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A novel GABAergic dysfunction in human Dravet syndrome

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Summary

Objective: Dravet syndrome is a rare neurodevelopmental disease, characterized by 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 γ-aminobutyric acid (GABAergic) impairment, as in other neurodevelopmental diseases, has been proposed as an additional mechanism, suggesting that seizures could be alleviated by GABAergic therapies. However, up to now the physiological mechanisms underlying the GABAergic dysfunction in Dravet syndrome 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 syndrome patients.

Methods: We transplanted in *Xenopus* oocytes cell membranes obtained from brain tissues of autopsies of Dravet syndrome patients, tuberous sclerosis complex patients as a pathological comparison, and age-matched controls. Additionally, experiments were performed on oocytes expressing human α1β2γ2 and α1β2 $GABA_A$ receptors. $GABA_A$ currents were recorded using the two-microelectrodes voltage-clamp technique. Quantitative real-time polymerase chain reaction, immunohistochemistry, and double-labeling techniques were carried out on the same tissue samples.

Results: We found (1) a decrease in GABA sensitivity in Dravet syndrome compared to controls, which was related to an increase in α4- relative to α1-containing $GABA_A$ receptors; (2) a shift of the GABA reversal potential toward more depolarizing values in Dravet syndrome, and a parallel increase of the chloride transporters NKCC1/KCC2 expression ratio; (3) an increase of $GABA_A$ currents induced by low doses of cannabidiol both in Dravet syndrome and tuberous sclerosis complex comparable to that induced by a classical benzodiazepine, flunitrazepam, that still persists in γ-less $GABA_A$ receptors.

Significance: Our study indicates that a dysfunction of the GABAergic system, considered as a 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.

G.R. and P.C. share first authorship.
Gabriele Ruffolo and Pierangelo Cifelli are contributed equally.
Eleonora Palma and Eleonora Aronica contributed equally for the last authorship.

KEYWORDS

cannabidiol, epilepsy, $GABA_A$ receptor, $GABA_A$ reversal potential, human brain tissue

1 | 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 cases a mutation of the *SCN1A* gene, codifying for the α1 subunit of the voltage-gated sodium channel gene (*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 γ-aminobutyric acid type A receptor ($GABA_AR$) subunit genes cause DS-like phenotypes in both animal models and humans.[2,4]

GABA is the main inhibitory neurotransmitter in the central nervous system, and its action is related to cellular chloride homeostasis, maintained in adults by the balance between the two chloride transporters, NKCC1, which is responsible for chloride influx, and KCC2, which 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 receptors containing α4–α6 and δ.[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 alleviate not only 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 whether 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 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 $GABA_ARs$ from human brain into *Xenopus* oocytes.[14] The advantage of this technique is the possibility of investigating human $GABA_ARs$ using a minimal amount of autoptic brain tissue of DS patients, bypassing the transcriptional and translational machinery of the host cell. It is noteworthy that the "microtransplanted" $GABA_ARs$ 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 quantitative real-time polymerase chain reaction (RT-PCR) 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 $GABA_ARs$ could have a key role in the pathophysiology of this disease as it has been shown for other neurodevelopmental disorders.[8–11]

Classical benzodiazepines (BDZs) are part of the first-line treatment of the disease, but their efficacy is

Key Points

- $GABA_A$ receptors can contribute, together with defective sodium channels, to brain hyperexcitability in Dravet syndrome
- GABA sensitivity is reduced in Dravet syndrome compared to age-matched controls, paralleling an increase of α4 relative to α1 $GABA_A$ subunit mRNAs
- $GABA_A$ reversal potential is shifted toward more depolarizing values in Dravet cortex where NKCC1/KCC2 expression ratio is increased
- Low doses of CBD can increase GABA currents in Dravet syndrome as in TSC patients, and this effect 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

negatively influenced by disease duration, which induces a decrease in BDZ sensitivity unless they are associated with other antiepileptic drugs (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 in both preclinical and clinical studies.[19,20] It is well known that CBD and cannabinoid derivatives can directly target $GABA_ARs$,[21,22] but the reason for their beneficial effect in DS patients is still partially unclear. Therefore, a further aim was to test CBD on human $GABA_ARs$ from DS patients to integrate clinical observations.

2 | MATERIALS AND METHODS

2.1 | 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 Appendix S1. All the autopsies were performed within 16 to 48 hours 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 no 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 Amsterdam University Medical Center Research Code provided by the Medical Ethics Committee and approved by the science committee of the University Medical Center Utrecht Biobank. For more details, see Appendix S1.

2.2 | 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 quantitative RT-PCR 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 hematoxylin and eosin staining and immunohistochemical staining for the routine analysis of cortical specimens.

2.3 | Immunohistochemistry

Immunohistochemistry was carried out as previously described[23] and as detailed in Appendix S1.

2.4 | RNA isolation and quantitative RT-PCR

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). The concentration and purity of RNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware). Sample RNA quality control was performed using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, California). An RNA integrity number > 6, comparable in controls and DS, was considered an indicator of RNA of good quality. Although our tissues were obtained at autopsy, we did not observe a correlation between tissue collection postmortem delay (see Table 1) and quantitative RT-PCR results.

Further details regarding quantitative RT-PCR analysis and primers are described in Table S1.

2.5 | Membrane preparation and injection

The preparation of human membranes, their injection in *Xenopus laevis*, and GABA current recordings in oocytes expressing human functional receptors were carried out as previously described[14] and as reported in Appendix S1. Injection procedures and experiments were performed by two different researchers blinded to which type of cell membranes they were working on.

2.6 | Electrophysiology in microtransplanted or cDNA-injected oocytes

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

GABA was always freshly dissolved in oocyte Ringer solution (OR) and, unless otherwise indicated, applied for 4 seconds to elicit inward currents (I_{GABA}). Dose-response relationships were performed as previously shown.[25] Further details on current-voltage relationships, solutions, measure of GABA reversal potential (E_{GABA}), curve fitting, drug treatments, and electrophysiological protocols are described in Appendix S1.

2.7 | 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 Appendix S1 for details regarding the statistical analysis.

3 | RESULTS

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

The application of 4 seconds of GABA (500 μmol/L, if not specified otherwise) in oocytes injected with DS membranes elicited I_{GABA} ranging from −6.3 to −396 nA (mean −69.8 ± 8.9 nA; Figure 1A; n = 58; #1-3, Table 1). This variability in current amplitude was unrelated to which patient was used for membranes injection, as the level of expression was very similar (for single patient data, see Table S2) in the different patients (the same amount of tissue was 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 $GABA_AR$ antagonist bicuculline (100 μmol/L; 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, Figure 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 seconds of GABA; the mean $T_{0.5}$ was 9.2 ± 3.5 seconds (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 seconds; Figure 1B, n = 10; $P > 0.05$, unpaired t test; #6-8; Table 1). Altogether, these results indicate that $GABA_ARs$ transplanted from DS brain tissue are functional and respond similarly to $GABA_ARs$ transplanted from controls.

It is a common idea that $GABA_ARs$ involved in tonic inhibition can contribute to regulating neuronal excitability.[27] Here, we tested various modulators acting on tonic extrasynaptic $GABA_A$ receptors (Table S4) to investigate whether 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 μmol/L Zn^{2+} pretreatment (Table S4) and that this effect was comparable to that observed in controls ($P > 0.05$; Figure 1C and Table S4).

Furthermore, we tested the steroids tetrahydrodeoxycorticosterone (THDOC) and delta selective compound 2, which are respectively an endogenous and an exogenous modulator of δ-containing $GABA_ARs$.[27,28] Both the

TABLE 1 Cases included: Clinical features

Patient	Age, y	Gender	Duration of epilepsy, y	Brain region	Type of seizures	Diagnosis/mut/cause of death	AEDs	PM
#1	8	M	7	T	FIAS/GS	DS/*SCN1A* mut; c.4834G>A p.Val1612Thr/heart failure	CLB, STP	24
#2	49	M	48	T	FIAS/GS	DS/*SCN1A* mut; c.5164A>G p.Thr1722Ala/heart failure	CLB, STP, VPA	20
#3	46	F	44	T	FIAS/GS	DS/*SCN1A* mut; c.677C>A p.Thr226Lys/ bronchopneumonia	CLB, STP, VPA	<48
#4	2	M	1.5	T	IS	TSC/c.4645C>T/myocardial infarction	TPM, LTG, CLB	24
#5	47	M	35	T	FAS	TSC/c.4909_4911delAAG/myocardial infarction	PHB, VPA, CBZ, CLB	24
#6	7	M	—	T	—	Intestinal ischemia		24
#7	61	M	—	T	—	Myocardial infarction		20
#8	38	M	—	T	—	Myocardial infarction		16

Patients #1-3: Dravet membranes from autopsies (temporal cortex); Patients #4 and 5: tissues from TSC patients (TSC2 mutation). All autopsies were performed within 48 hours after death. For one case of DS, the exact PM was not available (autopsy was performed within 48 hours). Sudden unexplained death in epilepsy was excluded in all cases. All patients in the Table have been used both for electrophysiological experiments and for quantitative real-time polymerase chain reaction analysis; Patients #3 and #8 have also been used for immunohistochemistry. Patients #6-8: tissues from controls without neurological diseases. The clinical cases and controls included in this study were selected from the databases of the Department of Neuropathology, Amsterdam University Medical Center, University of Amsterdam (Amsterdam, The Netherlands); Department of Neuropathology, University Medical Center Utrecht (Utrecht, The Netherlands); Division of Neuropathology, University College London Epilepsy Brain and Tissue Bank (London, UK); and the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders (Baltimore, MD). AEDs, antiepileptic drugs; CBZ, carbamazepine; CLB, clobazam; DS, Dravet syndrome; F, female; FAS, focal aware seizures; FIAS, focal impaired awareness seizures; GS, generalized seizures; IS, infantile spasms; LTG, lamotrigine; M, male; mut, mutation; PHB, phenobarbital; PM, postmortem hours; STP, stiripentol; T, temporal; TPM, topiramate; VPA, valproic acid.

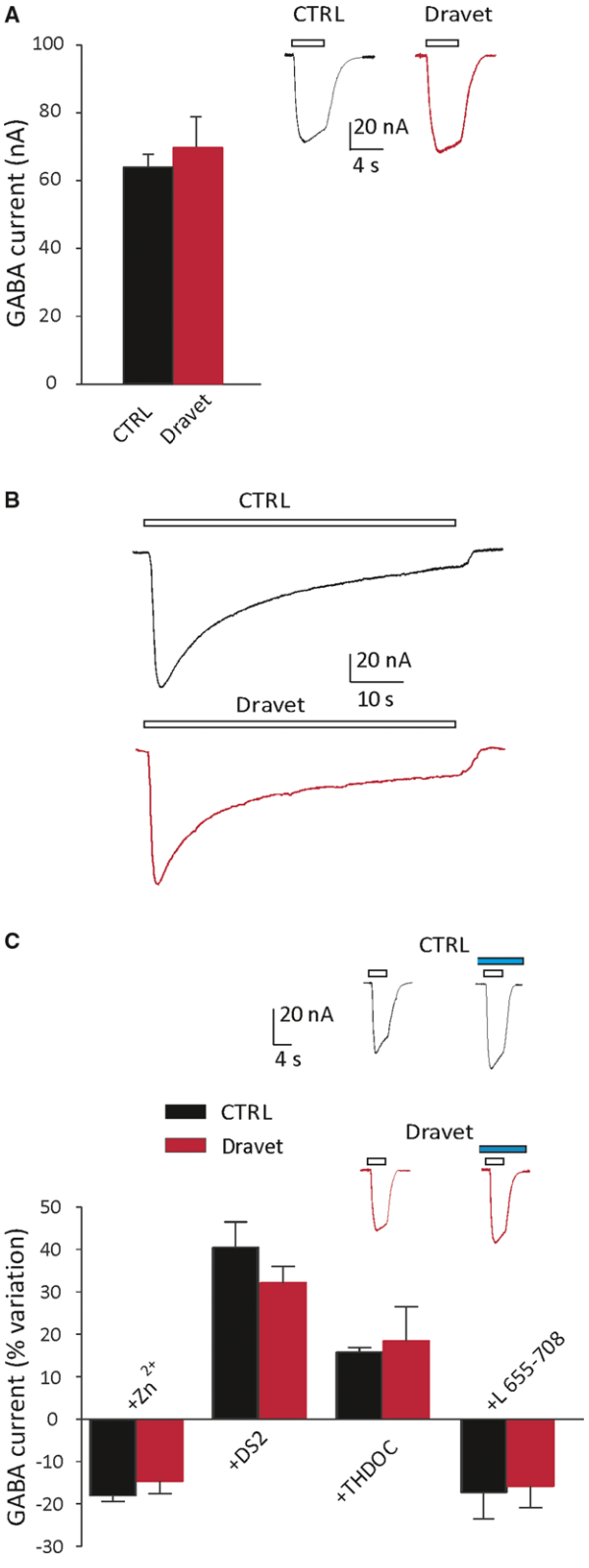

FIGURE 1 γ-Aminobutyric acid (GABA)-evoked currents from Dravet tissues. A, The bar graphs represent the mean ± standard error of the mean (SEM) of GABA-evoked current amplitude in oocytes injected with control (CTRL, Patients #6-8, Table 1, n = 45) or Dravet cortical membranes (Patients #1-3, Table 1, n = 58; Levene test for homogeneity of variance, $P > 0.05$). In the inset, sample currents (GABA 500 μmol/L) of experiments are shown. Here and in the other figures, if not otherwise indicated, GABA application was 500 μmol/L for 4 seconds (indicated with empty bars) and the holding potential was −60 mV. B, The sample current traces represent prolonged GABA application (500 μmol/L for 60 seconds) to evaluate the current decay both in CTRL (upper trace, Patient #8, Table 1) and Dravet syndrome (lower trace, Patient #2, Table 1). Note a similar current decay in the two current traces ($T_{0.5}$ = 9.9 seconds and 8.9 seconds for CTRL and Dravet syndrome, respectively). C, The bar graph (mean ± SEM) shows the effect of different GABA type A receptor ($GABA_AR$) modulators acting on $GABA_AR$ subunits that are relevant for the tonic inhibition in CTRL and Dravet syndrome as shown. The current amplitudes were normalized to the respective control currents before drug application. The raw current amplitudes relative to this graph are summarized (as nanoamperes) in Table S2. The inset shows representative currents of the experiments performed with tetrahydrodeoxycorticosterone (THDOC) in CTRL and Dravet syndrome patients; empty bars represent GABA application, whereas blue open bars represent THDOC, 1 μmol/L preapplied for 2 minutes. Note that all the modulators tested here had a similar effect in CTRL and Dravet tissues ($P > 0.05$). DS2, delta selective compound 2

compounds produced a significant increase of I_{GABA} amplitude, comparable with controls ($P > 0.05$; Figure 1C and Table S4). In line with these results, the test with the α5-containing inverse agonist L655-708 produced similar results. In addition, none of these compounds modified the I_{GABA} decay (data not shown). Our findings clearly show that, at least for the compounds tested here, the tonic contribution on I_{GABA} amplitude in DS is not statistically different from control patients.

Because our DS patients carried an *SCN1A* mutation, we tested whether we would be able to activate voltage-gated sodium channels. In 12 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).

3.2 | Decreased $GABA_A$ receptor sensitivity in DS patients

Previous experiments revealed that $GABA_AR$s sensitivity is modified in the drug-resistant epileptic brain[25,29] and recently in patients with febrile seizures.[30] To define the characteristics of the $GABA_AR$s in more detail, GABA dose–current response relations were obtained from DS and control patients in a different set of experiments (Figure 2A).

Whereas for the control tissues we confirmed a median effective concentration (EC_{50}) value comparable with results of previous experiments (EC_{50} = 61.6 ± 4.5 μmol/L, 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 μmol/L, $n_{H\ =}$ 1.1 ± 0.7; Figure 2A; n = 18; #1-3, Table 1). Thus, these findings indicate that the apparent affinity for GABA is significantly reduced in DS compared to controls. As a "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 μmol/L, n_H = 1.3 ± 0.1; n = 8; #4 and #5, Table 1) to that found for control experiments ($P > 0.05$, *t* test). Because 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 was significantly different between DS and controls (Figure 2B). Specifically, the expression α2 and 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. In contrast, no differences were found for α3 and α5 subunit mRNAs (Figure 2B). These differences may underlie the aforementioned decrease in GABA sensitivity.

3.3 | 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 E_{GABA} was significantly more depolarized in DS (−17.5 ± 1.3 mV, Figure 3A; n = 24; #1-3, Table 1) than in control samples (−23.2 ± 1.8 mV, Figure 3; n = 24, $P < 0.05$; #6-8, Table 1; for single patient data, see Table S2), 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 Table S2) and to that shown in previously published data.[10] In contrast, this altered E_{GABA} is not present in two temporal lobe epilepsy (TLE) cortical samples (see Table S3 and Figure S1). Furthermore, the altered E_{GABA} in DS is unlikely to be 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-hour treatment with a low concentration of selective NKCC1 blocker bumetanide (12 μmol/L).[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; Figure 3B). In contrast, bumetanide treatment in oocytes injected with control membranes did not significantly modify E_{GABA} (Figure 3B, inset), indicating that NKCC1 does not play a key role in determining E_{GABA} in control conditions.[10]

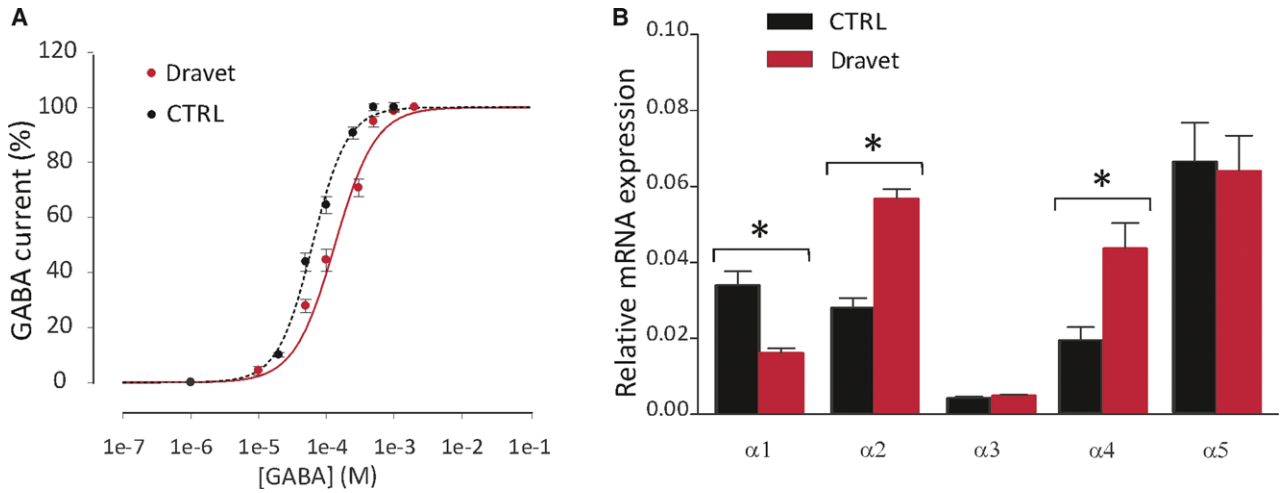

FIGURE 2 γ-Aminobutyric acid type A receptor ($GABA_AR$) apparent affinity is decreased in Dravet tissues. A, The graph shows the amplitudes (as mean ± standard error of the mean [SEM]) of currents evoked at different GABA concentrations, expressed as a percentage of the maximal current evoked and best fitted by Hill curves. The EC_{50} values and n_H were 134.5 ± 2.1 μmol/L and 1.1 ± 0.7 in oocytes injected with Dravet membranes (●, n = 18, Patients #1-3, Table 1) and 61.6 ± 4.5 μmol/L and 1.7 ± 0.2 in oocytes injected with control (CTRL) membranes (●, n = 18, #6-8, Table 1; $P < 0.05$, unpaired *t* test). B, Quantitative real-time polymerase chain reaction (PCR) analysis of α1-α5 $GABA_AR$ subunits revealing mRNA expression in Dravet tissues (Patients #1-3, Table 1) compared to CTRL (Patients #6-8, Table 1; *$P < 0.05$, α1, α2, α4, α5, 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

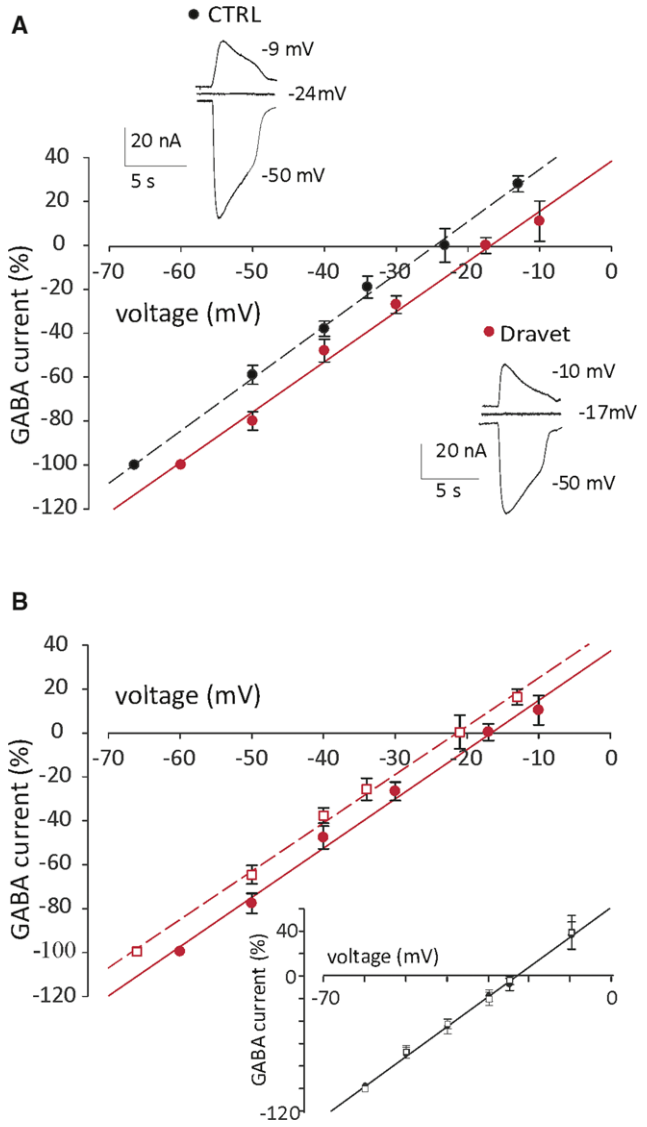

FIGURE 3 γ-Aminobutyric acid reversal potential (E_{GABA}) alteration in Dravet syndrome. A, Current-voltage (I-V) relationships from oocytes injected with membranes of control (CTRL) temporal cortex (●) and Dravet temporal cortex (●). The points represent means ± standard error of the mean SEM of peak 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 shows sample currents from the same experiments at the holding potentials as indicated (in millivolts). B, I-V relationships from oocytes injected with Dravet membranes before (□) and after (●) 2 hours of treatment with bumetanide (12 μmol/L). 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; Patients #1-3, Table 1). Inset shows I-V relationships from oocytes injected with control tissues before (●) and 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

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] We found a higher expression of NKCC1 in DS tissues compared to controls (1.3-fold increase; Figure 4A), but a clear lower expression of KCC2 (0.63-fold decrease; Figure 4A). Our findings demonstrate that the unbalance of chloride transporter expression (Figure 4A, inset) in DS may explain the change of E_{GABA}.

A down-regulation of KCC2 has been reported in focal cortical dysplasia,[32] in Rett syndrome,[9] and in the subiculum of patients with TLE[33]; 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 experiments: one DS and one age-matched control (#3 and #8, Table 1). Prominent neuropil staining was found in normal control adult cortex (Figure 4B). We also observed intrasomatic immunoreactivity with expression in cells containing $GABA_AR$ α1 subunit. It is noteworthy that in the DS patient the neuropil staining for KCC2 was decreased and a variable immunoreactivity was observed in neurons containing $GABA_AR$ α1 subunit (Figure 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 down-regulation of KCC2, together with the loss of function of sodium channel,[3] may be involved in the alteration of inhibitory tone of the network.

3.4 | Effect of CBD on GABA currents from patients affected by DS

DS is a highly drug-resistant form of epilepsy, and the use of new drugs in DS patients is a topic currently under the spotlight. 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 $GABA_A$ subunit expression (Table 1).[12,16,34] CBD is already giving promising results in both animal models and clinical trials.[19,20,35] Using the approach of membrane microtransplantation, we found that coapplication with 2 μmol/L CBD increased GABA-evoked currents in DS patients (+26.9 ± 4.7%; GABA 50 μmol/L; Figure 5A; n = 28; #1-3, Table 1).[22] The CBD effect was fast and completely reversible after 5 minutes of washing with oocyte Ringer solution (data not shown). In the same cells, we observed a similar potentiation using the classical BDZ flunitrazepam (FLU; +35.2 ± 5%; GABA 50 μmol/L, FLU 6 μmol/L; Figure 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 that in DS

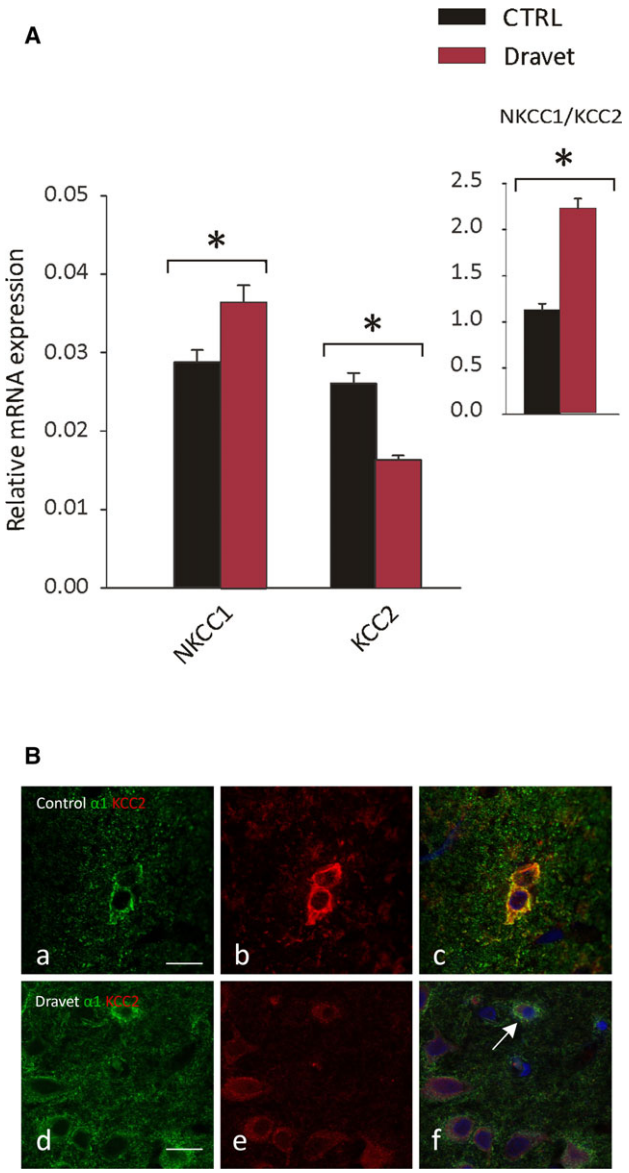

FIGURE 4 Chloride transporters in Dravet syndrome. A, quantitative real-time polymerase chain reaction (PCR) analysis of chloride cotransporters NKCC1 and KCC2. Inset shows NKCC1/KCC2 ratio in Dravet syndrome and in control (CTRL; $*P < 0.05$, Student *t* test). The error bars indicate the standard error of the mean from two replicates of PCR experiments, tested in duplicate. B, KCC2 immunoreactivity in control cortex and in that of a Dravet syndrome patient (confocal images). a-c, KCC2 immunoreactivity in control cortex (a, α1 γ-aminobutyric acid type A receptor [$GABA_AR$] subunit, green; b, KCC2, red; c, merged image; Patient #8, Table 1) showing colocalization with α1 $GABA_AR$ subunit. d-f, KCC2 immunoreactivity in the cortex of a Dravet syndrome patient (d, α1 $GABA_AR$ subunit, green; e, KCC2, red; f, merged image; Patient #3, Table 1) showing the colocalization with α1 $GABA_AR$ subunit (arrow in f). Scale bars for a-f = 40 μm. Note the decreased KCC2 staining in the neuropil in Dravet syndrome. See Appendix S1 for quantification

patients for both CBD (+29.0 ± 2.6%; GABA 50 μmol/L, CBD 2 μmol/L) and FLU (+38.4 ± 6%; GABA 50 μmol/L, FLU 6 μmol/L).

Additional experiments were performed using cortical membranes from two TSC patients. We found that CBD and FLU could induce an increase of I_{GABA} in oocytes injected with TSC cortical membranes (CBD = +28.8 ± 3.6%; GABA 50 μmol/L; n= 28; FLU = + 43.5 ± 12 %; GABA 50 micromol/L; n = 18; Figure 5B; #4 and #5, Table 1) that was similar in magnitude and way of action to that shown in DS patients.

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

We found that, whereas FLU produced a strong potentiation on α1β2γ2 $GABA_ARs$ (+149 ± 49%; GABA 5 μmol/L; Figure 5C; n = 8)[22] and was ineffective on α1β2 receptors (−8 ± 6.9%; GABA 1 μmol/L; Figure 5D; n = 8),[22] CBD maintained its effect on the composition of both $GABA_ARs$ (α1β2γ2: +49.8 ± 15.5%; GABA 5 μmol/L; Figure 5C; n = 10; α1β2: +28.6 ± 5.8%; GABA 1 μmol/L; Figure 5D; n = 8). We obtained similar results expressing α2-containing $GABA_ARs$ (Table S5). These last findings show that CBD is a powerful positive modulator of human $GABA_ARs$ and that it is still effective on γ-less $GABA_ARs$, confirming recently shown data.[22]

4 | DISCUSSION

Here we were able, for the first time, to record $GABA_A$-evoked currents from DS human brain tissue showing that (1) the GABA currents are very similar to those evoked using age-matched controls regarding amplitude, decay, and responses to the most common tonic inhibition modulators; (2) the apparent GABA affinity in DS is decreased compared to controls accompanied by a change of the expression of $GABA_AR$ α1, α2, and α4 subunits; (3) the GABA reversal potential is more depolarized in DS patients compared to age-matched controls; (4) there is altered expression of the chloride cotransporters NKCC1 and KCC2 in DS in favor of an increase of NKCC1/KCC2 ratio; and (5) CBD, similarly to FLU, can increase GABA currents in both DS and TSC tissues, and this potentiation persists on γ-less $GABA_ARs$.

Here, we obtained brain tissue from three DS patients and respective age-matched controls, all from autopsies. Postmortem samples represent the only source of control human tissue from patients without neurological diseases,

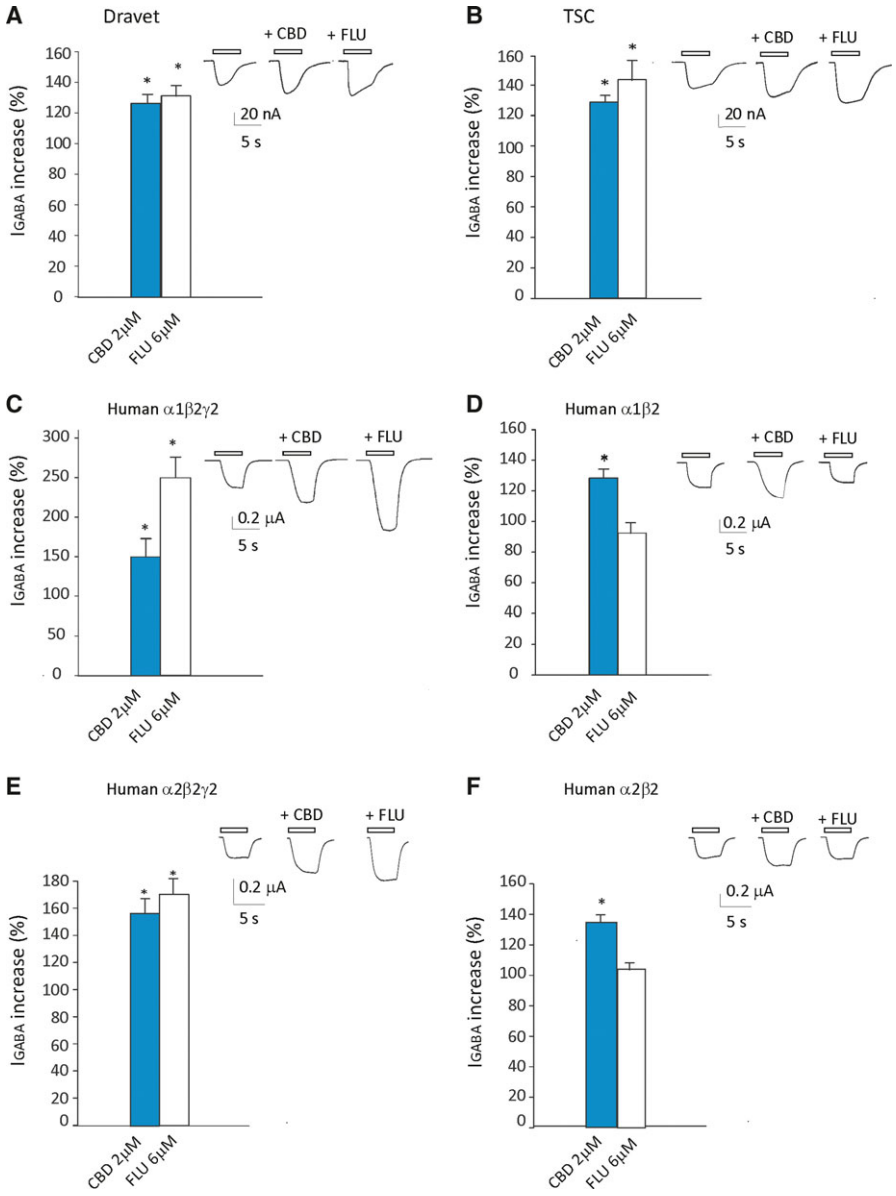

FIGURE 5 Cannabidiol (CBD) potentiates γ-aminobutyric acid (GABA)-evoked currents in Dravet syndrome, in tuberous sclerosis complex (TSC) brain tissues and in human α1β2γ2, α1β2, and α2β2γ2, α2β2 GABA type A receptors ($GABA_AR$s). A, B, Bar graphs show the GABA current amplitude increase induced by 2 μmol/L CBD and 6 μmol/L flunitrazepam (FLU) in oocytes injected with Dravet (A; n = 28; Patients #1-3, Table 1) and TSC (B; n = 28; Patients #4 and 5, Table 1) membranes. Bar graphs show the amplitudes (as mean ± standard error of the mean) of GABA inward currents (I_{GABA}) expressed as a percentage of the currents evoked before drug applications (50 ± 6 nA for Dravet syndrome and 55 ± 3.5 nA for TSC; $^*P < 0.05$ vs control currents, analysis of variance [ANOVA] and post hoc Holm-Sidak test; $P > 0.05$ CBD vs FLU application by ANOVA and post hoc Holm-Sidak test). Insets show representative sample currents of the experiments in control conditions (left) and after 7 seconds of exposure to CBD (center) and FLU (right). Empty bars represent GABA application (50 μmol/L, see Bakas et al[22]). C, D, Bar graphs show the GABA current amplitude increase induced by 2 μmol/L CBD and 6 μmol/L 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 ($^*P < 0.05$ vs control currents; $P < 0.05$ for CBD vs FLU application by ANOVA test). In D, mean control current = 0.43 ± 0.02 μA current ($^*P < 0.05$ vs control currents; $P < 0.05$ for CBD vs FLU application by ANOVA test). In insets, empty bars represent GABA application (C, GABA 5 μmol/L; D, 1 μmol/L). E, F, Bar graphs show the GABA current amplitude increase induced by 2 μmol/L CBD and 6 μmol/L 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 ($^*P < 0.05$ vs control currents; $P > 0.05$ for CBD vs FLU application by ANOVA test). In F, mean control current = 0.35 ± 0.02 μA ($^*P < 0.05$ CBD vs control currents; $P < 0.05$ CBD vs FLU application by ANOVA test). In insets, empty bars represent GABA application (GABA 10 μmol/L for both E and F). Note that CBD effect is higher in cDNA than membranes, likely due to the presence in membranes of many $GABA_AR$ subunit compositions that possess a different sensitivity to the drug

as 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 subjects with electrophysiological recordings. The microtransplantation approach enables the 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 factors 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, which failed to identify differences between DS and controls using α5 and δ subunit modulators. Obviously, we cannot exclude the involvement of other tonic $GABA_A$ subunits or 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 α1, which is necessary for a functional GABA binding site.[17] We found that, whereas α3 and α5 mRNAs are similarly expressed in DS and controls, α1, α2, and α4 are statistically different, α1 being down-regulated and both α2 and α4 being up-regulated. An increased α4 subunit is considered a hallmark of status epilepticus and both spontaneous and chronic seizures.[42–44] 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 different expression and/or function of one or both chloride transporters NKCC1 and KCC2 inducing a different NKCC1/KCC2 ratio that leads to altered chloride homeostasis.[5,45] Here, we found that this kind of dysfunction is present also in DS, inducing a bumetanide-sensitive shift of E_{GABA} toward more depolarized values. Of note, even if we are using the oocyte system as a 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 question is how E_{GABA} could be shifted by a small quantity of transplanted membranes. However, it should be noted that we previously showed 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 colocalizes 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 lowering the threshold of brain excitability.[12] However, we cannot exclude that the altered chloride homeostasis could be caused by 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] 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 in treating drug-resistant DS patients.[18,20,35] Although CBD can increase GABAergic transmission by acting on GABA release,[18] to date it is still an open question whether CBD could directly modulate $GABA_ARs$ 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] and (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 to test CBD on "real" pathological human $GABA_ARs$ of both these neurodevelopmental disorders. In addition, to exclude that CBD action on $GABA_ARs$ could be due to its binding to other transplanted receptors and/or accessory proteins, we expressed human α1β2γ2, α2β2γ2, and α1β2, α2β2 $GABA_ARs$, showing that CBD effect persists on all the different $GABA_ARs$ subunit compositions.[22] On the contrary, and as expected, the γ-less $GABA_ARs$ were fully insensitive to the BDZ FLU, which is well known for its potent action on $GABA_ARs$. 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 for clinicians is that CBD may restore GABAergic function and that DS patients could be treated with low doses of CBD, which have been proven also to ameliorate 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 a feature of "brain dysmaturity"[50] could be targeted by new therapeutic approaches with few side effects in these patients, who are already burdened by high drug load.

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DISCLOSURE

The authors have no conflicts of interest to report. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

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