NMDA Receptor Alterations in Neurons from Pediatric Cortical Dysplasia Tissue

The subunit composition of glutamate receptors affects their functional properties, and could contribute to abnormal electrophysiology in pediatric cortical dysplasia (CD). We examined electrophysiological responses and subunit assembly of N-methyl-D-aspartate (NMDA) receptors in acutely dissociated normal-appearing pyramidal and cytomegalic neurons from CD tissue and normal-appearing pyramidal neurons from non-CD tissue. In most cytomegalic and ~30% of normal-appearing pyramidal neurons from CD tissue, NMDA currents showed decreased Mg²⁺ sensitivity compared with neurons from non-CD tissue. Ifenprodil had less effect in CD pyramidal neurons from non-CD tissue. In most cytomegalic and cytomegalic neurons from CD tissue and normal-appearing neurons or heterotopias in the white matter. In severe CD, abnormal cells that could participate in the generation of epileptic activity. For example, compared with normal-appearing neurons, cytomegalic neurons displayed decreased Mg²⁺ sensitivity compared with normal-appearing neurons. Single-cell reverse transcriptase polymerase chain reaction showed that all megalic compared with normal-appearing neurons. Single-cell reverse transcriptase polymerase chain reaction showed that all megalic compared with normal-appearing neurons. Single-cell reverse transcriptase polymerase chain reaction showed that all megalic compared with normal-appearing neurons. Single-cell reverse transcriptase polymerase chain reaction showed that all megalic compared with normal-appearing neurons. Single-cell reverse transcriptase polymerase chain reaction showed that all megalic compared with normal-appearing neurons. Single-cell reverse transcriptase polymerase chain reaction showed that all megalic compared with normal-appearing neurons. Single-cell reverse transcriptase polymerase chain reaction showed that all megalic compared with normal-appearing neurons.

Introduction

Cortical dysplasia (CD) is a frequent pathological substrate in pediatric epilepsy, and is typically associated with epileptogenic EEG abnormalities (Marchal et al., 1989; Chugani et al., 1990; Mischel et al., 1995; Mathern et al., 1999a; Schwartzkroin and Walsh, 2000). CD is characterized by abnormal neuronal migration with cortical dyslamination and ectopic neurons or heterotopias in the white matter. In severe CD, cytomegalic neurons and ‘balloon’ cells are routinely observed (Taylor et al., 1971; Vinters et al., 1992; Crino et al., 2002). These aberrant cells could have a role in the generation of epileptic discharges and/or seizures (Mathern et al., 2000; Sprefico et al., 2000). This potential role is supported by the demonstration that cytomegalic neurons have increased immunoreactivity for glutamate receptor subunits, including those composing N-methyl-D-aspartate (NMDA) receptors (Kerfoot et al., 1999; Ying et al., 1999; Crino et al., 2001).

NMDA receptors are important in normal and pathologic neurological functions, such as synaptic plasticity, development, cell death, and epilepsy (Meldrum, 1992; Mody and MacDonald, 1995; Komuro and Rakic, 1998; Sattler and Tymianski, 2001; Carroll and Zukin, 2002). The NMDA receptor/channel complex is highly permeable to Ca²⁺, and its activation is voltage-dependent due to a Mg²⁺ blockade at hyperpolarized membrane potentials (Ascher and Nowak, 1986). These receptors are heteromeric proteins composed of three subunit families, NR1, NR2A-D and NR3 (Monyer et al., 1992; Ciabarra et al., 1995). In heterologous expression systems such as Xenopus oocytes, the NR1 subunit alone forms homomeric channels displaying very low amplitude currents (Moriyoshi et al., 1991). Co-expression of NR1 with NR2 subunits enhances the expression of functional channels (Monyer et al., 1992; Ishii et al., 1993; Cull-Candy et al., 2001). Mg²⁺ blockade is imparted mainly by NR2 subunits, but the sensitivity to Mg²⁺ varies depending on subunit composition. Thus, NMDA receptors that contain NR2C/D subunits are less sensitive to Mg²⁺ blockade than those containing NR2A/B (Monyer et al., 1992; Cull-Candy et al., 2001).

Cortical neurons mainly co-express NR1, NR2A and NR2B mRNAs as well as their corresponding proteins (Petralia et al., 1994; Schito et al., 1997; Scherzer et al., 1998; Conti et al., 1999). Abnormal composition and function of the NMDA receptor can lead to migration disorders, and may contribute to seizures seen in CD (Komuro and Rakic, 1993; Luhmann et al., 1998; DeFazio and Hablitz, 2000; Ramoa et al., 2001). Studies examining NMDA receptor proteins and mRNAs have described alterations in receptor composition in CD neurons with an increase in the expression and/or assembly of NR1/NR2 subunits (Ying et al., 1998a, 1999; Crino et al., 2001). However, little is known about the electrophysiological properties of dysplastic neurons and their responsiveness to NMDA in human cortical tissue (Mattia et al., 1995; Avoli et al., 1999).

Recently, we characterized the morphological and electrophysiological properties of abnormal cells in tissue from children with CD (Mathern et al., 2000; Cepeda et al., 2003). We described some of the active membrane properties of these abnormal cells that could participate in the generation of epileptic activity. For example, compared with normal-appearing pyramidal neurons, cytomegalic neurons displayed increased Ca²⁺ currents and influx when the membrane was depolarized (Cepeda et al., 2003). These findings led us to hypothesize that these cells may contribute to increased excita-
ability in CD. The present studies extended these observations and were specifically designed to examine NMDA receptors in CD and non-CD tissue. Correlated electrophysiological, morphological and molecular approaches were used to compare normal-appearing and cytomegalic neurons from CD tissue with normal-appearing pyramidal neurons from non-CD tissue. We characterized neuronal responses to NMDA, their sensitivity to Mg\textsuperscript{2+} and ifenprodil, an NMDA receptor antagonist, as well as the subunit composition of NMDA receptors.

Materials and Methods

Patient Groups

The protocol was approved by the review board of the Human Protection Research Committee at UCLA and parents gave informed consent to use clinical information from their children for research purposes. Neocortical samples were resected from 27 children with intractable seizures, 16 had CD and 11 had non-CD pathology as previously described (Kerfoot et al., 1999; Mathern et al., 2000; Cepeda et al., 2003). In the non-CD group, two patients had temporal lobe epilepsy, five had seizures due to an old cerebral infarct, two presented with seizures after head trauma, and two had Rasmussen’s encephalitis. In CD patients, the tissue was classified as least or most abnormal (LA and MA), based on electrocorticography, neuroimaging studies (magnetic resonance imaging, 1\textsuperscript{4}fluoro-2-deoxyglucose positron emission tomography), and gross physical appearance during resection. All patients were taking antiepileptic drugs, and the types of drugs were similar between CD and non-CD patients. Electrophysiological recordings were performed on brain tissue from eight of the 11 non-CD patients (mean ± age ± SEM in years: 8.8 ± 1.5; range 2.6–14.2) and from nine of the 16 CD patients (3.9 ± 1.5; 0.2–11.7). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on brain tissue from five non-CD patients (4.2 ± 1.2; 1.2–8.0) and from nine CD patients (1.5 ± 0.8; 0.3–7.8). Age was not significantly different between CD and non-CD patients in the electrophysiology group (\(P > 0.05\)), whereas age was significantly less in CD compared with non-CD patients in the RT-PCR studies (\(P = 0.005\)). Tissue from four patients (two CD and two non-CD) was studied using both electrophysiology and RT-PCR. Tissue was resected from frontal (40%), temporal (30%), and parietal cortex (30%). The brain regions sampled were similar in the non-CD and CD groups (\(\chi^{2}; P = 0.81\)).

Immunohistochemistry was performed on fronto-central cortex or frontal operculum from six CD patients (4.3 ± 1.9; 0.3–11.0) and from five non-CD patients (5.1 ± 2.8; 1.8–17). Average ages were not different from CD and non-CD groups (\(P = 0.57\)). Tissue from one of the above CD patients was studied by electrophysiology, RT-PCR, and immunohistochemistry.

Acute Neuron Dissociation and Electrophysiological Recordings

Cortical slices (350 µm) were used for acute dissociation. After 1 h incubation in standard artificial cerebrospinal fluid (ACSF) solution, a slice was placed in a low Ca\textsuperscript{2+}-isethionate solution (in mM): 140 Na\textsuperscript{+}-isethionate, 2 KCl, 2 MgCl\textsubscript{2}, 0.1 CaCl\textsubscript{2}, 25 glucose, 15 HEPES (pH 7.4, 300–305 mOsm). Small cortical pieces were dissected and placed in an oxygenated cell-stir chamber (Wheaton, Inc., Millville, NJ) containing Hank’s buffered salt solution (HBSS, Sigma Chemical Co. St Louis, MO) at 35 °C and the tube was frozen.

Whole-cell recordings used standard electrophysiological techniques. The internal pipette solution consisted of (in mM): 180 N-methyl-D-glucamine (NMDG), 40 HEPES, 2 MgCl\textsubscript{2}, 10 Ethylene glycol-bis-(\textbeta-aminomethyl ether)-N,N,N,N-tetraacetic acid (EGTA), 12 phosphocreatine, 2 Na\textsubscript{2}ATP, 0.2 Na\textsubscript{2}GTP, 0.1 leupeptin (pH 7.2–7.5 with H\textsubscript{3}SO\textsubscript{4}), 255–270 mOsm). The Mg\textsuperscript{2+} free external solution consisted of (in mM): 155 NaCl, 20 CaCl\textsubscript{2}, 5 BaCl\textsubscript{2}, 10 glucose, 10 HEPES, 0.02 glycine, 0.01 tetradotoxin (TTX, pH 7.5, 300–305 mOsm). Electrode resistance was typically 2–4 M\Omega in the bath. After seal rupture, series resistance (\(4–25 \text{ M}\Omega\)) was compensated (70–90%) and periodically monitored. Signals were recorded with an Axopatch 200A amplifier. Membrane capacitances and input resistances were measured by applying a 10 mV depolarizing step voltage command and using the membrane test function integrated in the pClamp8 software. (Axon Instruments, Foster City, CA). Drugs were applied with a nitrogen pressure ‘two-pipe’ system. The array of application capillaries (150 µm inner diameter) was positioned a few hundred micrometers from the cell under study. Solution changes were performed by changing the position of the array with a DC drive system controlled by a SF-77B perfusion system (Warner Instruments, Hamden, CT) synchronized by pClamp. The solution changes were complete within <100 ms. NMDA (10–500 µM) was applied for 3 s every 15–20 s. Responsiveness of cells to NMDA and the sensitivity to Mg\textsuperscript{2+} blockade were examined at two different holding potentials, –40 and –60 mV. The recovery from desensitization was examined by repeated applications (every 15 s) of pairs of NMDA pulses, a pre-test pulse (2 s duration, 100 µM, \(V_{\text{hold}} = –40 \text{ mV}\)) followed by a test pulse at 250 ms increments (from 250 to 2500 ms) after the end of the first pulse. Percent recovery values were calculated by computing the amplitude of the second peak as a percent of the amplitude of the first peak. To assess if NMDA receptors contained NR2B subunits, we tested the effect of ifenprodil (1 and 10 µM), an NMDA receptor antagonist with more selectivity for the NR1/NR2B subtype. Due to the clinical limitations in obtaining a large number of severe CD cases and consequently of cytomegalic neurons, not all electrophysiological experiments could be performed on the three cell groups. In order to be able to better interpret our results, we therefore used other strategies, such as RT-PCR and immunohistochemistry, to obtain convergent data to confirm our electrophysiological findings.

Cell Harvesting and RT-PCR

Similar dissociation techniques were used for RT-PCR, except that slices were treated with protease XIV (Sigma, 0.75 mg/ml) for 30 min at 35 °C. We used protease XIV to collect cells for RT-PCR because the yield of cells was higher than with papain, the enzyme we used for electrophysiology. The cell suspension was placed in a Petri dish containing RNase-free HEPES-buffered HBSS solution for 10–15 min. Individual cells at the bottom of the dish could be visualized under the microscope with an immersion objective and the entire cell was aspirated in previously baked electrodes filled with 8 µl of RNase-free water. The tip of the electrode was broken and the cellular content expelled into a tube containing 12 µl of reverse transcriptase (RT) mix containing 0.5 mM dNTPs, 1µl/reaction Sensiscript reverse transcriptase (Qiagen Inc., Valencia, CA), 0.01 mM random primers, 10 mM dithiothreitol (DTT), 0.05 U/ml RNase inhibitor (Promega, Madison, WI). The RT reaction was performed for 1 h at 37 °C and the tube was frozen.

PCR was performed using the following sets of primers designed from the human genome database (synthesis by IDT, Coralville, IA).

NSE (Neuronal Specific Enolase):[accession number NM_001975, Cerebral Cortex June 2004, V 14 N 6 635]
Homo sapiens neuronal enolase 2); sense, 5'-ACGTAGGACCATGTCATTGCTG-3' (position 1348); antisense, 5'-CATGGTCCTCCTCTAAACCTTC-3' (1888), predicted size 540 bp. NR1: (accession number NM_021560, Homo sapiens glutamate receptor, transcript variants NR1 (isofoms 3b and 4b of NR1-1, NR1-2, NR1-3 mRNA); sense, 5'-CATGGTGCCTCCTACATTGGAAC-3' (3272); antisense, 5'-AGCTTACGGGCGGCAAG-5' (3665), predicted size 391 bp. NR2A: (accession number NM_000833, Homo sapiens glutamate receptor GRIN2A, mRNA); sense, 5'-GTCCTTCTCAGGAGTGGG-3' (1492); antisense, 5'-AGTGACAGGGTGTTGATCA-3' (1948), predicted size 456 bp. NR2B: (accession number NM_000834, Homo sapiens glutamate receptor GRIN2C, mRNA); sense, 5'-TACACACCCCAAGAAGAAGGA-3' (1173); antisense, 5'-CAGAGGTTCACACATTTCC-3' (1484), predicted size 511 bp. NR2C: (accession number NM_000835, Homo sapiens glutamate receptor GRIN2C, mRNA); sense, 5'-CAAGAAGCTGGCCAGATGGTTC-3' (1547); antisense, 5'-ACTTGCGATATGGAAGAACCGGGG-3' (1922), predicted size 418 bp. The NR2D subunit was not examined in the present study because of its low abundance. The following RNA was used with each PCR as well as the data not shown.

The cDNAs of NSE, NR1, NR2A and NR2B were amplified individually with each set of primers; 3 µl of cell content in a total volume of 20 µl containing Hotstart Taq polymerase master mix (2.5 µl polymerase; 1.5 mM MgCl2; 200 µM each dNTP; Qiagen, Valencia, CA), 0.1 µM each primer, for 40 cycles (94°C, 45 s; 58°C, 45 s; 72°C, 1 min). When a subunit was not expressed, a multiplex PCR was run to ensure the cDNA was not present. In the first 25 cycle PCR, all cDNAs were amplified simultaneously. A 10 µl volume of cDNAs in a total volume of 50 µl containing Hotstart Taq polymerase master mix (3.0 µl polymerase; 1.8 mM MgCl2; 240 µM each dNTP; 0.1 µM each primer). The second PCR was performed for each set of primers using 5 µl of the first PCR product in a total volume of 20 µl (2.5 µU polymerase; 1.5 mM MgCl2; 200 µM each dNTP; 0.1 µM each primer). 35 cycles. Due to its low abundance, NR2C cDNA was always amplified in two successive PCRs. A 10 µl volume of each PCR as well as the 100 bp molecular weight marker were then run on a 2% agarose gel stained with ethidium bromide.

To test the efficiency of the PCR protocol, total RNA (90 pg) extracted from human neocortex was run with each RT-PCR. The housekeeping gene, neuron specific enolase (NSE) was run for each RT-PCR. The extracted human neocortex was run with each RT-PCR. The efficiency of the PCR protocol, total RNA (90 pg) extracted from human neocortex was run with each RT-PCR. The housekeeping gene, neuron specific enolase (NSE) was run for each RT-PCR. The extracted human neocortex was run with each RT-PCR. The housekeeping gene, neuron specific enolase (NSE) was run for each RT-PCR.

Immunohistochemistry
To study the expression of the NMDA receptor subunits at the protein level, immunohistochemical staining was performed for Neuron-specific nuclear protein (NeuN), NR1 and NR2B. As commercially available NR2A antibodies did not appear to work well in human tissue, we did not perform immunohistochemistry for NR2A. Tissue blocks adjacent to those used for electrophysiology and RT-PCR were fixed in 4% paraformaldehyde for 6–8 h, cryoprotected in increasing sucrose concentrations (20–30%) diluted in PBS, frozen, and stored at -80°C for later batch processing. Cryostat sections (50 µm) were rinsed in Tris-saline and incubated overnight at 4°C with mouse monoclonal antibodies (50 m/s Tris-saline, pH 7.4, 0.2% Triton X-100), 4% goat serum (Vector Laboratories, Burlingame, CA) against NeuN (MAB577, Chemicon, Temecula, CA) and rabbit polyclonal antibodies against NR1 or NR2B (Ab1516 and Ab1557P, Chemicon). Double immunofluorescence staining was performed with antibodies against NeuN (1:1000) and NR1 (1:500), and NeuN (1:1000) and NR2B (1:100). Sections were then washed three times in Tris-saline and incubated with goat antibodies conjugated either to Cy3 (1:500, Jackson ImmunoResearch, West Grove, PA) or to Alexa 488 (1:1000, Molecular Probes, Eugene, OR) in Tris-saline containing 4% normal goat serum and 0.05% Triton for 30 min at room temperature. After three washings with Tris-saline, sections were mounted on gelatin-coated slides and air-dried. They were cover-slipped with glycerol and analyzed by confocal laser scanning microscopy (LSM 510 META, Zeiss, Jena, Germany). To evaluate the number of neurons expressing NR1 and NR2B, cell counts were performed on 5–10 pictures taken with the confocal microscope. Colocalization of NeuN and NR2B was assessed on 50–550 cells for each tissue section.

Data Analyses
Data analyses were performed with Origin (Microcal Software, Northampton, MA) and pClamp software. Electrophysiological membrane properties and surface areas were compared among groups by one-way analyses of variance (ANOVA) followed by a Tukey test using Statistica software (SPSS Science, Chicago, IL). NMDA current characteristics were compared using a two-way ANOVA with one repeated measure followed by Bonferroni t-tests. The frequency distribution of cells expressing NMDA receptor subunits was compared using chi-square analysis (Statview Software, Cary, NC). Differences were considered statistically significant when P < 0.05.

Results

Electrophysiology

Cell Sampling and Classification

Only pyramidal-shaped cells (both normal-appearing and cytomegalic) were included in the present study. These cells were relatively easy to differentiate morphologically by their typical somatic shape and the presence of clear apical and basilar dendrites. Other cells, such as atypical (putative ‘balloon’ cells), interneurons, and glia were excluded (Cepeda et al., 2003). Based on pathology, somatic size and dendritic morphology, three groups of cells were examined and compared: non-CD normal-appearing, CD normal-appearing, and CD cytomegalic neurons (Fig. 1). In CD cases, both normal-appearing cells and cytomegalic neurons were visualized while in the non-CD group, only normal-appearing pyramidal neurons were found (Figs 1, 6 and 7). Cytomegalic cells were abnormally large, had thick initial dendritic segments and, in some cases, aberrant branches protruded from many parts of the soma (Fig. 1F). In CD patients, all abnormal-appearing cytomegalic neurons were from the MA sample sites. The somatic area of cytomegalic neurons was more than twice that of normal-appearing pyramidal neurons from CD and non-CD groups (Table 1).

Electrophysiological Properties

Passive membrane properties were measured in normal-appearing pyramidal cells from non-CD cases and normal-appearing pyramidal and cytomegalic cells from CD cases. There were no significant differences in membrane capacitance, input resistance, or time constants between normal-appearing pyramidal neurons from CD and non-CD cases. By contrast, cytomegalic neurons had significantly larger mean membrane capacitances and longer mean time constants due to their larger size (Table 1). The input resistance tended to be lower in cytomegalic neurons, but the difference was not statistically significant.

Characteristics of NMDA Currents

To determine the effective NMDA concentration for dissociated human cortical neurons, we tested increasing concentrations of NMDA on non-CD and CD normal pyramidal neurons in the absence of Mg2+ (Fig. 2). Short pulses of NMDA...
(10–500 µM, 3 s duration, $V_h = –40$ mV) induced inward currents in a concentration-dependent manner (Fig. 2A, B). At concentrations of 50 µM or higher, the NMDA response consisted of a fast peak, followed by a slowly desensitizing component. The EC$_{50}$ for non-CD cells was 56.4 ± 3.6 µM ($n = 4$) and for CD cells it was 41.5 ± 4.8 (µM) ($n = 5$). Because these values are not significantly different, in subsequent experiments we used a non-saturating concentration (100 µM) of NMDA. NMDA responses ($V_h = –40$ mV) in normal-appearing pyramidal cells were reduced by the addition of 50–500 µM Mg$^2+$ (53–87% block, respectively) to the extracellular solution (data not shown).

In Mg$^2+$-free solution, most pyramidal neurons from CD tissue showed similar NMDA current characteristics to those observed in non-CD neurons (Fig. 3A). As expected, NMDA currents were larger when the membrane was held at –60 than at –40 mV. There were no significant differences in the peak amplitude of NMDA currents or current densities between normal-appearing pyramidal neurons from CD and non-CD cases. By contrast, peak NMDA currents were larger in cytomegalic compared with normal-appearing pyramidal neurons from both CD and non-CD cases at holding potentials of –60 mV and –40 mV (Fig. 3A, B1, B3; $P < 0.05$). However, after normalizing to cell capacitance, NMDA current densities in cytomegalic neurons were significantly smaller compared with those of normal-appearing pyramidal cells from non-CD and CD groups (Fig. 3B2 and B4; $P < 0.01$), indicating that the receptor density or conductance per unit area may be reduced in cytomegalic neurons.

### NMDA Receptor Sensitivity to Mg$^2+$

NMDA responses in normal-appearing pyramidal cells from non-CD cases were decreased markedly by the addition of 50 µM Mg$^2+$ to the extracellular solution (average percent block was 72% at –60 mV and 55% at –40 mV) (Fig. 3C1, C2). The addition of 50 µM Mg$^2+$ also produced a significant blockade of NMDA currents in normal-appearing pyramidal cells from CD cases (average block was 53% at –60 mV and 33% at –40 mV). However, the Mg$^2+$ blockade was significantly smaller in normal-appearing pyramidal cells from CD compared with non-

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>Cm (pF)</th>
<th>Rm (GΩ)</th>
<th>Tau (µs)</th>
<th>Surface area (µm$^2$)</th>
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<tbody>
<tr>
<td>Non-CD normal-appearing ($n = 26$)</td>
<td>11.2 ± 1.5</td>
<td>1.0 ± 0.2</td>
<td>217.6 ± 29.0</td>
<td>204.7 ± 20.4</td>
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<tr>
<td>CD normal-appearing ($n = 27$)</td>
<td>13.9 ± 1.4</td>
<td>0.9 ± 0.2</td>
<td>234.7 ± 34.0</td>
<td>239.7 ± 28.0</td>
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<tr>
<td>CD cytomegalic ($n = 5$)</td>
<td>52.2 ± 14.5</td>
<td>0.8 ± 0.1</td>
<td>437.4 ± 221</td>
<td>483.0 ± 71.6</td>
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<tr>
<td>CD cytomegalic vs Non-CD</td>
<td>$P &lt; 0.001$</td>
<td>NS</td>
<td>$P &lt; 0.001$</td>
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<td>CD cytomegalic vs CD normal</td>
<td>$P &lt; 0.001$</td>
<td>NS</td>
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<td>Non-CD vs CD normal</td>
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Cm, membrane capacitance; Rm, membrane input resistance; Tau, membrane time constant.
CD cases ($P < 0.001$). Among the normal-appearing pyramidal cells of the CD group, 7/27 had Mg$^{2+}$ blockade below 20% (at –40 mV), and 5/15 had a blockade below 50% (at –60 mV). The other cells were in the range of non-CD values (above 30% at –40 mV), while 4/5 had a Mg$^{2+}$ blockade below 15% (average percent block was 9% at –60 mV and 11% at –40 mV). When normal-appearing and cytomegalic cells from CD tissue were combined, 11/32 of the cells had Mg$^{2+}$ blockade below 30% (at –40 mV) and 9/25 had a blockade below 50% (at –60 mV). The rest displayed Mg$^{2+}$ blockade similar to that of normal-appearing non-CD pyramidal cells. In the CD group, the distribution of cells with lower Mg$^{2+}$ sensitivity correlated with the presence of cytomegalic cells in the brain area studied ($\chi^2, P = 0.009$). In other words, cells that displayed low Mg$^{2+}$ sensitivity were always located in an area containing cytomegalic cells, based on histopathological examination.

**NMDA Current Recovery from Desensitization**

Recovery from desensitization, a measure that depends on receptor subunit composition (Vicini et al., 1998), was examined in the three groups of neurons by repeated applications of NMDA (see Materials and Methods). Normal-appearing neurons from non-CD and CD tissue displayed the same time course and percent NMDA current recovery (Fig. 4). In contrast, in cytomegalic neurons the time course was faster and the percent recovery was larger than in normal-appearing neurons. The differences were statistically significant at 250, 500, 750 and 1000 ms intervals ($P < 0.03$–0.001).

**Sensitivity to Ifenprodil**

Because NMDA receptors containing a functional NR2B subunit are more sensitive to ifenprodil, we tested the effect of this blocker (1 and 10 $\mu$M) on NMDA-induced currents (100 $\mu$M). In non-CD pyramidal cells ($n = 7$), ifenprodil (1 $\mu$M) blocked 49.4% while at 10 $\mu$M it blocked 73.5% of the peak NMDA current (data not shown). We tested 1 $\mu$M ifenprodil on CD normal-appearing pyramidal cells ($n = 6$). Ifenprodil blockade was significantly smaller in CD normal-appearing cells compared with non-CD neurons (26.5% vs 49.4% $P = 0.003$, Fig. 5A,B). Unfortunately, due to limitations of tissue availability, no cytomegalic cells were tested with ifenprodil.

**RT-PCR**

**Cell Classification**

RT-PCR was performed on 69 dissociated cells, 41 (59.5%) from the CD group and 28 (40.5%) from the non-CD group. The cells were collected from the same cortical areas (29 frontal, 27 temporal and 13 parietal) in the CD and non-CD group ($\chi^2, P = 0.6$). The number of cells collected per brain sample (2–10) was similar between non-CD and CD sites ($P = 0.6$). The 28 cells from the non-CD group were all normal-appearing pyramidal neurons. In the CD group, 31 cells (75.6%) were classified as normal-appearing pyramidal and 10 (24.4%) as cytomegalic neurons (Figs 1 and 6).

Dissociated cells from CD tissue had altered NMDA receptor subunit composition compared with non-CD cells (Fig. 6). In the non-CD group, 96% of the cells (27/28) co-expressed mRNAs for NR1, NR2A and NR2B, and one neuron expressed NR1 and NR2B but no NR2A mRNAs. In the CD group, by contrast, 19% (6/31) of the normal-appearing pyramidal cells lacked NR2B mRNA. The differences in the distributions of the three NMDA receptor subunits per cell were statistically significant in the non-CD compared with the CD group ($\chi^2, P = 0.008$). In the CD group, the distribution of cells lacking the NR2B subunit correlated with the presence of cytomegalic cells in the brain area studied ($\chi^2, P = 0.01$). Cells that lacked NR2B were always located in an area containing cytomegalic neurons. The differences in NMDA receptor subunit mRNAs did not depend on the location of the sample site (temporal, parietal or frontal; $P = 0.81$), nor did they correlate with the age at surgery ($P = 0.14$). Moreover, in the non-CD group, the three NMDA receptor subunit mRNAs were detected in all eight cells from the youngest (1.2 years) as well as the seven cells from the oldest patient (8.0 years). Brain tissue from two CD patients that was used for Mg$^{2+}$ sensitivity was also tested for RT-PCR. Although the recorded cells were different from the ones used for RT-PCR, we found that in these two CD cases two of four cells displayed low Mg$^{2+}$ sensitivity and 3 of 12 cells had no NR2B subunit.

RT-PCR for NR2C was performed on total RNA (100–300 pg) extracted from tissue samples of four cases (one non-CD, four
Figure 3. Effects of Mg2+ on NMDA currents in non-CD, CD normal-appearing neurons, and cytomegalic cells. (A) Traces show NMDA currents evoked in the absence of Mg2+, and in the presence of 50 µM Mg2+ in a non-CD cell (A1), two normal-appearing CD cells with different sensitivities to 50 µM Mg2+ (A2), and a cytomegalic cell (A3). In the non-CD cell, 50 µM Mg2+ blocked 55% of the NMDA current. In the normal-appearing CD cells, 50 µM Mg2+ blocked 36% of the current in the upper cell whereas the block of NMDA current was minimal in the lower cell. In the cytomegalic cell, 50 µM Mg2+ had almost no effect on the NMDA current. (B) Bar graphs illustrating mean ± SEM NMDA current amplitude in non-CD, CD and cytomegalic cells at –60 and –40 mV holding potentials in the absence (white) and presence (gray) of 50 µM Mg2+. (B1, B3) Peak current amplitude induced by NMDA (100 µM) was larger in cytomegalic cells compared with normal-appearing cells from non-CD and CD groups, whether Mg2+ was applied or not (P < 0.05). Mg2+ reduced the NMDA current in the normal-appearing cells from non-CD and CD groups (P < 0.001), but not in the cytomegalic cells. (B2, B4) NMDA current densities were smaller in cytomegalic cells compared with non-CD cells at –60 and –40 mV holding potentials (P < 0.01). Mg2+ reduced the current density in the normal-appearing cells of non-CD and CD groups (P < 0.001), but not in the cytomegalic cells. (C) Bar graphs illustrating the percent blockade produced by 50 µM Mg2+ in non-CD, CD normal-appearing and cytomegalic cells at –60 mV (C1) and –40 mV (C2) holding potentials. The percent of Mg2+ blockade was smaller in the cytomegalic cells compared with the normal-appearing cells of the non-CD and CD groups (P < 0.05). It was also significantly smaller in the normal-appearing cells of the CD compared with the non-CD group (P < 0.001). Asterisks indicate differences were statistically significant.
CD) and on 15 cells (five non-CD, eight CD normal and two CD cytomegalic). While the NR2C product was detected in total RNA extracted from tissue in the four patients, it was not detected in any individual cell (Fig. 6). This suggests that NR2C mRNA levels are very small relative to NR1 and NR2A/B in human brain tissue sampled in this study.

Immunohistochemistry
We used immunofluorescence to further examine expression of protein for NMDA receptor subunits. Antibodies against NeuN with NR1 and NeuN with NR2B were used on CD and non-CD tissue. In non-CD tissue, immunoreactivity for the NeuN antibody demonstrated neuronal expression and was visible in the soma as well as in part of the proximal dendrites (Fig. 7A). NR1 and NR2B antibodies also labeled the soma and some of the dendrites (Fig. 7B,E,H). At high magnification, NR1 and NR2B immunoreactivity showed puncta diffusely present in the soma and dendrites of cells (Fig. 7B,E,H). In cells from non-CD and CD patients, NR1 immunoreactivity was bright and observed in all NeuN-labeled cells, regardless of the cell type (normal or cytomegalic, Fig. 7B,C,E,F). In non-CD cells, NR2B immunoreactivity was detected in all neurons (Fig. 7D). In the non-CD tissue, NR2B immunolabeling was detected in 99.2% of NeuN positive cells (n = 2432). In CD tissue, NeuN-positive cells were nearly all positive for NR2B in four patients in which the sample sites did not contain cytomegalic cells (97.0%, n = 1344). However, in two CD patients where the sample sites contained cytomegalic cells, 50.5% of the NeuN positive neurons lacked NR2B immunoreactivity (n = 568) (Fig. 7K). The percentage of neurons containing NR2B in the sections containing cytomegalic neurons was significantly lower compared with non-CD and CD sections without cytomegalic neurons (P < 0.0001). In the sections containing cytomegalic neurons, some of the NeuN-positive cells (normal-appearing or cytomegalic) were positive for NR2B, sometimes very brightly, while some neurons of the same section showed NR2B immunoreactivity that could barely be detected, suggesting differential levels of NR2B expression within CD cells (Fig. 7K).

The brain tissue from one CD case was tested for electrophysiology, RT-PCR and immunohistochemistry. This case displayed reduced Mg²⁺ sensitivity in one out of three recorded cells, lack of NR2B in one out of four cells tested for RT-PCR and lack of immunoreactivity for NR2B subunit in 44% of NeuN positive cells.

Discussion
The present study demonstrates differences in NMDA receptor function and subunit expression as determined by in vitro electrophysiology, RT-PCR and immunohistochemistry in neurons from pediatric CD and non-CD tissue. Compared with non-CD tissue, there was reduced sensitivity to Mg²⁺ in dissociated cytomegalic and a sub-population of normal-appearing pyramidal neurons from CD tissue samples that contained cytomegalic cells. In addition, the electrophysiological findings provide evidence to support the hypothesis that a subpopulation of normal-appearing CD neurons may lack or have a reduced presence of NMDA receptors with a functional NR2B subunit, as
demonstrated by reduced ifenprodil sensitivity. RT-PCR and immunohistochemistry confirmed decreased NR2B subunit mRNA and protein expression in a subset of cytomegalic and normal-appearing cells from CD compared with non-CD cells. Alterations in Mg\(^{2+}\) sensitivity and NMDA receptor subunit composition was observed only in brain areas containing cytomegalic neurons, suggesting that normal-appearing and cytomegalic neurons from severe CD cases are similar with respect to these parameters. Taken together, these convergent findings support the concept that there are alterations in NMDA receptor subunit composition and function in a proportion of CD neurons. These alterations may be associated with decreased Mg\(^{2+}\) sensitivity of the receptor. Finally, cytomegalic neurons in CD tissue displayed increased peak NMDA currents. However, after accounting for cell size, NMDA current densities were smaller in cytomegalic cells compared with normal-appearing neurons, indicating that the density and/or conductance of NMDA receptors per unit area was decreased in these enlarged cells.

Comparison with Previous Human Studies

Previous studies using human brain tissue support the concept that NMDA receptors are important in the epileptogenic process, and subunit composition can be altered in human pathological epileptic substrates, such as hippocampal sclerosis in temporal lobe epilepsy (Ying et al., 1998b; Mathern et al., 1999b). Other studies have shown an increase of NR2A/B proteins and NR2B and NR2C mRNAs in dysplastic cells, suggesting that the presence of NR2 subunits in conjunction with NR1 could be implicated in hyperexcitability and seizures (Ying et al., 1998a; Kerfoot et al., 1999; Crino et al., 2001; White et al., 2001). In contrast, in human cases of nodular heterotopia, immunocytochemical studies demonstrated decreased NR2A/B expression that was localized to the heterotopic region (Battaglia et al., 2002), suggesting that subunit alterations are substrate specific. Changes in the co-assembly of NR1 with calmodulin, and NR1 with NR2A/B proteins in dysplastic cells from CD patients have also been reported.
again suggesting that NMDA receptor composition was changed in CD cells (Mikuni et al., 1999a,b; Ying et al., 1999).

Our results from pediatric CD patients compare and contrast with the morphological studies of NMDA receptors in adult CD tissue. For example, we observed that NMDA-induced peak currents were increased in cylomegalic neurons, supporting the hypothesis that, because of their increased size, they have a greater number of receptors per cell. This would be consistent with previous human studies showing increased somatic immunoreactivity for NR1 and NR2A/B subunits in dysplastic neurons (Ying et al., 1998a; Mikuni et al., 1999a). However, we also showed that the current density was reduced in cylomegalic neurons, suggesting that the receptor density per unit area may actually be reduced.

Figure 7. Colocalization of NeuN, NR1 and NR2B subunits in frontal cortex sections from a non-CD and a CD patient (tissue from the same CD patient as in Fig. 6), visualized by double-immunofluorescence staining and laser scanning confocal microscopy. Superposition of NeuN (green) and NR1 (red) or NeuN (green) and NR2B (red) are illustrated in C, F, J and L with sites of colocalization appearing yellow. (A) NeuN immunoreactivity is present in the soma and apical dendrites of pyramidal neurons in tissue sections from a non-CD patient. (B) NR1 immunoreactivity in the same section as in A. (C) In the non-CD patient, NR1 is extensively colocalized with NeuN in the soma and processes. (D) In the CD patient, cells are disorganized and abnormally large. (E) NR1 immunoreactivity in the same section as in D. (F) NR1 is extensively colocalized with NeuN in cylomegalic neurons. (G) NeuN immunoreactivity in a section from a non-CD patient. (H) NR2B immunoreactivity in the same section as in G. (I) Together, G and H show that NR2B is colocalized with NeuN in all the neurons of the section from a non-CD patient. (J) NeuN immunoreactivity in cylomegalic neurons. (K) NR2B immunoreactivity in the same section as in J. (L) Superposition of NeuN and NR2B shows more restricted colocalization in some processes of the CD case, and no NR2B immunoreactivity in the soma of one of the cylomegalic neurons (arrow) while the two other neurons are immunopositive for NR2B. Scale bar is 50 µm and refers to all panels.
We found decreased sensitivity to Mg$^{2+}$ in some normal CD neurons from non-CD tissue expressed NR1, NR2A and NR2B subunit mRNAs while a sub-population of neurons in the CD tissue did not express NR2B. In contrast to our findings, Crino et al. (2001) found a decrease of NR2A and an increase of NR2B mRNA in dysplastic cells of adult CD patients using single-cell microarray techniques. There are several methodological differences between these two human studies. We did not quantify the RT-PCR product, but instead performed the RT-PCR process on individual cells from non-fixed sources. As NR2B mRNA was absent in 22% of the CD neurons in the present study, a limited decrease of mRNA could be missed if the data from sampled neurons were pooled. Moreover, all the cortical tissue we sampled came from surgical resections in which the cells were alive at the time of harvest, while fixed tissue from autopsy brains with a 12 h post-mortem delay was used in the other study (Crino et al., 2001). Fixation protocols and post-mortem delay could, therefore, alter the RT-PCR process and account for the difference between the two studies.

The immunohistochemical findings showed NR1 and NR2B protein expression in almost 100% of non-CD neurons. This finding agrees with the literature reporting co-expression of NR1, NR2A and NR2B in cortical cells (Petralia et al., 1994; Wenzel et al., 1995; Conti et al., 1999; Valtschanoff et al., 1999). In previous immunohistochemical studies of adult CD patients, NR2A/B protein expression in the soma was not detected in normal cells, and NR2A/B subunits co-assembled with NR1 by Western blots only in dysplastic tissue (Ying et al., 1999; Najm et al., 2000). The differences between these studies and ours probably depend on the tissue source and fixation protocols. Our study is on CD tissue from children, which is often more dysplastic than adult cases. Also, the prior human study fixed cortical samples for 48 h, and NMDAR antigens, especially NR2, are very sensitive to fixatives that alter antibody epitopes (Fritschy et al., 1998). In our protocol we fixed the tissue for 6–8 h and used an antibody specific for NR2B rather than one for both NR2A/B. Furthermore, in the present study, the decrease of immunoreactivity for NR2B was limited to a sub-population of neurons in the cortical areas containing cytomegalic cells. A limited decrease of NR2B immunoreactivity is likely to be overlooked if an extended zone of CD containing cytomegalic cells is not examined. From a functional perspective, the presence of NMDA receptors devoid of NR2A/B subunits in non-dysplastic, putative normal neurons seems unlikely, first because native, homomeric NR1 receptors may not exist (Luo et al., 1997) and second because NR2C/D subunits are minimally expressed in the cerebral cortex (Conti et al., 1999; Sun et al., 2000).

**Loss of NR2B Subunits: Possible Implications**

We found decreased sensitivity to Mg$^{2+}$ in some normal-appearing CD neurons and most cytomegalic cells. NMDA receptor Mg$^{2+}$ sensitivity depends mainly on NR2A and NR2B subunits (Burnashev et al., 1992; Sakurada et al., 1993; Schoepfer et al., 1994). In CD cells, we also found decreased sensitivity to ifenprodil, a more selective NR1/NR2B blocker, suggesting that the decreased response to Mg$^{2+}$ may be related to alterations in NR2B subunit expression (Le Bourdelles et al., 1994; Tovar and Westbrook, 1999; Coughenour and Barr, 2001; Perin-Dureau et al., 2002). However, although convergent immunohistochemical data suggested reduced NR2B expression in cytomegalic neurons, because ifenprodil could not be tested on these cells, these results should be interpreted with caution.

Although the mechanisms leading to lower Mg$^{2+}$ sensitivity in a subset of CD neurons are still unclear, there are several potential explanations. One possibility is that decreased Mg$^{2+}$ sensitivity is associated with the lack or reduction of NR2B subunits. Although recombinant systems with binary NR1/NR2A receptors display the same Mg$^{2+}$ sensitivity as NR1/NR2B, sensitivity of ternary NR1/NR2A/NR2B receptors, the prevalent form expressed in vivo in cortical neurons (Luo et al., 1997), remains unknown. Furthermore, although some of the pharmacological properties of receptors formed from NR1/NR2B resemble those formed from NR1/NR2A subunits, there are differences in their interactions with NMDA receptor modulators such as spermidine (Lynch et al., 1995). The stoichiometry of NMDA receptors might also be important in determining Mg$^{2+}$ sensitivity, as it is for the deactivation time constant and ifenprodil sensitivity (Vicini et al., 1998; Tovar and Westbrook, 1999). NMDA receptors are proposed to be tetra- or pentameric structures with two to three NR1 and NR2 subunits (Premkumar and Auerbach, 1997). It is likely that native, neuronal NMDA receptors contain NR1, NR2A and NR2B in different combinations, resulting in intermediate deactivation time constants and ifenprodil sensitivities, and possibly different Mg$^{2+}$ sensitivities. Because there is subunit cooperativity within NMDA receptors (Regalado et al., 2001), lack of NR2B subunits may reduce Mg$^{2+}$ sensitivity by depriving the receptor of a potential binding site. Another possibility is the presence of NMDA receptors expressing NR2C subunits, which have low Mg$^{2+}$ sensitivity (Monyer et al., 1992). Although this receptor subunit is not very abundant in cerebral cortex, it is possible that in CD it may be differentially up-regulated. In fact, it has been reported that dysplastic neurons have increased expression of NR2C (Crino et al., 2001). In the present study, we were not able to detect NR2C cDNA in any non-CD or CD cells, while it was detected in total RNA extracted from tissue. This would support indications that NR2C subunit has a very low abundance in cortical cells and that it does not seem to be upregulated in single dissociated CD neurons.

A consequence of the decrease in NR2B subunits, would be the presence of more receptors containing NR2A subunits, which likely results in receptors with high conductance and opening probability, as described in recombinant receptors (Chen et al., 1999). However, the lack of NR2B subunits, which contain a glutamate binding site, may result in NMDA receptors that are less sensitive to this endogenous ligand, thus reducing their overall conductance. Studies in cultured cortical neurons have demonstrated that deletion of the C-terminal domain of the NR2B subunit produces a significant reduction in the current density of whole-cell currents mediated by non-synaptic NMDA receptors (Mohrmann et al., 2002).

In recombinant receptors expressed in HEK 293 cells, it has been shown that NR1/NR2A receptors recover much faster from desensitization than NR1/NR2B receptors (Vicini et al., 1998). In fact, compared to other NR2 subunits, NR2A subunits are unique in that they show the fastest deactivation and the most prominent desensitization (Dingledine et al., 1999). If NR2B subunits are missing in a number of cells from CD tissue, this will produce receptors that recover from desensitization much faster. Indeed, we observed that cytomegalic neurons
recover faster from repeated NMDA applications than normalappearing pyramidal neurons from CD and non-CD tissue. In conclusion, lack of NR2B subunits in cells from CD tissue may result in cells with a reduced conductance in response to NMDA, hence the reduced current density, and also in receptors that recover faster from desensitization.

Finally, an intriguing outcome of a reduction of NR2B subunits is the possibility of alterations in cortical synaptogenesis. In effect, NR2B subunits are instrumental in NMDA receptors targeting and clustering around nascent glutamatergic synapses (Mohrmann et al., 2002). This subunit is normally found in the cerebral cortex before birth (Ritter et al., 2001) and NR1/NR2B receptors are probably the first glutamate receptors expressed at functionally immature synapses (Tovar and Westbrook, 1999). Lack of this subunit could disrupt synaptogenesis and proper cellular connectivity. Further, neuronal migration is modulated by NMDA receptors (Komuro and Rakic, 1993). It is tempting to speculate that cortical abnormalities such as the presence of cytomegalic neurons after birth in severe CD is caused by altered interneuronal signaling, probably associated with the lack of NR2B subunits.

**Implications for Epileptogenesis in CD Tissue**

The present work is the first electrophysiological and morphological study that has examined NMDA currents in dissociated normal-appearing and cytomegalic neurons from pediatric CD. We demonstrate that some cytomegalic neurons and a proportion of normal-appearing neurons from CD tissue samples containing cytomegalic cells are less sensitive to Mg²⁺ blockade and have NMDA receptors with abnormal subunit composition. Reduced Mg²⁺ sensitivity indicates that the NMDA receptor can be activated by glutamate at relatively hyperpolarized membrane potentials. Animal studies have demonstrated that neurons have a higher probability to fire action potentials in low Mg²⁺ concentrations, even in non-epileptogenic networks (Jones and Lambert, 1990; Whittington et al., 1995; Quilichini et al., 2002). *In vitro*, audiogenic seizures can be induced in Mg²⁺-deficient mice and hippocampal neurons from kindled rats show decreased Mg²⁺ sensitivity (Bac et al., 1993; Kohr et al., 1998). Other studies have shown reduced voltage dependence and Mg²⁺ sensitivity in immature neurons (Ben-Ari et al., 1988; Morrisett et al., 1990). The fact that most cytomegalic neurons have reduced Mg²⁺ sensitivity suggests preservation of some electrophysiological immature features in these abnormal cells. This alteration may be relevant in the pathophysiology of infantile spasms (Avanzini et al., 2002).

In conclusion, a proportion of cytomegalic and normal-appearing pyramidal neurons in CD tissue may have intrinsic capabilities to induce NMDA currents at relatively hyperpolarized membrane potentials. Although the current density is reduced, because of their large size, the overall electrophysiological output of cytomegalic neurons may be increased. This, in conjunction with our previous findings demonstrating larger Ca²⁺ currents and influx in cytomegalic neurons (Cepeda et al., 2003), could help us understand altered neuronal excitability in CD.

**Notes**

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