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Phytocannabinoids as potential tools for ameliorating Rett Syndrome-like phenotype in Mecp2-null mice

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ABSTRACT

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder with a prevalence rate of 1 in 10,000 females. RTT patients have apparently normal perinatal development until about 6-18 months of age, after which they undergo a period of rapid regression, characterized by the appearance of autistic features, stereotypic hand movements and loss of language. RTT girls also have seizures during childhood, breathing arrhythmias, develop scoliosis and lose mobility between ages 1 and 4 years.

Recently, there has been growing interest in the therapeutic potential of phytocannabinoids in the context of neurological diseases. Interestingly, several preclinical and clinical data support the ability of some phytocannabinoids to modulate cognitive and motor functions, mood and neuronal excitability, all of which are altered in RTT. Despite this evidence, so far no studies have addressed the potential therapeutic application of phytocannabinoids in RTT.

Based on these premises, the aim of this project was to evaluate the ability of chronic administration of the phytocannabinoids, cannabidivarin (CBDV) and cannabidiolic acid (CBDA), to affect neurological and motor defects as well as cognitive deficits in a mouse model of RTT, namely Mecp2 knockout (KO) mice.

To this aim, Mepc2 KO mice were treated daily with CBDV (or relative vehicle) at the doses of 0.2, 2, 20 or 200 mg/kg or CBDA (or relative vehicle) at the dose of 2 and 20 mg/kg from postnatal day (PND) 28 to 66. During the whole treatment schedule, motor and neurological signs were scored while short- and long-term memory deficits were evaluated at PND 41, 56 and 66. 24 hours after the last injection, brain tissues were collected to investigate the presence of alterations on neurotrophic factors (BDNF and IGF-1), inflammatory markers (CD11b, GFAP and TNFα) as well as components of the endocannabinoid system.

The present findings provide for the first time direct evidence that CBDV and CBDA improve motor and neurological signs as well as cognitive deficits in Mecp2 KO mice. In particular, CBDV administration delays the appearance neurological and motor signs in Mecp2 KO mice in a time window between 5 and 7 weeks of age. Conversely, CBDA administration ameliorates motor signs only at later stages of the disease progression, i.e. 8 and 9 weeks of age. Remarkably, both phytocannabinoids exert a complete and enduring beneficial effect towards short- and long-term memory deficits in Mecp2 KO animals.

At the biochemical level, chronic treatments with CBDV and CBDA enhance the expression of both BDNF and IGF-1 and reduce microglia activation in the brain of Mecp2 KO mice. Moreover, Mecp2 deletion results in alterations in the endocannabinoid system that could likely sustain RTT-like phenotype, and chronic CBDV treatment further modulates them.

Although further studies are needed to directly assess the mechanism(s) through which CBDV and CBDA can improve RTT-like phenotype in Mecp2 KO mice, overall these findings suggest for the first time a potential therapeutic application of the phytocannabinoids CBDV and CBDA in the context of RTT.

INTRODUCTION

1. RETT SYNDROME

RETT syndrome (RTT) is a postnatal progressive neurodevelopmental disorder that represents the most common genetic cause of severe intellectual disability in females (Percy and Lane, 2005) with an incidence of approximately 1 in 10,000 live births (Chahrour and Zoghbi, 2007). The disorder is characterized by arrested development between 6 and 18 months of age, regression of acquired skills, loss of speech, stereotypic movements, microcephaly, seizures, and mental retardation.

Affected males with somatic mosaicism or an extra X chromosome have been described rarely (Moog et al. 2003). Although MECP2 mutations were initially thought to be prenatally lethal in males, it has been shown that MECP2 mutations actually cause a variable phenotype in male patients (Ravn et al. 2003). The phenotypes can be divided in different categories with presence of symptoms that are highly similar to classic RTT in females or mild or severe mental retardation.

Dr. Andreas Rett, an Austrian pediatrician, first described the syndrome in 1966. Rett noticed that, after 1 year of normal development, girls affected by RTT undergo a progressive regression characterized by motor dysfunctions, stereotyped hand movements and progressive deterioration of cognitive functions (Rett et al. 1966). A subsequent study by Hagberg and colleagues in 35 girls with a progressive encephalopathy from three different European countries (France, Portugal, and Sweden), also highlighted that the syndrome develops in four distinct phases (Hagberg et al. 1986). The first phase, called stagnation of development, appears after 7 to 18 months of age and it is characterized by a failure to meet major developmental milestones, such as word development, social interaction, and motor ability. This phase is further evidenced by microcephaly and growth delay. The second phase of the syndrome is characterized by a rapid deterioration that

results in loss of previously acquired motor abilities. Purposeful hand movements are replaced by stereotypies, such as hand clasping and wringing. Breathing abnormalities also often appear at this stage together with mental decline and apraxia. The last two phases of RTT, called pseudo-stationary phase and late motor deterioration, appear between 3 to 10 years of age and are characterized by seizures and scoliosis, accompanied by severe motor impairments, including loss of ambulation and parkinsonian features (Hagberg, 2005; Roze et al. 2007). At the central level, this clinical picture is associated with decreased brain size, most likely resulting from smaller neurons and reduced dendritic branching, whereas no signs of neuronal degeneration are observed in RTT patients (Armstrong, 2005).

In addition to the classic form, there are five recognized atypical RTT variants (Trevathan et al. 1988, Hagberg et al. 2001). These variants show some, but not all, diagnostic features of RTT and can be milder or more severe. Several variants of atypical RTT have been defined: 1) The *early-onset seizure type* (Hanefeld variant); 2) The *congenital variant* (Rolando variant) is the most severe form of atypical RTT; 3) The *'forme fruste'* is a milder variant of RTT; 4) The *late childhood regression form*; 5) The *preserved speech variant* (PSD or Zappella variant).

In the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV edition (1994), Rett syndrome was classified as one of five autism-related conditions, along with Asperger syndrome and childhood disintegrative disorder. So, it defined Rett syndrome as part of the spectrum but different from autism itself. In 2013, the American Psychiatric Association has published the fifth edition of the DSM-V that modifies the diagnostic criteria for autism spectrum disorder. In this current edition, with the growing understanding associated with the variability of symptoms, RTT is no longer considered as part of the autism spectrum

disorder. However, girls with RTT can also be diagnosed with autism if they meet the behavioral criteria.

The search for a gene for RTT was seriously hampered by the lack of familial cases, as 99% of cases of the syndrome are due to de novo mutations. However, linkage analysis studies on the few available familial cases allowed the identification of methyl-CpG binding protein 2 (MECP2) as the key gene responsible for the development of the syndrome (Tariverdian et al. 1987; Partington, 1988; Amir et al. 1999). Subsequent genetic studies confirmed that 95% of RTT patients show mutations in the MECP2 gene (Neul et al. 2008). The range of MECP2 mutations causing RTT includes missense, frameshift, nonsense mutations, and intragenic deletions.

MECP2 is a gene that maps on the X-chromosome, located in Xq28, and encodes for a nuclear protein of 53 kDa, which is found in various tissues in mammals (Meehan et al. 1989; Shahbazian, 2002), with the highest expression in the brain, where its levels are seven times higher in neurons than in glia, underscoring the importance of this protein in neuronal function (Shahbazian, 2002). Furthermore, the levels of MeCP2 in the mammalian brain increase over postnatal development, suggesting the importance of this protein for brain development and synapse maturation (Shahbazian, 2002; Balmer et al. 2002). It is the first discovered protein with the ability to regulate higher order chromatin structure and to bind methylated cytosine on DNA (Lewis et al. 1992). DNA methylation is a covalent modification of the genome, performed by specific enzymes, that provides an additional layer of information beyond the sequence of the genome; this modification appears to be critical in different process such as genomic imprinting, cellular differentiation and the silencing of transposable elements. (Smith et al. 2013; Schubeler, 2015).

1.1 MECP2 FUNCTIONS

Experimental evidence supports the role of MECP2 as a transcriptional repressor, transcriptional activator, chromatin organizer, regulator of alternative splicing, and miRNA processor (Lyst et al. 2015).

The MECP2 is encoded by a four-exon gene located at q28 on the X chromosome. MECP2 has two isoforms that differ in the N-terminus and in the distribution, which are generated by alternative splicing. Concerning the subcellular localization, both MECP2 isoforms are nuclear and co-localize with methylated heterochromatic foci in mouse cells. At the anatomical level, it has been demonstrated that the two isoforms have a differential distribution in the developing mouse brain (Dragich et al. 2007).

MECP2 is characterized by the presence of different domains. The first identified domain is the methyl CpG binding domain (MBD), which mediates a specific interaction between MECP2 and methylated cytosines followed by a guanine (Ohki et al. 2001). Recently, it has been highlighted that MECP2 is also able to interact with methylated cytosines in CpA-CpT and CpC dinucleotides context and to bind hydroximethyled cytosines, although this last interaction appears to be weaker in respect to those with methylated cytosine (Guo et al. 2014; Chen et al. 2015; Frauer et al. 2011; Valinluck et al. 2004).

The MDB domain is not the only portion of the protein able to bind the DNA (Ballestar et al. 2000). In fact, comparative computational analysis have identified three AT-hooks domains three AT-hooks in MECP2, which are specialized basic clusters that bind the minor groove of AT-rich DNA (Baker et al. 2013). Interestingly, the three AT-hooks domains of MECP2 exhibit a strong homology with the AT-hooks domain that are present in the high-mobility-group-AT-hook family of chromatin modulators (HMGA), who participate in different cellular

processes including regulation of gene expression and integration of retroviruses into chromosomes (Baker et al. 2013; Reeves and Beckerbauer, 2001).

Given the presence of an MBD, early functional studies of MECP2 operated under the assumption that MECP2 was likely a transcriptional repressor (Bird, 2002). However, in vivo transcriptional studies in animal models revealed that many genes are downregulated rather than upregulated in Mecp2 KO mice compared to WT controls, suggesting that MECP2 could also promote gene expression (Chahrour et al. 2008), probably via recruitment of the cAMP responsive elements binding protein 1 (CREB1) (Chahrour et al. 2008).

Thus, available evidence suggests the possibility that MECP2 could function as a transcriptional activator or repressor depending on the interactions involved in the recruitment of MECP2 to chromatin (Li et al. 2013; Ficz et al. 2011; Wu et al. 2011).

In addition to gene expression regulation, MECP2 appears to modulate processes such as alternative splicing and miRNA processing, indicating also a regulation at the post-transcriptional level (Kazazian, 1998; Han et al. 2004; Perepelitsa-Belancio et al. 2003).

Mecp2-related neurodevelopmental disorders might therefore be the results of misregulations of both transcription and other processes. However, the physiological roles of many of these interactions and their relevance to RTT pathology remain to be established.

1.2 MOUSE MODELS OF RETT SYNDROME

The identification of MECP2 mutations as the genetic cause of RTT led to the development of mouse models for studying the molecular basis of the pathology in the search for possible effective therapies. In 2001, two mouse models of RTT were generated on different backgrounds (Chen et al. 2001; Guy et al. 2001) and both models displayed

phenotypes resembling the human pathology. Similar to the human condition, after a period of normal development, Mecp2 KO mice undergo a progressive regression characterized by stereotyped movement, tremors, myoclonic jerks and seizures, often associated with severe motor impairments. Null male mice develop uncoordinated gait, breathing abnormalities and hindlimb clasping. Symptoms begin to emerge after 5 weeks of age and become more severe between 6 and 12 weeks of age, eventually leading to death (Chen et al. 2001; Guy et al. 2001; Katz et al. 2009). Mecp2 heterozygous female mice are viable, fertile and appear normal even in early adulthood. Unlike males, heterozygous female mice develop a similar phenotype but with a delayed onset (Stearns et al. 2007). Specifically, signs of the disease in female heterozygous mice appear around 6 months of age. In addition, the features of the phenotype in heterozygous females are much more variable than in hemizygous males but females tend to show a similar progression of phenotypes, though shifted in time. In the case of females, the worsening of the animal's condition does not lead to early natural death.

Other similarities between humans and animal models for RTT rely on the neuroanatomical signs. Indeed, microcephaly, neurons with smaller somas (Chen et al. 2001; Marchetto et al. 2010; Li et al. 2013), a decrease in dendritic complexity (Armstrong et al. 1995; Chapleau et al. 2009) and in synaptic plasticity (Asaka et al. 2006; Guy et al. 2007), dysregulation of neurotransmitter levels (Panayotis et al. 2011; Santos et al. 2010) and the absence of neurodegeneration are observed in Mecp2 KO mice compared to WT littermates (Robinson et al. 2012).

Unfortunately, the exact molecular consequences of the disruption of MECP2 functionality that lead to RTT are not fully understood. Interestingly, mutation of MECP2 restricted to neuronal lineage resulted in a phenotype indistinguishable from that of mice lacking

MECP2 in all tissues, suggesting that absence of normal protein function in neurons is sufficient to cause the disease (Chen et al. 2001; Guy et al. 2001).

Given that dysregulation in protein synthesis is a hallmark of many neurodevelopmental disorders associated with severe mental retardation, it has been proposed that alterations in protein synthesis and PI3K/AKT/mTor signaling pathway may also play a role in the pathogenesis of RTT (Krab et al. 2008; Hoeffer and Klann, 2010; Bear et al. 2008; Ehninger et al. 2008). The activity of the PI3K/AKT/mTor signaling pathway in the central nervous system (CNS) is crucial for synapse formation and functionality, as it regulates the morphology and arborization of dendritic spines as well as synaptic plasticity. Interestingly, all these aspects appear to be profoundly altered in RTT patients as well as in Mepc2 KO mice (Hoeffer and Klann, 2010; Kelleher et al. 2004; Jaworski and Shengt, 2006; Richter et al. 2009). A study by Ricciardi and colleagues (2011) investigated the presence of dysregulations of the PI3K/AKT/mTor signaling in a mouse model of RTT. The authors analyzed the phosphorylation status of the ribosomal protein s6 (rpS6), a key component of the 40S of ribosome and the final target of the PI3K/AKT/mTor pathway whose phosphorylation status can be correlated with the activation of protein synthesis (Pende et al. 2004; Roux et al. 2007). These data indicate that the phosphorylation status of rpS6 in Mecp2 KO mice is severely reduced compared to WT mice, suggesting that the PI3K/mTor signaling pathway is downregulated in Mecp2 KO mice.

In addition, convergent data indicate that the levels of Brain Derived Neurotrophic Factor (BDNF), one of the most intensively studied target gene involved in RTT pathogenesis, are significantly decreased in Mecp2 KO mice (Chahrour et al. 2008; Chang et al. 2006; Wang et al. 2006). BDNF is a neurotrophin involved in neuronal growth, maturation and differentiation, and plays also an important role in synaptic plasticity and learning and

memory processes. BDNF exerts its functions by binding to tropomyosin receptor kinase B (TrkB), leading to the activation of different intracellular pathways, including the PI3K-AKT pathway. In KO mice, BNDF overexpression is able to improve the core symptoms of RTT, including breathing abnormalities and motor dysfunction, and it increases animals' lifespan (Chang et al. 2006; Ogier et al. 2007; Deogracias et al. 2012). Unfortunately, the therapeutic potential of BDNF is hampered by its reduced ability to cross the blood brain barrier (BBB). Therefore, researchers are trying to identify alternative molecules that show the same positive effects but able to cross the BBB.

Another interesting molecule in the context of RTT is the insulin-like growth factor-1 (IGF-1), a peptide hormone that has an important role both in the periphery and in the CNS. It binds to IGF-1 receptors, promoting the activation of RAS-MAP pathway and PI3K-AKT pathway, thus regulating cellular proliferation, differentiation and stress resilience. Similar to BDNF, IGF-1 administration rescues many of the phenotypes present in Mecp2 KO mice but, from a therapeutic point of view, the advantage of using IGF-1 with respect to BDNF relies on its major capability to cross the BBB (Tropea et al. 2009).

Although RTT was thought to be caused exclusively by MeCP2 deficiencies in neurons, recent evidence provides a functional role of glial cells in the pathogenesis of the disorder. In 2009, Mandel and collaborator (Ballas et al. 2009) showed that the Mecp2 protein was present in all types of glia. Glial cells are composed of astrocytes, oligodendrocytes and microglia. Glia support and interact with neurons in innumerable ways, from providing the structural underpinnings and guidance of axons and dendrites to providing energy substrates necessary for neuronal function.

Indeed, evidence suggested that mice with Mecp2-null in glial cells showed behavioral and neural synaptic abnormalities that are similar to those in Mecp2-null mice, and studies

showed that the restoration of Mecp2 in glial cells can rescue some of these defects (Lioy et al. 2011; Nguyen et al. 2013; Cronk et al. 2015).

Despite the obvious limitation of animal models in the context of complex diseases such as RTT, the use of mouse models still represents a unique and essential tool to better characterize the molecular basis of the disease and to test possible new therapies.

2. THE ENDOCANNABINOID SYSTEM

The term 'endocannabinoid system' (EC system) refers to a neuromodulatory system involved in a variety of physiological processes, that are present both in the brain and periphery comprising the cannabinoid CB1 and CB2 receptors, their endogenous lipid ligands, endocannabinoids, such as the N-arachidonoylethanolamide (anandamide, AEA) and the 2-arachidonoylglycerol (2-AG), synthesized "on demand" as ligands for the cannabinoid receptors and the associated enzymatic machinery (transporters, biosynthetic and degradative enzymes) involved in the synthesis and degradation of endocannabinoids.

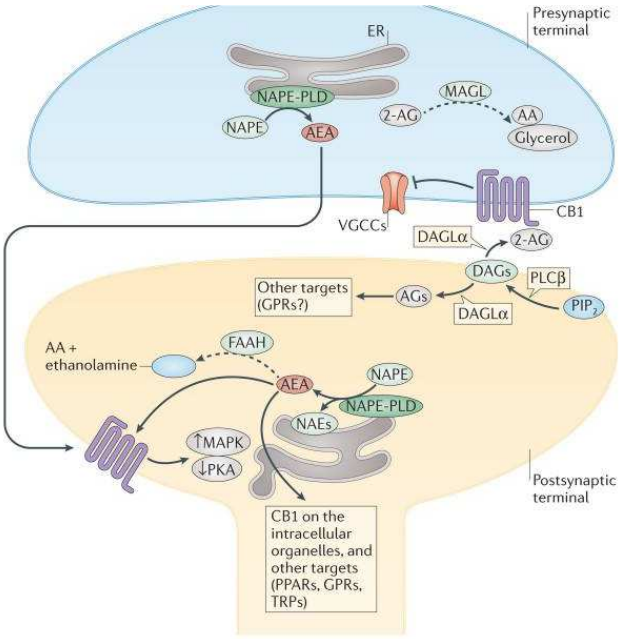

Figure 1. Main biosynthetic and inactivating enzymes in endocannabinoid signaling (Di Marzo et al. 2015).

Two types of cannabinoid receptors have been characterized, named CB1 and CB2 based on the order of their discovery (Matsuda et al. 1990; Munro et al. 1993), both belonging to the superfamily of G protein coupled receptors, whose activation mediates most of the effects of cannabinoid drugs. CB1 receptors are widely expressed in the CNS with low to moderate expression in periphery whereas CB2 receptors are predominately located in the

immune system, in particular in the spleen and tonsils and recent papers demonstrate that they may also be expressed in neurons (Brusco et al. 2008; Gong et al. 2006; Van Sickle et al. 2005). The CB1 receptor activation through both Gi/o proteins inhibits adenylyl cyclase activity, activates potassium channels and inhibits voltage-gated calcium channels, while the CB2 receptor is known only to couple to Gi proteins (Howlett, 2002). Some indirect evidence suggests the presence of additional cannabinoid receptors (GPR55, GPR119, PPAR) (Ryberg et al. 2007; O'Sullivan, 2007) but to date only CB1 and CB2 receptors are recognized by The International Union of Basic and Clinical Pharmacology.

Endogenous ligands for the cannabinoid receptors were discovered soon after their characterization. The two major known endogenous ligands are AEA and 2-AG (Devane et al. 1992; Stella et al. 1997; Sugiura et al. 2006). Both are arachidonic acid derivatives produced from phospholipid precursors postsynaptically through activity-dependent activation of specific phospholipase enzymes (Piomelli, 2003). Later on, a number of other endocannabinoid ligands have been identified including N- arachidonoyldopamine (NADA), N-arachidonoylglycerolether and O-arachidonoylethanolamine (De Petrocellis and Di Marzo, 2009).

These ligands do not share the same biosynthetic or metabolic pathways, indicating distinct mechanisms of regulation. Different pathways can produce AEA from the phospholipid precursors N-arachidonoyl-phosphatidylethanolamine, the most relevant being a direct conversion catalysed by an N-acyl-phosphatidylethanolamine selective phosphodiesterase. 2-AG is mainly synthesized through activation of phospholipase C and the subsequent production of diacylglycerol, which is rapidly converted to 2-AG by diacylglycerol lipase. After its re-uptake, AEA is hydrolysed by the enzyme fatty acid amide

hydrolase (FAAH), producing arachidonic acid and ethanolamine, while 2-AG is primarily metabolized by monoacylglycerol lipase (MAG lipase), leading to the formation of arachidonic acid and glycerol (Di Marzo and Petrosino, 2007). Apart from their well known binding to CB1 and CB2 receptors, endocannabinoids may also interact with non-CB1/non CB2 targets including the transient receptor potential vanilloid receptor type 1 (TRPV1) (Di Marzo and De Petrocellis, 2010), the 'orphan' G protein coupled receptor, GPR55 (Moriconi et al. 2010) and the peroxisome proliferator activated receptor, PPAR (Pistis and Melis, 2010), PPAR-α and PPAR-γ. However, CB1 and CB2 receptors are certainly the most known targets for AEA and 2-AG, which activate them with different affinity: AEA has the highest affinity in both cases, whereas 2-AG has the highest efficacy in both cases (McPartland et al. 2007).

Importantly, endocannabinoids are synthetized and released "on demand" by post synaptic cells through the cleavage of membrane phospholipid precursors in response to different physiological and pathological stimuli. Released endocannabinoids act as retrograde transmitters and traverse back across the synapse where they bind pre synaptically located CB1 receptors and reduce synaptic transmitter release (Freund et al. 2003).

On this basis, the EC system can be considered one of the major players in regulating the activity state of various neurotransmitters, and endocannabinoids are involved in several physiological functions. The role of the EC system in the programming of neural cells and in the developing nervous system, is well recognized as important. In the fetal brain, endocannabinoid levels and CB1 receptor distribution are spatially and temporally regulated (Berrendero et al. 1999). Indeed, AEA prevails in the early embryonic stages whereas 2-AG levels increase during differentiation to control neural progenitor cells.

Moreover, in progenitor cells, CB1 levels become upregulated while CB2 levels decrease (Palazuelos et al. 2012). Remarkably, endocannabinoids may modulate cell proliferation, fate and migration, as well as synaptogenesis of postmitotic neurons by allowing neuron migration and axon targeting and alterations of the EC system contribute to the pathogenesis of several psychiatric and neurological disorders.

3. THE PHYTOCANNABINOIDS

The plant *Cannabis sativa* and its preparations have been used for millennia for both recreational and medical purposes. More than 100 chemically and biosynthetically related cannabinoids have been identified in cannabis flowers in relative amounts depending on the plant variety. Only in the 20th century, the different active constituents of the plant were isolated and their chemical structure elucidated, including ∆-9-Tetrahydrocannabinol (∆-9-THC) and cannabidiol (CBD). From their identification, many studies were carried out in order to understand the pharmacological and the physiological effects of the different constituents (Mechoulam and Shvo, 1963; Gaoni, 1964; Dewey, 1986). These characteristic compounds are lipophilic metabolites defined as to “phytocannabinoids” to distinguish them from often structurally dissimilar but pharmacologically analogous endocannabinoids and synthetic cannabinoids.

3.1 CBD AND ITS POTENTIAL THERAPEUTIC APPLICATIONS

CBD is the major non-psychotropic component of the plant cannabis sativa and many studies have highlighted its potential anxiolytic, anti-depressant, anti-psychotic, anti-convulsant, anti-nausea, anti-oxidant, anti-inflammatory, anti-arthritic and anti-neoplastic properties (see for rewiev Pertwee et al. 2008).

Unlike THC, which has a high affinity for both CB1 and CB2 receptors, CBD has a low affinity for the classical cannabinoid receptors thus it does not elicit the classical effects

mediated by CB1 receptors activation such as hypo-locomotion, analgesia, catalepsy and hypothermia (Long et al. 2010; McPartland et al. 2015). This has prompt the investigation of its therapeutic potential in several pathological conditions.

CBD's therapeutic potential has been extensively studied in the context of neurodegenerative diseases, where it seems to exert beneficial effects via its anti-inflammatory and neuroprotective actions. Thanks to the presence of the phenol group in its chemical structure (Hampson et al. 1998; Marsicano et al. 2002), CBD seems to restore the balance between oxidant and anti-oxidant elements, thus promoting neuronal survival (Fernández-Ruiz et al. 2010). Moreover, the anti-inflammatory properties arise also from its modulatory activity on the nuclear factor-erythroid 2-related Factor 2 (nrf-2). Indeed, CBD promotes the activity of this transcription factor enhancing the expression of anti-oxidant and anti-inflammatory proteins (Fernández-Ruiz et al. 2010). In addition, CBD promotes neuroprotection via the modulation of microglial activation and migration, resulting in a reduction of pro-inflammatory mediators (Walter et al. 2003). Unlike for other phytocannabinoids, this action is independent from the interaction with the CB2 receptor (Fernández-Ruiz et al. 2007), but it occurs via the inhibition of NF-kB activity (Esposito et al. 2006; 2007). NF-kB is a transcriptional factor expressed in almost all cell types and it plays key roles in the modulation of inflammation, immune response and cell survival (Sen and Baltimore, 1986; Li and Verma, 2002; Kaltschmidt et al. 2005; Mattson, 2005; Ledoux and Perkins, 2014). It is activated in response to various insults, such as virus and bacterial infections and oxidative stress (Baeuerle and Henkel, 1994; Baldwin, 1996). When activated, NF-kB crosses the nuclear membrane and binds the DNA promoting the expression of various pro-inflammatory mediators such as inducible NO synthase (INos), interleukin-1, 6, 8 and chemokines (Duh et al. 1989, Kaltschmidt et al. 1993; Ahn and

Aggarwal, 2005; Gupta et al. 2010a, Gupta et al. 2010b). The inhibition of this transcription factor by CBD occurs through the interaction with specific kinase such as p38 MAP Kinase, blocking their translocation into the nucleus and the cascade that leads to the expression of pro-inflammatory mediators (Esposito et al. 2006), as well as through the interaction with nuclear receptors belonging to the PPAR family, PPAR-gamma in particular (Hill et al. 2012; Esposito et al. 2011).

Preclinical studies have been carried out to test the efficacy of CBD as a new possible therapy for neurodegenerative diseases such as Parkinson's (PD) and Alzheimer's (AD) disease. Both pathologies share the presence of a chronic inflammatory state in the central nervous system, which leads to neuronal suffering and death (Lee et al 2010; Qian et al. 2010). In AD, there is a progressive loss of cholinergic neurons due to the accumulation of beta-amyloid plaques and neurofibrillary tangles, that leads to a severe impairment in short and long-term memory functions (Perl et al. 2010). In contrast, PD is characterized by a progressive accumulation of alpha-synuclein oligomers that impairs dopaminergic neurons in the substantia nigra pars compacta causing severe motor disturbances but also psychotic episodes (Hindle et al. 2010). In both these pathologies, the antioxidant properties of CBD and its capability to reduce the activation of microglia seem to be very efficacious. Indeed, as shown by different studies, CBD is able to reduce the inflammatory processes that lead to the formation of the beta-amyloid plaques and alpha-synuclein oligomers, including phosphorylation of NF-kB by MAP 38 and increased iNos activity (Iuvone et al. 2004; Esposito et al. 2006; 2007). Interestingly, in PD the administration of CBD is also able to reduce PD-associated psychotic episodes (Zuardi et al. 2009).

CBD is currently under investigation for the treatment of epilepsy, a chronic neurological condition caused by defects in neuronal excitability that lead to altered neural network synchronization and seizure. CBD appears to be very effective in the treatment of hyper-excitability disorders as demonstrated both in preclinical and clinical studies (Jones et al. 2010). The beneficial effect of CBD in the context of epilepsy can be ascribed to its ability to modulate Ca^{2+} levels via its direct interaction with TRPV1 and TRPV2 receptors and to enhance GABAergic transmission, ultimately resulting in a reduction of spontaneous bursts (Iannotti et al. 2014; Consroe et al. 1982). Furthermore, it has been also shown that co-administration of CBD with some classical anti-epileptic drugs, such as phenytoin and phenobarbital, is able to potentiate their anticonvulsant effect (Jones et al. 2010).

The need for new therapies for hyper-excitability disorders arises from the observation that about 30% of patients do not respond to the classical anti-epileptic drugs, including patients diagnosed with Lennox-Gastaut and Dravet syndromes, which affect mostly children and are characterized by high frequency of seizure and severe neurodevelopmental problems (Oakley et al. 2011). Remarkably, CBD seems to be effective against convulsions in mouse models of Dravet syndromes (Devinsky et al. 2014).

3.2 OTHER PHYTOCANNABINOIDS

In the plant cannabis sativa, in addition to THC and CBD, there are other phytocannabinoids that show interesting pharmacological and therapeutic properties, such as ∆-9-tetrahydrocannabivarin (THCV), cannabichromene (CBC), cannabigerol (CBG), cannabidivarin (CBDV), ∆-9-tetrahydrocannabinolic acid (THCA), and cannabidiolic acid (CBDA). Although the pharmacology of these compounds has not yet been fully elucidated, experimental data suggest a complex pharmacological profile which involves

not only the EC system, but also other receptors, channels or intracellular targets (Pertwee, 2009).

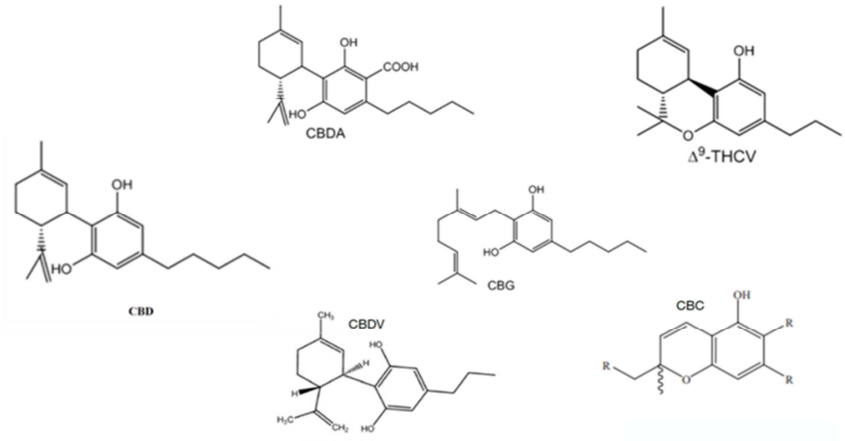

Figure 2. Chemical structures of the phytocannabinoids cannabidiol (CBD), ∆-9-tetrahydrocannabivarin (∆-9-THCV), cannabigerol (CBG), cannabidiolic acid (CBDA), cannabichromene (CBC) and cannabidivarin (CBDV).

Among them, THCV is the second most studied cannabinoid after CBD. This phytocannabinoid is able to antagonize CB1 receptors at low doses (Thomas et al. 2005), preventing also the effects induced by THC, such as hypothermia and anti-nociception (Pertwee, 2008; Englund et al. 2016). Moreover, THCV is a CB2 receptor agonist that reduces microglia cell activation and the production of pro-inflammatory cytokine, promoting neuroprotection in models of PD. In addition, THCV is also able to enhance the activity of 5-HT1A receptors, attenuating the positive and negative symptoms in animal models of schizophrenia (Cascio et al. 2015).

CBC is one of the most abundant phytocannabinoids present in the plant cannabis sativa (Turner et al. 1980; Russo, 2011), and it shows anti-inflammatory and analgesic properties. CBC's anti-inflammatory action occurs through the inhibition of the production of NO, IL-10 and IFN-gamma. In contrast, the analgesic action occurs via the stimulation of the descending anti-nociceptive pathway in the ventrolateral periaqueductal grey mediated by the activation of TRPA1 receptors (Romano et al. 2013; Maione et al. 2011).

CBG is the precursor of THC, CBD and CBC (Hill et al. 2012). This phytocannabinoid is a TRPV1 and TRPV2, TRPA1 and α2-adrenoceptor receptor agonist that possesses analgesic, anti-inflammatory, anti-oxidant and neuroprotective properties. The neuroprotective properties of CBG have been investigated in preclinical studies in the context of Huntington disease, a severe neurodegenerative disease caused by a mutation in the Huntingtin gene that produce the loss of glutamatergic neurons in the basal ganglia. Indeed, CBG administration in mouse models of HD increases the expression of BDNF and IGF-1 promoting a reduction in the aggregation of huntingtin (Giovannoni et al. 2009; Rock et al. 2011; Valdeolivas et al. 2015). Finally, this phytocannabinoid is also a potent TRPM8 and 5-HT1A antagonist with anti-emetic and anti-nausea properties (De Petrocellis et al. 2011).

CBDV is a non-psychoactive phytocannabinoid with a structure similar to CBD. Like CBD, it has low affinity for CB1 and CB2 receptors. Interestingly, CBDV, through the interaction with TRPV1 channels, exerts anti-epileptic effects, reducing seizures frequency and amplitude of bursts in the limbic areas (Hill et al. 2013; Iannotti et al. 2014).

THCA is a precursor of tetrahydrocannabinol (THC). Recent data reported that it has moderate affinity for human CB1 and CB2 receptors (Rosenthaler et al. 2014) and acts as a weak TRAP1 antagonist and a good TRPM8 antagonist (Ligresti et al. 2006).

Finally, CBDA is another non-psychoactive phytocannabinoid currently under investigation. It is the acid precursor of CBD (Potter et al. 2008; Mechoulam and Gaoni, 1965). Currently, little is known about the pharmacological actions of CBDA. There is already evidence, however, that CBDA shares the ability of CBD to activate the transient receptor potential (TRP) cation channels, TRPV1 and TRPA1, and to antagonize TRPM8 (De Petrocellis et al. 2008; 2011). Importantly, however, CBDA produces these effects with

significantly less potency than CBD (Rock et al. 2012; Bolognini et al. 2013). In addition, CBDA can act as a selective inhibitor of COX-2 (Takeda et al. 2014), an enzyme expressed by cells undergoing inflammation, although this finding has not been confirmed by subsequent studies (Ruhaak et al. 2011).

Overall, many phytocannabinoids possess interesting pharmacological properties that could be useful for the treatment of several pathological conditions. In addition, in preclinical models, all these compounds have been proven to be safe and well tolerated also at the higher doses tested, further supporting their potential exploitation for the treatment of human diseases.

AIM

Recent studies have highlighted the potential beneficial effects of some phytocannabinoids in the context of neurological diseases, including neurodegenerative disorders, hyperexcitability and in affective disorders, thanks to their anti-oxidant, anti-inflammatory and neuroprotective properties. Additionally, several preclinical and clinical data support the ability of some phytocannabinoids to modulate cognitive and motor functions, mood and neuronal excitability, all of which are altered in Rett syndrome (RTT). Despite the evidence suggestive of a potential therapeutic application on phytocannabinoids in RTT, so far no studies have addressed this possibility.

Cannabidivarin (CBDV) is a *n*-propyl analogue of cannabidiol (CBD) that lacks psychoactive activity (Hill et al. 2013). In vitro data shows that CBDV possesses low affinity and lacks appreciable functional activity at the cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). Little interaction with the enzymes responsible for the synthesis and degradation of endocannabinoids is reported (Bisogno et al. 2003; De Petrocellis et al. 2011) although purified CBDV is potentially a low potency inhibitor of the endocannabinoid membrane transporter and Diacylglycerol Lipase (DAGL), which would lead to inhibition of the biosynthesis of the endocannabinoid 2-AG. Moreover, CBDV may act through transient receptor potential (TRP) cation channels with agonistic actions at TRPV1 and TRPV2 channels (De Petrocellis et al. 2011; 2012), and antagonistic actions at TRPM 8 (De Petrocellis et al. 2011) in vitro.

Cannabidiolic acid (CBDA) is the acidic precursor of CBD. In harvested cannabis, CBDA gradually loses its carboxyl group to form CBD, a process that can be accelerated by heating or burning cannabis, as happens when it is smoked. Currently, little is known about the pharmacological actions of CBDA. There is already evidence, however, that CBDA shares the ability of CBD to activate TRPV1 and TRPA1 channels and to

antagonize TRPM8 (De Petrocellis et al. 2008; 2011). However, CBDA produces these effects with significantly less potency than CBD (Rock et al. 2012). In addition, CBDA can act as a selective inhibitor of COX-2 (Takeda et al. 2008), an enzyme expressed by cells undergoing inflammation, although this finding has not been confirmed by subsequent studies (Ruhaak et al. 2011).

In this project, we started our investigations using CBDV and CBDA, two compounds strictly related to CBD (CBDV is its propyl analogue while CBDA is its direct precursor) but whose therapeutic applications are more understudied in the context of neurological disorders. We aimed at testing for the first time the possibility that non-psychotomimetic phytocannabinoids, namely CBDV and CBDA, could be beneficial towards symptoms and signs associated with RTT. To this aim, chronic intraperitoneal treatments with CBDV (or relative vehicle) at the doses of 0.2, 2, 20 or 200 mg/kg or CBDA (or relative vehicle) at the dose of 2 and 20 mg/kg were carried out from PND 28 to PND 66 and their effects on motor and neurological symptom progression and cognitive deficits were monitored in Mecp2 KO mice, an animal model of RTT. Doses were chosen based on previous pharmacokinetic studies (Deiana et al. 2012) showing that CBDV at the dose of 60 mg/kg injected intraperitoneally was rapidly absorbed and readily detectable in the rat brain 30 minutes post-injection and its levels in the brain gradually declined until reaching values close to the detection threshold within 24 hours after dosing.

During the whole treatment schedule mice were scored every other day to evaluate the possible impact of phytocannabinoid treatments on motor symptoms (hindlimb clasping, gait, mobility) as well as neurological signs (breathing abnormalities, tremors) and general condition present in Mecp2 KO mice. Moreover, to assess the efficacy of phytocannabinoids administration in reverting short- and long-term memory deficits

present in KO mice, the NOR test was performed at different time points during pharmacological treatments, i.e. PND 41, 56 and 66.

Finally, 24 hours after the last injection, mice were sacrificed and brain tissues from Mecp2 WT and KO were collected to perform biochemical studies in order to assess the ability of phytocannabinoids to modulate same of the neurochemical markers known to be altered in RTT such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1) protein levels and their common downstream PI3K/Akt/mTOR signaling pathway. Furthermore, additional analysis were carried out in order to assess the levels of inflammatory markers, such as CD11b, TNFα and GFAP, as well as the status of the endocannabinoid system in the brain of Mecp2 WT and KO mice after phytocannabinoid administration.

Behavioral and biochemical results obtained in this study will provide important information about the effectiveness of CBDV and CBDA administration on symptom progression and cognitive deficits in the context of RTT.

MATERIALS AND METHODS

ANIMALS

The Mecp2-null mouse strain was generated according to Cobolli Gigli et al. (2016). C57BL/6 Mecp2 heterozygous females purchased from Jackson Laboratories (B6.129P2(C)-MeCP2tm1.1Bird/J) were crosses with CD1 WT male mice (Crl:CD1(ICR); Charles River) and kept backcrossing each generation of CD1 heterozygous females with new CD1 males for at least 10 generations. All animals were housed in groups of 4-5 in clear plastic cages on a 12 h light-dark cycle (lights on 8:00 AM) and in a temperature- (22 ± 2°C) and humidity-controlled environment (50 ±10%) with a plastic tube for environmental enrichment and had free access to food and water. Experimental procedures were performed in accordance with the guidelines released by the Italian Ministry of Health (D.L. 2014/26) and the European Community directives regulating animal research (2010/63/EU). Protocols were approved by the Italian Minister for Scientific Research and all efforts were made to minimize the number of animals used and their suffering.

GENOTYPING

Mouse genotypes were determined through PCR on genomic DNA purified from tail biopsy. Biopsies were obtained within the second and the third week of life.

Forward PCR primer sequences: 5'-ACCTAGCCTGCCTGTACTTT for the identification of the null allele, 5'-GACTGAAGTTACAGATGGTTGTG for the WT allele. Common reverse primer sequence: 5'- CCACCCTCCAGTTTGGTTTA. The obtained PCR products were: a single band of 450 base pairs (bp) for the Mecp2-null mice; a single band of 400 bp for the WT animals and the two bands for heterozygous females.

TREATMENT

CBDV treatment. A total of 112 littermate male mice from 4 different cohorts were used in the study, divided in 10 treatment groups: 22 for WT-vehicle, 10 for WT-CBDV 0.2 mg/kg, 13 for WT-CBDV 2 mg/kg, 12 for WT-CBDV 20 mg/kg, 3 for WT-CBDV 200 mg/kg, 11 for KO-vehicle, 6 for KO-CBDV 0.2 mg/kg, 13 for KO-CBDV 2 mg/kg, 14 for KO-CBDV 20 mg/kg and 8 for KO-CBDV 200 mg/kg.

CBDA treatment. A total of 37 littermate male mice were used in this study, divided in 6 treatment groups: 4 for WT-vehicle, 6 for WT-CBDA 2 mg/kg, 6 for WT-CBDA 20 mg/kg, 7 for KO-vehicle, 7 for KO-CBDA 2 mg/kg, 7 for KO-CBDA 20 mg/kg.

Pure CBDV (96.4%; CBD 3.3%) and pure CBDA (97%; CBGA 2.4%, CBD 0.4%), a kind gift of GW Pharmaceuticals (Cambridge, UK), was stored at -20°C and freshly prepared daily by dissolution in ethanol, kolliphor EL and saline (2:1:17 and 1:1:18 respectively). The treatments started at PND 28 (when animals were 4 weeks old) and lasted until PND 67 (when animals were 9 weeks old). Every day, mice were weighed and received an intraperitoneal injection of CBDV (or relative vehicle) at the doses of 0.2, 2, 20 or 200 mg/kg or CBDA (or relative vehicle) at the dose of 2 and 20 mg/kg between 9:00 a.m. and 11:00 a.m. (Figure 3).

BEHAVIORAL STUDIES

Score test

Neurological defects in Mecp2-null mice were evaluated using semi-quantitative observational scoring for hindlimb clasping, gait, breathing, tremor, mobility and general condition. Starting from postnatal day (PND) 28, Mecp2 WT and KO mice were scored every other day to evaluate the effect of chronic phytocannabinoids, CBDV and CBDA, treatment on motor symptoms as well as neurological signs and general condition.

Each of the six symptoms was scored from 0 to 2 (0 corresponds to the symptom being absent or the same as in the WT animal; 1 when the symptom was present; 2 when the symptom was severe) as previously described by Guy et al. (2007) and Szczesna et al. (2014). Specifically:

Mobility: the mouse is observed when placed on bench, then when handled gently and scored as follows: 0 = As WT, 1 = Reduced movement when compared with WT: extended freezing period when first placed on bench and longer periods spent immobile. 2 = No spontaneous movement when placed on the bench; mouse can move in response to a gentle prod or a food pellet placed nearby.

Gait: 0 = As WT. 1 = Hind legs are spread wider than WT when walking or running with reduced pelvic elevation, resulting in a 'waddling' gait. 2 = More severe abnormalities: tremor when feet are lifted, walks backward or 'bunny hops' by lifting both rear feet at once.

Hindlimb clasping: mouse observed when suspend by holding base of the tail. 0 = Legs splayed outward. 1 = Hindlimbs are drawn toward each other (without touching) or one leg is drawn into the body. 2 = Both legs are pulled in tightly, either touching each other or touching the body.

Tremor: mouse observed while standing on the flat palm of the hand. 0 = No tremor. 1 = Intermittent mild tremor. 2 = Continuous tremor or intermittent violent tremor.

Breathing: movement of flanks observed while animal is standing still. 0 = Normal breathing. 1 = Periods of regular breathing interspersed with short periods of more rapid breathing or with pauses in breathing. 2 = Very irregular breathing-gasping or panting.

General condition: mouse observed for indicators of general well-being such as coat condition, eyes and body stance. 0 = Clean shiny coat, clear eyes, and normal stance. 1 =

Eyes dull, coat dull/ungroomed, and somewhat hunched stance. 2 = Eyes crusted or narrowed, piloerection, and hunched posture.

If at any point an animal scored 2 out of 2 for any of the last three behaviors, or if the animal lost 20% of its body weight during the experiment, it was killed.

Novel object recognition (NOR) test

The test was performed on PND 28, 41, 56 and 66 (0, 14, 29 and 39 days after starting treatments). The experimental apparatus used for the NOR test was an open-field box (43x43x32 cm) made of Plexiglas, placed in a dimly illuminated room. Animals performed each test individually as in Zamberletti et al. 2015 using retention times of 30 minutes (short-term memory) and 24 hours (long-term memory). During the test phase the time spent exploring the familiar object (Ef) and the new object (En) was recorded separately by two observers blind to the groups and the discrimination index was calculated as follows:

$$(En-Ef)/(En+Ef) \times 100.$$

BIOCHEMICAL STUDIES

Western blot

Mice were sacrificed by cervical dislocation on PND 68, 24 hours after the last phytocannabinoid injection. The brains were quickly removed, immediately frozen in liquid nitrogen and stored at -80°C until processing.

The experiments were carried out as previously reported (Zamberletti et al. 2015). Briefly, equal amounts total protein lysates from hemisected brains (50 µg) were run on a 14% SDS-polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes and blocked for 2 hours before incubation overnight at 4°C with the following antibodies: rabbit polyclonal anti-BDNF (1:1000; Millipore, Italy), goat polyclonal anti-IGF-1 (1:1000; Relia Tech GmbH, Germany), rabbit polyclonal anti-pAKT Ser473 (1:1000; Cell

Signaling, Danvers, MA), rabbit polyclonal anti-pS6 (1:1000; Cell Signaling, Danvers, MA), rabbit polyclonal anti-pERK1/2 (1:1000; Cell Signaling, Danvers, MA), rabbit polyclonal anti-NAPE-PLD (1:3000; Cayman Chemical, Ann Arbor, MI), rabbit polyclonal anti-FAAH (1:2000; Cayman Chemical, Ann Arbor, MI), goat polyclonal anti-DAGLα (1:1000; Abcam, Cambridge, UK), rabbit polyclonal anti-MAGL (1:1000; Cayman Chemical, Ann Arbor, MI), rabbit polyclonal anti-GFAP (1:1000; Sigma Aldrich, Italy), rabbit polyclonal anti-TNFalpha (1:1000; Relia Tech GmbH, Germany), rabbit polyclonal anti-CD11b (1:1000; Novus Biologicals, USA). Bound antibodies were detected with horseradish peroxidase (HRP) conjugated secondary anti-rabbit or anti-goat antibody (1:2000-1:5000; Chemicon International, Temecula, CA). For normalization, the blots were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL) and re-blotted with mouse anti-β-actin monoclonal antibody (1:10000; Sigma Aldrich, Italy), rabbit polyclonal anti-total AKT (1:1000; Abcam, Cambridge, UK), rabbit polyclonal anti-total S6 (1:1000; Cell Signaling, Danvers, MA) or rabbit polyclonal anti-total ERK1/2 (1:1000; Cell Signaling, Danvers, MA) overnight at 4°C. Bound antibodies were visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) and bands were detected with a GBOX XT camera (Syngene, Cambridge, UK). Optical density of the bands was quantified using Image Pro Plus 7.0 software (MediaCybernetics, Bethesda, MD, USA), normalized to β-actin and expressed as arbitrary units.

Lipid extraction and endocannabinoid measurement

Hemisected brains were dounce-homogenized and extracted with chloroform/methanol/Tris-HCl (50 mM, pH 7.4) (2:1:1, v/v) containing internal deuterated standards for AEA, PEA, OEA, and 2-AG, quantification by isotope dilution [8-AEA, d4-PEA, d4-OEA, and d5-2-AG, (Cayman Chemical)], respectively. The lipid-containing

organic phases were then purified by open bed chromatography on silica and fractions were obtained by eluting the column with 99:1, 90:10, and 50:50 (v/v) chloroform/methanol. Fractions eluted with chloroform/methanol 90:10 were collected, the excess solvent evaporated with a rotating evaporator, and aliquots analyzed by isotope dilution-LC/atmospheric pressure chemical ionization/MS carried out in the selected ion monitoring mode by using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2020) quadrupole mass spectrometer via a Shimadzu atmospheric pressure chemical ionization interface. MS detection was performed by using values of m/z 356 and 348 (molecular ions + 1 for d8-AEA and AEA), m/z 304 and 300 (molecular ions + 1 for d4-PEA and PEA), m/z 330 and 326 (molecular ions + 1 for d4-OEA and OEA) and m/z 384 and 379 (molecular ions +1 for d5-2-AG and 2-AG). AEA, PEA, OEA, and 2-AG levels were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas.

Lipid amounts expressed as picomoles were then normalized per gram or milligram of wet tissue.

Statistics

For CBDV experiments, score data were analyzed non-parametrically using Pearson's Chi-square test with Yates correction. NOR data and biochemical results were expressed as mean ± SEM and analyzed by factorial- and main effects ANOVA, respectively. Calculations were carried out using Statistica 7.0 and R (R Core Team, 2013) Statistical Software. Bonferroni's post-hoc test was used to control for multiple comparisons.

CBDA behavioral and biochemical data were expressed as mean ± SEM and first analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. Calculations were carried out using Prism 5.0 software (GraphPad Software, San Diego, CA). A more

detailed and accurate statistical analysis is now ongoing (Pearson's Chi-square test with Yates correction for qualitative score data and three-way ANOVA analysis for NOR results). Survival curves were generated and compared using the log-rank method and the level of statistical significance was set at $p<0.05$.

RESULTS

1. BEHAVIORAL DATA

1.1 Effect of chronic CBDV treatment in Mecp2 WT and KO mice

Effect of chronic CBDV treatment on motor and neurological sign progression in Mecp2-deficient mice

Figure 4A shows the effect of chronic CBDV on tremors, hindlimb clasping, mobility, breathing, general condition and gait in KO mice over time. No effect of genotype or treatment on motor and neurological signs was observed in 4-week-old Mecp2 KO mice (data not shown). CBDV, at all doses tested, did not affect any of the considered parameters when given to WT mice (Figure 4).

Tremors

Male Mecp2 KO mice exhibited tremors beginning from 5 weeks of age. CBDV administration significantly reduced tremors in Mecp2 KO mice at 5 ($\chi^2_{[8]}$=12.765, p=0.012) and 6 ($\chi^2_{[8]}$=16.422, p=0.036) weeks of age, but not later. Doses of 2, 20 and 200 mg/kg showed similar efficacy (p<0.001) whereas the lowest dose of CBDV tested, 0.2 mg/kg, was ineffective and both appearance and intensity of tremors were similar in KO-CBDV 0.2 mg/kg mice compared to KO-vehicle animals.

Hindlimb clasping

Mecp2 KO mice showed a significant increase in hindlimb clasping starting from 6 weeks of age. CBDV treatment significantly attenuated hindlimb clasping in KO mice at 6 weeks of age ($\chi^2_{[8]}$=22.814, p=0.0036). This effect was reached at doses of 2, 20 and 200 mg/kg (p<0.05, 0.01). In contrast, CBDV was ineffective at the lowest dose tested, 0.2 mg/kg.

Breathing and gait

Breathing and gait abnormalities were evident in Mecp2 KO mice starting from 7 weeks of age. Breathing ($\chi^2_{[8]}$=22.405, p=0.0042) and gait ($\chi^2_{[8]}$=16.275, p=0.039) were improved

by CBDV in Mecp2 KO mice at the starting of the symptoms only, that is 7 weeks of age. Once again, doses of 2, 20 and 200 mg/kg were equally efficacious ($p<0.05$, 0.01, 0.001) whereas the lowest dose tested was completely ineffective.

Mobility and general condition

Impaired mobility and deterioration of general condition in KO mice was observed starting from 7 weeks of age. CBDV administration at all doses tested did not affect both parameters in KO mice during the whole treatment schedule.

Total Symptom Score

Individual behavior scores were combined into a total symptom score for each mouse (Figure 4B). Total symptom score was significantly increased in KO mice treated with vehicle starting from 5 weeks of age compared to WT mice and continued to increase until the end of the observation period. Significant effects were observed on total symptom score at 6 ($\chi^2_{[8]}=19.617$, $p=0.0037$) and 7 ($\chi^2_{[8]}=21.732$, $p=0.0041$) weeks of age. Treatment with CBDV 2, 20 and 200 mg/kg showed similar efficacy in attenuating motor and neurological deficits in KO mice. Indeed, all doses of CBDV significantly improved total symptom score in KO mice at 6 ($p<0.001$) and 7 ($p<0.001$) weeks of age, although CBDV's beneficial effect was lost at later stages of the disease progression (i.e. 8 and 9 weeks of age). In contrast, CBDV 0.2 mg/kg did not affect the progression of symptoms over the entire observation period.

Effect of chronic CBDV treatment on survival in Mecp2-deficient male mice

The percentage survival of Mecp2 KO and WT mice treated with CBDV or vehicle is shown in figure 5, where the results are plotted as Kaplan–Meier survival curves and expressed as the percentage of surviving mice over total mice with respect to time expressed as weeks. No lethality was observed in WT mice treated with either vehicle or

CBDV. In contrast, survival in KO mice treated with vehicle was only 38.5% by 9 weeks of age. All doses of CBDV administered from PND 28 onward were able to increase survival rates in Mecp2 KO mice at the end of the observation period compared to vehicle-treated littermates (KO-CBDV 0.2 mg/kg: 66.6%; KO-CBDV 2 mg/kg: 61.5%; KO-CBDV 20 mg/kg: 71.4%; KO-CBDV 200 mg/kg: 87.5%), although this effect reached statistical significance only at the higher dose tested, 200 mg/kg ($p=0.0433$; log Rank test).

Effect of chronic CBDV treatment on short- and long-term memory deficits in Mecp2-deficient male mice

Chronic administration of all doses of CBDV did not affect both short- and long-term memory in WT animals.

Short-term memory

Figure 6A shows the effect of chronic CBDV treatment on short-term memory in Mecp2 WT and KO mice at different time points before and during CBDV administration.

Three-way ANOVA revealed significant effects of genotype ($F_{[1,281]}=38.298$, $p<0.0001$), treatment ($F_{[4,281]}=33.091$, $p<0.0001$) and genotype x treatment interaction ($F_{[4,280]}=24.974$, $p<0.0001$) on short-term memory. In Mecp2 KO mice, a cognitive impairment in short-term memory was already present at PND 28, a time point when the motor symptoms were not yet present. Indeed, the discrimination index was significantly reduced in KO mice with respect to WT littermates at this time point ($p<0.001$). The cognitive impairment in short-term memory was consistently present in KO mice and was maintained until adulthood. In fact, KO animals showed significant reductions of the discrimination index at PND 41 ($p<0.001$), 56 ($p<0.001$) and 66 ($p<0.001$) compared to WT mice. Interestingly, at each of these time points, administration of three different doses of CBDV (2, 20 and 200 mg/kg) completely and significantly counteracted the cognitive impairment in short-term memory

present in KO mice ($p<0.001$). In contrast, chronic CBDV treatment at the lowest dose tested, 0.2 mg/kg, did not rescue the cognitive impairment in KO mice, the discrimination index being still significantly reduced at PND 41 ($p<0.001$), 56 ($p<0.001$) and 66 ($p<0.001$) compared to WT mice.

Long-term memory

Figure 6B shows the effect of chronic CBDV treatment on long-term memory in Mecp2 WT and KO mice at different time points before and during CBDV administration.

Three-way ANOVA revealed significant effects of genotype ($F_{[1,280]}=163.45$, $p<0.0001$), treatment ($F_{[4,280]}=22.819$, $p<0.0001$) and genotype x treatment interaction ($F_{[4,281]}=25.409$, $p<0.0001$) on long-term memory. Similar to what was observed for short-term memory, Mecp2 KO animals showed a significant cognitive impairment in long-term memory at PND 28, a pre-symptomatic phase of the disease. Indeed, the discrimination index was significantly reduced in KO mice with respect to WT littermates ($p<0.001$). The cognitive impairment in Mecp2 KO mice was maintained until adulthood, with the discrimination index of KO mice treated with vehicle being reduced at PND 41 ($p<0.001$), 56 ($p<0.001$) and 66 ($p<0.001$) respectively compared to WT mice.

Interestingly, at each of these time points, administration of CBDV 2, 20 and 200 mg/kg significantly rescued the cognitive impairment in long-term memory present in KO mice ($p<0.001$). As already shown for short-term memory, the lowest dose of CBDV (0.2 mg/kg) was not able to recover this cognitive impairment present in KO mice, the discrimination index being still reduced at PND 41 ($p<0.01$), 56 ($p<0.001$) and 66 ($p<0.05$) compared with WT mice.

1.2 Effect of chronic CBDA treatment in Mecp2 WT and KO mice

Effect of chronic CBDA treatment on motor and neurological sign progression in Mecp2-deficient male mice

Figure 7A shows the effect of chronic CBDA on tremors, hindlimb clasping, mobility, breathing, general condition and gait in KO mice over time. No effect of genotype or treatment on motor and neurological sign was observed in 4-week-old Mecp2 KO mice (data not shown). CBDA, at all doses tested, did not affect any of the considered parameters when given to WT mice (Figure 7).

Tremors

Male Mecp2 KO mice exhibited tremors beginning from 5 weeks of age as revealed by two-way ANOVA analysis (genotype effect, 5 wks: $F_{[2,31]=}69.20$; $p<0.0001$; 6 wks: $F_{[2,30]=}2577$; $p<0.0001$; 7 wks: $F_{[2,28]=}11270$; $p<0.0001$; 8 wks: $F_{[2,26]=}939.3$; $p<0.0001$; 9 wks: $F_{[2,24]=}621.4$; $p<0.0001$). CBDA per se, at all doses tested, did not have any effect when administered to WT mice. In Mecp2 KO mice, chronic administration of both doses of CBDA 2 and 20 mg/kg significantly reduced tremors in KO mice only at 9 weeks of age (treatment effect and genotype x treatment interaction: $F_{[2,24]}= 4.197$; $p=0.0273$).

Hindlimb clasping

Mecp2 KO mice show an increase in hindlimb clasping starting from 5 weeks of age (genotype effect, 5 wks: $F_{[2,31]=}23.22$; $p<0.0001$; 6 wks: $F_{[2,30]}=49.91$; $p<0.0001$; 7 wks: $F_{[2,28]}=59.62$; $p<0.0001$; 8 wks: $F_{[2,26]}=110.4$; $p<0.0001$; 9 wks: $F_{[2,24]}=136$; $p<0.0001$). CBDA per se, at all doses tested, did not have any effect when given to WT mice and chronic administration of CBDA 2 and 20 mg did not affect this parameter in Mecp2 KO mice during the whole treatment schedule.

Mobility

Impaired mobility was observed in KO mice starting from 7 weeks of age (genotype effect, 7 wks: $F_{[2,28]=}18.05$; p=0.0002; 8 wks: $F_{[2,26]=}62.83$; p<0.0001; 9 wks: $F_{[2,24]=}161.3$; p<0.0001). CBDA treatment per se did not have any effect when administered to WT mice. In Mecp2 KO mice, chronic CBDA at the dose of 20 mg/kg significantly attenuated mobility deficits in KO mice at 8 and 9 weeks of age (treatment effect and genotype x treatment interaction: 8 wks: $F_{[2,26]}$= 3.174; p=0.0529; 9 wks: $F_{[2,24]}$= 3.716; p=0.0393). In contrast, CBDA 2 mg/kg did not affect the development of this symptom in KO mice at any time.

Breathing

Breathing abnormalities were evident in Mecp2 KO mice starting from 7 weeks of age (genotype effect, 7 wks: $F_{[2,28]=}12.57$; p=0.0014; 8 wks: $F_{[2,26]=}176.3$; p<0.0001; 9 wks: $F_{[2,24]=}144.2$; p<0.0001). As with the other behavioral signs, CBDA per se did not have any effect on breathing abnormalities when given to WT animals. Administration of CBDA at doses of 2 and 20 mg/kg did not affect this parameter during the entire treatment period.

General Condition

General condition of KO mice treated with vehicle started to worsen from 7 weeks of age compared to WT animals (genotype effect, 7 wks: $F_{[2,28]=}20.25$; p=0.001; 8 wks: $F_{[2,26]=}125.6$; p<0.0001; 9 wks: $F_{[2,24]}$=217.2; p<0.0001). At all doses tested, CBDA per se had no effect when administered to WT animals. CBDA did not exert any beneficial effect on the general condition in Mecp2 KO mice during the whole treatment schedule.

Gait

Gait abnormalities were present in Mecp2 KO mice starting from 6 weeks of age (genotype effect, 6 wks: $F_{[2,30]=}16.62$; p=0.0003; 7 wks: $F_{[2,28]=}76.30$; p<0.0001; 8 wks: $F_{[2,26]=}380.9$; p<0.0001; 9 wks: $F_{[2,24]=}973$; p<0.0001). CBDA per se did not affect this parameter when

given to WT mice. Administration of CBDA only at a dose of 20 mg/kg significantly attenuated gait deficits in KO mice at 8 and 9 weeks of age (treatment and genotype x treatment interaction: 8 wks: $F_{[2,26]}$= 6.215; p=0.0062; 9 wks: $F_{[2,24]}$= 7.959; p=0.0022), whereas CBDA 2 mg/kg did not affect this symptom in KO mice at any time.

Total Symptom Score

To obtain an overview of the effect of CBDA on progression of the motor and neurological sign in Mecp2 KO animals individual behavior scores were combined into a total symptom score for each mouse (Figure 7B). Total symptom score was significantly increased in KO mice treated with vehicle starting from 5 weeks of age compared to WT mice and continued to increase until the end of the observation period (genotype effect, 5 wks: $F_{[2,31]=}$88.67; p<0.0001; 6 wks: $F_{[2,30]=}$124.7; p<0.0001; 7 wks: $F_{[2,28]=}$229.1; p<0.0001; 8 wks: $F_{[2,26]=}$528.8; p<0.0001; 9 wks: $F_{[2,24]=}$755.6; p<0.0001). Two-way ANOVA revealed a significant effect of treatment and treatment x genotype interaction on total symptom score at 8 and 9 weeks of age (8 wks: $F_{[2,26]}$= 3.721; p=0.0379; 9 wks: $F_{[2,24]}$= 5.070; p=0.0146). As outlined by the total symptom score graph, treatment with CBDA 20 mg/kg significantly improved total symptom score in KO mice at later stages of the disease progression (8 and 9 weeks of age). In contrast, CBDA 2 mg/kg had no significant effect on the progression of symptoms over the entire treatment period.

Effect of chronic CBDA treatment on survival in Mecp2-deficient male mice

The percentage survival of Mecp2 KO and WT mice treated with CBDA (2 and 20 mg/kg) or vehicle is shown in figure 8, where the results are plotted as Kaplan–Meier survival curves and expressed as the percentage of surviving mice over total mice with respect to time expressed as weeks. No lethality was observed for WT mice treated with vehicle or CBDA. In contrast, survival in KO mice treated with vehicle was 57.14% at 9 weeks of age.

Chronic CBDA treatment at the higher dose tested, 20 mg/kg, was able to increase survival rates in Mecp2 KO mice at the end of the observation period compared to vehicle-treated littermates (85.71%), although this effect did not reach statistical significance. On the contrary, CBDA at dose of 2 mg/kg did not affect this parameter in Mecp2 KO mice. Indeed, the survival rate being the same in the/this treatment group compared to KO-vehicle littermates (57.14%).

Effect of chronic CBDA treatment on short- and long-term memory deficits in Mecp2-deficient male mice

Chronic administration of all doses of CBDA per se did not affect both short- and long-term memory in WT animals, the discrimination index being similar in all the experimental groups analyzed.

Short-term memory

Figure 9A shows the effect of chronic CBDA treatment on short-term memory in Mecp2 WT and KO mice at different time points before and during CBDA administration.

As expected, a cognitive impairment in short-term memory was already present in KO animals at PND 28, a time point when the motor symptoms were not present. Indeed, the discrimination index was significantly reduced by about 185% in KO mice with respect to WT littermates. Two-way ANOVA analysis revealed significant effects of genotype, treatment and genotype x treatment interaction on short-term memory at PND 41 (genotype, $F_{[2,30]}=30.05$, $p<0.0001$; treatment, $F_{[2,30]}=4.163$, $p=0.0254$; genotype x treatment interaction, $F_{[2,30]}=5.021$, $p=0.0132$), 56 (genotype, $F_{[2,27]}=25.20$, $p<0.0001$; treatment, $F_{[2,27]}=3.687$, $p=0.0384$; genotype x treatment interaction, $F_{[2,27]}=3.122$, $p=0.0603$) and 66 (genotype, $F_{[2,24]}=50.28$, $p<0.0001$; treatment, $F_{[2,24]}=7.134$, $p=0.0037$; genotype x treatment interaction, $F_{[2,24]}=10.29$, $p=0.0006$). This cognitive impairment in

short-term memory was constantly present in KO mice and it was maintained until adulthood. In fact, KO animals showed significant reductions of the discrimination index by about 183, 153 and 179%, at PND 41, 56 and 66 respectively compared to WT mice.

At each considered time point, administration of the two doses of CBDA (2 and 20 mg/kg) completely and significantly counteracted the cognitive impairment in short-term memory present in KO mice.

Long-term memory

Figure 9B shows the effect of chronic CBDA treatment on long-term memory in Mecp2 WT and KO mice at different time points before and during CBDA administration.

Similar to what was observed for short-term memory, KO animals showed a significant cognitive impairment in long-term memory at PND 28. Indeed, the discrimination index was significantly reduced by about 149% in KO mice with respect to WT littermates.

Two-way ANOVA analysis revealed significant effects of genotype, treatment and genotype x treatment interaction on long-term memory at PND 41 (genotype, $F_{[2,30]}=46.79$, $p<0.0001$; treatment, $F_{[2,30]}=2.454$, $p=0.1031$; genotype x treatment interaction, $F_{[2,30]}=6.997$, $p=0.0032$), 56 (genotype, $F_{[2,27]}=56.40$, $p<0.0001$; treatment, $F_{[2,27]}=0.8938$, $p=0.4208$; genotype x treatment interaction, $F_{[2,27]}=2.400$, $p=0.1098$) and 66 (genotype, $F_{[2,24]}=144.4$, $p<0.0001$; treatment, $F_{[2,24]}=19.26$, $p<0.0001$; genotype x treatment interaction, $F_{[2,24]}=21.52$, $p<0.0001$). This cognitive impairment was maintained until adulthood, the discrimination index of KO mice treated with vehicle being reduced by about 147, 156 and 227%, at PND 41, 56 and 66 respectively compared to WT mice.

Chronic administration of the two doses of CBDA (2 and 20 mg/kg) ameliorated the cognitive impairment in long-term memory present in KO mice, and this effect reached

statistical significance at PND 41 and 66, whereas a non-significant trend was observed at PND 56.

2. BIOCHEMICAL DATA

2.1 Effect of chronic CBDV treatment in Mecp2 WT and KO mice

Effect of chronic CBDV treatment on BDNF, IGF-1 protein levels and downstream PI3K/AKT/mTOR and ERK1/2 pathways in hemisected brains of Mecp2-deficient male mice

CBDV at all doses tested did not affect the expression of all the considered biochemical markers when given chronically to WT mice.

Two-way ANOVA analysis revealed significant effects of genotype ($F_{[4,38]}$=4.773, p=0.0032) and genotype x treatment interaction ($F_{[4,38]}$=2.709, p=0.0444) on IGF-1 levels. IGF-1 levels were significantly reduced in Mecp2 KO mice treated with vehicle compared to WT animals ($p<0.05$). CBDV administration dose-dependently rescued IGF-1 expression in KO mice. Indeed, CBDV 2, 20 and 200 mg/kg significantly increased IGF-1 levels in KO mice ($p<0.05$, 0.01), whereas a non-significant trend (p=0.0864) was observed after administration of the lowest dose of 0.2 mg/kg (Figure 10A).

Statistical analysis showed significant effects of genotype ($F_{[4,38]}$=5.677, p=0.0220), treatment ($F_{[4,38]}$=2.677, p=0.0455) and genotype x treatment interaction ($F_{[4,38]}$=9.997, p=0.0081) on BDNF expression. BDNF protein levels were significantly reduced in Mecp2 KO mice treated with vehicle with respect to WT littermates ($p<0.01$). Interestingly, chronic administration of CBDV at the dose of 0.2 mg/kg did not affect BDNF expression in KO mice, its levels being still significantly reduced compared to WT-vehicle animals ($p<0.05$). In contrast, chronic CBDV at the doses of 2, 20 and 200 mg/kg significantly restored BDNF levels in KO mice, to a similar degree ($p<0.05$, Figure 10B).

Finally, figure 10C, represents the effect of chronic CBDV administration on pAKT Ser473, rpS6 and ERK1/2 phosphorylation in the brain of Mecp2 WT and KO mice. Significant effects of genotype and genotype x treatment interaction were also found on pAKT ($F_{[4,26]}$=3.967, p=0.0570; $F_{[4,26]}$=6.574, p=0.0009) and rpS6 phosphorylation ($F_{[4,29]}$=23.83, p<0.0001; $F_{[4,29]}$=2.702, p=0.0500). Phosphorylation levels of AKT were significantly reduced in Mecp2 KO mice compared to WT animals (p<0.05). Chronic CBDV administration dose dependently recovered pAKT levels in Mecp2 deficient mice, reaching statistical significance at doses of 20 and 200 mg/kg (p<0.01, 0.001). In contrast, the lowest dose of CBDV, 0.2 mg/kg, did not affect pAKT Ser473 levels in Mecp2 deficient mice, the levels of which were still decreased compared to controls (p<0.05).

Mecp2 deletion significantly reduced rpS6 phosphorylation within the adult brain compared to WT littermates (p<0.001). Chronic CBDV treatment at doses of 2, 20 and 200 mg/kg significantly increased rpS6 phosphorylation levels in KO mice (p<0.05, 0.01). In contrast, the lowest dose tested, 0.2 mg/kg, was ineffective, rpS6 phosphorylation being similar in KO-vehicle and KO-CBDV 0.2 mice (p<0.05). Neither genotype nor CBDV treatment affected ERK1/2 phosphorylation.

Effect of chronic CBDV treatment on endocannabinoid contents in Mecp2-deficient male mice

Figure 11A shows the effects of chronic CBDV administration on the levels of the endocannabinoids AEA and 2-AG as well as the N-acylethanolamines PEA and OEA in the brain of Mecp2 WT and KO mice. No statistically significant differences in AEA, OEA PEA and 2-AG levels were found between the two genotypes, although a trend for the increase of AEA (p=0.0613) and OEA (p=0.0817) was noticed. Statistical analysis revealed significant effects of genotype x CBDV treatment interaction on AEA

($F_{[4,20]}$=5.859, p=0.045) and 2-AG ($F_{[4,20]}$=5.375, p=0.041) levels as well as non-significant trend on OEA ($F_{[4,20]}$=8.787, p=0.067) content. CBDV administration at doses of 2, 20 and 200 mg/kg significantly increased AEA levels in Mecp2 KO mice with respect to WT-vehicle mice (p<0.05, 0.001, 0.01). A similar effect of CBDV was also observed on OEA levels. Indeed, chronic CBDV treatment at doses of 2, 20 and 200 mg/kg significantly enhanced OEA content (p<0.01, 0.001, 0.05). Chronic CBDV administration significantly reduced 2-AG levels in the brain of KO mice at doses of 20 and 200 mg/kg compared to vehicle-treated animals (p<0.05, 0.01). CBDV treatment also showed a trend toward reduction of 2-AG levels in WT mice. Finally, CBDV treatment did not affect PEA content in the brain of both WT and KO animals.

Effect of chronic CBDV treatment on endocannabinoid synthetic and degrading enzymes in Mecp2-deficient male mice

Figure 11B depicts the effect of chronic CBDV treatment on the protein levels of the main endocannabinoid synthetic and degrading enzymes in the brain of Mecp2 WT and KO mice.

Two-way ANOVA analysis revealed significant effects on NAPE-PLD (genotype, $F_{[4,32]}$=5.724, p=0.0228; genotype x treatment interaction, $F_{[4,32]}$=3.227, p=0.0247), FAAH (genotype, $F_{[4,32]}$=11.14, p=0.0022; treatment, $F_{[4,32]}$=1.782, p=0.1576) and DAGLα (treatment, $F_{[4,32]}$=11.75, p<0.0001) levels in the hemisected brains of KO mice. The expression of the main AEA synthetic enzyme NAPE-PLD was significantly increased in male Mecp2 KO mice after chronic treatment with CBDV at the highest dose tested, 200 mg/kg (p<0.01). In contrast, CBDV treatment at doses of 0.2, 2 and 20 mg/kg did not affect NAPE-PLD levels both in Mecp2 WT and KO mice. Deletion of Mecp2 resulted in a significant reduction of FAAH levels in vehicle-treated mice (p<0.05). Chronic CBDV

administration at all doses tested did not rescue FAAH expression in Mecp2 KO mice. In addition, chronic treatment with CBDV 0.2 and 20 mg/kg showed a trend towards a reduction in FAAH expression in WT mice, although the effect did not reach statistical significance. CBDV treatment also affected the expression of DAGLα, a key enzyme in the biosynthesis of the endocannabinoid 2-AG. In fact, chronic CBDV administration significantly reduced DAGLα protein levels at all doses tested both in WT ($p<0.01$) and in KO mice ($p<0.001$). No effect of either genotype or CBDV treatment was observed on the 2-AG degrading enzyme MAG lipase.

Effect of chronic CBDV treatment on CB1 and CB2 receptors in Mecp2-deficient male mice

Figure 11C shows the effect of chronic CBDV treatment on the protein levels of CB1 and CB2 receptors in the brain of Mecp2 WT and KO mice.

Two-way ANOVA analysis revealed significant effects of genotype and genotype x treatment interaction on CB1 ($F_{[4,32]}=19.89$, $p<0.0001$; $F_{[4,32]}=3.429$, $p=0.0193$) and CB2 ($F_{[4,32]}=4.662$, $p=0.0384$; $F_{[4,32]}=3.834$, $p=0.0118$) levels. CB1 protein levels were significantly increased in Mecp2 KO mice treated with vehicle compared to WT controls ($p<0.001$). Chronic CBDV treatment 2, 20 and 200 mg/kg significantly reduced CB1 receptor expression in KO mice ($p<0.05$), whereas the 0.2 mg/kg dose was ineffective. CBDV at all doses tested did not affect CB1 receptor levels when chronically administered to WT animals.

CB2 receptor expression was significantly enhanced in Mecp2 KO mice compared to WT controls ($p<0.001$). Chronic CBDV administration at doses of 2, 20 and 200 mg/kg significantly rescued CB2 receptor expression in KO mice ($p<0.001$), without affecting its

levels in WT littermates. In contrast, CBDV 0.2 mg/kg was not able to normalize CB2 receptor expression in Mecp2 KO animals.

Effect of chronic CBDV treatment (20 mg/kg) on glial markers in hemisected brains of Mecp2-deficient male mice

Figure 12 shows the effect of chronic CBDV treatment (20 mg/kg) on the protein levels of the microglia marker, CD11b, the astrocyte marker, GFAP, and the pro-inflammatory cytokine, TNFα, in the brain of Mecp2 WT and KO mice.

Statistical analysis shows main effects of genotype ($F_{[1-12]}$=6.966, p=0.0216) and treatment ($F_{[1-12]}$=4.756, p=0.0498) on CD11b levels. In the brain of Mecp2 KO mice, CD11b expression was significantly increased by about 60% compared to WT littermates. Chronic CBDV treatment at the dose of 20 mg/kg was able to reduce CD11b levels in KO mice, without affecting the expression of this marker when administered to controls.

Two-way ANOVA revealed a significant effect of CBDV treatment also on GFAP levels ($F_{[1-12]}$=6.540, p=0.0251). Indeed, CBDV showed a trend towards increasing GFAP expression both in WT and KO mice. Finally, neither genotype nor treatment affected TNFα levels in the brain.

2.2 Effect of chronic CBDA treatment in Mecp2 WT and KO mice

Effect of chronic CBDA treatment on BDNF and IGF-1 protein levels in hemisected brains of Mecp2-deficient male mice

In order to investigate the ability of CBDA to modulate brain levels of BDNF and IGF-1 in Mecp2 KO mice, animals were sacrificed 24 hours after the last CBDA (or vehicle) injection and western blot studies were carried out on protein extracts obtained from hemisected brains.

Figure 13 shows the effect of chronic CBDA treatment on BDNF and IGF-1 protein levels in Mecp2 WT and KO mice.

Two-way ANOVA revealed significant effects of genotype and genotype x treatment interaction on BDNF (genotype: $F_{[2\text{-}18]}$=4.629, p=0.0453; interaction: $F_{[2\text{-}18]}$=4.6115, p=0.0241) and IGF-1 (genotype: $F_{[2\text{-}18]}$=3.288, p=0.0607; interaction: $F_{[2\text{-}18]}$=9.514, p=0.0015) levels. As expected, Mecp2 deletion resulted in a reduction of both BDNF and IGF-1 by about 66% and 50% compared to controls. Chronic CBDA treatment did not affect the expression of the two neurotrophins when administered to WT mice. In contrast, both doses of CBDA showed a similar efficacy in increasing BDNF and IGF-1 expression in Mecp2 KO mice.

Effect of chronic CBDA treatment on glial markers in hemisected brains of Mecp2-deficient male mice

Figure 14 shows the effect of chronic CBDA treatment (2 and 20 mg/kg) on the protein levels of the microglia marker, CD11b, the astrocyte marker, GFAP, and the pro-inflammatory cytokine, TNFα, in the brain of Mecp2 WT and KO mice.

Preliminary data seem to confirm that in the brain of Mecp2 KO mice, CD11b expression was increased compared to WT littermates. Chronic CBDA treatment (2 and 20 mg/kg) seems to reduce CD11b levels in KO mice, without affecting the expression of this marker when administered to controls.

In contrast, chronic CBDA administration did not affect GFAP and TNFα levels in the brain of both WT and KO mice.

DISCUSSION

BEHAVIORAL RESULTS: motor and neurological signs

The present behavioral analyses confirm our previous findings (Cobolli Gigli et al. 2016) showing that Mecp2 KO male mice from a CD1 background show normal development for about the first month of life. Starting from 5 weeks of age, these mice develop severe and progressive motor deficits and neurological abnormalities, including tremors, hindlimb clasping, gait and breathing abnormalities, mobility impairments and a worsening of their general condition. Due to the severity of symptoms, about 50% of Mecp2 KO mice dies by 9 weeks of age. Furthermore, these mice display cognitive impairments both in short- and long-term memory that are already present at 4 weeks of age, a phase of the disease when motor and neurological impairments are still absent.

We here show for the first time that both CBDV and CBDA ameliorate neurological and motor signs in Mecp2 KO mice at different stages of the disease progression. In particular, CBDV significantly delays the appearance of tremors, hindlimb clasping as well as breathing and gait abnormalities between 5 and 7 weeks of age, but its beneficial effect is lost at later stages of the disease progression (i.e. 8 and 9 weeks of age). Unlike CBDV, chronic administration of CBDA in Mecp2 KO mice partially attenuates tremors, mobility and gait deficits only at later stages of the disease progression, i.e. 8 and 9 weeks of age, a time point when CBDV's beneficial effect is lost.

Remarkably, chronic administration of both phytocannabinoids shows a trend towards increasing survival rates in Mecp2 KO mice at the end of the observation period.

From a pharmacological point of view, CBDV and CBDA do not show a linear dose-response curve, at least in the dose ranges we tested, but they elicit an "all-or-none" response. Doses of CBDV of 2, 20 and 200 mg/kg show similar efficacy in ameliorating

RTT-like phenotype in Mecp2 mice, whereas the lowest dose tested, 0.2 mg/kg, is completely ineffective. Similarly, CBDA is effective only at the dose of 20 mg/kg.

Importantly, both CBDV and CBDA chronic treatments do not affect motor and neurological signs when chronically administered to WT animals, suggesting the safety and tolerability of these compounds.

BEHAVIORAL RESULTS: short- and long-term memory

Remarkably, both phytocannabinoids show efficacy in reverting short- and long-term memory deficits in Mecp2 KO mice, although CBDV does so more potently that CBDA.

In particular, chronic CBDV administration at doses of 2, 20 and 200 mg/kg completely recovers short- and long-term memory deficits during the whole treatment schedule, and this improvement persists at later stages of the disease progression, when CBDV's efficacy towards neurological and motor signs is lost.

Similarly, both doses of CBDA, 2 and 20 mg/kg, are able to improve short-term memory deficits in KO mice at each time point considered. The beneficial effect of CBDA is also evident on long-term memory deficits: chronic CBDA treatment at both doses tested significantly attenuates long-term memory deficits in Mecp2 KO mice, reaching statistical significance at PND 41 and 66.

BIOCHEMICAL RESULTS: neurotrophic factors

Besides behavioral analyses, we also carried out biochemical studies in hemisected brains of Mecp2 WT and KO mice to find possible molecular correlates of the effects observed at behavioral level.

BDNF is one of the best-characterized target of Mecp2 regulation (Chen et al. 2003), which is known to play an important role in neuronal and synaptic maturation (Carvalho et al. 2008) as well as in synaptic plasticity mechanisms underlying learning and memory in

the developing and adult CNS (Cunha et al. 2010). Expression of BDNF is decreased in mouse models of RTT (Chen et al. 2003; Chang et al. 2006; Li et al. 2014; Martinowich et al. 2003) and overexpression or enhancement of BDNF levels in vivo relieve some symptoms of the mutant phenotype (Chang et al. 2006; Deogracias et al. 2012; Johnson et al. 2012; Kline et al. 2010; Kron et al. 2014; Ogier et al. 2007; Schmid et al. 2012; Roux et al. 2012). Unfortunately, the therapeutic potential of BDNF is limited by its poor efficiency at crossing the blood–brain barrier (Pardridge et al. 1994) hence possible therapeutic strategies must rely on the identification of agents capable of indirectly stimulating BDNF levels.

In this context, the recent demonstrated ability of the phytocannabinoid CBD to restore BDNF expression in animal models of hepatic encephalopathy (Magen et al. 2010) and meningitis (Barichello et al. 2012), suggests that also its sister compound CBDV as well as its direct precursor CBDA could possess this feature. Accordingly, our data support CBDV's and CBDA's potentials to modulate BDNF levels in an animal model of RTT. Importantly, here we show for the first time that CBDV chronic treatment elevates the levels of BDNF in the hemisected brain of Mecp2 KO mice at behaviorally efficacious doses. In particular, chronic CBDV at doses of 2, 20 and 200 mg/kg significantly restores BDNF levels in KO mice, to a similar degree. In contrast, CBDV at the dose of 0.2 mg/kg does not affect BDNF expression in KO mice. A similar effect is also present after CBDA administration. Indeed, both doses tested, 2 and 20 mg/kg, significantly enhance BDNF levels in the brain of Mecp2 KO mice. Although we cannot rule out the possibility that more subtle effects could be present in specific brain regions, the ability of CBDV and CBDA to increase BDNF levels on the overall brain of Mecp2 KO mice can represent an important

feature in the context of RTT, which is characterized by profound synaptic dysfunctions across a broad range of brain areas (Della Sala et al. 2014).

In addition to BDNF, IGF-1 is another neurotrophic factor that holds great promise as therapeutic agent in RTT. Like BDNF, IGF-1 is widely expressed in the brain during normal development; it promotes neuronal cell survival as well as synaptic maturation and plasticity (Dyer et al. 2016). In mouse models of RTT, administration of both IGF-1 and its truncated form (1-3)IGF-1 reverses many of the features of the RTT phenotype (Chen et al. 2007; Castro et al. 2014; Della Sala et al. 2016; Tropea et al. 2009). Different from BDNF, IGF-1 crosses the blood–brain barrier. Early studies in RTT patients have demonstrated the tolerability and safety of IGF-1 as a potential treatment (Pini et al. 2012; 2014; 2016; Khwaja et al. 2014) and Phase 2 clinical trials using IGF-1 in RTT are currently ongoing.

Of note, in addition to their effect on BDNF, both CBDV and CBDA treatments also enhance IGF-1 levels in the brain of Mecp2 KO mice. Indeed, chronic CBDV treatment at doses of 2, 20 and 200 mg/kg restores IGF-1 levels in KO mice and a similar effect is also present after CBDA administration at doses of 2 and 20 mg/kg.

Given that both BDNF and IGF-1 modulates neuroplasticity and adaptive processes underlying learning and memory (Cunha et al. 2010; Dyer et al. 2016) and normalization of BDNF levels by CBD was associated with improved cognitive performance in animal models (Magen et al. 2010; Barichello et al. 2012), we speculate that CBDV- and CBDA-mediated enhancement of BDNF and IGF-1 levels could likely contribute to their shared ability to ameliorate short- and long-term memory deficits in Mecp2 KO mice. In line with this hypothesis, CBDV-induced enhancements of BDNF and IGF-1 occur at a time point when it is no longer effective towards neurological signs but it still recovers short- and

long-term memory impairments in Mecp2 KO mice. In addition, a close association between CBDA-induced normalization of BDNF and IGF-1 levels and its pro-cognitive effects in Mecp2 deficient mice is suggested by the observation that these effects are observed at both doses tested, which at behavioral level share similar efficacy only towards cognitive signs.

Although both BDNF and IGF-1 signal via PI3K/AKT and MAPK/ERK pathways to affect neuronal maturation and survival (Tropea et al. 2006; Yoshii et al. 2007; Zheng et al. 2004), there is direct evidence of the involvement of the AKT pathway in RTT. Indeed, it has been demonstrated that reduced phosphorylation of rpS6 in Mecp2 deficient mice is specific to signaling via AKT and not ERK1/2 kinases (Ricciardi et al. 2011). The PI3K/AKT/mTOR pathway is crucially implicated in a broad range of physiological functions, such as cell growth, proliferation, metabolism and protein synthesis (Yu et al. 2016). This pathway has been widely investigated in the pathogenesis of cognitive dysfunction and neurological diseases.

In this thesis, we first evaluated and completed investigations of the effect of chronic CBDV administration on PI3K/AKT pathway while CBDA experiments are still ongoing. We found that CBDV-mediated increases of BDNF and IGF-1 are associated with the normalization of their common downstream AKT/mTOR signaling pathway whereas no effects of either genotype or CBDV treatment are observed on ERK1/2 expression. This suggests that the beneficial effect of CBDV on RTT-like phenotype might arise from its ability to increase the levels of both growth factors that, in turn, synergistically stimulate downstream PI3K/AKT/mTOR intracellular signaling pathway to normalize rpS6 phosphorylation.

BIOCHEMICAL RESULTS: glia markers

The symptoms of RTT are mainly attributed to neuronal dysfunction. Indeed, converging evidence supports that neuronal, morphological and functional abnormalities are involved in RTT pathogenesis. In vitro and in vivo, Mecp2-deficient neurons show fewer dendritic spines and reduced arborization (Zhou et al. 2006; Smrt et al. 2007; Palmer et al. 2008). Moreover, selective re-expression of Mecp2 effectively rescued synaptic and behavioral abnormalities in Mecp2-null mice (Luikenhuis et al. 2004).

However, increasing evidence suggests that white matter abnormalities and glial cell dysfunction might contribute to RTT phenotype. Recently, it was reported that Mecp2-null astrocytes are incapable of supporting the normal development of co-cultured WT neurons (Williams et al. 2014) and Mecp2-null microglia and astrocytes are toxic to WT neurons mainly via disruption of glutamate clearance and release (Maezawa et al. 2009; Maezawa and Jin, 2010). These findings clearly suggest that RTT is not simply a disease of neurons alone, but a more complex disease in which glial cells might play a pivotal role in the pathological process, possibly via modulation of neuronal function.

Also in light of the proven anti-oxidant and anti-inflammatory properties of phytocannabinoids, we evaluated whether CBDV and CBDA administration affected glia cells in Mecp2 KO mice. Hence, we monitored the protein levels of the microglia marker, CD11b, the astrocyte marker, GFAP, as well as the pro-inflammatory cytokine, TNFα, in the brain of Mecp2 WT and KO mice after chronic phytocannabinoid administration.

We found that CD11b expression is significantly increased in the brain of Mecp2 KO mice compared to WT littermates, suggesting an activation of microglia cells in the mouse model. Interestingly, chronic treatments with both CBDV and CBDA reduce CD11b expression in Mecp2 KO mice. In particular, CBDV significantly rescues CD11b at the

dose of 20 mg/kg and a similar effect is observed after administration of CBDA at doses of 2 and 20 mg/kg.

These results suggest that both phytocannabinoids could dampen microglia activation in the context of RTT, and this effect might contribute to their ability to ameliorate the behavioral phenotype. In particular, the observation that CBDV reduces microglia activation at the end of the treatment schedule (when it was still able to rescue only short- and long-term memory deficits) and CBDA does so at both doses tested (that share the ability to ameliorate only cognitive impairments) is suggestive of an association between phytocannabinoids-mediated reduction of microglia activation and their pro-cognitive properties.

BIOCHEMICAL RESULTS: EC system

No studies have thus far investigated the possible involvement of the EC system in the pathogenesis of RTT. Thus, in this research project, we also evaluated for the first time whether Mecp2 deletion could result in alterations of the brain EC system and we also investigated whether phytocannabinoids could affect components of the EC system in the brain of Mecp2 KO mice. We collected data on the effect of chronic CBDV treatment on the different components of this neuromodulatory system, whereas experiments after chronic CBDA treatment are still ongoing.

Mecp2 deletion results in a reduction of the degrading enzyme FAAH in the brain which is associated with a trend towards increasing AEA and OEA levels compared to WT littermates. More interestingly, Mecp2 deletion significantly upregulates the expression of brain CB1 and CB2 receptors in Mecp2 deficient mice. Increased CB1 and CB2 receptor levels in the absence of Mecp2 suggest the possibility that Mecp2 could function as a repressor of CNR1 and CNR2 gene expression. However, this is a merely speculative

hypothesis and specific studies are needed to assess whether Mecp2 can act as a transcriptional repressor of these genes. However, dysregulation of CB1 receptor might also be a direct consequence of reduced BDNF levels. Indeed, it has been shown that CB1 receptor function is highly potentiated in mice lacking one copy of the BDNF gene (De Chiara et al. 2010), suggesting a functional interplay between BDNF and cannabinoid CB1 receptors.

Chronic CBDV treatment is able to modulate components of the endocannabinoid system in the brain of Mecp2 KO mice. Chronic CBDV administration at doses of 2, 20 and 200 mg/kg increases AEA levels and elevates OEA content in Mecp2 mutant mice, effects that are associated with reduced FAAH expression and, only for the highest dose tested, i.e. 200 mg/kg, with enhancement of NAPE-PLD expression. Furthermore, chronic CBDV treatment greatly reduces 2-AG levels both in Mecp2 WT and KO mice, via reduction of DAGLα expression, consistent with previous findings demonstrating CBDV's ability to inhibit DAGLα in vitro (Bisogno et al. 2003; De Petrocellis et al. 2011). Thus, CBDV modulates endocannabinoid content through different mechanisms: it enhances AEA and OEA levels mainly by inhibiting their degradation whereas it reduces 2-AG by inhibiting its synthesis.

Chronic CBDV treatment at doses of 2, 20 and 200 mg/kg significantly normalizes the levels of both receptors in the brain of Mecp2 deficient mice. As previous reports suggest that CBDV binds to CB1 and CB2 receptors with only very weak affinity (Iannotti et al. 2014; Hill et al. 2012; Hill et al. 2013; Amada et al. 2013; Rosenthaler et al. 2014), we propose that normalization of CB1 receptor expression following chronic CBDV treatment in Mecp2 KO mice may occur indirectly as a consequence of its ability to modulate endocannabinoid signaling. Nevertheless, CBDV-induced normalization of CB1 receptor in

Mecp2 KO mice could also be secondary to its ability to increase BDNF levels. In fact, it has been shown that BDNF potently inhibits CB1 receptor function, by modulating cholesterol metabolism and membrane lipid raft function (De Chiara et al. 2010). In support of this hypothesis, administration of a CBDV dose that did not restore BDNF levels (0.2 mg/kg) also failed to normalize CB1 receptor expression.

CONCLUSIONS

The present findings provide for the first time direct evidence that CBDV and CBDA are able to improve motor and neurological signs as well as cognitive deficits in Mecp2 KO mice.

In particular, CBDV administration delays neurological and motor signs in Mecp2 KO mice in a time window between 5 and 7 weeks of age. Conversely, CBDA administration ameliorates motor signs only at later stages of the disease progression, i.e. 8 and 9 weeks of age. Remarkably, both phytocannabinoids exert a complete and enduring beneficial effect towards short- and long-term memory deficits in Mecp2 KO animals.

At the biochemical level, chronic treatments with CBDV and CBDA enhance the expression of both BDNF and IGF-1 and reduce microglia activation in the brain of Mecp2 KO mice. It is tempting to speculate that the effect of CBDV and CBDA on BDNF and IGF-1 as well as on microglia cells might contribute to their ability to ameliorate cognitive impairments whereas the molecular basis of phytocannabinoid-mediated amelioration of neurological and motor signs has not been identified yet. Future experiments will be aimed at assessing the hypothesis that CBDV and CBDA may skew microglia cells towards a neuroprotective phenotype, promoting BDNF and IGF-1 release (Derecki et al. 2013) that ultimately might act on neurons to modulate synaptic plasticity processes underlying learning and memory functioning.

Finally, we here showed that Mecp2 deletion also results in profound changes in the brain EC system that could likely sustain RTT-like phenotype, suggesting that modulation of the EC signaling might represent a potential strategy to ameliorate RTT-related behavioral and molecular parameters.

Although further studies are clearly needed to directly assess the mechanism(s) through which CBDV and CBDA can improve RTT-like phenotype in Mecp2 KO mice, overall the present findings suggest for the first time a potential therapeutic application of the phytocannabinoids CBDV and CBDA in the context of RTT.

FIGURES

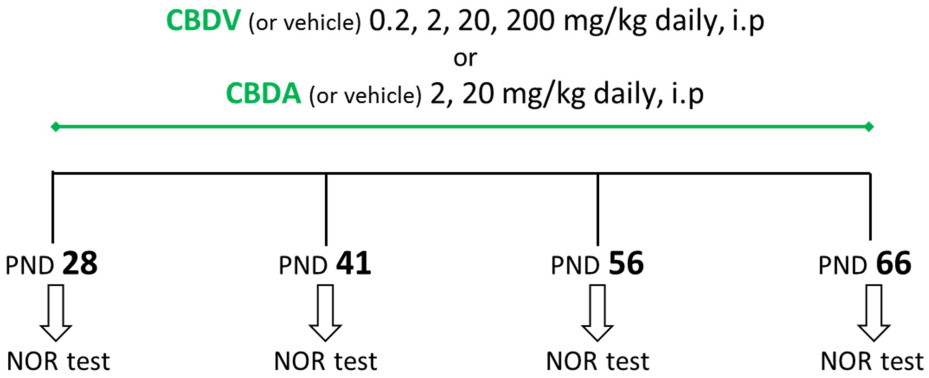

Figure 3. Treatment schedule. CBDV (or relative vehicle) at the doses of 0.2, 2, 20 and 200 mg/kg or CBDA (or relative vehicle) at doses of 2 and 20 mg/kg were administered daily starting from PND 28 to PND 67. At PND 28, 41, 56 and 66 the Novel Object Recognition (NOR) test was performed to assess the presence of cognitive deficits.

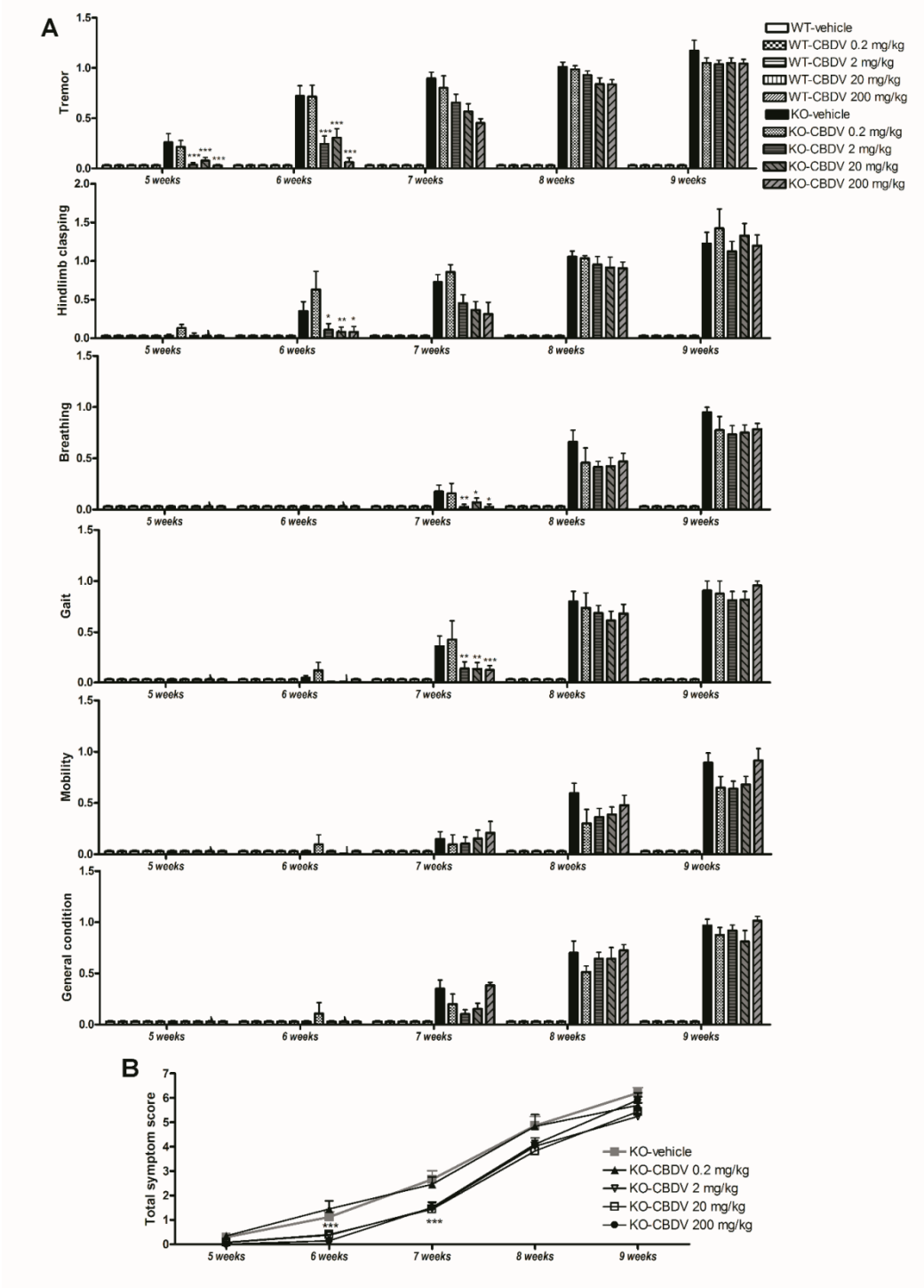

Figure 4. Effect of chronic CBDV (0.2, 2, 20, 200 mg/kg/day, PND 28-67) treatments on the Mecp2 KO phenotype. (**A**) Plots showing "Score Test" after CBDV treatment related to tremor, hindlimb clasping, breathing, gait, mobility and general condition. (**B**) Plot of total average symptom scores showing "Score Test" in Mecp2 KO-treated mice after CBDV treatment. The number of animals used in each experiment was 22 for WT-vehicle, 10 for WT-CBDV 0.2 mg/kg, 13 for WT-CBDV 2 mg/kg, 12 for WT-CBDV 20 mg/kg, 3 for WT-CBDV 200 mg/kg, 11 for KO-vehicle, 6 for KO-CBDV 0.2 mg/kg, 13 for KO-CBDV 2 mg/kg, 14 for KO-CBDV 20 mg/kg and 8 for KO-CBDV 200 mg/kg. Data represent mean ± SEM and were analyzed non-parametrically using Pearson's Chi-square test with Yates correction. *p<0.05, **p<0.01, ***p<0.001 vs KO-vehicle mice.

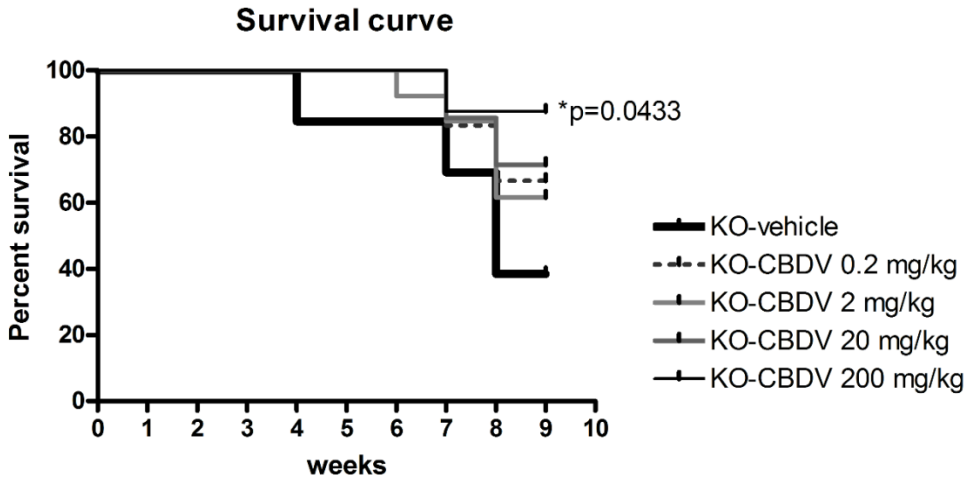

Figure 5. Effect of chronic CBDV (0.2, 2, 20, 200 mg/kg/day, PND 28-67) treatments on survival in Mecp2 KO mice. Kaplan-Meier survival curves of CBDV-treated Mecp2 KO mice compared to KO-vehicle littermates.

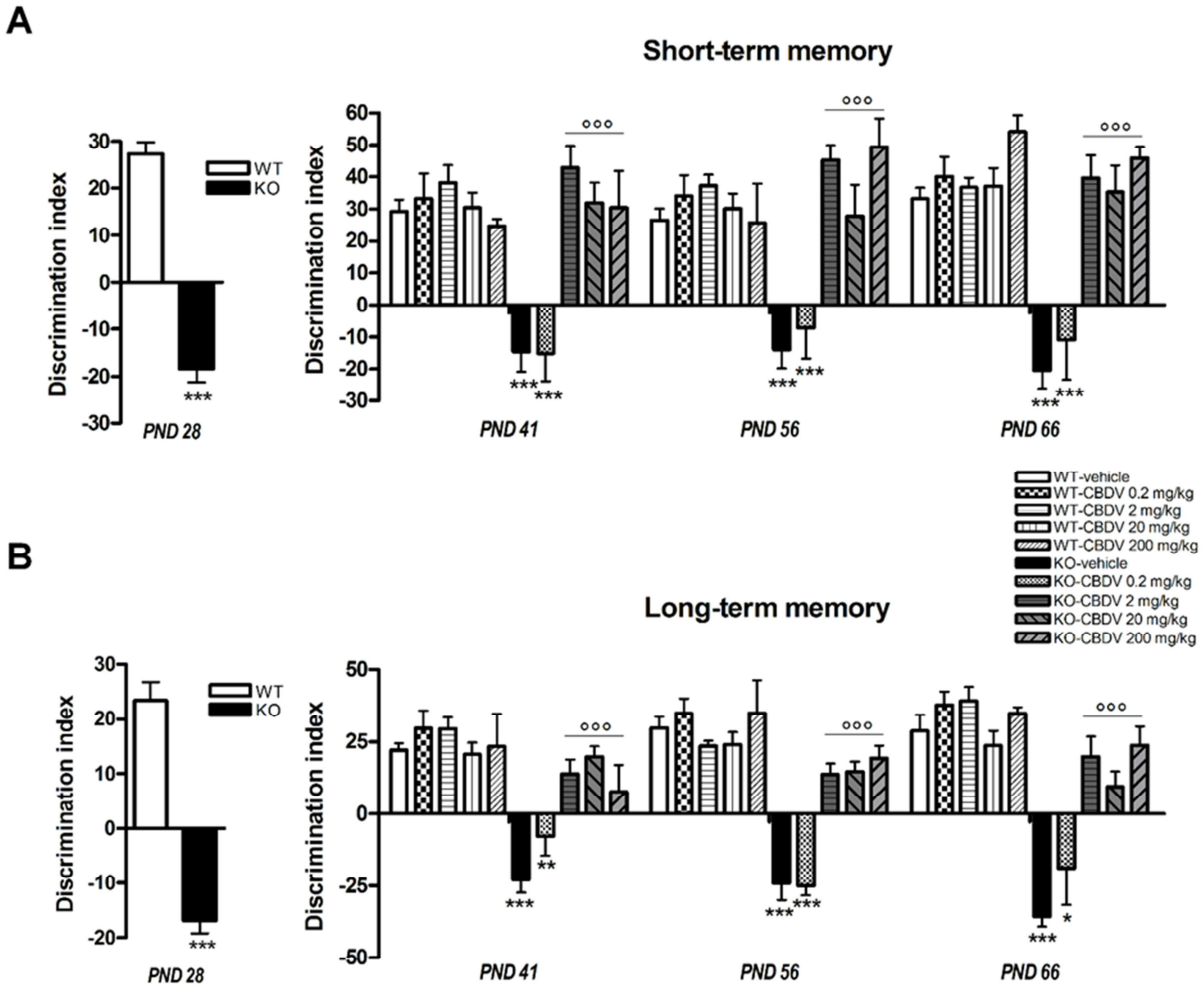

Figure 6. Effect of chronic CBDV (0.2, 2, 20, 200 mg/kg/day, PND 28-67) treatments on (**A**) short-term memory (retention time 30 minutes) and (**B**) long-term memory (retention time 24 hours) in WT and KO mice as measured through the NOR test. The test was performed at PND 28, 41, 56 and 66. Data are expressed as mean ± S.E.M. and were analyzed using a three-way ANOVA followed by Bonferroni post-hoc test. *p<0.05, **p<0.01, ***p<0.001 vs WT-vehicle; °°°p<0.001 vs KO-vehicle.

Number of animals. PND 28: 40 for WT mice and 35 for KO mice. **WT** mice (PND 41, 56, 66: 22 for WT-vehicle, 10 for WT-CBDV 0.2 mg/kg, 13 for WT-CBDV 2 mg/kg, 12 for WT-CBDV 20 mg/kg, 3 for WT-CBDV 200 mg/kg). **KO** mice (PND 41: 11 for KO-vehicle, 6 for KO-CBDV 0.2 mg/kg, 13 for KO-CBDV 2 mg/kg, 14 for KO-CBDV 20 mg/kg and 8 for KO-CBDV 200 mg/kg. PND 56: 11 (short-term) and 10 (long-term) for KO-vehicle, 5 for KO-CBDV 0.2 mg/kg, 11 for KO-CBDV 2 mg/kg, 12 for KO-CBDV 20 mg/kg and 7 for KO-CBDV 200 mg/kg. PND 66: 5 for KO-vehicle, 4 for KO-CBDV 0.2 mg/kg, 8 for KO-CBDV 2 mg/kg, 10 (short-term) and 9 (long-term) for KO-CBDV 20 mg/kg and 6 (short-term) and 5 (long-term) for KO-CBDV 200 mg/kg).

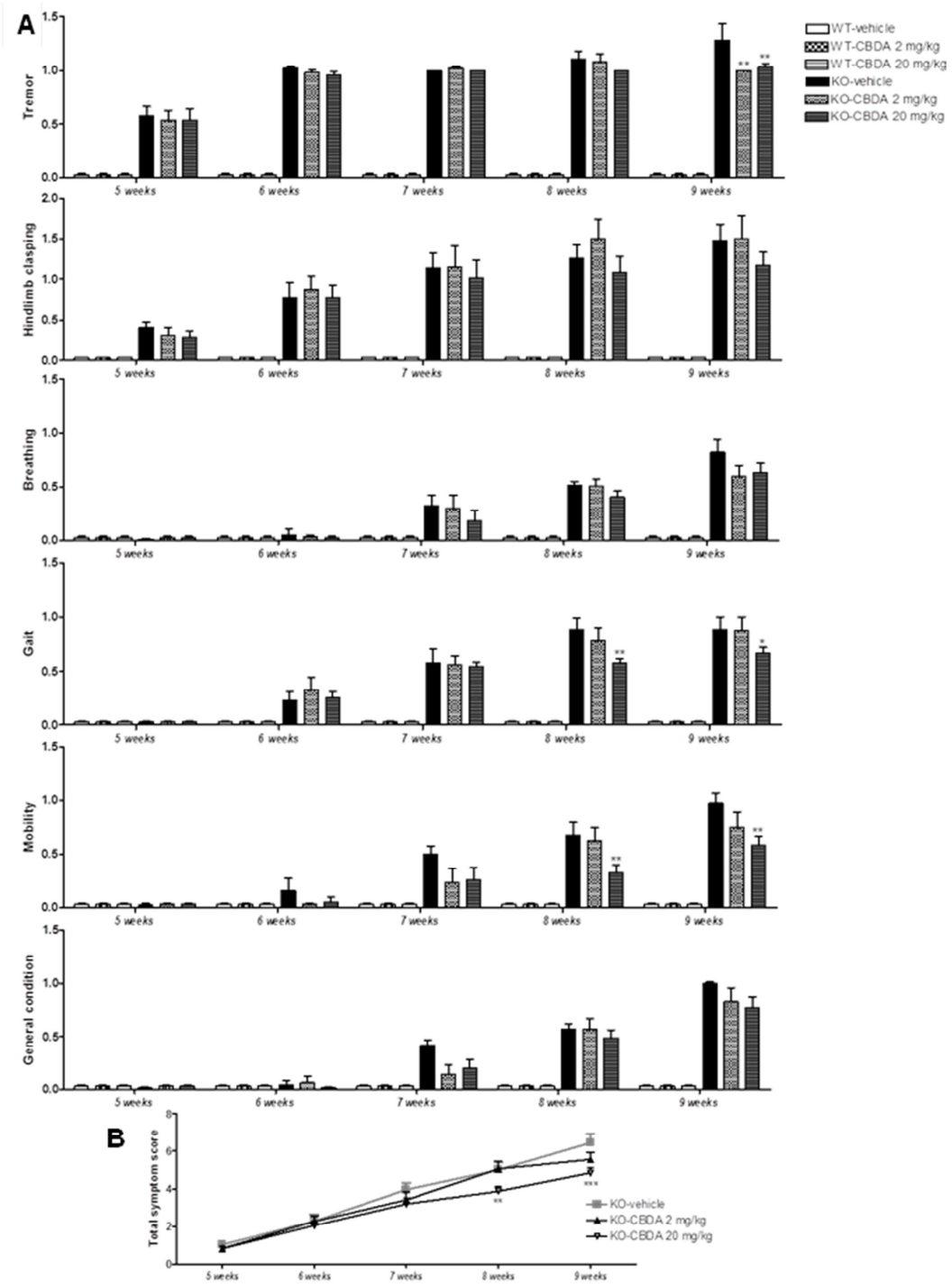

Figure 7. Effect of chronic CBDA (2, 20 mg/kg/day, PND 28-67) treatments on the Mecp2 KO phenotype. (**A**) Plots showing "Score Test" after CBDA treatment related to tremor, hindlimb clasping, breathing, gait, mobility and general condition. (**B**) Plot of total average symptom scores showing "Score Test" in Mecp2 KO-treated mice after CBDA treatment. The number of animals used in each experiment was 4 for WT-vehicle, 6 for WT-CBDA 2 mg/kg, 6 for WT-CBDA 20 mg/kg, 7 for KO-vehicle, 7 for KO-CBDA 2 mg/kg, 7 for KO-CBDA 20 mg/kg. Data represent mean ± SEM and were analyzed using a two-way ANOVA followed by Bonferroni post-hoc test. *p<0.05, **p<0.01, ***p<0.001 vs KO-vehicle mice.

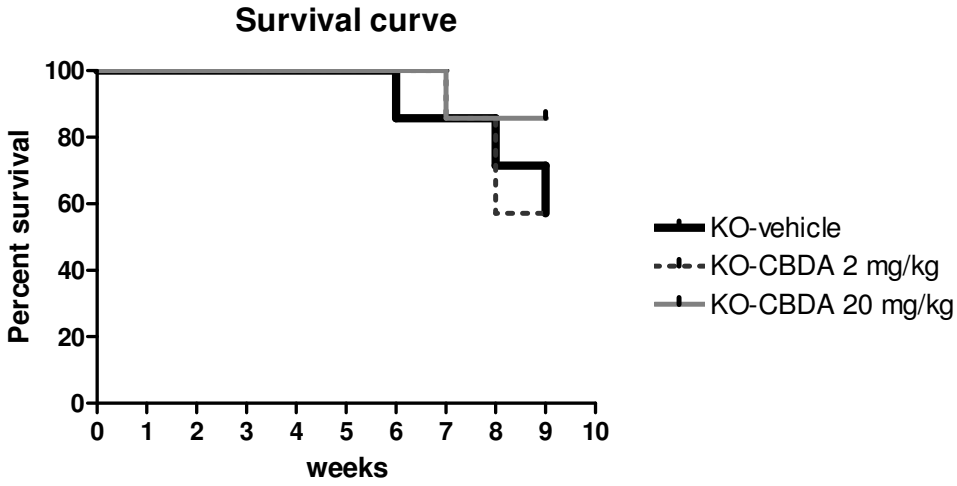

Figure 8. Effect of chronic CBDA (2, 20 mg/kg/day, PND 28-67) treatments on survival in Mecp2 KO mice. Kaplan-Meier survival curves of CBDA-treated Mecp2 KO mice compared to KO-vehicle littermates.

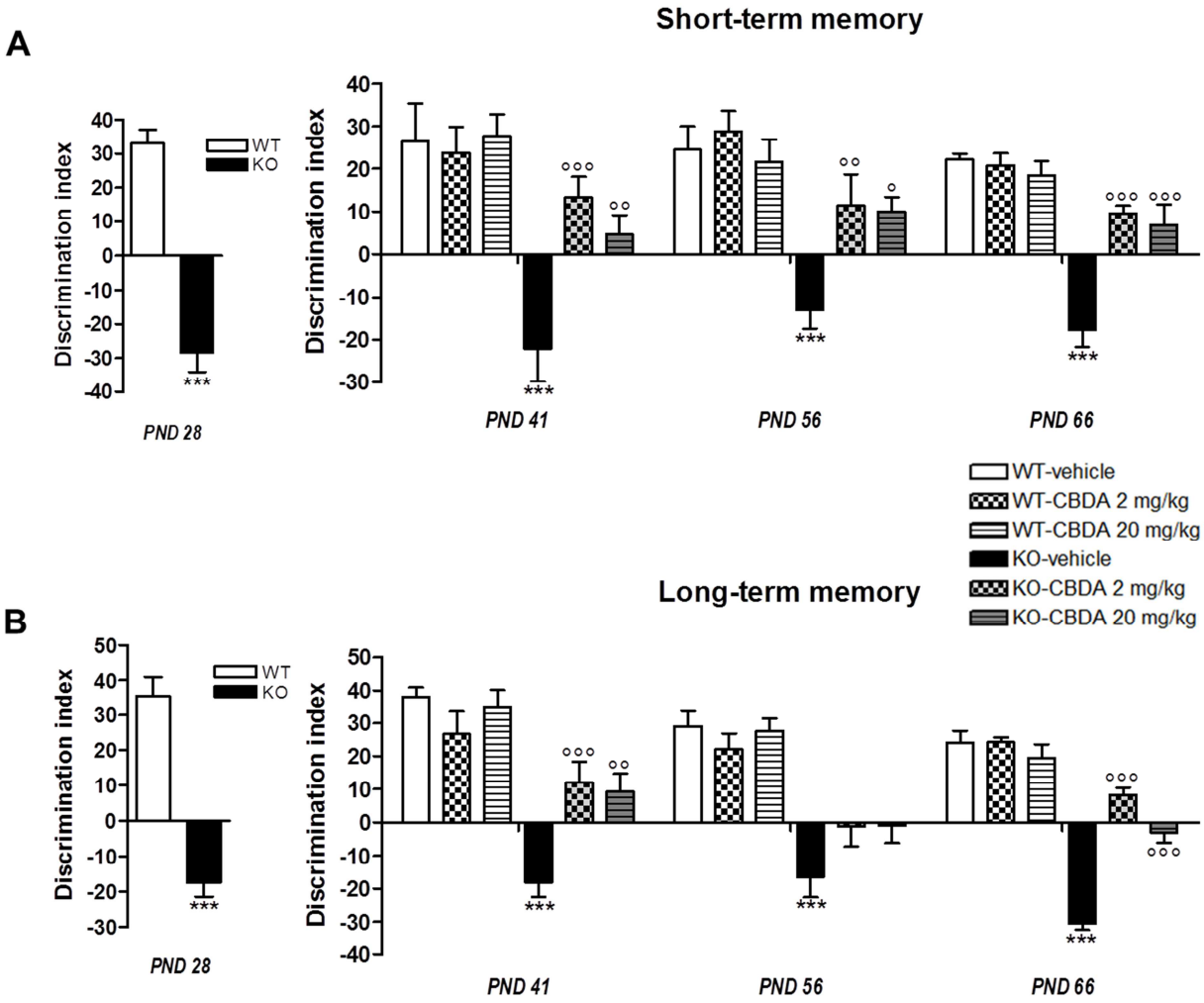

Figure 9. Effect of chronic CBDA (2, 20 mg/kg/day, PND 28-67) treatments on (**A**) short-term memory (retention time 30 minutes) and (**B**) long-term memory (retention time 24 hours) in WT and KO mice as measured through the NOR test. The test was performed at PND 28, 41, 56 and 66. Data are expressed as mean ± S.E.M. and were analyzed using a two-way ANOVA followed by Bonferroni post-hoc test. ***p<0.001 vs WT-vehicle; °p<0.01; °°p<0.01; °°°p<0.001 vs KO-vehicle.
Number of animals. PND 28: 9 for WT mice and 13 for KO mice. **WT** mice (PND 41, 56, 66: 4 for WT-vehicle, 6 for WT-CBDA 2 mg/kg, 6 for WT-CBDA 20 mg/kg). **KO** mice (PND 41: 7 for KO-vehicle, 7 for KO-CBDA 2 mg/kg, 7 for KO-CBDA 20 mg/kg. PND 56: 5 for KO-vehicle, 6 for KO-CBDA 2 mg/kg, 6 for KO-CBDA 20 mg/kg. PND 66: 4 for KO-vehicle, 4 for KO-CBDA 2 mg/kg, 6 for KO-CBDA 20 mg/kg).

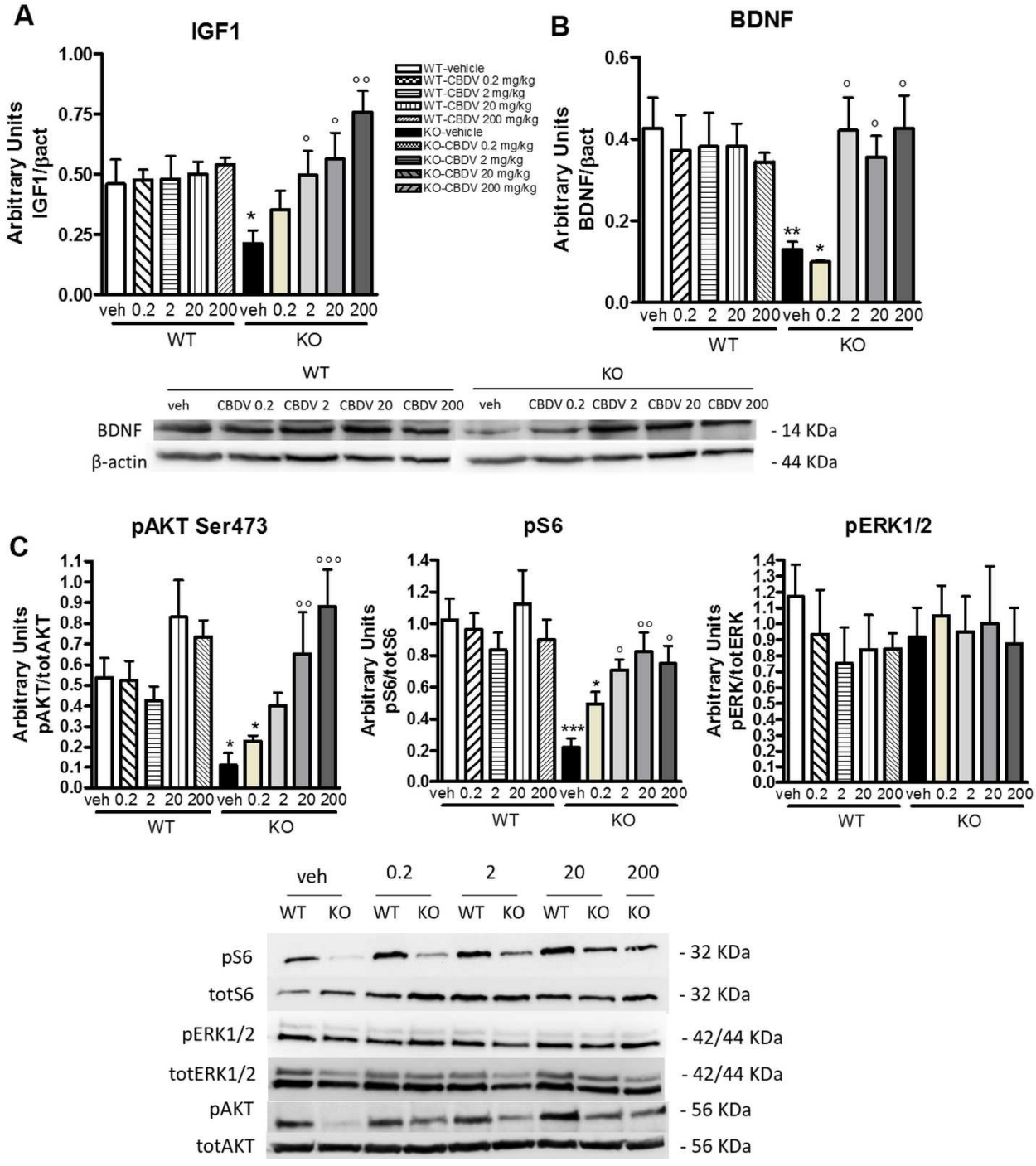

Figure 10. Effect of chronic CBDV (0.2, 2, 20, 200 mg/kg/day, PND 28-67) treatments on protein levels of (**A**) Insulin-Like Growth Factor-1 (IGF-1), (**B**) Brain-Derived Neurotrophic Factor (BDNF) and their common downstream pathway (**C**) pAkt Ser473 and its downstream effector rpS6 and ERK1/2 phosphorylation in hemisected brains of WT and KO Mecp2 mice. Measurements were carried out on total protein lysates from hemisected brains. Representative blot images correspond to the results of one experiment out of four. Data are expressed as mean ± SEM of 3-5 animals per group. *p<0.05, **p<0.01, ***p<0.001 vs WT-vehicle; °p<0.05, °°p<0.01; °°°p<0.001 vs KO-vehicle. Two-way ANOVA followed by Bonferroni post-hoc test.

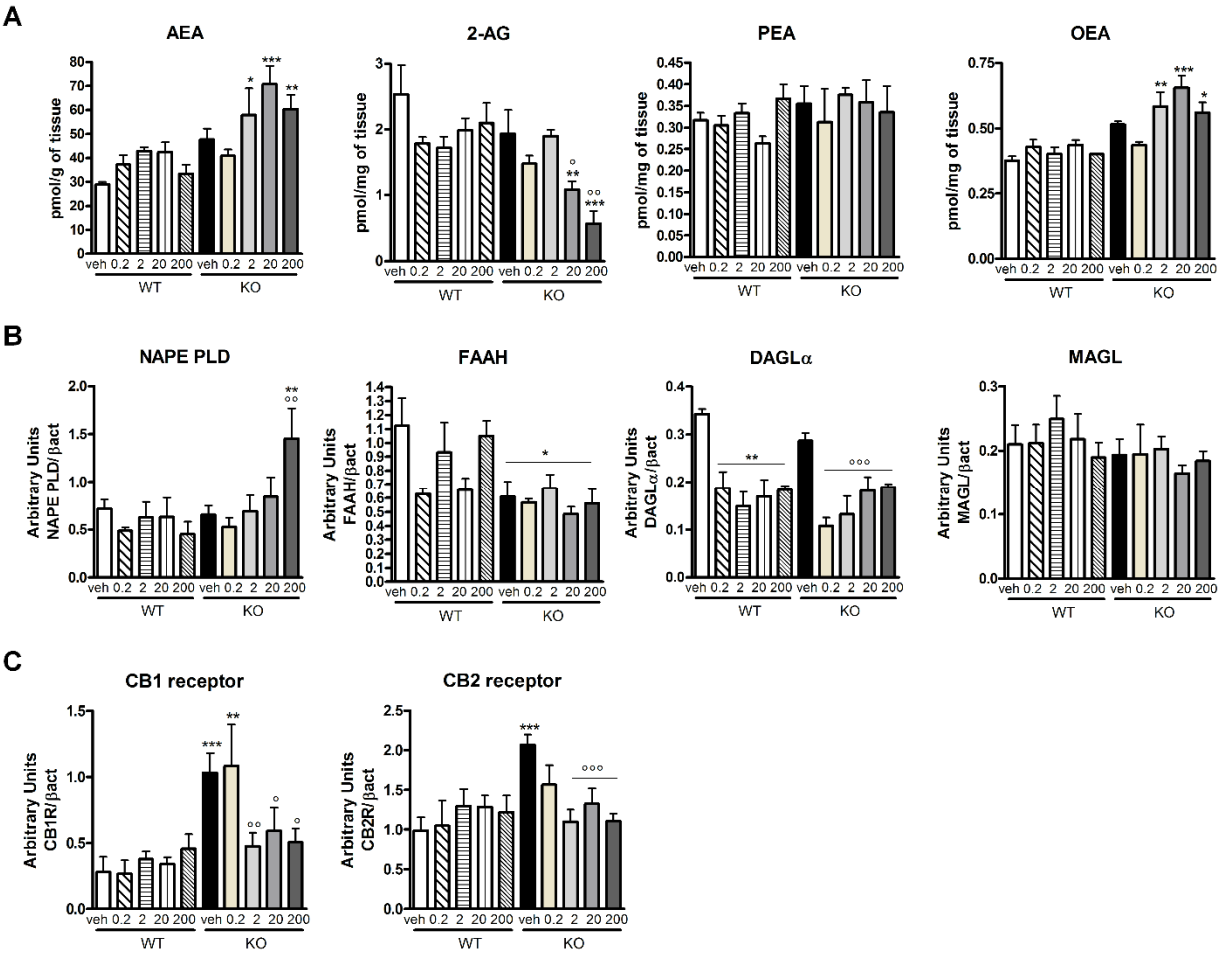

Figure 11. Effect of chronic CBDV (0.2, 2, 20, 200 mg/kg/day, PND 28-67) treatments on (**A**) AEA, 2-AG, PEA and OEA contents, (**B**) protein levels of the main endocannabinoid synthetic and degrading enzymes NAPE-PLD, DAGLα, FAAH and MAGL and (**C**) CB1 and CB2 receptors in hemisected brains of WT and KO Mecp2 mice. Data are expressed as mean ± SEM of 3-6 animals per group. *p<0.05, **p<0.01, ***p<0.001 vs WT-vehicle; °p<0.05, °°p<0.01; °°°p<0.001 vs KO-vehicle. Two-way ANOVA followed by Bonferroni post-hoc test.

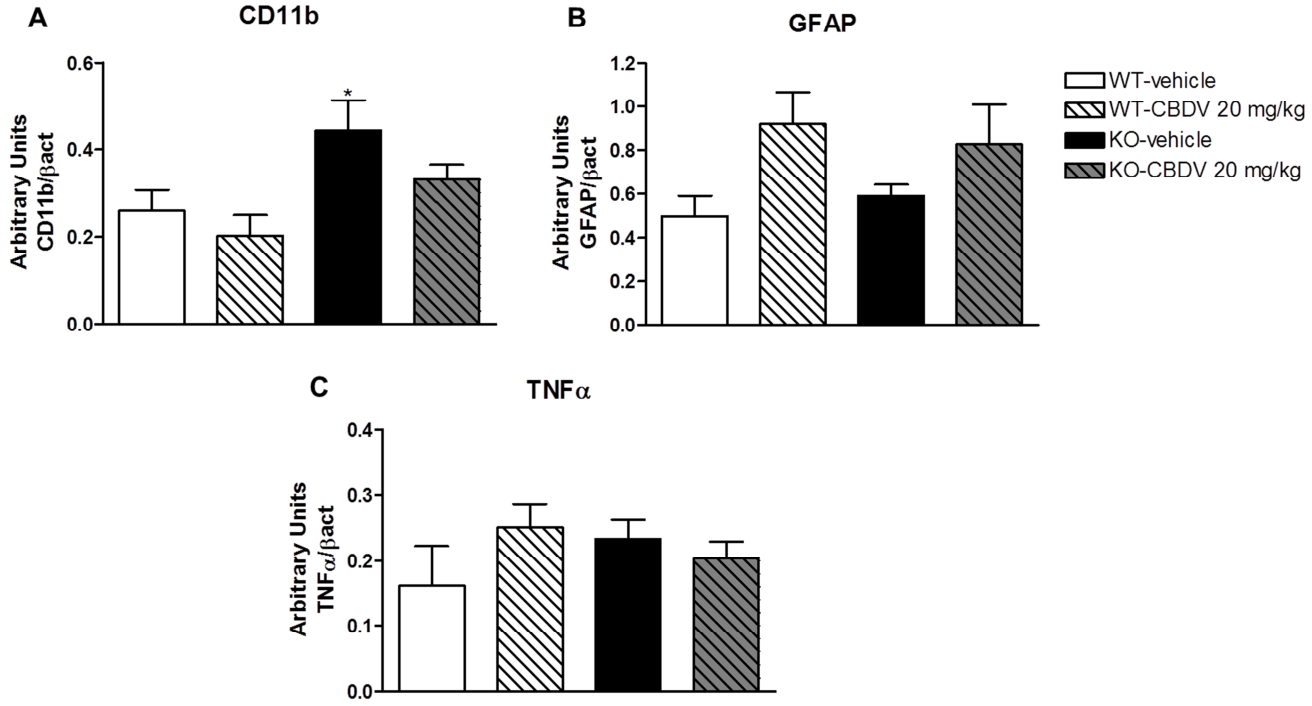

Figure 12. Effect of chronic CBDV (20 mg/kg/day; PND 28-67) treatment on protein levels of (**A**) CD11b, (**B**) GFAP and (**C**) TNFα in hemisected brains of WT and KO Mecp2 mice. Data are expressed as mean ± SEM of 4 animals per group. *p<0.05 vs WT-vehicle. Two-way ANOVA followed by Bonferroni post-hoc test.

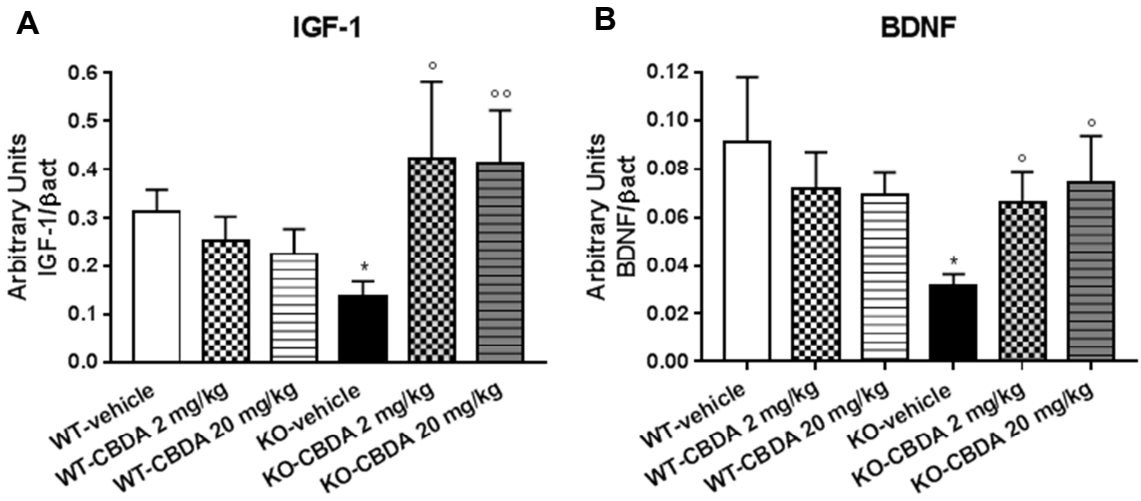

Figure 13. Effect of chronic CBDA (2, 20 mg/kg/day, PND 28-67) treatments on protein levels of (**A**) Insulin-Like Growth Factor-1 (IGF-1), (**B**) Brain-Derived Neurotrophic Factor (BDNF) in hemisected brains of WT and KO Mecp2 mice. Measurements were carried out on total protein lysates from hemisected brains. Data are expressed as mean ± SEM of 4 animals per group. *p<0.05 vs WT-vehicle; °p<0.05, °°p<0.01 vs KO-vehicle. Two-way ANOVA followed by Bonferroni post-hoc test.

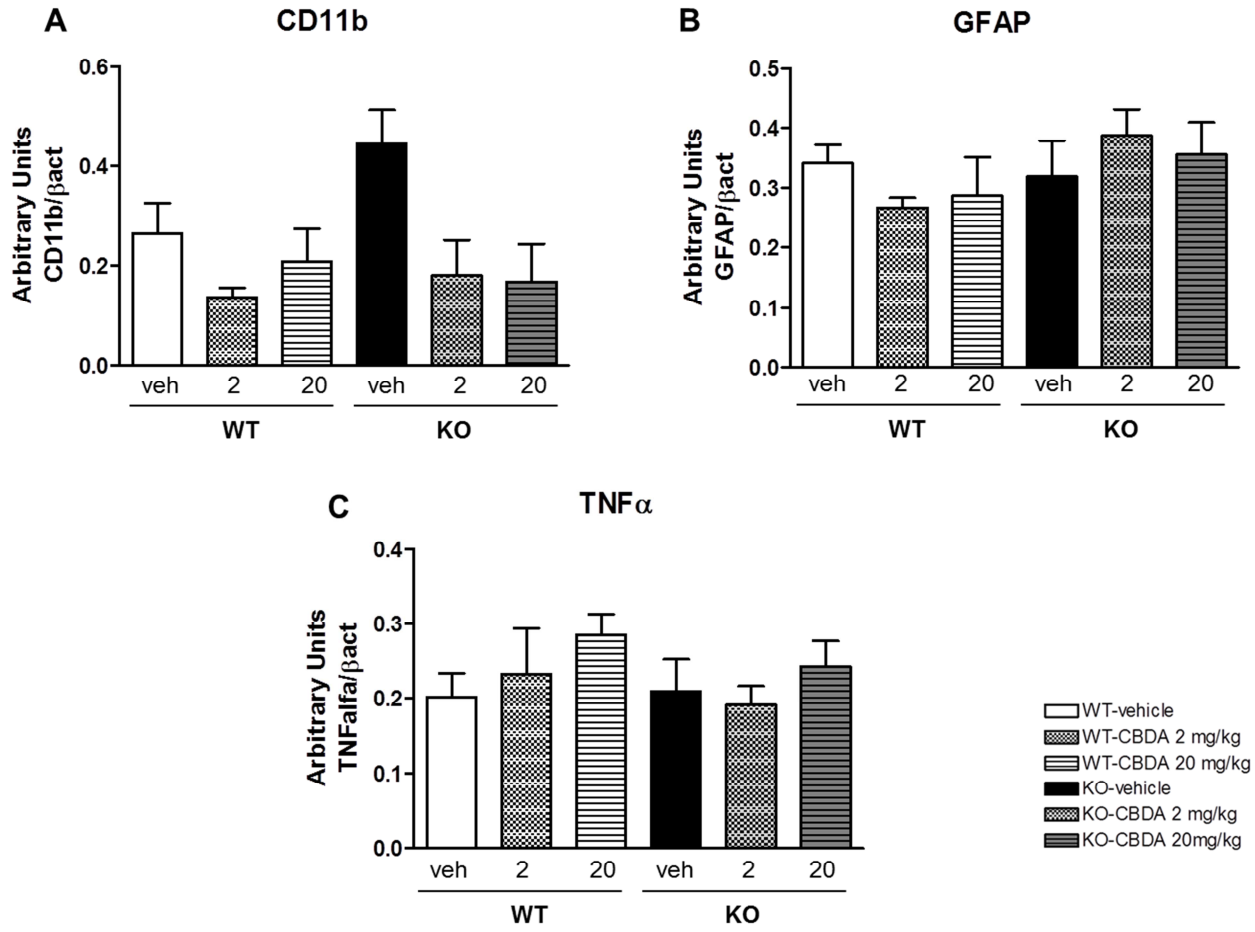

Figure 14. Effect of chronic CBDA (2, 20 mg/kg/day, PND 28-67) treatments on protein levels of (**A**) CD11b, (**B**) GFAP and (**C**) TNFα in hemisected brains of WT and KO Mecp2 mice. Data are expressed as mean ± SEM of 3-4 animals per group.

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- P. Prini, F. Rusconi, E. Zamberletti, M. Gabaglio, F. Penna, M. Fasano, E. Battaglioli, D. Parolaro, T. Rubino
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