SOCIETÀ ITALIANA DI FARMACOLOGIA

MODELLO PER INVIO RELAZIONE DI METÀ E FINE PERIODO

NOME E COGNOME: Valentina Satta

UNIVERSITÀ: Complutense University- Madrid

DIPARTIMENTO (in caso di borsa per soggiorno all'estero specificare l'ente presso cui si è svolta la ricerca): Faculty of Medicine, Department of Biochemistry and Molecular Biology III

TUTOR (in caso di borsa per soggiorno all'estero specificare il tutor dell'ente presso cui si è svolta la ricerca): Profesor Javier Fernandez Ruiz

TIPOLOGIA DI BORSA RICEVUTA: Borse di Studio per progetti di ricerca in ambito farmacologico bandite dalla SIF con l'appoggio incondizionato di MSD Italia.

TIPOLOGIA DI RELAZIONE: Relazione finale

TITOLO DELLA RELAZIONE: Possible dysregulation of the endocannabinoid signaling system in Dravet syndrome

Background

Dravet Syndrome (DS) is a rare genetic epileptic syndrome caused by mutations in the human SCN1A gene encoding the voltage-gated sodium channel subunit Nav1.1, whose function contributes to the rising phase of the action potential in neurons (Bender et al., 2012). DS is characterized by febrile and afebrile, generalized and unilateral, clonic or tonic-clonic seizures that occur in the first year of life. DS is often accompanied by cognitive impairment, behavioral disorders and has higher incidence of sudden unexpected death (Guerrini, 2012). Most of antiepileptic agents are poorly effective, although recent anecdotal data have shown some promise with the phytocannabinoid cannabidiol (CBD), which target the endocannabinoid system (eCBS) but also other elements outside this system. These data are based on the observation by parents of a seizure reduction after the medication of DS children with a cannabis preparation containing a high CBD amount (Porter and Jacobson, 2013). Despite the mere anecdotal value of these observations, CBD formulated as Epidiolex® by the British company GW Pharmaceuticals has received the orphan indication by the FDA (USA), and more recently the European EMA, for clinical studies in DS and related syndromes, and preliminary data obtained from on-going clinical trials have indicated a relatively relevant number of patients being seizure free or with notable reductions after the treatment with Epidiolex®. The molecular mechanism responsible of CBD efficacy in DS is not known, although it could involve some targets that have been found to be sensitive to this compound in other neurological disorders.

Aim

Since we hypothesized that the apparent efficacy of CBD in DS may be due to the normalization of an underlying dysregulation of the endocannabinoid signaling system, the first part of my project concerned to investigate the possible dysregulation of the endocannabinoid signaling in patients affected by DS. In particular, using lymphocytes isolated from blood of DS patients, we analyzed the gene expression of different related elements including receptors (e.g. CB1 and CB2 receptors as well as GPR55, GPR18, TRPV1 and TRPV2 receptors) and enzymes (e.g. FAAH, MAGL, NAPE-PLD, DAGL). We only found a significance up-regulation of the gene expression of CB2 receptors. This up-regulation have been found also in postmortem brain of epileptogenic developmental pathologies (Zurolo et al., 2010) and in other neurological disorders associated with 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 (Xu et al., 2013). For this reason, the second part of the study focused on the analysis of the gene expression of

- proteins related to inflammation such as CD70, a marker of lymphocyte activation, the cytokines TNF-α and IL-1β, inducible NOS and the proinflammatory enzyme COX-2 and the receptor PPAR-γ;
- different transcription factors (e.g. NFκB, Akt, Nrf-2, Keap-1, β- arrestin-1, β-arrestin-2) that may be related to CB2 receptor signaling.

Since the therapeutic effects of CBD seen in other neurological disorders have been related to other elements outside the endocannabinoid system (Fernández-Ruiz et al., 2013), we investigated if changes in the expression of genes coding for these elements are present in patients affected by DS. For this purpose DS patient lymphocytes were used to analyze gene expression for:

- ion channels, e.g. voltage-dependent calcium channel α-1h subunit (CACNA1h);
- transmitter receptors, e.g. serotonin-1A receptor (5HT1A), adenosine 2A receptor (A2A);
- transporters for glutamate (e.g. GLT-1, GLAST), GABA (e.g. GABAT), dopamine (e.g. DAT), serotonin (e.g. 5HTT), and adenosine (e.g. equilibrative nucleoside transporter (ENP)).

Materials and Methods

As previously described in the first report, our study was carried out in control and DS patient lymphocytes (peripheral blood mononuclear cells) isolated from blood cells using a density gradient centrifugation method. Then we analyzed the gene expression of different elements using the quantitative real-time PCR (qPCR-RT). 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 disclosed all ethical aspects related to this study.

Results

Given the elevation found in CB2 receptor gene expression in lymphocytes of DS patients, which was shown in the first report, and the fact that this receptor has been linked to the control of inflammatory processes, we analyzed the expression of some inflammation-related genes (Figure 1). Those encoding the PPAR-γ receptors (which are also activated by CBD) and the cytokines TNF-α and IL-1β showed certain trends towards an increase in DS patients, in particular in the case of IL-1β (p=0.08), but not in the case of COX-2. This was in agreement with the up-regulation of CB2 receptors, as well as with the elevation in gene expression of CD70 (Figure 1), a marker of lymphocyte activation ($p<0.05$). We were unable to measure inducible NOS because its expression was below the level of sensitivity (data not shown).

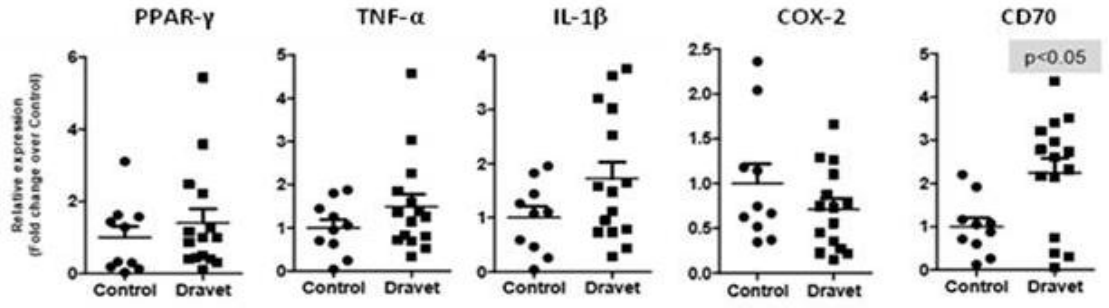
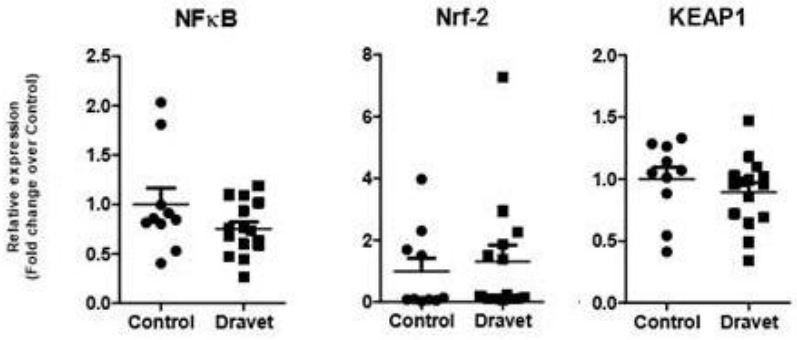


Figure 1: Gene expression for PPAR-γ nuclear receptors, the cytokines TNF-α and IL-1β, the proinflammatory enzyme COX-2 and the marker of lymphocyte activation CD70 measured by qRT-PCR in lymphocytes obtained from DS and control subjects. Values correspond to fold of change over controls and are expressed as means ± SEM of 15 DS and 10 controls. Data were assessed by the analysis of variance followed by the Bonferroni multiple comparison.

We also analyzed different transcription factors and intracellular signals (NFKB, Nrf-2, KEAP1, Akt1, β-Arrestin 1 and β-Arrestin 2) that are also involved in inflammatory responses and directly, or indirectly, related to CB2 receptor signaling, but we did not find any difference between control and Dravet patients (see Figure 2).



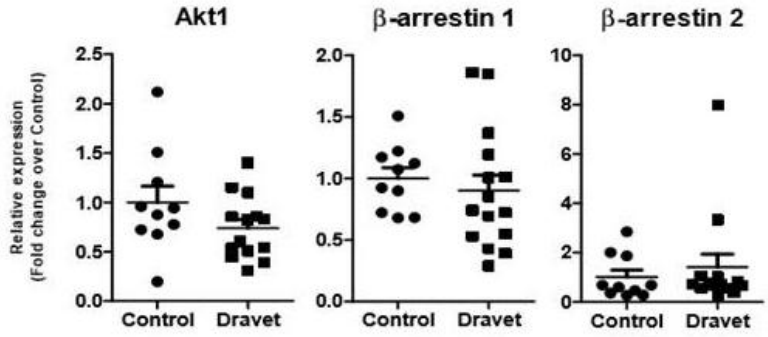


Figure 2: Gene expression for different intracellular signaling proteins (NFκB, Nrf-2, Keap-1, Akt, β-arrestin-1 and -2) measured by qRT-PCR in lymphocytes obtained from DS and control subjects. Values correspond to fold of change over controls and are expressed as means ± SEM of 15 DS and 10 controls. Data were assessed by the analysis of variance followed by the Bonferroni multiple comparison.

Next, we follow with the analysis of some potential targets for CBD all of them outside the endocannabinoid system. As can be shown in Fig. 3, significant difference was found between patients with DS and control subjects in the gene expression of the CACNA1h, confirmed by the analysis of variance followed to the Bonferroni multiple comparison ($F(13,154)=2.872$, $p<0.005$). By contrast, no changes were found in the gene expression of A2A receptors and of adenosine transporter (ENT).

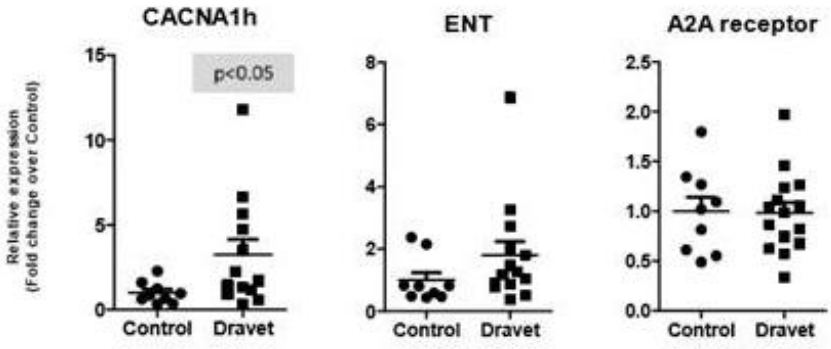


Figure 3: Gene expression for ion channels (CACNA1h), adenosine transmitter transporters (ENT) and receptor (A2A), measured by qRT-PCR in lymphocytes obtained from DS and control subjects. Values correspond to fold of change over controls and are expressed as means ± SEM of 15 DS and 10 controls. Data were assessed by the analysis of variance followed by the Bonferroni multiple comparison.

Moreover, no change was found in the gene expression of glutamate transporters (GLAST and GLT-1), and dopamine transporter (Figure 4), although a trend towards a reduction ($p=0.10$) was found for the serotonin transporter in DS patients (Figure 4). We were unable to measure the genes for the 5HT1A receptor and the GABA transporter, because their expression was below the level of sensitivity in the lymphocytes (data not shown).

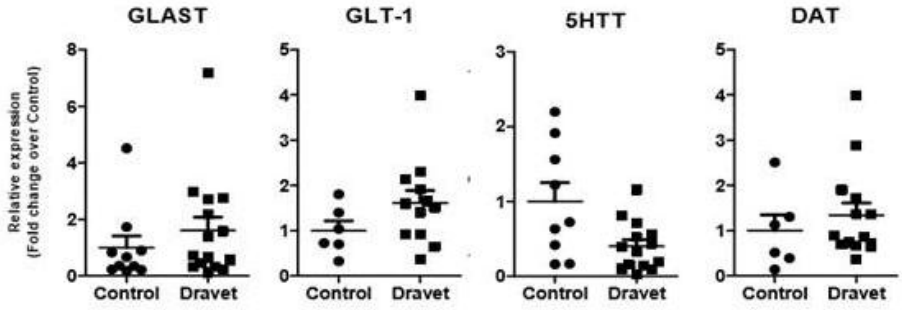


Figure 4: Gene expression for different transmitter transporters (GLAST, GLT-1, 5HTT, DAT) measured by qRT-PCR in lymphocytes obtained from DS and control subjects. Values correspond to fold of change over controls and are expressed as means ± SEM of 15 DS and 10 controls. Data were assessed by the analysis of variance followed by the Bonferroni multiple comparison

It is important to also mention that all data obtained in the lymphocytes of the cohort of DS patients have been recently published (Rubio et al., 2016).

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. Our general 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. Since, in the first part of the project, we found an elevated gene expression for the CB2 receptor in DS lymphocytes and CB2 receptor has been strongly linked, among others, with the regulation of glial activation and associated inflammatory events, we hypothesized that inflammation could have an influence in DS, although this possibility has not been fully demonstrated in previous studies (Xu et al., 2013; Catarino et al., 2011). In addition to the elevation of CB2 receptor gene expression, we found that other inflammation-related markers, e.g. PPAR-γ receptors and proinflammatory cytokines, also showed certain trends towards an increase, although they did not reach statistically significance. These trends were due to subsets of patients having significantly higher expression levels than the mean of the control group. 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 CB2 receptors. We expect that a similar proinflammatory response might also occur in the brain, possibly eliciting an elevation of CB2 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 CB2 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 it was also observed here. All these data point to the potential role that the activation of CB2 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 (Fernández-Ruiz et al., 2011; Iuvone et al., 2009). All these questions are currently under investigation and will benefit from the availability of mouse models of DS (Oakley et al., 2011). In the second part of my project, the most important finding was an increase in the 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).

In conclusion, our data showed changes in a few potential CBD targets, e.g. the serotonin transporter and, in particular, the CACNAC1h, in DS patients. They also proved the existence of an up-regulation of CB2 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 CB2 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.

References

- Bender AC, Morse RP, Scott RC et al. SCN1A mutations in Dravet syndrome: impact of interneuron dysfunction on neural networks and cognitive outcome. Epilepsy Behav 2012; 23: 177-186.

- Guerrini R. Dravet syndrome: The main issues. Eur J Pediat Neurol 2012; 16: S1-S4.

- Porter BE, Jacobson C. Report of a parent survey of cannabidiol-enriched cannabis use in pediatric treatment-resistant epilepsy. Epilepsy Behav 2013; 29: 574-577.

- Zurolo E, Iyer AM, Spliet WG et al. CB1 and CB2 cannabinoid receptor expression during development and in epileptogenic developmental pathologies. Neuroscience 2010; 170: 28-41.

- Xu D, Miller SD, Koh S. Immune mechanisms in epileptogenesis. Front Cell Neurosci 2013; 7: 195 28.

- Fernández-Ruiz J, Sagredo O, Pazos MR et al. Cannabidiol for neurodegenerative disorders: important new clinical applications for this phytocannabinoid? Br J Clin Pharmacol 2013; 75: 323-333.

- Frankel WN. Genetics of complex neurological disease: challenges and opportunities for modeling epilepsy in mice and rats. Trends Genet 2009; 25: 361-367.

- Yang K, Su J, Hu Z et al. Serotonin transporter (5-HTT) gene polymorphisms and susceptibility to epilepsy: a meta-analysis and meta-regression. Genet Test Mol Biomarkers 2013; 17: 890-897 27.

- Catarino CB, Liu JY, Liagkouras I et al. Dravet syndrome as epileptic encephalopathy: evidence from long-term course and neuropathology. Brain 2011; 134: 2982-3010.

- Borst J, Hendriks J, Xiao Y. CD27 and CD70 in T cell and B cell activation. Curr Opin Immunol 2005; 17: 275-281.

- Shipkova M, Wieland E. Surface markers of lymphocyte activation and markers of cell proliferation. Clin Chim Acta 2012; 413: 1338-1349.

- Fernández-Ruiz J, Romero J, Velasco G et al. Cannabinoid CB2 receptor: a new target for controlling neural cell survival? Trends Pharmacol Sci 2007; 28: 39-45.

- Siniscalco D, Sapone A, Giordano C et al. Cannabinoid receptor type 2, but not type 1, is up-regulated in peripheral blood mononuclear cells of children affected by autistic disorders. J Autism Dev Disord 2013; 43: 2686-2695.

- Molloy CA, Morrow AL, Meinzen-Derr J et al. Elevated cytokine levels in children with autism spectrum disorder. J Neuroimmunol 2006; 172: 198-205.

- Fernández-Ruiz J, Moreno-Martet M, Rodríguez-Cueto C et al. Prospects for cannabinoid therapies in basal ganglia disorders. Br J Pharmacol 2011; 163: 1365-1378.

- Iuvone T, Esposito G, De Filippis D et al. Cannabidiol: a promising drug for neurodegenerative disorders? CNS Neurosci Ther 2009; 15: 65-75 38.

- Oakley JC, Kalume F, Catterall WA. Insights into pathophysiology and therapy from a mouse model of Dravet syndrome. Epilepsia 2011; 52: 59-61.

- Rubio M, Valdeolivas S, Piscitelli F et al. Analysis of endocannabinoid signaling elements and related proteins in lymphocytes of patients with Dravet syndrome: Relevance to cannabinoid-based therapies under investigation. British Journal of Clinical Pharmacology 2016.