identified the endocannabinoid system (ECS) as a strategic biological component with key functional relevance in memory (Puighermanal *et al.*, 2009; Busquets-Garcia *et al.*, 2014). The ECS is a neuromodulatory system playing an important part in brain function due to its role in modulating synaptic transmission (Lutz, 2020). The ECS is composed by receptors, ligands (endocannabinoids) and the enzymes participating in the synthesis and degradation of the endocannabinoids (Zou and Kumar, 2018). Endocannabinoids are believed to mediate retrograde synaptic signaling in the central nervous system being released from the postsynaptic terminal after depolarization. Released endocannabinoids bind to the presynaptic cannabinoid receptors and this finally results in the release inhibition of different neurotransmitters (Wilson and Nicoll, 2002).

Using Ts65Dn mice, the cannabinoid type-1 receptor (CB1R) was identified as a target to improve deficits in cognitive performance in young-adult individuals (2-4 months of age) of the model (Navarro-Romero *et al,.* 2019). In fact, pharmacological CB1R inhibition with rimonabant (Rinaldi-Carmona *et al.*, 1994), a CBR1 antagonist/inverse agonist, restored memory deficits, synaptic plasticity, and adult neurogenesis in this mouse model. Unfortunately, available orthosteric antagonists such as rimonabant, can also act as inverse agonists in a dose-dependent

manner, producing a series of adverse effects at high doses (Landsman *et al.*, 1997; Silvestri and Di Marzo, 2012). Indeed, the commercialization of rimonabant (Acomplia®) was stopped because to psychiatric side effects in a subpopulation of obese subjects (Christensen *et al.*, 2007; Rucker *et al.*, 2007). Other approaches to target and inhibit CB1R function have been recently discovered. Indeed, CB1R over-activation was found to increase the concentration of the steroid hormone pregnenolone in the brain (Vallée *et al.*, 2014). Pregnenolone binds to a specific allosteric site on the CB1R different from the orthosteric site targeted by classical antagonists, and operates as an endogenous signaling specific inhibitor of the CB1R. Pregnenolone, however, cannot be employed as a pharmacological treatment since it is poorly available, has a short half-life, and is converted to downstream active steroids (Vallée *et al.*, 2014). In contrast, AEF, a new pregnenolone-derived compound from Aelis farma, was designed to present improved properties over endogenous pregnenolone, making feasible to use it *in vivo*.

In this study, we have determined the effect of a long-term pharmacological administration of AEF in memory function in young, adult, and middle-aged Ts65Dn mice following the same cohort of mice during a sustained exposure to the drug.

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). Procedures were approved by the local ethical committee (Comité Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB) and local authorities (Generalitat de Catalunya).

All experimental mice were bred at the Barcelona Biomedical Research Park (PRBB) Animal Facility. 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 purchased from The Jackson Laboratory. Euploid littermates of Ts65Dn mice served as wild-type (WT) controls.

Mice were housed in Plexiglas cages with a maximum of 4 males or 5 female mice per cage in a temperature-controlled (21°C ± 1°C) (mean ± range) and humidity-controlled (55% ± 10%) environment. Lighting was maintained at 12 h cycles (light on at 8 AM; light off at 8 PM). All the experiments were conducted during light phase in an experimental room at the animal facility. Food and water were available *ad libitum*. All behavioral experiments were performed by an observer blind to the genotype and treatment. Number of animals was calculated to finally separate them in two groups for

different purposes: to later perform transcriptomic or histologic analysis.

Drug treatments

AEF (a gift from Aelis Farma) was diluted in water and 2-Hydroxypropyl-β-cyclodextrin (0.3 %) in a final concentration of 0.05μg/ml. The compound was administered through the drinking bottle, and a placebo solution was used in mouse littermates as control/vehicle condition. Mice were included in the study at the age of weaning (postnatal day 21, PND21).

Behavioral tests

All behavioral tests were performed in a sound-attenuated room with dim illumination. A digital camera on top of the maze was used to record the sessions.

Novel object-recognition test: Novel object-recognition memory was performed following a previously described protocol (Puighermanal *et al.*, 2009). Briefly, novel object-recognition memory was assessed in a V-shape maze with dim illumination (3-5 lux). This task consists in 3 different phases (habituation, familiarization/training, and test) performed on 3 consecutive days for 9 min. On day 1, mice were habituated to the empty V-maze. Next day, in the familiarization/training phase, mice were introduced in the V-maze where 2 identical objects were presented.

Finally, the test was performed 24 h later, where 1 of the familiar objects was replaced for a novel object and the exploration time for both objects was recorded. Object exploration was defined as orientation of the nose toward the object at a distance < 2 cm. A discrimination index (DI) was calculated as the difference between the time spent exploring either the novel (Tn) or familiar (Tf) object divided by the total time spent exploring both objects: DI = (Tn − Tf) / (Tn + Tf).

Locomotor activity: Locomotor activity was assessed for 120 min. Individual locomotor activity boxes (9 × 20 × 11 cm) (Imetronic) were used in a low luminosity environment (5 lux). The total activity was detected by infrared sensors.

Elevated-plus maze test: The elevated plus maze test was performed in a black Plexiglas apparatus with four arms (29 cm long x 5 cm wide), 2 open and 2 closed, set in cross from a neutral central square (5 cm x 5 cm) elevated 30 cm above the floor and indirectly illuminated from the top (40–50 lux in the open arms/4–6 lux in the close arms). 5-min test sessions were performed, and total number of entries and the percentage of time spent in the open arms were used as a measure of anxiety-like behavior.

Open field-test: Mice were placed in an experimental arena (Plexiglas box, 90×70) for 5-min test session to assess locomotor activity and anxiety-like behavior. Periphery and internal zones of same area were established, and percentage of time spent in the

internal zone was used as a measure of anxiety-like behavior. Smart v3.0 (Panlab) software was used to control the videotracking system.

Experimental design and statistical analysis

Sample size choice was based on previous studies (Busquets-Garcia *et al.*, 2013, 2016) and it is indicated in figure legends for each experiment. Data were analyzed with GraphPad Software using unpaired Student's t-test or two-way ANOVA for multiple group comparisons. Subsequent *post-hoc* analysis (Bonferroni) was used when significance in interaction between factors was found. Comparisons were considered statistically significant when $p < 0.05$. Outliers (±2 s.d. from the mean) were excluded.

Results

Sustained oral AEF administration improves memory performance and does not modify general condition in young and young-adult Ts65Dn mice

We sought to evaluate if a long-term sustained pharmacological intervention targeting CB1R with a low dose of AEF may enhance memory function in Ts65Dn mice. From PND21 to 10 months of age, a cohort of male and female mice (Ts65Dn and WT littermates) received the treatment (AEF or vehicle) through the drinking water. During this period, behavioral responses were studied to determine different aspects of general activity, including the treatment's effectiveness at the cognitive level (Figure 1A).

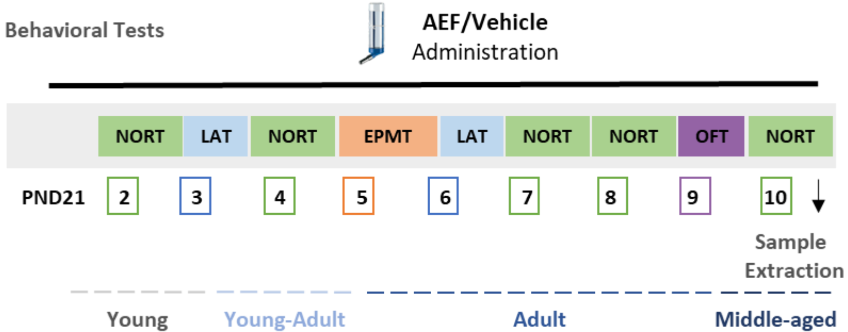

Figure 1. Schematic representation of the experimental protocol. Months of age are indicated for each behavioral observation. EPMT = elevated plus maze test, LAT = locomotor activity test, NORT = novel object-recognition test, OFT = open field test, PND21 = postnatal day 21.

At 2 months of age cognitive performance was tested with the novel object-recognition (NOR) test. At this time, we saw that young-adult Ts65Dn male mice administered with vehicle had a deficit in this task, as was previously described. (Reeves *et al.*, 1995; Fernandez *et al.*, 2007b). On the other hand, Ts65Dn male mice treated with AEF presented a better long-term memory performance in NOR test (Figure 2A and 2B). This observation confirms that long-term exposure to AEF is still able to restore memory performance in a mouse model of DS. This finding indicates that AEF treatment can improve memory function in a young mouse model of DS.

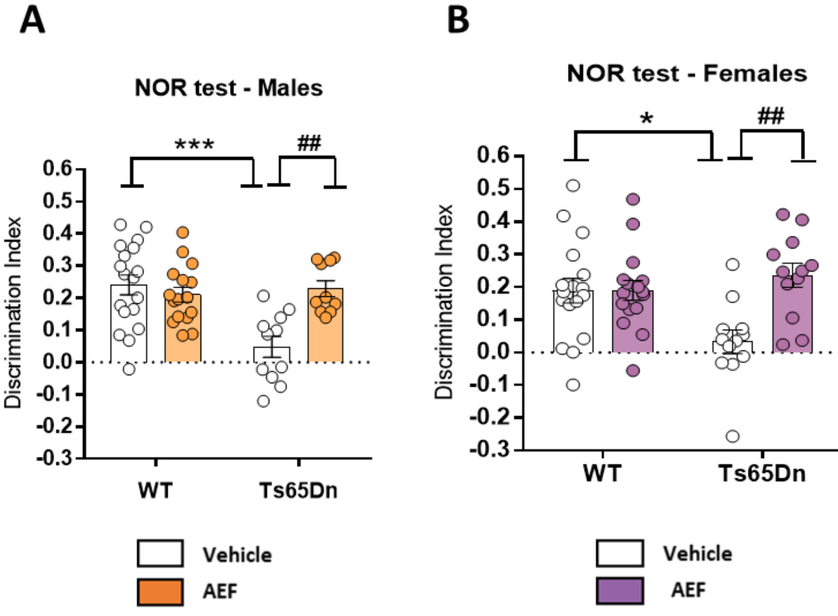

Figure 2. Long-term CB1R inhibition with AEF improves memory performance in young male and female Ts65Dn mice. (A-B) Discrimination index in novel object-recognition test (NORT) of WT and Ts65Dn male (A) and female (B) mice of 2 months of age treated with VEH or AEF (males: WT VEH, n=18; WT AEF, n=16; Ts65Dn VEH, n=11; Ts65Dn AEF, n=10; females: WT VEH, n=17; WT AEF, n=16; Ts65Dn VEH, n=12; Ts65Dn AEF, n=12). Distribution of individual data with mean ± s.e.m. * $p < .05$, *** $p < .001$ (genotype effect); ## $p < .01$, (treatment effect) by Bonferroni *post hoc* test following two-way ANOVA.

The treatment's effect on locomotor activity was also assessed. We found that Ts65Dn male and female mice have a hyperlocomotor phenotype, which was unaffected by AEF (Figure 3A and 3B).

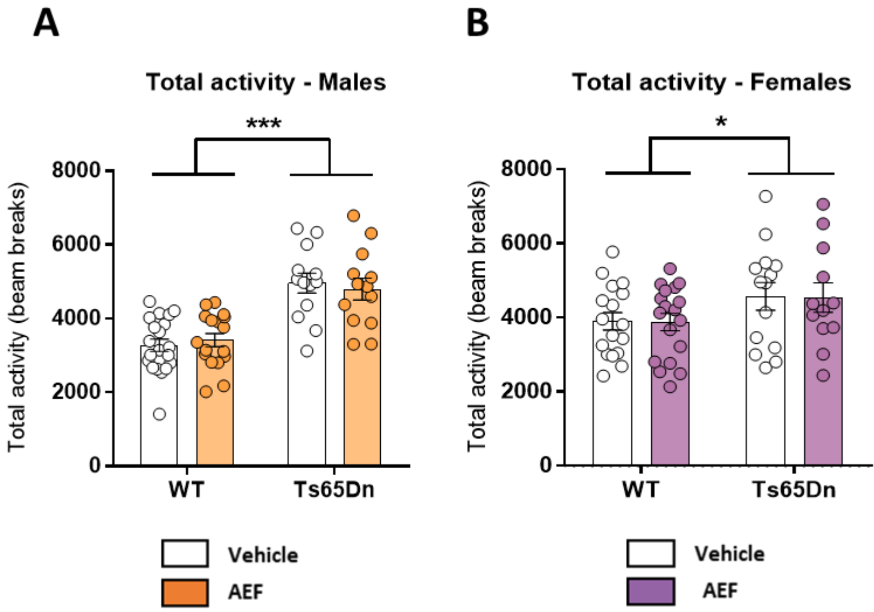

Figure 3. Long-term CB1R pharmacological inhibition with AEF does not modify locomotor activity in young-adult male and female Ts65Dn mice. (A-B) Total activity measured as number of beam breaks of male (A) and female (B) WT and Ts65Dn mice of 3 months of age treated with VEH or AEF (males: WT VEH, n=20; WT AEF, n=17; Ts65Dn VEH, n=13; Ts65Dn AEF, n=13; females: WT VEH, n=17; WT AEF, n=18; Ts65Dn VEH, n=14; Ts65Dn AEF, n=12). Distribution of individual data with mean ± s.e.m. * $p < .05$, *** $p < .001$ (genotype effect) following two-way ANOVA.

Then, we assessed whether a sustained long-term administration of AEF would maintain its effectiveness improving memory performance in adult Ts65Dn mice. To this end, NOR memory performance was tested at 4 months of age. Interestingly, over the months of treatment, no tolerance was developed since long-term memory improvement was detected in the same cohort of AEF-treated male and female young-adult Ts65Dn mice (Figure 4A and 4B).

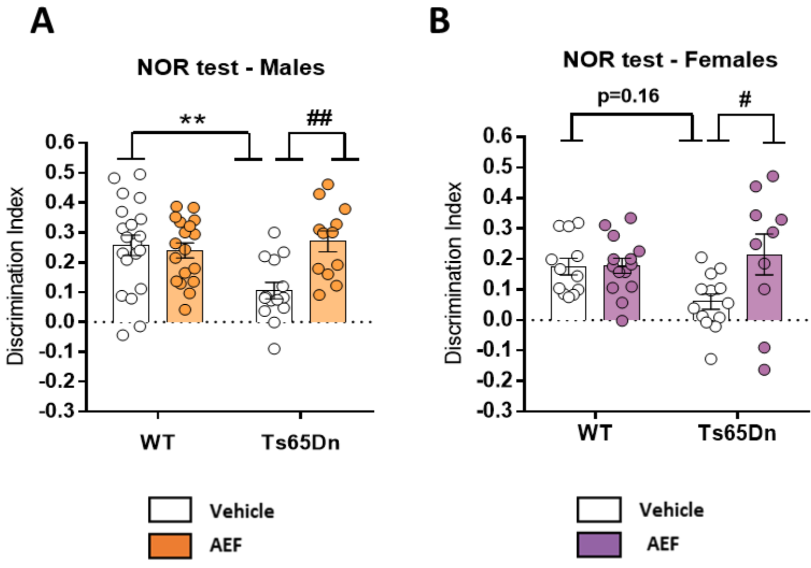

Figure 4. Long-term CB1R inhibition with AEF improves memory performance in young-adult male and female Ts65Dn mice. (A-B) Discrimination index in novel object-recognition test (NORT) of WT and Ts65Dn male (A) and female (B) mice of 4 months of age treated with VEH or AEF (males: WT VEH, n=20; WT AEF, n=18; Ts65Dn VEH, n=14; Ts65Dn AEF, n=12; females: WT VEH, n=12; WT AEF, n=14; Ts65Dn VEH, n=13; Ts65Dn AEF, n=10). Distribution of individual data with mean ± s.e.m. ** $p < .01$, (genotype effect); # $p < .05$, ## $p < .01$, (treatment effect) by Bonferroni *post hoc* test following two-way ANOVA.

Then, the elevated plus maze task was used to examine the anxiety like behavior of WT and Ts65Dn. We showed that Ts65Dn male and female mice displayed a low anxiety-like phenotype, which was unaffected by AEF treatment (Figure 5A and 5B) without altering the number of total entries (Figure 5C and 5D). All together, these data suggest that chronic AEF treatment recovers NOR memory in young and young-adult Ts65Dn mice without affecting other behavioral traits.

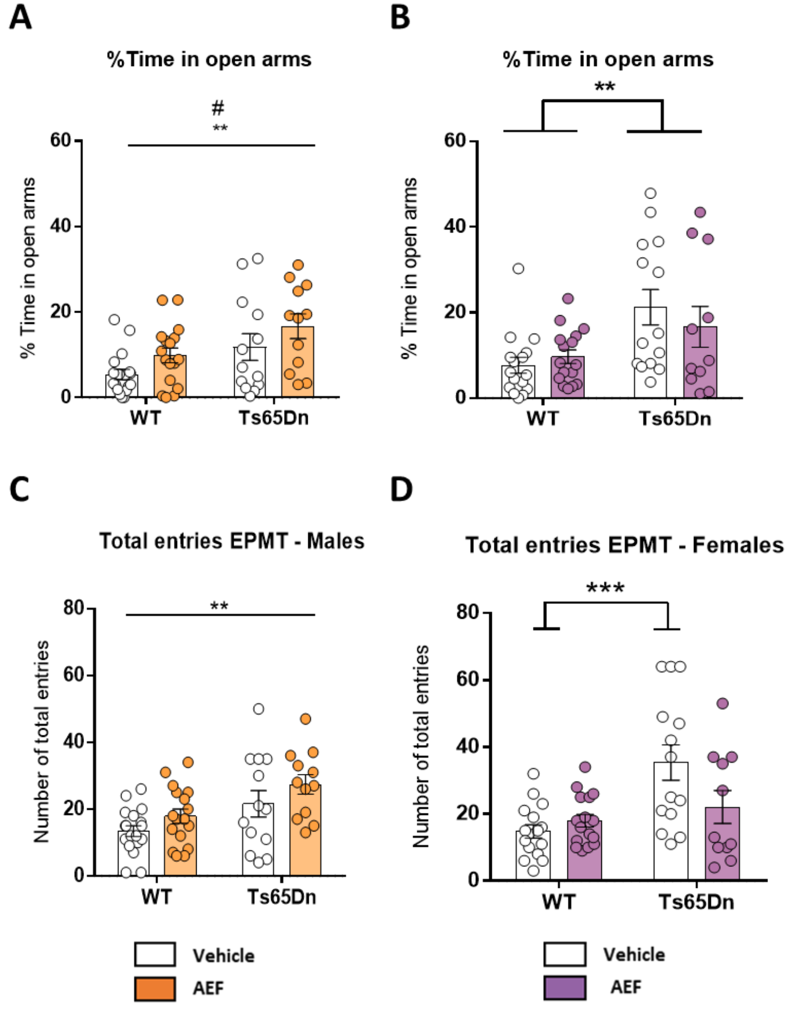

Figure 5. Long-term CB1R pharmacological inhibition with AEF does not modify anxiety-like behavior in male and female Ts65Dn mice. (A-B) Percentage of time in open arms and number of total entries in the elevated plus maze test of male (A) and female (B) WT and Ts65Dn mice of 5 months of age treated with VEH or AEF (0.5 mg/kg/day) (males: WT VEH, n=18; WT AEF, n=18; Ts65Dn VEH, n=13; Ts65Dn AEF, n=12; females: WT VEH, n=17; WT AEF, n=18; Ts65Dn VEH, n=14; Ts65Dn AEF, n=11). Distribution of individual data with mean ± s.e.m. ** $p < .01$; *** $p < .001$ (genotype effect); # $p < .05$ (treatment effect) by Bonferroni *post hoc* test following two-way ANOVA.

Sustained oral AEF administration improves memory performance and does not modify general condition in adult and middle-aged Ts65Dn mice.

Given the positive results observed with AEF in young-adult Ts65Dn mice, we wondered whether the treatment would result efficacious in this same cohort of adult if maintained into middle-age. First, we assessed whether chronic long-term administration of AEF would alter locomotor activity. We found, as observed a few months before, that Ts65Dn male and female mice displayed a hyperlocomotor phenotype, which was unaffected in mice under AEF treatment (Figure 6A and 6B).

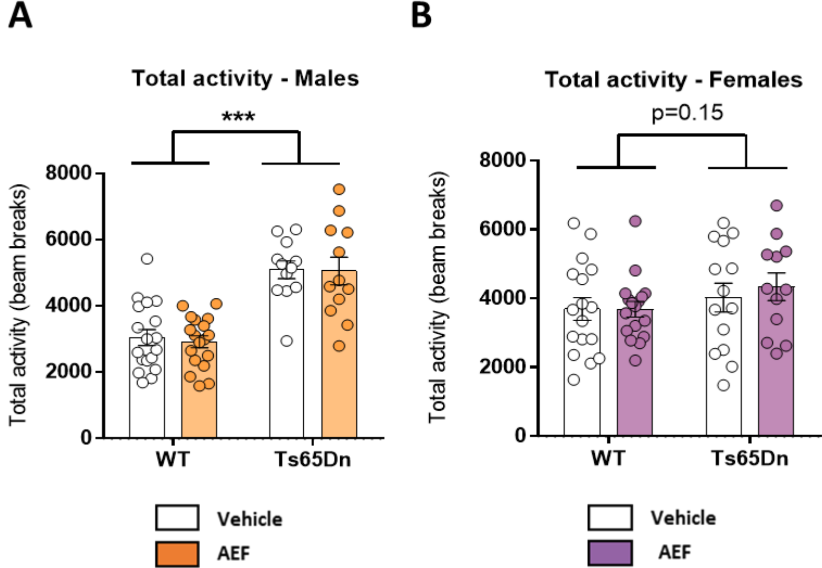

Figure 6. Long-term CB1R pharmacological inhibition with AEF does not modify locomotor activity in adult male and female Ts65Dn mice. (A-B) Total activity measured as number of beam breaks of male (A) and female (B) WT and Ts65Dn mice of 6 months of age treated with VEH or AEF (males: WT VEH, n=18; WT AEF, n=18; Ts65Dn VEH n=12; Ts65Dn AEF n=12; females: WT VEH, n=17; WT AEF, n=17; Ts65Dn VEH n=14; Ts65Dn AEF n=12). Distribution of individual data with mean ± s.e.m. *** $p < .001$ (genotype effect) following two-way ANOVA.

Then, we assessed AEF's efficacy in improving short-term and long-term NOR memory in adulthood. At 7 months of age, we observed that male and female Ts65Dn mice treated with AEF presented normalized short-term memory levels in the NOR test (Figure 7A and 7B).

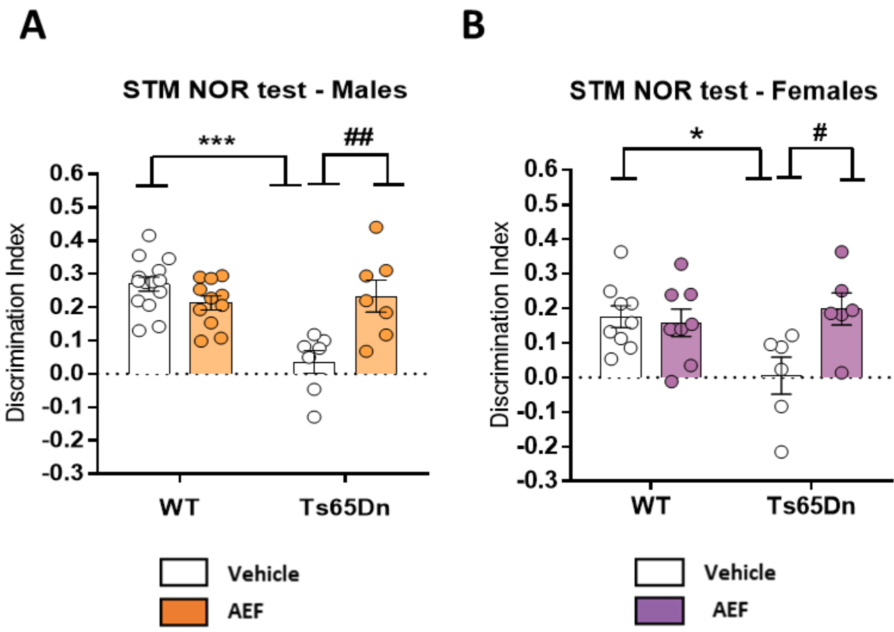

Figure 7. Long-term CB1R inhibition with AEF improves short-term memory performance in adult male and female Ts65Dn mice. (A-B) Discrimination index in novel object-recognition test (NORT) of WT and Ts65Dn male (A) and female (B) mice of 7 months of age treated with VEH or AEF (males: WT VEH, n=14; WT AEF, n=11; Ts65Dn VEH, n=7; Ts65Dn AEF, n=7; females: WT VEH, n=9; WT AEF, n=8; Ts65Dn VEH n=6; Ts65Dn AEF, n=6). Distribution of individual data with mean ± s.e.m. * $p < .05$, *** $p < .001$ (genotype effect); # $p < .05$, ## $p < .01$ (treatment effect) by Bonferroni *post hoc* test following two-way ANOVA.

Furthermore, AEF also improved long-term memory in male and female Ts65Dn mice at 8 months of age (Figure 8A and 8B).

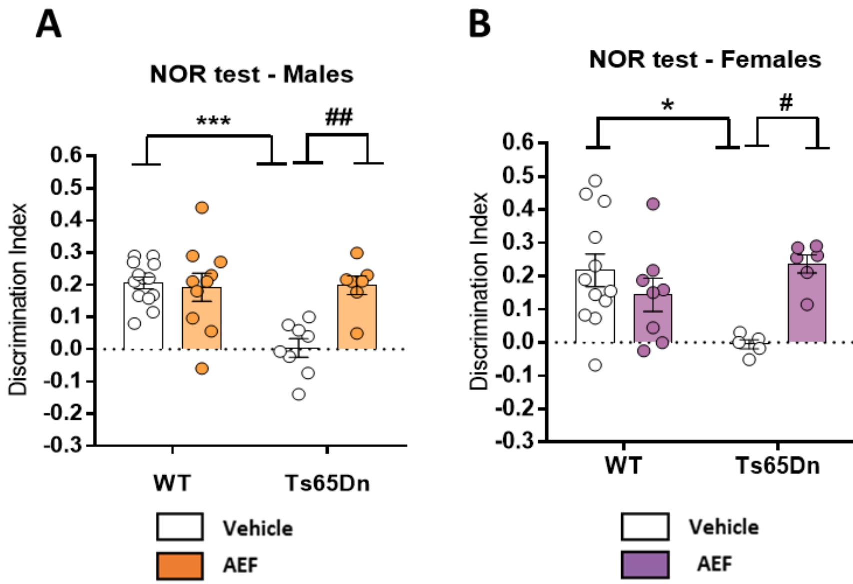

Figure 8. Long-term CB1R inhibition with AEF improves long-term memory performance in adult male and female Ts65Dn mice. (A-B) Discrimination index in novel object-recognition test (NORT) of WT and Ts65Dn male (A) and female (B) mice of 8 months of age treated with VEH or AEF (males: WT VEH, n=13; WT AEF, n=10; Ts65Dn VEH n=8; Ts65Dn AEF, n=7; females: WT VEH, n=12; WT AEF, n=8; Ts65Dn VEH, n=5; Ts65Dn AEF, n=6). Distribution of individual data with mean ± s.e.m. * $p < .05$, *** $p < .001$ (genotype effect); # $p < .05$, ## $p < .01$ (treatment effect) by Bonferroni *post hoc* test following two-way ANOVA.

At this point, anxiety-like behavior was tested with the open field test. In this test, we observed that AEF treatment did not have a major impact in anxiety like-behavior in Ts65Dn mice (Figure 9A and 9B) and did not alter the hyperlocomotor phenotype in the trisomic mice (Figure 9C and 9D).

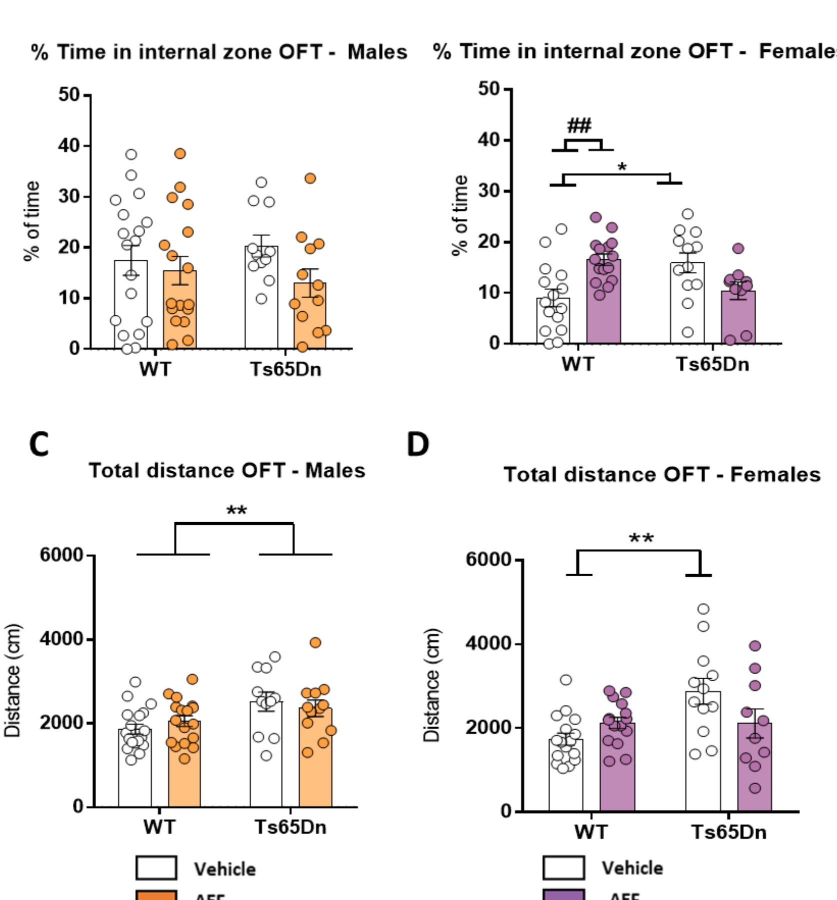

Figure 9. Long-term CB1R pharmacological inhibition with AEF does not modify anxiety-like behavior in male and female Ts65Dn mice. (A-B) Percentage of time in open arms and number of total entries in the elevated plus maze test of male (A) and female (B) WT and Ts65Dn mice of 9 months of age treated with VEH or AEF (0.5 mg/kg/day) (males: WT VEH, n=18; WT AEF, n=17; Ts65Dn VEH, n=12; Ts65Dn AEF, n=12; females: WT VEH, n=17; WT AEF, n=16; Ts65Dn VEH n=12; Ts65Dn AEF, n=11). Distribution of individual data with mean ± s.e.m. * $p < .05$, ** $p < .01$ (genotype effect); ## $p < .01$ (treatment effect) by Bonferroni *post hoc* test following two-way ANOVA.

Finally, we tested AEF efficacy in enhancing memory in the middle-aged Ts65Dn and WT. At this age, the trisomic mice show a noticeable neurodegenerative phenotype that resembles that reported in middle-aged DS individuals. To test memory performance, we analyzed long-term NOR memory in Ts65Dn mice at 10 months of age. Remarkably, no tolerance to the pro-cognitive effect of AEF in trisomic mice was developed throughout the months of therapy, with memory enhancement being shown in the same cohort of AEF-treated male and female Ts65Dn mice (Figure 10A and 10B).

Overall, our findings suggest that chronic/sustained long-term AEF therapy restores short-term and long-term NOR memory in adult Ts65Dn mice with no impact in the hyperlocomotor phenotype nor in the low anxiety-like behavior. These results suggest the relevance of analyzing the brain samples obtained at the end of the long-term treatment in the search for the effects of the AEF treatment in the well-described neurological features of middle-aged trisomic mice.

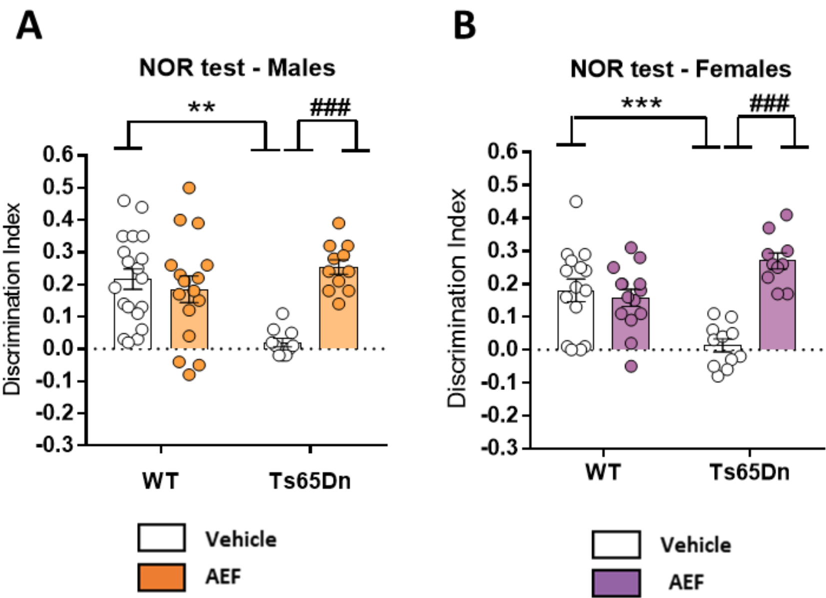

Figure 10. Long-term CB1R inhibition with AEF improves memory performance in middle-aged male and female Ts65Dn mice. (A-B) Discrimination index in novel object-recognition test (NORT) of WT and Ts65Dn male (A) and female (B) mice of 10 months of age treated with VEH or AEF (males: WT VEH, n=19; WT AEF, n=16; Ts65Dn VEH, n=10; Ts65Dn AEF, n=11; females: WT VEH, n=15; WT AEF, n=14; Ts65Dn VEH, n=11; Ts65Dn AEF, n=10). Distribution of individual data with mean ± s.e.m. ** $p < .01$, *** $p < .001$ (genotype effect); ### $p < .001$ (treatment effect) by Bonferroni *post hoc* test following two-way ANOVA.

Discussion

Intellectual disability, defined as learning and memory deficits, is the most prevalent and limiting feature of DS. Nevertheless, there is currently no effective treatment that prevents the genetic alterations of DS, and only palliative therapies can be devised in the near future. As a result, it is crucial finding and developing innovative treatment options. In this study, we described that sustained long-term therapy with a CB1R negative allosteric modulator recovered hippocampal-dependent memory in Ts65Dn mice of various ages.

We previously described that CB1R expression and function were upregulated in young-adult Ts65Dn mice (Navarro-Romero *et al.*, 2019). Additionally, a sub-chronic treatment with the CB1R antagonist/inverse agonist rimonabant (Rinaldi-Carmona *et al.*, 1994) improved memory performance, neuronal plasticity and adult neurogenesis in young-adult Ts65Dn mice (Navarro-Romero *et al.*, 2019). However, rimonabant is not the most appropriate compound for clinical testing since it was associated to psychiatric dose-dependent side effects in obese subjects (Christensen *et al.*, 2007; Rucker *et al.*, 2007). In this study, we have used a pregnenolone-derived compound with improved properties, AEF (Aelis Farma), as a possible alternative translational approach for memory improvement in DS. First, we found that a chronic exposure to AEF enhances memory performance of young and young-adult Ts65Dn mice. Additionally, the treatment with AEF did not induce anxiety-

like behavior and do not modulate locomotor activity in the same cohort of mice. Remarkably, according to previous research, CB1R activation raises brain pregnenolone levels, which has a negative feedback effect on CB1R activity (Vallée *et al.*, 2014). In fact, pregnenolone, through acting as a CB1R signaling-specific inhibitor, decreases numerous of Δ^9- tetrahydrocannabinol effects such as memory impairment (Vallée *et al.*, 2014; Busquets-Garcia *et al.*, 2017). Based on these findings, pregnenolone appears to act as a signaling-specific negative allosteric modulator. Taking this into account and since CB1R activity is higher in Ts65Dn mice hippocampus (Navarro-Romero *et al.*, 2019), AEF may be rescuing NOR memory in Ts65Dn correcting this overactivation. Supporting this hypothesis, it has been proposed that pregnenolone does block specific signaling pathways generated by CB1R activation in contrast of orthosteric antagonists: while pregnenolone does not affect cannabinoids' ability to reduce cAMP, it does completely block CB1R-dependent regulation of the ERK pathway and mitochondrial processes (Vallée et al, 2014). This novel mechanism of action implies that pregnenolone-derived drugs might be utilized to treat conditions defined by high CB1R receptor activation (Vallée *et al.*, 2014; Busquets-Garcia *et al.*, 2018a).

Importantly for this project, allosteric modulators may result in reduced receptor desensitization and/or behavioral tolerance in some cases since allosteric activity doesn't always activate cellular pathways that lead to receptor desensitization and downregulation

(May *et al.*, 2007). Then, we tested whether long-term CB1R pharmacological inhibition with AEF was effective in improving memory performance in adult and middle-aged Ts65Dn mice. Notably, AEF treatment increased recognition memory function in both male and female adult and middle-aged Ts65Dn mice, showing the absence of tolerance mechanisms. Importantly, long-term treatment with AEF did not alter anxiety-like behavior nor locomotor activity in this same cohort of mice. According to these findings, long-term AEF therapy enhanced memory function in Ts65Dn at an age when a pronounced neurodegenerative phenotype is present (Granholm *et al.*, 2000; Salehi *et al.*, 2009; Hamlett *et al.*, 2015). Further studies would be needed to determine whether AEF has an impact in neurodegenerative molecular processes in Ts65Dn mice.

Rimonabant exhibits inverse agonist properties on CB1R at high doses, which is thought to be the origin of some of the antagonist's side effects (Landsman *et al.*, 1997; Silvestri and Di Marzo, 2012). Then, in addition to allosteric modulators, drugs without the inverse agonist characteristics of rimonabant, such as neutral agonists or peripherally restricted molecules that may lack central effects, are other possible pharmacological alternatives under study (Ruiu *et al.*, 2003; Khurana *et al.*, 2017). Regardless, as therapeutic compounds, allosteric modulators may have several advantages. Allosteric drugs can be signaling-specific, influencing only a fraction of the receptor's functions having a more targeted activity than orthosteric

compounds (Christopoulos and Kenakin, 2002; Ross, 2007; Kenakin, 2012). Other possible advantages are preservation of spatial and temporal characteristics of receptor activation, and reduced side effects (Conn *et al.*, 2009).

Together, our findings support CB1R as a druggable target for improving cognitive function in the context of DS. Furthermore, our data point the efficacy of a CB1R-pharmacological strategy with translational potential for long-term memory enhancement in DS.

Objective 3

To investigate auricular transcutaneous vagus nerve stimulation as a method for memory enhancement in naïve mice and in a mouse model of fragile X syndrome.

Article #3

Auricular transcutaneous vagus nerve stimulation improves memory persistence in naïve mice and in an intellectual disability mouse model

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Auricular transcutaneous vagus nerve stimulation improves memory persistence in naïve mice and in an intellectual disability mouse model

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A B S T R A C T

Background: Vagus nerve stimulation (VNS) using non-invasive approaches have attracted great attention due to their anti-epileptic, anti-depressive and pro-cognitive effects. It has been proposed that auricular transcutaneous VNS (atVNS) could benefit intellectual disability disorders, but preclinical data supporting this idea is limited.
Objective: To develop an atVNS device for mice and to test its efficacy on memory performance in naïve mice and in a mouse model for intellectual disability.
Methods: Naïve outbreed CD-1 mice and a model for fragile X syndrome, the *Fmr1* knockout (*Fmr1*KO), were used to assess the effect of atVNS in the novel object-recognition memory performance.
Results: We found that atVNS significantly improves memory persistence in naïve mice. Notably, atVNS was efficacious in normalizing the object-recognition memory deficit in the *Fmr1*KO model.
Conclusion: Our data show that atVNS improves memory persistence in naïve mice and in a model of intellectual disability and support further studies taking advantage of preclinical mouse models of cognitive disorders.
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Introduction

The vagus nerve (cranial nerve X) contains mostly afferent fibers (80%) carrying sensorial inputs from visceral organs and superficial areas [1]. Vagus nerve electrostimulation (VNS) through invasive approaches has shown effectiveness in controlling refractory epilepsy, as a co-adjuvant in the treatment of major depression, and the improvement of cognitive performance [1,2]. The innervation of the external ear is supplied by a heterogeneous distribution of cranial branchial nerve and somatic cervical nerves [3]. The helix of the auricle is mainly supplied by the auriculotemporal nerve (91%) and to a minor extent by the great auricular nerve (9%) [3], while the *cymba conchae* is entirely (100%) supplied by the auricular branch of the vagus nerve (ABVN) [3], which makes it a readily accessible site for electrostimulation. In rats, the ABVN emerges from the superior ganglion [4] and terminates in the nucleus of the tractus solitarius (NTS) of the brainstem [5]. As the therapeutic effects of invasive VNS involve the activation of the NTS, the ABVN has gained interest as a target for non-invasive auricular transcutaneous vagus nerve stimulation (atVNS) [1].

Neurodevelopmental disorders resulting in intellectual disability may benefit from non-invasive VNS. Unfortunately, adequate VNS set-ups combined with relevant behavioral outcomes are not currently available for well-established animal models of cognitive disorders which hampered the possibility of producing a thorough preclinical assessment. Fragile X syndrome is the most common monogenic cause of inherited intellectual disability and autism produced by the silencing of the *FMR1* gene [6]. The constitutive knockout mouse for the *Fmr1* gene (*Fmr1*KO) [7] shows significant cognitive alterations [8,9] and has been long

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used as a well-accepted tool for experimental therapeutic assessment [10].

In this study, we designed, produced and tested the effect on memory persistence of a non-invasive auricular stimulator for mice. Our data further support the relevance of atVNS in cognitive modulation in naive mice and in a model of intellectual disability.

Materials and methods

Animals

Young-adult male CD-1 mice (10–12 weeks old) were purchased from Charles River Laboratories (France). Young-adult *Fmr1*KO mice (12–14 weeks old) and wildtype (WT) littermates in a C57BL/6 J congenic background (B6.129P2-*Fmr1*^{tm1Cgr}/J) [7] were bred at the Barcelona Biomedical Research Park (PRBB) Animal Facility. All animal procedures were conducted in accordance with the standard ethical guidelines (European Communities Directive 2010/63/EU). Mice were housed in a temperature-controlled (21 ± 1 °C) and humidity-controlled (55 ± 10%) environment. Lighting was maintained at 12 h cycles (on at 8 a.m. and off at 8 p.m.). Food and water were available *ad libitum*. Mice were handled for 1 week before starting the experiment. All behavioral experiments were performed by experimenters blind to the experimental conditions.

Electrode system

The electrode prototype was based in a description of a setup for atVNS in rat [5]. Briefly, silver wires with a diameter of 0.5 mm were mounted on a newly designed transparent methacrylate surface and fixed with epoxy resin (Fig. 1A).

Stimulation parameters

Rectangular bipolar pulses were delivered with a Beurer EM49 stimulator (Beurer, Germany). The stimulation parameters were: 1 mA, 20 pulses/second, 30 s ON and 5 min OFF, total length of 30 min, with 330 μs pulse width [5,11].

Current delivery was monitored using a Hantek DSO8060 oscilloscope (Qingdao Hantek Electronic, China), measuring the voltage drop across an external reference resistance R_{ref} in series with the electrode system Z_{load}. Then, the current was calculated as $I = V/R_{ref}$, where I is the delivered current and V the voltage drop calculated across the external reference resistance R_{ref}.

Electrostimulation procedure

Mice were anesthetized with isoflurane (2% induction; 1.5% maintenance) in 0.8 L/min O_2 during 30 min. Normothermic conditions were maintained during anesthesia with a heating pad. For atVNS condition, electrodes were placed in the *concha* of the left ear (Fig. 1B) to avoid cardiac complications, as the right branch of the vagus nerve innervates the sinoatrial node and can have undesirable effects on heart rate [12]. For sham condition, electrodes were placed on the *helix* of the left auricle, outside of the ABVN innervated area. For the “no stimulation” condition mice were anesthetized, but no electrical stimulation was delivered. True atVNS, sham stimulation and no stimulation procedures were performed immediately after the familiarization phase of the novel object-recognition test.

Novel object-recognition memory test

Object-recognition memory was assayed as described previously [13] (Fig. 1C). Briefly, on the first day, mice were habituated to an empty V-shaped maze (V-maze) for 9 min (habituation phase). Then, on the second day, mice were introduced into the V-maze where two identical objects were presented for 9 min (familiarization phase). Immediately after the familiarization phase, mice were exposed to one of the following conditions: (atVNS condition, sham condition or no stimulation) as described above. Then, on the third day, the memory persistence test was performed. To this end, one familiar and one novel object were presented to the mice for 9 min in the V-maze. In naïve CD-1 mice, object-recognition

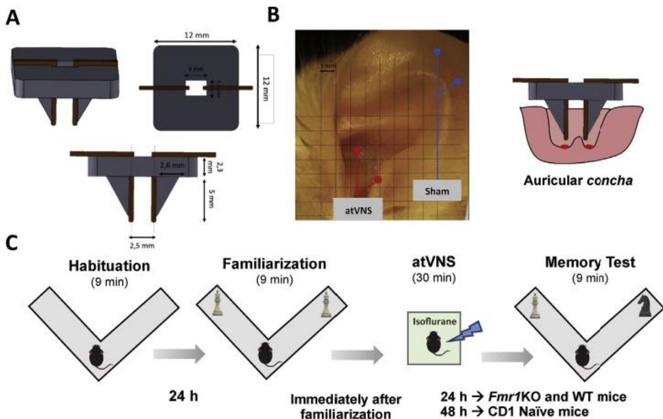
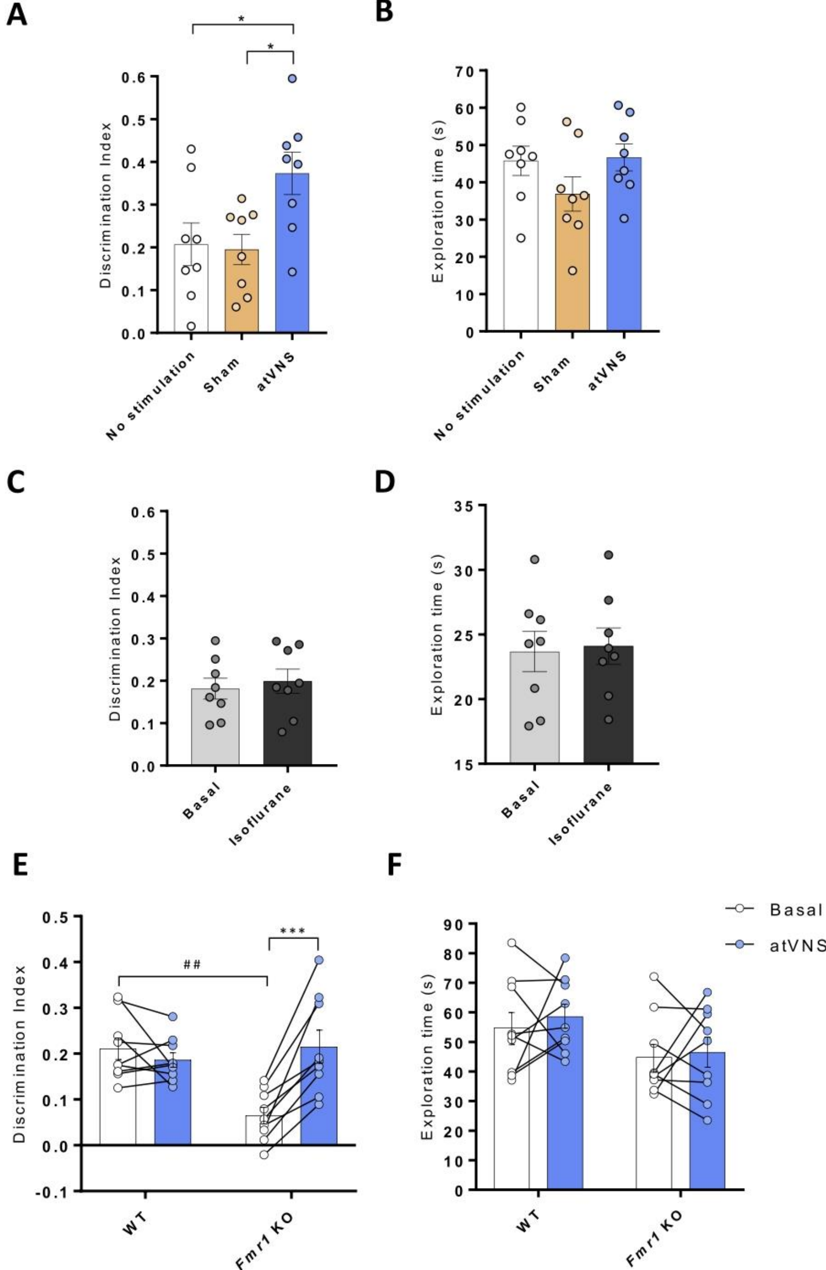


Fig. 1. Experimental design. (A) atVNS prototype design and dimensions. **(B)** Sites of electrostimulation in the auricle for true atVNS and sham condition (top panel) and detail on the position of electrodes on the true atVNS condition (bottom panel). **(C)** Different phases of the novel object-recognition memory test performed in a V-maze (top view). Electrostimulation was performed immediately after the familiarization phase, and memory performance was assessed 48 h later to measure improvements in object-recognition memory persistence in naïve mice, or 24 h later in the Fmr1KO model of fragile X syndrome.


A
B
C
D
E
F
Discrimination Index
Exploration time (s)
No stimulation
Sham
atVNS
Basal
Isoflurane
WT
Fmr1 KO
##
***
Basal
atVNS

memory was tested 48 h after the familiarization phase, a time at which memory persistence enhancements can be measured. In the case of the fragile X syndrome model, memory persistence was tested 24 h after the familiarization phase, at a time when Fmr1KO mice show a significant memory impairment [14]. Time exploring both novel and familiar objects was considered as the time mice spent within 2 cm from the object and with their nose facing it. The exploration time was used to calculate the discrimination index (DI): the difference between the exploration time for the novel and the familiar object related to the time exploring both objects. Higher discrimination indexes were considered to reflect greater object-recognition memory persistence.

Statistical analysis

Data were analyzed with Statistica Software using one-way analysis of variance (ANOVA) or repeated-measures two-way ANOVA for multiple group comparison. Subsequent *post-hoc* analysis (Newman-Keuls) was used when required (significant interaction between factors). Comparisons were considered statistically significant when $p < 0.05$. Data are represented as mean ± standard error of the mean (s.e.m.).

Results

atVNS improves novel object-recognition memory performance in naïve mice

We first assessed the effect of atVNS in cognitive function in naïve CD-1 mice. atVNS condition showed a significantly improvement in object-recognition memory performance at 48 h as revealed by the discrimination index values for the different experimental groups (atVNS = 0.37 ± 0.05; Sham = 0.19 ± 0.03, p = 0.01; No stimulation = 0.21 ± 0.05, p = 0.03) (Fig. 2A), while sham condition reproduced the data on discrimination indexes obtained in mice that did not receive any electrostimulation (Sham = 0.19 ± 0.03; No stimulation = 0.21 ± 0.05, p = 0.84) (Fig. 2A). No differences were observed in overall exploration during the memory test phase (Fig. 2B).

To discard possible confounding effects of anesthesia in the consolidation of novel object-recognition memory delivered after the familiarization session, we assessed object-recognition performance in anesthetized and non-anesthetized naïve mice. Discrimination indexes corresponding to object-recognition memory tested 48 h showed no difference between experimental groups (Basal = 0.18 ± 0.02; Isoflurane = 0.20 ± 0.03, p = 0.65) (Fig. 2C). As expected, there were no differences in exploratory behavior during the memory test phase (Fig. 2D).

atVNS improves novel object-recognition memory performance in Fmr1KO mice

We used the *Fmr1*KO as a model of intellectual disability. In this case, a cohort of *Fmr1*KO and WT littermates was first investigated for their novel object-recognition memory performance 24 h after the familiarization phase in basal conditions (WT Basal = 0.21 ± 0.02; *Fmr1*KO Basal = 0.065 ± 0.02, p = 0.00016) (Fig. 2E), a time known to show clear genotype differences in discrimination indexes [14]. Two weeks later, all mice were submitted to atVNS to assess the effect of electrostimulation. Notably, atVNS had a significant effect in novel object-recognition memory in *Fmr1*KO mice compared to previous basal levels (*Fmr1*KO Basal = 0.065 ± 0.02; *Fmr1*KO atVNS = 0.21 ± 0.03, p = 0.0003), while it did not modify the performance of WT littermates (WT Basal = 0.21 ± 0.02; WT atVNS = 0.19 ± 0.02, p = 0.34) (Fig. 2E). The improvement was observed in almost every *Fmr1*KO animal analyzed (Fig. 2E). No changes were observed in the overall exploration of experimental groups at the time of the memory test (Fig. 2F).

Discussion

This study describes a novel non-invasive transcutaneous vagus nerve stimulation method for mice with a direct impact on memory performance.

Vagus nerve stimulation has emerged as a therapy for the treatment of drug-resistant epilepsy and refractory major depression, since vagal afferents onto the brainstem convey relevant inputs to numerous brain areas deregulated in both pathological states. Among those, brain regions such as the amygdala, the prefrontal cortex and the hippocampus, are also relevant for attention and memory [15]. In agreement, previous studies have revealed the modulation of memory function using invasive and non-invasive approaches of VNS in animal models and in humans [16–18], but, to the best of our knowledge non-invasive transcutaneous approaches had not been assessed in mouse models.

We run atVNS under normothermic conditions and using a low dose of isoflurane to prevent alterations in hippocampal signaling pathways relevant for cognition [19,20]. Our anesthesia conditions revealed no alteration of memory performance compared to non-anesthetized mice, ruling out this step as a potential bias in our behavioral results.

Notably, atVNS in the *concha* of the left external ear in naïve CD-1 mice improved memory retention compared to no stimulation or to sham stimulation conditions. These results are reminiscent of previous reports in other species using invasive techniques for the enhancement of memory retention [21] and point to a potential role of endogenous modulators such as noradrenaline, which extracellular concentrations are enhanced by VNS in rodents [22,23]. We then tested the potential of our atVNS protocol in a well-characterized mouse model of fragile X syndrome, the Fmr1KO model [7], which shows a poor object-recognition memory performance 24 h after familiarization. This marked phenotype of Fmr1KO mice, which can be improved through pharmacological interventions [24,25], was also normalized through atVNS. Discrimination index of WT littermates was not affected after atVNS, since the novel object-recognition test presents a ceiling effect. Therefore, it is not possible to reveal an improvement in the performance of the task in models that do not display a deficit in the test phase 24 h after the familiarization phase.

Together, these results further confirm the potential of atVNS in modulating memory retention in naïve mice, and as a therapeutic tool worth exploring in the context of neurodevelopmental disorders, as previously proposed for invasive forms of VNS [26]. Future studies should focus on the cellular and molecular outcomes of atVNS to elucidate the mechanisms involved in the pro-cognitive effects of this non-invasive technique.

Fig. 2. atVNS improves object-recognition memory persistence in naïve and *Fmr1*KO mice. Discrimination index **(A)** and total exploration time **(B)** in novel object-recognition test (NORT) for atVNS, sham and not stimulated naïve mice (atVNS condition, $n = 8$; Sham condition, $n = 8$; No stimulation condition, $n = 8$). Discrimination index **(C)** and total exploration time **(D)** in NORT for naïve mice in basal and anesthetized conditions (Basal, $n = 8$; Isoflurane, $n = 8$). Discrimination index **(E)** and total exploration time **(F)** in NORT for WT and *Fmr1*KO mice under basal conditions (basal) and after the same mice received atVNS (atVNS) (WT, $n = 9$; *Fmr1*KO, $n = 9$). *$p < 0.05$, ***$p < 0.001$ (electrostimulation effect); ##$p < 0.01$ (genotype effect) by one-way ANOVA (A, B, C, D) and repeated measures two-way ANOVA (E, F).

Author contribution section

A.V.-O. participated in experimental design, conducted and analyzed behavioral experiments and wrote the manuscript.

C.B.-P. participated in experimental design, conducted and analyzed behavioral experiments and wrote the manuscript.

M.D.-G. designed and generated auricular transcutaneous vagal nerve stimulator and wrote the manuscript.

R.M. participated in the supervision and experimental design, funded the project and revised the manuscript.

A.I. participated in the supervision and stimulator design and generation, funded the project and revised the manuscript.

A.O. conceptualized, participated in experimental design, supervised, funded the project and wrote the manuscript.

All authors reviewed and approved the final version of the manuscript

Declaration of competing interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brs.2019.12.024.

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Discussion

The general aim of this thesis was to investigate novel pharmacological and electroceutical approaches for memory enhancement in preclinical models of intellectual disability. Combining the use of behavioral, cellular, and biochemical approaches with the use of genetic mouse models, we have focused on three specific objectives.

In the first two objectives we wanted to expand our understanding of the ECS modulation as a therapeutic approach in DS. In **objective 1** we have studied the relevance of CB1R as a target in DS by revealing its overexpression in human hippocampal samples. Furthermore, we obtained evidence of the efficacy of a long-term CB1R inhibition with rimonabant, a CB1R antagonist/inverse agonist, in enhancing memory function in the Ts65Dn mouse model of DS. We also determined that while maintaining cognitive functionality, rimonabant treatment does not prevent neurodegeneration in these animals but reduces microglial reactivity. In **objective 2** we wanted to expand our understanding of the CB1R as a druggable target for memory improvement in DS in a more translational manner. To this aim, we used the novel negative allosteric modulator AEF (Aelis Farma), to rescue memory performance from young to middle-aged Ts65Dn mice with no observable side effects. Finally, in **objective 3**, we assessed a non-invasive approach for VN stimulation to enhance memory performance in naïve but also in a Fragile X syndrome mouse model.

In this section, we will further discuss the main results obtained in each of the three objectives of this thesis. Since both **objective 1** and **objective 2** study CB1R directed pharmacological approaches for memory improvement in DS, will be discussed in the same section.

1. Study of long-term treatments directed to CB1R as a therapeutic approach for memory improvement in animal models of Down syndrome.

We focused our efforts on DS, a condition in which intellectual disability is the primary limiting factor in individuals' daily lives. There are currently no gold-standard treatments available to help subjects with DS with cognitive impairment. As a result, there is a pressing need to identify novel targets that can help to alleviate such characteristics. We concentrated our efforts on the ECS because it plays a crucial role in memory and regulates multiple pathways that appear to underpin DS impairments (Augustin and Lovinger, 2018).

CB1R expression and function were previously characterized as being upregulated in young-adult Ts65Dn trisomic mice (Navarro-Romero *et al.*, 2019), but whether this was also found in subjects with DS was not investigated. We compared CB1R immunodetection in the hippocampus of older subjects with DS to controls to achieve this goal. We concentrated our attention on the hippocampus, which is involved in memory processing and appears to be particularly affected in subjects with DS (Pennington *et al.*, 2003).

When the entire hippocampus was evaluated, we observed evidence of a non-significant increase in CB1R protein expression, as well as a specific significant enhancement of CB1R immunodetection in the dentate gyrus. These findings support the hypothesis that inhibiting or attenuating CB1R activity may be a viable strategy for treating memory impairment in DS, as seen in preclinical investigations (Navarro-Romero *et al.*, 2019).

For the first time to our knowledge, we revealed a dysregulation in the expression of CB1R in the hippocampus of subjects with DS, suggesting CB1R as a promising target for long-term therapy to boost hippocampal-dependent memory. Interestingly, 4 out of 5 of the subjects with DS participants in this research had AD. CB1R expression in AD brains has been studied in a variety of ways. CB1R has been proven to be unchanged (Lee *et al.*, 2010; Ahmad *et al.*, 2014), upregulated (Manuel *et al.*, 2014), and downregulated (Westlake *et al.*, 1994). It is possible, then, that increased hippocampus CB1R expression is a heretofore unrecognized trait in subjects with DS, strengthening the rationale for CB1R-blocking therapies. This result is in consonance with other studies that have found an increase in the *Cnr1* gene expression in hippocampal samples of young DS subjects (Andrea Contestabile, personal communication). Additionally, a prior study looked at the expression of CB1R, CB2R, and FAAH in the cortex of young subjects with DS. None of these ECS components' expression were found to differ in this DS population from that of control individuals (Núñez *et al.*,

2008). More research is needed to determine whether CB1R overexpression, as we observed, is found just in the hippocampus, if it is a common feature of DS in younger DS cases, and if it is associated to neurodegenerative disease in this population.

We also found CB1R expression increased in the hippocampus homogenates of middle-aged Ts65Dn trisomic mice. Interestingly, Ts65Dn trisomic mice exhibit symptoms of a neurodegenerative phenotype at this age (Hamlett *et al.*, 2015). Indeed, our findings are consistent with a previous study in the adult Ts65Dn mouse hippocampus, that found increased CB1R protein expression at the proteome of subsynaptic compartments (Gómez de Salazar *et al.*, 2018). The molecular processes that underpin CB1R overexpression are yet unknown. Because the *Cnr1* gene, which codes for CB1R, is located at MMU4, Ts65Dn mice do not have it in trisomy. Genes or non-coding regions in trisomy in Ts65Dn mice, on the other hand, may interact directly or indirectly to modify CB1R expression, affecting processes such as transcription, splicing, methylation, and receptor turnover. Alternatively, the availability of endocannabinoids may influence CB1R expression levels, pointing to a modulation of CB1R secondary to other dysregulated biological processes (Laprairie et al., 2012).

In the mouse model, we then investigated whether a long-term CB1R pharmacological inhibition intervention would be effective in improving memory performance. Notably, recognition memory performance was preserved in both male and female young-adult

and middle-aged Ts65Dn trisomic mice, despite the length of the treatment. Notably, tolerance development has been pinpointed as a key adverse feature reducing the effectiveness of other treatments aimed at improving cognition in other intellectual disability disorders such as fragile X syndrome (Stoppel *et al.*, 2021). Our findings of pharmacological long-term CB1R attenuation further support the importance of CB1R in addressing cognitive impairment in DS, as observed in previous shorter sub-chronic treatment studies (Navarro-Romero *et al.*, 2019). Additionally, such results open the door to testing this long-term approach in other intellectual disability disorders where low concentrations of CB1R inhibitors have been found to be effective ameliorating pathological traits, such as in fragile X syndrome (Busquets-Garcia *et al.*, 2013; Gomis-González *et al.*, 2016). Nevertheless, rimonabant was utilized in our study as a well-established CB1R antagonist (Rinaldi-Carmona *et al.*, 1994). Since higher dosages of rimonabant were discovered to lower food intake and food reinforcing characteristics, this compound was introduced to the market as an obesity therapy (Acomplia®, Sanofi Aventis) (Pacher, 2006). However, due to psychiatric adverse effects affecting a subset of obese people, Acomplia® was pulled off the market (Christensen *et al.*, 2007; Rucker *et al.*, 2007). Rimonabant possesses inverse agonist characteristics on CB1R at high doses, which is thought to be the origin of some of the antagonist's side effects (Landsman *et al.*, 1997; Silvestri and Di Marzo, 2012). Therefore, it is worth mentioning that our study used a long-term rimonabant treatment, at a dosage 20 times lower than that used to

demonstrate anti-obesity characteristics in diet-induced obesity (Martín-García *et al.*, 2010). We found this low-dose treatment was well tolerated and had no effect on body weight in mice when compared to vehicle-treated animals.

Nonetheless, to target CB1R with alternate translational possibilities for obesity, agents without the inverse agonist characteristics of rimonabant, such as neutral agonists, negative allosteric modulators, or peripherally-restricted molecules that may lack central effects, are being studied (Ruiu *et al.*, 2003; Khurana *et al.*, 2017; Lu *et al.*, 2019). These are novel agents' worth studying in the context of intellectual disability.

Other study found that in 11-month-old Ts65Dn trisomic mice, blocking the metabolizing enzyme monoacylglycerol lipase (MAGL) with JZL184 has positive effects on cognitive function and synaptic plasticity (Lysenko *et al.*, 2014). Although the method utilized in our work with middle-aged Ts65Dn mice appears to be contradictory with this study, both rimonabant and JZL184 treatments have been demonstrated to impair CB1R functioning, with JZL184 treatment in mice, or mice knockout for MAGL, causing CB1R desensitization due to the additional 2-AG available (Schlosburg *et al.*, 2010; Bernal-Chico *et al.*, 2015). As a result, both therapies may diminish CB1R functioning in the long-term, even though different mechanisms. Moreover, the possibility that JZL184 is functioning through a CB1R-independent mechanism cannot be discarded. In this regard, prior research in AD animal models revealed that JZL184 has anti-

inflammatory properties that are mediated by a CB1R-independent mechanism (Chen *et al.*, 2012; Piro *et al.*, 2012).

Adults with DS, show neuropathological changes similar to AD, including degeneration of LC-NE and BFCN (Ballard *et al.*, 2016b; Fortea *et al.*, 2021), which are also seen in middle-aged Ts65Dn mice (Granholm *et al.*, 2000; Salehi *et al.*, 2009; Hamlett *et al.*, 2015). As a result, examining the neurodegenerative features of middle-aged Ts65Dn mice following rimonabant administration was a prime objective, especially given the favorable effect on behavioral results. Interestingly, Ts65Dn treated with rimonabant showed a significant LC-NE cell loss and emerging cholinergic impairment to a comparable degree as Ts65Dn treated with vehicle. According to the decreased LC-NE cell density, our findings exclude out rimonabant's anti-degenerative effect but support its functional effects on cognition. There are already reports of experimental therapies that improve memory performance without rescuing neurodegeneration in Ts65Dn. For instance, the NMDA antagonist memantine enhances cognition in numerous hippocampal-dependent learning and memory tests in Ts65Dn but does not modulate the loss of BFCN or LC-NE neurons (Costa *et al.*, 2008; Rueda *et al.*, 2010; Lockrow *et al.*, 2012).

Endocannabinoids are dysregulated in subjects with AD and in mouse models of the pathology, which contribute to the illness's development, according to previous studies (reviewed in Cristino *et al.*, 2020). According to these data, there are evidence of

endocannabinoid tone increase and decrease linked to a reduction in pathology (Aso and Ferrer, 2014; Manuel *et al.*, 2014; Aso *et al.*, 2018). In Tg2576 mice, APP/PS1 mice (Aso *et al.*, 2013), and animals given intracerebral injections of Aβ (Ramírez *et al.*, 2005; Martín-Moreno *et al.*, 2011), CB1R and/or CB2R agonists improved memory and/or cognitive deficits. Activation of CB1R by endocannabinoids, on the other hand, protected mice from Aβ-induced memory impairment (Mazzola *et al.*, 2003), suggesting that while CB1R activation inhibits neurotoxicity, it may worsen long-term consequences (such as reduced acetylcholine signaling) that lead to cognitive impairment. Furthermore, as variations between male and female BFCN neurons been reported in Ts65Dn (Granholm *et al.*, 2002) and women with DS (Coppus *et al.*, 2010), it would be important to investigate neurodegeneration in female Ts65Dn mice treated with rimonabant.

We investigated other neurological parameters related to cognitive function, such as neuroinflammation, since long-term rimonabant therapy prevented memory deficits in the Ts65Dn cohort. Neuroinflammation is a key contributor to neurodegenerative disorders (Colonna and Butovsky, 2017; Yin *et al.*, 2017), including AD in DS (Wilcock, 2012; Wilcock *et al.*, 2015; Flores-Aguilar *et al.*, 2020; Pinto *et al.*, 2020). Reductions in microglial reactivity have also been linked to benefits in learning and memory, according to recent research (Pinto et al., 2020). We discovered that Ts65Dn animals had an increase in microglial soma size, which is linked with a

reactive type of microglia (Helmut *et al.*, 2011), and this is consistent with prior investigations in Ts65Dn microglial populations (Hunter *et al.*, 2004b). Surprisingly, rimonabant-treated Ts65Dn trisomic mice displaying regular recognition memory had a microglial body area that was equivalent to control mice. This anti-inflammatory effect of rimonabant could be because of a direct effect onto microglial cells or could be secondary to the modulation of rimonabant on neuronal circuits that promote microglial reactivity. Remarkably, the microglial soma area of these mice was shown to be associated to memory performance in the NOR test. Microglial activation and cholinergic cell death have been associated in previous research (Hunter *et al.*, 2004b). This was not the case in our research. One possibility is that microglial alterations sensitive to rimonabant are restricted to the hippocampus region, with little effect on degenerative areas such as the basal forebrain. In this regard, microglial cells respond to local environmental cues, that could support independent regional alteration. Together, our data point to a relationship between rimonabant therapy and enhanced memory performance with a parallel modulation of hippocampal microglial morphology.

Given the difficulties of using rimonabant in future clinical trials, alternative pharmacological methods with more translational potential are required. To this aim, we employed AEF (Aelis Farma), a pregnenolone-derived molecule with enhanced pharmacokinetic and pharmacodynamic characteristics, as an alternative

translational approach for improving memory in DS (Lu *et al.*, 2019). Pregnenolone is the inactive precursor to all neurosteroid hormones, and its possible functional effects have largely gone unexplored (Baulieu *et al.*, 2001; Ratner *et al.*, 2019). Pregnenolone, on the other hand, cannot be used as a therapy due to its poor availability, short half-life, and rapid conversion to active steroids downstream (Vallée *et al.*, 2014). For this reason, the biotechnology company Aelis Farma's created a number of synthetic pregenolone-derivates, named AEF compounds (WO2012/160006Al; WO2014/083068Al; WO2019/162328AI patents) (Raux *et al.*, 2021). These molecules have a better profile than pregnenolone, with a longer half-life, no steroid conversion, and high absorption after oral administration, being possible to use it in *in vivo* studies (Raux *et al.*, 2021).

First, we found that persistent AEF exposure (from PND21) improves memory function in young and young-adult Ts65Dn mice. Furthermore, in the same cohort of mice, AEF administration did not cause anxiety-like behavior or modify locomotor activity. Interestingly, pregnenolone is produced endogenously after THC administration, and acts as a CB1R negative allosteric modulator (Vallée *et al.*, 2014). As a signaling-specific inhibitor of the CB1R, pregnenolone reduces many of THC's effects such as the increase in food intake, alterations in cognitive function and endophenotypes that are frequently linked to psychotic-like symptoms (Vallée *et al.*, 2014; Busquets-Garcia *et al.*, 2017). Taking these previous

information into consideration, and the fact that CB1R activity is higher in the hippocampus of Ts65Dn mice (Navarro-Romero *et al.*, 2019), AEF was hypothesized to rescue NOR memory in Ts65Dn mice by compensating this overactivation.

Since allosteric sites do not always engage physiological pathways necessary for receptor desensitization and downregulation, allosteric modulators may lack additional unwanted properties over their targets (May *et al.*, 2007). Then in adult and middle-aged Ts65Dn mice, we evaluated whether long-term pharmacological treatment with AEF was beneficial in enhancing memory performance. AEF therapy improved recognition memory function in both male and female adult and middle-aged Ts65Dn mice, indicating the absence of any tolerance for its pro-cognitive effects. In this same cohort of mice, long-term therapy with AEF had no effect on anxiety-like behavior or locomotor activity. All together, these results demonstrate that CB1R targeting with a negative allosteric modulator could produce memory enhancement in DS. Remarkably, this new approach for CB1R inhibition could present a stronger translational potential than CB1R drugs acting in orthosteric sites (Lu *et al.*, 2019). Targeting allosteric sites can have various advantages, including increased subtype selectivity (Conn *et al.*, 2009), preservation of spatial and temporal characteristics of receptor activation, and reduced side effects (Conn *et al.*, 2009; Burford *et al.*, 2013). In addition, allosteric drugs have delimited effects compared to orthosteric drugs since they affect just a part of

the receptor's functions (Ross, 2007; Conn *et al.*, 2009; Kenakin, 2012). Because allosteric activity frequently has a ceiling effect, it may be used to produce ligands with less adverse effects than orthosteric ligands, which can lead to unwanted side effects as concentrations increase (Raux *et al.*, 2021). Interestingly, allosteric binding sites are under less evolutionary pressure than orthosteric sites, and this allows to target them with a better subtype selectivity (Melancon *et al.*, 2012). However, this can produce those allosteric sites do not conserve a strong sequence similarity between species. Then, allosteric modulators working on a certain specie may not be similarly effective in others (Khurana *et al.*, 2017). Thus, targeting allosteric sites might aid the development of receptor subtype-specific drugs, but can also present difficulties in finding and characterizing allosteric modulators across species (Conn *et al.*, 2009). Thus, preclinical pharmacodynamic and safety studies in animal models can be different than the clinical condition. Interestingly, it has been demonstrated that pregnenolone also inhibited the increase in p-ERK1/2 and the decrease in cellular and mitochondrial respiration induced by THC in cells expressing human CB1R (Vallée *et al.*, 2014).

Remarkably, pregnenolone has a specific binding site in CB1R that is topographically different from orthosteric ligand sites (Vallée *et al.*, 2014). However, other studies demonstrated that high dosages of pregnenolone reduced equilibrium binding of rimonabant but did not diminish THC-induced phosphorylation of ERK1/2 in cell lines

expressing human CB1R (Khajehali *et al.*, 2015). Further research will be needed to resolve this disparity in results around pregnenolone.

According to our findings, long-term AEF therapy improved memory performance in Ts65Dn mice at an age when a neurodegenerative phenotype was present (Granholm *et al.*, 2000; Salehi *et al.*, 2009; Hamlett *et al.*, 2015). Interestingly, lipoxin A4, that has been recently described as negative allosteric modulator of CB1R (Straiker *et al.*, 2015; Raux *et al.*, 2021), has been found to protect neuronal cells against the neurotoxicity caused by Aβ given its anti-inflammatory properties (Pamplona *et al.*, 2012). To see if AEF influences neurodegenerative molecular pathways in Ts65Dn mice, more research will be required. So far, there are few investigations on the biological effects of pregnenolone due to its weak activity on the conventional targets of neurosteroids; nonetheless, certain animal studies show that pregnenolone has impacts on anxiety, cognition, and memory (Eser *et al.*, 2008; Zheng, 2009). In fact, pregnenolone has been shown to increase cognitive performance in a variety of memory tasks in animal studies (Vallée, 2016). This memory improvement has been mainly shown in avoidance paradigms (Flood *et al.*, 1992), a food search task (Isaacson *et al.*, 1994), and a working memory paradigm (Melchior and Ritzmann, 1996) in mice. Additionally, pregnenolone enhanced the synaptic plasticity of memory-related brain regions in old rats, increased brain cholinergic activity, and showed to promote improvements of

learning and memory in old rats (Bu and Zu, 2014). Indeed, pregnenolone has been shown to alter microtubule-associated proteins (Murakami *et al.*, 2000; Weng *et al.*, 2013). These proteins are implicated in synaptic plasticity (Penazzi *et al.*, 2016), and pregnenolone has been demonstrated to accelerate microtubule polymerization, implying a function in brain development and aging (Vallée, 2016). Additionally, pregnenolone was originally utilized as an anti-inflammatory treatment in humans in the 1940s, and placebo-controlled human studies with pregnenolone showed substantial increases in mood, overall well-being, psychomotor performance, and learning in normal controls evaluated under stressful settings, with little side effects (Pincus and Hoagland, 1945).

Together, negative allosteric modulators, with their distinct method of action, have been hypothesized to treat conditions defined by excessive CB1R activation (Vallée *et al.*, 2014; Busquets-Garcia *et al.*, 2017). Indeed, a phase II clinical trial was already completed (NCT03717272) to assess the efficacy of a pregnenolone derivative (AEF0117) on cannabis addiction. This compound had previously demonstrated efficacy in preclinical approaches (Busquets-Garcia *et al.*, 2018a).

Then, in addition to allosteric modulators, there are other compounds that do not have the inverse agonist properties of rimonabant such as neutral agonists or peripherally limited compounds that may lack central adverse effects (Ruiu *et al.*, 2003;

Khurana *et al.*, 2017). In fact, a subchronic therapy with the neutral antagonist NESS0327, for example, has already been shown to improve memory function in young-adult Ts65Dn mice in our previous study (Navarro-Romero *et al.*, 2019). On the other hand, targeting peripheral CB1R might be an intriguing way to avoid the central adverse effects that have been described when targeting CB1R systemically, as well as concerns with blood-brain barrier permeability in the development of novel therapeutic drugs (Pardridge, 2012).

Importantly, our research has some limitations that should be considered. The Ts65Dn mouse model is trisomic for 90 ortholog genes to those reported in HSA21, as well as for another 35 coding genes that are not found in trisomy in subjects with DS, which may influence the findings in this study. Although the Ts65Dn model's construct validity isn't the best compared to other DS mouse models described so far, it accurately reproduces the majority of the phenotypes seen in DS patients of various ages (Aziz *et al.*, 2018). Unfortunately, there is no ideal DS mouse model. Other models, such as the Dp16, which expresses three copies of 119 genes orthologues on MMU16, while lacking any extra non-DS related genes, and the TTS, which is trisomic for all three syntenic areas homologous to HSA21, have superior construct validity (Yu *et al.*, 2010a, 2010b; L. Zhang *et al.*, 2014). Surprisingly, both mouse models exhibit abnormalities that are less severe than those shown in the Ts65Dn model, and they lack several of the traits seen in

people with DS (Belichenko *et al.*, 2015; Aziz *et al.*, 2018). This difference could be due to the fact that, unlike Ts65Dn mice, the extra genetic material in the Dp16 and TTS models is not contained on a freely segregating chromosome. Alternatively, the use of human induced pluripotent stem cells obtained from subjects with DS is an emerging promising approach for studying DS neurobiology (Takahashi and Yamanaka, 2006). Human brain organoids produced from induced pluripotent stem cells provide a complementing technique to animal models for researching the neurological basis of DS and discovering therapeutic targets, as species differences restrict the resemblance of animal models to human biology (Weick *et al.*, 2013; Hibaoui and Feki, 2015; Mizuno *et al.*, 2018; Rueda *et al.*, 2020). The use of both animal models and iPSCs together will certainly result in more accurate information, thus improving the predictive validity of preclinical studies.

Another potential limitation is that in people with DS, maintaining a specific treatment for such a long time would be difficult. Treatments for shorter periods of time that have long-term effects, for example, would need to be researched. Because most of the neuron proliferation and maturation occurs during the prenatal and early postnatal periods (Semple *et al.*, 2013; Stagni *et al.*, 2015), more research is needed to determine whether starting the treatment at a younger age, such as the time of weaning, or even during the prenatal period, would result in greater benefits. For instance, prenatal therapy might have a significant influence,

potentially affecting the entire brain's development and having long-term consequences. Some studies have already demonstrated its efficacy in prenatal or perinatal treatments such as fluoxetine (Guidi *et al.*, 2014b), choline (Moon *et al.*, 2010), neurotrophic factors (Toso *et al.*, 2008; Incerti *et al.*, 2012), and epigallocatechin-3-gallate (Guedj *et al.*, 2009).

Despite several preclinical research, there are no authorized therapies for intellectual disability in DS (Hamlett *et al.*, 2015; Stagni *et al.*, 2015b; Rueda *et al.*, 2020). We aimed to optimize the translational potential of our work by considering a variety of factors:

- We looked at CB1R expression in the DS human brain, finding some interesting parallels with preclinical models.
- We conducted our research using the Ts65Dn, the most widely used preclinical model for DS, whose predictive validity was recently demonstrated for innovative experimental techniques to improve memory function in subjects with DS (de la Torre *et al.*, 2014; Torre *et al.*, 2016). Furthermore, the Ts65Dn mouse model is the only one with a well-documented neurodegenerative phenotype, which is essential for assessing the long-term implications of our treatment (Herault *et al.*, 2017; Antonarakis *et al.*, 2020).
- We compared the effectiveness of rimonabant in male and female mice, obtaining similar findings.

- We used two different CB1R-targeted pharmacological methods, one of which has a potential translational profile.

Overall, our findings support the use of CB1R taming to maintain cognitive function and avoid neurological impairments in DS, and they add to our knowledge of the long-term effects of CB1R inhibition.

2. Study of auricular transcutaneous vagus nerve stimulation in memory persistence in naïve mice and in a mouse model of fragile X syndrome.

Previous research has shown that invasive and non-invasive VNS techniques can modulate memory performance in animal models and humans (Clark *et al.*, 1999; Ghacibeh *et al.*, 2006a; Vonck *et al.*, 2014; Jacobs *et al.*, 2015; Sun *et al.*, 2017), but non-invasive transcutaneous approaches have not been evaluated in mouse models to our knowledge. In this study, we described a non-invasive transcutaneous VNS (tVNS) method for memory improvement in naïve mice and in a mouse model of FXS.

VNS has emerged as a therapy for the treatment of drug-resistant epilepsy and refractory severe depression since vagal afferents onto the brainstem convey significant inputs to multiple brain regions affected in both disease conditions (Woodbury and Woodbury, 1990; Rush *et al.*, 2000; Ben-Menachem, 2002; Johnson and Wilson, 2018). In addition, attention and memory are also influenced by

these brain regions such as the amygdala, prefrontal cortex, and hippocampus (Grimonprez *et al.*, 2015). However, a clear direct effect in memory of atVNS mice was still not studied.

To this aim, we designed a device adapted for the stimulation of the auricular branch of the VN in mice. We operated atVNS in a normothermic environment with a low dosage of isoflurane to avoid changes in cognition-relevant hippocampal signaling pathways (Cohen and Stackman, 2015; Hao and Wang, 2017). When compared to non-anesthetized mice, our anesthetic settings had no effect on memory performance, ruling out this step as a possible source of bias in our behavioral results. In naive CD-1 mice, atVNS in the concha of the left external ear increased memory retention relative to no stimulation or sham stimulation. These findings mirror previous studies in humans, according to limited clinical studies in invasive VNS (Clark *et al.*, 1999; Ghacibeh *et al.*, 2006a, 2006b) and tVNS trials (Jacobs *et al.*, 2015; Klaming *et al.*, 2020). These clinical studies found that only when stimulation was delivered during the learning phase, not during the recall phase, memory performance was improved. This suggests that VNS has no effect on memory retrieval, but rather on memory consolidation, resulting in increased retention power (Ghacibeh *et al.*, 2006b).

Invasive VNS treatment has also been shown to enhance quality of life in people with neurodevelopmental problems in a number of studies (Engineer *et al.*, 2017). We then assessed our atVNS methodology in a well-characterized mouse model of FXS, the *Fmr1*

KO animal (Bakker *et al.*, 1994), which exhibits poor object-recognition memory 24 h after familiarization. This distinct phenotype of *Fmr1* KO mice, which can be improved by pharmacological interventions (Busquets-Garcia *et al.*, 2013; Gomis-González *et al.*, 2016), was also normalized by atVNS. Because the novel object-recognition test reaches ceiling discrimination index values in the case of WT littermates when memory is assessed 24 hours after training, WT performance seems unaffected following atVNS. Therefore, the assessment of atVNS efficacy should be performed under challenging conditions, where there is room for improvement.

VNS may give some symptomatic alleviation for subjects with ASD, according to several lines of evidence (Warwick *et al.*, 2007; Levy *et al.*, 2010; Hull *et al.*, 2015; Manning *et al.*, 2016). The parasympathetic system is often dysregulated in ASD and decreased vagal tone is common in this population, specially described in FXS (Hall *et al.*, 2009; Klusek *et al.*, 2013; Cohen *et al.*, 2015). Reduced vagal activity has been linked to autistic behaviors and language difficulties (Roberts *et al.*, 2006; Klusek *et al.*, 2013), whereas increased vagal activity has been linked to improved communication outcomes later in life (Watson *et al.*, 2010). VNS treatment has already been demonstrated to overcome a lack of vagal response (Peña *et al.*, 2014), indicating that it may be useful in neurodevelopmental diseases characterized by altered parasympathetic activity. Research in patients demonstrates that

VNS reduces anxiety measures (George *et al.*, 2008). The dual action of VNS in providing both a brain plasticity reinforcing stimulus and soothing parasympathetic input through activation of descending pathways is a unique characteristic that may give advantages not yet available with pharmaceutical interventions (Fanselow, 2013). Indeed, drugs that engage neuromodulatory systems can improve learning, but they also produce anxiety because they block parasympathetic activation. Anxiolytic medications, on the other hand, tend to interfere with plasticity and reduce the benefits of rehabilitation (Engineer *et al.*, 2017).

In this study we performed atVNS during memory retention, but whether it is effective in learning or memory recall is currently being investigated. Interestingly, preliminary research suggests that combining VNS with cognitive treatment may be beneficial. Furthermore, preclinical and clinical data suggests that combining bursts of VNS with certain actions or sensory events can help with some disorder rehabilitation (Hays, 2016). Unlike open-loop delivery of 30 sec long stimulation trains for epilepsy, new VNS applications employ short 0.5 sec trains administered in time with particular events during a training or rehabilitative paradigm. The VNS-dependent activation of plasticity-enabling neuromodulatory circuits in this paradigm is used to reinforce the neuronal activity associated with rehabilitation (Hays, 2016). Interestingly, when VNS is combined with fear conditioning extinction paradigm, it affects the production and activation of synaptic plasticity proteins as

CaMKII, Arc, and GluN2B (Alvarez-Dieppa *et al.*, 2016). This combination accelerates the extinction of a conditioned fear response in an animal model of anxiety, which is consistent with these alterations in plasticity-associated proteins (Peña *et al.*, 2013, 2014).

To further understand the processes behind atVNS's pro-cognitive benefits, future study should focus on the cellular and molecular outcomes. Some studies are focused on the relevance of the noradrenergic and cholinergic systems in VNS treatment (De Ridder *et al.*, 2014). Specifically, the VN stimulates the *locus coeruleus*, the major source of norepinephrine in the central nervous system, resulting in strong, phasic neuronal activity (Hulsey *et al.*, 2017). VNS increases norepinephrine levels in the hippocampus and cortex, which is consistent with VNS-dependent noradrenergic system involvement (Roosevelt *et al.*, 2006; Raedt *et al.*, 2011). Furthermore, VNS raises levels of brain-derived neurotrophic factor (BDNF) (Follesa *et al.*, 2007), a neurotrophin associated to neural plasticity that is dysregulated in people with ASD (Ricci *et al.*, 2013). Remarkably, the function of these neuromodulatory systems is altered in several neurodevelopmental diseases, including Down syndrome, Rett syndrome, and FXS (Yates *et al.*, 1983; German *et al.*, 1992; Taneja *et al.*, 2009). Considering this, the effectiveness of atVNS might be advantageous for various neurodevelopmental disorders.

In our study, one single session of atVNS was enough to produce pro-cognitive effects. Future research should determine the effectiveness of long-term atVNS therapy in naïve mice and in mouse models of neurodevelopmental disorders. In this regard, there has not been much research into how long the effects of tVNS remain once the stimulation phase is finished. The therapeutic results are monitored concurrently in most clinical studies, which include daily stimulation sessions over the length of the experiment (Yap *et al.*, 2020). Longer treatment periods have been linked to improved therapeutic outcomes in several studies (He *et al.*, 2013; Yuan and Silberstein, 2016; Liu *et al.*, 2018). These trials, however, did not provide a follow-up to assess if the benefits of tVNS were long-lasting or persisted after the treatment period ended (Yap *et al.*, 2020).

Together, these findings support the potential of atVNS in altering memory retention in naive mice and as a therapeutic approach worth examining in the context of neurodevelopmental disorders. Future research should concentrate on the cellular and molecular results of atVNS to better understand the processes underlying its pro-cognitive effects.

Conclusions

The findings presented in this thesis allow stating the following conclusions:

1. CB1R expression is enhanced in human *post-mortem* hippocampal samples of aged subjects with Down syndrome.
2. CB1R expression is enhanced in hippocampus of middle-aged Ts65Dn mouse model of Down syndrome.
3. Long-term treatment with a low dose of the CB1R antagonist/inverse agonist rimonabant, improves memory performance in young-adult and middle-aged male and female Ts65Dn mice.
4. Rimonabant does not modify locomotor activity and anxiety-like behavior in the same cohort of Ts65Dn mice where object recognition memory is restored.
5. Rimonabant treatment does not modify the degeneration of adrenergic and cholinergic neurons in middle-aged Ts65Dn mice.
6. Rimonabant treatment normalizes to control values the microglia cell morphology in the hippocampus of middle-aged Ts65Dn mice.
7. Long-term treatment with the CB1R negative allosteric modulator AEF improves object recognition memory performance from young to middle-aged male and female Ts65Dn mice.

8. AEF does not modify locomotor activity and anxiety-like behavior in the same cohort of Ts65Dn mice where memory is improved.
9. A functioning auricular transcutaneous vagus nerve device can be employed for preclinical experiments on vagus nerve stimulation in mice.
10. Acute auricular transcutaneous vagus nerve stimulation improves object-recognition memory persistence in naïve mice.
11. Acute auricular transcutaneous vagus nerve stimulation improves object-recognition memory persistence in the Fmr1KO mouse model of fragile X syndrome.

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Annex

Article #1

Cannabinoid type-1 receptor blockade restores neurological phenotypes in two models for Down syndrome

Alba Navarro-Romero, Anna Vázquez-Oliver, Maria Gomis-González, Carlos Garzón-Montesinos, Rafael Falcón-Moya, Antoni Pastor, Elena Martín-García, Nieves Pizarro, Arnau Busquets-Garcia, Jean Michel Revest, Pier Vincenzo Piazza, Fátima Bosch, Mara Dierssen, Rafael de la Torre, Antonio Rodríguez-Moreno, Rafael Maldonado*, Andrés Ozaita*.

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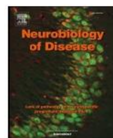

Cannabinoid type-1 receptor blockade restores neurological phenotypes in two models for Down syndrome

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ABSTRACT

Intellectual disability is the most limiting hallmark of Down syndrome, for which there is no gold-standard clinical treatment yet. The endocannabinoid system is a widespread neuromodulatory system involved in multiple functions including learning and memory processes. Alterations of this system contribute to the pathogenesis of several neurological and neurodevelopmental disorders. However, the involvement of the endocannabinoid system in the pathogenesis of Down syndrome has not been explored before. We used the best-characterized preclinical model of Down syndrome, the segmentally trisomic Ts65Dn model. In male Ts65Dn mice, cannabinoid type-1 receptor (CB1R) expression was enhanced and its function increased in hippocampal excitatory terminals. Knockdown of CB1R in the hippocampus of male Ts65Dn mice restored hippocampal-dependent memory. Concomitant with this result, pharmacological inhibition of CB1R restored memory deficits, hippocampal synaptic plasticity and adult neurogenesis in the subgranular zone of the dentate gyrus. Notably, the blockade of CB1R also normalized hippocampal-dependent memory in female Ts65Dn mice. To further investigate the mechanisms involved, we used a second transgenic mouse model overexpressing a single gene candidate for Down syndrome cognitive phenotypes, the dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A). CB1R pharmacological blockade similarly improved cognitive performance, synaptic plasticity and neurogenesis in transgenic male Dyrk1A mice. Our results identify CB1R as a novel druggable target potentially relevant for the improvement of cognitive deficits associated with Down syndrome.

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1. Introduction

Down syndrome, derived from the complete or partial trisomy of human chromosome 21 (Hsa21), is the main genetic cause of intellectual disability affecting 1 in 700–1000 live births (Dierssen, 2012; Parker et al., 2010). Nowadays, intellectual disability is the most limiting feature of Down syndrome subjects' daily life, a condition for which there is no available treatment in the clinical practice, although partially effective treatments are under investigation (Gardiner, 2014). Down syndrome subjects display low intelligence quotients, and learning and memory deficits, especially in hippocampus-related cognitive domains (Dierssen, 2012; Pennington et al., 2003).

Based on the homology of the Hsa21 to the mouse chromosomes Mmu16, Mmu17 and Mmu10, several Down syndrome trisomic mouse models have been developed (Dierssen, 2012; Herault et al., 2017). The most studied is the Ts65Dn mouse which carries an extra copy of part of the mouse chromosome 16 resulting in trisomy of around 90 conserved protein-coding gene orthologues to Hsa21 (Reeves et al., 1995). Additionally, Down syndrome has been modeled by overexpressing individual genes, such as the *dual specificity tyrosine-phosphorylation-regulated kinase 1A* (*Dyrk1A*), a gene localized in HSA21q22.2 proposed to contribute to the cognitive phenotype of the syndrome (Altafaj et al., 2001). Both, the Ts65Dn and the TgDyrk1A mouse models show a marked cognitive impairment with deficits in hippocampal-dependent memory (Altafaj et al., 2001; Fernandez et al., 2007; Reeves et al., 1995). Several mechanisms have been proposed to underlie these cognitive deficits, including alterations in hippocampal synaptic plasticity, adult neurogenesis of the hippocampal dentate gyrus and excitatory/inhibitory balance of neuronal circuits (Clark et al., 2006; Contestabile et al., 2013; Kleschevnikov et al., 2004; Pons-Espinal et al., 2013). Interestingly, the endocannabinoid system (ECS), a neuromodulatory system involved in synaptic homeostasis and plasticity fine-tunes all of these processes (Alger, 2002; Augustin and Lovinger, 2018; Marsicano and Lafenêtre, 2009; Monory et al., 2015; Prenderville et al., 2015) but its possible role in the cognitive deficits of Down syndrome has not yet been explored. The ECS is composed of two main cannabinoid receptors, the cannabinoid type-1 and type-2 receptors (CB1R and CB2R, respectively), their endogenous ligands (endocannabinoids) and the enzymes involved in endocannabinoid metabolism (Mechoulam and Parker, 2013). The ECS is involved in 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 have a direct impact on cognitive function (Akirav, 2011; Busquets-Garcia et al., 2015; Zanettini et al., 2011).

In this study, we revealed the involvement of the ECS in the neurological alterations of two relevant mouse models for Down syndrome. Together we pinpointed the CB1R as a target to restore the characteristic cognitive phenotypes on both models.

2. Materials and methods

2.1. 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). Procedures were 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. Transgenic mice for *Dyrk1A* (TgDyrk1A) and WT littermates were obtained as described previously (Altafaj et al., 2001). 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; only males were used for TgDyrk1A experiments.

Mice were housed in Plexiglas cages with a maximum of 4 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 h light/dark cycle (light on at 8 am; light off at 8 pm) in an experimental room at the animal facility. 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.

2.2. Drug treatment

Rimonabant (Sanofi-Aventis) and NESS 0327 (Cayman Chemical, Ann Arbor) were diluted in 5% ethanol, 5% Cremophor EL and 90% saline. They were injected in a volume of 10 ml/kg of body weight. Drugs were administered daily by intraperitoneal (i.p.) injection during 7 days. The test phase of the cognitive tasks, electrophysiological recordings and neurogenesis analysis were performed 24 h after last injection of the 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 hydrocloride (Sigma) were mixed and dissolved in 5% ethanol and 95% distilled water.

2.3. Behavioral tests

Novel object-recognition test (NORT). It was performed following a protocol previously described (Puighermanal et al., 2009). Briefly, on day 1 mice were habituated to a V-shaped maze for 9 min. On day 2, two identical objects (familiar objects) were located at the end of each corridor for 9 min 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. A 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). Object exploration was defined as the orientation of the nose towards the object at a distance of < 2 cm. A higher discrimination index is considered to reflect greater memory retention for the familiar object. Mice that explored < 10 s 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 6th and 7th respective days of treatment. Test was performed 24 h after the last administration.

Novel place-recognition test (NPRT). Mice were first habituated for 10 min to an empty open field during two consecutive days. On day 3 mice were trained for 10 min 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 in 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 < 5 s 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 h after the last administration.

Locomotor activity. Locomotor activity was assessed for 30 min after 3 days of treatment 24 h after the last drug administration. Individual locomotor activity boxes (9 × 20 × 11 cm) (Imetronic) were used in a low luminosity environment (5 lx). The number of horizontal movements was detected by a line of photocells located 2 cm above the floor.

2.4. Immunoblot

Brain tissue was rapidly dissected, immediately frozen on dry ice and stored at −80 °C until used. Samples were processed following a protocol previously described (Ozaita et al., 2007) to obtain cytosolic and solubilized membrane proteins. The primary antibodies used were: anti-CB1R (rabbit and guinea pig, 1:500, CB1-Rb-Af380 and CB1-GP-Af530 Frontier Institute Co.Ltd), anti-FAAH (mouse, 1:1200, 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 or anti-guinea pig antibodies 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).

2.5. RNA extraction and reverse transcription

Hippocampal tissues were rapidly dissected and stored at −80 °C until used. Isolation of total RNA was performed using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. Total RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed with 100 ng of total RNA from each animal to produce cDNA in a 20-μl reaction using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. The cDNAs from brain tissues were diluted 1:2 and stored at −20 °C until use.

2.6. Quantitative real-time PCR analysis

Real-time PCR was carried out in a 10 μl reaction using SYBR Green PCR Master Mix (Roche) according to the manufacturer's protocol with a QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems). The following primers previously described (Cutando et al., 2013; Puighermanal et al., 2017) specific for mouse were used: *Cnr1* (sense, 5′- CCTGGGAAGTGTCATCTTTGT -3′; antisense, 5′- GGTAACCCCACC CAGTTTGA -3′) and *β-actin* (sense, 5'-CGTGAAAAGATGACCCAGA TCA-3′; antisense, 5′- CACAGCCTGGATGGCTACGT-3′). Quantification was performed by using the comparative CT Method (ΔΔCT Method). All the samples were tested in triplicate and the relative expression values were normalized to the expression value of *β-actin*. The fold change was calculated using the eq. $2^{(-\Delta\Delta Ct)}$.

2.7. Electrophysiological recordings

Hippocampal slices were prepared following a protocol previously described (Andrade-Talavera et al., 2016). Mice were anesthetized with isoflurane (2%) and decapitated for slice preparation. Briefly, after decapitation, the whole brain, containing the two 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 h before use. All experiments were carried out at room temperature (22–25 °C). 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 > 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 (50 μM) and NBQX (10 μM) to block NMDA and AMPA/Kainate receptors, respectively. Mean amplitude of eEPSCs and eIPSCs was quantified the last 4 min where the effect of WIN55,212–2 was maximum before recovery.

Field excitatory postsynaptic potentials (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). Extracellular recording electrodes were filled with the solution I. A stimulus-response curve (1–160 μA, mean of five fEPSPs at each stimulation strength) was compiled for the different mice used.

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 min. Long-term potentiation was induced by a theta burst stimulation (TBS) protocol consisting in 5 episodes of 10 train stimulus at 5 Hz, each one with 4 pulses at 100 Hz. Recordings lasted 60 and 120 min after LTP induction. For paired-pulse ratio (PPR) experiments, two fEPSPs were evoked 40 ms apart for 0.5 min at baseline frequency (6 times) at the beginning of the baseline recording and again 2 h after applying the plasticity protocol. The PPR was expressed as the slope of the second fEPSP divided by the slope of the first fEPSP.

Data were analyzed using the Clampfit 10.2 software (Molecular Devices). The last 5 min 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 min post-tetanus period with the mean fEPSP slope during the baseline period and calculating the percentage change from 5 last min.

2.8. 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-acyl glycerols were quantified: N-arachidonoylethanolamine or anandamide (AEA), N-docosatetraenoylethanolamine (DEA), N-docosahexaenoylethanolamine (DHEA), 2-arachidonoyl glycerol (2-AG), 2-linoleoyl glycerol (2-LG) and 2-oleoyl glycerol (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. 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 < 5 min 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 min by placing the tubes in a rocking mixer. Tubes were centrifuged at 1700*g* over 5 min at room temperature. The lower organic phase was transferred to clean glass tubes, evaporated under a stream of nitrogen in a 39 °C 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 °C). Chromatographic separation was carried out with a Waters C18-CSH column (3.1 × 100 mm, 1.8 µm particle size) maintained at 40 °C with a mobile phase flow rate of 0.4 ml/min. 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 and data were expressed as a percentage of control group (WT).

2.9. Stereotaxic surgery and AAV9 vector injection

To knockdown CB1R expression we used an adeno-associated viral serotype 9 (AAV9) vector-mediated 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). 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 (Paxinos and Franklin, 2004). Mice were anesthetized with a ketamine (75 mg/kg)/medetomidine (1 mg/kg) mixture before they underwent stereotaxic surgery and received a bilateral hippocampal injection. The injection of 0.5 µl of AAV9-shSC (control, 5.03×10^{13} vector genomes/ml) or AAV9-shCB1R (AAV9-shRNACB1A: 6.48×10^{13} vector genomes/ml plus AAV9-shCB1B: 1.1×10^{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). 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/min by using a microinfusion pump (Harvard Apparatus, Holliston) during 2 min. After infusion, the injection cannula was left in place for an additional period of 10 min to allow the fluid to diffuse and to prevent reflux, then it was slowly withdrawn during 10 additional min. After surgery, anesthesia was reversed by a subcutaneous injection of atipamezole (2.5 mg/kg). In addition, mice received an i.p. injection of gentamicine (1 mg/kg) and a subcutaneous injection of the analgesic meloxicam (2 mg/kg). For tropism studies, we used an AAV9 vector expressing GFP cDNA (4.3×10^{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.

2.10. Rimonabant detection

Rimonabant was quantified in plasma and brain samples obtained from mice treated for 7 days with vehicle or rimonabant. Samples were obtained 24 h after the last vehicle or rimonabant administration. Plasma samples were obtained from trunk blood recovered with EDTA (1 mM). For the analysis of these samples, 5 µl of the internal standard (I.S., methachlorophenylpiperazine, 1 µg/ml) was mixed with 50 µl of plasma and proteins were precipitated by using 100 µl of acetonitril. After separating and evaporating the organic phase, extracts were reconstituted with a mixture of 50:50 methanol-milliQ water. Brain tissues where weighted and homogenized with a 1 ml Wheaton glass homogenizer in two steps: first by adding 400 µl of HCOOH 0.1%, followed by a second homogenization with 800 µl of acetronitril. The mixture was centrifuged 10 min at 15,700*g*, 4 °C, and supernatant was stored at −20 °C until used. Then, 50 µl of the supernatant were processed following the same protocol described for plasma samples.

Samples were analyzed in an Acquity UPLC System (Waters Associates) coupled to a mass spectrometer (QuattroPremier, Watters Associates). Chromatographic separation was carried out in an Acquity BEH C18 (100 mm × 2.1 mm i.d., 1.7 µm) (Waters Associates) at a flow rate of 0.4 ml/min. Ammonium formate (1 mM)-HCOOH 0.01% (A) and MeOH with ammonium formate (1 mM)-HCOOH 0.01% (B) were used as mobile phases. After keeping 40% B for 0.5 min, the gradient was increased to 95% B in 3 min and maintained at 95% B for 1 min after going back to initial conditions. Detection of analytes was done by the selected reaction monitoring (SRM) method, being the transitions used for identification and quantification (in bold) for each compound as follows: 465→84, 99, **365** (rimonabant); 197→44, 118, **154** (I.S.).

2.11. Immunofluorescence and cell quantification

Four hours after the last administration of rimonabant, 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. For detections we used the following primary antibodies: anti-BrdU (mouse, 1:150, B8434, Sigma-Aldrich) and anti-Ki67 (rabbit, ab15580, 1:150, Abcam). 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). 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. Brains were removed and post-fixed overnight at 4 °C in the same fixative solution. The next day, brains were moved to PB 0.1 M at 4 °C. Coronal brains sections (50 µm) 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 °C until they were used for immunodetections. For BrdU detection, DNA denaturalization was required. For this purpose, slices were pretreated with 2 N HCl at 37 °C for 30 min. Then, free-floating sections were incubated with 0.1 M borate buffer pH = 8.5 for 15 min to neutralize the pH. Afterwards, slices were rinsed in 0.9% NaCl PB (PBS),

blocked in a solution containing 3% normal donkey serum and 0.3% Triton X-100 in 0.1 M PBS (NDS-T-PBS) at room temperature for 2 h and incubated in the same solution with primary antibodies at 4 °C. Forty-eight hours later, slices were rinsed with 0.1 M PBS and incubated with secondary antibodies in NDS-T-PBS for 2 h at room temperature. 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 (20×) 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.

2.12. Experimental design and statistical analysis

Mice were randomly assigned to experimental groups. Sample size choice was based on previous studies (Busquets-Garcia et al., 2013, 2016) and it is listed in figure legends for each experiment. 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 $p < .05$. Outliers (± 2 s.d. from the mean) were excluded.

3. Results

3.1. CB1R function is enhanced at hippocampal excitatory terminals of Ts65Dn mice

We first analyzed the expression of the main components of the ECS in the hippocampus of young-adult Ts65Dn male mice. Protein expression of the most abundant cannabinoid receptor in the brain, CB1R, was increased in comparison to wild-type (WT) mice (Fig. 1A) (Student's *t*-test: $p = .0003$). Conversely, the *Cnr1* mRNA levels remained unaltered in hippocampus of Ts65Dn mice (Fig. S1A). We also assessed CB1R protein expression in the rest of the brain and no changes were observed between WT and Ts65Dn mice (Fig. S1B). In addition, the expression of the main enzymes involved in the synthesis (N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D, NAPE-PLD and diacylglycerol-α lipase, DAGLα) and degradation (fatty acid amide hydrolase, FAAH and monoacylglycerol lipase, MAGL) of the main endocannabinoids, 2-AG and anandamide, was unaffected in the hippocampus of Ts65Dn mice (Fig. S1C).

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 evoked excitatory postsynaptic currents (eEPSCs) (mean ± s.e.m., WT = 76.44 ± 10.80 and Ts65Dn = 43.87 ± 7.94, Student's *t*-test: $p = .031$), but had no effect on evoked inhibitory postsynaptic currents (eIPSCs) in trisomic mice compared to controls (mean ± s.e.m., WT = 67.27 ± 10.73 and Ts65Dn = 63.4 ± 8.23) (Fig. 1, B-C). To determine whether the enhanced inhibitory effect on the amplitude of eEPSCs produced by WIN55,212–2 had a postsynaptic or a presynaptic 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. We calculated the ratio of both CVs and plotted the observed change in the mean EPSC amplitude (M) 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) (Fig. 1D). Together, these data revealed an overall increased function of CB1R at excitatory terminals of CA1 hippocampal region in Ts65Dn mice.

We also determined the levels of endocannabinoids (arachidonoylethanolamine, AEA and 2-arachidonoylglycerol, 2-AG), and related N-acylethanolamine (N-docosatetraenoylethanolamine, DEA and N-docosahexaenoylethanolamine, DHEA) and 2-monoacylglycerol (2-linoleoylglycerol, 2-LG and 2-oleoylglycerol, 2-OG) compounds in hippocampus and whole brain. AEA, DEA and DHEA were decreased in hippocampus (AEA: $p = .008$; DEA: $p = .015$; DHEA: $p = .047$) and DHEA was also decreased in whole brain ($p = .008$) (Table 1).

3.2. 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; Busquets-Garcia et al., 2015; Zanettini et al., 2011), we next evaluated the role of hippocampal CB1R overexpression in the cognitive impairment of Ts65Dn mice. To this aim, we used a set of adeno-associated viral serotype 9 (AAV9) vectors, as previously described (Guegan et al., 2013). AAV9-shRNAs against CB1R (shCB1R) or AAV9-scramble shRNA (control, shSC) were bilaterally injected into the hippocampus (Fig. 2A depicts AAV9 tropism and hippocampal diffusion). Three weeks after the infusion, we assessed hippocampal-dependent memory using the novel object-recognition test (NORT). Then, we obtained brain samples to analyze CB1R protein levels from hippocampal homogenates. Intra-hippocampal infusion of shCB1R, but not shSC, significantly reduced cognitive impairment in the NORT (two-way ANOVA interaction: $F(1,23) = 7.51$, $p = .011$; post hoc Newman-Keuls: WT shSC vs Ts65Dn shSC $p = .009$; Ts65Dn shSC vs Ts65Dn shCB1R $p = .011$) (Fig. 2B) and reduced the expression of CB1R in hippocampus of Ts65Dn mice (Student's *t*-test: $p = .035$) to the level observed in WT mice (Fig. 2, C and D). These improvements were not related to modifications in the exploratory behavior since total object exploration times did not change significantly among experimental conditions (Fig. S2A). shCB1R infusion also reduced CB1R expression in WT mice ($p = .048$) without effects on NORT performance (Fig. 2, B and E). Thus, the normalization of CB1R expression in hippocampus was sufficient to rescue hippocampal memory deficits in Ts65Dn mice.

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

We then tested whether a pharmacological intervention was suitable to treat memory deficits in Down syndrome using systemic administration of the CB1R specific antagonist/inverse agonist rimonabant in Ts65Dn mice. We administered rimonabant (1 mg/kg, intraperitoneal injection (i.p.)) for 7 days. Twenty-four hours after the last administration, the test phase of the NORT was performed (Fig. 3A). By that time, the presence of rimonabant was detected in plasma and brain samples of treated mice (1.5–6 ng/ml and 100–200 pg/mg of wet tissue, respectively; $n = 6$). Sub-chronic treatment of rimonabant improved object-recognition memory of male and female Ts65Dn mice (male: two-way ANOVA, interaction, $F(1,31) = 8.42$, $p = .0068$; post hoc Newman-Keuls, WT VEH vs Ts65Dn VEH $p = .0025$; Ts65Dn VEH vs Ts65Dn RIM $p = .0028$; female: two-way ANOVA, interaction, $F(1,38) = 4.13$, $p = .048$; post hoc Newman-Keuls, WT VEH vs Ts65Dn VEH $p = .0006$; Ts65Dn VEH vs Ts65Dn RIM $p = .0011$) (Fig. 3, B and C). Given this positive result, we also assessed place-recognition which is more dependent on the hippocampus itself with less influence of the cortico-hippocampal system (Barker and

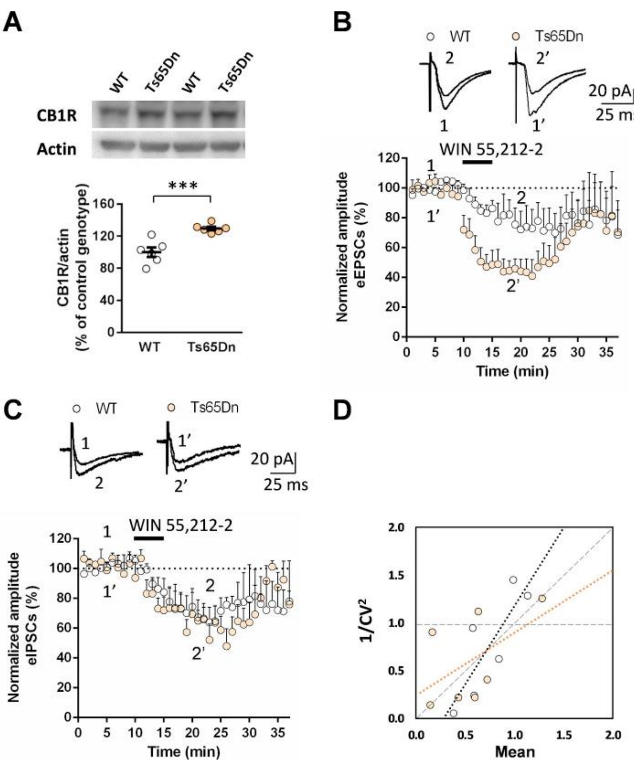


Fig. 1. CB1R function is enhanced at hippocampal CA1 excitatory terminals of Ts65Dn mice. (A) Representative immunoblots and quantification of CB1R in hippocampus from WT and Ts65Dn mice (WT, $n = 6$; Ts65Dn, $n = 6$). Actin immunodetection was used as housekeeping control. (B) Average time course of eEPSCs amplitude in WT and Ts65Dn slices during baseline and after bath application of WIN 55,212-2 (300 nM). Inset, traces show eEPSCs during baseline (1, 1′) and after (2, 2′) bath application of WIN 55,212-2 (WT, $n = 6$; Ts65Dn, $n = 7$). (C) Average time course of eIPSCs amplitude in WT and Ts65Dn slices during baseline (1, 1′) and after (2,2′) bath application of WIN 55,212-2 (300 nM) (WT, $n = 5$; Ts65Dn, $n = 5$). (D) 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. *** $p < .001$ (genotype effect) by Student's t-test.

Table 1
Relative levels of endocannabinoids and related compounds in hippocampal and whole brain homogenates of Ts65Dn and WT controls.

	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

Data is expressed as mean ± s.e.m. (hippocampus: WT, $n = 18$; Ts65Dn, $n = 15$; whole brain: WT, $n = 9$; Ts65Dn, $n = 8$). * $p < .05$, ** $p < .01$ (genotype effect) by Student's t-test.

Warburton, 2011; Warburton and Brown, 2015) using the novel place-recognition test (NPRT). Notably, rimonabant administration also rescued the phenotype of Ts65Dn mice in the NPRT (two-way ANOVA, interaction, $F(1,34) = 4.14$, $p = .049$; post hoc Newman-Keuls, WT VEH vs Ts65Dn VEH $p = .0014$; Ts65Dn VEH vs Ts65Dn RIM $p = .0125$) (Fig. 3D). No significant differences were detected in total object exploration times among the different experimental groups in neither of the two tests (Fig. S2, B-D). Furthermore, rimonabant treatment did not affect locomotor activity neither in WT nor in Ts65Dn mice (Fig. S2E).

We also assessed the effect of a CB1R neutral antagonist, NESS 0327 (Ruiu et al., 2003), over memory deficits of Ts65Dn mice. A sub-chronic treatment of NESS 0327 (0.1 mg/kg, 7 days), also improved NORT memory performance in Ts65Dn mice (two-way ANOVA, interaction: $F(1,20) = 5.85$, $p = .025$; post hoc Newman-Keuls, WT VEH vs Ts65Dn VEH $p = .014$; Ts65Dn VEH vs Ts65Dn NESS 0327 $p = .043$) (Fig. 3E) indicating that CB1R antagonists without CB1R inverse agonist profile are also able to normalize memory deficits in Ts65Dn mice. Again, alterations in total object exploration times were not observed among the different experimental groups (Fig. S2F). Interestingly, none of the memory tests revealed an effect of the treatment in WT mice (Fig. 3, B-E) demonstrating that blocking CB1R specifically improves hippocampal-dependent memory in Ts65Dn mice.

3.4. 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 Down syndrome cognitive impairment (Clark et al., 2006; Kleschevnikov et al., 2004), we assessed whether pharmacological CB1R targeting could also rescue those phenotypes. We studied long-term potentiation (LTP) elicited by theta-burst stimulation (TBS) which is reduced in hippocampal CA3-CA1 synapses of Ts65Dn mice (Costa and Grybko, 2005). We analyzed early-LTP and late-LTP (*E-*

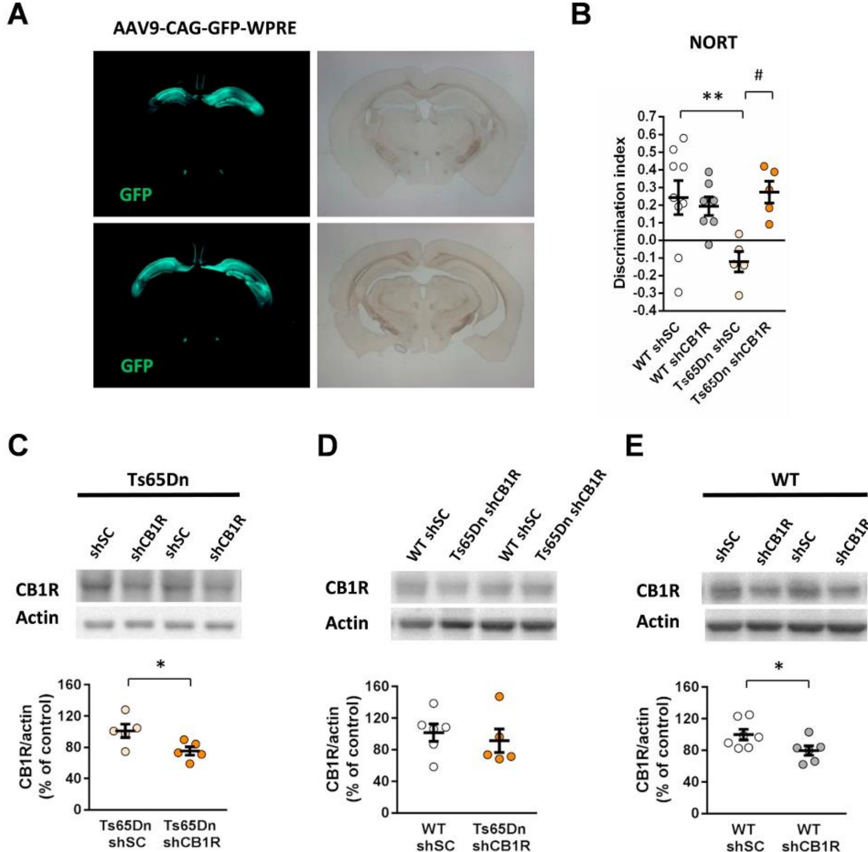


Fig. 2. Injection of AAV9-shRNA against CB1R knocks down its expressions and normalizes memory deficits of Ts65Dn mice. (A) Representative images from two coronal planes of a mouse showing localization and diffusion of AAV9 expressing GFP cDNA injected in the same stereotaxic coordinates than AAV9-shSC/CB1R. (B) Discrimination index in novel object-recognition test (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, $n = 5$). (C) Representative immunoblots and quantification of CB1R in hippocampus from Ts65Dn mice injected with shSC or shCB1R (Ts65Dn shSC, $n = 5$; Ts65Dn shSCB1R, $n = 5$). (D) 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, $n = 5$). (E) 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. Distribution of individual data with mean ± s.e.m. * $p < .05$, ** $p < .01$ (genotype effect); # $p < .05$ (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA (B) and by Student's t-test.

LTP and L-LTP; 60 and 120 min 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 h before slice collection). As expected, overall LTP was decreased in Ts65Dn mice compared to WT mice treated with vehicle. Interestingly, rimonabant administration normalized the Ts65Dn deficit in L-LTP (E-LTP, two-way ANOVA, interaction: $F(1,23) = 4.24$, $p = .0508$; L-LTP, two-way ANOVA, interaction: $F(1,23) = 4.815$, $p = .038$; post hoc Newman-Keuls, WT VEH vs Ts65Dn VEH $p = .021$; Ts65Dn VEH vs Ts65Dn RIM $p = .046$) (Fig. 4, A and B; see Fig. S3, A and B, for control groups), again with no effect of the treatment was revealed in WT mice. Changes in LTP were not due to differences in basal synaptic transmission as similar input/output curves were observed between experimental groups (Fig. S3, C and D). To determine the site of expression of LTP, we analyzed paired-pulse facilitation ratios (PPRs) during baseline and 120 min after the application of the induction protocol. The analysis of PPRs before and after LTP did not show differences in any of the experimental groups (Fig. S3E) suggesting that this form of LTP is postsynaptically expressed.

In addition, we studied adult neurogenesis, since Ts65Dn mice show a reduction in cell proliferation (Belichenko and Kleschevnikov, 2011; Clark et al., 2006), that 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 (i.p., last administration 24 h before brain perfusion) showed a decreased number of Ki67 + cells in the subgranular zone of the dentate

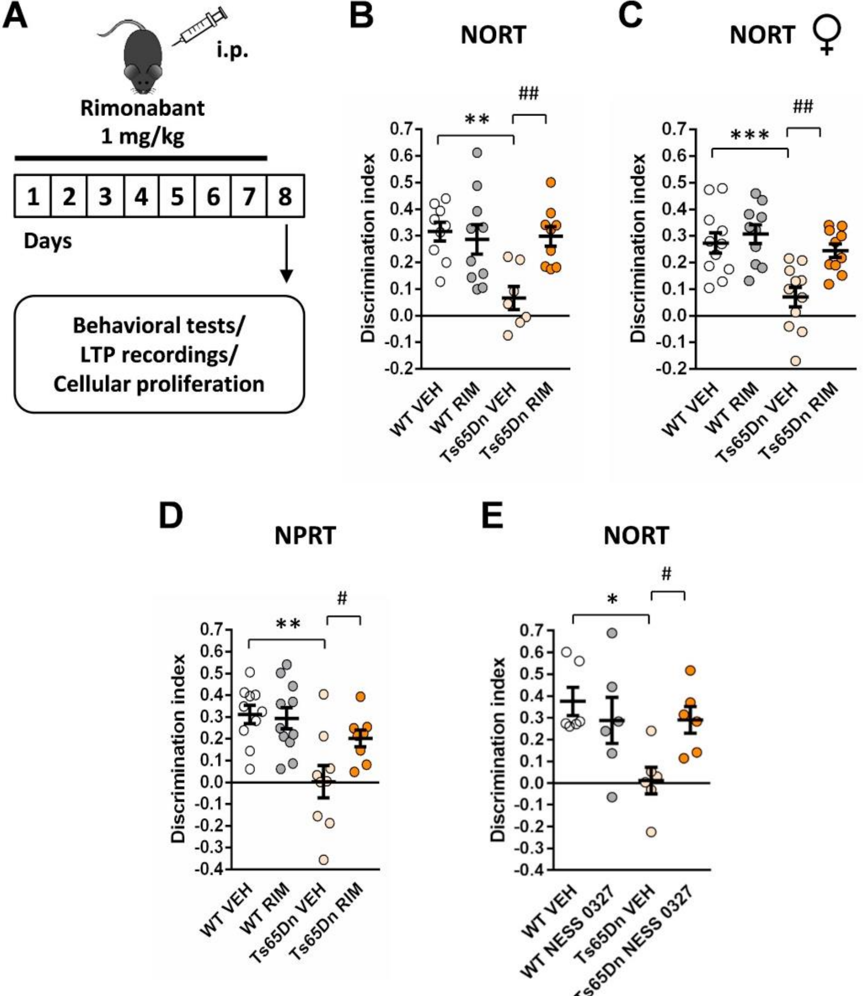
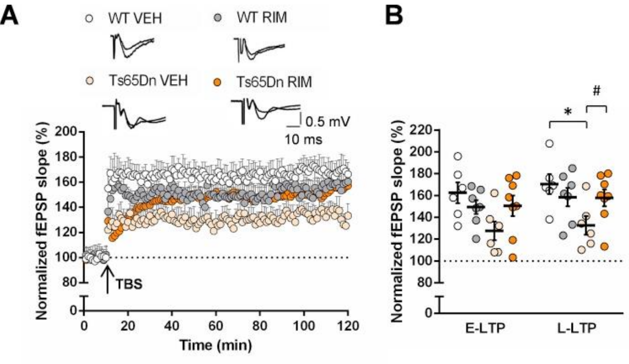


Fig. 3. Pharmacological targeting of CB1R improves hippocampal-dependent memory in Ts65Dn mice. (A) Schematic representation of the experimental protocol. (B-C) Discrimination index in novel object-recognition test (NORT) after a sub-chronic treatment with vehicle (VEH) or rimonabant (RIM) (1 mg/kg) of male (B) and female (C) mice (males: WT VEH, $n = 9$; WT RIM, $n = 10$; Ts65Dn VEH, $n = 7$; Ts65Dn RIM, $n = 9$; females: WT VEH, $n = 11$; WT RIM, $n = 10$; Ts65Dn VEH, $n = 11$; Ts65Dn RIM, $n = 10$). (D) Discrimination index in novel place-recognition test (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, $n = 8$). (E) Discrimination index in NORT after a sub-chronic treatment with vehicle or NESS 0327 (0.1 mg/kg) (WT VEH, $n = 6$; WT NESS 0327, $n = 6$; Ts65Dn VEH, $n = 6$; Ts65Dn NESS 0327, $n = 6$). Distribution of individual data with mean ± s.e.m. * $p < .05$, ** $p < .01$, *** $p < .001$ (genotype effect); # $p < .05$, ## $p < .01$ (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA.

gyrus (Fig. 4C). Treatment with rimonabant (1 mg/kg) rescued this phenotype, with Ki67+ cell counts similar to WT controls, without modifying Ki67+ cell counts in WT mice (two-way ANOVA, interaction: $F(1,17) = 7.544$, $p = .013$; post hoc Newman-Keuls, WT VEH vs Ts65Dn VEH $p = .034$; Ts65Dn VEH vs Ts65Dn RIM $p = .049$) (Fig. 4C). Thus, sub-chronic administration of rimonabant normalized defective synaptic plasticity and cellular proliferation in the hippocampus of Ts65Dn mice.

3.5. CB1R pharmacological targeting is effective in the transgenic model overexpressing Dyrk1A

Dyrk1A overexpression in mice recapitulates cognitive deficits and brain alterations of Down syndrome (Altafaj et al., 2001; Pons-Espinal



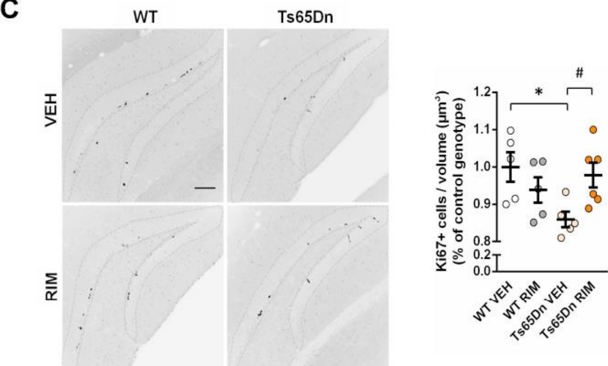


Fig. 4. Pharmacological targeting of CB1R improves hippocampal synaptic plasticity and cellular proliferation in dentate gyrus of Ts65Dn mice. (A) Average time courses of the change in the slope of fEPSP in hippocampal slices from mice treated for 7 days with vehicle (VEH) or rimonabant (RIM) (1 mg/kg). Traces represent samples of fEPSPs recorded for each experimental group before and after TBS. (B) Average LTP of the last 5 min of recordings in *E*-LTP and L-LTP (60 and 120 min post-tetanus period respectively) (WT VEH, $n = 6$; WT RIM, $n = 7$; Ts65Dn VEH, $n = 6$; Ts65Dn RIM, $n = 8$). (C) Representative grey scale confocal images and average density of Ki67+ cells in the subgranular zone of the dentate gyrus of mice treated for 7 days with VEH or RIM (1 mg/kg) (WT VEH, $n = 5$; WT RIM, $n = 5$; Ts65Dn VEH, $n = 5$; Ts65Dn RIM, $n = 6$) (scale bar = 100 μm). Distribution of individual data with mean ± s.e.m. * $p < .05$ (genotype effect); # $p < .05$ (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA.

et al., 2013). In addition, normalization of the dosage of this gene in Ts65Dn mice rescues cognitive, LTP and cellular proliferation deficits (García-Cerro et al., 2014) indicating that *Dyrk1A* overexpression is involved in the phenotypes normalized after CB1R blockade. Therefore, we investigated the protein expression of the CB1R on the hippocampus of a transgenic model overexpressing only this kinase, the TgDyrk1A mice (Altafaj et al., 2001). We observed an increase of CB1R in hippocampus of TgDyrk1A in comparison to WT mice (Student's *t*-test: $p = .02$) (Fig. S4). We also studied the expression of the main endocannabinoid metabolic enzymes and we did not observed differences between WT and TgDyrk1A mice (Fig. S4). Then, we assessed whether CB1R inhibition also improves memory deficits in TgDyrk1A mice. These mice showed a marked deficit in NORT that was rescued after rimonabant treatment (1 mg/kg, i.p., 7 days). A reduced dose of rimonabant (0.1 mg/kg) was almost as effective in improving cognitive performance of this model (two-way ANOVA, interaction: $F(1,41) = 12.08$, $p = .000008$; post hoc Newman-Keuls, WT VEH vs TgDyrk1A VEH $p = .0001$; TgDyrk1A VEH vs TgDyrk1A RIM 0.1 mg/kg $p = .0002$; TgDyrk1A VEH vs TgDyrk1A RIM 1 mg/kg $p = .0001$) (Fig. 5A). In addition, TgDyrk1A mice also displayed deficits in the NPRT that were normalized after a sub-chronic treatment of rimonabant (1 mg/kg) (two-way ANOVA, interaction: $F(1,49) = 5.66$, $p = .021$; post hoc Newman-Keuls, WT VEH vs TgDyrk1A VEH $p = .018$; TgDyrk1A VEH vs TgDyrk1A RIM $p = .012$) (Fig. 5B). Notably, NESS 0327 treatment (0.1 mg/kg, 7 days) also improved memory performance in TgDyrk1A mice (two-way ANOVA, interaction: $F(1,23) = 4.73$, $p = .04$; post hoc Newman-Keuls, WT VEH vs TgDyrk1A VEH $p = .025$; TgDyrk1A VEH vs TgDyrk1A NESS 0327 $p = .017$) (Fig. 5C) further supporting CB1R as a relevant target in the cognitive improvement of this mouse line. Alterations in total object exploration times were not observed among the different experimental conditions (Fig. S5, A-C).

We also studied the effect of rimonabant after treatment withdrawal in TgDyrk1A mice. Remarkably, the beneficial effects of rimonabant treatment were observed after 2 but not 4 weeks of washout in NORT (2 weeks: two-way ANOVA, interaction: $F(1,20) = 4.53$, $p = .045$; post hoc Newman-Keuls, WT VEH vs TgDyrk1A VEH $p = .0005$; TgDyrk1A VEH vs TgDyrk1A RIM $p = .001$) (Fig. S6, A-D).

We next 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 while after LTP induction a transient facilitation of fEPSPs slope was clearly observed in TgDyrk1A mice similar to wild-type mice (Fig. 6A). Sub-chronic rimonabant administration (7 days, 1 mg/kg, i.p.) normalized L-LTP in TgDyrk1A mice and did not have any effect on WT mice (L-LTP, two-way ANOVA, interaction: $F(1,30) = 8.761$, $p = .0059$; post hoc Newman-Keuls, WT VEH vs TgDyrk1A VEH $p = .0066$; TgDyrk1A VEH vs TgDyrk1A RIM $p = .001$) (Fig. 6, A and B; see Fig. S7, A and B for control groups). No differences in basal synaptic transmission were observed between

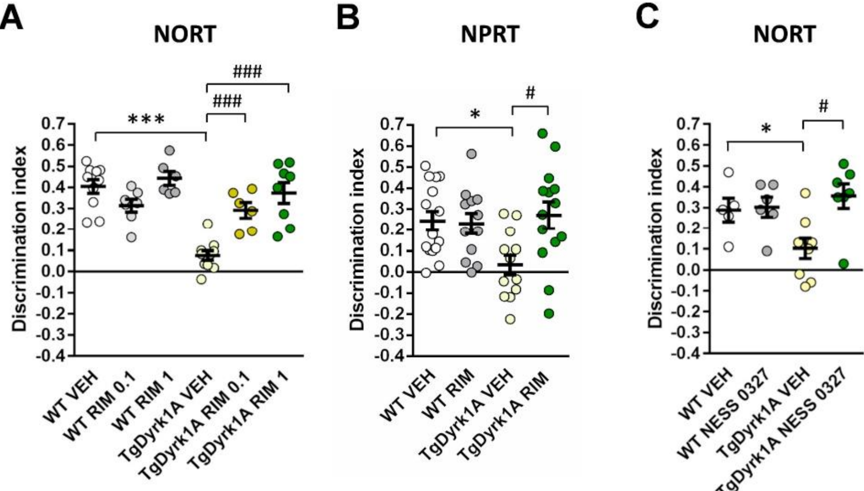


Fig. 5. CB1R targeting improves hippocampal-dependent memory in TgDyrk1A mice. (A) Discrimination index of WT and TgDyrk1A mice in novel object-recognition test (NORT) after 7 days of treatment with vehicle (VEH) or rimonabant (RIM) 0.1 mg/kg or 1 mg/kg (WT VEH, $n = 10$; WT RIM 0.1 mg/kg, $n = 7$; WT RIM 1 mg/kg, $n = 6$; TgDyrk1A VEH, $n = 10$; TgDyrk1A RIM 0.1 mg/kg, $n = 6$; TgDyrk1A RIM 1 mg/kg, $n = 8$). (B) Discrimination index in novel place-recognition test (NPRT) after 7 days of treatment with VEH or RIM (WT VEH, $n = 15$; WT RIM, $n = 12$; TgDyrk1A VEH, $n = 12$; TgDyrk1A RIM, $n = 14$). (C) Discrimination index of mice after 7 days of treatment with VEH or NESS 0327 (0.1 mg/kg) in NORT (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. * $p < .05$, *** $p < .001$ (genotype effect); # $p < .05$, ### $p < .001$ (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA.

experimental conditions, as input/output curves were similar (Fig. S7, C and D). No differences were observed in the PPRs before and after LTP in any of the experimental groups indicating that this form of LTP is postsynaptically expressed (Fig. S7E).

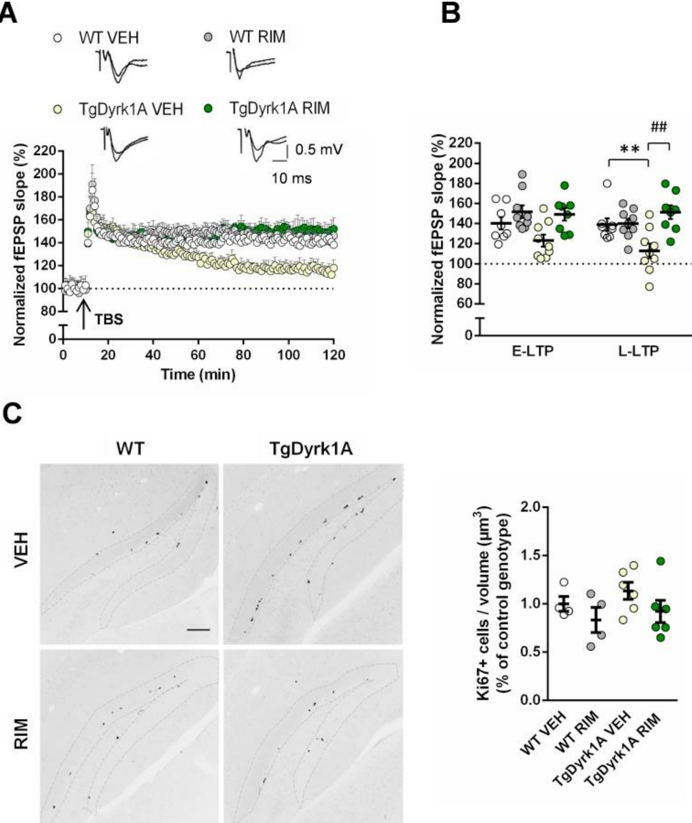
TgDyrk1A mice also showed defects in adult neurogenesis. Conversely to what occurs in Ts65Dn mice, we observed a trend towards an increase of Ki67+ cells in TgDyrk1A mice, though this modification did not reach significance (Fig. 6C). Since this increase seems to be secondary to a cell cycle arrest (Pons-Espinal et al., 2013) rather than an increase in cell proliferation rate, we analyzed the progenitors exiting the cell cycle. To address this specific issue in TgDyrk1A, we injected a set of rimonabant (1 mg/kg)/vehicle treated mice (7 days, i.p.) with a single dose of the DNA intercalating agent BrdU, 24 h before brain perfusion to in vivo label 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 (two-way ANOVA, interaction: $F(1,15) = 21.14$, $p = .0003$; post hoc Newman-Keuls, WT VEH vs TgDyrk1A VEH $p = .205$; TgDyrk1A VEH vs TgDyrk1A RIM $p = .0009$) (Fig. 6D). This indicates that rimonabant rescued the decrease in cell cycle exit in TgDyrk1A mice. Therefore, repeated CB1R inhibition normalized hippocampal L-LTP and progenitor cell proliferation derived from *Dyrk1A* overexpression.

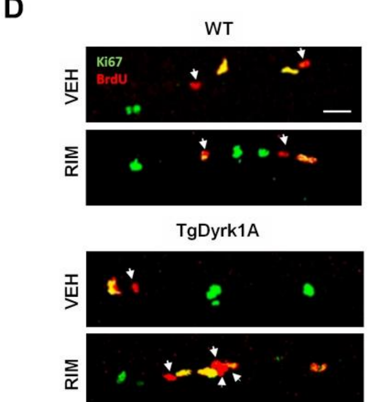
4. Discussion

In this study, we identified CB1R as a relevant target to improve hippocampal-dependent memory, long-term synaptic plasticity and adult neurogenesis in two relevant models for Down syndrome, the segmentally trisomic Ts65Dn model and the transgenic TgDyrk1A model.

We first analyzed the main protein components of the ECS in the hippocampus of Ts65Dn mice since this brain area is especially impaired in Down syndrome subjects (Lott and Dierssen, 2010; Pennington et al., 2003). We revealed an increased protein expression of CB1R restricted to the hippocampus. Such increase was functionally relevant at excitatory terminals and not at inhibitory ones. Hippocampal CB1R is not distributed equally in the different cell types, presenting low expression levels on glutamatergic terminals and astrocytes, and high levels exclusively on cholecystokinin-expressing GABAergic terminals (Han et al., 2012; Kano et al., 2009; Kawamura et al., 2006). However, it has been described that the levels of expression do not correlate with their functional relevance. Glutamatergic CB1R, but not CB1R in other cells, reduced excitotoxicity in a model of epilepsy (Marsicano et al., 2003; Monory et al., 2006), and showed more effective coupling to G-protein signaling than GABAergic CB1R (Steindel et al., 2013). These data reflect the relevance of glutamatergic CB1R for hippocampal circuit activity, which function is affected in Ts65Dn mice. In this regard, an imbalance of excitatory/inhibitory neuronal circuit activity has been proposed to contribute to the cognitive deficits of the Ts65Dn mice (Best et al., 2012; Kurt et al., 2004; Potier et al., 2014; Souchet et al., 2015). It is plausible that the increased inhibition of glutamatergic neurotransmission elicited by excess glutamatergic CB1R function in Ts65Dn hippocampus would contribute to this imbalance.

Regarding the levels of the most studied hippocampal endocannabinoids, we found a decrease of AEA and no changes on 2-AG in 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 it may involve alterations in alternative metabolism pathways (Ueda et al., 2013). Changes in neuronal activity happening in the Ts65Dn





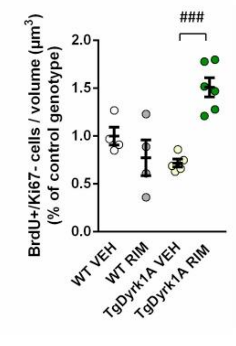


Fig. 6. Pharmacological targeting of CB1R improves hippocampal synaptic plasticity and the number of progenitor cells exiting the cell cycle in TgDyrk1A mice. (A) Average time courses of the change in the slope of the fEPSP in hippocampal slices from mice treated for 7 days with vehicle (VEH) or rimonabant (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 min of recordings in *E*-LTP and L-LTP (60 and 120 min post-tetanus period respectively) (WT VEH, $n = 8$; WT RIM, $n = 9$; TgDyrk1A VEH, $n = 9$; TgDyrk1A RIM, $n = 8$). (C) Representative grey scale confocal images and average density of Ki67 + cells in the subgranular zone of the dentate gyrus of mice treated for 7 days with VEH or RIM (1 mg/kg) (WT VEH, $n = 4$; WT RIM, $n = 4$; TgDyrk1A VEH, $n = 6$; TgDyrk1A RIM, $n = 6$) (scale bar = 100 μm). (D) Representative confocal images and average density of BrdU +/Ki67− cells in the subgranular zone of the dentate gyrus of mice treated for 7 days with VEH or RIM (1 mg/kg) (WT VEH, $n = 4$; WT RIM, $n = 4$; TgDyrk1A VEH, $n = 5$; TgDyrk1A RIM, $n = 6$) (scale bar = 20 μm). Distribution of individual data with mean ± s.e.m. ** $p < .01$ (genotype effect); ## $p < .01$, ### $p < .001$ (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA.

hippocampus may also have an effect over AEA levels (Kim and Alger, 2010) contributing to the observed differences. Additionally, we cannot discard that decreased levels of AEA in hippocampus of Ts65Dn mice could lead to a decrease in biological responses dependent on this endocannabinoid such as hippocampal synaptic plasticity mediated by transient receptor potential channel 1 (TRPV1) (Chávez et al., 2010). However, it is unlikely that the decrease in AEA levels can compensate the over-expression of CB1R in the Ts65Dn hippocampus. In fact, it is 2-AG the endocannabinoid that mediates major forms of hippocampal synaptic plasticity through the activation of CB1R including depolarization-induced suppression of inhibition (DSI), depolarization-induced suppression of excitation (DSE) and inhibitory long-term depression (iLTD) (Chevaleyre and Castillo, 2003; Hashimotodani et al., 2013; Straiker and Mackie, 2005).

Alterations of AEA in Ts65Dn mice would be anatomically restricted since AEA levels detected in whole brain homogenates were not different from those in wild-type mice. Notably, our results of endocannabinoid content in whole brain extracts from young-adult Ts65Dn mice are complementary to those previously described in middle-aged 9-to-11-month-old Ts65Dn mice reporting enhanced levels of 2-AG (Lysenko et al., 2014). Middle-aged Ts65Dn mice present an Alzheimer's disease-like neuropathology including age-dependent cognitive decline, cholinergic neurodegeneration in the basal forebrain, increased levels of amyloid precursor protein (APP), amyloid-β peptide and tau hyperphosphorylation (Hamlett et al., 2016) although amyloid plaques are absent. In this regard, an increase in 2-AG but not AEA has been described after stereotaxic injection of beta-amyloid peptide in naïve rats (van der Stelt et al., 2006). Therefore, specific increase in 2-AG levels observed in middle-age Ts65Dn mice could be related to the associated Alzheimer's disease-like neuropathology. Alternatively, the relative increase in older Ts65Dn mice may be secondary to age-dependent changes, since alterations in the expression and activity of endocannabinoid metabolic enzymes and particularly, in MAGL and DAGLα, have been described in aged rodents (Pascual et al., 2013; Piyanova et al., 2015).

We next 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 over-expression of CB1R is contributing to cognitive deficits of Ts65Dn mice. This result was reproduced by pharmacological approaches using the CB1R specific antagonist/inverse agonist rimonabant and the neutral antagonist NESS 0327 further supporting the relevance of this new target to tackle cognitive impairments in Down syndrome. The administration of rimonabant in Ts65Dn mice did not alter CB1R expression in hippocampus *(data not shown)*. This is consistent with previous findings in rats where a longer treatment of rimonabant at a dose of 10 mg/kg did not modify the hippocampal density of CB1R (Martín-García et al., 2010). This observation indicates that the effect of the treatment would not depend upon changes in the expression of the receptor.

The improvement on cognitive function of Down syndrome mouse models with the CB1R inhibition was accompanied by changes in synaptic plasticity and adult neurogenesis. CB1R sub-chronic blockade restored impaired LTP in CA3-CA1 synapses. Notably, conditional deletion of CB1R in glutamatergic or GABAergic neurons was shown to increase or decrease hippocampal LTP, respectively (Monory et al., 2015), indicating that CB1R changes in either population is sufficiently to alter long-term plasticity. These results further support the participation of glutamatergic CB1R enhanced function in the hippocampal LTP alterations of Ts65Dn mice, and the beneficial effect of CB1R sub-chronic blockade over this phenotype.

Several compounds that normalize adult neurogenesis in Ts65Dn mice (Bianchi et al., 2010; Contestabile et al., 2013) also normalize LTP and associated memory functions. We evaluated adult neurogenesis and we showed a decrease in cell proliferation in young Ts65Dn mice, which is consistent with previous results (Belichenko and Kleschevnikov, 2011; Clark et al., 2006). Notably, rimonabant treatment normalized alterations in adult neurogenesis in Ts65Dn without modifying that in WT mice. Although several studies have addressed the role of CB1R in adult neurogenesis, there is no general consensus since the effects depend on the precise experimental conditions (Prenderville et al., 2015). In this regard, it is plausible that the main effect of sub-chronic CB1R blockade in Ts65Dn mice is indirectly associated to the regulation of adult neurogenesis through the modification of local network activity in the dentate gyrus of the hippocampus driving to cell proliferation (Lehmann et al., 2005).

Our study used a well characterized mouse model of the HSA21 trisomy, but the perfect Down syndrome mouse model does not exist. It is important to mention that 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 Down syndrome individuals. Therefore, although the construct validity of the Ts65Dn model is not perfect, it recapitulates most of the phenotypes observed in Down syndrome patients at different ages (Aziz et al., 2018). Other models with better construct validity, such as the Dp16 or the TTS, show phenotypes milder than those found in the Ts65Dn model and they do not present some of the phenotypes observed in Down syndrome patients, probably due to the lack of a freely segregating chromosome (Aziz et al., 2018; Belichenko et al., 2015). In order to investigate whether our findings were not limited to the Ts65Dn model, we turned to a model in which *Dyrk1A* is overexpressed, the TgDyrk1A model (Altafaj et al., 2001). This mouse model was constructed using the inducible sheep metallothionein-Ia promoter and, although spatial and temporal regulation of the transgene may not match with the endogenous *Dyrk1A* gene, it was demonstrated that the expression in cortex of DYRK1A in this model is similar to Down syndrome cerebral fetal tissue (Toiber et al., 2010). Moreover, this model reproduces several Down syndrome phenotypes (Altafaj et al., 2001; Martínez de Lagrán et al., 2004; Pons-Espinal et al., 2013).

DYRK1A gene is directly associated to the characteristic neurological phenotype in Down syndrome subjects. Normalization of *Dyrk1A* gene dosage in Ts65Dn mice have revealed a key role of this protein in memory performance, hippocampal synaptic plasticity, adult neurogenesis and neuronal circuit excitatory/inhibitory imbalance (Ahn et al., 2006; García-Cerro et al., 2014; Souchet et al., 2014). Interestingly, we observed an increase of CB1R in hippocampus of TgDyrk1A mice indicating a link between DYRK1A and CB1R expression. In addition, as previously described, TgDyrk1A mice showed a significant impairment in the NORT (de la Torre et al., 2014) and also in the NPRT. We also found a decrease of hippocampal LTP in this mouse model in agreement with those Down syndrome models sharing the trisomic expression of *Dyrk1A* (Belichenko et al., 2009, 2015; Costa and Grybko, 2005; Siarey et al., 2005). In addition, normalizing *Dyrk1A* dosage on Ts65Dn mice increases LTP to control values (García-Cerro et al., 2014), which fit with our results on LTP obtained in the TgDyrk1A model. CB1R blockade rescued the performance of TgDyrk1A mice in both NORT and NPRT and deficits in LTP. Interestingly, in agreement with previous reports (Pons-Espinal et al., 2013), we observed a non-significant increase on Ki67+ cells and a decrease on BrdU+/Ki67− cells in TgDyrk1A mice which may be explained by a reduction on cell cycle exit. According to a previous study, this reduction is secondary to an elongation of G2 phase during progenitor cell cycle (Pons-Espinal et al., 2013), which in fact, has also been described in Ts65Dn mice (Contestabile et al., 2007). Therefore, both Ts65Dn and TgDyrk1A models may present decreased cell proliferation and cell cycle exit, but probably at different extents, which explains the different phenotype observed with the proliferative marker Ki67. Notably, CB1R sub-chronic blockade in the TgDyrk1A model significantly facilitated cell cycle exit of neuronal precursors. Together, these findings strongly indicate that CB1R signaling is implicated in the deficits derived from the increase on *Dyrk1A* gene dosage.

Different compounds have been tested in preclinical studies of

Down syndrome. In fact, another approach targeting the ECS (JZL184 administration), improved cognitive performance and synaptic plasticity of 9-to-11-months old Ts65Dn mice (Lysenko et al., 2014). Beneficial effects of JZL184 inhibiting 2-AG metabolism in middle-aged Ts65Dn mice may be specific for the age-associated neurodegeneration and neuroinflammation. In this sense, previous studies in mouse models of Alzheimer's disease showed that JZL184 produces anti-inflammatory effects which may be obtained through a CB1R-independent mechanism (Chen et al., 2012; Piro et al., 2012). Although several compounds have improved memory deficits in Down syndrome mouse models, most of them have produced marginally positive results in clinical trials (Hart et al., 2017). We took several considerations to maximize the translational potential of our study. First, we used two mouse models whose predictive validity has been recently demonstrated for novel experimental approaches to improve intellectual performance in Down syndrome individuals (de la Torre et al., 2016, 2014). Second, we assed three different methods directed to the same target, CB1R, one genetic approach and two pharmacological approaches (rimonabant and NESS 0327). Third, although most experiments were carried out in male mice, we also assessed hippocampal-dependent memory after CB1R blockade in female mice, showing comparable gender results.

Altogether, our study highlights the effects of targeting CB1R, both genetically and pharmacologically, in two young-adult mouse models of relevance for Down syndrome, with an improvement in cognitive performance, synaptic plasticity and adult neurogenesis. These preclinical evidences pinpoint CB1R as a novel target worth exploring in the context of Down syndrome cognitive deficits.

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Conflict of interest

A.B.-G., R.M. and A.O. declare intellectual property of the patent PCT/EP2013/055728. The remaining authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2019.01.014.

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Supplementary material

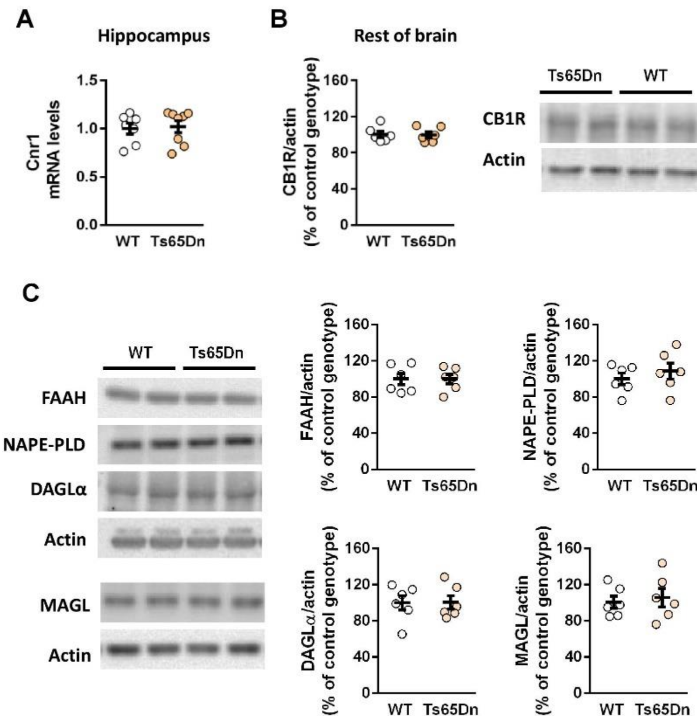

Figure S 1. Expression of CB1R and the main endocannabinoid-metabolizing enzymes in Ts65Dn mice. (A) Analysis of *Cnr1* mRNA expression in hippocampus of WT and Ts65Dn mice. (B) Quantification and representative images of CB1R protein expression levels of whole brain samples where hippocampus was removed. (C) Quantification and representative images of hippocampal expression levels of the main enzymes involved in the synthesis and inactivation of endocannabinoids including fatty acid amide hydrolase (FAAH), N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD), diacylglycerol-α lipase (DAGLα) and monoacylglycerol lipase (MAGL). Actin was used as the housekeeping control (WT, n=6; Ts65Dn, n=6). Distribution of individual data with mean ± s.e.m. Statistical significance was calculated by Student's t-test.

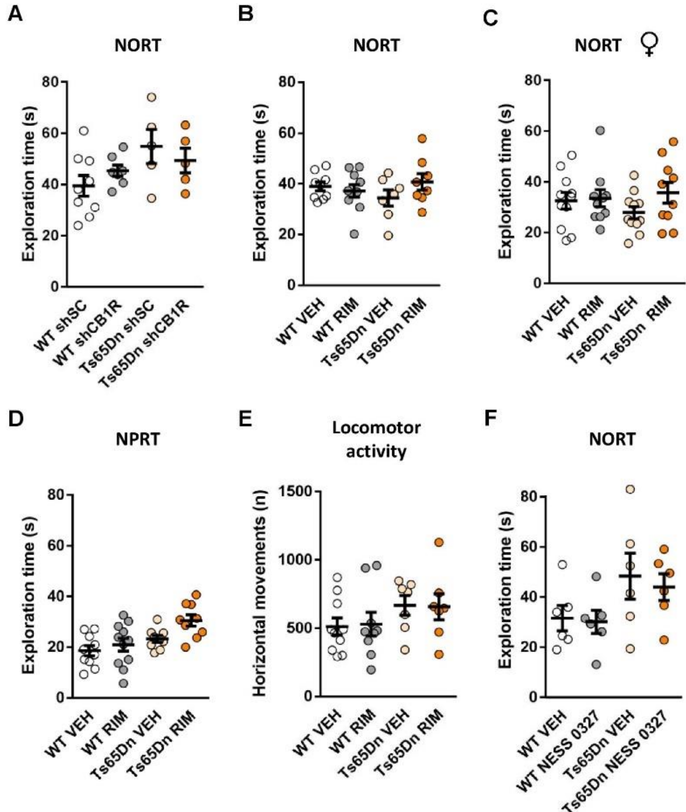

Figure S 2. Total object exploration times mice in hippocampal-dependent memory tasks and locomotor activity after pharmacological targeting of CB1R. (A) Total object exploration times during test phase of novel object-recognition test (NORT) of mice injected with shSC- and shCB1R-containing adenoassociated viral vectors**.** (B-C) Total object exploration times during test phase of NORT of male (B) and female (C) mice treated with vehicle (VEH) or rimonabant (RIM). (D) Total object exploration times during test phase of novel place-recognition test (NPRT) of mice treated with vehicle or rimonabant. (E) Photocell counts of locomotor activity during a 30 min period of WT and Ts65Dn mice treated with vehicle or rimonabant (WT VEH, n=10; WT RIM, n=9; Ts65Dn VEH, n=7; Ts65Dn RIM, n=7). (F) Total object exploration times during test phase of NORT 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.

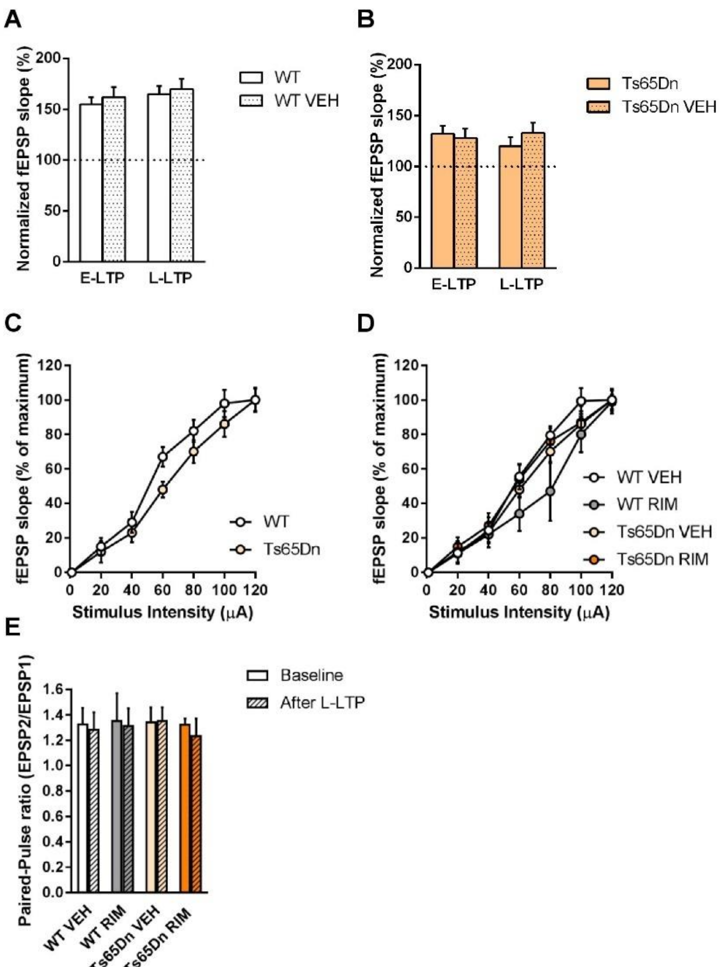

Figure S 3. Effect of vehicle treatment over LTP and basal transmission and paired-pulse facilitation on WT and Ts65Dn mice. (A-B) Average LTP of the last 5 min of recordings in E-LTP and L-LTP (60 and 120 min post-tetanus period, respectively) in hippocampal slices from naïve mice and mice treated with vehicle (VEH), WT (A) and Ts65Dn (B) (n=6-8). (C) Stimulation input/output curves of naïve WT and Ts65Dn mice. (D) Stimulation input/output curves of WT and Ts65Dn mice treated with VEH and rimonabant (RIM). (E) Paired-pulse facilitation before (baseline) and after L-LTP in WT and Ts65Dn mice treated with VEH or RIM. Data are shown as the mean ± s.e.m.

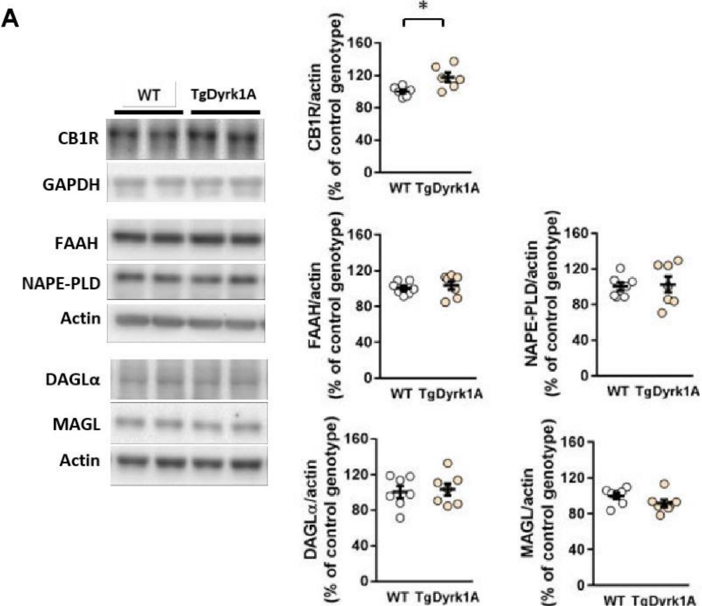

Figure S 4. Hippocampal expression of CB1R and the main endocannabinoid-metabolizing enzymes in the TgDyrk1A mouse. (A) Quantification and representative images of hippocampal protein levels of CB1R and the main endocannabinoid-metabolizing enzymes including fatty acid amide hydrolase (FAAH), N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD), diacylglycerol-α lipase (DAGLα) and monoacylglycerol lipase (MAGL). Actin or GAPDH was used as the housekeeping control (WT, n=6-7; TgDyrk1A, n=6-7). Distribution of individual data with mean ± s.e.m. * $p < 0.05$ by Student's t-test.

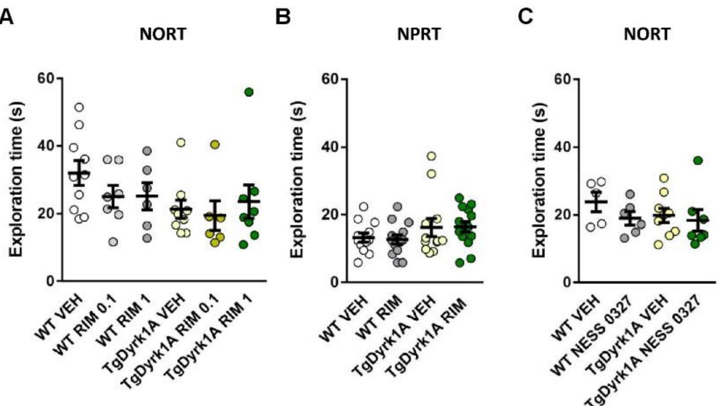

Figure S 5. Total object exploration times of TgDyrk1A mice in hippocampal-dependent tasks. (A-B) Total object exploration time during test phase of novel object-recognition test (NORT) (A) and novel place-recognition test (NPRT) (B) of mice treated for 7 days with vehicle (VEH) or rimonabant (RIM) (0.1 mg/kg and 1 mg/kg). (C) Total exploration times of mice treated with vehicle or NESS 0327 (0.1 mg/kg). Distribution of individual data with mean ± s.e.m. Statistical significance was calculated by two-way ANOVA.

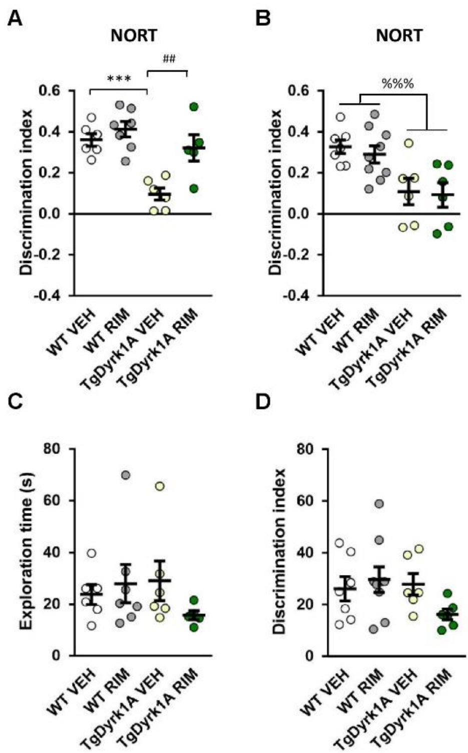

Figure S 6. Effect of rimonabant withdrawal in TgDyrk1A mice in the novel object-recognition test. (A-B) Discrimination index after 2 weeks (A) and 4 weeks (B) of vehicle (VEH) or rimonabant (RIM) (1 mg/kg) treatment withdrawal in novel object-recognition (NORT) (2 weeks; WT VEH, n=6; WT RIM, n=7; TgDyrk1A VEH, n=6; TgDyrk1A RIM, n=5; 4 weeks; WT VEH, n=7; WT RIM, n=9; TgDyrk1A VEH, n=6; TgDyrk1A RIM, n=6). (C-D) Total object exploration times during test phase of NORT after 2 weeks (C) and 4 weeks (D) of treatment withdrawal. Distribution of individual data with mean ± s.e.m. *** $p < 0.001$ (genotype effect); ## $p < 0.001$ (treatment effect) by Newman-Keuls post hoc test following two-way ANOVA and by main effect of the genotype (%%% $p < 0.0001$) with non-significant interaction (B).

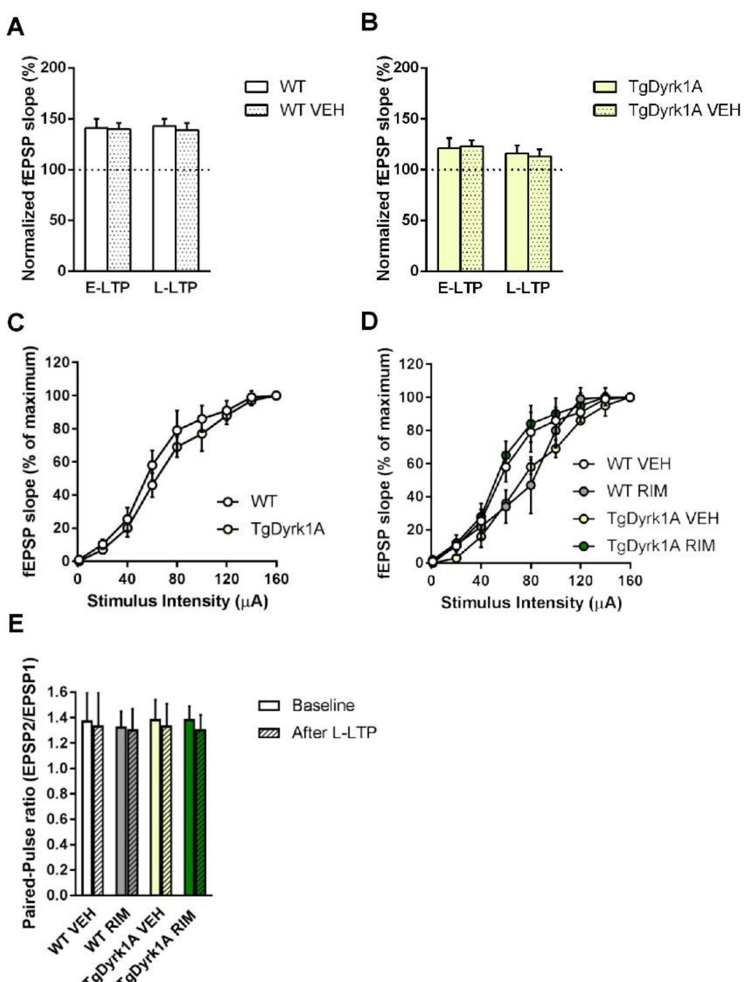

Figure S 7. Effect of vehicle treatment over LTP and basal transmission and paired-pulse facilitation of WT and TgDyrk1A mice. (A-B) Average LTP of the last 5 min of recordings in E-LTP and L-LTP (60 and 120 min post-tetanus period respectively) in hippocampal slices from naïve mice and mice treated with vehicle (VEH), WT (A) and TgDyrk1A (B) (n=8-12). (C) Stimulation input/output curves of naïve WT and TgDyrk1A mice. (D) Stimulation input/output curves of WT and TgDyrk1A mice treated with VEH and rimonabant (RIM). (E) Paired-pulse facilitation before (baseline) and after L-LTP in WT and TgDyrk1A mice treated with VEH or RIM. Data are shown as the mean ± s.e.m