Dysfunction of Synaptic Inhibition in Epilepsy Associated with Focal Cortical Dysplasia

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Focal cortical dysplasia (FCD) is a common and important cause of medically intractable epilepsy. In patients with temporal lobe epilepsy and in several animal models, compromised neuronal inhibition, mediated by GABA, contributes to seizure genesis. Although reduction in GABAergic interneuron density has been reported in FCD tissue samples, there is little available information on the resulting physiological changes in synaptic inhibition and the potential contribution of these changes to epileptogenesis in the dysplastic human brain. Using visualized whole-cell patch-clamp recordings from identified neurons in tissue slices obtained from patients with FCD, we demonstrate that GABA_A-receptor-mediated inhibition is substantially altered in regions of dysplasia. These alterations include a significant reduction in IPSC frequency and a potentially compensatory decrease in transporter-mediated GABA reuptake function; the latter is marked by a significant increase in the decay-time constant for evoked and spontaneous IPSCs and a lack of effect of the GABA transporter-inhibitor 1-[2[[[(diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride on IPSC kinetics. Immunohistochemical staining revealed a scattering of GABAergic interneurons across dysplastic cortex and striking reductions in GABA transporter expression. Together, these results suggest that profound alterations in GABA-mediated synaptic inhibition play an essential role in the process of epileptogenesis in patients with FCD.

Key words: dysplasia; epilepsy; human brain slices; inhibition; GABA; GAT

Introduction

GABA is the main inhibitory neurotransmitter in the CNS and acts on both postsynaptic GABA_A and presynaptic GABA_B receptors. GABA_A receptors mediate the majority of GABA-dependent fast synaptic inhibition in the mammalian brain. Moreover, GABAergic inhibition plays a critical role in controlling normal circuit function (McCormick, 1989), limiting the spread of epileptiform discharges (Miller and Ferrendelli, 1990; Telfeian and Connors, 1998), regulating the intrinsic burst-firing properties of neurons (Isokawa-Akesson et al., 1989), and modulating the activation of glutamatergic synapses (Ots and Trussell, 1996). Impairment in the GABAergic system can lead to neuronal hyperexcitability that contributes to epileptogenesis (De Deyn et al., 1990; Tasker and Dudek, 1991). Alteration in GABA function may result from normal GABA receptors participating in abnormal circuitry or from loss of a subpopulation of inhibitory GABAergic interneurons. Epilepsy-associated alterations in the function and molecular composition of postsynaptic GABA receptor subunits have also been reported (Brooks-Kayal et al., 1998; Loup et al., 2000). Experimental models of temporal lobe epilepsy (TLE) are perhaps one of the best characterized exam-

ples in which loss of GABAergic interneurons is associated with reduced IPSC frequency (Kobayashi and Buckmaster, 2003; Austin and Buckmaster, 2004) and altered inhibitory circuit function (Zappone and Sloviter, 2004). Evidence from human studies further suggest that alteration in GABA-mediated inhibition contributes to the pathophysiology of TLE (Buhl et al., 1996; Gibbs et al., 1996; Arellano et al., 2004), but little is known about the function of GABA-mediated inhibition in human focal cortical dysplasia (FCD) (D'Antuono et al., 2004).

Malformations of cortical development are increasingly recognized as an underlying pathology in children with medically intractable epilepsy (Mischel et al., 1995; Porter et al., 2003). Seizures associated with these malformations are often resistant to available antiepileptic drugs and an understanding of how dysplastic neurons communicate is far from complete. The little we do know about synaptic function in a malformed brain is derived primarily from animal model research. For example, rodent models of cortical dysplasia exhibit a reduction in IPSC frequency (Zhu and Roper, 2000; Calcagnotto et al., 2002; Chen and Roper, 2003) and reduced numbers of parvalbumin-positive interneurons (Rosen et al., 1998; Roper et al., 1999). Inhibitory dysfunction in a malformed brain might result from altered arrangement of GABAergic inputs, loss of interneurons, or abnormal neurotransmitter reuptake in regions of dysplasia. If any of these alterations occur in humans with FCD, postsynaptic GABA-mediated synaptic function would appear to be substantially altered in dysplastic tissue. Such changes (if in the direction of reduced inhibition) might contribute to epileptogenesis. Inter-

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estingly, long-term reductions in interneuron density were noted in tissue samples from patients with FCD (Spreafico et al., 1998a,b, 2000; Garbelli et al., 1999; Alonso-Nanclares et al., 2005) but have not been correlated with epileptogenic changes in synaptic inhibition, although anatomical findings suggest a physiological condition of disrupted cortical function. To address whether GABA-mediated function is altered in a dysplastic brain, we examined IPSCs, interneuron distribution, and GABA-transporter (GAT) expression in neocortical tissue obtained during surgery from patients with medically intractable epilepsy and FCD. For comparison, we measured the same parameters in neocortical tissue obtained from patients undergoing surgery for medically refractory epilepsy who did not exhibit FCD.

Materials and Methods

Patients and tissue collection. Neocortical samples were obtained from patients who underwent surgery in the Department of Neurological Surgery at the University of California, San Francisco (UCSF) for medically intractable seizures associated with type II FCD (Palmini et al., 2004) ($n = 7$; patients aged 17–39 years; mean, 30 years) or mesial temporal lobe sclerosis (MTLS) (control patients; $n = 9$; patients aged 18–39 years; mean, 29 years). Tissue from an area with normal histology (i.e., nondysplastic) was taken from one patient with FCD as an additional control. All of the tissue specimens were from brain tissue that was removed as routine treatment for the patients' condition. The UCSF Committee on Human Research approved the protocol. Patients were taking a variety of antiepileptic drugs that were discontinued or reduced during the days preceding surgery (no clear bias toward a specific drug regimen was observed in either patient group) (Table 1). In FCD patients, tissue samples were obtained from regions identified as dysplastic on magnetic resonance imaging and confirmed *post hoc* by neuropathology. Within the suspected region of FCD, the most epileptogenic area was identified by using electrocorticographic (ECoG) recording (Fig. 1). In MTLS cases, nonspiking, nondysplastic neocortical tissue was used for comparison. Thus, although these patients had an epilepsy phenotype, the tissue chosen was as far away from the seizure focus (typically the hippocampus) as possible within the area to be resected. Neuropathological analysis of resected brain tissue revealed, in MTLS patients, a mild-to-moderate degree of gliosis, whereas cortical lamination was always preserved. In contrast, structural disruption along with the presence of large, aberrant neurons was seen in all tissue samples obtained from FCD patients. Table 1 summarizes the clinical and pharmacological data for all patients.

Slice preparation. Brain tissue was removed and placed in ice-cold oxygenated slicing medium, an artificial CSF (sACSF) consisting of the following (in mM): 220 sucrose, 3 KCl, 1.25 NaH_2PO_4 , 2 MgSO_4 , 26 NaHCO_3 , 2 CaCl_2 , and 10 dextrose (295–305 mOsm). A sample containing cortical tissue was then rapidly transported to the laboratory and cut on a vibroslicer within 10–20 min of resection. Slices 300 μm thick were cut in 4°C oxygenated (95% O_2 , 5% CO_2) sACSF and transferred immediately to a holding chamber where they remained submerged in oxygenated recording medium (nACSF) consisting of the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4 , 2 MgO , 26 NaHCO_3 , 2 CaCl_2 , and 10 dextrose (295–305 mOsm). Slices were held at 37°C for 45 min and then allowed to come to room temperature. For each experiment, an individual slice was gently transferred to a submersion-type recording chamber where it was continuously perfused with oxygenated nACSF at room temperature.

Electrophysiology. Whole-cell voltage-clamp recordings were obtained

Table 1. Patient data

Patient	Age (years)	FCD	AEDs	Brain location
1	18	No	Valproic acid, clonazepam	Temporal
2	17	Yes	Carbamazepine, levetiracetam	Frontal
3	46	Yes ^a	Phenytoin	Parietal
4	38	No	Lamotrigine, levetiracetam	Temporal
5	22	No	Carbamazepine, phenytoin	Temporal
6	24	Yes	Carbamazepine, topiramate	Frontal
7	19	Yes	Oxcarbazepine, phenobarbital, zonisamide	Frontal
8	33	Yes	Phenytoin, carbamazepine	Frontal
9	19	No	Oxcarbazepine	Temporal
10	32	No	Topiramate, clonazepam	Temporal
11	39	No	Lamotrigine, carbamazepine	Temporal
12	24	No	Carbamazepine, levetiracetam	Temporal
13	39	No	Lamotrigine, zonisamide, carbamazepine	Temporal
14	32	No	Valproic acid, levetiracetam	Temporal
15	33	No	Lamotrigine, topiramate	Temporal
16	39	Yes	Oxcarbazepine, primidone, lamotrigine	Temporal
17	33	Yes	Phenytoin	Temporal

AEDs, Antiepileptic drugs.

^aFCD patient in which normal cortex was recorded as a control.

from visually identified neurons using an infrared differential interference contrast (IR-DIC) video microscopy system (Stuart et al., 1993) (Fig. 2B). Patch electrodes (3–7 M Ω) were pulled from 1.5-mm-outer-diameter borosilicate glass capillary tubing (World Precision Instruments, Sarasota, FL) using a micropipette puller (Sutter P-87; Sutter Instrument, Novato, CA), coated with Sylgard (Dow Chemical, Midland, MI), and fire polished. Intracellular patch-pipette solution for whole-cell recordings contained the following (in mM): 120 Cs-glucuronate, 10 HEPES, 11 EGTA, 11 CsCl₂, 1 MgCl₂, 1.25 N-ethyl bromide quaternary salt, 2 Na₂-ATP, and 0.5 Na₂-GTP, pH 7.25 (285–290 mOsm). Cells were filled with biocytin and analyzed *post hoc* (Fig. 2B). To isolate GABAergic IPSCs, slices were perfused with nACSF containing 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 50 μM D-(-)-2-amino-5-phosphonopivalic acid (D-APV). Miniature IPSCs (mIPSCs) were recorded in ACSF supplemented with 1 μM tetrodotoxin (TTX). IPSCs were recorded as outward currents at a holding potential of 0 mV, which is near the reversal potential for glutamatergic postsynaptic currents (Otis and Mody, 1992; Kobayashi et al., 2003). In some cells, spontaneous IPSCs (sIPSCs) and evoked IPSCs (eIPSCs) were recorded at different holding potentials (−75 and −30 mV), because the decay kinetics can vary as a function of membrane potential (Otis and Mody, 1992). IPSCs were recorded from pyramidal cortical cells from FCD specimens ($n = 25$ cells) (Fig. 2B) or from pyramidal cells from normal cortical areas from MTLS specimens ($n = 25$ cells) and from a nondysplastic area in one patient with FCD (additional control).

eIPSCs were elicited at 0.1 Hz using a monopolar electrode placed in the white matter. Low-frequency (0.1 Hz), 100 μs pulses were applied, and their intensity was increased until the threshold for eliciting a detectable monosynaptic eIPSC was reached. Stimulus intensity was then increased to two times the threshold and maintained at this intensity for the entire experiment. Because the recording and stimulating electrodes were separated by relatively large distances (e.g., recording electrode in layers II/III and stimulating electrode in the white matter), the decay-time constant of evoked IPSCs could be attributed, at least in part, to the release of GABA at multiple synapses situated on distal dendrites. The peak of the evoked response was examined (at a fast time resolution) to ensure that the measured response, a delay >5 ms, did not arise from shock artifact. Evoked and spontaneous IPSCs were abolished by bath application of 10 μM bicuculline ($n = 10$ cells) to confirm a role for postsynaptic GABA_A receptors. Voltage and current were recorded with an Axopatch 1D amplifier (Molecular Devices, Union City, CA) and monitored on an oscilloscope. Whole-cell voltage-clamp data were low-pass filtered at 1 kHz (−3 dB; eight-pole Bessel), digitally sampled at 10 kHz, and monitored with pClamp software (Molecular Devices) running on a Pentium computer (Dell Computer, Round Rock, TX). The whole-cell capacitance was similar for cells sampled in control and FCD tissue

Figure 1. Representative magnetic resonance imaging (MRI), ECoG, and histology from a patient with FCD. **A**, Coronal MRI section shows an area of FCD in the left frontal lobe. R, Right; L, left. **B**, ECoG showing almost continuous paroxysmal activity recorded from the surface of the FCD. Bipolar montage in rows of 4: rows 1–3 represent the superior frontal lobe with anterior (1) and posterior (3) localization. Rows 4–6, 7–9, and 10–12 represent subsequent inferior locations. Rows 13 and 14 represent referential recordings from monopolar electrodes placed in the bone edges with anterior (13) and posterior (14) localization. This ECoG shows epileptogenic activity mainly in rows 1, 2, 4, and 5, indicating a superior anterior focus. This corresponds precisely to the area of abnormality observed on the MRI. **C**, Hematoxylin and eosin (H&E) staining of tissue from FCD (left) and control (right) patients showing all neocortical layers; the dysplastic tissue has lost the layered architecture that is seen in normal tissue and also has many bizarre cells present throughout (magnification, 100 $\times$). **D**, H&E stain of tissue at the cortico-white matter (WM) junction from a patient with FCD (left) and a control patient (right); note the excess number of cells in the FCD specimen (magnification, 200 $\times$).

(30–43 pF), and the whole-cell access resistance was monitored carefully throughout the recordings. Cells were rejected if values changed by >25% (or exceeded 20 M Ω); only recordings with stable series resistance of <20 M Ω were used for IPSC analysis.

Immunohistochemistry. Surgically removed tissue from both control and FCD patients were embedded in paraffin. The paraffin blocks (seven for controls and 30 for FCD) were cut into 5- μ m-thick sections, and these samples were used for immunostaining for interneuronal markers. The sections were deparaffinized in xylene and rehydrated in five washes with decreasing concentrations of ethanol (100–70%). Antigen retrieval was performed subsequently to enhance the final signal. Slides were immersed in antigen retrieval solution (DakoCytomation, Carpinteria, CA), pH 6, in a steamer for 20 min. After several rinses with PBS, the slides were treated with 3% H₂O₂ and, after blocking in lamb serum, were incubated with the primary antibody overnight at 4°C. Antibodies used were anti-calbindin (mouse; Sigma, St. Louis, MO), anti-parvalbumin (mouse; Sigma), and anti-glutamate decarboxylase

(GAD) 65/67 (rabbit; used to label the terminals and cell bodies of GABAergic interneurons; Sigma). Antibodies were visualized by using the ABC biotin-diaminobenzidine detection system (Vector Laboratories, Burlingame, CA).

Staining for GABA transporters was done on fresh, frozen surgical tissue. One-half of each of the surgical specimens was used for the electrophysiology experiments, and the other half was fixed overnight in 4% paraformaldehyde, cryo-protected in 30% sucrose, and frozen. The frozen blocks were cut into 10- μ m-thick sections. Immunostaining, without antigen retrieval, was performed as described above using antibodies against GAT-1 and GAT-3, the two transporters found in normotopically organized cortex (Conti et al., 2004) (rabbit; Novus Biologicals, Littleton, CO). For all immunohistochemical experiments, tissue samples from control and FCD were processed simultaneously. We included rodent tissue for each antibody used to confirm that the staining protocol was working during each immunostaining run. In addition, for the GABA transporter immunostaining experiments, we also stained equivalent tissue (from the same tissue block) with neuronal-specific nuclear protein, a neuron-specific antibody, to ensure sample quality.

We could not systematically quantify changes in the cells labeled by the interneuronal markers used, because we did not have equivalent pieces of tissue for all patients and serial sections through the focal cortical dysplasia were not available. However, we followed a qualitative protocol for analysis to identify any differences between control tissue and tissue from patients with FCD. Three separate investigators viewed each slide specimen and examined the following parameters for each slide: (1) intensity of staining, (2) the density of cells stained (high vs low), and (3) localization of labeled cells. The figures presented are representative of these evaluations.

Data analysis. Spontaneous IPSCs were measured using Mini Analysis 5.2.5 software (Synaptosoft, Decatur, GA). Each event was selected manually based on rise time, amplitude, and decay properties. Between 100 and 200 individual events were analyzed for each cell. Analysis was performed twice, first by an investigator blind to the status of the experiment and then

by the experimenter. The frequency, interevent interval, amplitude, 10–90% rise time, and area were measured. Evoked IPSCs were analyzed using Clampfit (Molecular Devices). Kinetic analysis of the IPSCs was performed with a single-exponential function. Results are presented as mean $\pm$ SEM. Data before and after drug application were analyzed using a Student's *t* test on the SigmaStat program (Jandel Scientific, San Rafael, CA). A one-way ANOVA was used to compare the results between different cell types. Significance level was set as *p* < 0.05, unless otherwise noted.

Results

Reduced IPSC in focal cortical dysplasia

To examine the possibility that GABA-mediated synaptic inhibition is compromised in a cortical malformation, we recorded IPSCs in the presence of 50 μ M D-APV and 20 μ M DNQX. sIPSC

frequency was significantly lower for pyramidal cells in dysplastic cortex (1.9 ± 0.2 Hz; $n = 25$) than for pyramidal cells in control cortex taken from patients with MTL (3.1 ± 0.3 Hz; $n = 25$; ANOVA, $p < 0.001$) (Fig. 2). Representative spontaneous and miniature IPSCs for each cell type are shown for direct comparison in Figure 2A. The shift toward higher interevent intervals in the cumulative histogram signifies that inhibition is reduced (Fig. 2D). The measured peak amplitudes and sIPSC 10–90% rise times showed no significant differences among cells from the two groups (amplitude: FCD, 27.3 ± 3.5 pA; control, 28.3 ± 2.8 pA; rise time: FCD, 6.3 ± 0.5 ms; control, 5.4 ± 0.4 ms) and were suggestive of normal postsynaptic GABA-receptor function. To obtain an overall measure of inhibitory tone, we next performed a charge transfer calculation. By measuring the charge carried by individual sIPSC events as area under the current curve (Vreugdenhil et al., 2003; Di Paolo et al., 2004), a cumulative inhibitory current over a specified period of time could be calculated and compared between FCD and control tissue. For cells sampled in FCD tissue this value was 291 ± 18 pA·ms ($\times 100$), significantly smaller than that calculated for controls [600 ± 54 pA·ms ($\times 100$); $p < 0.001$].

To begin to distinguish whether reduced sIPSC frequency could be attributed to loss of inhibitory interneurons or to a reduced probability of presynaptic GABA release, we evaluated miniature IPSCs recorded in the presence of $1 \mu\text{M}$ TTX, $50 \mu\text{M}$ D-APV, and $20 \mu\text{M}$ DNQX (Fig. 2A). Consistent with spontaneous IPSC findings, mIPSCs recorded from pyramidal cells in dysplastic tissue had significantly lower frequencies (1.2 ± 0.3 Hz; $n = 7$) compared with mIPSCs recorded on control cells (2.5 ± 0.5 Hz; $n = 7$; ANOVA, $p < 0.05$) (Figs. 2A,C,D). Again, the mIPSC measured peak amplitudes and 10–90% rise times from FCD and control cortex showed no significant differences (amplitude: FCD, 15.2 ± 1.5 pA; control, 13.8 ± 0.6 pA; rise time: FCD, 3.8 ± 0.5 ms; control, 4.9 ± 0.3 ms). These findings suggest that GABA-mediated synaptic inhibition is functionally reduced in patients with FCD.

Alterations in interneuron distribution in focal cortical dysplasia

To determine whether the decrease in IPSC frequency in FCD is associated with interneuronal abnormalities, we used immunohistochemistry to compare the density and distribution of interneurons between FCD and nondysplastic tissue specimens. We used an antibody against GAD to label the terminals and cell bodies of GABAergic interneurons (Hiscock et al., 2000). The

Figure 2. Whole-cell visualized patch-clamp recording in cortical control and dysplastic cells. **A**, sIPSCs and mIPSCs (mini IPSC) of cortical pyramidal cells from control and FCD groups. **B**, IR-DIC imaging (top) and biocytin filling (bottom) of a pyramidal neuron in cortical tissue sample resected from patients with FCD. **C**, Plots of frequency of sIPSCs and mIPSCs of cortical pyramidal cells from control (Con) and FCD groups. Note the significantly lower sIPSCs and mIPSCs frequencies for dysplastic cells. **D**, Cumulative probability plots of interevent intervals of sIPSCs and mIPSCs on control (black) and FCD (gray) cells. Note that the curve of the interevent interval for FCD is shifted to the right (lower frequency), compared with controls. Error bars indicate SEM. * $p < 0.001$; ** $p < 0.05$; ANOVA.

general GAD-expression pattern did not indicate a clear difference in overall distribution of interneurons between FCD and nondysplastic tissue specimens (Figs. 3A,D). However, when we looked at the expression of parvalbumin and calbindin, Ca^{2+} binding proteins that can also serve as markers for subpopulations of GABAergic interneurons (Van Brederode et al., 1990; del Rio and DeFelipe, 1996), we observed a striking difference in the regionalization of these cell types. In nondysplastic neocortical control tissue, calbindin- and parvalbumin-positive cells were confined primarily to neocortical layers II/III (Figs. 3B,C). In contrast, tissue from patients with FCD showed an abnormal distribution of these cells throughout all cortical layers; some areas contained small clusters of interneurons, whereas in many regions, calbindin- and parvalbumin-positive cells were scarce (Figs. 3E,F).

Figure 3. Immunostaining of dysplastic tissue with interneuronal markers reveals abnormal interneuronal distribution. Expression of GAD (**A**, **D**), calbindin (**B**, **E**), and parvalbumin (**C**, **F**) shows no significant change in the overall numbers of interneurons in dysplastic tissue (compare **A**, **D**) but does show loss of regionalization of a subpopulation of interneurons (compare **B**, **C** and **E**, **F**). The insets at higher magnification do not reveal any severe changes in morphology (original magnification, 40 $\times$).

Figure 4. Changes in decay-time constant of sIPSCs and mIPSCs from cortical control pyramidal cells compared with those from dysplastic cells. Superimposed traces of sIPSCs (**A**) and mIPSCs (Mini IPSC; **D**) of cells from control (black) and FCD (gray) groups are shown. Graphs of decay-time constant of sIPSCs (**B**) and mIPSCs (**E**) of cortical pyramidal cells from control and FCD groups are shown. Note the significantly prolonged sIPSCs decay for dysplastic cells (compared with control cells). Representative event histograms of sIPSCs (**C**) and mIPSCs (**F**) decay-time constant from control (black) and FCD (gray) groups are shown. Decay-time constant are plotted for 100 individual events. Note that sIPSCs from dysplastic neurons have longer decay-time constant than control cells. Error bars indicate SEM. * $p < 0.05$; ANOVA. Con, Control.

Figure 5. Changes in decay-time constant of eIPSCs from cortical control cells compared with those from dysplastic cells. Representative eIPSC recordings obtained from control pyramidal cells (black; **A**) and dysplastic cells (gray; **B**) and superimposed traces of eIPSCs of cells from control (Con; black) and FCD (gray; **C**) tissue. Note the significantly prolonged eIPSCs decay for dysplastic cells (compared with control cells; inset graph). Error bars indicate SEM. ** $p < 0.001$; ANOVA.

Dysfunctional GABA transport in FCD

Our recent analysis of hippocampal heterotopia in an animal model of malformation reported a potentially “compensatory” increase in IPSC decay time and corresponding decrease in GABA reuptake (Calcagnotto et al., 2002). To examine whether a similar change occurs in FCD patients, additional analysis of IPSC kinetics was performed. The decay-time constant for sIPSCs in dysplastic neurons was 67% greater than that measured in control cells (FCD, 10.3 ± 1.7 ms, $n = 18$; control, 6.5 ± 0.3 ms, $n = 25$; ANOVA, $p < 0.05$) (Figs. 4A,B); no significant differences in the decay-time constant were noted for mIPSCs (FCD, 5.7 ± 1.0 ms, $n = 7$; control, 4.7 ± 1.2 ms, $n = 7$) (Figs. 4D,E). To describe more fully the difference in sIPSC decay times, event histograms were constructed for representative cells (Fig. 4C). A significant shift toward longer decay-time constant for dysplastic-neuron sIPSCs was noted in comparison with control cells (Fig. 4C); no shift was noted for mIPSCs (Fig. 4F). IPSCs recorded from cells at different holding potentials (-75 and -30 mV; $n = 6$) also revealed a slower decay-time constant for dysplastic cells when compared with those measured in control cells (data not shown). Next, we examined the kinetics of evoked IPSCs. In the presence of DNQX and D-APV, white-matter stimulation consistently elicited a GABA_A-receptor-mediated IPSC. Comparison of IPSCs evoked in control ($n = 34$) and dysplastic neurons ($n = 22$) also revealed a clear prolongation in current decay in dysplastic neurons (FCD, 112.5 ± 19.5 ms, $n = 26$; control, 38.5 ± 2.4 ms, $n = 34$; ANOVA, $p < 0.001$) (Fig. 5). If the prolonged IPSCs result from altered GABA transport and reuptake, we would expect application of a GAT inhibitor to increase the duration of evoked (or spontaneous) GABA responses on normal cells but produce little (or no) effect on dysplastic neurons. To test this possibility, we examined the kinetic properties of IPSCs in the presence of 1-[2-[(diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetra-hydro-3-pyridinecarboxylic acid hydrochloride (NO-711), a selective GAT-1 inhibitor (Borden et al., 1994). Bath application of NO-711 (50 μ M) had no effect on the decay-time constant for eIPSCs recorded from dysplastic neurons ($n = 8$) (Fig. 6A,B). As expected, NO-711 produced a significant prolongation of the decay-time constant for

spontaneous IPSCs (Fig. 6C,D). In contrast, bath application of NO-711 (50 μ M) had no effect on the decay-time constant for eIPSCs recorded from control neurons ($n = 8$) (Fig. 6A,B). As expected, NO-711 produced a significant prolongation of the decay-time constant for

Figure 6. Dysplastic neurons show a GABA transporter defect. **A**, Average traces of eIPSC before (control and FCD; black) and after (gray) application of NO-711. **B**, Graphs of effect of NO-711 (gray) on the decay-time constant of eIPSCs for control (Con) and dysplastic (black) cells. NO-711 did not significantly alter the eIPSC decay-time constant on dysplastic cells but increased the eIPSC decay-time constant on control cells. Error bars indicate SEM. $^{**}p < 0.001$; Student's *t* test.

eIPSCs recorded from control cells ($n = 12$) (Fig. 6*A,B*). Similarly, bath application of NO-711 ($50 \mu\text{M}$) had no effect on the decay-time constant for spontaneous IPSCs recorded from dysplastic neurons ($n = 3$; two patients) but produced a 295% prolongation in decay-time for sIPSCs recorded from control cells ($n = 3$; two patients).

To further explore a role for GABA transporters in the decay changes observed, we immunohistochemically examined the expression of two transporters found in normotopically organized cortex (i.e., GAT-1 and GAT-3). For both transporters, we observed a dramatic decrease in immunoreactivity throughout the FCD tissue. Puncta in FCD specimens were not as large as in control tissue, and the dense distribution of GAT transporters that typically outline neurons was essentially absent (Fig. 7). The observed reductions in transporter function and/or expression could impair GABA reuptake in malformed cortex and prolong GABA-mediated synaptic events (i.e., increase the IPSC decay-time constant) in dysplastic neurons (Fig. 6).

Discussion

Although physiological alterations in GABA-mediated inhibition are well described in animal models of epilepsy (Kamphuis et al., 1989; Roper et al., 1999; Austin and Buckmaster, 2004), studies in human samples from epileptic patients have mainly addressed the molecular and anatomical changes associated with the GABAergic system. For example, one of the earliest examinations of tissue resected from TLE patients reported a reduction in somatostatin-immunoreactive hilar interneurons (de Lanerolle et al., 1989). Subsequent studies in human tissue obtained at surgery (Spreafico et al., 1998a; Williamson et al., 1999; Thom et al., 2004; Wittner et al., 2005) or rodent models (Roper et al., 1999; Austin and Buckmaster, 2004) consistently describe a loss of specific interneuron subpopulations. In TLE, the surviving GABAergic interneurons can make new inhibitory synapses with granule cells (Davenport et al., 1990; Mathern et al., 1995; Andre et al., 2001), provide a robust paired-pulse inhibition at the extracellular level (Buckmaster and Dudek, 1997), and are associated with alterations in postsynaptic GABA_A receptor expression (Brooks-Kayal et al., 1998). Many of these findings have been replicated in rodent TLE models. An interesting and potentially

controversial recent observation suggesting reduced IPSC frequency on dentate granule cells was made by Kobayashi and Buckmaster (2003) in a well characterized rodent TLE model. Whether tissue samples from patients with medically intractable FCD also exhibit altered levels of GABA-mediated inhibition was examined here for the first time. The main findings of our study on GABAergic inhibition in human FCD are (1) decreased IPSC frequency and decreased cumulative GABA-mediated synaptic currents in regions of FCD that could be related to changes in the density and/or distribution of interneurons within the malformed cortex and (2) increased duration of GABAergic events that are associated with a decrease in GABA-transporter expression in FCD.

Despite considerable research attention the exact mechanism(s) by which seizures are generated in a dysplastic brain remain unknown. Not surprisingly, it has been suggested that aberrant expression of NMDA receptors play a role in seizure genesis. Immunohistochemical analysis of adult FCD tissue indicates that NR2A/B subunits coassemble with NR1 in dysplastic, but not nondysplastic, pyramidal neurons (Babb et al., 1998; Najm et al., 2000). Using single-cell amplification techniques, Crino et al. (2001) reported a decrease of NR2A and an increase of NR2B mRNA levels in dysplastic cells from fixed adult FCD samples. Both findings are consistent with a conclusion that glutamate-mediated excitation is enhanced. Electrophysiological findings from one sampling of pediatric FCD patients, however, indicate a decrease in NMDA-mediated current density for cyto-megalic neurons, decreased Mg^{2+} and ifenprodil sensitivity, and reduced NR2B immunoreactivity (Andre et al., 2004). Whether synaptic excitation is increased (or decreased) in regions of dysplasia and whether altered glutamate receptor-mediated excitation plays a critical role in FCD-associated epilepsy cannot be determined from these disparate findings. Analysis of field potentials in tissue slices obtained from FCD patients has indicated a capability to generate GABA-mediated synchronous potentials after exposure to a convulsant agent (4-aminopyridine) (Avoli et al., 2003; D'Antuono et al., 2004). Unfortunately, the epileptogenic significance of these findings is unclear, given that similar potentials can be elicited in normal tissue slices exposed to this agent. Overall, these studies highlight the emphasis (thus far) on sophisticated functional analysis of glutamate-mediated excitation in a dysplastic brain, a level of analysis that has not yet been applied to GABAergic systems.

Reduced GABAergic inhibition in focal cortical dysplasia

Our observed reduction in the frequency of inhibitory events recorded on pyramidal cells within dysplastic tissue (with no clear difference in IPSC amplitude) is consistent with a presynaptic alteration of synaptic inhibition. Electrophysiological recordings such as these provide direct evidence for reduced GABA-mediated inhibition in FCD patients. Furthermore, this decreased inhibition, even in the absence of additional defects, could be sufficient for the generation of seizures, because even small reductions in GABA-mediated function were shown to induce abnormal electrical discharge (Powell et al., 2003; Cobos et al., 2005). Interestingly, a reduction in IPSC frequency was reported in a rodent model of cortical dysplasia (caused by prenatal irradiation) featuring hyperexcitability and rare spontaneous seizures (Zhu and Roper, 2000; Chen and Roper, 2003; Kellinghaus et al., 2004). In both human tissue and experimental models, this reduced IPSC frequency could result from a decreased release of GABA at presynaptic terminals or simply from an impairment of GABAergic terminals and/or cells within the FCD. A presynaptic

Figure 7. Dysplastic tissue from patients with FCD showed a decrease of cortical GABA transporters. Immunostaining for GAT-1 in control tissue (**A**) and tissue from a patient with focal cortical dysplasia (**B**) showed a loss of the transporter protein in the abnormal tissue. The expression of GAT-3 in control tissue (**C**) and FCD tissue (**D**) was similar (magnification, 200 $\times$; inset magnification, 400 $\times$).

disturbance is suggested by our finding of abnormally distributed GABAergic interneurons in dysplastic cortex, including clusters separated by gaps containing few or no GABAergic cells. Moreover, the reduced synaptic inhibition observed on dysplastic pyramidal neurons (Fig. 2) does not require a frank loss of GABAergic interneurons but could result from a diffuse (or limited) distribution of inhibitory inputs corresponding to the scattering of interneurons across a wide region of dysplastic cortex or lack of synapses between clustered GABAergic terminals and the cell bodies of dysplastic cells (Garbelli et al., 1999).

Our findings are entirely consistent with previous anatomical studies reporting a “widespread decrease” in GAD-immunoreactive neurons in dysplastic tissue (Spreafico et al., 1998a,b, 2000). As additional examples of this potentially common interneuron defect, a reduction in immunoreactivity against parvalbumin and calcium-binding proteins was also noted in dysplastic cortex from irradiated rats (Roper et al., 1999), from patients with nodular neuronal heterotopia (Hannan et al., 1999), in the hippocampi of experimental models of TLE (Houser et al., 1986; Ribak et al., 1986), and from patients with TLE (Ferrer et al., 1992, 1994; DeFelipe et al., 1993; Marco et al., 1996). Because quantitative analysis or staining of serial sections throughout the entire dysplastic area has not been performed in human tissue studies, we cannot infer from histological data alone that these alterations are homogeneous in the dysplastic region. At best, these studies represent “snapshots” of how interneurons may be distributed in a dysplastic brain. A more conservative interpretation of these anatomical snapshots is that they suggest reduced numbers (or uneven distribution) of GABA-containing interneurons in regions of FCD and that this reduction could be directly associated with observed changes in synaptic physiology. From this work, we hypothesize that altered inhibitory circuitry is a contributing factor to the epileptic phenotype observed in FCD patients.

Impairment of GABA transport in focal cortical dysplasia

Another interesting finding in our study was the prolongation of GABA-evoked synaptic currents in pyramidal cells within the FCD. This increase in IPSC duration could be attributed to a presynaptic increase of GABA released by the interneuron terminals, postsynaptic changes in GABA_A receptors, or changes in GABA transporters. However, the larger IPSC decay-time in FCD tissue is unlikely to be produced by the increased release of GABA, because the IPSC frequency is decreased and interneurons are abnormally distributed throughout the cortical regions. Although alterations in postsynaptic GABA_A receptors cannot be ruled out at this time, our observations would indicate that the impairment of GABA transport reuptake with consequent increased neurotransmitter levels in the extracellular space around dysplastic neurons causes the prolonged IPSC decay-time constant in the dysplastic areas. That the decay-time constant for miniature IPSCs did not change could be attributed to the idea that GABA clearance for mIPSCs (and thus kinetics) are more closely related to diffusion

than GABA reuptake. This could also explain why GABA uptake blockers, such as tiagabine, normally have no effect on mIPSC kinetics (Thompson and Gahwiler, 1992; Isaacson et al., 1993). The blockage of GABA transporters hinders the proper removal of GABA from the synaptic cleft, thereby prolonging the decay-time for spontaneous and evoked events (Isaacson et al., 1993; Soudijn and van Wijngaarden, 2000). As we demonstrated here, application of a GABA-transport inhibitor did not alter the eIPSC decay-time constant in cells from FCD tissue but did prolong the eIPSC decay time in pyramidal cells from control tissue, as would be expected for a normal cell. This finding is corroborated by our observation of a qualitative decrease in GAT-1 and GAT-3 expression within the dysplastic region. We suggest that a decrease in GAT function could be a compensatory mechanism to reduce the hyperexcitability associated with a dysplastic lesion. Indeed, compensatory impairment of GABA-transporter expression was reported in a previous study from this laboratory of a rodent model featuring nodular heterotopias in the hippocampal formation (Calcagnotto et al., 2002). Although not interpreted as compensatory at the time, similar GAT transporter defects were described in hippocampal tissue samples obtained from TLE patients (Patrylo et al., 2001) and patients with FCD (Spreafico et al., 2000). Clearly, it will be necessary to elucidate the developmental timing of IPSC and transporter defects to determine whether a loss of inhibition precedes changes in GAT transporter function.

Conclusion

Animal models have limitations in how well they replicate the human disease and how directly applicable they are for analysis of complex neurological disorders such as epilepsy. Despite these inherent limitations, it is interesting that some of the electrophysiological findings reported here have been described previously in rodent models of cortical malformation (Zhu and Roper, 2000; Calcagnotto et al., 2002; Chen and Roper, 2003). Human tissue

samples, on the other hand, limit experimental design in that strict age-matched control samples from nonepileptic patients are rarely available for comparison. Nonetheless, obtaining a better understanding of how seizures can be generated in dysplastic human neocortex ultimately requires examination of available human tissue samples. By demonstrating a reduced inhibitory drive to dysplastic neurons, our data fit with a long-standing conclusion that reduced inhibition (i.e., inadequate control of excitation) results in epilepsy (De Deyn et al., 1990; Jefferys and Whittington, 1996; Macdonald and Kapur, 1999; Cossart et al., 2005). This work, like much of what we have learned from previous animal and human studies, strongly suggests a critical role for GABA-mediated inhibition in the overall control of neural excitability. Thus, our findings provide a new perspective on the epileptogenic process in dysplastic brain tissue and suggest potential therapeutic targets (e.g., alternative methods to augment inhibition via cell replacement) for the treatment of intractable epilepsy associated with focal cortical dysplasia.

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