Table 1. Primer sequences for qPCR

Name	Gene ID	Forward primer	Reverse primer	Amplicon (nt)
EF1α	EEF1A1	ATCCACCTTTGGGTCGCTTT	CCGCAACTGTCTGTCTCATATCAC	51
HPRT	HPRT1	TGCTGGTGAAAAGGACCCCACG	TGGCGATGTCAATAGGACTCCAGA	223
GABAA α1	GABRA1	TCCCCGGTCTTAAGAGATCC	ACCTGGACTTTTCCTCATCG	74
GABAA α2	GABRA2	TTACGATAATCGGCTTAGACCAG	TGGTCACGTAGATGTTAGTGAAGAC	71
GABAA α3	GABRA3	CCATGTTGTTGGGACAGAGAT	TTGAGATGGAAGTGGGTTGTC	75
GABAA α4	GABRA4	GTCAGTTTCGCCCTCCTG	GCGGGTGAAATTTTCTGTG	105
GABAA α5	GABRA5	AGGTGTCCTTTTGGCTGAAC	CTGAGGGTCGTCATGGTCA	85
NKCC1	SLC12A2	TGACTTGAGAGAAGGTGCACAG	TGTTTGGCTTCATACGACCA	65
KCC2	SLC12A5	GCCACGCTTTCGATATTACC	GCATGGCTACCAGTGCATAA	104

Table 2. $GABA_A$ evoked currents and reversal potential in epileptic and control patients

P #	I_{GABA} [n] (nA)	E_{GABA} [n] (mV)
1	-66.6 ± 10 [18]	-17.1 ± 1.5 [8]
2	-76.8 ± 11 [15]	-17.9 ± 1.3 [7]
3	-65.9 ± 14 [25]	-17.5 ± 1.1 [9]
4	-63.1 ± 14 [8]	-19.9 ± 1.0 [8]
5	-31.5 ± 7 [8]	-19.4 ± 1.0 [8]
6	-71.1 ± 13 [11]	-23.7 ± 2.0 [8]
7	-60.8 ± 8 [22]	-23.0 ± 1.8 [9]
8	-60.5 ± 11 [12]	-22.8 ± 1.6 [7]
9	-78.8 ± 4 [8]	-23.3 ± 0.9 [8]
10	-77.0 ± 3 [8]	-24.8 ± 0.8 [8]

Values of GABA evoked current amplitudes (I_{GABA}; GABA concentration 500 μM, holding potential, -60 mV) and GABA current reversal potential (E_{GABA}) as the mean ± SEM of the values obtained

from a given patient as indicated. Dravet patients #1-3; TSC patients #4-5, Table 1 in the main text; TLE patients #9-10, see Table 3 below; control patients #6-8 see Table 1 in the main text. [n] Represents the number of oocytes.

Table 3. TLE patients: clinical features

P#	Age(yrs)/sex	Epilepsy onset	Surgical Zone	Seizure type	Seizures/month	Pathology	Medication
#9	F/54	12	R-T	FIAS/GS	8	HS	CBZ, LTG, PHB
#10	F/54	15	R-T	FAS/FIAS	24	HS	CBZ, VPA, CLB

Patients #9-10: TLE patients with hippocampal sclerosis (HS); T, temporal; L, left; R, right.

CBZ, carbamazepine; CLB, clobazam; LTG, lamotrigine; PHB, phenobarbital; VPA, valproic acid.

FAS, focal aware seizure; FIAS, focal impaired awareness seizure; GS, generalized seizures;

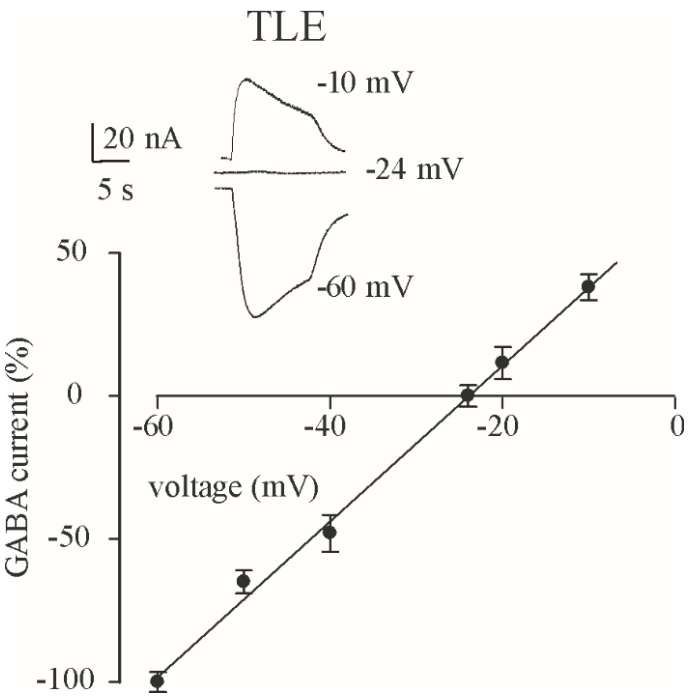

Fig. 1 Supplemental: E_{GABA} in TLE patients. Current-voltage (I-V) relationships from oocytes injected with membranes of temporal cortex obtained from two drug-resistant TLE patients (●). The points represent means ± SEM of peak GABA currents normalized to I_{max} that inverted at -24 ± 3.8 mV (●, I_{max}= 77 ± 8 nA ; n=12 , patients #9-10, Table 3 Supplemental). (*inset*) Sample currents from the same experiments at the holding potentials as indicated (in millivolts).

Table 4. $GABA_A$Rs modulators of tonic inhibition

	Action	Control	Dravet	n
Zn^{2+} 40 μM	Tonic receptors blocker	27.1 *vs* 22.2 p<0.05	47.6 *vs* 40.6 p<0.05	10
DS2 10 μM	δ-containing PAM	17.0 *vs* 23.8 p<0.05	22.3 *vs* 29.4 p<0.05	10
THDOC 1 μM	δ-containing PAM	33.7 *vs* 39.0 p<0.05	58.4 *vs* 69.1 p<0.05	20

L655-708 50 μM	α5-containing inverse agonist	64.0 *vs* 52.9 p<0.05	63.3 *vs* 53.3 p<0.05	23

Mean I_{GABA} amplitude (nA) before and after treatment with different modulators of tonic inhibition at the concentration as shown. GABA concentration was 100 μM for DS2, L655-708 and THDOC and 500 μM for Zn^{2+}. Oocytes were pre-incubated for two min with the different compounds and maintained at -60 mV of holding potential. THDOC, 5α-pregnane-3α,21-diol-20-one; PAM, positive allosteric modulator. Patients #1-3 and #6-8, Table 1 main text. For Zn^{2+} and THDOC statistical analysis was performed using Mann-Whitney Rank Sum test.

Methods

Patients

The TSC cases included in this study were obtained from the archives of the Departments of Neuropathology of the Academic Medical Center (AMC, Amsterdam University Medical Centers) and University Medical Center Utrecht (UMCU, Utrecht); both patients had severe intellectual disability, autism, and epilepsy. All the three Dravet patients presented mutations affecting the SCN1A gene and showed severe intellectual disabilities. The predominant seizure types observed were focal impaired awareness seizures and generalized seizures, and all patients were resistant to maximal doses of different anti-epileptic drugs (Table 1, main text). Epilepsy duration was calculated as the interval in years from the age at seizure onset to the age at tissue sampling. 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, a 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. In detail, patient #1's tissue was obtained through a BioBank (NICHD Brain and Tissue Bank) and no additional information regarding this patient were retrievable from NICHD. Patient #2, the tissue was collected directly in the operating room during the autopsy at the Department of Neuropathology of AMC (Amsterdam), snap frozen in loco and subsequently shipped to Rome in dry ice. This patient suffered from intellectual disability and autism, and died following a heart failure. Patient #3 came from the University College of London, Epilepsy Brain and Tissue Bank, and data relative to both the tissue and the patient have been previously published (Brain 2011: 134; 2982–3010 | 2982; PM1/EP039).

All the TSC (#4-5) and controls (#6-8) cases were selected in the tissue bank of Department of Neuropathology of the Academic Medical Center (AMC, University of Amsterdam) and additional

data can be found in the table of patients of Ruffolo et al., 2016 (patients #11 and #16) and in the corresponding “patients” section.

RNA isolation and real-time qPCR

To evaluate the expression of $GABA_A$ (α1-5), NKCC1and KCC2 mRNA in control and DS tissues, 5 µg of total RNA for each sample was reverse-transcribed into cDNA using oligo dT primers (Eurogentec Nederland, Maastricht, The Netherlands). Primers for quantitative RT-PCR (see Supplementary Table 1) were designed using the Universal Probe Library Assay Design Center (Roche Applied Science, https://lifescience.roche.com/en_nl/brands/universal-probe-library.html#assay-design-center) on the basis of the reported mRNA sequences. For each PCR, a mastermix was prepared on ice, containing per sample: 1 µl cDNA, 2.5 µl of FastStart Reaction Mix SYBR Green I (Roche Applied Science, Indianapolis, IN, USA), 0.4 µM of both reverse and forward primers. Furthermore, a non-template control was included for each primerset. The final volume was adjusted to 5 µl with H_2O (PCR grade). The LightCycler® 480 Real-Time PCR System (Roche Applied Science, Basel, Switzerland) was used with a 384-multiwell plate format. The cycling conditions were carried out as follows: initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 65°C for 10 s and extension at 72°C for 15 s. The fluorescent product was measured by a single acquisition mode at 72°C after each cycle. For distinguishing specific from non-specific products and primer dimers, a melting curve was obtained after amplification by holding the temperature at 65°C for 60 s followed by a gradual increase in temperature to 95°C at a rate of 0.1°C s^{-1}, with the signal acquisition mode set to continuous. Quantification of data was performed using LinRegPCR in which a baseline correction and window-of-linearity are determined for each sample separately, followed by a linear regression analysis on the Log (fluorescence) per cycle to fit a straight line through the PCR data set. The slope of this line is used to determine the PCR efficiency of each individual sample. The mean PCR efficiency per amplicon and the Ct value per sample are used to calculate a starting concentration N_0 per sample, which is expressed in arbitrary fluorescence units (*Ramakers et al., 2003; Ruijter et al., 2009*). The starting concentration N_0 of each specific product was then divided by the geometric mean of the starting concentrations N_0 of the reference genes elongation factor 1α (EF1α) and hypoxanthinephosphoribosyltransferase 1 (HPRT) and this ratio was compared between groups.

PCR reference genes

EF1A **control** **DS**

P#	PM		P#	PM	

6	**24**	7,13859E-06	**1**	**24**	4,6522E-06
7	**20**	5,22289E-06	**2**	**20**	1,58172E-05
8	**16**	8,43E-06	**3**	**<48**	4,97525E-06
		6,93036E-06			8,48157E-06

HPRT control DS

P#	**PM**		**P#**	**PM**	
6	**24**	1,02E-06	**1**	**24**	1,21712E-06
7	**20**	8,81E-07	**2**	**20**	1,58172E-05
8	**16**	2,44E-07	**3**	**<48**	6,8557E-07
		7,16E-07			8,4118E-07

Immunohistochemistry

Immunohistochemistry was carried out as previously described (*Aronica et al., 2007*) using a KCC2 rabbit polyclonal antibody raised against the N-terminal fusion protein (residues 932– 1043) of KCC2 (Upstate Biotechnology, Lake Placid, NY, USA; 1:200). Sections, after incubation with the primary Ab (KCC2) combined with anti-$GABA_AR$ α1 subunit (mouse clone BD24, Merck Millipore, Amsterdam, The Netherlands; 1:50) were incubated for 2 h at RT with Alexa Fluor ® 568-conjugated anti-rabbit IgG and Alexa Fluor® 488 anti-mouse IgG (1:100, Fisher Scientific, Landsmeer, The Netherlands). Sections were then analyzed using Leica Confocal Microscope TCS SP8 X (Leica, Son, The Netherlands).

Human brain tissue fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 6 µm, mounted on pre-coated glass slides (Star Frost, Waldemar Knittel, Braunschweig, Germany) and processed for immunohistochemical staining. Sections were deparaffinated in xylene, rinsed in ethanol (100%, 96%, 70%) and incubated for 20 minutes in 0.3% hydrogen peroxide diluted in methanol. Antigen retrieval was performed using a pressure cooker in 10 mM sodium citrate, pH 6.0 at 121°C for 10 minutes. Slides were washed with phosphate-buffered saline (PBS; pH 7.4) and incubated overnight with primary antibody (KCC2 (Upstate Biotechnology, Lake Placid, NY, USA; 1:200) at 4°C. For single labeling, staining was performed using 3'-3'-diaminobenzidine (DAB) substrate solution (1:10 in 0.05 M Tris-HCl, pH 7.6; ImmunoLogic, Duiven, The Netherlands) with 0.015% H_2O_2. The reaction was stopped by washing with distilled water after which sections were dehydrated in alcohol and xylene and coverslipped. For double-

labeling of KKC2 with anti-$GABA_AR$ $\alpha 1$ subunit (mouse clone BD24, Merck Millipore, Amsterdam, The Netherlands; 1:50) and after overnight incubation at 4°C and rinsing, sections were incubated with secondary antibodies, Alexa Fluor 488 donkey anti-mouse IgG (H+L) and Alexa Fluor 568 goat anti-rabbit IgG (H+L) (1:200, Invitrogen, Eugene, OR, USA) for two hours at room temperature and washed in PBS. Sections were coverslipped with Vectashield with DAPI (H-1200, Vector laboratories Inc., Burlingame, CA, USA). Fluorescent microscopy was performed using a Leica Confocal Microscope TCS SP8-X (Leica, Son, The Netherlands).

KCC2 quantification (Fig. 4B)

The digital images were processed by Fiji (ImageJ2). Color threshholding was performed based on the HSB color space (red signal allowed to pass: HUE= 228-255; SATURATION=0-255; BRIGHTNESS=38-255). The mean grey value as equivalent of the mean intensity was used for graphical representation.

Con	Dravet
27,98	24,024
27,735	22, 735
28	22,32
28	23

Membrane preparation and injection

Tissues were homogenized using a Teflon glass homogenizer with 2 ml of assay buffer of the following composition (in mM): 200 glycine, 150 NaCl, 50 ethylene glycol tetraacetic acid (EGTA), 50 ethylenediaminetetraacetic acid (EDTA), 300 sucrose; 20 µl protease inhibitors (Sigma Aldrich Inc., St. Louis, MO, USA); pH 9 (adjusted using NaOH). The homogenate was centrifuged for 15 min at 9,500 g. The supernatant was collected and centrifuged for 2 h at 105 g at 4°C. The pellet was washed, re-suspended in 5 mM glycine and used directly, or aliquoted and stored at -80°C for later use. The use of female *Xenopus laevis* frogs conformed to institutional policies and guidelines of the Italian Ministry of Health (no. authorization 78/2015-PR). In a set of experiments intranuclear injection of human complementary DNA (cDNA) in *Xenopus* oocytes has been performed. We used α1, β2 and γ2 GABA subunits cDNAs enclosed in pcDNA3 vectors and performed injection in two different combinations (α1β2γ2 and α1β2). Human cDNA used in the present study was a kind gift by Dr. Keith Wafford.

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 (Conti et al., 2011). The oocytes were placed in a recording chamber (0.1 mL volume) and perfused continuously with oocyte Ringer solution (OR: NaCl 82.5 mM; KCl 2.5 mM; CaCl 2 2.5 mM; MgCl 2 1 mM; Hepes 5 mM, adjusted to pH 7.4 with NaOH) at room temperature (20 – 22 C°). The perfusion was controlled by computer (Biologique RSC-200; Claix, France) through a gravity driven system (9-10 ml/min).

GABA was always freshly dissolved in OR and where otherwise indicated applied for 4 s to elicit inward currents (I_{GABA}).

The concentration of agonist used in each experiment is always specified in the results section. When performing dose-response relationships we used different concentrations of GABA, ranging from 1 μM to 2 mM, and these doses have been determined basing on the total absence of response at the lower dose tested and the presence of a plateau phase at the maximum dose in the curve. GABA pulses were applied every 4 minutes to avoid receptor desensitization and to determine the half-maximal effect (EC_{50}) our data was fitted to Hill equations, using least-square routines as previously described (*Palma et al., 2002*)

Unless otherwise stated the recordings were performed at a holding potential (V_H) of -60 mV. When constructing current-voltage (I-V) relationships the V_H was stepped for 2-4 minutes at the desired value before applying the neurotransmitter. For these experiments, electrodes were filled with K-Acetate (3 M – *Conti et al., 2011*) to reduce the leakage of high concentration of Cl^- from electrodes into the oocytes. However, the experiments gave same results when KCl filling solution was used (not shown).The GABA reversal potential (E_{GABA}) was determined by fitting the I-V relationships with a regression curve-fitting software (Sigmaplot 12 software). When necessary the curve-fitting and E_{GABA} determination took place before and after pretreatment with Bumetanide (Bum – 2h; *Palma et al., 2006*). The I_{GABA} decay was defined as the time required for the current to decay from its peak to the half-peak value ($T_{0.5}$). In some experiments GABA was co-applied with other drugs after a short pre-incubation (L655-708, selective inverse agonist of $GABA_A$Rs containing the α5 subunit (*Atack et al.,2006)* ; THDOC and DS2, positive modulators of the "tonic" δ-containing $GABA_A$Rs (*Palma et al., 2007, Jensen et al., 2013*); Zn^{2+}, which inhibits preferentially γ2-lacking $GABA_A$Rs (*Palma et al., 2007*). In order to test the effect of flunitrazepam (FLU) or cannabidiol (CBD) on GABA currents amplitude, the cells were preincubated for 10 seconds with these drugs before the coapplication of 4 s of GABA (4s, 1 or 5 μM in cDNA experiments; the EC_5 values reported for these $GABA_A$Rs subunit composition; *Bakas et al. 2017*) (7s, 150 μM in membranes experiments) plus FLU or CBD *(Palma et al., 2002)*. In these cases, when reporting data, we express the percentage of increase or decrease of the GABA-evoked current relative to the first GABA application that

preceded the exposition to the drug. In few experiments, voltage-gated sodium channels were elicited by clamping the microtransplanted oocytes at -100 mV and giving brief depolarizing pulses (100 ms). Leak and capacitive transient transients were removed by subtraction with records obtained in presence of 300 nM TTX preapplied 1 min before giving the stimulation pulses (*Nguyen et al., 1998; Miledi et al., 2004*). All the drugs were purchased by Sigma Aldrich (USA) except all the modulators of tonic inhibition (by Tocris Bioscience, Bristol, UK). Cannabidiol was purchased by THC Pharma (Frankfurt, Germany) while flunitrazepam was a kind gift of Dr. Letizia Antonilli, Sapienza University of Rome.

Statistics

Before data analysis, normal distribution was assessed with Shapiro-Wilk test and Levene test. According to the result parametric (Student's t-test) or non-parametric (Wilcoxon signed rank test, Mann-Whitney rank sum test) tests have been used. Data in figure 5 were statistically analyzed using analysis of variance [ANOVA] and *post hoc* Holm–Sidak test as appropriated. The statistical analysis of the data was performed with Sigmaplot 12 software, and differences between two data sets were considered significant when $p < 0.05$.

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