Molecular alterations in epilepsy-associated malformations of cortical development
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Differential expression patterns of chloride transporters, $\text{Na}^+-\text{K}^+-2\text{Cl}^-$-cotransporter and $\text{K}^+-\text{Cl}^-$-cotransporter, in epilepsy-associated malformations of cortical development

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Molecular alterations in epilepsy-associated malformations of cortical development
ABSTRACT

Malformations of cortical development are recognized causes of chronic medically intractable epilepsy. An increasing number of observations suggests an important role for cation-chloride co-transporters (CCTs) in controlling neuronal function. Deregulation of their expression may contribute to the mechanisms of hyperexcitability that lead to seizures. In the present study, the expression and cell-specific distribution of the Na^+/K^+-2Cl^-cotransporter (NKCC1) and the K^+-Cl^-cotransporter (KCC2) were studied immunocytochemically in different developmental lesions, including focal cortical dysplasia (FCD) type IIB (n=9), hemimegalencephaly (HMEG, n=6) and ganglioglioma (GG, n=9) from patients with medically intractable epilepsy and in age-matched controls. In normal control adult cortex, NKCC1 displayed low neuronal and glial expression levels. In contrast, KCC2 showed strong and diffuse neuropil staining. Notable glial immunoreactivity (IR) was not found for KCC2. NKCC1 was highly expressed in the majority of FCD, HMEG and GG specimens. NKCC1 IR was observed in neurons of different size, including large dysplastic neurons, in balloon cells (in FCD and HMEG cases) and in glial cells with astrocytic morphology. The immunoreactivity pattern of KCC2 in FCD, HMEG and GG specimens was characterized by less neuropil staining and more intrasomatic IR compared with control. KCC2 IR was observed in neurons of different size, including large dysplastic neurons, but not in balloon cells or in glial cells with astrocytic morphology. Double-labeling experiments confirmed the differential cellular distribution of the two CCTs and their expression in GABA\textsubscript{A} receptor (α1 subunit)-positive dysplastic neurons. The cellular distribution of these CCTs, with high expression of NKCC1 in dysplastic neurons and altered subcellular distribution of KCC2, resembles that of immature cortex and suggests a possible contribution of CCTs to the high epileptogenicity of malformations of cortical development.

INTRODUCTION

Malformations of cortical development (MCDs) are recognized causes of medically intractable epilepsy [29, 69, 205, 234]. A recent classification scheme of MCDs includes focal cortical dysplasia (FCD; with balloon cells; type IIB), glioneuronal tumors (such as ganglioglioma, GG) and hemimegalencephaly (HMEG) among the disorders of proliferation (with abnormal cell types) [13]. Accordingly, recent studies suggest that these MCDs share common pathogenetic mechanisms [67, 331]. Several studies, based on electrocorticographical, immunocytochemical and electrophysiological observations support the intrinsic epileptogenicity of these MCDs [29, 71, 235-238]. In the attempt to detect the still unclear underlying cellular mechanism(s) of epileptic activity in MCDs, attention has been focused on the alterations of the balance between excitation and inhibition and particularly on the local pathways of excitatory amino acid synaptic transmission (for reviews see [205, 377]). Recent evidence in human epileptogenic tissue indicates that human dysplastic tissue may retain immature properties, displaying mechanisms of seizure generation similar to that observed during development in the immature brain (for reviews see [377, 436]). Accordingly, electrophysiological studies performed in brain slices from FCD tissue show immature GABA receptor-mediated responses. GABA receptor-mediated synchronization
appears to be involved in the mechanism leading to in vitro ictal activity in human FCD and this hypothesis is also supported by pharmacological manipulations of GABA type A receptors [377, 436, 437]. The paradoxical excitatory action of GABA observed in the immature brain [438, 439] depends on the relatively high intracellular chloride ion content, which is critically regulated by the cation-Cl cotransporters (CCTs; [352, 353]. Interestingly, in both rodent and human brain the CCTs (Na⁺-K⁺-2Cl⁻ cotransporter, NKCC1, and K⁺-Cl⁻ cotransporter, KCC2) are developmentally regulated [440-442]. In particular, the strong expression of NKCC1 early during development is considered to sustain the excitatory action of GABA and facilitate seizures in the immature brain [442]. Deregulation of CCT levels with upregulation of NKCC1 has been recently reported in brain specimens from temporal lobe epilepsy (TLE) patients [443]. The present histological study analyzed the expression of both NKCC1 and KCC2 in a large series of developmental lesions, including FCD (type IIB), GG and HMEG from patients with medically intractable epilepsy. We report the specific cellular distribution of the two CCTs in both neuronal and glial components of these developmental lesions and discuss the potential role of CCTs in the epileptogenesis of MCDs.

**MATERIALS AND METHODS**

**Subjects**
The cases included in this study were obtained from the files of the Departments of Neuropathology of the Academic Medical Center (University of Amsterdam) and the University Medical Center in Utrecht. We examined a total of 24 specimens removed from patients undergoing surgery for severe FCD (type IIB; n= 9), HMEG (n=6) and GG (n=9). Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki. Two neuropathologists reviewed all cases independently. For the GG, we used the revised WHO classification of tumors of the nervous system [250]. For the FCD, we followed the classification system proposed by Palmini et al. for grading the degree of FCD [15]. Normal-appearing control cortex/white matter from temporal region was obtained at autopsy from 6 adult control patients (male/female: 3/3; mean age 31; range 17-41) without history of seizures or other neurological diseases. All autopsies were performed within 10 h after death. We also included control material from patients with age < 10 years (1 month, 2 months, 3 months, 6 months, 2 years and 8 years). We also selected 3 cases (GG) that contained sufficient amount of perilesional zone (normal-appearing cortex/white matter adjacent to the lesion), for comparison with the autopsy specimens. This material represents good disease control tissue, since it is exposed to the same seizure activity, drugs, fixation time, and age and gender are the same. The clinical features (derived from the patient’s medical records) are summarized in Table 1. Seizures were resistant to maximal tolerated doses of antiepileptic drugs. All patients underwent presurgical evaluation [144]. In all patients the lesion was localized by brain MRI; electroencephalographic recordings were performed to detect the epileptogenic area. We classified the postoperative seizure outcome according to Engel [145]. Class I consisted of patients who remained completely seizure-free and class II includes patients who are almost seizure free or have rare or nocturnal seizures only. Follow-up period ranged from 1 to 15 years.
Table 1. Summary of clinical features of MCD patients

<table>
<thead>
<tr>
<th>Patient/sex/age</th>
<th>Lesion</th>
<th>Duration of epilepsy</th>
<th>Seizure type</th>
<th>Location</th>
<th>Engel's class</th>
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</thead>
<tbody>
<tr>
<td>1/M/10 (years)</td>
<td>FCD</td>
<td>10 (years)</td>
<td>CPS</td>
<td>Temporal</td>
<td>I</td>
</tr>
<tr>
<td>2/M/14</td>
<td>FCD</td>
<td>4</td>
<td>CPS/SGS</td>
<td>Temporal</td>
<td>I</td>
</tr>
<tr>
<td>3/M/11</td>
<td>FCD</td>
<td>9</td>
<td>CPS/SGS</td>
<td>Temporal</td>
<td>I</td>
</tr>
<tr>
<td>4/M/21</td>
<td>FCD</td>
<td>21</td>
<td>CPS/SGS</td>
<td>Temporal</td>
<td>I</td>
</tr>
<tr>
<td>5/M/24</td>
<td>FCD</td>
<td>17</td>
<td>CPS</td>
<td>Temporal</td>
<td>I</td>
</tr>
<tr>
<td>6/F/16</td>
<td>FCD</td>
<td>9</td>
<td>CPS/SGS</td>
<td>Temporal</td>
<td>I</td>
</tr>
<tr>
<td>7/F/29</td>
<td>FCD</td>
<td>10</td>
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<td>I</td>
</tr>
<tr>
<td>8/F/26</td>
<td>FCD</td>
<td>14</td>
<td>CPS/SGS</td>
<td>Temporal</td>
<td>II</td>
</tr>
<tr>
<td>9/F/24</td>
<td>FCD</td>
<td>17</td>
<td>CPS</td>
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</tr>
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<td>11/M/16</td>
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<td>CPS</td>
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<tr>
<td>12/M/26</td>
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<tr>
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<td>24</td>
<td>CPS</td>
<td>Temporal</td>
<td>I</td>
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<tr>
<td>16/F/35</td>
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</tr>
<tr>
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<tr>
<td>18/F/24</td>
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<td>Temporal</td>
<td>II</td>
</tr>
<tr>
<td>19/F/3 (months)</td>
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<td>3 (months)</td>
<td>IF</td>
<td>CH/R</td>
<td>I</td>
</tr>
<tr>
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<td>HMEG</td>
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<td>IF</td>
<td>CH/R</td>
<td>I</td>
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<tr>
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<td>HMEG</td>
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<td>IF</td>
<td>CH/L</td>
<td>II</td>
</tr>
<tr>
<td>24/F/24</td>
<td>HMEG</td>
<td>24</td>
<td>CPS/SGS</td>
<td>CH/L</td>
<td>II</td>
</tr>
<tr>
<td>24/M/96</td>
<td>HMEG</td>
<td>96</td>
<td>CPS/SGS</td>
<td>CH/L</td>
<td>II</td>
</tr>
</tbody>
</table>

Age of FCD and GG patients in years; HMEG in months, CPS, complex partial seizures; SGS, secondary generalized seizures; IF, infantile spasm; CH, cerebral hemisphere; R/L, right/left.

Tissue preparation

Tissue was fixed in 10% buffered formalin and embedded in paraffin. To avoid differences in labeling related to technical variables such as tissue fixation, we used the same fixation protocol for both autopsy and surgical material; small samples of selected cortical regions (temporal cortex) were collected at autopsy and immediately fixed in formalin for 24 h (same fixation time used for the surgical specimens). Paraffin-embedded tissue was sectioned at 6 µm, mounted on organosilane-coated slides (SIGMA, St. Louis, MO, USA) and used for immunocytochemical reactions as described below. Frozen tissue from control cortex, FCD and GG (stored at -80°C) was used for western blot analysis.

Antibody characterization

Glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000), vimentin (mouse clone V9; DAKO; 1:1000), neuronal nuclear protein (NeuN; mouse clone MAB377, Chemicon, Temecula, CA, USA; 1:2000), synaptophysin (mouse clone Sy38; DAKO; 1:200), CD34 (mouse clone QBEnd10; Immunotech, Marseille, Cedex, France; 1:600), human leukocyte antigen (HLA)-DP, -DQ, -DR (mouse clone CR3/43; DAKO; 1:400), and HLA-DR (mouse clone Ta1b5; Sigma; 1:100) were used in the routine immunocytochemical analysis of FCD, GG and HMEG specimens to document the presence of a heterogeneous population of cells. For the detection of the CCTs the following antibodies (Abs) were used: NKCC1
rabbit polyclonal Ab raised against a 22 amino acid peptide sequence near the C-terminus of NKCC1 ([444]; Chemicon; 1:30); KCC2 rabbit polyclonal Ab raised against the N-terminal fusion protein (residues 932-1043) of KCC2 (Upstate Biotechnology, Lake Placid, NY, USA; 1:200). By western blot analysis NKCC1 protein was detectable as a single band of approximately 170 kDa, whereas KCC2 labeled a band at approximately 140 kDa corresponding to the glycosylated form of KCC2 and an upper band above 200 kDa corresponding to an aggregate form of the protein [445, 446]. The specificity of these Abs against CCTs was tested by preincubating each Ab with 100-fold excess of the corresponding antigenic peptide (22 amino acid peptide from NKCC1, C-terminus and a 18 amino acid peptide of KCC2, N-terminal). All immunoreactive bands disappeared after preadsorption with the corresponding peptide (Fig. 1).

Figure 1. Expression in total homogenates from control (CTX), FCD and GG specimens
Expression of β-actin (as reference protein) is shown in the same protein extracts. Immunoreactive bands for NKCC1 and KCC2 in control cortex (1), FCD (2) and GG (3) were completely abolished by preadsorption of the antibody with the corresponding peptide.

For immunoblot analysis, human normal cortex, FCD and GG samples were homogenized in lysis buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, 0.4 mg/ml Na-orthevanadate, 5 mM EDTA (pH 8.0), 5 mM NaF and protease inhibitors (cocktail tablets, Roche Diagnostics, Mannheim, Germany). Protein content was determined using the bicinchoninic acid method [187]. For electrophoresis, equal amounts of protein (50 μg/lane) were separated by sodium dodecylsulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis. Separated proteins were transferred to nitrocellulose paper by electroblotting for 1 h and 30 min (BioRad, Transblot SD, Hercules, CA, USA). After blocking for 1 h in TBST (20 mM Tris, 150 mM NaCl, 1% Tween, pH 7.5)/5% non-fat dry milk, blots were incubated overnight in TBST/5% non-fat dry milk containing the primary antibody (anti-NKCC1 1:300 or anti-KCC2, 1:2000). After several washes in TBST, the membranes were incubated in TBST/5% non-fat dry milk, containing a goat anti-rabbit Ab coupled to horseradish peroxidase (1:2500; DAKO) for 1 h. After washes in TBST, immunoreactivity was visualized using lumi–light PLUS western blotting substrate (Roche Diagnostics, Mannheim, Germany). Expression of β-actin (monoclonal mouse, Sigma; 1:50.000) was used as reference protein.
Immunocytochemistry
Immunocytochemistry was carried out as previously described [113, 256]. Both autopsy and surgical specimens were placed into sodium citrate buffer (0.01 M, pH 6.0) and heated in a microwave oven (650 W for 10 min). Single-label immunocytochemistry was performed using the ready-for-use Powervision peroxidase system (Immunologic, Duiven, The Netherlands) and 3,3’-diaminobenzidine as chromogen. Sections were counterstained with hematoxylin. Sections incubated without the primary Ab or with excess of the antigenic peptide were essentially blank. For double-labeling studies, sections, after incubation with the primary antibodies NKCC1 or KCC2 combined with NeuN, vimentin or anti-GABA\_R α1 subunit (mouse clone BD24; Chemicon; 1: 50), were incubated for 2 h at RT with Alexa Fluor® 568-conjugated anti-rabbit IgG and Alexa Fluor® 488 anti-mouse IgG (1:100, Molecular Probes, The Netherlands). Sections were then analyzed by means of a laser scanning confocal microscope (Bio-Rad, Hercules, CA, USA; MRC1024) equipped with an argon-ion laser. Labeled tissue sections were examined by two observers with respect to the presence or absence of specific immunoreactivity (IR) for the different markers. Two representative paraffin sections per case were immunoperoxidase-stained for NKCC1 and KCC2 and assessed by two investigators independently; a consensus score was obtained. As previously reported [113] we rated the degree of CCT staining on a semi-quantitative three-point scale where IR was defined as: -, not present; +, weak; ++ strong (Table 2). Neuronal cell bodies were differentiated from glia and glioneuronal balloon cells on the basis of morphology. Only neurons in which the nucleolus could be clearly identified were included. Balloon cells have eccentric nuclei and ballooned opalescent eosinophilic cytoplasm. Reactive astrocytes were detected on the basis of the morphology and the upregulation of GFAP and vimentin. Sections stained with NeuN, GFAP and vimentin adjacent to those used for the CCT staining were also studied.

RESULTS
Human material and histological features
The clinical features of the cases included in this study are summarized in Table 1. All patients had a history of chronic pharmacoresistant epilepsy. The FCD cases included in this study have all the histopathological features of severe (type IIB) FCD, including laminar disorganization, neuronal heterotopia and presence of immature neurons, giant neurons, dysmorphic neurons and balloon cells [15]. GG were composed histologically of a mixture of atypical neuronal cells and neoplastic astrocytes and showed a broad spectrum of histopathological features [29]. The neuronal component, variable in amount, was represented by cells with abnormal orientation and shape, vesicular nuclei and prominent nucleoli. The glial component of GG consisted mainly of fibrillary astrocytes with strong immunoreactivity (IR) for GFAP. The GGs included in this study were not associated with cortical dysplasia. The HMEG specimens included in our study are all from non-syndromic HMEG cases (Table 1). Microscopically, the cortical pathological findings comprised cortical dislamination, presence of large dysmorphic neurons and balloon cells. Neurons were also heterotopically located in the subcortical regions. These features were previously reported in both syndromic and non-syndromic HMEG cases [28, 447, 448].
Expression of NKCC1 in normal human brain and perilesional areas

Low NKCC1 neuronal expression was found in normal control adult cortex (Fig. 2A). The expression pattern was similar during childhood (age < 10 years), but the intensity of neuronal staining (cortical pyramidal neurons) was higher in control cortex of patients with age < 1 year, in agreement with previous observations ([442, 449]; Fig. 2B-C). The majority of the resting glial cells did not show detectable levels of NKCC1 IR (Fig. 2D), except in 3 cases in which a weak IR was observed in a few astrocytes within the subcortical white matter (Table 2). Histologically normal peritumoral cortex (of GG patients) displayed a pattern of IR similar to that observed in control adult cortex, with weak or undetectable NKCC1 IR (Fig. 3G). The specificity of the immunolabeling is supported by the specific band detected by western blot analysis of human adult control cortex (30 years; Fig. 1; [442]).

Figure 2. Cell-type distribution of NKCC1 IR in focal cortical dysplasia type IIB

Panel A: Histologically normal adult cortex (CTX; 30 year old patient) showing neuronal distribution of NKCC1 with weak IR (high magnification of a pyramidal neuron is shown in the insert). Panel B: low expression of NKCC1 is observed in cortical pyramidal neurons of a 2 year old child. Panel C: strong NKCC1 IR is observed in cortical pyramidal neurons of a 2 month old child. Panel D: normal subcortical white matter (Wm) of an adult patient, showing no detectable NKCC1 glial labeling. Panels E-I: NKCC1 in FCD (type IIB). Panel E: dysplastic cortex (low magnification) with disorganized radial and laminar organization and strong NKCC1 staining. Panels F-G: NKCC1 IR in balloon cells localized in the cortex, as well as in the white matter (arrows). Inserts in F: expression of NKCC1 (a, red) with vimentin (b, VIM; green) in a group of balloon cells (c, merged image). Panels H-I: NKCC1 IR within the neuronal component of the dysplastic cortex. Note strong IR in dysplastic neurons of different size and shape (arrows) and weak IR in few astroglial cells (arrowheads in I). Inserts in H: merged image, showing expression of NKCC1 (red) with NeuN (green) in a dysplastic neuron. Inserts in I: co-localization (yellow; arrow) of NKCC1 (red) with GABA_A (α1 subunit; green); the arrowhead indicates a NKCC1-positive cell without GABA_A (α1 subunit) expression. Single-labeled sections are counterstained with hematoxylin. Scale bar in A: A: 250, µm; B-C: 50 µm; D: 100 µm; E: 400 µm; F-I: 40 µm.
Expression of NKCC1 in MCDs

NKCC1 labeling was highly represented in surgical specimens from patients with different MCD. NKCC1 IR was encountered in both the neuronal and the glial components of FCD, HMEG and GG tissue specimens (Table 2; Figs. 2 and 3). On western blot, homogenates from adult FCD and GG cases (25 years, GG and 24 years, FCD) displayed a denser band than that observed in control adult cortex (Fig. 1).

NKCC1 in FCD

NKCC1 IR was encountered in all the FCD specimens examined in both neuronal and non-neuronal cells (Table 2; Fig. 2). The neuronal NKCC1 IR pattern (with labeling of cell bodies and processes) was similar to control cortex, with enrichment in the population of dysplastic neurons (Fig. 2H, I). Among all specimens analyzed, 78% of cases displayed strong neuronal NKCC1 IR (Table 2). Double-labeling experiments confirmed expression in neurons (NeuN-positive cells; Fig. 2H) and in cells containing GABA_A receptor subunit α1 (Fig. 2I, insert). In all the FCD cases moderate to strong NKCC1 IR was detected in balloon cells, localized within the dysplastic area in both cortex and with matter regions (Table 2 and Fig. 2F, G). Double-labeling experiments confirmed NKCC1 expression in vimentin-positive balloon cells (Fig. 2F, insert). Expression of NKCC1 was also detected in glial cells. Astrocytes immunoreactive for NKCC1 were observed in all FCD cases (Table 2). Three cases showed strong staining in astrocytes and the remaining 6 cases displayed weak astroglial NKCC1 IR (Table 2; Fig. 2I). Double-labeling experiments confirmed expression in reactive astrocytes (vimentin-positive cells; data not shown).

Figure 3. Cell-type distribution of NKCC1 IR in HMEG and GG

Panels A-D: NKCC1 IR in the cortex of HMEG specimens. Panel A: dysplastic HMEG cortex (low magnification) with strong neuronal NKCC1 staining. Panels B-C: NKCC1 IR within the neuronal component of the dysplastic cortex in neurons of different size and shape (arrows). Inserts in B: expression of NKCC1 (red) with NeuN (green) in a dysplastic neuron. Panel D: NKCC1 IR in a balloon cell localized in the white matter (arrow). Panels E-G: NKCC1 IR in GG. Note IR within neuronal (arrows in E-F) and glial (arrowheads) cells. Insert in E: merged image, showing expression of NKCC1 (red) with vimentin (VIM; green) in a tumor astrocyte. Insert in F: merged image, showing co-localization (yellow; arrow) of NKCC1 (red) with GABA_A R (α1 subunit; green); the arrowhead indicates a GABA_A R-positive cell without NKCC1 expression. Panel G: merged image, showing almost undetectable expression of NKCC1 (red) in neuronal cells (NeuN—positive cells; green) within the peritumoral cortex. Single-labeled sections are counterstained with hematoxylin. Scale bar in A: 250 µm; B-D, F-G: 40 µm; E: 100 µm.
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NKCC1 in HMEG
NKCC1 IR was encountered in all the HMEG specimens examined in both neuronal and non-neuronal cells (Table 2; Fig. 3A-D). The neuronal NKCC1 IR pattern was similar to that observed in FCD specimens, with strong labeling of cell bodies and processes in all the 6 specimens included in this study (Fig. 3A-C). Double-labeling experiments confirmed expression in neurons (NeuN-positive cells; Fig. 3B, inserts) and in cells containing the GABA<sub>α</sub> receptor subunit α1 (not shown). In 4 out of 6 HMEG cases with age < 1 year (2, 3, 6, 7 months), both the pattern and intensity of the staining was similar to the age-matched controls. However, in the 2 HMEG patients with age > 1 year (2 years and 8 years) the expression of NKCC1 in neurons was higher than that observed in the age-matched control cortex (Fig. 3A-B, 2 year old HMEG case). In all the 5 HMEG cases containing balloon cells moderate to strong NKCC1 IR was observed in this cell type (Table 2; Fig. 2D). Expression of NKCC1 was also detected in glial cells. Astrocytes immunoreactive for NKCC1 were observed in 5 out 6 HMEG cases (Table 2).

Table 2. NKCC1 and KCC2 distribution in different cellular types in cases of FCD, HMEG and DNT (% of cases with immunoreactive cells)

<table>
<thead>
<tr>
<th>Control (n=6)</th>
<th>FCD (n=9)</th>
<th>HMEG (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons</td>
<td>Astrocytes</td>
<td>Neurons</td>
</tr>
<tr>
<td>NKCC1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>83</td>
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</tr>
<tr>
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<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Control: adult control (mean age 31); FCD, focal cortical dysplasia; HMEG, hemimegalencephaly; GG, ganglioglioma; NKCC1 and KCC2 staining; -, no; +, weak; ++, strong staining. * neuropil, ** few astrocytes within the subcortical white matter, ***somatic staining.

NKCC1 in GG
NKCC1 IR was encountered in all the GG specimens examined in both neuronal and non-neuronal cells (Table 2; Fig. 3E-F). The neuronal NKCC1 IR pattern (with labeling of cell bodies and processes) was similar to that observed in dysplastic neurons in both FCD and HMEG specimens, with strong IR detected in 8 out of 9 specimens (Fig. 3E-F; Table 2). Double-labeling experiments confirmed expression in cells containing GABA<sub>α</sub> receptor subunit α1 (Fig. 3F, insert). Expression of NKCC1 was also detected in glial cells. Tumor astrocytes immunoreactive for NKCC1 were observed in all GG cases (Table 2). 78 % of cases displayed strong astroglial NKCC1 IR (Table 2; Fig. 3E). Double-labeling experiments confirmed expression in astrocytes (vimentin-positive cells; Fig. 3E, insert).

Expression of KCC2 in normal human brain and perilesional areas
Strong KCC2 neuronal expression, with prominent neuropil staining, was found in normal control adult cortex (Fig. 4A-B). The expression pattern was similar during childhood (age < 10 years), but the intensity of cortical neuropil staining was lower in control cortex of patients with age < 1, in agreement with previous observations ([442, 449]; Fig. 4C-D).
Before 1 year of age the neuropil staining was low or not detected (Fig. 4D) and an intrasomatic IR was observed in several neurons (data not shown). Resting glial cells, in both white and gray matter, did not express KCC2 (Fig. 4E). Histologically normal peritumoral cortex (of GG patients) displayed a pattern of IR similar to that observed in control adult cortex, with strong neuropil staining (Fig. 5G). The specificity of the immunolabeling is supported by the specific band detected by Western blot analysis of human adult control cortex (30 years; Fig. 1; [442]).

**Expression of KCC2 in MCDs**

KCC2 labeling was represented within the neuronal component of surgical specimens from patients with different MCDs (Table 2; Figs. 4 and 5). On Western blot, homogenates from adult FCD and GG cases (24 years, FCD and 25 years, GG), displayed lighter bands compared with that observed in control adult cortex (Fig. 1).

**KCC2 in FCD**

KCC2 IR was encountered in neuronal cells, in all the FCD specimens examined (Table 2; Fig. 4). The neuronal KCC2 IR pattern was different to that observed in control cortex. The neuropil staining was decreased, whereas the intensity of staining of the cell bodies was increased within the population of dysplastic neurons (Fig. 4F-J). Among all specimens analyzed, 67% of cases displayed strong neuronal somatic KCC2 IR (Table 2). Double-labeling experiments confirmed expression in neurons (NeuN-positive cells; Fig. 4K-L) and in cells containing the GABA<sub>A</sub> receptor subunit α1 (Fig. 4M). KCC2 IR was observed also in small-sized neurons within the dysplastic cortex (Fig. 4I, L). Expression of KCC2 was not detected in glial or balloon cells (Fig. 4G). Double-labeling experiments confirmed absence of KCC2 IR in vimentin-positive glial cells (Fig. 4N).

**KCC2 in HMEG**

KCC2 IR was encountered in neuronal cells, in all the HMEG specimens examined (Table 2; Fig. 5A-C). The neuronal KCC2 IR pattern is similar to that observed in FCD specimens, with reduction of the neuropil staining and increase of the somatic staining in dysplastic neurons (Fig. 5A-C). Strong labeling of cell bodies was observed in 5 out of 6 specimens included in the study (Table 2; Fig. 5B). KCC2 IR was observed also in small-sized neurons within the dysplastic cortex (Fig. 5C). Double-labeling experiments confirmed expression in neurons (NeuN-positive cells; Fig. 5B, insert) and in cells containing the GABA<sub>A</sub> receptor subunit α1 (not shown). In 4 out of 6 HMEG cases with age < 1 year (2, 3, 6, 7 months), both the pattern and intensity of the staining was similar to the age-matched controls. However, in the 2 HMEG patients with age > 1 year (2 and 8 years) the expression of KCC2 in neuropil was lower than that observed in the age-matched control cortex (Fig. 5A-C; 2 year old HMEG case). KCC2 was not detected in glial or balloon cells (Table 2).
Figure 4. Cell-type distribution of KCC2 IR in FCD type IIB
Panels A and B: histologically normal cortex (CTX; 30 year) showing diffuse and strong neuropil KCC2 IR. Panel C: strong KCC2 IR in the cortex of a 2 year old child. Panel D: undetectable KCC2 IR in a large number of neurons within the cortex of a 2 month old child. Panel E: normal subcortical white matter (Wm) of an adult patient, showing absence of detectable KCC2 glial labeling. Panels F-N: KCC2 in FCD (type IIB). Panel F: dysplastic cortex (low magnification) with disorganized radial and laminar organization and strong KCC2 intrasomatic IR and reduced neuropil staining. Panels G-J: KCC2 IR in dysplastic neurons of different size and shape (arrows), including small size neurons (arrows in I). Balloon cells (arrowheads in G) did not display KCC2 IR. Panels K-N: merged images of double-labeling with NeuN, GABA_A (α1 subunit) and vimentin. Panels K-L: co-localization (yellow) of KCC2 (red) with NeuN (green) in neurons of different size and shape. Panel M: co-localization (yellow) of KCC2 (red) with GABA_A (α1 subunit; green). Panel N: no co-localization of KCC2 (red) with vimentin (VIM; green). Single-labeled sections are counterstained with hematoxylin. Scale bar in A: A and F: 400 µm; B: 60 µm; C-E, G-N: 40 µm.

Figure 5. Cell-type distribution of KCC2 IR in HMEG and GG
Panel A: dysplastic HMEG cortex (low magnification) with weak neuropil KCC2 staining. Panels B-C: KCC2 intrasomatic staining within the neuronal component of the dysplastic cortex (in neurons of different size and shape; arrows). Insert in B: merged image showing expression of KCC2 (red) in NeuN-positive (green) dysplastic neurons. D: GG (low magnification) with weak neuropil KCC2 staining. Insert in D shows that there is no colocalization of KCC2 with vimentin-positive astrocytes (arrowheads). Panel E: high magnification showing intrasomatic staining within the neuronal component of the tumor. Insert in E: merged image showing expression of KCC2 (red) with NeuN (green). Panel F: merged image showing co-localization (yellow; arrow) of KCC2 (red) with GABA_A (α1 subunit; green); the arrowhead indicates a KCC2-positive cell without GABA_A IR; the double arrowhead indicates a GABA_A receptor (α1 subunit)-positive cell without KCC2 expression. Panel G: merged image, showing strong neuropil KCC2 IR expression within the peritumoral cortex. Single-labeled sections are counterstained with hematoxylin. Scale bar in A: A and D: 400 µm; B-C and E-G: 40 µm.
**KCC2 in GG**
KCC2 IR was encountered in 6 out of 9 GG specimens examined in neuronal cells (Table 2; Fig. 5D-F). The neuronal KCC2 IR pattern with labeling of cell bodies and reduced neuropil staining was similar to that observed within the dysplastic cortex of both FCD and HMEG specimens (Fig. 5D-E; Table 2). Double-labeling experiments confirmed expression in neurons (NeuN-positive cells; Fig. 5E, insert) and in cells containing the GABA<sub>α</sub> receptor subunit α1 (Fig. 5F). Expression of KCC2 was not detected in glial cells. Double-labeling experiments confirmed absence of KCC2 IR in vimentin-positive tumor astrocytes (Fig. 5D, insert).

**DISCUSSION**
MCDs are important causes of pediatric medically intractable epilepsy. Recently, particular attention has been focused on the possible role of CCTs in ictogenesis [442, 443, 450, 451]. In the present study, we show an abnormal expression of both NKCC1 and KCC2 in MCDs that could contribute to increased network excitability in these developmental lesions. The cell-specific distribution of the two CCTs and the significance of these findings for the epileptogenicity of different MCDs are discussed below.

**CCTs and neuronal cells of MCD specimens**
Recent observations, in both rodent and human brain, indicate that NKCC1 and KCC2 are expressed in neuronal cells and that their expression is developmentally regulated with opposite patterns [442, 449, 452-454]. In human cortex, high NKCC1 and low KCC2 neuronal expression is observed before the end of the first year of life [442, 449]. In our study we could confirm this developmental pattern of expression in the temporal cortex for both CCTs. In addition, we show high neuronal expression of NKCC1 in specimens of patients with different MCDs. Strong IR for NKCC1 is observed in dysplastic neurons of FCD and HMEG specimens, as well as in the aberrant neuronal component of GG. The persistence of high neuronal levels of NKCC1 in MCDs from patients with age > 1 year supports the hypothesis of delayed cortical maturation in the pathogenesis of MCDs, containing abnormal and immature neuronal cells (for reviews see [13, 436]). Increased levels of NKCC1 mRNA are also observed in the subiculum of temporal lobe epilepsy (TLE) patients and in experimental TLE models [443, 455]. Since all cases examined were associated with epilepsy, we cannot exclude that chronic seizure activity could contribute to the strong NKCC1 expression observed in the neuronal component of MCD specimens. However, upregulation of neuronal NKCC1 was not observed in the perilesional area of GG specimens. Thus, the strong neuronal expression of NKCC1 may represent an intrinsic and immature feature of MCDs associated with intractable epilepsy. NKCC1 is known to facilitate the accumulation of Cl<sup>-</sup> in neuronal cells, producing a GABA receptor-mediated excitatory response, which is characteristic of the immature brain [352, 353, 438, 439]. Immature GABA receptor-mediated responses have been detected in specimens of FCD patients [377, 436, 437]. Moreover, it has been recently shown that NKCC1 facilitate seizures in the developing brain [442]. Thus, the high expression of NKCC1 in neuronal cells (including GABA<sub>α</sub> receptor (α1 subunit)-positive dysplastic neurons) observed in MCD specimens may support the involvement of this CCT in the epileptogenicity of these lesions. Future studies focusing on the co-localization of CCTs with other GABA<sub>α</sub> receptor
subunits, may shed light on the functional interaction between GABA$_A$ receptor and CCTs in both physiological and pathological conditions.

We also report changes in the neuronal expression pattern of KCC2 in MCDs. In FCD, HMEG and GG specimens of adult epileptic patients, we observed a reduction of the KCC2 neuropil staining. However, dysplastic neurons displayed strong KCC2 IR with a predominant intrasomatic staining. This pattern of IR may reflect the persistence in MCDs of a feature of the immature brain. Accordingly, low neuropil staining for KCC2 was observed before the first year of life. Strong neuropil staining has been reported in adult rat hippocampus as a result of the developmental increased expression of this CCT in principal neurons. In these cells KCC2 appears to be primarily located in dendritic spine heads, and at a much lower level on the somata and dendritic shafts [456]. However, further ultrastructural and functional studies are required to better investigate this abnormal subcellular expression and to detect whether altered distribution of KCC2 may affect GABA receptor-mediated responses in epilepsy-associated developmental lesions.

**CCTs and glial cells of MCD specimens**

Expression of NKCC1, but not of KCC2, has also been reported in glial cells [457, 458]. In the present study, we show that both reactive astrocytes in FCD and HMEG and tumor astrocytes in GG express NKCC1. Astrocytes are highly represented within developmental lesions associated with epilepsy and several studies support the role of astroglial cells in the hyperexcitability leading to epilepsy (for review see [459]). In astrocytes, in addition to its role in the regulation of intracellular Cl$^-$ levels, NKCC1 may also contribute to K$^+$ clearance and maintenance of intracellular Na$^+$ levels [458, 460]. Recent studies suggest that NKCC1 activation may lead to astrocyte swelling and glutamate release in pathological conditions associated with gliosis (for review see [460]). Thus, the upregulation of NKCC1 in the astroglial component of MCDs could represent an additional mechanism underlying the epileptogenicity of these lesions.

**CCTs and balloon cells of MCD specimens**

Balloon cells represent an important cellular component of severe FCD (type IIB) and HMEG [13, 15, 448]. Their nature (glial or neuronal) and their role in the epileptogenicity of MCDs is controversial [72, 205, 281, 282]. We report that balloon cells express NKCC1, but not the neuronal-specific CCT, KCC2. Recent studies support the idea that balloon cells represent the result of a failure of differentiation of radial glia [72, 461]. Thus, the presence of NKCC1 in balloon cells reflects the known expression of this CCT in both glial, as well as neuronal cells.
CONCLUSION

Our observations indicate an abnormal expression of CCTs in MCDs associated with intractable epilepsy. The expression patterns of NKCC1 and KCC2 resemble the expression patterns that are observed in the immature brain and support the hypothesis of a failure of developmental maturation in the pathogenesis of different MCDs (FCD, HMEG and GG). Whether the reported deregulation of CCTs may actively contribute to the epileptogenicity of MCDs, via modulation of GABA receptor-mediated responses, cannot be concluded from this immunocytochemical study. Studies of pharmacological modulation of CCTs, performed in surgical resected tissue will be essential to test this hypothesis and to better understand the role of CCTs in epilepsy-associated pathologies.

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