A novel GABAergic dysfunction in human Dravet syndrome

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Summary

Objectives

Dravet syndrome is a rare neurodevelopmental disease, characterized by a general cognitive impairment and severe refractory seizures. The majority of patients carry the gene mutation SCN1A, leading to a defective sodium channel that contributes to pathogenic brain excitability. A GABAergic impairment, as in other neurodevelopmental diseases, has been proposed as additional mechanism, suggesting that seizures could be alleviated by GABAergic therapies. However, up to now the physiological mechanisms underlying the GABAergic dysfunction in Dravet are still unknown due to the scarce availability of this brain tissue. Here we studied, for the first time, human $GABA_A$ evoked currents using cortical brain tissue from Dravet patients.

Methods

We transplanted in *Xenopus* oocytes cell membranes obtained from brain tissues of autopsies of Dravet patients, tuberous sclerosis complex (TSC) patients as pathological comparison, and age-matched controls. Additionally, experiments were performed on oocytes expressing human $\alpha1\beta2\gamma2$ and $\alpha1\beta2$ $GABA_A$Rs. $GABA_A$ currents were recorded using the two-microelectrodes voltage-clamp technique. Quantitative RT-PCR, immunohistochemistry and double-labelling techniques were carried out on the same tissue samples.

Results

We found: (i) a decrease in GABA sensitivity in Dravet compared to controls, which was related to an increase in $\alpha4$- relative to $\alpha1$-containing $GABA_A$ receptors; (ii) a shift of the GABA reversal potential towards more depolarizing values in Dravet, and a parallel increase of the chloride

transporters NKCC1/KCC2 expression ratio; (iii) an increase of $GABA_A$ currents induced by low doses of cannabidiol both in Dravet and TSC comparable to that induced by a classical benzodiazepine, flunitrazepam, and that still persists in γ-less $GABA_A$ receptors.

Significance

Our study indicates that a dysfunction of the GABAergic system, considered as feature of brain immaturity, together with defective sodium channels, can contribute to a general reduction of inhibitory efficacy in Dravet brain, suggesting that $GABA_A$ receptors could be a target for new therapies.

Key Point Box

- $GABA_A$ receptors can contribute, together with defective sodium channels, to brain hyperexcitability in Dravet syndrome.
- GABA sensitivity is reduced in Dravet compared to age-matched controls, paralleling an increase of α4- relative to α1 $GABA_A$ subunits mRNAs.
- $GABA_A$ reversal potential is shifted towards more depolarizing values in Dravet cortex where NKCC1/KCC2 expression ratio is increased.
- low doses of CBD can increase GABA currents in Dravet as in TSC patients and this effect still persists in both human γ-less $GABA_A$ receptors and control tissues.
- impaired $GABA_A$ mediated transmission could represent an additional target for new therapies in drug-resistant Dravet epileptic patients.

Introduction

Dravet syndrome (DS) is a rare infantile encephalopathy (with an incidence of 1:40,000) characterized by the onset of severe drug-resistant epilepsy during the first years of life, often accompanied by serious developmental and cognitive impairment. [1,2] The cause of DS is in 70-80 % of the cases a mutation of the SCN1A gene, codifying for the α1 subunit of the voltage gated sodium channel (VGSC). However, other genes related to DS-like phenotypes have been identified in a small percentage of cases.[2] All these mutations produce altered channels that are probably the primary cause of epileptic phenotype and intellectual disabilities in DS. [2,3] Nonetheless, mutations in the $GABA_A$ receptor ($GABA_AR$) subunits genes cause DS-like phenotypes both in animal models and humans. [2,4]

GABA is the main inhibitory neurotransmitter in the central nervous system (CNS) and its action is related to cellular chloride homeostasis, maintained in the adults by the balance between the two chloride transporters, NKCC1 that is responsible for chloride influx and KCC2 that is responsible for chloride efflux.[5] $GABA_A$ function is complicated by the existence of "phasic" synaptic and "tonic" extrasynaptic inhibition; the former mainly involving α1−α3, β1−β3 and γ2 subunits, whereas the latter involves α4−α6 and δ containing receptors. [6] A GABAergic impairment has been reported in DS cases with VGSC mutations [7] ; in patients with Down syndrome [8] ; in Rett syndrome [9] and in tuberous sclerosis complex (TSC) patients.[10, 11] Altogether, these studies suggest abnormal features of "brain dysmaturity" in neurodevelopmental diseases and lead to the hypothesis that GABAergic therapies could not only alleviate the seizures but also the cognitive impairment. [12] Although it is an accepted hypothesis that the loss of function of VGSC in the GABAergic interneurons induces a decrease of GABAergic neuronal firing[3], it is still unknown if an additional GABAergic mechanism may be altered in human DS as in other neurodevelopmental diseases .[12]

The lack of human tissue-based research in DS is a consequence of low availability of fresh viable tissues due to the rarity of the disease and the infrequency of resection of brain tissue. The use of induced pluripotent stem cell (iPSC)-derived neurons from patients led to important results, even if with some discrepancies (i.e., an increase or deficit in the sodium current density), probably due to differences in neuronal differentiation protocols. [3,13] To overcome the limited availability of DS tissues, one approach to study GABAergic transmission in rare human epileptic diseases is the microtransplantation of $GABA_A$Rs from human brain into *Xenopus* oocytes. [14] The advantage of this technique is the possibility to investigate human $GABA_A$Rs using a minimal amount of autoptic brain tissue of DS patients, bypassing the transcriptional and translational machinery of the host cell. Noteworthy, the "microtransplanted" $GABA_A$Rs retain their native characteristics. [15] Here, we performed voltage-clamp recordings on *Xenopus* oocytes "microtransplanted" with membranes from cortical samples of human DS brains to study GABA-evoked currents and compared these to membranes from age-matched control patients. The electrophysiological experiments were also supported by qPCR analysis and immunohistochemistry that were performed on the same samples. Our first aim was to show that DS is not a condition exclusively dependent on the malfunction of sodium channels, but that $GABA_A$Rs could have a key role in the pathophysiology of this disease as it has been shown for other neuropathologies. [8-11]

Classical benzodiazepines (BDZ) are part of the first-line treatment of the disease but their efficacy is negatively influenced by disease duration that induces a decrease of BDZ-sensitivity, unless they are associated with other AEDs. [16, 17] Therefore, new compounds are currently under investigation and among these are the cannabis derivatives. [18] In particular, cannabidiol (CBD) is being studied both in pre-clinical and clinical studies.[19, 20] It is well-known that CBD and cannabinoids derivatives can directly target $GABA_A$Rs, [21, 22] but the reason of their beneficial effect in DS patients is still

partially unclear. Therefore, a further aim was to test CBD on human $GABA_AR$s from DS patients to integrate clinical observations.

Methods

Patients

The source of human tissues and the clinical characteristics derived from the patients' medical records are summarized in Table 1. In the text, the number of patients used in each experiment is reported, and referred to using the symbol #. See also Supporting Information, methods. All the autopsies were performed within 16 to <48 h after death with the acquisition of appropriate written consent for brain autopsy and subsequent use for research purposes. Cases were included as controls only when there was no known history of epilepsy, normal cortical structure for the corresponding age and without significant brain pathology. Informed consent was obtained for the use of brain tissue for research purposes. Tissue was obtained and used in accordance with the Declaration of Helsinki and the AMC Research Code provided by the Medical Ethics Committee and approved by the science committee of the UMC Utrecht Biobank. For more details see Supporting information.

Tissue preparation

Brain tissue from control, TSC and DS patients was snap frozen in liquid nitrogen and stored at -80°C until further use (RNA isolation for qPCR and membrane preparation). Part of the frozen tissue (5-10 mg) was shipped on dry ice by courier to the University of Rome. Additional tissue was fixed in 10% buffered formalin and embedded in paraffin. Representative sections of all specimens were processed for haematoxylin and eosin staining and immunohistochemical staining for the routine analysis of cortical specimens.

Immunohistochemistry

Immunohistochemistry was carried out as previously described [23] and as detailed in Supporting information, methods.

RNA isolation and real-time qPCR

For RNA isolation, frozen material was homogenized in Qiazol Lysis Reagent (Qiagen Benelux, Venlo, The Netherlands). Total RNA was isolated using the miRNeasy Mini kit (Qiagen Benelux, Venlo, The Netherlands). The concentration and purity of RNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Sample RNA quality control was performed using an Agilent 2100 bioanalyzer. A RIN (RNA Integrity Number) value greater than 6 comparable in controls and DS, was considered as indicator of RNA of good quality. Although our tissues are obtained at autopsy, we did not observe correlation between tissue collection post-mortem delay (see Table 1) and qPCR results.

Further details regarding qPCR analysis and primers are described in Supporting information and Table 1.

Membrane preparation and injection

The preparation of human membranes, their injection in *Xenopus laevis*, GABA current recordings in oocytes expressing human functional receptors was carried out as previously described [14] and as reported in Supporting information, methods. Injection procedures and experiments were performed by two different researchers blinded to which type of cell membranes they were working on.

Electrophysiology in microtransplanted or cDNA injected oocytes

From 12 to 48 h after injections, we recorded membrane currents from voltage-clamped *Xenopus laevis* oocytes using two microelectrodes filled with 3M KCl. [24] The use of female *Xenopus laevis* frogs conformed to institutional policies and guidelines of the Italian Ministry of Health (no. authorization 78/2015-PR).

GABA was always freshly dissolved in oocyte's Ringer (OR) and where otherwise indicated applied for 4 s to elicit inward currents (I_{GABA}). Dose-response relationships were performed as previously shown. [25] Further details on current-voltage relationships (I-V), solutions, measure of GABA reversal potential (E_{GABA}), curve fitting, drug-treatments and electrophysiological protocols are described in Supporting information, methods.

Statistics

Data are reported as mean ± standard error of the mean (SEM). Unless otherwise indicated, numbers (n) refer to oocytes used in each experiment. For all the experiments in which a comparison between the DS, TSC and control groups was made, the statistical analysis has been done on age-matched patients. See Supporting information, methods for details about the statistics analysis.

Results

GABA-evoked currents in *Xenopus* oocyte microtransplanted with DS tissues.

The application of 4s GABA (500 μM, if not specified otherwise) in oocytes injected with DS membranes, elicited inward currents (I_{GABA}) ranging from -6.3 to -396 nA, mean -69.8 ± 8.9 nA

(Fig. 1A; n = 58; # 1-3, Table 1). This variability in current amplitude was unrelated to which patient was used for membranes injection since the level of expression was very similar (for single patient data see Supporting information, Table 2) in the different patients (same amount of tissue used for each sample), but it may be due to a different efficiency in the expression of the oocytes as previously reported. [26]

These currents were blocked by the $GABA_A$Rs antagonist bicuculline (100 μM; not shown) indicating that we are recording genuine $GABA_A$ currents. For comparison, we injected cortical membranes from control patients (# 6-8, Table 1) and we found a comparable I_{GABA} amplitude (-64.1 ± 3.8 nA, Fig. 1A; n = 45, Levene test for homogeneity of variance, $p>0.05$).

Then, we measured the current decay ($T_{0.5}$) applying long pulses of 60 s GABA: the mean $T_{0.5}$ was 9.2 ± 3.5 s (n = 10; # 1-3, Table 1) in oocytes injected with DS membranes. This value was not different from that found in controls ($T_{0.5}$ = 9.9 ± 3.7 s; Fig 1B, n = 10; $p > 0.05$, unpaired t-test; # 6-8; Table 1). Altogether, these results indicate that $GABA_A$Rs transplanted from DS brain tissue are functional and respond similarly to $GABA_A$Rs transplanted from controls.

It is a common idea that $GABA_A$Rs involved in tonic inhibition can contribute to regulate neuronal excitability.[27] Here, we tested various modulators acting on tonic extrasynaptic $GABA_A$ receptors (Supporting information, Table 4) to investigate if the tonic inhibition in DS could be modified. [16] As a first step, we used Zn^{2+} to study the "rough" contribution of highly Zn^{2+}-sensitive tonic receptors to the GABA-evoked currents. We found that in DS-injected oocytes the I_{GABA} amplitude was reduced by 40 μM Zn^{2+} pretreatment (Supporting information, Table 4) and that this effect was comparable to that observed in controls ($p > 0.05$; Fig. 1C; Supporting information, Table 4).

Furthermore, we tested the steroid THDOC and DS2, respectively an endogenous and an exogenous modulator of δ-containing $GABA_A$Rs. [27, 28] Both the compounds produced a significant increase of

I_{GABA} amplitude, comparable with controls. ($p > 0.05$; Fig. 1C; Supporting information, Table 4). In line with these results, the test with the α5-containing inverse agonist L655-708 produced similar results. In addition, all these compounds did not modify the I_{GABA} decay (data not shown). Our findings clearly show that, at least for the compounds here tested, the tonic contribution on I_{GABA} amplitude in DS is not statistically different from control patients.

Since our DS patients carried a SCN1A mutation, we tested if we would be able to activate voltage-gated sodium channels. In twelve oocytes microtransplanted with DS or control cortical membranes (#1-3; 6-8, Table 1) we did not record sodium currents large enough to perform statistically relevant electrophysiological experiments (data not shown).

Decreased $GABA_A$-receptor sensitivity in DS patients.

Previous experiments revealed that $GABA_A$Rs sensitivity is modified in the drug-resistant epileptic brain [25, 29] and recently in patients with febrile seizures. [31] To define the characteristics of the $GABA_A$Rs in more detail, GABA dose–current response relations were obtained from DS and control patients in a different set of experiments (Fig. 2A).

While for the control tissues we confirmed an EC_{50} value comparable with results of previous experiments (EC_{50}=61.6 ± 4.5 μM, n_H = 1.7 ± 0.2; n = 18; #6-8, Table 1) [29] , the EC_{50} estimated for the DS samples was significantly higher (134.5 ± 2.1 μM, n_H = 1.1 ± 0.7; Fig. 2A; n = 18; #1-3, Table 1). Thus, these findings indicate that the apparent affinity for GABA is significantly reduced in DS compared to the controls. As "pathological" comparison we performed the same experiments using TSC patients and we found a value of EC_{50} very close (EC_{50}=71.7 ± 5.0 μM, n_H = 1.3 ± 0.1; n = 8; #4-5, Table 1) to that found for controls experiments ($p>0.05$, t-test). Since the GABA sensitivity is determined by the binding sites between the α and the other subunits, we decided to perform

quantitative RT-PCR analysis to evaluate the expression of most common α $GABA_AR$ subunits (as relative mRNAs) in the DS patients compared to controls. Interestingly, we found that the expression of mRNAs encoding α1, α2 and α4 subunits were significantly different between DS and controls (Fig. 2B). Specifically, the expression α2–, α4–subunit mRNAs was significantly higher (2.0 and 2.3-fold increase respectively), whereas the expression of α1-subunit mRNA was significantly lower (0.5-fold decrease) in DS compared to controls. On the contrary, no differences were found for α3– and α5- subunit mRNAs (Fig. 2B). These differences may underlie the aforementioned decrease in GABA sensitivity.

GABA-evoked current reversal potential in DS patients

The contribution of altered GABAergic transmission to neurodevelopmental pathologies due to an unbalance of chloride homeostasis has been confirmed by several studies. [10,11] Interestingly, we found that the GABA current reversal potential (E_{GABA}) was significantly more depolarized in DS (-17.5 ± 1.3 mV, Fig. 3A, n = 24, # 1-3, Table 1) than in control samples (-23.2 ± 1.8 mV, Fig. 3; n = 24, $p<0.05$; #6-8, Table 1; for single patient data see Supporting information, Table 2), suggesting that in DS patients GABA is less inhibitory than in controls. Of note, this altered E_{GABA} was very similar to that measured here for two TSC patients (see Supporting information, Table 2) and to that showed in previously published data[10]. On the contrary, this altered E_{GABA} is not present in two TLE cortical samples (see Supporting information, Table 3 and supplemental Fig.1). Furthermore, the altered E_{GABA} in DS is unlikely due to a contribution of HCO_3^- ions that are absent in our solutions and blockade of carbonic anhydrase by acetazolamide did not affect E_{GABA} value (not shown) as previously shown. [10,24]

In line with previous studies, where it was demonstrated that an alteration of E_{GABA} can be due to an altered expression of NKCC1, we performed another set of experiments in DS injected oocytes to measure E_{GABA} after 2 h treatment of low concentration of Bumetanide (12 μM, Bum, a selective NKCC1 blocker). [24,31] In these experiments, we found a statistically significant restoration of E_{GABA} to a value closer to control samples (from -17.0 ± 0.7 mV to – 21.0 ± 1.3 mV; p=0.007; n = 12; # 1-3, Table 1; Fig. 3B). On the contrary, the Bum treatment in oocytes injected with control membranes did not modify significantly the E_{GABA} (Fig. 3B, Inset), indicating that NKCC1 does not play a key role in determining E_{GABA} in control conditions. [10]

In order to better investigate the E_{GABA} shift, we performed quantitative RT-PCR experiments to measure mRNA expression of chloride transporters in brain, namely KCC2 and NKCC1. [5] Indeed, we found a higher expression of NKCC1 in DS tissues compared to controls (1.3-fold increase; Fig. 4A), but a clear lower expression of KCC2 (0.63-fold decrease; Fig. 4A). Our findings demonstrate that the unbalance of chloride transporters expression (Fig. 4A, *inset*) in DS may justify the change of E_{GABA}.

A downregulation of KCC2 has been reported in focal cortical dysplasia [32], in Rett syndrome [9] and in the subiculum of patients with temporal lobe epilepsy [33] (TLE), thus, to strengthen the quantitative RT-PCR data, we performed immunohistochemistry using brain tissue from patients from whom we had enough tissue to perform these kind of experiments: one DS and one age-matched control (#3 and #8 Table 1). A prominent neuropil staining was found in normal control adult cortex (Fig. 4B). We also observed intrasomatic immunoreactivity (IR) with expression in cells containing $GABA_AR$ α1 subunit. Noteworthy, in the DS patient the neuropil staining was decreased and a variable IR was observed in neurons containing $GABA_AR$ α1 subunit (Fig. 4B).

Despite the low number of patients due to the rarity of the disease and to the low availability of human tissue (in the range of 5-10 mg for each patient), our results suggest that the downregulation of KCC2, together with the loss of function of sodium channel[3], may be involved in the alteration of inhibitory tone of the network.

Effect of cannabidiol on GABA currents from patients affected by Dravet syndrome

Dravet syndrome is highly drug-resistant form of epilepsy and the use of new drugs in DS patients is a topic currently under the spotlight. Up to date, stiripentol is a GABAergic drug very effective in combination with valproate and clobazam and also in immature brain where it does not depend upon the $GABA_A$ subunits expression (Table 1). [12,16,34] CBD is already giving promising results in both animal models and clinical trials. [19,35,20] Using the approach of membrane microtransplantation, we found that co-application with 2 μM CBD increased the GABA-evoked currents in DS patients (+ 26.9 ± 4.7 %; GABA 50 μM; Fig. 5A; n = 28; # 1-3, Table 1). [22] The CBD effect was fast and completely reversible after 5 min of washing with OR (data not shown). In the same cells, we observed a similar potentiation using the classical BDZ flunitrazepam (FLU), (+ 35.2 ± 5 %; GABA 50 μM; FLU 6 μM, Fig. 5A; n = 28; $p>0.05$; #1-3, Table 1).[25] Moreover, in oocytes injected with control tissues, we measured an increase in GABA currents very similar to DS patients both for CBD (+ 29.0 ± 2.6 %; GABA 50 μM; CBD 2 μM) and FLU (+ 38.4 ± 6 %; GABA 50 μM; FLU 6 μM).

Additional experiments were performed using cortical membranes from two TSC patients. We found that CBD could induce an increase of I_{GABA} in oocytes injected with TSC cortical membranes (+ 28.8 ± 3.6 %; GABA 50 μM; Fig. 5B**;** n = 28; # 4,5 Table 1) that was similar as magnitude and way

of action to FLU (+ 43.5 ± 12 %; GABA 50 μM; Fig. 5B**;** n = 18; # 4,5 Table 1) and to that shown in DS patients.

It is well known that the BDZ bind with high affinity to $GABA_A$Rs at α/γ subunits interface [17] thus we wanted to investigate if the CBD effect could be comparable to a classical BDZ such as FLU that is well-known as potent positive allosteric modulator of $GABA_A$Rs. To this purpose, we intranuclearly injected oocytes with human cDNAs encoding for α1β2γ2 and α1β2 $GABA_A$Rs in order to test both the aforementioned compounds both on most common $GABA_A$Rs subunit composition and on a $GABA_A$R lacking the BDZ binding site. [36]

We found that, while as expected, FLU produced a strong potentiation on α1β2γ2 $GABA_A$Rs (+ 149 ± 49 %; GABA 5 μM Fig. 5C ; n = 8) [22] and was ineffective on α1β2 receptors (- 8 ± 6.9 %; GABA 1 μM, Fig. 5D; n = 8;)[22], CBD maintained its effect on both $GABA_A$Rs composition (α1β2γ2: + 49.8 ± 15.5 %; GABA 5 μM; Fig 5C; n = 10; α1β2: + 28.6 ± 5.8 %; GABA 1 μM; Fig. 5D; n = 8). We obtained similar results expressing α2 containing $GABA_A$Rs (Supporting information, Table 5). These last findings show that CBD is a powerful positive modulator of human $GABA_A$Rs and that it is still effective on γ-less $GABA_A$Rs confirming data recently shown. [22]

Discussion

Here we were able, for the first time, to record $GABA_A$ evoked currents from DS human brain tissue showing that: *i)* the GABA currents are very similar to those evoked using age-matched controls as amplitude, decay, and responses to the most common tonic inhibition modulators; *ii*) the apparent GABA affinity in DS is decreased compared to controls accompanied by a change of the expression of $GABA_A$Rs α1, α2 and α4 subunits; *iii)* the GABA reversal potential is more depolarized in DS patients respect to age-matched controls; *iv*) there is an altered expression of chloride cotransporters,

NKCC1 and KCC2 in DS in favour of an increase of NKCC1/KCC2 ratio; *v)* the CBD, similarly to FLU, can increase GABA currents both in DS and TSC tissues and this potentiation still persists on γ-less $GABA_A$Rs.

Here, we obtained brain tissue from three DS patients and respective age-matched controls, all from autopsies. Post-mortem samples represent the only source of control human tissue from patients without neurological diseases, since no relevant histological differences from the surgical tissues have been previously reported. [10,24,26]

To our knowledge, there are no studies using fresh human slices or organotypic cultures from DS with electrophysiological recordings. The micro-transplantation approach enables study of functional receptors from tissues of rare human diseases. [14] Although the exact glial or neuronal origin of the transplanted membrane patches is unknown, we were able to measure the "whole", glial and neuronal GABAergic responses as shown for TSC and TLE. [10,26] The recent idea that the impairment of GABAergic transmission could be a hallmark of neurodevelopmental syndromes is very fascinating and validated by several papers. [9,10,12,37] In addition, a defective GABAergic system could be detrimental for the occurrence of seizures in all these syndromes becoming one of the most relevant factor contributing to epileptogenesis. [10,11,38] In line with this evidence, we focused our attention on $GABA_A$ mediated transmission in DS that, together with a defective sodium channel on the GABAergic interneurons could synergistically affect the threshold for seizures in DS.[3] The inhibition due to tonic $GABA_A$ receptors when altered could contribute to the hyperexcitability in DS as shown for TLE. [39, 40] However, this hypothesis was not supported by our experiments that failed to identify differences between DS and controls using α5− and δ- subunits modulators. Obviously, we cannot exclude the involvement of other tonic $GABA_A$ subunits or an altered GABA release. However, we identified a reduction of GABA sensitivity (as 2.1 fold decrease of EC_{50}) in

DS as one possible explanation for GABA impairment. A similar reduction was observed in TLE patients and juvenile myoclonic epilepsy. [29, 41] Therefore, it is likely that a decrease of GABA sensitivity is dependent on differential expression of the most common α subunits, especially the α1, necessary for a functional GABA binding site. [17] We found that, while α3 and α5 mRNAs are similarly expressed in DS *versus* controls, α1, α2 and α4 are statistically different: α1 being downregulated and both α2 and α4 upregulated. Indeed, an increased α4 subunit is considered a hallmark of both status epilepticus, spontaneous and chronic seizures [42,43,44,]. A common trait of several neurodevelopmental syndromes is an immaturity of GABAergic transmission that in the normal human brain is fully developed during the first years of life. [38] In many cases GABA behaves as less hyperpolarizing or clearly depolarizing as shown in a model of Down syndrome [8], in TSC [10,11] and in Rett syndrome. [9] These modifications are caused by a different expression and/or function of one or both chloride transporters NKCC1 and KCC2 inducing a different ratio NKCC1/KCC2 that leads to an altered chloride homeostasis. [5,45] Here, we found that this kind of dysfunction is present also in DS inducing a BUM-sensitive shift of E_{GABA} towards more depolarized values. Of note, even if we are using oocyte's system as tool to measure E_{GABA}, the reliability of this approach has been demonstrated by studies of E_{GABA} shifts in subiculum of TLE patients [33] ; in peritumoral and TSC cortical tissues [10,24] *;* and in human brain slices. [11,46] An intriguing argument is how E_{GABA} could be shifted by a small quantity of transplanted membranes. However, it should be noted that we previously shown that few patches of membranes containing $GABA_AR$ and transporters are incorporated in the oocytes leading to "local perturbations" of chloride homeostasis. [14,24] Interestingly, from our results an increase of NKCC1/KCC2 ratio in DS patients with the KCC2 protein that co-localizes with α1 $GABA_AR$ subunit on the interneurons is evident. Therefore, our study clearly suggests that in human DS GABA is less inhibitory and contributes, together with the

reduced GABA sensitivity and with the loss of function of sodium channels, to lower the threshold of brain excitability. [12] However, we cannot exclude that the altered chloride homeostasis could be caused by a reduced function of one or both transporters caused by an imbalance of phosphorylation/dephosphorylation cellular mechanisms of these proteins,[5] or by changes induced by recurrent seizures. [47] Although it should be noted that the alteration of E_{GABA} in the cortex of DS is shared with TSC and other developmental diseases, but not with TLE cortex[33] , suggesting that this alteration is not common to all the drug- resistant epilepsies.

Further experiments will better elucidate this specific point. Another open issue concerning DS is the well-known refractoriness to standard therapies. [16] Recently, CBD seems to be very helpful to treat drug-resistant DS patients. [18,20,35] Although CBD can increase GABAergic transmission by acting on GABA release [18] , up to date it is still an open question if CBD could directly modulate $GABA_A$Rs in DS patients. Here we show that CBD at low concentrations can increase $GABA_A$ currents similarly to a classical BDZ in DS brain. The effect was fast and very similar to that obtained in TSC. Here we compared the CBD effect on DS with TSC for three main reasons: 1. TSC is a genetic neurodevelopmental disease with epileptic phenotype and cognitive impairment [48] ; 2. TSC presents a GABAergic impairment [10,11] ; 3. CBD has been proposed as possible adjuvant therapy for drug-resistant epilepsy in TSC. [49] Notably our study, to our knowledge, is the first testing CBD on “real” pathological human $GABA_A$Rs of both these neurodevelopmental disorders. In addition, to exclude that CBD action on $GABA_A$Rs could be due to its binding to other transplanted receptors and/or accessory proteins, we expressed human $\alpha1\beta2\gamma2$, $\alpha2\beta2\gamma2$ and $\alpha1\beta2$, $\alpha2\beta2$ $GABA_A$Rs showing that CBD effect persists on the different $GABA_A$Rs subunit compositions. [22] On the contrary, and as expected, the γ-less $GABA_A$Rs were fully insensitive to the BDZ flunitrazepam that is well-known for its potent action on $GABA_A$Rs. Altogether, our results suggest that the observed CBD beneficial

effects in DS clinical therapy due to its capability to indirectly decrease brain excitability, could involve a direct modulation of $GABA_A$Rs leading to an ameliorated inhibitory function. A take home message from our results to clinicians is that CBD may restore the GABAergic function and that DS patients could be treated with low doses of CBD that has been proven to ameliorate also the social behaviour in DS models.[19] In conclusion, we describe for the first time a new additional GABAergic dysfunction in DS that could exacerbate the occurrence and progression of ictogenic mechanisms. Furthermore, this GABAergic defect as feature of "brain dysmaturity" [50] could be targeted by new therapeutic approaches with few side-effects in these patients that are already burdened by high drug load.

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Figure legends

Figure 1 GABA-evoked currents from Dravet tissues. (A) The bar graphs represent the mean±SEM of GABA-evoked currents amplitude in oocytes injected with control (CTRL, patients #6-8, Table 1, n=45) or Dravet cortical membranes (patients #1-3, Table 1, n=58) as shown; Levene test for homogeneity of variance, $p>0.05$. (*inset*) Sample currents (GABA 500 μM) of experiments shown. Here and in the other figures, if not otherwise indicated, GABA application was 500 μM for 4s (indicated with empty bars) and the holding potential was -60 mV. **(B)** The sample current traces represent prolonged GABA application (500 μM for 30 s) to evaluate the current decay both in CTRL (upper trace, patient # 8, Table 1) and Dravet (lower trace, patient #2, Table 1). Note a similar current decay in the two current traces ($T_{0.5}$= 9.9 s and 8.9 s for CTRL and Dravet respectively). **(C)** The bar graph (as mean±SEM) shows the effect of different $GABA_AR$s modulators acting on $GABA_AR$s subunits that are relevant for the tonic inhibition in CTRL and Dravet as shown. The current amplitudes were normalized to the respective control currents before drug application. The raw current amplitudes relatives to this graph are summarized (as nA) in Supporting information, Table 2. (*inset*) Representative currents of the experiments performed with THDOC in CTRL and Dravet patients; empty bars represent GABA application, while blue open bars represent THDOC, 1 μM pre-applied for 2 min. Note that all the modulators tested here had a similar effect in CTRL and Dravet tissues ($p>0.05$).

Figure 2 $GABA_AR$ apparent affinity is decreased in Dravet tissues. (A) The graph shows the amplitudes (as mean ± SEM) of currents evoked at different GABA concentrations, expressed as a percentage of the maximal current evoked and best fitted by Hill curves. The EC_{50} values and n_H

were 134.5 ± 2.1 μM and 1.1 ± 0.7 in oocytes injected with Dravet membranes (●, n = 18; patients #1-3, Table 1) and 61.6 ± 4.5 μM and 1.7 ± 0.2 in oocytes injected with control membranes (●, n = 18; #6-8, Table 1) ($p < 0.05$, unpaired t-test). **(B)** quantitative RT-PCR analysis of α1 to α5 $GABA_AR$ subunits revealing mRNA expression in Dravet tissues (patients #1-3, Table 1) compared to CTRL (patients #6-8, Table 1) as shown (* = $p < 0.05$, α1-2,4-5, Mann Whitney rank sum test, α3 unpaired t-test). The error bars indicate the mean ± SEM from two replicates of PCR experiments, tested in duplicate. Note the clear differences in α1, α2 and α4 expression.

Figure 3. E_{GABA} alteration in Dravet (A) Current-voltage (I-V) relationships from oocytes injected with membranes of control temporal cortex (●) and Dravet temporal cortex (●). The points represent means ± SEM of peak GABA currents normalized to I_{max} that inverted at -23.2 ± 1.8 mV (●, I_{max}= 60±9 nA ; n=24, patients #6-8, Table 1) and at -17.5 ± 1.3 mV (●, I_{max}= 56±8 nA; n=24, Mann-Whitney Rank sum test, $p<0.05$; patients #1-3, Table 1). (*inset*) Sample currents from the same experiments at the holding potentials as indicated (in millivolts). **(B)** I-V relationships from oocytes injected with Dravet before (●) and after (□) 2 h of treatment with bumetanide (12 μM). Dravet currents inverted at -17.0 ± 0.7 mV before (I_{max} = 75.9 ± 13 nA, patients #1-3, Table 1) and -21± 1.3 mV after (I_{max} = 68.1 ± 7.8 nA) bumetanide treatment ($p=0.007$, paired t-test; n = 12; # 1-3, Table 1). *(inset*) I-V relationships from oocytes injected with control tissues before (●) after bumetanide (□). [E_{GABA} = -23.1 ± 1 mV; I_{max} = 51.6 ± 15.8 nA (●) ; E_{GABA} = -23.3 ± 0.7 mV; I_{max} = 68.1 ± 7.8 nA (□), $p > 0.05$, paired t-test, patients #6-8, Table 1)]. Note that for control tissues there is no E_{GABA} shift after bumetanide treatment.

Figure 4. Chloride transporters in Dravet. (A) quantitative RT-PCR analysis of chloride cotransporters NKCC1 and KCC2. *(inset)* NKCC1/KCC2 ratio in DS and in CTRL (* = $p < 0.05$, student's t-test). The error bars indicate the ± SEM from two replicates of PCR experiments, tested in duplicate. KCC2 immunoreactivity in control cortex and in that of a Dravet patient. **(B)**: (confocal images). A-C: KCC2 immunoreactivity in control cortex (A, α1 $GABA_AR$ subunit, green; B, KCC2, red; C, merged image; patient #8, Table 1) showing co-localization with α1 $GABA_AR$ subunit. (D-F): KCC2 immunoreactivity in the cortex of a Dravet patient (D, α1 $GABA_AR$ subunit, green; E, KCC2, red; F, merged image; patient #3, Table 1) showing the co-localization with α1 $GABA_AR$ subunit. (arrow in F). Scale bar A-F: 40 μm. Note the decreased KCC2 staining in the neuropil in Dravet. See Supporting information for quantification.

Figure 5. Cannabidiol potentiates GABA-evoked currents in Dravet, TSC brain tissues and in human α1β2γ2, α1β2, and α2β2γ2, α2β2 $GABA_A$ receptors. The bar graphs show the GABA current amplitude increase induced by 2 μM CBD and 6 μM FLU in oocytes injected with Dravet (**A,** n=28; patients #1-3, Table 1) and TSC (**B,** n=28; patients #4,5, Table 1) membranes. In the figure bar graphs show the amplitudes (as mean ± SEM) of I_{GABA} expressed as a percentage of the currents evoked before drug applications (50±6 nA for Dravet and 55±3.5 nA for TSC; *, $p<0.05$ respect to control currents, [ANOVA] and *post hoc* Holm–Sidak test; $p>0.05$ CBD versus FLU application by [ANOVA] and *post hoc* Holm–Sidak test). (*inset*). Representative sample currents of the experiments in control conditions (*left*) and after 7 s exposure to CBD (*center*) and FLU (*right*). Empty bars represent GABA application (50 μM, *see Bakas et al., 2017*).

In **C** and **D,** bar graphs show the GABA current amplitude increase induced by 2 μM CBD and 6 μM FLU in oocytes intranuclearly injected with human α1β2γ2 $GABA_AR$ subunits (**C,** n=10, CBD;

n=8, FLU) or human α1β2 $GABA_AR$ subunits (**D**, n=8, CBD; n=8, FLU). In **C,** mean control current = 0.5 ± 0.01 μA current; *, p<0.05 respect to control currents; p<0.05 for CBD versus FLU application by [ANOVA] test; in **D** mean control current= 0.43 ± 0.02 μA current; *, p<0.05 respect to control currents; p<0.05 for CBD versus FLU application by [ANOVA] test. Inset, empty bars, as in **A**, GABA 5 μM (**C**) and 1 μM (**D**).

In **E** and **F**, bar graphs show the GABA current amplitude increase induced by 2 μM CBD and 6 μM FLU in oocytes intranuclearly injected with human α2β2γ2 $GABA_AR$ subunits (**E**, n=11, CBD; n=11, FLU) or human α2β2 $GABA_AR$ subunits (**F**, n=11, CBD; n=11, FLU). In **E**, mean control current= 0.2 ± 0.014 μA current; *, p<0.05 respect to control currents; p>0.05 for CBD versus FLU application by [ANOVA] test; in **F** mean control current = 0.35 ± 0.02 μA; *, p<0.05 CBD respect to control currents; p<0.05 CBD versus FLU application by [ANOVA] test. Inset, empty bars, as in **A**, GABA 10 μM (**E,F**). Note that CBD effect is higher in cDNAs respect to membranes likely due to the presence in membranes of many $GABA_ARs$ subunit compositions that possess a different sensitivity to the drug.

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