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Enhanced GABAergic Network and Receptor Function in Pediatric Cortical Dysplasia Type IIB Compared with Tuberous Sclerosis Complex

Carlos Cepeda^{1,*}, Véronique M. André^{1,*}, Jason S. Hauptman^{1,2}, Irene Yamazaki¹, My N. Huynh², Julia W. Chang², Jane Y. Chen¹, Robin S. Fisher¹, Harry V. Vinters³, Michael S. Levine¹, and Gary W. Mathern^{1,2}

¹Intellectual and Developmental Disabilities Research Center, David Geffen School of Medicine, University of California Los Angeles

²Department of Neurosurgery, Section of Neuropathology, David Geffen School of Medicine, University of California Los Angeles

³Department of Neurology, Section of Neuropathology, David Geffen School of Medicine, University of California Los Angeles

Abstract

Tuberous sclerosis complex (TSC) and cortical dysplasia Type IIB (CDIIB) share histopathologic features that suggest similar epileptogenic mechanisms. This study compared the morphological and electrophysiological properties of cortical cells in tissue from pediatric TSC (n=20) and CDIIB (n=20) patients using whole-cell patch clamp recordings and biocytin staining. Cell types were normal-appearing and dysmorphiccytomegalic pyramidal neurons, interneurons, and giant/balloon cells, including intermediate neuronal-glial cells. In the cortical mantle, giant/balloon cells occurred more frequently in TSC than in CDIIB cases, whereas cytomegalic pyramidal neurons were found more frequently in CDIIB. Cell morphology and membrane properties were similar in TSC and CDIIB cases. Except for giant/balloon and intermediate cells, all neuronal cell types fired action potentials and displayed spontaneous postsynaptic currents. However, the frequency of spontaneous glutamatergic postsynaptic currents in normal pyramidal neurons and interneurons was significantly lower in CDIIB compared with TSC cases and GABAergic activity was higher in all neuronal cell types in CDIIB. Further, acutely dissociated pyramidal neurons displayed higher sensitivity to exogenous application of GABA in CDIIB compared with TSC cases. These results indicate that, in spite of similar histopathologic features and basic cell membrane properties, TSC and CDIIB display differences in the topography of abnormal cells, excitatory and inhibitory synaptic network properties, and GABA_A receptor sensitivity. These differences support the notion that the mechanisms of epileptogenesis could differ in patients with TSC and CDIIB. Consequently, pharmacologic therapies should take these findings into consideration.

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Address for Correspondence: Carlos Cepeda, Ph.D. Intellectual and Developmental Disabilities Research Center Semel Institute for Neuroscience, Room 58-258 UCLA School of Medicine 760 Westwood Plaza Los Angeles, CA 90024 Tel: (310) 206-0861 Fax: (310) 206-5060 ccepeda@mednet.ucla.edu.

*These authors contributed equally.

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Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant disease linked to *TSC1* and *TSC2* mutations. The proteins encoded by these genes, hamartin and tuberlin respectively, are involved in cellular migration, proliferation, and differentiation in multiple organs *via* a negative regulation of the mTOR pathway (Crino et al., 2006). In the brain, TSC is associated with the presence of cortical and subcortical tubers, as well as subependymal giant cell astrocytomas and nodules. The histopathology of cortical tubers shows severe laminar disorganization and the presence of dysmorphic-cytomegalic neurons and giant cells, suggesting that tubers are areas of malformed cerebral cortex. Epilepsy occurs in up to 85% of TSC patients at some point in their lives (Curatolo et al., 2008; Sparagana and Roach, 2000). Seizures can begin as early as the first day of life, are often associated with infantile spasms, and can be frequently refractory to pharmacologic treatment (Chu-Shore et al 2010).

Focal cortical dysplasia (CD) of Taylor is another malformation of cortical development that is associated with refractory epilepsy (Taylor et al., 1971). It is the most common histopathologic substrate in infants and young children undergoing surgery for the treatment of epilepsy (Lerner et al., 2009; Mathern et al., 1999). According to the new consensus classification of CD (Blumcke et al., 2011), CD Type I (CDI) is characterized by mild cytoarchitectural abnormalities and CD Type II (CDII) by the additional presence of dysmorphic-cytomegalic neurons (CDIIA) and balloon (CDIIB) cells. Giant cells in TSC and balloon cells in CDIIB are morphologically indistinguishable (Blumcke et al., 2011). Hence, CDIIB and TSC share a number of histopathologic similarities (Gumbinger et al., 2009; Mackay et al., 2003; Vinters et al., 1993).

The morphology and electrophysiology of normal and abnormal cells in pediatric CD cases have been examined (Andre et al., 2007; Cepeda et al., 2006; Cepeda et al., 2003; Mathern et al., 2000). However, little is known about the electrophysiological properties of similar cortical cells in tissue from TSC patients (Cepeda et al., 2003; Wang et al., 2007). The aim of the present study was to compare the morphological and electrophysiological properties of normal and abnormal cells in the cerebral cortex of pediatric TSC and CDIIB patients. Based on our previous anatomical studies demonstrating differences in cortical neuronal densities between TSC and CD cases (Chandra et al., 2007) we hypothesized that, despite similar histopathology, there would be differences in network properties when comparing cells from TSC with CDIIB cases. A preliminary report on changes in excitatory synaptic activity based on a smaller cohort of TSC and CD patients and without separation of CDIIA and CDIIB types was published recently (Cepeda et al., 2010).

Methods

The research protocols were approved by the Institutional Review Board of the Human Protection Research Committee at the University of California Los Angeles (UCLA) and informed consent to use the surgically resected tissue for research was obtained through the parents or legal guardians.

Patient cohorts

Consecutive patients with TSC (n=20) who underwent surgery from 1997 to 2009 were included. All patients had cortical tubers as demonstrated by high resolution magnetic resonance imaging (MRI) and Fluoro-2-deoxyglucose positron emission tomography (FDG-PET) evaluations and verified by histopathologic examination. Genetic analysis of brain samples was obtained from 12 patients (Qin et al., 2010). Nine cases had *TSC2* mutations, 1 case had a *TSC1* mutation, and 2 had no recognizable *TSC1* or *TSC2* mutation. The cells

from TSC cases were compared with those from CDIB cases (n=20) displaying similar clinical features (Table 1).

Pre-surgical evaluation and clinical information

The clinical protocols used to evaluate patients have been described in detail elsewhere (Cepeda et al., 2005a; Mathern et al., 1999). The standardized evaluation included clinical history and neurological examinations, and interictal and ictal scalp EEG video-recordings. Neuroimaging studies included MRI and FDG-PET (Salamon et al., 2008). Clinical data were abstracted from the medical records and included: age at seizure onset, age at surgery, history of infantile spasms, type of operation (hemispherectomy, multilobar, lobar/focal), side of operation, gender, seizure frequency presurgery (daily or weekly), number of antiepileptic drugs (AEDs) at surgery, seizure freedom and duration of follow-up (Hemb et al., 2010). Epilepsy duration was calculated as the interval in years from age at seizure onset to age at surgery. Also collected was the region of the brain sampled for *in vitro* cellular electrophysiological studies.

Sample selection and *in vitro* electrophysiology

Neocortical sample sites were excised for *in vitro* electrophysiological and histological evaluation based on abnormal neuroimaging and electrocorticography (ECoG) assessments. Tissue samples were classified as most (MA) and least abnormal (LA) according to published criteria (Cepeda et al., 2003). Sample sites (~2 cm³) were removed microsurgically and directly placed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 130, NaHCO₃ 26, KCl 3, MgCl₂ 5, NaH₂PO₄ 1.25, CaCl₂ 1.0, glucose 10 (pH 7.2-7.4). Within 5-10 min, slices (350 µm) were cut (Microslicer, DSK Model 1500E or Leica VT1000S) and placed in ACSF for at least 1h (in this solution CaCl₂ was increased to 2 mM and MgCl₂ was decreased to 2mM). Slices were constantly oxygenated with 95% O₂-5% CO₂ (pH 7.2-7.4, osmolality 290-300 mOsm, at room temperature). Methodological details for cell visualization and identification have been published elsewhere (Cepeda et al., 2003). Patch electrodes (3-6 MΩ) were filled with (in mM) Cs-methanesulfonate 125, NaCl 4, KCl 3, MgCl₂ 1, MgATP 5, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 9, HEPES 8, GTP 1, phosphocreatine 10 and leupeptine 0.1 (pH 7.25-7.3, osmolality 280-290 mOsm) for voltage clamp recordings or K-gluconate 140, HEPES 10 MgCl₂ 2, CaCl₂ 0.1, EGTA 1.1, and 2 K₂ATP (pH 7.25-7.3, osmolality 280-290 mOsm) for current clamp recordings. Electrodes also contained 0.2% biocytin (Sigma, St. Louis, MO) in the internal solution to label visualized cells. The access resistance ranged from 8-20 MΩ. Liquid junction potentials (~6 mV) were not corrected. After the experiment, the slice was fixed in 10% formaldehyde and processed according to published protocols (Horikawa and Armstrong, 1988).

Cells were initially held at -70 mV in voltage clamp mode. At this holding potential, passive membrane properties in slices were determined by applying a depolarizing step voltage command (10 mV) and using the membrane test function integrated in the pClamp (version 8) software (Axon Instruments, Foster City, CA). This function reports membrane capacitance (in pF), input resistance (in MΩ or GΩ) constant (in ms or µs). After characterizing the basic membrane properties of the cell, spontaneous excitatory (E) and inhibitory (I) postsynaptic currents (PSCs) were recorded for 3 min. The membrane current was filtered at 1 kHz and digitized at 200 µs using Clampex (Axon Instruments). Spontaneous EPSCs were isolated by holding the membrane at -70 mV, which closely corresponds to the resting membrane potential (RMP). At this potential glutamate currents mediated by α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors are maximized and the γ-aminobutyric acid (GABA) receptor-mediated currents are minimized. In some cases, the GABA_A receptor blocker bicuculline (BIC, 10 µM) was added to block

residual GABA currents. IPSCs were isolated by holding the membrane at +10 mV. At this potential GABA currents are maximized and glutamate currents are minimized. In some cases, CNQX (10 μ M) and AP5 (50 μ M) were added to block possible residual glutamate currents. Miniature (m) IPSCs were isolated by addition of TTX (1 μ M) to the external solution.

Spontaneous PSCs were analyzed off-line using the Mini Analysis Program (Jaejin Software, Leonia, NJ, USA). The threshold amplitude for the detection of an event was adjusted above root mean square noise level. This software was used to calculate EPSC/IPSC frequency and amplitude for synaptic events, and to construct amplitude-frequency and inter-event interval histograms. Frequencies were expressed as number of events per second (Hz). EPSC kinetic analysis was performed using the Mini Analysis Program.

Dissociated neurons

A detailed description of the methods used to obtain dissociated cells from human tissue samples has been published (Andre et al., 2004). Briefly, a slice was placed in an oxygenated cell-stir chamber (Wheaton, Millville, NJ) and enzymatically treated for 25-30 min with papain (0.625 mg/ml; Calbiochem, La Jolla, CA) at 35°C in a HEPES-buffered Hank's balanced salt solution (Sigma, St. Louis, MO). The tissue was rinsed with a low Ca^{2+} HEPES-buffered Na-isethionate solution and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension was plated into a Petri dish containing a HEPES-buffered salt solution. Whole-cell voltage clamp recordings were obtained using an internal pipette solution that contained (in mM): 175 N-methyl-D-glucamine (NMDG), 40 HEPES, 2 MgCl_2 , 10 EGTA, 12 phosphocreatine, 2 Na_2ATP , 0.2 Na_2GTP , and 0.1 leupeptin. The Mg^{2+} -free external solution consisted of (in mM): 127 NaCl, 20 CsCl, 2 CaCl_2 , 5 BaCl_2 , 10 glucose, 10 HEPES, and 0.0003 TTX. Signals were detected with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Drugs were applied through capillaries positioned 400-500 μ m from the cell using a pressure-driven fast perfusion system (SF-77B; Warner Instruments, Hamden, CT) synchronized by pClamp. GABA (Sigma, St. Louis, MO) was applied for 3 sec. An interval of 60 sec between each application was adequate to avoid GABA current run down.

Data analysis

Values are presented as mean $\pm$ standard deviation (SD) or standard error of the mean (SEM). Differences were considered statistically significant if $p < 0.05$. Statistical differences between groups were examined using Student's *t* or Mann-Whitney Rank Sum tests, appropriate one-way or two-way ANOVAs followed by Bonferroni *post hoc* tests, and Kolmogorov-Smirnov (K-S) for distributions using the Sigmastat or Statview (SAS, Cary, NC) software. For dissociated neurons analyses were performed with Origin (Microcal Software, Northampton, MA) and pClamp software. Membrane properties and GABA current characteristics were compared by a one-way or two-way ANOVA followed by Fisher's PLSD tests. For EC_{50} measurements, currents induced by increasing concentrations of GABA were plotted semi-logarithmically, and fitted with the Hill equation. The comparison between clinical and experimental data was performed using ANOVA, *t*-tests, Chi-square, or linear correlations where appropriate (cf. Hemb et al., 2010).

Results

TSC and CDIB cohorts and clinical findings

The TSC pediatric population comprised 20 cases (12 females) ranging in age from 1.1 to 10.1 yr. All TSC cases had one or more cortical tubers by MRI (Fig. 1A, left panel). Generalized and partial seizures occurred in all but one case that presented with complex

partial seizures, and infantile spasms occurred in 15 cases (75%). The CDIIIB group comprised 20 cases (11 females) ranging in age from 0.2 to 9.2 yr. Generalized and partial seizures were seen in all cases and a history of infantile spasms was documented in 7 patients (35%). All CDIIIB patients had positive neuroimaging involving cortical malformations (Fig. 1A, right panel).

Most of the clinical characteristics were similar between TSC and CDIIIB groups (Table I). There were no significant differences in age at seizure onset, age at surgery, gender, side of operation, seizure frequency before surgery, number of AEDs at the time of surgery, percent of patients seizure-free after surgery, and duration of follow-up ($p>0.07$). Epilepsy duration was longer ($p=0.026$) and the frequency of infantile spasms was higher ($p=0.011$) in the TSC compared with the CDIIIB group. By neuroimaging, 4 patients had hemimegalencephaly, of which 1 was in the TSC and 3 were in the CDIIIB group ($p=0.373$). This association, although rare, is not unprecedented (Guerra et al., 2007). More patients with CDIIIB underwent hemispherectomy compared to the TSC group ($p=0.009$). Seizure freedom was obtained in 77.5% of this combined cohort without surgical mortality.

At the time of surgery all patients were taking AEDs, and a total of 13 AEDs were used in mono- ($n=2$) and poly-therapy ($n=38$). In descending order, the most common AEDs were: topiramate ($n=16$; 40%), levetiracetam ($n=13$; 32.5%), carbamazepine ($n=13$; 32.5%), lamotrigine ($n=12$; 30%), phenobarbital ($n=9$; 22.5%), zonisamide ($n=8$; 20%), vigabatrin ($n=7$; 17.5%), and clorazepate ($n=6$; 15%). Comparing patients with TSC and CDIIIB, there were no differences in patients taking a particular AED ($p>0.08$, Chi-square) with one exception. At the time of surgery, more patients with TSC were taking vigabatrin ($n=7$) compared to those with CDIIIB ($n=0$, $p=0.004$). However, it should be noted that only 35% of patients with TSC were taking vigabatrin at the time of surgery.

Tissue samples and principal pathologic findings

In the 20 TSC patients, a total of 30 cortical tissue samples were examined (16 from the right side). They were from the frontal ($n=9$), temporal ($n=10$), and parietal ($n=11$) regions. The majority of samples were classified as MA ($n=25$) and contained tissue from the tuber. Fewer samples ($n=5$) were classified as LA, and were at the edge of the tuber and part of the planned resection. All children had confirmed tubers upon histopathologic examination using Haematoxylin & Eosin, Nissl and Bielschowsky staining (Fig. 1B, left panels). The main findings included cortical dyslamination, pyramidal neuron misorientation, and the presence of dysmorphic-cytomegalic neurons. Cell clustering and heterotopic neurons were also a common finding. Giant cells with pale, glassy, eosinophilic cytoplasm were found in all cases, mostly in white matter but in many cases also extending to the cortical mantle. Astrogliosis and microcalcifications were observed in some TSC cases.

In the 20 CDIIIB patients, 29 cortical tissue samples were examined (11 from the right side). The majority of samples were classified as MA ($n=21$) and a few samples ($n=8$) were classified as LA. They were from the frontal ($n=16$), temporal ($n=7$), and parietal ($n=6$) regions. There were no differences in sampled regions between the TSC and CDIIIB groups ($p=0.356$, Chi-square, Table I). Similar to TSC cases, the histopathology of CDIIIB cases showed severe architectural abnormalities, neuronal disarray and abnormal polarity, neuronal cytomegaly and balloon-type gemistocytic astrocytes restricted in three cases to the white matter (Fig. 1B, right panels).

Morphology of individual cells in TSC and CDIIIB

A total of 156 cells were recorded electrophysiologically from TSC tissue and 65% were successfully labeled with biocytin (Fig. 2). Based on previously published criteria (Cepeda

et al., 2003) cells were classified, in descending order of frequency, as normal-appearing pyramidal neurons (n=87, including 13 immature-looking cells), giant cells (n=47), interneurons (n=14) and cytomegalic pyramidal neurons (n=8). As expected, the average area of cytomegalic pyramidal neurons and giant cells was larger than the somatic area of normal-appearing pyramidal neurons ($p < 0.05$, Table II). The somata of most giant cells were round but others were elongated. They did not have axons and their processes (7-20) had a wavy, hair-like appearance (Supplementary Fig. 1). Overall these cells resembled enlarged astrocytes (Sosunov et al., 2008). However, three cells looked more neuronal-like with somata similar to those of pyramidal neurons but whose processes appeared like those of typical giant/balloon cells (Fig. 4A). We identified these as intermediate neuronal-glial cells (cf. Blumcke et al., 2011; Talos et al., 2008).

In the CDIIB cohort we recorded 129 cells and, as in TSC, the same types of cells were found (Fig. 3) but with different frequency. Sampled cells were normal-appearing pyramidal neurons (n=74, including 6 immature-looking pyramidal cells), cytomegalic pyramidal neurons (n=25), balloon cells (n=16, including one intermediate neuronal-glial cell), and interneurons (n=14). Thus, in our experimental conditions, we observed more giant cells in TSC than balloon cells in CDIIB samples ($p = 0.028$), whereas more dysmorphic-cytomegalic neurons were observed in CDIIB than in TSC samples ($p = 0.032$; Table 1). There were no differences in somatic areas and overall morphologic characteristics between cell types from TSC and CDIIB samples based on biocytin measurements.

Passive and active membrane properties of normal and abnormal cell types in TSC and CDIIB

Although there were differences in membrane capacitance, input resistance and time constant among cell types within TSC or CDIIB tissue ($p < 0.001$, one-way ANOVA), these properties did not differ when comparing the same cell types between TSC and CDIIB tissue (Table II). In both TSC and CDIIB, interneurons had the smallest membrane capacitance and highest input resistance, whereas cytomegalic pyramidal neurons had the largest capacitance and lowest input resistance. Giant/balloon cells also had very large capacitance but their input resistance was variable, with some cells having very low ($\sim 20 \text{ M}\Omega$) and others very high ($\sim 1 \text{ G}\Omega$) values. This variability may reflect morphological differences and the predominance of a glial (low input resistance) over a neuronal-like (high input resistance) phenotype (Talos et al., 2008).

The RMP of normal-appearing pyramidal neurons and interneurons was similar, whereas cytomegalic pyramidal neurons and giant/balloon cells were more hyperpolarized (Table II). Action potentials could be evoked in all neurons but not in giant/balloon cells. The current-voltage (IV) relationships were fairly typical for normal-appearing and cytomegalic pyramidal neurons, with inward rectification in the hyperpolarizing direction. Giant/balloon cells displayed an almost linear relationship in the hyperpolarizing direction but in the depolarizing direction they showed strong rectification, probably caused by the opening of delayed rectifier K^+ channels (Fig. 2, arrow).

In normal-appearing and cytomegalic pyramidal neurons repetitive action potentials were readily elicited once the threshold for action potential firing was reached. In interneurons the action potential duration was generally shorter than in normal-appearing pyramidal neurons and the amplitude of the action potential afterhyperpolarization was larger (Fig. 2, arrow). Action potentials could never be elicited in balloon cells, even at highly depolarized membrane potentials. There were no significant differences in resting membrane potentials, firing properties, or current-voltage relationships of normal pyramidal neurons from TSC and CDIIB cases (Figs. 2-3).

In voltage clamp recordings, normal-appearing and cytomegalic pyramidal neurons had prominent inward currents including persistent and fast Na^+ currents, as well as transient and/or slowly inactivating Ca^{2+} currents. Most interneurons had negligible Ca^{2+} currents and fast-firing interneurons displayed repetitive Na^+ currents (not shown). Giant/balloon and intermediate cells (Fig. 4B and C) did not display inward currents. In contrast, small outward currents were commonly observed in these cells.

Spontaneous glutamatergic currents in TSC and CDIIIB

Except for the giant cells, all neurons from TSC cases displayed spontaneous EPSCs. Normal-appearing pyramidal neurons and interneurons displayed the highest frequencies of spontaneous EPSCs (average 2.95 ± 0.5 and 4.6 ± 0.9 Hz, respectively), whereas cytomegalic pyramidal neurons generally displayed low frequencies (average 1.8 ± 0.5 Hz). The frequency of spontaneous EPSCs was significantly higher in normal-appearing pyramidal neurons from TSC compared with CDIIIB cases ($p=0.029$, Mann-Whitney Rank Sum test) (Fig. 5A and 5B inset). Amplitude-frequency histograms showed that increased frequencies occurred at most amplitude bins, encompassing low-, medium- and large-amplitudes (Fig. 5B). Cumulative inter-event interval (the reciprocal of frequency) probability distributions between normal pyramidal neurons from TSC and CDIIIB cases also were different ($p<0.05$, K-S, Fig. 5C). In contrast, cumulative amplitude probability histograms were similar, suggesting differences were probably due to presynaptic mechanisms.

The EPSC frequency of interneurons from TSC was significantly higher compared to interneurons from CDIIIB ($p=0.012$, Student's *t*-test). Amplitude-frequency histograms demonstrated that differences occurred across most amplitude bins and cumulative inter-event interval distributions were significantly different ($p<0.0001$, K-S). Although the average frequency of spontaneous EPSCs in cytomegalic neurons from TSC and CDIIIB was not different ($p=0.995$) due to the low number of cytomegalic pyramidal neurons sampled from TSC cases, the cumulative inter-event interval probability distributions were different ($p=0.001$, K-S). The kinetics of spontaneous synaptic events, including rise time, decay time and half-amplitude duration, of normal and cytomegalic pyramidal neurons and interneurons were similar between TSC and CDIIIB groups (Table III).

Spontaneous GABAergic currents in TSC and CDIIIB

GABA_A receptor-mediated spontaneous currents were relatively abundant in all neuronal types from TSC cases (normal pyramidal 8.0 ± 1.3 Hz, cytomegalic 10.8 ± 3.6 Hz, and interneurons 6.7 ± 0.6 Hz) (Fig. 6A). As expected, giant/balloon cells displayed no spontaneous GABA synaptic activity. The average frequency of spontaneous IPSCs in normal-appearing pyramidal neurons from CDIIIB cases was higher than in cells from TSC cases ($p=0.018$, Mann-Whitney Rank Sum test). Amplitude-frequency histograms showed that increased frequencies occurred at most amplitude bins, but were more evident in the larger amplitude bins (>25 pA) and were significantly different for events >50 pA (Fig. 6B). Similarly, the average IPSC frequencies in cytomegalic pyramidal neurons and interneurons from CDIIIB tended to be higher than in TSC samples, but because of the low numbers of cells sampled and high variability between cells the differences did not reach statistical significance. Reflecting increased frequencies, cumulative inter-event interval probability distributions demonstrated a significant leftward shift in pyramidal neurons ($p<0.0001$, K-S) and interneurons ($p=0.0152$, K-S), but only marginal in cytomegalic pyramidal neurons ($p=0.0699$), from CDIIIB compared to TSC cases (Fig. 6C). In contrast, cumulative amplitude probability histograms were similar (not shown). Finally, the kinetic properties of spontaneous IPSCs were not different in cells from TSC and CDIIIB cases although there was a trend for normal and cytomegalic pyramidal neurons from CDIIIB cases to display slower decay times (Table III).

In a few normal pyramidal cells from TSC (n=4) and CDIIB (n=4) cases mIPSCs were isolated by addition of TTX to the external solution (not shown). The difference in average frequency between both groups remained after TTX (TSC 1.5 ± 0.3 Hz and CDIIB 2.6 ± 0.4 Hz, $p < 0.05$). Cumulative inter-event interval probability distributions were also different ($p < 0.0001$, K-S, not shown). In contrast, the cumulative amplitude probability histogram and kinetics were similar, suggesting the involvement of presynaptic mechanisms (Redman, 1990).

GABA-glutamate synaptic ratio

In the subset of cases where the frequency of glutamate and GABA synaptic activities could be measured in the same cell, an index ratio was obtained by dividing the average GABA frequency by the average glutamate frequency for each patient (TSC, n=7 and CDIIB, n=9). This analysis included 56 cells from TSC (50 normal pyramidal, 2 cytomegalic, and 4 interneurons) and 72 cells from CDIIB (48 normal pyramidal, 13 cytomegalic, and 11 interneurons). The average GABA-glutamate ratio in TSC cases was lower (4.2 ± 0.9) than in CDIIB cases (10.2 ± 0.8 , $p < 0.001$) confirming that, relative to glutamate, GABA synaptic activity is higher in CDIIB compared with TSC cases.

GABA_A receptor function in dissociated normal-appearing pyramidal neurons from TSC and CDIIB cases

To examine postsynaptic GABA_A receptor sensitivity in TSC and CDIIB cases we used the isolated cell preparation. After recording the membrane properties, GABA was exogenously applied to normal-appearing pyramidal neurons (n=15, from 5 TSC cases and n=13, from 5 CDIIB cases). In all cells a clear apical dendrite and some basilar dendrites were present (Fig. 7A, left panels). No differences in capacitance, input resistance or time constant were found between cells from TSC and CDIIB cases (Table II). Application of increasing concentrations of GABA induced typical outward currents ($V_{\text{hold}} = -40$ mV) with a fast peak followed by a rapidly desensitizing current more evident at the higher concentrations (Fig. 7A, right panel). Peak current amplitudes and current densities at different GABA concentrations were larger in CDIIB compared with TSC pyramidal neurons at all concentrations tested (1, 10, 100 and 1000 μM) (Fig. 7B). Furthermore, the EC_{50} was significantly lower in cells from CDIIB (17.6 ± 2.2 μM) compared with TSC cases (30.3 ± 4.5 μM), indicating increased GABA_A receptor sensitivity in CDIIB pyramidal neurons (Fig. 7C).

Correlation with Clinical Data

Clinical variables that differed between TSC and CDIIB cases, including epilepsy duration, history of infantile spasms and use of vigabatrin (Table 1), were compared with glutamate and GABA synaptic activities and GABA_A receptor sensitivity. Results showed that none of these clinical variables was associated with differences in synaptic activity or GABA_A receptor sensitivity ($p > 0.08$). In other words, the observed differences were not confounded by identified clinical variables, including use of AEDs, between TSC and CDIIB cases.

Discussion

This study found that, despite similarities in cell type, morphology, and basic membrane properties, there are differences in cell distribution and network connectivity between TSC and CDIIB patients. Within the cortex, giant/balloon cells were more frequently sampled in TSC compared with CDIIB, whereas dysmorphic-cytomegalic neurons were more common in CDIIB. Glutamatergic synaptic activity was lower and GABAergic synaptic activity was higher in CDIIB compared with TSC cases and, in the dissociated cell preparation, normal pyramidal neurons had more robust responses to exogenous GABA application in CDIIB.

We also characterized a small number of intermediate neuronal-glial cells in TSC and CDIIIB cases and found that they were similar to giant/balloon cells as they lack the ability to generate action potentials. These results were not associated with different clinical variables, including use of AEDs, supporting the idea that the findings were intrinsic to cell and network properties of patients with TSC and CDIIIB. Collectively, these results support that notion that, comparing TSC and CDIIIB patients, there are unique cell and network properties that should be taken into consideration when contemplating mechanisms of epileptogenesis and possible pharmacologic treatment for these two epilepsy syndromes.

Cell morphology, distribution, and membrane properties

Similar histopathology and cell types have been described in cortical tissue from CDIIIB and TSC cases (Andre et al., 2007; Cepeda et al., 2003; Sisodiya et al., 2009), but no electrophysiological studies had directly compared cells from these two etiologies. In both TSC and CDIIIB normal-appearing pyramidal neurons were the predominant cell type and there were no differences in the frequency of occurrence of interneurons and immature cells. In contrast, balloon cells were more difficult to find in CDIIIB, probably because they tend to cluster in the white matter, and dysmorphic-cytomegalic neurons were found more frequently. This observation suggests that the developmental migration patterns and final destination of abnormal dysmorphic-cytomegalic neurons and giant/balloon cells are different between TSC and CDIIIB cases. This topographic variation could lead to differential synaptic arrangements and mechanisms of epileptogenesis. This observation is supported by our previous anatomical findings that neuronal density in superficial cortical layers and white matter is higher in CDIIIB compared with TSC, whereas neuronal density in deep cortical layers is decreased in TSC (Chandra et al., 2007).

Balloon cells in CDIIIB and giant cells in TSC have been difficult to classify due to their neuronal-glial morphology. Immunocytochemical studies have shown that giant/balloon cells express neuronal and glial markers alone or in combination (Englund et al., 2005; Farrell et al., 1992; Mathern et al., 2000), as well as a wide variety of embryological markers (Crino et al., 1996; Fukutani et al., 1992; Hirose et al., 1995; Thom et al., 2005; Ying et al., 2005). Anatomical studies first suggested that balloon cells were undifferentiated, mimicking features of neuroembryonic development (Huttenlocher and Heydemann, 1984; Machado-Salas, 1984; Probst and Ohnacker, 1977; Trombley and Mirra, 1981). Electrophysiological recordings demonstrated that giant/balloon and neuronal-glial cells from TSC and CDIIIB are unable to generate action potentials, (Cepeda et al., 2005b; Cepeda et al., 2003), suggesting their role in epileptogenesis is probably minor (Boonyapisit et al., 2003). In contrast, dysmorphic-cytomegalic pyramidal neurons, both in TSC and CDIIIB, have the potential to sustain epileptiform activity due to their hyperexcitability (Cepeda et al., 2005b). This differential contribution is supported by studies demonstrating cell-specific alterations in glutamate and GABA receptor subunit gene transcription and protein expression in dysmorphic neurons and giant cells in TSC (Crino et al., 2001; Talos et al., 2008; White et al., 2001).

Synaptic activity and GABA_A receptor sensitivity

At the circuit level, this study demonstrated that glutamatergic tone was lower in CDIIIB compared with TSC cases, while the GABAergic tone was higher in CDIIIB. In slices from CDIIIB tissue the frequency and sometimes the amplitude of spontaneous IPSCs were increased and, in dissociated normal pyramidal neurons, the sensitivity of GABA_A receptors was higher. Thus, in pediatric CDIIIB cases cortical cellular hyperexcitability cannot be due to decreased GABAergic inputs or GABA_A receptor sensitivity alone. If there is reduced glutamate activity in CDIIIB, one possibility is that, because of the relative immaturity of neuronal circuits, GABA may act as an excitatory neurotransmitter (Cepeda et al., 2007;

Cherubini et al., 1991). The cause of cortical hyperexcitability in TSC cases is more difficult to grasp as we found no clear decreases in IPSC or increases in EPSC frequencies (cf. Cepeda et al., 2005a). A study in one patient with a *TSC2* mutation but no recognizable tubers reported increased excitation after pharmacological blockade of GABA_A receptors (Wang et al., 2007), supporting the hypothesis that inhibitory circuits are relatively intact in TSC. However, this manipulation is likely to increase cortical excitability even in non-pathologic tissue. Further, the frequency of spontaneous EPSCs was not reported, precluding a direct comparison with the present data.

What could be the etiology of increased GABA_A receptor function in CDIIIB compared with TSC? In terms of spontaneous synaptic activity, higher frequency of IPSCs and lower frequency of EPSCs are reminiscent of immature circuits and support the dysmature cerebral developmental hypothesis of severe CD (Cepeda et al., 2006). While on average the TSC cohort was slightly younger, the age difference was not significant between groups, and age at surgery did not correlate with IPSC or EPSC frequency. Higher GABA inputs could also be contributed by the recently characterized cytomegalic interneurons (Andre et al., 2007) and by supernumerary cells in superficial layers and white matter observed in CDIIIB cases (Chandra et al., 2007), particularly if, as in rodents, layer I neurons are mostly GABAergic (Wozny and Williams, 2011). This differential network connectivity argues for diverse epileptogenic and propagation mechanisms in TSC and CDIIIB. With regard to the possible mechanism of GABA_A receptor up-regulation in CDIIIB, although data from mIPSCs suggest increased probability of neurotransmitter release, our study can not rule out a concomitant increase in the number of release sites. At this point, we think that the most parsimonious explanation is that both presynaptic (increased probability of release and number of release sites), as well as postsynaptic (increased surface expression and different stoichiometry of postsynaptic GABA_A receptors) mechanisms are probably involved. Additional studies are warranted to further characterize GABAergic receptor functions in CDIIIB.

Up-regulation of GABA relative to glutamate activity in severe CD (CD Type IIA/B) compared to non-CD or mild CD (CD Type I) cases, was previously reported by our group (Andre et al., 2008; Cepeda et al., 2005a). The fact that the same difference occurred when comparing CDIIIB versus TSC indicates that synaptic activity and GABA_A receptor sensitivity in TSC more closely resembles non-CD and CD Type I cases and further underscores that the dysmature cerebral developmental hypothesis seems to be more relevant to CDIIIB cases (Cepeda et al., 2006).

Clinical Relevance

It has been suggested that TSC and CDIIIB can be considered similar pathological entities (Crino, 2007; Wong, 2008). If correct, then common treatments can be used for both etiologies. However, as shown in the present and previous studies (Baybis et al., 2004; Miyata et al., 2004), some distinctions exist between TSC and CDIIIB tissue. Because of higher GABA tone in pediatric CDIIIB, and the possibility that in this pathology GABA is excitatory (Cepeda et al., 2007), utilization of drugs that increase GABA may be less effective (Kahle and Staley, 2008). In contrast, in TSC drugs that increase GABA could be more useful, a possible reason why vigabatrin is fairly successful in controlling infantile spasms in TSC patients (Camposano et al., 2008). These findings at the cellular and network levels will need to be confirmed in clinical trials. However, our results should be taken into consideration when proposing clinical pharmacological studies using agents that alter the mTOR pathway and chloride-transporters in cases of TSC and CDIIIB.

The reader should be aware of limitations of this human study when interpreting the results. For example, we compared findings from pediatric and not adult surgical cases. It is possible

that different results might be obtained in tissue from older TSC and CDIIB patients (Calcagnotto et al., 2005; Wang et al., 2007). Furthermore, our study compared two abnormal etiologies in refractory patients undergoing epilepsy neurosurgery, and non-epileptic normal control tissue was not ethically available for this human study. While the lack of correlation between clinical variables and experimental findings in our cohort would indicate that these variables did not make a major contribution, with larger cohorts more subtle correlations could be discovered, including use of certain AEDs, longer seizure duration, and a history of infantile spasms, which might be confounding factors. Our results should be replicated in other clinical studies and appropriate experimental systems. In addition, the relative density and topography of abnormal cells in TSC and CDIIB cases need to be validated using non-biased cell counting techniques in histology specimens. Even with these potential problems, this study indicates that there are differences in network properties and GABA_A receptor sensitivity in TSC and CDIIB cases that should be taken into consideration to further understand the mechanisms of epileptogenesis in these two refractory and surgically-treated epilepsy syndromes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Manuscript Highlights

1. First characterization of normal and abnormal cells in Tuberous Sclerosis Complex
2. Differential distribution of abnormal cells in the cerebral cortex of TSC and CD Type IIB cases.
3. GABA synaptic activity significantly increased in CD Type IIB compared to TSC cases.
4. Electrophysiology of intermediate cells, demonstrating properties similar to balloon cells.

Fig. 1.

A. Representative axial MRI scans of children with refractory epilepsy from TSC (left) and CDIIB (right). Both children were 7 yr of age at the time of surgery. There are multiple cortical tubers (dashed circles) in the patient with TSC and one focal area of cortical dysplasia in the left temporal lobe in the child with CDIIB. In the TSC case the left frontal tuber was removed and both children are seizure free post-surgery. **B.** Left panels: Section of a tuber (originating from a 19 month-old male) showing abundant giant cells and disorganized collections of dysmorphic neurons. Arrows in left panel indicate giant cells in which the nucleus shows coarse chromatin, a pattern often seen in astrocytes. Right panels: Representative fields from a resection (originating from a 9 year-old female) showing features of CD type IIB. Note a balloon cell (arrow, left panel) with glassy eosinophilic cytoplasm, and dysmorphic neurons (arrows, right panel). The balloon cell shows 'retraction' of cytoplasm from the neuropil (images are from sections stained with H&E). Scale bar represents 50 μm and applies to all panels.

Fig. 2.

Morphology and electrophysiology of TSC cell types revealed by biocytin labeling and electrophysiology using whole-cell patch clamp recordings in current clamp mode. Left panels are examples of biocytin-filled normal pyramidal (A), cytomegalic (B), interneuron (C), and giant (D) cells. Calibration bar in C also applies to panels A and B. Middle panels illustrate hyperpolarizing and depolarizing voltage deflections induced by negative and positive current pulses. Note the large amplitude action potential after-hyperpolarization in the interneuron (arrow), and the strong rectification in the depolarizing direction in the giant cell (arrow). No action potentials could be evoked in giant cells despite high depolarizing current intensity. Right panels show the current-voltage relationships for each cell.

Fig. 3.

Morphology and electrophysiology of CDIIB cell types revealed by biocytin labeling and whole-cell patch clamp recordings in current clamp mode. Left panels are examples of biocytin-filled normal pyramidal (A), cytomegalic (B), interneuron (C), and balloon (D) cells. Calibration in D applies to all panels. Middle panels illustrate hyperpolarizing and depolarizing voltage deflections induced by negative and positive current pulses. Note large amplitude action potential after-hyperpolarizations in the interneuron (arrow). No action potentials could be evoked in balloon cells despite high positive current intensity. Right panels show the current-voltage relationships for each cell. The properties by cell type are similar to those from TSC cases (Fig. 2).

Fig. 4.

A. Camera lucida drawings from biocytin-filled cells with hybrid or neuronal-glia morphology from a TSC and a CD IIB case. Under IR-DIC the somata of these cells looked similar to that of pyramidal neurons. Only the lack of inward currents during electrophysiological recordings and the absence of spines and axons after biocytin processing allowed classification of these cells in the intermediate cell type category. **B.** Traces show that even highly depolarized voltage steps were unable to induce Na^+ and Ca^{2+} inward currents. Due to the large size of these cells, the capacitive artifacts at the onset and offset of the step voltage commands could not be compensated completely. **C.** The graphs are current-voltage relationships indicating only an incipient outward current at depolarized potentials.

Fig. 5.

A. Spontaneous EPSC activity recorded in voltage clamp mode at a holding potential of -70 mV (mostly glutamatergic) in different cell types from TSC and CDIIB tissue samples. Normal pyramidal neurons and interneurons from TSC cases displayed higher frequencies than cytomegalic pyramidal neurons. In comparison, synaptic activity was low in the same cell types from CDIIB cases. Giant/balloon cells from TSC and CDIIB had no spontaneous synaptic activity. **B.** Amplitude-frequency histograms of synaptic glutamate activity recorded in normal and cytomegalic pyramidal neurons and interneurons from TSC and CDIIB samples. Across amplitude bins, the frequency of spontaneous events was consistently higher in cells from TSC cases. The difference in the lower amplitude bins was significant. Insets show the average frequencies of the 3 types of cells in TSC and CDIIB. The frequencies were significantly higher in normal pyramidal neurons and interneurons from TSC samples. **C.** Cumulative inter-event interval histograms comparing the probability distributions in normal pyramidal, cytomegalic and interneurons from TSC and CDIIB samples. (In all panels asterisks indicate statistically significant differences of at least $p < 0.05$).

Fig. 6.

A. Spontaneous synaptic activity recorded in voltage clamp mode at a holding potential of +10 mV (mostly GABAergic) in different cell types from TSC and CDIIB tissue samples. In contrast to glutamatergic activity, the frequencies of spontaneous GABAergic currents were higher in CDIIB compared to TSC cases, particularly in normal pyramidal neurons and interneurons. **B.** Amplitude-frequency histograms of synaptic GABAergic activity recorded in normal and cytomegalic pyramidal neurons and interneurons from TSC and CDIIB samples. Across amplitude bins, the frequency of spontaneous events was consistently higher in cells from CDIIB cases. The difference in amplitude bins >50 was significant ($p < 0.01$) in the pyramidal neuron group. Insets show the average frequencies of the 3 types of cells in TSC and CDIIB. The frequencies were higher in normal pyramidal neurons from CDIIB samples ($p = 0.018$). Although a similar trend was seen for cytomegalic pyramidal neurons and interneurons, the low number of cells precluded reaching statistical significance. **C.** Cumulative inter-event interval histograms comparing the probability distributions in normal pyramidal, cytomegalic and interneurons from TSC and CDIIB samples.

Fig. 7.

A. Responses to exogenous application of GABA in dissociated normal pyramidal neurons from TSC and CDIIB samples. Top left panels are examples of pyramidal neurons from a TSC and a CDIIB case. Traces on the right are outward currents induced by increasing concentrations of GABA at a holding potential of -40 mV. At the higher concentrations, the response consisted of a fast peak followed by a rapidly desensitizing current. **B.** Average peak currents and current densities at increasing concentrations of GABA. At all concentrations the response was larger in cells from CDIIB compared to TSC samples. **C.** In addition, the EC_{50} was significantly reduced in cells from CDIIB samples (right bar graph), indicating higher sensitivity of GABA receptors.

Table I

Clinical Characteristics of TSC and CDIIIB Cohorts

Clinical Variable	TSC (n=20)	CDIIB (n=20)	P value
Age Seizure Onset (Yrs)	0.3±0.3	0.8±1.3	P=0.080
Age at Surgery (Yrs)	4.0±2.8	2.8±2.5	P=0.194
Epilepsy Duration (Yrs)	3.7±2.8	2.0±1.6	P=0.026
History of Infantile Spasms (%)	75%	35%	P=0.011
Side of Surgery (% Left)	45%	60%	P=0.342
Gender (% Female)	60%	55%	P=0.749
Presurgery Seizure Frequency			P=0.072
Daily or Greater	85%	100%	
Weekly	15%	0%	
Number of AEDs * Presurgery	2.6±0.7	3.1±1.0	P=0.108
Type of Operation: %			P=0.009
Hemispherectomy	10%	50%	
Multilobar	30%	5%	
Lobar/Focal	60%	45%	
Percent Seizure-Free Postsurgery	75%	80%	P=0.705
Follow Up Duration (Years)	2.1±1.1	2.1±1.3	P=0.99
Primary Sample Site: %			P=0.356
Frontal	45%	65%	
Temporal	30%	20%	
Parietal	25%	15%	
Total Cells Sampled Per Case	8.2±4.7	6.4±2.3	P=0.133
Cell Subtypes Per Case			
Normal Pyramids	3.9±3.1	3.4±1.9	P=0.515
Cytomegalic Neurons	0.4±0.6	1.2±1.6	P=0.032
Immature Neurons	0.7±1.0	0.3±0.6	P=0.147
Interneurons	0.7±0.9	0.7±0.7	P=0.891
Balloon Cells	2.5±2.3	0.7±1.8	P=0.028
Intermediate Neuronal-Glia	0.1±0.4	0.05±0.2	P=0.657

* AEDs = antiepileptic drugs

Table II

Cell Membrane Properties in Slices					
	Cm (pF)	Ri (MΩ)	Tau (ms)	RMP (mV)	Somatic Area (μm ²)
Pyramidal					
TSC (n=87)	132±7	284±48	2.3±0.2	-65±2	176±10
CDIB (n=74)	152±9	202±23	2.5±0.1	-62±2	219±20
Cytomegalic					
TSC (n=8)	309±21	51±2	4.8±0.3	-69±1	520±163
CDIB (n=25)	353±14	58±6	4.5±0.2	-65±2	380±33
Interneuron					
TSC (n=14)	73±14	329±106	0.9±0.1	-63±1	187±24
CDIB (n=14)	59±9	523±119	1.1±0.3	-58±5	207±42
Balloon					
TSC (n=47)	244±16	210±46	3.2±0.3	-74±3	445±48
CDIB (n=16)	251±25	148±28	2.9±0.4	NM	339±45

Membrane Properties of Dissociated Pyramidal Neurons			
	Cm (pF)	Ri (GΩ)	Tau (μs)
Pyramidal			
TSC (n=15)	25.3±1.8	1.4±0.2	333±52
CDIB (n=13)	28.5±2.5	1.1±0.2	294±30

NM, Not Measured.
Data are presented as mean ± SEM.

Table III

Kinetics of Spontaneous EPSCs				
	<u>Rise (ms)</u>	<u>Decay (ms)</u>	<u>Half-width (ms)</u>	<u>Amplitude (pA)</u>
<u>TSC</u>				
Pyramidal	1.7±0.1	7.4±0.3	8.2±0.3	10.1±0.4
Cytomegalic	2.9±0.7	9.4±1.9	9.3±1.9	11.8±1.1
Interneuron	2.2±0.8	5.8±1.3	7.7±2.2	11.6±3.4
<u>CDIIB</u>				
Pyramidal	1.9±0.2	7.7±0.4	9.3±0.5	10.6±0.4
Cytomegalic	2.5±0.3	10.8±1.3	12.5±1.3	12.3±1.2
Interneuron	1.5±0.5	7.6±2.7	8.3±2.7	12.6±1.5

Kinetics of Spontaneous IPSCs				
	<u>Rise (ms)</u>	<u>Decay (ms)</u>	<u>Half-width (ms)</u>	<u>Amplitude (pA)</u>
<u>TSC</u>				
Pyramidal	2.8±0.3	19.0±2.0	18.7±1.5	23.5±1.1
Cytomegalic	3.8±0.3	31.5±9.7	21.9±1.7	25.8±4.5
Interneuron	0.8±0.2	6.9±1.0	6.6±0.8	20.5±1.4
<u>CDIIB</u>				
Pyramidal	2.8±0.2	23.9±3.1	20.5±1.2	26.2±1.5
Cytomegalic	3.8±0.1	35.3±12.0	20.2±0.4	26.9±1.0
Interneuron	1.0±0.3	7.4±2.4	7.8±2.2	20.0±2.7

Data are presented as mean ± SEM.