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Altered inhibition in Tuberous Sclerosis and Type IIb cortical dysplasia

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Abstract

Objective—The most common neurological symptom of tuberous sclerosis complex (TSC) and focal cortical dysplasia (FCD) is early-life refractory epilepsy. As previous studies have shown enhanced excitatory glutamatergic neurotransmission in TSC and FCD brains, we hypothesized that neurons associated with these lesions may also express altered GABA_A receptor (GABA_AR)-mediated inhibition.

Methods—Expression of the GABA_AR subunits $\alpha 1$ and $\alpha 4$, the Na⁺-K⁺-2Cl[−] (NKCC1), and the K⁺-Cl[−] (KCC2) transporters in human TSC and FCD Type II specimens were analyzed by Western blot and double label immunocytochemistry. GABA_AR responses in dysplastic neurons from a single case of TSC were measured by perforated-patch recording and compared to normal-appearing cortical neurons from a non-TSC epilepsy case.

Results—TSC and FCD Type IIb lesions demonstrated decreased expression of the GABA_AR $\alpha 1$, increased NKCC1 and decreased KCC2 levels. In contrast, FCD Type IIa lesions showed decreased $\alpha 4$, and increased expression of both NKCC1 and KCC2 transporters. Patch clamp recordings from dysplastic neurons in acute slices from TSC tubers demonstrated excitatory GABA_AR responses that were significantly attenuated by the NKCC1 inhibitor bumetanide, in contrast to hyperpolarizing GABA_AR-mediated currents in normal neurons from non-TSC cortical slices.

Interpretation—Expression and function of GABA_ARs in TSC and FCD IIb suggests the relative benzodiazepine insensitivity and more excitatory action of GABA compared to FCD IIa. These factors may contribute to resistance of seizure activity to anticonvulsants that increase

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GABAergic function, and may justify add-on trials of the NKCC1 inhibitor bumetanide for the treatment of TSC and FCD Type IIb related epilepsy.

INTRODUCTION

Cortical malformations are a major cause of pediatric focal epilepsy and are often associated with other disorders of intellectual development, including cognitive impairments and autism¹⁻³. Tuberous Sclerosis Complex (TSC) and focal cortical dysplasia (FCD) are two major classes of cortical malformations. TSC is a multiorgan autosomal dominant disorder due to inactivating mutations in the TSC1 and TSC2 genes⁴. In contrast, the various phenotypes of FCD are not associated with consistent genetic mutations, suggesting a role of fetal, perinatal, or early postnatal insults^{1,5}.

TSC cortical tubers are well-defined dysplastic lesions and are often the source of focal epileptiform activity that can also generalize⁶. Loss of TSC1/TSC2 function in TSC results in hyperactivation of the mammalian target of rapamycin (mTOR) pathway and subsequent increased protein synthesis, abnormal cellular differentiation and growth^{6,7}. Cortical tubers are characterized by areas of disrupted cortical and subcortical architecture, and the presence of enlarged cells that strongly express the phospho-ribosomal protein S6 (pS6) marker of mTOR Complex1 (mTORC1) activation⁸⁻¹⁰. In addition to being comprised of abnormal cell types, tuber cells express altered receptor patterns for the excitatory neurotransmitter glutamate^{8,11,12}, which may contribute to the highly refractory nature of these epileptogenic lesions.

Similar to TSC, sporadically occurring focal cortical dysplasias (FCDs) contain a variety of cellular abnormalities, including clusters of dysplastic cells. Classification of FCDs distinguishes two major categories: mild malformations of cortical development (MCD) and focal cortical dysplasia (FCD), subdivided into further subtypes^{13,14}. FCD Type II includes Type IIa (cortical dyslamination and dysmorphic neurons) and Type IIb (cortical dyslamination, dysmorphic neurons and balloon cells)¹³. FCD Type IIb has several striking histopathological features similar to TSC cortical tubers, which raises the possibility of common mechanisms responsible for structural abnormalities and epileptogenesis. Balloon cells and dysplastic neurons in FCD Type IIb also show evidence of mTORC1 pathway upregulation^{10,15} that is not observed in FCD Type IIa¹⁶. Additionally FCD Type IIb tissue has been reported to express similar patterns of dysregulated glutamate receptors, such as decreased NR2A and high NR2B levels, as that observed in TSC¹⁷⁻¹⁹.

Epilepsy in cortical malformations is thought to occur as a result of altered excitatory:inhibitory balance. There has been considerable interest in the status of the inhibitory γ -aminobutyric acid (GABA) neurotransmitter, given the aforementioned abnormalities observed in the glutamate receptor systems. Conventional antiepileptic drugs (AEDs) mainly target GABA_A receptors (GABA_ARs), yet are largely ineffective in patients with refractory epilepsy secondary to TSC or FCD²⁰, suggesting that altered inhibition in the dysplastic brain may contribute to seizure generation and epileptogenesis.

GABA_ARs are Cl⁻-permeable ion channels formed by an association of five subunits, and each subunit confers specific functional and pharmacological properties to the receptor^{21,22}. Expression of the $\alpha 1$ subunit is mainly responsible for the benzodiazepine sensitivity, and notably this isoform is developmentally regulated and expressed at low levels in the immature brain when seizures can be refractory to conventional AEDs²³.

GABA_AR function is also dependent on the transmembrane Cl⁻ gradient, which is regulated by two major transporters: the Na⁺-K⁺-2Cl⁻ (NKCC1) and the K⁺-Cl⁻ (KCC2). In adult neurons, intracellular Cl⁻ concentration is maintained at low levels due to the high KCC2

expression, therefore activation of these receptors results in a net Cl^- influx and subsequent hyperpolarization²⁴. In contrast, immature neurons accumulate Cl^- due to an age-specific upregulation of NKCC1 and a relative deficiency in KCC2^{25,26}, and therefore the receptor activation produces a net Cl^- efflux and membrane depolarization. Not surprisingly, barbiturates that enhance GABA_AR conductance are ineffective in suppressing electrographic seizures in human neonates²⁷. While experimental data demonstrate that combined therapy with barbiturates and bumetanide, a potent NKCC1 inhibitor, increases the efficacy of GABAergic inhibition in the neonatal brain^{26,28}, a recent case report shows that add-on bumetanide therapy decreased seizure activity in a neonatal patient²⁹.

There is accumulating evidence that human dysplastic tissue retains immature characteristics, including deficient cellular differentiation and maturation, as well as immature glutamatergic neurotransmitter receptors^{8,30}. Therefore, we hypothesized that TSC cortical tubers and FCD Type II lesions express immature GABA_AR phenotypes, which may contribute to epileptogenesis and the relative resistance to conventional AEDs. We specifically examined the differential expression patterns of GABA_AR subunits $\alpha 1$ and $\alpha 4$, and of Cl^- transporters NKCC1 and KCC2 within TSC tubers and FCD lesions, relative to control non-epileptic tissue. In addition, we performed perforated-patch recordings to analyze the GABA_AR responses in TSC dysplastic neurons, in comparison with normal-appearing cortical neurons within non-TSC epileptic tissue.

SUBJECTS/MATERIALS AND METHODS

Human subjects

In this study, we examined a total of 24 cortical tissue samples from TSC and FCD epilepsy patients (16 females and 8 males; mean age 10.3 years) and 10 control cases (6 females and 4 males; mean age 21.3 years). The main clinical and neuropathological characteristics of the study population are summarized in Table 1. TSC specimens were collected either from patients who underwent surgery for medically intractable epilepsy (n=8), or were obtained at autopsy (n=6). All patients were clinically diagnosed with TSC and presented a history of epilepsy. Postmortem TSC specimens (see Supplemental Methods) were obtained as a whole block containing both the diseased tissue and the surrounding normal-appearing cortex (a representative tissue block is shown in Supplemental Figure 1A–B). Tuberal tissue was dissected from within the distinct margins of the lesion, while the non-tuberal tissue was selected from areas of normal-appearing cortex well outside the lesion. Of note, we specifically avoided “perituberal” tissue within the border zone between the tuber and normal-appearing cortex, as the extent of neuropathological abnormalities in this area may vary among subjects. Consistent with its normal appearance, non-tuberal tissue demonstrated the same degree of mTORC1 activation as the control cortex (Supplemental Figure 1C). The non-tuberal tissue served as an internal control to correct for potential clinical variables, such as duration of epilepsy, seizure frequency or antiepileptic medication.

All FCD samples were obtained following epilepsy surgery for medically intractable epilepsy (n=10). The histopathological examination revealed dysplastic features consistent with FCD Type IIa (n=6) and Type IIb (n=4)¹³. The control group included cortical samples collected at autopsy from cases with normal neurological history (non-epilepsy controls; n=9), as well as non-dysplastic cortical tissue surgically removed due to medically intractable temporal lobe epilepsy (epilepsy control; n=1). This sample, devoid of dysplastic neurons, served as control for the electrophysiology experiments. All surgical specimens were obtained from the Departments of Neurosurgery at Children’s Hospital and Brigham and Women’s Hospital, Boston, MA. For these cases, additional clinical information (age at seizure onset, duration of epilepsy, seizure type and frequency, antiepileptic medication) is

presented in Supplemental Table 1. The autopsy samples were obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD and the Department of Neuropathology at Brigham and Women's Hospital, Boston, MA. The postmortem interval (PMI) in all cases was 24 hours or less (mean 19.3 hours for TSC cases and 14.1 hours for controls). All tissues were collected and handled in accordance with the guidelines of the hospitals' Institutional Review Boards.

Western Blot Analysis

Frozen cortical samples were processed for either membrane or whole cell protein extraction, as previously published⁸ (see Supplemental Methods). Protein levels were calculated as percentage of the mean expression levels of control samples run on the same blot (100%). Means (of minimum 3 cases/group) were compared by two tailed t-tests and differences between groups were considered significant at $p < 0.05$.

Immunocytochemistry

Paraformaldehyde (4%)-fixed tissue sections were stained using standard protocols⁸ (see Supplemental Methods). Sections were analyzed by epifluorescence microscopy (Zeiss Axioscope, Germany). Digital images were acquired with a Spot digital camera and Spot Software 4.5 (Diagnostic Instruments, Sterling Heights, MI), and processed using Adobe Photoshop 12.0 (Adobe Systems, San Jose, CA).

Cortical brain slice preparation

Biopsy cortical tissue from one TSC (Table 1, Case 1) and one non-TSC epilepsy patient (Table 1, Case 31) was transported to the laboratory and, within 10–15 min from resection, 300 μ m thick slices were prepared and maintained as described in the Supplemental Methods.

Perforated-patch recordings

Perforated-patch recordings were obtained from identified dysplastic and non-dysplastic human cortical neurons by using an Axopatch 200A amplifier (Axon Instruments) with a sampling frequency of 10 kHz after low-pass filtering at 1 kHz, and the pClamp9 software (Clampex; Molecular Devices). The experimental procedures are described in detail in the Supplemental Methods. Statistical significant differences were established at $p < 0.05$ using the Kolmogorov-Smirnov tests, or paired two-tailed t-test.

RESULTS

Differential expression levels of GABA_A receptor $\alpha 1$ and $\alpha 4$ subunits in human TSC cortex

The expression of GABA_AR subunits $\alpha 1$ and $\alpha 4$ in TSC cortical tubers, non-tuberal tissue and non-epileptic controls was quantified by Western blot (Figure 1). Cortical tubers demonstrated a significant reduction in membrane-expressed $\alpha 1$ subunit (Figure 1A) when compared to controls ($59 \pm 7\%$ of control, $n=9$, $p < 0.01$), while there was no significant difference in the expression of $\alpha 4$ subunit ($113 \pm 23\%$ of control, $n=9$, $p=0.6$; Figure 1B). The resulting $\alpha 4:\alpha 1$ ratio was significantly increased in cortical tubers relative to controls (2.42 ± 0.65 fold, $n=8$, $p < 0.05$; Figure 1C). In contrast, non-tuberal TSC tissue revealed $\alpha 1$ and $\alpha 4$ expression levels comparable to controls ($78 \pm 12\%$ of control, $n=6$, $p=0.6$ for $\alpha 1$, and $84 \pm 10\%$ of control, $n=6$, $p=0.31$ for $\alpha 4$; Figure 1A–B). Similarly, there was no significant difference in $\alpha 4:\alpha 1$ ratio between the non-tuberal tissue and controls (1.53 ± 0.28 fold increase, $n=6$, $p=0.58$; Figure 1C). Decreased $\alpha 1$ expression and increased $\alpha 4:\alpha 1$ ratio in cortical tubers correlated with upregulated mTORC1 signaling, as demonstrated by increased pS6 (Ser 235/236) levels ($316 \pm 60\%$ of control, $n=8$, $p < 0.01$; Supplemental Figure

1). Altered GABA_AR subunit composition in TSC cortical tubers may lead to reduced regional inhibition and decreased receptor responsiveness to benzodiazepine.

As deficient GABAergic inhibition may also result from reduced number of interneurons and/or decreased inhibitory terminals, we next quantified the tuberal and non-tuberal expression levels of the glutamic acid decarboxylase -67 (GAD-67), a GABA-converting enzyme selectively expressed in inhibitory neurons. Interestingly, we found no significant changes in GAD-67 expression in cortical tubers ($82 \pm 10\%$ of control, $n=9$, $p=0.30$) and non-tuberal tissue ($99 \pm 13\%$ of control, $n=6$, $p=0.94$; data not shown), relative to non-epileptic controls.

Altered NKCC1 and KCC2 expression levels in human TSC cortex

Western blot analysis for NKCC1 and KCC2 was similarly performed on membrane protein extracts from TSC cortical tubers, non-tuberal TSC tissue and non-epileptic control cortex (Figure 2A–B). Cortical tubers demonstrated a significant upregulation of NKCC1 expression ($133 \pm 15\%$ of control, $n=10$, $p<0.05$; Figure 2A), accompanied by a significant reduction in KCC2 levels ($59 \pm 6\%$ of control, $n=10$, $p<0.001$; Figure 2B). Unlike cortical tubers, the non-tuberal tissue showed significantly lower NKCC1 expression ($74 \pm 10\%$ of control, $n=6$, $p<0.05$), but KCC2 levels comparable to controls ($84 \pm 9\%$ of control, $n=6$, $p=0.19$). Consistently, the NKCC1: KCC2 ratios were significantly higher in cortical tubers (1.78 ± 0.25 fold increase, $n=10$, $p<0.01$), while the ratios were similar to control levels in the non-tuberal tissue (1.08 ± 0.21 fold, $n=6$, $p=0.6$; Figure 2C). These data suggest that hyperactivation of mTORC1 within cortical tubers (Supplemental Figure 1C) leads to dysregulation of Cl[−] transport, and that activation of GABA_ARs within these lesions might contribute to membrane depolarization.

Cell-specific expression of NKCC1 and KCC2 transporters in human TSC cortical tubers

As our western blot analysis demonstrated significant changes in the membrane expression of Cl[−] transporters (Figure 2), we next aimed to evaluate the cell-specific staining pattern of NKCC1 (Figure 3) and KCC2 (Figure 4) in TSC lesions. In addition, as we observed that dysregulated NKCC1 and KCC2 expression correlated regionally with the degree of mTORC1 activation (Supplemental Figure 1C), we next compared the cellular specificity of NKCC1 and KCC2 with that of pS6 (Supplemental Figure 2). Double labeling with pS6 and neuronal and glial markers (NeuN, SMI 311, vimentin and GFAP) demonstrated marked increases in pS6 expression in the majority of dysplastic neurons (Supplemental Figure 2A–C, small arrows), giant cells (Supplemental Figure 2D–F and J–L, arrowheads), dysplastic astrocytes (arrowheads in Supplemental Figure 2A–C and G–I), but not in normal-sized neurons (Supplemental Figure 2G–I, small arrows) and reactive astrocytes (Supplemental Figure 2J–L, small arrows) ($n=4$). The selective activation pattern of mTORC1 in dysplastic cells was consistently observed in all cortical tubers analyzed, and is in agreement with previous publications^{8,10,15,16}. Cellular expression pattern of NKCC1 differed from pS6, as the transporter was highly expressed in cytomegalic dysplastic neurons (Figure 3A–C, arrowheads), giant cells (Figure 3D–F, arrowheads) and dysplastic astrocytes (Figure 3A–C, small arrows), but also in normal-sized neurons (Figure 3D–F, small arrows and Figure 3G–I) and reactive astrocytes (Figure 3J–L) ($n=4$). This pattern suggests that within the tuber, in addition to mTORC1 dysfunction, the ictal activity may secondarily induce changes in NKCC1 expression in pS6 negative normal sized neurons and reactive astrocytes. In contrast, in the predominantly normal appearing adjacent cortex both NKCC1 and pS6 expression were very low or undetectable ($n=3$; data not shown).

While NKCC1 is widely expressed in both neuronal and glial cell types, KCC2 has been shown to be exclusively expressed in neurons³¹. Indeed, we found that in TSC cortical

tubers KCC2 was only expressed in dysplastic and normal neurons, but not in undifferentiated giant cells or dysplastic astrocytes. Consistent with our western blot data showing decreased membrane-bound expression for this transporter (Figure 2), we found a marked diffuse decrease in KCC2 perisomatic neuropil staining within cortical tubers (Figure 4) (n=4). A striking observation was the abnormally high intrasomatic labeling pattern in the cytomegalic dysplastic neurons (Figure 4A–C, arrowheads), which was not observed in normal appearing neurons (Figure 4D–F, arrowheads). The intracellular KCC2 expression in dysplastic neurons correlated at a cellular level with upregulated mTORC1 signaling (Supplemental Figure 2). Similar KCC2 expression pattern was previously reported in normal developing neurons, in which the transporter is accumulated in the cytoplasm prior to its incorporation into the cellular membrane³². These immunocytochemical data further suggest that altered Cl[−] transporter expression in TSC dysplastic neurons could in turn compromise Cl[−] homeostasis and reverse GABA-mediated inhibition to excitation.

Decreased BDNF expression in TSC cortical tubers

Brain derived neurotrophic factor (BDNF) plays a critical role in the formation, maturation and stabilization of inhibitory synapses during normal CNS development^{33,34} and its expression is highest in the immature brain³⁵. As the expression profiles of GABA_AR subunits and Cl[−] transporters in TSC dysplastic neurons are compatible with an immature status, we next aimed to determine if there were differences in endogenous BDNF expression in the tuberal and non-tuberal tissue relative to non-epileptic controls that may contribute to these changes. Western blot quantification demonstrated that BDNF levels (Figure 5) were significantly downregulated in cortical tubers (66±7% of control, n=9, p<0.01), while non-tuberal tissue was not different from control tissue (86±8% of control, n=6, p=0.23). Our data thus support the hypothesis that the relative BDNF deficiency in TSC cortical tubers, along with constitutive upregulation of mTORC1 signaling, might contribute to the maintenance of an immature transcriptional pattern in these lesions.

Evidence for Cl[−] transport dysfunction in human TSC cortical neurons

Given the protein expression changes in cortical tubers, we performed electrophysiological analysis of GABA_AR responses in biopsy tissue from a single human TSC case, in comparison to an epilepsy control case without evidence of cellular dysplasia. To maintain the intact intracellular Cl[−] concentration, we used the gramicidin perforated patch-clamp recording technique in all experiments. We first examined spontaneous GABA_AR-mediated post-synaptic currents (PSCs) in normal-appearing cortical neurons of acute cortical slices from the epilepsy control tissue (Figure 6A). As shown in Figure 6A₁, the neuron had a resting membrane potential of −60.4 mV and an input resistance of 262 MΩ. With a holding potential of −60 mV, we observed both inward excitatory PSCs mediated by AMPA/NMDA receptors as evidenced by complete blockade with the specific receptor antagonists NBQX and DL-AP5, as well as outward inhibitory GABA_AR-mediated PSCs, isolated by blocking AMPA and NMDA receptor-mediated PSCs (Figure 6A₂). The reversal potential of GABA_AR-mediated PSCs in the normal appearing neurons were close to −70 mV, and were completely abolished by bath application of 60μM picrotoxin (PTX), confirming that they were mediated by GABA_ARs.

We next performed recordings in acute TSC cortical tuber slices from dysplastic neurons under guidance of DIC imaging (Figure 6B₁). In contrast to the normal neurons in epilepsy control tissue, these dysplastic neurons exhibited depolarizing GABA_AR-mediated spontaneous PSCs (Figure 6B₂), consistent with NKCC1 and KCC2 expression pattern observed by western blot and immunocytochemical analysis. Current clamp recordings revealed that the resting membrane potential of dysplastic cortical neurons was −58.7 mV

with an input resistance of 238 M Ω . GABA_AR-mediated PSCs were isolated by blocking AMPA and NMDA receptor-mediated PSCs under voltage clamp mode with a holding potential of -60 mV, close to the resting membrane potential. Spontaneous PSCs were completely abolished by bath application of 60 μ M PTX, demonstrating that they were mediated by GABA_ARs. GABA_AR-mediated PSCs were excitatory with relatively high frequency and amplitude (sPSC amplitude: 29.1 pA; sPSC frequency: 2.8 Hz), indicating an excitatory GABA function and depolarized GABA reversal potential, or high [Cl⁻]_i (Figure 6B₃). Post-recording immunocytochemical double labeling confirmed increased pS6 and NKCC1, and decreased KCC2 expression in these cells (data not shown).

We next tested the effects of the NKCC1 blocker bumetanide ²⁸ on GABA_AR mediated excitatory PSCs in human TSC slices. Bath application of 10 μ M bumetanide (BUM) effectively suppressed PSC amplitude (50.2% of control) and frequency (53.6% of control) (Figure 6B₂-B₅). This is consistent with previously published data showing an inhibitory effect of bumetanide on depolarized GABA reversal potential in hippocampal neurons during neonatal seizures ²⁸. Our data confirm the functional dysregulation of Cl⁻ transport in human TSC dysplastic neurons and suggest that preventing GABA mediated excitation in TSC dysplastic cortex with bumetanide may represent a new therapeutic strategy for seizure control in TSC patients.

Comparison analysis between TSC and FCD Type II

As the severe focal cortical dysplasias (FCD Type II) share numerous histopathological, clinical and biochemical features of TSC, including altered mTORC1 signaling ², it is possible that they also present similar alterations in GABA_AR expression and function. To test this hypothesis, we next performed a comparative analysis of GABA_AR subunits, and Cl⁻ transporters NKCC1 and KCC2 in FCD Type IIa and Type IIb tissue (Figure 7). Western blot quantification of GABA_AR subunits α 1 and α 4 in FCD IIa cases demonstrated no changes in the expression of α 1 subunit compared to control (112 $\pm$ 41% of control, n=6, p=0.69). However, the α 4 subunit was actually down regulated (49 $\pm$ 8% of control, n=4, p<0.05; Figure 7A), with a significant decrease in the α 4: α 1 ratio compared to non-epileptic controls (0.64 $\pm$ 0.21 fold, n=4, p<0.05; Figure 7B). In contrast, FCD Type IIb cases showed a significant reduction in α 1 subunit expression (34 $\pm$ 8% of control, n=4, p<0.01), but no significant differences in α 4 levels (81 $\pm$ 23% of control, n=4, p=0.57; Figure 7A), resulting in a significant increase in the α 4: α 1 ratio (2.94 $\pm$ 0.8 of control, n=3, p<0.001; Figure 7B), similar to the pattern observed in TSC cortical tubers.

Unlike cortical tubers where we found only a slight decrease in GAD-67 levels, here we found a marked reduction in GAD-67 expression in both FCD Type IIa (25 $\pm$ 6% of controls, n=3, p<0.05) and FCD Type IIb lesions (36 $\pm$ 12% of controls, n=4, p<0.05; data not shown). These data are consistent with previous observations of reduced number of interneurons in cortical dysplasia ³⁶, relative to TSC cortical tubers where the interneurons appear to be less affected ¹⁰.

Next we quantified the expression levels of NKCC1 and KCC2 in both types of FCD II samples. In FCD Type IIa, both NKCC1 and KCC2 were significantly unregulated (252 $\pm$ 62% of control, n=4, p<0.001 for NKCC1, and 193 $\pm$ 46% of control, n=6, p<0.01 for KCC2; Figure 7C), with no significant change in the NKCC1: KCC2 ratio compared to controls (1.83 $\pm$ 0.6 fold, n=4, p=0.06; Figure 7D), suggesting rather hyperpolarizing and inhibitory GABA_ARs. In contrast, FCD Type IIb cases showed a significant increase in NKCC1 expression (138 $\pm$ 21% of control, n=3, p<0.05) with a significant reduction in KCC2 levels (40 $\pm$ 7% of control, n=5, p<0.001; Figure 7C). Similar to TSC cortical tubers, the NKCC1:KCC2 ratio in FCD Type IIb was significantly higher relative to controls

(3.6 ± 0.7 fold increase, $n=3$, $p<0.001$; Figure 7D), suggesting that GABA_ARs could have the capacity to be depolarizing in this disorder, consistent with previous observations³⁷.

Double-label immunocytochemical staining of FCD Type IIa and Type IIb showed critical differences in KCC2 expression patterns in the setting of high NKCC1 expression (Figure 8). In FCD Type IIa, KCC2 demonstrated high neuropil and membrane expression in the majority of neurons (Figure 8B–C, small arrows) ($n=3$), while in FCD Type IIb lesions there was a marked decrease in KCC2 staining in the neuropil around neuronal cell bodies (Figure 8D–F) ($n=3$). In contrast, similar to TSC cortical tubers, the dysplastic neurons in FCD Type IIb lesions demonstrated the same KCC2 accumulation in the cytoplasm (Figure 8E–F, arrowheads), while no membrane or cytoplasmic KCC2 staining was observed in balloon cells (data not shown). NKCC1 expression was not different between FCD IIa and IIb. In FCD Type IIa, dysplastic and nondysplastic neurons, as well as most reactive astrocytes showed high NKCC1 levels ($n=4$, data not shown). Similarly, in FCD Type IIb NKCC1 was highly expressed in dysplastic neurons, balloon cells, immature and normal appearing neurons and reactive astrocytes, resembling the pattern seen in TSC cortical tubers ($n=4$, data not shown).

In summary, this comparative analysis shows striking similarities between TSC and FCD Type IIb with respect to GABA_AR subunits, NKCC1 and KCC2 expression patterns, which are distinct from FCD Type IIa. These findings suggest possible common, or at least partially overlapping pathophysiological mechanisms of epileptogenesis in the first two entities.

DISCUSSION

In this study we demonstrate that TSC cortical tubers and FCD Type IIb lesions express similar alterations in GABA_AR subunit composition, as well as dysregulated NKCC1 and KCC2 levels that may result in altered inhibitory function and subsequent epileptogenesis. These changes are not seen in FCD type IIa, suggesting potentially shared mechanisms of epileptogenesis for TSC and FCD Type IIb. In addition, this is the first demonstration of an effect of bumetanide in human brain slices, and we show that dysplastic neurons from one TSC patient display excitatory GABA_AR responses that are effectively attenuated by the NKCC1 inhibitor bumetanide. Thus, our data may justify further consideration of clinical trials using FDA- approved NKCC1 inhibitors as adjunctive therapy for refractory epilepsy in TSC and FCD Type IIb, but not FCD Type IIa.

Differential postsynaptic GABA_A receptor alpha subunit alterations in TSC, FCD Type IIb and FCD Type IIa

Our study demonstrates striking similarities in the expression of GABA_AR subunits in TSC and FCD Type IIb lesions. We show that $\alpha 1$ subunit expression is significantly decreased with resultant increases in $\alpha 4:\alpha 1$ ratios in both pathologies. While these data are the first to report altered GABA_AR subunit protein expression in TSC, our findings are consistent with previous reports of decreased $\alpha 1$ mRNA levels in TSC cortical tubers¹². The reported changes in GABA_AR subunit protein in FCD Type IIb cases is consistent with previously reported electrophysiological and pharmacological properties of cytomegalic neurons from FCD Type II lesions^{36,38}. Longer GABA_AR kinetics, decreased sensitivity to benzodiazepines (zolpidem), and increased responses to bretazenil in FCD Type II neurons, relative to neurons from FCD Type I or non-dysplastic cortex, is consistent with decreased expression of $\alpha 1$ relative to $\alpha 3$, $\alpha 4$, or $\alpha 5$ GABA_AR subunits²¹. In contrast, we show the opposite pattern in FCD Type IIa cases, with a significant reduction in $\alpha 4$ subunit expression and decreased $\alpha 4:\alpha 1$ ratio. These changes are suggestive of better responsiveness of FCD Type IIa patients to benzodiazepines compared to FCD Type IIb and

TSC cases. Interestingly, unlike TSC and FCD Type IIb lesions, FCD Type IIa is characterized by a milder cellular dysplasticity, including the absence of balloon cells, and is usually associated with a less severe seizure phenotype, such as later onset and lower seizure frequency (Supplemental Table 1)^{13,39}.

Numerous animal studies have shown that alterations in GABA_AR α subunits are associated with development of chronic epilepsy. In rodents, status epilepticus (SE) induces acute decreases in $\alpha 1$ expression and upregulation of $\alpha 4$ (increased $\alpha 4:\alpha 1$ ratios), which correlate with profound changes in GABA_AR function such as slower kinetics and decreased benzodiazepine sensitivity⁴⁰. Importantly, these changes precede the development of epilepsy, while overexpression of $\alpha 1$ subunit (decreased $\alpha 4:\alpha 1$ ratios), reduces the incidence of spontaneous seizures after SE by 60%⁴¹, supporting the role of this subunit in mediating refractory epilepsy and/or epileptogenesis.

Dysregulation of NKCC1 and KCC2 expression consistent with altered Cl⁻ homeostasis in TSC and FCD Type IIb, but not FCD Type IIa

Here we show for the first time that in TSC cortical tubers the expression of Cl⁻ transporters NKCC1 and KCC2 is significantly altered, specifically we found increases in NKCC1 expression associated with downregulated KCC2 levels (increased NKCC1: KCC2 ratios). At a cellular level, NKCC1 expression was increased in neurons and glia. Compared to normal neuronal expression of KCC2 that is confined to the cellular membrane, dysplastic neurons in TSC cortical tubers showed accumulation of KCC2 in the cytoplasm, and relative depletion in the neuropil. Interestingly, this specific cellular pattern we report here in TSC cortical tubers was previously observed in FCD Type IIb^{42,43}, further supporting the idea that both pathologies may share the same mechanisms of epileptogenesis. In contrast, KCC2 membrane expression was actually increased in FCD Type IIa lesions, consistent with prior reports^{44,45}, and might be related to their lower refractoriness, or may represent a compensatory mechanism to prevent development of epilepsy⁴⁶.

The subcellular localization of KCC2 protein most critically regulates its function, and previous developmental studies have demonstrated that KCC2 is constitutively expressed in neurons even during the early stages when GABA_A receptors are depolarizing. However, in neonatal neurons KCC2 is mostly expressed within the cytoplasm, but gradually inserted into the membrane with increasing age, which coincides with the reversal of GABA polarity³². Targeting of KCC2 to the neuronal cell surface is critically regulated by protein kinase (PKC), which is one of the known downstream targets of mTOR Complex 2 (mTORC2). PKC-dependent phosphorylation of KCC2 inhibits its endocytosis, and at the same time increases the membrane-bound transporter activity⁴⁷. In TSC, mTORC2 function is reduced, which has been shown to decrease PKC activity⁴⁸. This mechanism may provide a link between TSC1/TSC2 deficiency and altered expression and function of KCC2 in human cortical tubers. This also raises the possibility that signaling molecules that activate PKC signaling pathways may restore neuronal Cl⁻ homeostasis in these lesions.

Decreased GABAergic inhibition mediated by depolarizing GABA_ARs may represent a critical mechanism of epileptogenesis in TSC and FCD Type IIb lesions. Increases in the expression of NKCC1 relative to KCC2 (increased NKCC1: KCC2 ratios) have been previously associated with depolarizing GABA responses and implicated in the pathophysiology of various types of seizures, including neonatal seizures and temporal lobe epilepsy^{26,49,50}. In addition, it has been shown that KCC2 deficiency leads to increased neuronal excitability and enhanced seizure susceptibility^{51,52}.

Evidence for depolarizing GABA_A receptors in TSC cortical tubers

While our expression data suggested a shift of GABA polarity towards excitation in TSC cortical neurons, we were also able to demonstrate GABA_AR mediated excitatory postsynaptic responses in dysplastic neurons from one TSC case. Using whole-cell patch clamp recordings, a previous study by Cepeda et al.³⁷ has reported depolarized GABA reversal potentials and excitatory GABA responses in severe FCD lesions, compared to mild FCD or non-dysplasia cases. However, the whole-cell recording technique may cause the dialysis disruption of $[Cl^-]_i$ that critically regulates GABA reversal potential. Gramicidin-perforated patch recordings were applied in the present study to keep $[Cl^-]_i$ intact⁵³, and as expected the normal appearing neurons in non-TSC tissue showed inhibitory GABA_AR-mediated PSCs at resting membrane potential. In contrast, slices from a TSC tuber exhibited excitatory GABA_AR-mediated PSCs at resting membrane potential, indicating a depolarized GABA reversal potential and high $[Cl^-]_i$ in dysplastic neurons. Importantly, the specific NKCC1 blocker bumetanide effectively attenuated these GABA_AR-mediated responses, supporting a potential role for abnormally high NKCC1:KCC2 membrane ratios as promoting network hyperexcitability. Thus, our data suggests that excitatory GABA responses in TSC may contribute to the relative refractoriness of GABAergic antiepileptic drugs in this patient population, and that bumetanide may represent a novel adjuvant therapeutic strategy for seizure control in TSC.

Epileptogenic mechanisms in TSC and FCD Type IIb may be different from FCD IIa

The expression patterns of the of GABA_ARs and Cl^- transporters suggesting that lesional expression profiles in TSC and FCD Type IIb cases are persistent immature transcriptional phenotypes^{37,54}, similar to patterns observed for glutamate receptors^{8,18}. Alternatively, acute post-ictal changes in GABA_AR kinetics and subunit expression as well as upregulation of NKCC1 with simultaneous downregulation of KCC2 have been previously reported in rodent epilepsy models in adult rats^{40,50,55}, raising the possibility that ongoing seizure activity may be in part responsible for altered expression and function of GABA_ARs in TSC and FCD Type IIb. However, as the additional analysis of TSC non-tuberal tissue revealed no significant changes compared to controls, this mechanism cannot fully explain the observed changes.

The mechanisms that regulate the development of GABAergic inhibition are still not fully elucidated. Immature GABA_ARs are characterized by an abundance of $\alpha 2$ and $\alpha 4$ subunits, while the incorporation of $\alpha 1$ coincides with brain maturation²³. At the same time, in the developing brain, GABA_ARs are depolarizing, a feature that has multiple age-specific physiologic functions, including the control of neuronal differentiation and dendritic development^{56,57}. In addition, these receptors enable NMDARs function, otherwise inhibited by a voltage-dependent Mg^{2+} blockade, facilitating the excitatory synapse formation in the neocortex⁵⁸. In contrast, the switch in the action of GABA from excitatory to inhibitory, which in human neocortex occurs by the end of the first year of life²⁶, coincides with augmentation of GABAergic synapses⁵⁹. This suggests that in the human dysplastic TSC and FCD Type IIb lesions, the persistence of depolarizing GABA_ARs might not just compromise GABAergic inhibition, but also might be responsible for the maintenance of immature glutamate receptor profiles (GluR2 lacking AMPA receptors and NR2B/NR3A containing NMDA receptors) and enhancement of glutamate receptor functions during epileptogenesis⁸.

Importantly, both Cl^- transporters and GABA_ARs are regulated, among other factors, by BDNF. Transgenic mice over-expressing BDNF demonstrate higher rates of GABAergic synaptogenesis and GABA production, as well as increased KCC2 expression³³, while heterozygous BDNF knockout mice demonstrate reduced efficiency of GABAergic

inhibition, consistent with an immature status³⁴. Our data showing a significant reduction in endogenous BDNF levels in TSC suggest a potential role of this neurotrophin in abnormal GABAergic synaptic maturation in these lesions.

Implications for treatment

The most common AEDs action involves modulation of GABAergic function, either by modulating the post-synaptic GABA_A receptors (barbiturates and benzodiazepine), or by acting on synaptic GABA availability (vigabatrin). Our data suggests that GABA_ARs in TSC and FCD IIB lesions are not only likely to be insensitive to benzodiazepine but are also depolarizing, so drugs that increase GABAergic function may fail to suppress seizures or even induce hyperexcitability. In neonatal rat brain, combined therapy with barbiturates and an NKCC1 inhibitor such as bumetanide increases the efficacy of GABAergic inhibition²⁸, and a clinical trial testing the safety and efficacy of bumetanide/phenobarbital therapy in neonatal seizures is currently ongoing (clinicaltrials.gov/NCT00830531). Similar to the neonatal patient population, a more specific therapeutic approach to control seizures in TSC and FCD Type IIB patients might be needed, such as combining bumetanide with an AED that enhances GABA_A receptor function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Differential expression of GABA_A receptor subunits in human cortical tubers and non-tuberal cortex

A. Western blot quantification of GABA_AR subunit α1 demonstrates a significant decrease in cortical tubers, but levels comparable to control in the non-tuberal cortex. **B.** Quantitative western blot analysis for GABA_AR subunit α4 shows normal expression levels in cortical tubers and in the non-tuberal cortex. **C.** α4:α1 ratios are significantly increased in TSC cortical tubers, but not in the non-tuberal tissue. Histograms represent averaged optical density normalized to actin, and expressed relative to the mean control values. Insets are the representative western blots for individual proteins. *: $p < 0.05$. Error bars indicate S.E.M.

Figure 2. Dysregulated expression of Cl^- transporters NKCC1 and KCC2 in cortical tubers and non-tuberal human cortex

A. Membrane-expressed NKCC1 transporter is significantly increased in cortical tubers, while downregulated in the non-tuberal cortex when compared to control. **B.** Western blot analysis for KCC2 shows decreased expression in cortical tubers and expression levels comparable to controls in the non-tuberal cortex. **C.** NKCC1:KCC2 ratios are significantly increased in TSC cortical tubers, but no different from controls in the non-tuberal tissue. Histograms represent averaged optical density normalized to actin, and expressed relative to the mean control values. Insets are the representative western blots for individual proteins. *: $p < 0.05$. Error bars indicate S.E.M.

Figure 3. Cell-specific expression of NKCC1 in human cortical tubers

A–C. Double-label immunocytochemistry for non-phosphorylated neurofilament SMI 311 and NKCC1 demonstrates high NKCC1 expression in dysplastic neurons (arrowheads), as well as in other non-neuronal dysplastic cells (small arrows). D–F. NKCC1 is highly expressed in vimentin positive giant cells (arrowheads) and vimentin negative normal-sized neurons (small arrows). G–I. Strong NKCC1 immunoreactivity is present in NeuN expressing disoriented immature neurons. J–L. GFAP-expression reactive astrocytes within cortical tubers are also highly NKCC1 positive. Scale bars represent 20μm in all panels.

Figure 4. Differential cellular distribution of KCC2 in human cortical tubers

A–C. NeuN positive immature and dysplastic neurons within the tuber demonstrate very low KCC2 expression surrounding the cell body and proximal dendrites. Dysplastic neurons show intense KCC2 immunoreactivity in the cytoplasm (**B–C**, arrowheads). **D–F.** Normal appearing NeuN neurons in the adjacent cortex demonstrate high perisomatic KCC2 expression, and low or no intracellular labeling (**E–F**, arrowheads). Insets (**D1–F1**) show corresponding higher magnification of individual cells. Scale bars are 20mm for **A–F** and 10 mm for **D1–F1**.

Figure 5. Decreased BDNF expression in TSC cortical tubers

Western blot quantification of whole cell homogenates demonstrates a significant decrease in endogenous BDNF levels in cortical tubers, but levels comparable to control in the non-tuberal cortex. Histograms are averaged optical density normalized to actin, and expressed relative to the mean control values. Insets are the representative western blots for individual proteins. *: $p < 0.05$. Error bars indicate S.E.M.

Figure 6. Dysplastic neurons in TSC cortical tubers present depolarizing GABA_A receptor responses that are bumetanide sensitive

A. Inhibitory GABA_A receptor responses in cortical *ex vivo* slices from a non-TSC epilepsy case. **A₁.** Representative differential interference contrast (DIC) image of normal-appearing neurons recorded in cortical *ex vivo* slices. **A₂.** First panel: Representative current traces of perforated-patch voltage clamp recordings at a holding potential of -60 mV. Note that both inward excitatory PSCs and outward inhibitory PSCs presented at -60 mV holding potential. Insert traces show individual outward PSCs in detail at expanded time scale. Second panel: Outward inhibitory PSCs were removed by increasing the holding potential to -70 mV. Third panel: Inward excitatory sPSCs were blocked by bath application of AMPA and NMDA receptor antagonists: 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX, $10 \mu\text{M}$), and DL-2-Amino-5-phosphonopentanoic acid (DL-AP5, $100 \mu\text{M}$) at a holding potential of -70 mV. Fourth panel: Outward inhibitory GABA_A receptor mediated PSCs appeared when the holding potential is decreased from -70 mV to -60 mV. Fifth panel: Outward inhibitory GABA_A receptor mediated PSCs were completely abolished by bath application of picrotoxin (PTX, $60 \mu\text{M}$). Insert traces show individual outward PSCs in detail at expanded time scale. **B.** Bumetanide-sensitive depolarizing GABA_A receptor responses in dysplastic neurons in TSC cortical tubers. **B₁.** Representative DIC image of dysplastic neurons in cortical *ex vivo* slices from a TSC patient. **B₂.** First panel: Representative current traces of perforated-patch voltage clamp recordings at a holding potential of -60 mV. Second panel: GABA_AR-mediated sPSCs were isolated by bath application of AMPA and NMDA receptor antagonists: NBQX ($10 \mu\text{M}$),

and DL-AP5 (100 μ M). Third panel: Bath application of bumetanide (BUM, 10 μ M) significantly attenuated GABA_AR mediated sPSCs. Fourth panel: Spontaneous PSCs were completely abolished by bath application of picrotoxin (PTX, 60 μ M). **B₃**. Normalized cumulative distribution of GABA_AR sPSCs demonstrates a significant decrease in amplitude by bumetanide ($p < 0.01$, *Kolmogorov-Smirnov* test). **B₄** and **B₅**. Both amplitude (**B₄**) and frequency (**B₅**) of GABA_AR mediated sPSCs are effectively inhibited by bath application of bumetanide. Scale bars represent 20 μ m in **A₁** and **B₁**.

Figure 7. Altered GABAergic inhibition in focal cortical dysplasia (FCD) Type IIa and Type IIb
A. Western blot quantification of GABA_AR subunits α 1 and α 4 demonstrate a significant α 1 decrease in FCD Type IIb, and a significant α 4 downregulation in FCD Type IIa. **B.** The relative abundance of α 4 subunit (α 4:α 1 ratio) is significantly increased in FCD Type IIb and decreased in FCD Type IIa. **C.** Quantitative western blot analysis for Cl⁻ transporters NKCC1 and KCC2 demonstrates increased NKCC1 and decreased KCC2 levels in FCD Type IIb, in contrast to increased NKCC1 and upregulated KCC2 in FCD Type IIa. **D.** This pattern results in significantly increased NKCC1:KCC2 ratios in FCD Type IIb. Histograms represent averaged optical density normalized to actin, and expressed relative to the mean control values. Dotted lines in **A.** and **C.** represent the mean control levels. Insets are the representative western blots for individual proteins. *: $p < 0.05$. Error bars indicate S.E.M.

Figure 8. Sub-cellular KCC2 distribution in human FCD Type II lesions

A–C. NeuN positive dysplastic neurons in FCD Type IIa lesions demonstrate high perisomatic KCC2 localization, but no cytoplasmic expression (B–C, small arrows). **D–F.** In contrast, SMI 311 expressing dysplastic neurons in FCD Type IIb lesions show very low perisomatic KCC2 expression, but diffuse KCC2 staining in the cytoplasm (E–F, arrowheads). Scale bars are 50µm for A–F.

TABLE 1
Study Population

Overview of the Clinical and Neuropathological Characteristics

Case #	Gender	Age (Years)	Diagnosis	Brain Region
1	F	1.4	TSC	Parieto-occipital Tuber
2	M	1.6	TSC	Parietal Tuber
3	F	2.5	TSC	Temporal Tuber
4	F	3	TSC	Frontal Tuber
5	F	3	TSC	Occipital Tuber and Non-tuberal Cortex
6	F	3.8	TSC	Occipital Tuber
7	M	4.8	TSC	Parietal Tuber
8	F	5	TSC	Temporal Tuber
9	M	7	TSC	Temporal Tuber
10	F	7	TSC	Parietal Tuber and Non-tuberal Cortex
11	F	20	TSC	Frontal Tuber and Non-tuberal Cortex
12	M	22	TSC	Parietal Tuber and Non-tuberal Cortex
13	F	22	TSC	Parietal Tuber and Non-tuberal Cortex
14	F	31	TSC	Frontal Tuber and Non-tuberal Cortex
15	M	1.2	FCD IIb	Occipital and Frontal Lesions
16	F	3	FCD IIb	Frontal Lesion
17	M	3	FCD IIb	Temporal Lesion
18	F	4	FCD IIb	Parieto-occipital Lesion
19	F	2.5	FCD IIa	Frontal Lesion
20	M	5	FCD IIa	Temporal Lesion
21	F	11	FCD IIa	Temporal Lesion
22	M	12	FCD IIa	Occipital Lesion
23	F	18	FCD IIa	Temporal Lesion
24	F	20	FCD IIa	Temporal Lesion
25	M	1	Control	Parieto-occipital Lobe
26	F	3	Control	Parieto-occipital Lobe
27	F	4.5	Control	Occipital Lobe
28	M	7.8	Control	Occipital Lobe
29	F	20	Control	Frontal Lobe
30	M	20	Control	Parietal Lobe
31	F	28	Control *	Temporal Lobe
32	M	31	Control	Frontal Lobe
33	F	41	Control	Parieto-occipital Lobe
34	F	57	Control	Parieto-occipital Lobe

Abbreviations: M, male; F, female; TSC, tuberous sclerosis complex; FCD, focal cortical dysplasia (Type IIa, IIb);

* denotes the epilepsy control case used for comparison in the electrophysiology studies.