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ORIGINAL ARTICLE

Analysis of endocannabinoid signaling elements and related proteins in lymphocytes of patients with Dravet syndrome

Marta Rubio[1,2,3,a], Sara Valdeolivas[1,2,3,a], Fabiana Piscitelli[4], Roberta Verde[4], Valentina Satta[1,2,3], Eva Barroso[5,6], Marisol Montolio[7,8], Luis Miguel Aras[7,9], Vincenzo Di Marzo[4], Onintza Sagredo[1,2,3,b] & Javier Fernández-Ruiz[1,2,3,b]

[1]Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Instituto Universitario de Investigación en Neuroquímica, Universidad Complutense, Madrid, Spain
[2]Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain
[3]Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain
[4]Endocannabinoid Research Group, Institute of Biomolecular Chemistry, Consiglio Nazionale delle Ricerche, Pozzuoli, Naples, Italy
[5]Instituto de Genética Médica y Molecular (INGEMM), Hospital Universitario La Paz, Universidad Autónoma de Madrid, IdiPAZ, Madrid, Spain
[6]Centro de Investigación Biomédica en Red sobre Enfermedades Raras (CIBERER), Instituto de Salud Carlos III, Madrid, Spain
[7]Dravet Syndrome Foundation, Madrid, Spain
[8]Departamento de Biología Celular, Facultad de Biología, Universidad de Barcelona, Barcelona, Spain
[9]Servicio Navarro de Salud, Osasunbidea, Estella, Spain

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Correspondence
Javier Fernández-Ruiz or Onintza Sagredo, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, Madrid 28040, Spain.
Tel: 34913941450 or 34913941454; Fax: 34913941691; E-mail: jjfr@med.ucm.es or onintza@med.ucm.es

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[a]Both authors share the first authorship.

[b]Both authors share the senior authorship and conducted the statistical assessment of the data.

Abstract

Cannabidiol (CBD) reduces seizures in childhood epilepsy syndromes including Dravet syndrome (DS). A formulation of CBD has obtained orphan drug designation for these syndromes and clinical trials are currently underway. The mechanism responsible for CBD effects is not known, although it could involve targets sensitive to CBD in other neurological disorders. We believe of interest to investigate whether these potential targets are altered in DS, in particular whether the endocannabinoid system is dysregulated. To this end, lymphocytes from patients and controls were used for analysis of gene expression of transmitter receptors and transporters, ion channels, and enzymes associated with CBD effects, as well as endocannabinoid genes. Plasma endocannabinoid levels were also analyzed. There were no differences between DS patients and controls in most of the CBD targets analyzed, except an increase in the voltage-dependent calcium channel α-1h subunit. We also found that cannabinoid type-2 (CB_2) receptor gene expression was elevated in DS patients, with no changes in other endocannabinoid-related receptors and enzymes, as well as in plasma levels of endocannabinoids. Such elevation was paralleled by an increase in CD70, a marker of lymphocyte activation, and certain trends in inflammation-related proteins (e.g., peroxisome proliferator-activated receptor-γ receptors, cytokines). In conclusion, together with changes in the voltage-dependent calcium channel α-1h subunit, we found an upregulation of CB_2 receptors, associated with an activation of lymphocytes and changes in inflammation-related genes, in DS patients. Such changes were also reported in inflammatory disorders and may indirectly support the occurrence of a potential dysregulation of the endocannabinoid system in the brain.

Abbreviations

2-AG, arachidonoylglycerol; 5HT1A, 5-hydroxytriptamine 1A receptor; 5HTT, serotonin transporter; A2A, adenosine 2A receptor; CBD, cannabidiol; CNS, central nervous system; COX-2, cyclooxygenase-2; DAGL, diacylglycerol lipase; DAT,

dopamine transporter; DS, Dravet syndrome; GABAT, GABA transporter; GLAST, glutamate-aspartate transporter; GLT-1, glutamate transporter type-1; GPR18, G protein-coupled receptor 18; GPR55, G protein-coupled receptor 55; GUSB, glucuronidase-B; IL-1, interleukin-1; Keap-1, Kelch-like ECH-associated protein 1; LC-APCI, liquid chromatography/atmospheric pressure chemical ionization; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-arachidonoyl-phosphatidylethanolamine-phospholipase D; NFκB, nuclear factor of κ-light polypeptide gene enhancer in B-cells; NOS, nitric oxide synthase; Nrf-2, nuclear factor, erythroid 2-like 2; OEA, oleylethanolamide; PEA, palmitoylethanolamide; PPAR, peroxisome proliferator-activated receptor; TNF, tumor necrosis factor; TRPA1, transient receptor potential ankyrin type-1; TRPV1, transient receptor potential vanilloid type-1; TRPV2, transient receptor potential vanilloid type-2.

Introduction

Dravet syndrome (DS), also known as severe myoclonic epilepsy of infancy, was described in 1978 by Charlotte Dravet (Dravet 1978). DS is a rare genetic pediatric epilepsy whose incidence is estimated in 1:20,000 subjects and it occurs more often in males than in females (2:1) (Hurst 1990). DS typically presents around 5–8 months of age with febrile seizures that progress to severe partial or generalized tonic–clonic seizures, myoclonic seizures, and episodes of status epilepticus. It is often accompanied by cognitive impairment, behavioral disturbances with hyperactivity, and sometimes autistic traits (Guerrini 2012). In approximately 60–80% of patients, DS is caused by a mutation in *SCN1A*, the gene encoding the α-subunit of the voltage-gated sodium channel NaV1.1 whose function contributes to the rising phase of the action potential (Bender et al. 2012). The use of mouse models of DS has improved our understanding of those mechanisms by which mutations in *SCN1A* gene may lead to seizure activity and cognitive dysfunction (Cheah et al. 2012). Loss of function in Nav1.1 causes impaired firing of GABAergic interneurons which results in an imbalance between excitation and inhibition, leading to seizures (Cheah et al. 2012), although a direct alteration in excitatory pyramidal neurons has been also proposed (Mistry et al. 2014). In both cases, these functional deficits likely alter the normal function of these neural networks known to be critical to cognitive functions (Bender et al. 2012). Despite these advances, DS pathogenesis remains poorly understood and only one drug, stiripentol, has been approved specifically for the treatment of this syndrome, although not in USA. In general, patients are treated with combinations of classic antiepileptic drugs (Kassaï et al. 2008) and ketogenic diet (Laux and Blackford 2013), but they remain largely pharmacoresistant. Therefore, novel therapies are urgently needed for this epileptic syndrome.

Preliminary data suggest that a promising therapy for DS may be the use of cannabidiol (CBD) (Porter and Jacobson 2013; Devinsky et al. 2014a), which targets indirectly the endocannabinoid system and directly proteins outside this signaling system (Fernández-Ruiz et al. 2013). CBD had been already investigated for its anticonvulsant properties in seizure models of adult epilepsy (Hill et al. 2012), but the first evidence of its potential for infantile epileptic syndromes was collected in 2013, when a number of US families having children affected by DS or related epileptic syndromes used CBD-enriched cannabis to attenuate the frequency and intensity of epileptic episodes. A follow-up survey collected their experience indicating a >80% reduction in seizure frequency in approximately 42% of children treated with CBD-enriched cannabis (Porter and Jacobson 2013), although the preparation also included certain quantities of Δ^9-tetrahydrocannabinol which has dual effects on epileptic seizures (Hill et al. 2012). Despite the anecdotal nature of these noncontrolled data, and that the issue had not been investigated in DS mouse models yet, an oral formulation of CBD developed by the British company GW Pharmaceuticals received Orphan Drug Designations by the US Food and Drug Agency and by the European Medical Agency for the treatment of childhood epilepsy syndromes. Then, clinical trials were initiated in the US and Europe (www.gwpharma.com/Epidiolex.aspx), and preliminary data indicate that a relevant number of patients achieved seizure disappearance or experienced a notable reduction in their seizure frequency after the treatment with CBD, as has been recently disclosed in the American Epilepsy Society 2014 meeting (Devinsky et al. 2014b).

The most intriguing aspect of these clinical studies is that they were initiated with no previous preclinical or clinical evidence of the potential of CBD for DS and related syndromes, other than its well-known anticonvulsant properties studied in preclinical models of adult epilepsy (Hill et al. 2012) and also in an old clinical trial that included treatment-resistant patients (Cunha et al. 1980). In this context, our present study attempted as a first objective to investigate the changes occurring in DS in different

pharmacological targets that have been directly or indirectly related to numerous therapeutic effects of CBD seen in other neurological disorders (Fernández-Ruiz et al. 2013), so that we can collect any evidence that relates changes in a specific target to the expected benefits of CBD in DS. These targets included certain transmitter receptors and transporters, as well as different enzymes and ion channels, which are not related to the endocannabinoid system but have the additional interest of having been related to epileptic states too (Hill et al. 2012; Fernández-Ruiz et al. 2013). This is in relation to recent evidence showing that CBD may be active at micromolar concentrations on Na_V (Hill et al. 2014; Patel et al. 2014) and transient receptor potential vanilloid type-1 (TRPV1) (Iannotti et al. 2014) channels, although it is not clear whether this activity underlies the anticonvulsant properties of CBD. We also included genes encoding for a few elements within the endocannabinoid signaling system, despite CBD being an atypical cannabinoid with poor activity in relation to classic cannabinoid type-1 (CB_1) and type-2 (CB_2) receptors (Fernández-Ruiz et al. 2013). Nevertheless, we wanted, as a second objective, to investigate how DS affects the endocannabinoid signaling system. This may be an important goal given the increasing evidence supporting a role of this signaling system in epilepsy (Hill et al. 2012), as well as in other neurological disorders in which a dysregulated endocannabinoid signaling has been related to specific events within disease pathogenesis and/or to an adaptive response derived from disease progression (Massa et al. 2010; Álvaro-Bartolomé and García-Sevilla 2013), in both cases having an influence on potential cannabinoid-based therapies.

Our study was carried out in control and DS patient lymphocytes. These immune cells are frequently used in different neurological disorders to recapitulate changes in specific markers that may also occur in the central nervous system (CNS) (Arosio et al. 2014). However, the changes found in these cells may be also interpreted as a response derived from the influence of the disease on the own immune system, in particular, from a repetitive seizure activity that is expected, for example, to generate inflammatory responses. Lymphocytes were used to analyze gene expression for: (i) ion channels, for example, voltage-dependent calcium channel α-1h subunit (CACNA1h); (ii) transmitter receptors, for example serotonin-1A receptor (5HT1A), adenosine 2A receptor (A2A); and (iii) transporters for glutamate (e.g., glutamate transporter type-1 [GLT-1], glutamate–aspartate transporter [GLAST]), GABA (e.g., GABA transporter [GABAT]), dopamine (e.g., Dopamine transporter [DAT]), serotonin (e.g., 5HTT), and adenosine (e.g., equilibrative nucleoside transporter). In most of the cases, these proteins were selected because they have been shown to be related to CBD action (Fernández-Ruiz et al. 2013). Lymphocytes were also used to analyze gene expression for different endocannabinoid-related elements including receptors (e.g., CB_1 and CB_2 receptors, G protein-coupled receptor 55 [GPR55], G protein-coupled receptor 18 [GPR18], TRPV1, transient receptor potential vanilloid type-2 [TRPV2], transient receptor potential ankyrin type-1 [TRPA1]) and enzymes (e.g., *N*-arachidonoyl-phosphatidylethanolamine-phospholipase D [NAPE-PLD], fatty acid amide hydrolase [FAAH], diacylglycerol lipase [DAGL], and monoacylglycerol lipase [MAGL]), as well as for some proteins downstream the endocannabinoid signaling (e.g., NFκB, Akt, nuclear factor, erythroid 2-like 2 [Nrf-2], Keap-1, β-arrestin-1, β-arrestin-2) and also related to inflammation (e.g., peroxisome proliferator-activated receptor-γ [PPAR-γ], tumor necrosis factor-α [TNF-α], interleukin-1β [IL-1β], inducible NOS, cyclooxygenase-2 [COX-2], CD70). In addition, we have analyzed the levels of endocannabinoids and related lipid derivatives in the plasma of DS patients. We assume that changes in gene expression do not necessarily reflect subsequent changes in protein levels, and this represents a potential limitation of our study. However, the isolation of RNA samples for qRT-PCR analysis was the best and only way to analyze the maximal number of potential disease-related markers in blood lymphocytes, whose collection is necessarily limited in children due to reasons of availability and ethical concerns.

Materials and Methods

Patient recruitment

All studies were conducted in peripheral blood mononuclear cells (approximately 85% are lymphocytes), and plasma samples obtained from DS patients ($n = 15$; recruited by the Dravet Syndrome Foundation, Spain). The relatively low number of patients accounts for the low incidence of DS, which is a rare disease Moreover, the disease affects infantile ages, which makes more complicated the recruitment due to logistic and ethical reasons. The numbers were also limited because we tried to get a certain homogeneity in the patient cohort, for example, with all individuals bearing confirmed mutations in *SCN1A* gene. They were compared with control subjects ($n = 10$), who were non-DS individuals including some nonaffected relatives who do not bear the mutation and have not undergone clinical diagnosis of DS. They were matched by age and gender to DS patients. The study was approved by the "Comité Etico de Investigación Clínica," IRYCIS, Madrid, Spain (code 389-13), and, in all cases, parents/legal representatives approved and signed an informed consent that discloses all ethical aspects related to this study. The major characteristics

(age at sampling, gender, age at diagnosis, and some clinical information) of patients and control subjects are shown in Table 1.

Biological sampling

Blood samples from DS patients or control subjects were collected in BD Vacutainer Cell Preparation Tubes containing sodium citrate (BD, Franklin Lakes, NJ). Tubes were inverted 8–10 times and centrifuged (1800 *g*) at room temperature for 25 min. Times between blood collection and centrifugation were always shorter than 30 min. After centrifugation, the plasma was collected and stored at −80°C for analysis of endocannabinoids and related *N*-acylethanolamines. The layer containing the lymphocytes was also collected and centrifuged again (300 *g*) at room temperature for 10 min. After this second centrifugation, the supernatant was removed and discarded, and the cell pellet stored at −80°C for analysis of gene expression.

Real-time qRT-PCR analysis

Total RNA was extracted from isolated lymphocytes using SurePrep™ RNA/Protein Purification kit (Fisher BioReagents, Fair Lawn, NJ). The total amount of RNA extracted was quantitated by spectrometry at 260 nm and its purity was evaluated by the ratio between the absorbance values at 260 and 280 nm, whereas its integrity was confirmed in agarose gels. To prevent genomic DNA contamination, DNA was removed and single-stranded complementary DNA was synthesized from 0.2 μg of total RNA using a commercial kit (Rneasy Mini Quantitect Reverse Transcription, Qiagen, Izasa, Madrid, Spain). The reaction mixture was kept frozen at −20°C until enzymatic amplification. Quantitative real-time PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) to quantify mRNA levels for those genes of interest (see details in Table 2; the nomenclature of all investigated targets including receptors, ion channels, transporters, etc., conforms to BJP's Guide to Receptors and Channels [Alexander et al. 2011]) using glucuronidase-B (GUSB) expression as an endogenous control gene for normalization (the expression of this gene was similar in DS cases and controls and it was maintained relatively constant within the two groups: CT values in controls: 27.30 ± 0.46 [10]; in DS: 27.29 ± 0.33 [15]). The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems) and the threshold cycle (Ct) was calculated by the instrument's software (7300 Fast System, Applied Biosystems). Values were expressed as fold change over the control group and were presented as mean ± SEM.

Analysis of endocannabinoid levels

Plasma samples were dounce-homogenized and extracted in 5 vol of chloroform/methanol/Tris–HCl 50 mmol/L (2:1:1) containing 5 pmol of $[^2H]_8$ anandamide and 10 pmol of $[^2H]_4$ palmitoylethanolamide (PEA), $[^2H]_2$ oleylethanolamide (OEA) and $[^2H]_5$ 2-arachidonoylglycerol (2-AG) (Cayman Chemicals, Ann Arbor, MI). The lipid-containing organic phase was dried down in a rotating evaporator, weighed, and prepurified by open-bed chromatography on silica gel. Lyophilized extracts were resuspended in chloroform/methanol 99:1 by vol. The solutions were then purified by open-bed chromatography on silica as described (Bisogno et al. 1997). Fractions eluted with chloroform/methanol 9:1 by vol. (containing anandamide, 2-AG, OEA, and PEA) were collected and the excess solvent evaporated with a rotating evaporator, and aliquots analyzed by isotope dilution-liquid chromatography/atmospheric pressure chemical ionisation/mass spectrometry (LC-APCI–MS) carried out under conditions described previously (Marsicano et al. 2002) and allowing the separations of 2-AG, anandamide, OEA, and

Table 1. Major characteristics of patients and control subjects included in this study.

Group	Age at sampling in years (lowest and highest ages)	Gender (M/F)	Major clinical characteristics
Control subjects (n = 10)	14.1 ± 1.8 (9–29)	6/4	Not relevant
Dravet patients (n = 15)	11.2 ± 1.7 (2–26)	9/6	• Age at onset of disease: <12 months • Frequent seizures triggered by fever, light, and/or intense emotions • Frequent motor, speech, and cognitive deficits • Medication: some antiepileptic agents (in most of the cases valproate and/or clobazam, and stiripentol in a few cases), but not taking CBD • Frequent pharmacoresistance

CBD, cannabidiol.

Table 2. List of genes analyzed and Taqman probes used in qRT-PCR determinations.

Gene encoding for	Taqman probe reference
Glutamate-aspartate transporter (GLAST)	Hs00188193_mL
Glutamate transporter type-1 (GLT-1)	Hs01102423_mL
GABA transporter (GABAT)	Hs01104475_mL
Serotonin transporter (5HTT)	Hs00984349_mL
Dopamine transporter (DAT)	Hs00997364_mL
Equilibrative nucleoside transporter (ENT)	Hs01085704_gL
Adenosine 2A receptor (A2A)	Hs00169123_mL
5-hydroxytriptamine 1A receptor (5HT1A)	Hs00265014_sL
Voltage-dependent calcium channel α-1h subunit (CACNA1h)	Hs00234934_mL
Cannabinoid receptor type-1 (CB_1)	Hs01038522_sL
Cannabinoid receptor type-2 (CB_2)	Hs00275635_mL
G protein-coupled receptor 55 (GPR55)	Hs00995276_mL
G protein-coupled receptor 18 (GPR18)	Hs01649814_mL
Transient receptor potential vanilloid type-1 (TRPV1)	Hs00218912_mL
Transient receptor potential vanilloid type-2 (TRPV2)	Hs00901640_mL
Transient receptor potential ankyrin type-1 (TRPA1)	Hs00175798_mL
Peroxisome proliferator-activated receptor-γ (PPAR-γ)	Hs00234592_mL
Tumor necrosis factor-α (TNF-α)	Hs99999043_mL
Interleukin-1β (IL-1β)	Hs99999029_mL
Cluster of differentiation 70 (CD70)	Hs00174297_mL
Cyclooxygenase-2 (COX-2)	Hs00153133_mL
Inducible nitric oxide synthase (iNOS)	Hs01075529_mL
Nuclear factor of κ-light polypeptide gene enhancer in B-cells (NFκB)	Hs00765730_mL
Nuclear factor, erythroid 2-like 2 (Nrf-2)	Hs00975691_gL
Kelch-like ECH-associated protein 1 (Keap-1)	Hs00202227_mL
v-Akt murine thymoma viral oncogene homolog 1 (Akt)	Hs00178289_mL
β-arrestin type-1 (ARR1)	Hs00244527_mL
β-arrestin type-2 (ARR2)	Hs01034132_mL
Cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1)	Hs02382916_sL
N-arachidonoyl-phosphatidylethanolamine-phospholipase D (NAPE-PLD)	Hs00419593_mL
Fatty acid amide hydrolase (FAAH)	Hs01038660_mL
Diacylglycerol lipase (DAGL)	Hs00391374_mL
Monoacylglycerol lipase (MAGL)	Hs00200752_mL
Glucuronidase-B (GUSB)	Hs00939627_mL

PEA. MS detection was carried out in the selected ion monitoring mode using *m/z* values of 356 and 348 (molecular ion +1 for deuterated and undeuterated anandamide), 384.35 and 379.35 (molecular ion +1 for deuterated and undeuterated 2-AG), 304 and 300 (molecular ion +1 for deuterated and undeuterated PEA), and 328 and 326 (molecular ion +1 for deuterated and undeuterated OEA). The amounts of all endocannabinoids were expressed as pmol/mL of plasma.

Statistics

Data were assessed by analysis of variance followed by the Bonferroni multiple comparison using series of related genes (e.g., CBD targets, endocannabinoid genes, inflammation-related markers, downstream signals).

Results

Characteristics of the cohort of DS patients investigated

All patients recruited for this study ($n = 15$) had been diagnosed of DS with confirmed mutations in the *SCN1A* gene, and, in all cases, the disease appeared during the first 12 months of life. Subject ages ranged from 2 to 26 years, with an average of 11.2 years at the time of recruitment (Table 1), a range very similar to the currently active GW Pharma clinical trials with CBD which ranged also from 2 to 26 years with a mean age of 10.8 years (www.gwpharma.com/Epidiolex.aspx). Two-thirds of the subjects were male and one-third were female (Table 1). We did not observe any correlation of data of gene expression or plasma levels with age or gender (data not shown), possibly because the number of patients was not sufficiently higher to conduct such correlation analyses. In the case of age, it should be noted that we included two patients with an age >20 years (also in controls), but they did not show any particular differences in the values of different genes analyzed in comparison with younger patients. The major clinical characteristics of these patients consisted of frequent seizures (clonic, tonic–clonic, myoclonic) triggered, among other causes, by fever, light and/or intense emotions, as well as significant motor, speech, and cognitive deficits (Table 1). All patients were taking antiepileptic medications, in most of the cases valproate and/or clobazam, and in a few cases stiripentol and other alternatives, but not CBD, although most of the cases exhibited pharmacoresistance which is typical of DS patients (Kassaï et al. 2008). Given the existence of this previous medication, it is possible that changes we found in lymphocyte gene expression may be related to or at least influenced by such medication, although the differences in the combination of medicines among patients are important, which makes this potential influence poorly probable.

Analysis of gene expression in lymphocytes obtained from DS patients

Lymphocytes from DS patients and control subjects were used first for analysis of genes encoding ion channels or transmitter receptors and transporters that have been related to the benefits found with CBD in a number of

neurological disorders (Fernández-Ruiz et al. 2013). The analysis of variance followed by the Bonferroni multiple comparison confirmed the existence of statistically significant differences ($F(13, 154)=2.872$, $P < 0.005$), which were evident in the elevated levels of gene expression for the CACNA1h in DS patients (Fig. 1). No changes were found for A2A receptors, adenosine transporter, glutamate transporters (GLAST and GLT-1), and dopamine transporter (Fig. 1), although a trend toward a reduction ($P = 0.10$) was found for the serotonin transporter in DS patients (Fig. 1). We were unable to measure the genes for the 5HT1A receptor and GABA transporter, because their expression was below the level of sensitivity in the lymphocytes (data not shown).

As regards gene products encoding endocannabinoid-related receptors ($F(9, 124)=2.596$, $P < 0.005$), we found the CB_2 receptor to be significantly elevated in DS patients, with no changes in GPR55, GPR18, TRPV1, and TRPV2 (Fig. 1). We were unable to measure the gene products for the CB_1 and TRPA1 receptors because their expression was below the level of sensitivity (data not shown). Given that the CB_2 receptor has been linked to the control of inflammatory processes, we then analyzed the expression of some inflammation-related genes ($F(11, 149)=3.217$, $P < 0.01$). Those encoding the PPAR-γ receptors (which are also activated by CBD) and the cytokines TNF-α and IL-1β (Fig. 2) showed certain trends toward an increase in DS patients, in particular, in the case of IL-1β ($P = 0.08$), but not in the case of COX-2 (Fig. 2). This was in agreement with the upregulation of CB_2 receptors, as well as with the elevation in gene expression of CD70 (Fig. 2), a marker of lymphocyte activation. We were unable to measure inducible NOS because its expression was below the level of sensitivity (data not shown).

We also analyzed intracellular signals downstream the endocannabinoid signaling, but we did not find any differ-

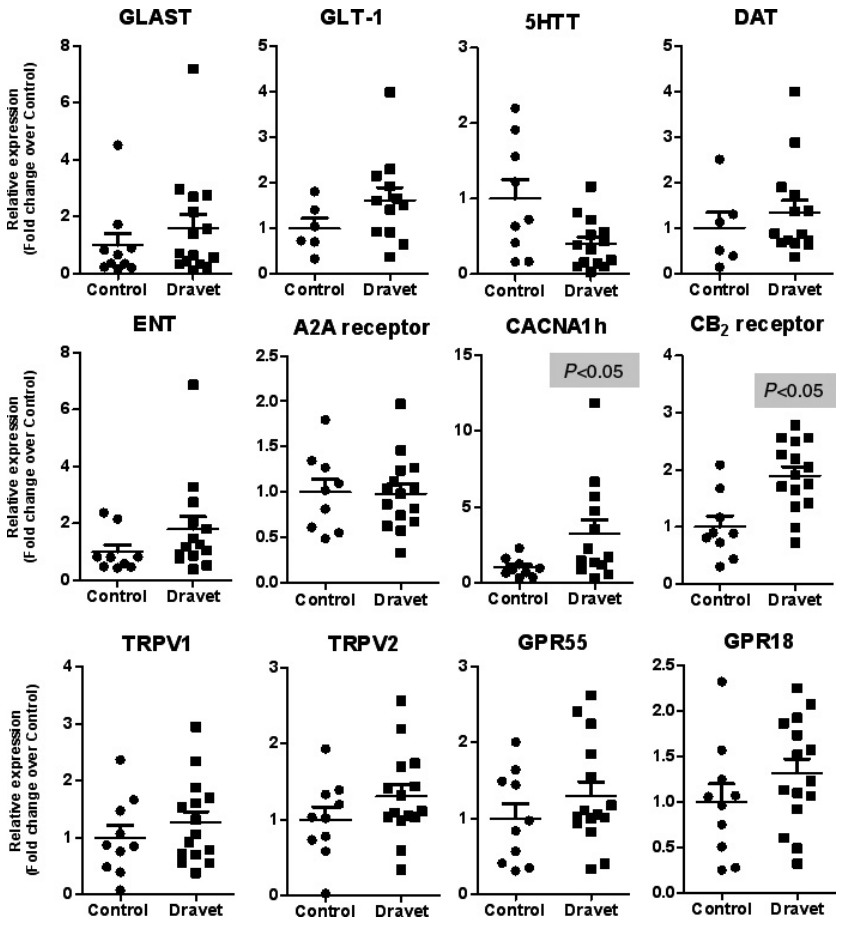

Figure 1. Gene expression for different transmitter transporters (glutamate-aspartate transporter [GLAST], GLT-1, 5HTT, Dopamine transporter (DAT), and ENT) and receptors adenosine 2A receptor (A2A receptor), ion channels (CACNA1h) and endocannabinoid-related receptors (CB_2 receptors, GPR55, GPR18, TRPV1, and TRPV2), measured by qRT-PCR in lymphocytes obtained from Dravet syndrome (DS) and control subjects. Values correspond to fold of change over controls and are expressed as means ± SEM of 15 DS and 10 controls. Data were assessed by the analysis of variance followed by the Bonferroni multiple comparison.

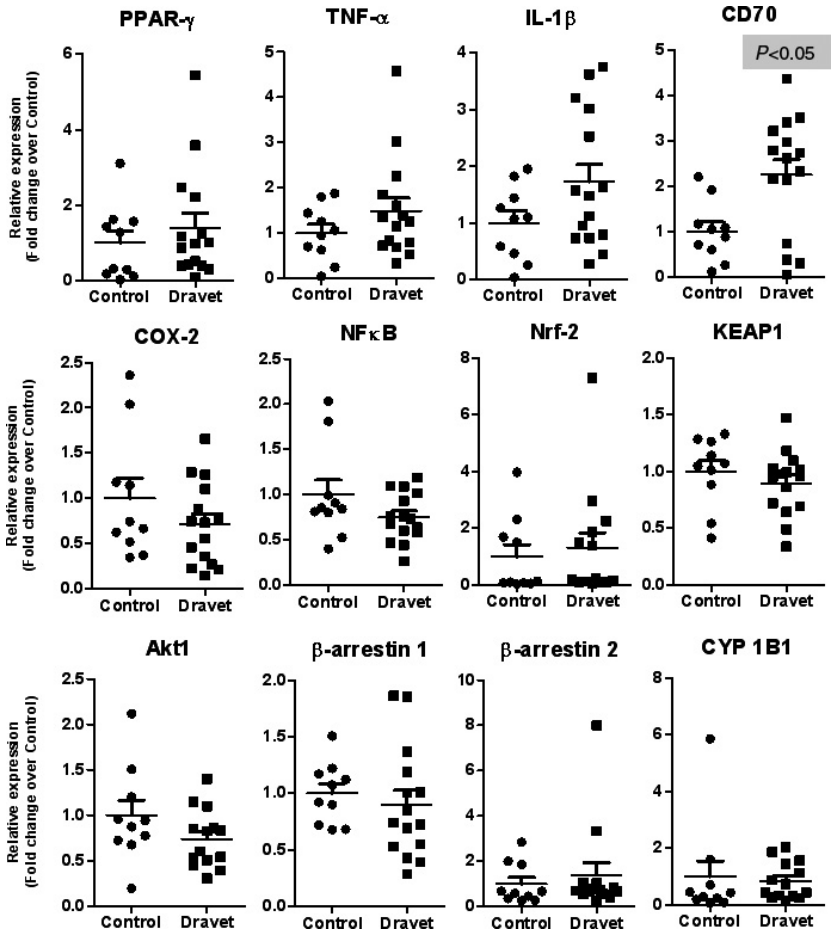

Figure 2. Gene expression for peroxisome proliferator-activated receptor-γ (PPAR-γ) nuclear receptors, the cytokines Tumor necrosis factor-α (TNF-α) and IL-1β, the marker of lymphocyte activation CD70, the proinflammatory enzyme COX-2, different intracellular signaling proteins (NFκB, nuclear factor, erythroid 2-like 2 (Nrf-2), Keap-1, Akt, β-arrestin-1 and -2), and the P450 member CYP1B1, measured by qRT-PCR in lymphocytes obtained from Dravet syndrome (DS) and control subjects. Values correspond to fold of change over controls and are expressed as means ± SEM of 15 DS and 10 controls. Data were assessed by the analysis of variance followed by the Bonferroni multiple comparison.

ence in transcription factors such as NFκB or Nrf-2, its inhibitory protein Keap-1, the kinase Akt, and the receptor regulatory proteins β-arrestin-1 and -2 ($F(11, 148)$=0.4891, ns; see Fig. 2). We analyzed cytochrome P450 1B1 enzyme isoform, which has been found to be inhibited by CBD (Yamaori et al. 2010), although its expression was similar in DS and controls (Fig. 2). Lastly, we analyzed the gene expression of endocannabinoid-related enzymes, for example, NAPE-PLD, DAGL, FAAH, and MAGL, whose level of expression, however, was also similar in DS and control subjects ($F(7, 99) = 0.9307$, ns; see Fig. 3).

Analysis of plasma levels of endocannabinoids and related mediators in DS patients

In order to determine potential changes in the levels of endocannabinoids and related *N*-acylethanolamines, we measured anandamide, 2-AG, PEA, and OEA in the plasma samples from DS patients and control subjects. However, we did not find any changes in these signaling lipid derivatives (Fig. 3) in agreement with the data of gene expression for metabolic enzymes of endocannabinoids and related *N*-acylethanolamines in lymphocytes of DS patients and controls (Fig. 3).

Discussion

Recent anecdotal and clinical experience with pharmacological preparations based on CBD has led to high expectations for having a new antiepileptic agent with efficacy in DS and related syndromes. However, there is no rationale to explain the presumed efficacy of CBD in these pediatric syndromes other than the observations derived from studies conducted in adult epilepsy, which included both preclinical studies carried out in seizure models (Hill et al.

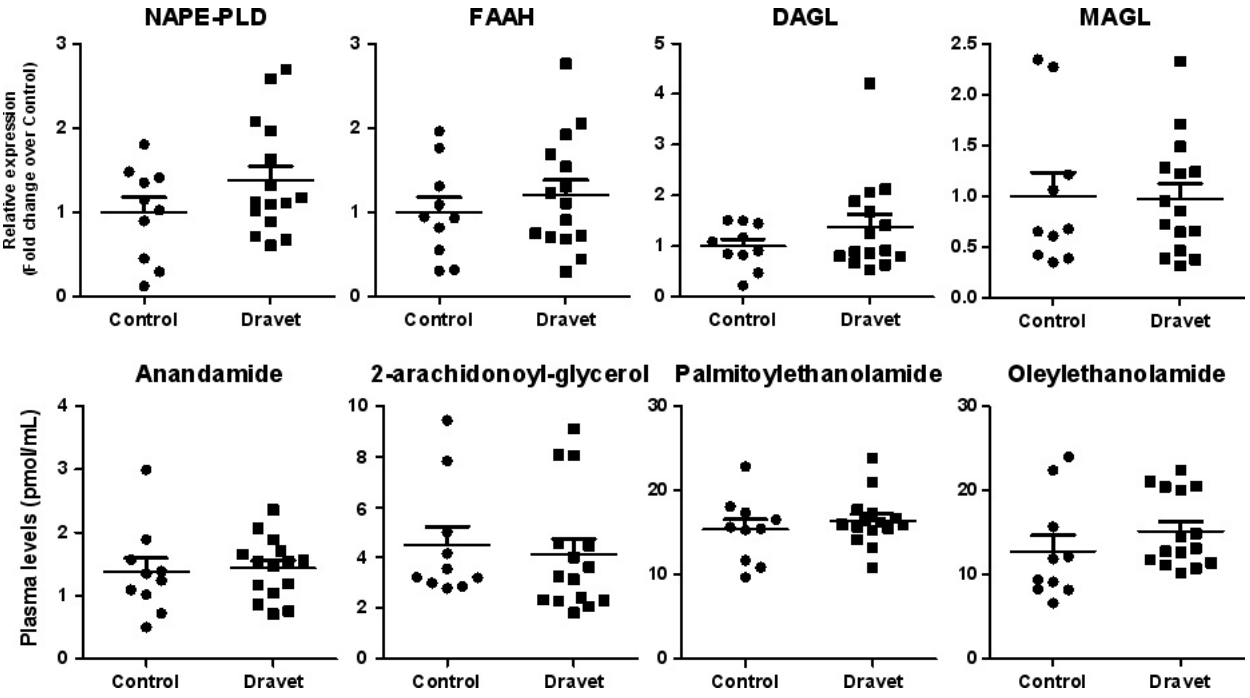

Figure 3. Gene expression for different endocannabinoid-related enzymes, including NAPE-PLD, fatty acid amide hydrolase (FAAH), diacylglycerol lipase (DAGL), and monoacylglycerol lipase [MAGL], measured by qRT-PCR in lymphocytes obtained from Dravet syndrome (DS) and control subjects, and concentrations of anandamide, 2-arachidonoyl-glycerol, palmitoylethanolamide, and oleylethanolamide measured in the corresponding plasma samples from DS and control subjects. Values correspond to fold of change over controls (gene expression data) or pmol/mL of plasma (endocannabinoids), and are expressed as means ± SEM of 15 DS and 10 controls. Data were assessed by the analysis of variance followed by the Bonferroni multiple comparison.

2012) and the unique clinical trial conducted with CBD in adult epileptic patients (Cunha et al. 1980). Because these syndromes have been proved to be refractory to all currently existing antiepileptic medications, there is a need to find new medications and also to understand the specific mechanism(s) involved in their effects. Our study represents a first step toward building the lacking preclinical and clinical understanding of the mechanisms driving CBD activity in these syndromes, together with some recent studies providing incipient evidence on the possible mechanisms underlying CBD effects in DS (Hill et al. 2014; Iannotti et al. 2014; Patel et al. 2014).

Our first objective was to analyze the changes that the disease may produce in some pharmacological targets (e.g., transmitter receptors and transporters, ion channels) related to the benefits obtained with CBD in a number of neurological disorders (Fernández-Ruiz et al. 2013), using analysis of their gene expression in patient and control lymphocytes. The only change we found was an increase in CACNA1h, which has been proposed as a candidate gene for epilepsy (Frankel 2009). In fact, gain-of-function mutations in this gene have been related to infantile forms of epilepsy (Frankel 2009), which is in agreement with our results. Another interesting change affected the serotonin transporter gene, whose expression tended to be lower in DS patients, a result that may be related to some recent evidence indicating that serotonin transporter gene polymorphisms may increase susceptibility to epilepsy (Yang et al. 2013).

Our study also included the analysis of some genes encoding proteins of the so-called endocannabinoid system, although these proteins, except for FAAH, have not been particularly associated with the effects of CBD, which may be considered an atypical cannabinoid in relation to its activity at the classic endocannabinoid receptors and enzymes. In this case, our objective was to determine the changes that the disease may produce in specific elements of the endocannabinoid system, rather than to find potential targets within this system that may explain the antiseizure effects of CBD. We hypothesized the existence of a potential dysregulation of the endocannabinoid signaling system in DS patients, as found in other disorders (Massa et al. 2010; Álvaro-Bartolomé and García-Sevilla 2013), and reflected in impairments in endocannabinoid levels that may be caused by changes in their synthesizing and degrading enzymes, as well as in impairments in the density and/or activity of endocannabinoid receptors. Our major observation was

an elevated gene expression for the CB_2 receptor in DS lymphocytes, although we did not find any change in endocannabinoid levels in the plasma of DS patients, nor any change in the gene expression for the major enzymes involved in the synthesis and degradation of endocannabinoids. In the brain, the CB_2 receptor has been strongly linked, among others, with the regulation of glial activation and associated inflammatory events, which may support the idea that inflammation could have an influence in DS, although this possibility has not been fully demonstrated in previous studies (Catarino et al. 2011; Xu et al. 2013). Elevated CB_2 receptor gene expression in lymphocytes has been also found in other neurological disorders such as multiple sclerosis (Sánchez López et al. 2015) and autism (Siniscalco et al. 2013), although the issue has not been investigated, to the best of our knowledge, in epilepsy. It is interesting to note that in the case of multiple sclerosis, the elevation found in lymphocytes (Sánchez López et al. 2015) recapitulated a similar response measured in CNS structures (Fernández-Ruiz et al. 2007) and was reversed by progressive interferon therapy (Sánchez López et al. 2015). This allows lymphocyte CB_2 expression to be used as a peripheral biomarker for monitoring treatment efficacy, something that could be of interest also for DS.

In addition to the elevation of CB_2 receptor gene expression, we found that other inflammation-related markers, for example, PPAR-γ receptors and proinflammatory cytokines, also showed certain trends toward an increase, although they did not reach statistical significance. These trends were due to subsets of patients having significantly higher expression levels than the mean of the control group. We examined whether these subsets of patients exhibited some particular characteristics in relation to their clinical data (e.g., age, gender, seizure frequency, type of medication), but we did not find any positive correlation, possibly also due to the relatively less number of subjects employed in this study. An interesting observation was that these responses were paralleled by an increase in gene expression for CD70 (the Ki24 antigen), a surface receptor which serves as a marker for lymphocyte proliferation and activation (Borst et al. 2005; Shipkova and Wieland 2012). Therefore, we may hypothesize that lymphocytes are activated in DS patients, which may presumably cause the elevation of CB_2 receptors. In this respect, it is important to note that our data (elevated CB_2 receptor and CD70 gene expression) may be also interpreted as a mere reflection of specific changes occurring in the immune system and not necessarily occurring in the brain, a fact that may be normal given the frequent seizure activity experienced by these patients which should trigger peripheral inflammation-related responses. Therefore, our data should be taken with caution. However, we expect that a similar proinflammatory response might also be demonstrated in the brain, possibly eliciting an elevation of CB_2 receptors in glial elements in which they are frequently located (Fernández-Ruiz et al. 2007). This question is important in view of the increasing evidence in support of a role of non-neuronal components of the CNS, such as glial elements and infiltrated peripheral cells, in the pathogenesis of epilepsy (Xu et al. 2013). In support of this, an elevation in the immunostaining for CB_2 receptors in microglial cells has been recently described in postmortem brain of epileptogenic developmental pathologies (Zurolo et al. 2010), whereas children having autistic features, which also appear in DS, showed elevated levels of this receptor in lymphocytes (Siniscalco et al. 2013) as in our study. A previous study described increased levels of proinflammatory cytokines in these children (Molloy et al. 2006), as was also observed here. All these data point to the potential role that the activation of CB_2 receptors may have in these disorders, including DS, and this potential would support the possibility to develop cannabinoid-based therapies with more benefits for the treatment of DS than just an antiepileptic activity. For example, they may be potentially effective in delaying/reducing the occurrence of motor, speech, and cognitive deficits, which are also important disabling consequences of this pathology, given their well-known anti-inflammatory and neuroprotective properties largely demonstrated in preclinical models of neurodegenerative disorders (Iuvone et al. 2009; Fernández-Ruiz et al. 2011). All these questions are currently under investigation and will benefit from the availability of mouse models of DS (Oakley et al. 2011).

In summary, our data showed changes in a few potential CBD targets, for example, the serotonin transporter and, in particular, the CACNAC1h, in DS patients. They also proved the existence of an upregulation of CB_2 receptors in lymphocytes, associated with the activation of these cells, in DS patients, similar to the responses found with this receptor in neuroinflammatory disorders. In the case that such response also occurs in the brain and that CB_2 receptors are involved in the physiopathology of DS, this finding would extend the possible cannabinoid-based therapies for this disease, not only for the reduction of seizures, but particularly for the control of inflammation and other responses underlying cognitive and motor deficits. Importantly, our study uncovers a dysregulation of the endocannabinoid system in patients with DS that might shed light on the biology of this drug refractory syndrome.

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Author Contributions

L. M. A., M. M., O. S., and J. F.-R. designed the study. M. R., S. V., V. S., E. B., F. P., and R. V. performed the sample analysis. V. D., O. S., and J. F.-R. were involved in data interpretation (including statistical assessment). J. F.-R. drafted the manuscript that was revised, corrected, and approved by all authors.

Disclosures

All authors declare that they have no conflicts of interest in relation to this work as they have indicated in the corresponding disclosure form. The PI confirms that he has collected all these disclosure forms which are available from the editors on request.

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