Induced Expression of NMDAR2 Proteins and Differential Expression of NMDAR1 Splice Variants in Dyslastic Neurons of Human Epileptic Neocortex

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Abstract. Immunocytochemistry was used to study the expressions of glutamate receptor subunit proteins for NMDAR2A/B, NMDAR1 splice variants, and AMPA GluR2/3 in human brain resected for intractable epilepsy associated with cortical dysplasia. NMDAR2A/B intensely labeled dysplastic neurons showing staining in both the cell bodies and dendritic profiles. However, nondysplastic neurons were not immunoreactive to NMDAR2A/B. The antibody selective to NMDAR1 splice variants of NR1-1a, -1b, -2a, and -2b labeled dysplastic neurons, but few nondysplastic neurons. In contrast, the antibody to splice variants of NR1-1a, -1b, 2a, -2b, -3a, -3b, -4a, and -4b labeled both dysplastic and nondysplastic neurons. The different labeling patterns by these two antibodies indicate that variants of NMDAR1-3a, -3b, -4a, and -4b are present in nondysplastic neurons. Both dysplastic neurons and nondysplastic neurons were immunoreactive to AMPA GluR2/3, but denser immuno-reactivity was observed in dysplastic neurons. We also found that the locations of dysplastic neurons labeled by NMDAR2A/B were related to focal epileptic EEG seizure onsets or spiking and to focal behavioral seizure types. Our results suggest that there is hyperexcitability of dysplastic cortical regions, at least in part, from the presence of NMDAR2 subunits and selectively expressed NMDAR1 splice variants in dysplastic neurons.

Key Words: Cortical dysplasia; Epilepsy; Glutamate receptors; GluR2/3; Hippocampal sclerosis; Immunocytochemistry; NMDA subunits.

INTRODUCTION

Cortical dysplasias or displaced neurons, including disoriented or giant cortical neurons, were first reported in adult epileptics in 1971 by Taylor et al (1), who identified them in temporal neocortices resected for routine surgical treatment of temporal lobe seizures. Taylor et al (1) speculated that these dysplasias were small cerebral malformations that possibly indicated neuronal migration disorders. In subsequent decades, microscopic studies of epileptic dysplastic neocortex resected from children and adults have confirmed that there are many features of migrational disorders of both neurons and glia that would date these cortical dysplasias to prenatal, perinatal, or genetic causes (for classification schemata, see 2). Our study has been limited to the mature neocortex, i.e. non-infants with no gross structural malformations. The term “cortical dysplasia” used in our study is defined as microscopically identified abnormal cortical cytoarchitecture such as dislocated cortical layers and aberrantly shaped neurons. These cortical abnormalities cannot be identified by palpation or direct vision. The adult cortical dysplasia epileptic population is of particular interest because the lesions appear to be nondestructive, nonprogressive, not limited to one lobe, and because they may be limited to only a few cortical layers in one lobe.

Glutamate is the predominant excitatory neurotransmitter in the brain (3, 4). It is now well-established that postsynaptic glutamate receptor changes play a fundamental role in human hippocampal epilepsy. Increased AMPA receptor mRNAs were detected in surgically dissected epileptic human hippocampi (5). Several immunocytochemical studies have demonstrated increased AMPA and NMDA receptor subunit protein densities in the fascia dentata of human hippocampal epilepsy (6–8). These studies suggest that altered glutamate excitatory receptors lead to excessive activation of glutamate transmission and may contribute to human hippocampal seizures. The main hypothesis in this study is that dysplastic neurons would have increased densities of both NMDA and AMPA receptor subunit proteins.

Rapid excitatory signal transduction is mediated by activating the postsynaptic glutamate receptors. Electrophysiologic and pharmacologic studies (for reviews see 9, 10, 11) have identified several different classes of glutamate receptors based on their selective agonist activation and selective antagonist blockade. Ionotrophic AMPA receptors transmit fast excitatory synaptic potentials, while NMDA receptors have ligand-gated ion channels with voltage-dependent properties and prolonged neuronal depolarization. Recent molecular techniques for functional receptor expression have identified receptor subunits that comprise specific functional glutamate receptors (for review see 12, 13). To date, the AMPA receptor comprises 4 receptor subunit proteins, termed GluR1–GluR4. The NMDA receptor has 2 families: NMDAR1 and NMDAR2, with the NMDAR1 gene generating 8 alternate splicing variants. The NMDAR2 gene has 4 gene products, NR2A–NR2D subunits.

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**Fig. 1.** Photomicrographs of A: normal, nondysplastic neocortex of inferior temporal gyrus from a patient (1108962) with only severe hippocampal sclerosis (no extrahippocampal pathology). B: patient (0108971) with mild dysplastic neocortex of the inferior temporal gyrus with focal cortical dysplasia localized in the inferior temporal gyrus with coexisting severe hippocampal sclerosis. In A, the well-defined, six-layered neocortical pattern is typical of human neocortex (24), with well preserved vertical
GLUTAMATE RECEPTORS IN NEOCORTICAL DYSPLASIA

TABLE 1
Primary Antibodies Used to Label Glutamate Receptor Subtype Proteins

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Code</th>
<th>Type</th>
<th>Concentration</th>
</tr>
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<tr>
<td>GluR2/3</td>
<td>Chemicon</td>
<td>AB1506</td>
<td>Rabbit IgG</td>
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<tr>
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<td>Chemicon</td>
<td>AB1516</td>
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<td>Chemicon</td>
<td>AB1548</td>
<td>Rabbit IgG</td>
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</tbody>
</table>

TABLE 2
Differential Antigenic Recognition of NR1 Splice Variants by Two Sources of NR1 Antibodies (21)

<table>
<thead>
<tr>
<th>Splice variants</th>
<th>Antibody</th>
<th>NR1-1a</th>
<th>NR1-1b</th>
<th>NR1-2a</th>
<th>NR1-2b</th>
<th>NR1-3a</th>
<th>NR1-3b</th>
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<tr>
<td>NR1 Chemicon</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>NR1 Pharmingen</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

We have examined the expressions of various glutamate receptor subunit proteins using immunocytochemistry. We were able to identify cortical dysplasias and single dysplastic neurons using Nissl stains. We also related our immunocytochemical findings in cortical dysplasia to preoperative EEG localization and seizure types. Our results demonstrated that there were induced expressions of NR2 proteins and differential expressions of splice variants of NR1-3a, -3b, -4a, and -4b in dysplastic neurons. The expressions of AMPA GluR2/3 subunit proteins were increased in dysplastic neurons. The locations of cortical dysplasias detected by Nissl stain and NR2 immunocytochemical labeling were related to EEG seizure onsets or spiking and to focal onset behavior seizure types. This suggests that subunits in glutamate receptor transmission are related to a hyperexcitable response to glutamate in cortical dysplasias that have focal EEG epileptic spikes and seizure onsets.

MATERIALS AND METHODS

Patients with intractable seizures were routinely evaluated in the Comprehensive Epilepsy Program at The Cleveland Clinic Foundation with diagnostic preoperative epilepsy surgery protocols that have been published in detail previously (14). Briefly, all patients had well-documented drug-refractory epilepsy despite various comprehensive trials of anticonvulsant therapies. Presurgical evaluations included complete medical histories, neurologic and physiologic examinations, extensive neuropsychologic tests, intracarotid sodium amytal tests of language and memory, interictal and ictal scalp-sphenoidal EEG, cranial CT, and magnetic resonance imaging. Positron emission tomography was performed on some of the subjects. When necessary, invasive EEG/CCTV monitoring was used to localize epileptogenic regions by chronic recordings from large subdural grid electrode arrays and to relate the EEG locations and patterns to the observed seizure behaviors. The patients with cortical dysplasias (CD) were not infants; their ages ranged from 5–65 years, (mean = 26, median = 30, mode = 19).

The present study of glutamate receptors was limited to consecutive neocortical resections (n = 30) from June 1996 to February 1997, the time period when all antibodies were used simultaneously on all neocortical specimens resected. In patients with hippocampal sclerosis (HS), the percentage of Ammon’s horn cell loss obtained by averaging the percentage of cell loss in the subfields of Ammon’s horn (CA1, CA2, CA3, and CA4) was used to define the severity of hippocampal sclerosis. For example: HS = cell loss greater than 40%; mild HS = cell loss between 39% and 20%; and non-HS = less than 20% cell loss. Of those 30, (a) 17 had cortical dysplasias (57%), (b) 9 of the 17 CDs had mild HS (5) or typical HS (4) (i.e., 53% dual lesions), and (c) 13 of the 30 had no evidence of CDs (43%). All of those 13 had hippocampal sclerosis alone as the sole pathology and no evidence of CDs in the overlying temporal neocortex, which was removed for surgical access to the hippocampus and used as the nondysplastic comparison cortex.

TISSUE PREPARATION

The cortical dysplasias described in this study were obtained from 17 patients who underwent partial lobectomies including the frontal lobe, central region, motor strip, parietal lobe, temporal lobe, and occipital lobe. Representative portions of the specimens were received fresh from the neurosurgeon, and several blocks, 5–7 mm thick, were made in the coronal plane to broadly sample the large neocortical resections. The remaining parts of and horizontal laminations. However, in the dysplastic neocortex (B), both vertical and horizontal laminations are disrupted. The hexalaminar pattern was less clearly distinguished and the large neurons are disoriented. Cortical layers are indicated by Arabic numbers. Scale bar: 200 µm. WM, white matter. Cresyl blue violet stain.
Fig. 2. Photomicrographs from the inferior temporal gyrus of a patient (0625961) with a region of nondysplastic cortex (A) that was located distinctly remote from a focal dysplastic cortical region (B). Both A and B are taken from the same 30-μm-thick section stained with cresyl echt violet and are representative of neurons in layer 5 of normal pyramidal neurons (A) and the dysplastic neurons (B), which are approximately 1.5 cm away, that are also present in layer 5. Figure 2A shows normal pyramidal
<table>
<thead>
<tr>
<th>Patient code#</th>
<th>Age at surgery</th>
<th>Location of CD</th>
<th>Hippocampal pathology</th>
<th>EEG sz onset or spiking</th>
<th>Seizure type</th>
</tr>
</thead>
</table>
| Extratemporal neocortex (n = 5):  
0614961       | 15 Frontal     | N/A            | Frontal-central szs    | Generalized tonic      |              |
| 1022961       | 32 Frontal     | N/A            | Frontal szs            | Generalized tonic-clonic|              |
| 0306971       | 11 Frontal     | N/A            | Frontal-central spks   | Generalized tonic-clonic|              |
| 0802961       | 20 Motor area  | N/A            | Frontal-parietal spks* | Bilateral              |              |
| 1122961       | 22 Occipital   | N/A            | Occipital-parietal szs*| Complex motor → generalized tonic-clonic |              |
| Temporal neocortex (n = 7), frontal (n = 1) with mild HS (n = 3):  
0807961       | 23 Temporal    | N/A            | Not available          | Absence                |              |
| 0919861       | 65 Temporal    | N/A            | Not available          | Partial complex        |              |
| 0115971       | 10 Temporal    | N/A            | Temporal szs           | Complex motor          |              |
| 0821961       | 49 Temporal    | Mild HS (26%)  | Temporal szs*          | Psychomotor → generalized tonic-clonic |              |
| 0121971       | 29 Frontal and temporal | Mild HS (32%) | Fronto-temporal szs*   | Generalized tonic-clonic|              |
| 0625961       | 35 Temporal    | Mild HS (32%)  | Mesial temporal spks    | Psychomotor → generalized tonic-clonic |              |
| 0131971       | 38 Temporal    | Mild HS (32%)  | Temporal szs            | Paroxysmal              |              |
| Temporal (n = 5), parietal (n = 1), with HS (n = 5):  
0812961       | 23 Parietal and temporal | HS (46%) | Parietal-temporal szs* | Tonic generalized motor |              |
| 0731961       | 15 Temporal    | HS (55%)       | Mesial temporal spks; frontal-temporal spks | Psychomotor only |              |
| 0807962       | 5 Temporal     | Mild-HS (25%)  | Parietal-occipital; temporal spks | Hypomotor → hypermotor |              |
| 0827961       | 12 Temporal    | HS (57%)       | Lateral and mesial temporal szs | Complex motor → generalized tonic-clinic |              |
| 0108971       | 30 Temporal    | HS (70%)       | Mesial temporal spks; temporal spks | Psychomotor → generalized |              |
| Control (non-CD) temporal with HS (1 of 13):  
1108961       | 34 None        | HS             | Mesial temporal spks    | Psychomotor only        |              |

Note that the EEG seizure onset (szs), spiking (spks), and dominant seizure types as diagnosed prior to surgery were related to the locations of CDs or HS. Subdural grids shown by asterisks. NA = Not resected; Psychomotor seizure type was partial complex, i.e. included automatisms; * = also the location of tissue resection.

the specimen were submitted to the Department of Pathology for an independent pathologic diagnosis. For immunocytochemistry (ICC) of receptor proteins, preliminary tests used tissue blocks that were immersion fixed for 3 different durations: 24, 36, and 48 hours in 4% paraformaldehyde at 4°C. The best staining results were obtained when the blocks were fixed for 36–48 hours. Therefore, all tissue blocks used in this study were fixed for those periods. Table 3 in the Results section shows that the hippocampus was also removed and analyzed in 9 of 12 temporal lobes with CDs. One of the 13 non-CD temporal lobectomies (all of which have HS) is listed because the temporal neocortex is illustrated in figures in the Results section as normal. All hippocampi were processed with cresylecht violet stain for cell counts to quantify HS (see 15) and with Timm histochemistry to test for pathognomonic evidence of mossy fiber sprouting (see 16).

**Antibody Characterization**

Table 1 summarizes the primary antisera used in this study, which have been well characterized in several studies (17–20). The specificity of each antibody has neurons in layer 5 with clear nuclei, stained nucleoli, and unstained dendrites; i.e. only short segments of apical dendrites (arrowheads) are stained, and the basilar dendrites are not visible. The apical dendrites are appropriately positioned towards the pial surface. The dysplastic neurons (B) are aberrant in shape (arrows), their somata are darkly stained, and long segments of apical (arrowheads) and basal dendrites (double arrows) are stained. These disoriented pyramidal neurons have apical dendrites pointing in all directions. Scale bar: 20 μm.
Fig. 3. Normal nondysplastic temporal neocortex from a patient (1108962) with only hippocampal sclerosis and no neocortical pathology. Adjacent sections were immunocytochemically stained with NR2 (A), NR1 (B), or GluR2/3 (C). In A, there are virtually no NR2 immunoreactive cells in any of the 6 layers. An area in layer 3 (box) stained by NR2 is magnified ×12.3 (D). In B, the NR1 antibody (Pharminen, Inc.) labeled neurons distributed throughout layers 2, 3, 4, 5, and 6. Pyramidal cell
been demonstrated by Western blot analysis in those studies. Except for antibodies GluR2/3 (AB1506, Chemicon Inc.) and NMDAR2A/B (AB1548, Chemicon), each antibody was found to be selective for its receptor subunit. The two primary antibodies labeling NMDAR1 differed significantly as described below, and the specificity of the subunits recognized by each are shown in Table 2. These different subunits proteins identified unique characteristics of receptor proteins in dysplastic neurons. The polyclonal antibody NMDAR1 (AB1516, Chemicon Inc.) was raised against a 30–amino acid polypeptide corresponding to C-terminal residues 909–938 (19). The monoclonal antibody NMDAR1 (54.1, Pharmingen Inc.) was directed against a fusion protein corresponding to amino acids 660–811, which is included in the putative second intracellular loop between the third and fourth transmembrane regions (18). The NMDAR2 antibody (AB1548) was raised in rabbit against a 20–amino acid synthetic peptide corresponding to the C-terminus of NMDAR2A subunit (20); however, Western blot analysis of transfected cells showed that this antibody can recognize both NR2A and NR2B subunits equally. The polyclonal antibody GluR2/3 (AB1506, Chemicon Inc.) was raised against synthetic peptides corresponding to the carboxyl terminus of these peptides and was shown to recognize both GluR2 and GluR3 subunits that have nearly identical carboxyl terminal sequences (17). The optimal concentration of each primary antibody was determined (Table 1) through a series of tests and was unchanged thereafter to ensure that comparisons could be made between subsequent cortical sections without apparent variance, as long as all the other standardized reagents and timing protocols were held constant. Of course, for a single cortical specimen, comparisons between different receptor immunoreactive labeling were valid because all reagents except the primary antibodies were identical throughout the tissue processing.

Immunocytochemistry

Since the resected neocortices were not malformed (e.g., hemimegenccephalic), the focal cortical dysplastic regions were not detectable by gross visual observation, and the whole coronally dissected tissue was used for sequential histologic and alternating immunocytochemical staining. Cryostat sections were cut at 30 μm and placed in individual 3 ml tissue culture wells containing 0.05 M Tris buffered saline (TBS, pH 7.6). TBS was used as the rinsing buffer throughout the immunocytochemical (ICC) staining procedure. For certain comparisons of the immunostaining patterns among different antibodies, sequential tissue sections were arranged to react with every other antibody in all possible combinations. The ICC protocol was similar to previously described procedures (22, 23). Free floating sections were processed for ICC immediately following cryostat sectioning: (a) TBS rinses (3 changes) between each step: 5 min in 3% H2O2/10% methanol in TBS; (b) 60 min in a blocking solution of 1.5% normal serum in TBS; (c) 18 hours overnight at room temperature in primary antisera diluted in TBS containing 1% normal goat serum for antibodies raised in rabbit and with 1% normal horse serum for antibodies raised in mouse (optimal concentration of each antibody is shown in Table 1); (d) 35 min in diluted biotinylated species-specific anti-IgG (goat anti-rabbit; horse antimouse; ABC kit, Vector Labs). To visualize the immunoreactive complex, the sections were reacted for 8 min in 0.05% 3,3′-diaminobenzidine (DAB) tetrahydrochloride and 0.01% H2O2 in TBS. The DAB step was done in Petri dishes to avoid tissue folding and uneven staining. The reaction was terminated by transfer of sections to ice-cold TBS. The tissue sections were then mounted on chromium alum gelatin-coated slides, air-dried for several hours, and coverslipped. At alternating intervals, adjacent to the ICC in neocortex and hippocampal sections, cryostat sections were cut and CV stained, 30 μm thick for routine cytolgy and 10 μm thick for cell density counts that define HS (15). In all the hippocampal resected, routine Timm-stained histochemistry (16) was done to test for hippocampal sclerosis (HS), and the findings are itemized in Table 3. Control experiments for immunocytochemistry were performed by omission of primary antibodies using the same staining protocol as mentioned above; no specific immunocytochemical stainings were seen in the absence of specific primary antibodies.

RESULTS

Cortical Dysplasias, EEGs, and Seizure Types

Table 3 summarizes the relationship between the locations of the cortical dysplasias, the evidence of EEG seizure onset or spiking in nearby neocortex, and the behavioral seizure types. In the first 5 patients, all remote from the temporal lobe, both EEG seizure onsets and
Fig. 4. Photomicrographs demonstrating that dysplastic neurons are immunoreactive to NR2 (B, D, E, F). Cresylecht violet-stained dysplastic neurons (A, C). Examples in adjacent regions of layer 3 of the parietal lobe from one patient (0812961) showing the aberrant cytoarchitecture of dysplastic neurons: darkly stained, long segments of apical (arrowheads) and basilar dendrites (double arrows). NR2-stained sections (B, D) adjacent to CV sections (A, C) show that NR2 labeled only the dysplastic neurons. The somata, extremely long apical dendrites (arrowheads), and basilar dendrites (double arrows) are intensely labeled by NR2 in...
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spiking were recorded from the regions of the cortical dysplasias. The behavioral seizures in these 5 patients were exclusively typical of suprasylvian motor seizures. By comparison, in the last 2 groups having temporal, frontal, or parietal lobe CDs, the epileptiform EEG was localized to those neocortices or to the medial temporal (hippocampal) region because there was often dual pathology (CDs plus HS), as seen in the last 5 patients. In these last 2 groups, the behavioral seizure classification was less clear because some patients had complex motor seizures, others had psychomotor seizures (hippocampal seizure onset), and most had both types, usually with secondarily generalized motor seizures. By contrast, in the 13 cases with HS only and no temporal lobe CDs, such severe secondarily generalized motor seizures were controlled by anticonvulsants. For the 17 cases with cortical dysplasia, MRI scans of 16 patients were available for review and revealed cortical abnormalities in 9 of the patients, and PET studies of 6 patients were available for analysis and showed hypometabolism (nonquantitative data) in 5 patients and hypermetabolism in one patient. Among these 6 patients, MRI scans failed to demonstrate cortical abnormalities in 5 cases.

Characterization of Dysplastic Neocortex

Figures 1 and 2 illustrate the main features of normal temporal neocortex compared with dysplastic temporal neocortex. Nondysplastic neocortex is defined as those regions with well-preserved vertical and horizontal laminations. Such normal regions were identified in the temporal neocortex of patients who had typical hippocampal sclerosis and no other pathologies, especially no evidence of cortical dysplasia by careful histological examination. Other control comparisons were possible from the neocortical regions distant to focal cortical dysplasias. Normal neocortex has 6 layers (see Fig. 1A) with neurons arranged in both vertical and horizontal laminations. The densest packed cells are found in layers 2 and 4, the large neurons are in 3, 5, and 6, and the vertical spacing together give the appearance of a "rain fall" lamination. Dysplastic neocortex is identified by its lack of laminar organization, lacking both the horizontal differentiation of neuron sizes and the vertical columnar spacing. In this example (Fig. 1B), which is a case of mild cortical dysplasia, only layer 2 neurons appear less disorganized; however, they are less well-packed and larger than normal (see Fig. 1A). Dysplastic neurons appear larger, more darkly stained, and lack the unipolar orientation of apical dendrites toward the pial surface, (compare Figs. 2A and B). Figure 2A shows the normal spacing, orientation, and density for cresyl violet (CV) staining of layer 5 pyramidal neurons and nearby glia in the temporal cortex. Approximately 1.5 cm away, in the same 30-μm-thick section, there are dysplastic neurons in layer 5 (Fig. 2B) that have aberrant spacing, denser CV staining in apical and basilar dendrites that show the disorganized orientations, and multiple secondary processes, (see Fig. 2B, double arrows).

Because the focal cortical dysplasias had no macroscopic polymicrogyria or lissencephaly, only clusters or laminae of disorganized neurons, large segments of each resected cortex (whole coronally oriented tissue sections) were sampled and stained to detect the small CDs. Very large cortical dyslaminations or more focal CDs could be found limited to certain layers or to portions of a gyrus in all types of cortex (see Table 3). Many cases had CDs limited to layers II and III, with normal neurons in lower cortical layers. Single or a few normal-appearing neurons located in the white matter were seen in every surgically resected cortical specimen, including the 13 cases with HS alone. Frequently, normal-appearing neurons were located close to the gray-white junction. Therefore, the CDs were not necessarily limited to or greater in layers V and VI. There was no relation between the locations and numbers of these white matter neurons to any features of the gray matter dysplasias. From the specimens available in our study, the transition between dysplasia and normal cortex is gradual. Neurons in the border zones were normal in shape and not darkly stained; however, the neurons were not properly laminated.

Glutamate Receptor Subtypes NR2, NR1, and GluR2/3 in Normal Cortex

In nondysplastic cortex, verified by CV-stained adjacent sections, neurons were not immunoreactive to NR2, as shown in Figures 3A and D. The lack of NR2 staining in nondysplastic neurons was not related to the quality of the NR2 antibody, but rather, it was due to the absence of NR2 proteins because the NR2 antibody did stain pyramidal neurons in the subicular complex of this patient

both parietal and temporal lobe dysplastic neurons. Figures E and F are highly magnified illustrations that demonstrate the detailed cytoarchitecture of NR2-labeled dysplastic cortical neurons from one patient (0812961) who had profound cortical dysplasia distributed throughout several gyri including temporal and parietal lobes. Figure E shows dysplastic neurons in layer 5 from the posterior temporal lobe, Figure F shows dysplastic neurons in layer 3 from the parietal lobe. NR2 intensely labeled large cell bodies, long and thick segments of apical dendrites (arrowheads), basilar dendrites (double arrows) and complex secondary dendritic processes branching out from the somata and apical dendrites (double arrowheads). Scale bars: 60 μm in A, B, C, D; 20 μm in E, F.
Fig. 5. Photomicrographs (A, B) from a region of dysplastic parietal lobe of a patient (0812961) showing 2 adjacent sections, one (A) with NR2-labeled dysplastic neurons, and the other, which is only 30 μm away (B), with a cortex that is almost entirely immunoreactive to GluR2/3. In A, the NR2-labeled dysplastic neurons are focally distributed only in layer 2 and the in upper part of layer 3. The deeper cortical layers 3, 4, 5, and 6 have normal neuronal laminae and are free of NR2-labeled neurons, which was verified in the adjacent CV-stained section (data not show). As traced by landmarks of tissue cavities (C) and blood vessels (bv), clusters of dysplastic neurons labeled by NR2 (A) are also GluR2/3 immunoreactive, as shown in B. Note that, in contrast to the NR2 staining pattern, neurons in the deeper layers of cortex (normal cortex) are labeled by GluR2/3 in normal cortical laminar patterns that are preserved despite the cortical dysplasia in layers 2 and upper layer 3. Cortical layers are indicated.
Fig. 6. Photomicrographs of 3 adjacent sections from the middle temporal gyrus of a patient (0115971) with NR2 labeled dysplastic neurons (C, F in layer 3) that are also immunoreactive to both NR1 antibodies ([Chemicon: B, E] and [Pharmin: A, D]). Note that in C, the NR2-labeled cluster of dysplastic neurons is focally distributed in layer 3, forming a triangular area at the right bottom of C. In comparison, NR1 (Chemicon: B) labeled the same cluster of dysplastic neurons, showing a very similar distribution. In contrast to the labeling patterns shown in C and B, the other NR1 antibody (Pharminen) labeled both the dysplastic (triangular area) and the nondysplastic neurons in layer 3 (A). The darkest, selectively labeled clusters of dysplastic neurons in A have a distribution almost identical to that in B and C. Representative dysplastic neurons (boxed areas) labeled by antibodies of NR2 (F), NR1 (Chemicon: E), and NR1 (Pharminen: D) demonstrate the detailed cytoarchitectural staining patterns. Note that all three of the antibodies labeled the dysplastic somata densely, but the dendritic processes (arrowheads) labeled by NR2 are much longer than those labeled by either of the NR1 antibodies. Cortical layers are indicated by arabic numbers. Scale bars: 200 μm in A, B, C; 60 μm in D, E, F.

by Arabic numbers. Figures C and D demonstrate at high magnification the cytoarchitecture of dysplastic neurons from layer 2 labeled by NR2 (C) and GluR2/3 (D). Note that NR2 labels long apical dendrites (arrowheads) and numerous smaller dendrites (double arrows). GluR2/3 stained dysplastic neurons somata intensely, but less so the dendritic processes (apical dendrites, arrowheads; basilar dendrites, double arrowheads). GluR2/3 stained neurons in fewer numbers and less densely than those neurons stained by NR2. Scale bar: 200 μm in A, B; 20 μm in C and D.
Fig. 7. Photomicrographs of 4 adjacent sections from the middle temporal gyrus of a patient (0115971) demonstrating that NR2-labeled dysplastic neurons (D, H in layers 5 and 6) are also immunoreactive to NR1 (Chemicon: C, G), NR1(Pharmlingen: B, F), and AMPA GluR2/3 (A, B). Note that in D, the NR2-labeled dysplastic neurons are located in layers 5 and 6. With a similar staining pattern, NR1 (Chemicon: C) labeled dysplastic neurons in layers 5 and 6, and a few neurons in layers 3 and 4. In contrast, NR1(Pharmlingen: B) and GluR2/3 (A) darkly labeled dysplastic neurons in layers 5 and 6, and less darkly labeled most nondysplastic neurons in layers 3 and 4. The boxed areas represent clusters of dysplastic neurons in layer 6, which are magnified ×4.25 in E (GluR2/3), F (NR1, Pharmlingen), G (NR1, Chemicon), and H (NR2). Labeled apical dendrites are indicated by arrowheads. Cortical layers are indicated by arabic numbers. C = tissue cavity. Scale bars: 200 μm in A, B, C, and D; 60 μm in E, F, G, and H.
with HS. That is, the subiculum was a positive control in all 21 cases with the hippocampus resected. When even higher concentrations than 0.5 \(\mu g/ml\) were used (see Table 1), NR2 did not label normal cortical neurons despite the intense labeling by NR2 of the dysplastic neurons (see Figs. 4–7). It is also important to state that even for specimens with focal CDs, normal neurons in the nearby cortex had no NR2 immunoreactivity. In Figures 3B and C, the NR1-IR (Pharmingen, Inc.) and GluR2/3-IR (Chemicon, Inc.) adjacent sections (within 30 \(\mu m\)) show a pattern found in all the human nondysplastic neocortex. At this low-power magnification, the distributions of NR1 and GluR2/3-IR neurons have a similar pattern. The large population of neurons in layers 2, 3, 5, and 6 are stained by both antibodies, but glia are not stained. In layer 4, small neurons were less stained by both NR1 and GluR2/3, with much less GluR2/3-IR. At higher magnifications (Figs. 3E, F), the cellular staining in layer 3 for NR1 and GluR2/3 is similar. The somata are well-stained, short segments of vertically-oriented apical dendrites from the somata are stained (arrowheads), and basilar dendrites are faintly stained. With the NR1 (Chemicon), only a few large layer 3 pyramidal cells were stained (data not shown).

**NR2 Expression in Cortical Dysplasia Neurons**

Two main features of dysplastic cortex are laminar disorganization and aberrantly-shaped, darkly stained neurons. In contrast to the nondysplastic neurons, which were not immunoreactive to NR2, dysplastic neurons identified by CV-stained adjacent sections were darkly stained by NR2 as shown in Figure 4. Figures 4A and C show dysplastic neurons sampled from layer 3 in two different regions of the same parietal lobe. These figures demonstrate that the sizes or shapes of dysplastic neurons (Fig. 4A, C) were variable and different from one region to another. However, they have one thing in common: the aberrantly shaped cell bodies and their apical and basilar dendritic processes are darkly stained by CV. The adjacent NR2-IR mirror sections (Fig. 4B, D) showed that the CV-stained dysplastic neurons are immunoreactive to NR2, which demonstrates an unexpected upregulation or new expression of NR2 receptor proteins. When comparing the CV to the NR2 stains of the dysplastic neurons, the CV- and NR2-stained dysplastic neurons have similar cell shapes, dendritic processes, and orientations of their dendritic processes. Figures 4E and F illustrate at higher magnification the typical cytoarchitectural features of NR2-IR dysplastic neurons sampled from dysplastic regions. The cell bodies are darkly stained, the nuclei are clear, and the NR2-stained apical dendrites are very long. There are widespread basilar dendrites and several smaller dendritic branches that also show NR2 expression. The unique staining pattern of NR2-IR dysplastic neurons was highly definitive and consistent, virtually identical in the frontal lobe, central regions, parietal lobe, temporal lobe, and the occipital lobe. The cellular staining pattern with NR2 (see Fig. 4E, F) demonstrates that NR2 proteins label the entire neuron and its dendritic processes.

**Expression of GluR2/3 in NR2-IR Dysplastic Neurons**

To determine if NR2-IR dysplastic neurons also contain the AMPA GluR2/3 receptor proteins, adjacent sections (30 \(\mu m\) apart) were immunocytochemically processed with NR2 and GluR2/3 antibodies. Figure 5 illustrates a representative example of a focal cortical dysplasia limited to layers 2 and the upper part of layer 3 where dysplastic neurons have both NR2 and GluR2/3 immunoreactivity. Tissue landmarks, such as tissue cavi ties and blood vessels, were used to trace the laminar clusters of dysplastic neurons in NR2- and GluR2/3-stained sections. Figure 5A clearly shows that clusters of NR2-IR dysplastic neurons were focally distributed in layer 2 and some larger NR2-IR dysplastic neurons were found singly interspersed in the upper part of layer 3. Neurons in the deep layers were not stained by NR2 (Fig. 5A) and were determined to be nondysplastic neurons on examination by CV staining (data not shown). As traced by the tissue landmarks, the GluR2/3 immunostaining (Fig. 5B) labeled both the dysplastic neurons (layer 2 and upper part of layer 3, similar to NR2) and the normal-appearing, deep-layered neurons (Fig. 5B), which were not NR2-IR (Fig. 5A). Figure 5C and D compare at high magnification the dysplastic neurons in layer 2 that are NR2-IR (C) or GluR2/3-IR (D). The NR2 proteins are more densely distributed on the dysplastic somata, dendrites and branches than the GluR2/3-IR proteins, which nevertheless are abnormal.

**Differential Expression of NR1 Splice Variants on Dysplastic Neurons**

By using two well-characterized NR1 antibodies (Pharmingen, Inc. and Chemicon Inc.), each recognizing different groups of NR1 splice variants (see Table 2 for details), adjacent tissue sections (30 \(\mu m\) apart) were simultaneously processed for NR1 splice variants, NR2, and GluR2/3 to further define unique receptor proteins on dysplastic cortical neurons. It is known that NR1 proteins must act with NR2 subunits to form a functional NMDA receptor complex. Figure 6 illustrates that NR2-IR dysplastic neurons also selectively express NR1 splicing variants. Figure 6C shows that clusters of NR2-IR dysplastic neurons are mainly distributed at the right bottom of the picture as indicated by the triangular area (layer 3). Similarly, the NR1 antibody (B: Chemicon, Inc., recognizing only splice variants of 1a, 1b, 2a, and 2b) also labeled layer 3 dysplastic neurons distributed like...
those shown in Figure 6C (NR2). By comparison, the
NR1 antibody (A: Pharmingen, Inc., recognizing splice
variants of 1a, 1b, 2a, 2b, 3a, 3b, 4a, and 4b) labeled
the layer 3 dysplastic neurons, but also labeled nondysplastic
neurons. These findings that nondysplastic neurons are
labeled by NR1 (A: Pharmingen, Inc.) but not labeled by
NR1 (B: Chemicon, Inc.) suggests that proteins of NR1
variants 3a, 3b, 4a, and 4b are expressed in normal neu-
rons, while NR1 variants 1a, 1b, 2a, and 2b are highly
expressed in dysplastic neurons because both NR1 anti-
odies had the first 4 splice variants. Figures 6D, E, and
F show the boxed areas at higher magnifications to il-
lustrate the cytoarchitecture of labeled dysplastic neurons.
NR2, NR1 (Chemicon, Inc.), and NR1 (Pharmingen, Inc.)
labeled somata; however, neither NR1 antibody labeled
dendritic processes as densely as the NR2 antibody. It is
not known which or how many NR1 variant proteins
among variants of 1a, 1b, 2a, and 2b are present on dys-
plastic neurons.

Figure 7 compares the labeling patterns of GluR2/3
(A), NR1 (B: Pharmingen, Inc.), NR1 (C: Chemicon,
Inc.), and NR2 (D) in adjacent 30-μm sections from a
focal cortical dysplasia. The patterns of NR2 and NR1
(Chemicon, Inc.) are similar (Fig. 7C, D), labeling dys-
plastic neurons in layers 5 and 6. NR1 (Chemicon, Inc.)
also stained a few nondysplastic neurons in layer 3. By
contrast, the labeling patterns of NR1 (B: Pharmingen,
Inc.) and GluR2/3 (A) labeled dysplastic neurons in lay-
ers 5 and 6 and labeled most nondysplastic neurons in
layers 3 and 4. The boxed areas at high magnifications
(E, F, G, and H) demonstrate that all 4 antibodies labeled
the dysplastic neurons more intensely than the nondys-
plastic neurons. However, the dendritic shafts and pro-
cesses are best labeled by NR2. Hence, these data pre-
sented on dysplastic neurons in identified epileptic
neocortex suggests that dysplastic neurons had increased
 glutamate receptor proteins for NR2, NR1, and GluR2/3.

DISCUSSION

Histologic Features of Epileptic Cortical Dysplasias

As mentioned in the introduction, our study did not
include infant cortical dysplasias that were associated
with intractable severe seizures and did not have mal-
formed or atrophied hemispheres or gyri. The mean age
of patients in our study was 26 years. We did not find
gross pathologic alterations in these adult dysplastic
neocortices. A few scattered, normal-appearing neurons in
the white matter were not considered to be cortical dys-
plasia because they were seen in every surgical specimen
including the cases with HS alone, indicating that these
neurons represent a minor nonspecific neuronal migration
disorder. Such a concept is supported by the evidence from
morphometrical studies on autopsy specimens (24). The most striking microscopic abnormalities identified in
cresylecht violet stains were cortical dyslaminations, dis-
oriented neurons, and, most interestingly, very dark Nissl
body staining of those dysplastic neurons. Those disori-
ented, misshapen, often enlarged neurons had darkly
stained, long apical branches and basilar dendritic pro-
cesses (see Figs. 1, 2, and 4).

Cell bodies and dendritic processes of dysplastic neu-
rons darkly stained by cresylecht violet indicate increased
distributions of Nissl substances in these sites and suggest
that dysplastic neurons more efficiently synthesize or
post-translationally modify receptor proteins not only in
their cell bodies, but also in their dendritic processes. An
in vitro study (26) has demonstrated that dendrites can
synthesize and glycosylate proteins. Gazzaley et al (27)
found increased NMDAR1 mRNA hybridization levels
throughout the entire molecular layer of hippocampus
within 5 days after perforant path denervation of the
granule cell outer dendrites. Our previous ICC studies of
neoinnervation and upregulated receptor proteins for
AMPA (6) and for NMDAR2 (7) showed that receptor
proteins were upregulated in the granule cell dendrites in
both human and kainic acid rat hippocampal epilepsy. All
human hippocampal epileptics, regardless of age, had
these increased AMPA and NMDA receptors. All of this
information supports the suggestion that dysplastic neu-
rons in human epileptic cortical dysplasias may synthe-
size receptor proteins in the dendritic processes. Second-
ary, it is likely that glutamate receptor proteins would be
induced to have greater densities in the transmembrane
domains of dysplastic neurons as a postsynaptic mech-
nism for epileptic hyperexcitability. The expressions of
NR2 subunit proteins and selective NR1 splice variants
in dysplastic neurons were unexpected findings as dis-
cussed below.

Aberrant Expressions of NR2 Proteins in Dysplastic
Neurons

The most important and reliable finding from this study
was that in the epileptic neocortex, dysplastic neurons
expressed NR2 proteins on somata, dendrites, and even
small processes, while normal-appearing neurons had no
NR2-IR. The NR2-IR dysplastic neurons were identified
by both Nissl and NR2 stains (see Fig. 4). Dysplastic
neurons were not necessarily distributed throughout all
the layers of the resected epileptic cortex. They were usu-
ally limited to a few cortical layers, with the other layers
having normal-appearing neurons (see Fig. 5). The lim-
ited vertical laminar organizations of dysplastic neurons
were usually associated with limited horizontal clusters
of dysplasias across gyri. Hence, in physiologically-
verified epileptic neocortex (see Table 3), restricted or
focal cortical dysplasia may have been sufficient to form
a synaptic basis for focal seizure genesis.

Dysplastic neurons were identified by Nissl stain as
well as by immunocytochemical staining for NR2, NR1,
and AMPA GluR2/3 proteins; however, the boundaries of a cortical dysplasia region could be better-defined by NR2-IR (see Fig. 5). For example, the Nissl stain of Figure 1 shows that almost the entire gyrus is dyslamimated, and the high magnifications of Figure 2 better identify the dense, anomalous Nissl staining of disoriented neurons. However, the NR2 stains differentially and more densely label the somata, apical, basilar dendrites, and other processes to clearly define the upregulated NR2 glutamate receptor type not found on normal cortical somata or dendrites (see Figs. 4–7). The NR2 staining of very small foci of dysplastic neurons, e.g. limited to layer 2, (see Fig. 5) may not be detected as a true cortical dysplasia with routine Nissl stains and diagnostic light microscopy or by other immunostainings. Finally, since cresylecht violet stains Nissl in both normal and dysplastic neurons of all sizes, dysplastic neurons are best identified by NR2 stains because normal cortical neurons are not NR2-IR.

Expressions of NR-1 Subunit Proteins in Neocortex

Molecular cloning of glutamate NMDA receptors have revealed 2 NMDA receptor gene families, designated as NR2 and NR1. The NR2 gene has 4 gene products sharing considerable homology with each other and constituting 4 subunits: NR2A, NR2B, NR2C, and NR2D (28, 29, 30, 31). The NR1 gene expresses a functional receptor with a weak response activated by glutamate in the Xenopus receptor (32). By contrast, when members of the NR2 gene family were expressed as a homomeric or any heteromeric configuration, none of the 4 NR2 subunits assembled into functional ion channels. However, when the NMDA receptor is composed of various heteromeric combinations of NR1 and NR2 subunits, it produced NMDA-induced currents 100 times larger than the homomeric NR1 channels (31). A crucial question relevant to the possible epileptogenesis of NR2-expression in dysplastic neurons is whether or not these dysplastic neurons must also express NR1 subunit proteins. Using immunocytochemical staining of adjacent sections with specific NR2 and NR1 antibodies, our results clearly demonstrated that aberrant cortical dysplastic neurons expressed both NR2 and NR1 receptor subunit proteins. Possessing NR1 subunit proteins in the NR2-expressed dysplastic neurons suggests that these dysplastic neurons have the potential to be hyperexcitable instead of only being a marker of nonspecific arrest or disorganization of neuronal migration.

Differential Expressions of NR1 Splice Variants on Cortical Neurons

The NR1 gene has been demonstrated to have at least 8 variants by alternate splicing (33, 34), designated as NR1-1a, 1b, 2a, 2b, 3a, 3b, 4a, and 4b. Figures 6 and 7 demonstrate different expressions of NR1 splice variants on normal and epileptic dysplastic neurons. Although we know of no studies yet published on the densities of NR1 mRNA 1 in human epileptic neocortex, in situ hybridization on normal rats demonstrated that there was a dense distribution of NR1 mRNA in all cortical regions (31). The NR1 antibody from Pharmingen was raised against a peptide corresponding to amino acids 660–811, which recognizes all 8 splice variants. The Chemicon NR1 antibody was raised against a short peptide with amino acids 864–900, which recognizes only the 4 splice variants NR1-1a, 1b, 2a, and 2b. In the normal neocortex, there are striking differences between these 2 NR1 antibodies. NR1 (Chemicon) only faintly labeled large pyramidal neurons in layer 3. By comparing the different distinct staining patterns and subtracting the splicing variants recognized by these 2 different antibodies, we conclude that only the NR1 splicing variants NR1-3a, 3b, 4a, and 4b are well expressed on normal cortical neurons; however, NR1-1a, 1b, 2a, and 2b are not. The 4 splice variants of NR1 (Chemicon) labeled dysplastic neurons. However, as expected, the NR1 (Pharmingen) with all 8 variants labeled both normal and dysplastic cortical neurons. By visual comparison, (see Figs. 6, 7) dysplastic neurons labeled by NR1-Pharmingen were darker than the normal cortical neurons. The positive labeling by NR1-Chemicon and the more intense labeling by NR1-Pharmingen of the dysplastic neurons suggest that the expression of splicing variants NR1-1a, 1b, 2a, and 2b were induced in the dysplastic neurons, and those epitopes would be detected by either NR1 antibody. Possible molecular mechanisms for the specificity of expression of certain NR1 splicing variants in dysplastic neurons are not yet known; however, the alterations in glutamate receptor expressions noted in our study could contribute to physiological abnormalities.

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