Correlation Between Clinical and Histologic Findings in the Human Neonatal Hippocampus After Perinatal Asphyxia

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Abstract

Hypoxic ischemic encephalopathy after perinatal asphyxia is a major cause of mortality and morbidity in infants. Here, we evaluated pathologic changes in the hippocampi of a cohort of 16 deceased full-term asphyxiated infants who died from January 2000 to January 2009. Histochemical and immunocytochemical results for glial and neuronal cells were compared between cases with or without seizures and to adult and sudden infant death syndrome cases (n = 3 each). All asphyxiated infants displayed neuronal cell damage and reactive glial changes. Strong aquaporin-4 immunoreactivity was seen on astroglial cells within hippocampi in 50% of cases. In patients with seizures, the expression of metabotropic glutamate receptors was increased in glial cells. Cases with seizures displayed increased microglial activation and greater expression of the inflammatory markers interleukin 1β and complement 1q compared with those in cases without seizures. All cases with seizures displayed alterations in the blood-brain barrier, as assessed by immunohistochemistry for albumin. These findings confirm the complex cascade of cellular and molecular changes occurring in the human neonatal hippocampus after perinatal asphyxia. These changes may contribute to seizure development leading to secondary brain damage. These data may aid in the development of therapeutic targets for neonatal seizures.

Key Words: Asphyxia, Astrocytes, Epilepsy, Glutamate, Hippocampus, Inflammation, Neonate.

INTRODUCTION

Perinatal asphyxia is a major cause of neonatal hypoxic ischemic encephalopathy (HIE), neonatal mortality, and long-term neurodevelopmental disabilities, including cerebral palsy, delayed mental development, neuropsychiatric disorders (e.g., schizophrenia and attention-deficit hyperactivity disorder), and epilepsy (1–4). The developing brain is highly prone to seizures, and severe neonatal seizures have been associated with a detrimental neurodevelopmental outcome (5, 6).

During the last decade, our understanding of the pathogenesis of neonatal brain injury has greatly increased. Several hypotheses have been put forward to explain the progression of the brain damage and development of neonatal seizures after perinatal asphyxia. Both experimental and clinical studies suggest the involvement of multiple mechanisms including alterations in the balance between excitation and inhibition, development of cerebral edema, and activation of CNS immune responses (7–9).

Several studies support involvement of glutamate-mediated excitotoxicity, pointing particularly to the role of ionotropic glutamate receptors (iGluRs), which are ligand-gated ion channels (including the N-methyl-D-aspartate [NMDA] and non-NMDA receptor subtypes) and metabotropic glutamate receptors (mGluRs). The mGluRs are a family of G-protein-linked receptors regulating a variety of intracellular signaling systems critically affecting hippocampus-dependent synaptic plasticity and memory (10, 11). Metabotropic glutamate receptors, particularly Group I mGluRs (mGluR1 and mGluR5), have also been studied for their potential involvement in cell death after perinatal asphyxia (9, 12).

A large majority of neonates with severe perinatal asphyxia develop cytotoxic and vasogenic edema in the brain parenchyma (8). Recent studies support a key role of aquaporin proteins, particularly aquaporin-4 (AQP4), in both the onset and clearance of cerebral edema after neonatal asphyxia (8, 13–15).

Activation of astrocytes and cells of the microglia/macrophage lineage, followed by induction of inflammatory pathways, has all been suggested to play important roles in the pathogenesis of neonatal HIE (9, 16, 17). Moreover, increasing evidence supports the role of inflammatory mediators in the etiopathogenesis of seizures, exerting both acute and long-term effects on seizure threshold (18, 19).

The complex cascade of cellular and molecular changes occurring in the human brain after perinatal asphyxia, particularly in brain regions such as the hippocampus, which is very sensitive to hypoxic ischemic insults and critically involved in...
the generation of seizures, remains uncharacterized, and autopsy findings are valuable for identifying pathologic changes that are not detected by brain imaging techniques (20). A recent study comparing antemortem cranial magnetic resonance imaging (MRI) with postmortem histopathologic examination of infants with HIE after perinatal asphyxia demonstrated that MRI underestimated the extent of injury to the hippocampus (21).

In the present study, we evaluated hippocampal pathology after perinatal asphyxia in a cohort of 16 infants using histochemical and immunocytochemical glial and neuronal markers, including evaluation of both iGluRs and mGluR (groups I and II), AQP4, inflammatory mediators (e.g. interleukin 1β [IL-1β] and complement 1q [C1q]), and albumin. Possible correlations of clinical data and histopathologic findings were investigated. Our major aim was to investigate the cascade of cellular and molecular changes occurring in human neonatal hippocampi after perinatal asphyxia. We hypothesize that changes in glial cells may contribute to seizure development, leading to secondary brain damage in infants with HIE.

**MATERIALS AND METHODS**

**Patients**

The Neonatology Intensive Care Unit database of the Emma Children’s Hospital/Academic Medical Centre in Amsterdam, the Netherlands, was searched for cases with perinatal asphyxia. The study period extended from January 2000 until January 2009. During this period, we did not yet use controlled hypothermia as the standard of care for asphyxiated newborns. Clinical data were retrieved from digital or written files. Digitally stored electroencephalography (EEG) recordings and imaging results were retrieved for each case. The autopsy specimens of the patients were obtained from the Department of Neuropathology of the Academic Medical Center (University of Amsterdam, Amsterdam, The Netherlands) (Table; Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A565). In our hospital, autopsy was offered to all parents of deceased asphyxiated neonates without any selection based on clinical or other criteria. We excluded cases with chromosomal anomalies, major CNS malformations, postmortem autolysis, intraventricular hemorrhages, severe hydrocephalus, and meningitis or ventriculitis. We only included specimens displaying a normal hippocampal structure for the corresponding age. For control cases, we included autopsy results of normal-appearing hippocampi from 3 adult patients and 3 sudden infant death syndrome cases, we included autopsy results of normal-appearing hippocampal structure for the corresponding age. For control cases, we included autopsy results of normal-appearing hippocampi from 3 adult patients and 3 sudden infant death syndrome cases.

**Tissue Preparation**

Brains were fixed in 10% buffered formalin for 3 weeks before gross examination, cutting, and tissue processing for paraffin sections. Formalin-fixed, paraffin-embedded tissue

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**TABLE. Clinical Features and Imaging and EEG Findings**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (male/total)</td>
<td>8/16</td>
</tr>
<tr>
<td>Gestational age, mean (range), weeks</td>
<td>40.4 (39–42)</td>
</tr>
<tr>
<td>Delivery, n = 16</td>
<td></td>
</tr>
<tr>
<td>Normal vaginal delivery</td>
<td>3</td>
</tr>
<tr>
<td>Vacuum extraction</td>
<td>7</td>
</tr>
<tr>
<td>Cesarean section</td>
<td>6</td>
</tr>
<tr>
<td>Birth weight, mean ± SE, g</td>
<td>3,577 ± 112.4</td>
</tr>
<tr>
<td>HIE severity, n = 16</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>5</td>
</tr>
<tr>
<td>Severe</td>
<td>11</td>
</tr>
<tr>
<td>Multiorgan failure</td>
<td>6/16</td>
</tr>
<tr>
<td>Persistent acidosis (pH &lt; 7.20 for &gt; 24 hours)</td>
<td>7/16</td>
</tr>
<tr>
<td>Seizures</td>
<td></td>
</tr>
<tr>
<td>Clinical seizures</td>
<td>9/16</td>
</tr>
<tr>
<td>Confirmed by EEG</td>
<td>4/9</td>
</tr>
<tr>
<td>Only visible on EEG</td>
<td>1/16</td>
</tr>
<tr>
<td>Age at death, mean (range), days</td>
<td>3.8 (1–11)</td>
</tr>
<tr>
<td>Cerebral ultrasound abnormalities, n = 16</td>
<td></td>
</tr>
<tr>
<td>Edema</td>
<td>8</td>
</tr>
<tr>
<td>Cortical infarcts</td>
<td>4</td>
</tr>
<tr>
<td>Normal</td>
<td>4</td>
</tr>
<tr>
<td>MRI abnormalities, n = 7</td>
<td></td>
</tr>
<tr>
<td>Thalamus/basal ganglia/internal capsule</td>
<td>5</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>4</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2</td>
</tr>
</tbody>
</table>

(1 representative paraffin block per case containing the midbody of the hippocampus was sectioned at 6 µm and mounted on precoated glass slides (Star Frost; Waldemar Knittel GmbH, Barunschweig, Germany). Sections of all specimens were processed and stained with hematoxylin and eosin (H&E), Luxol fast blue, and Nissl stains.

**Antibody Characterization and Immunohistochemistry**

Antibodies specific for the following were used for immunohistochemistry: glial fibrillary acidic protein (GFAP) polyclonal rabbit, 1:4000; DAKO, Glostrup, Denmark), anti-human leukocyte antigen (HLA)-DP, DQ, DR (mouse clone CR3/43, 1:400; DAKO), AQP4 (rabbit polyclonal antibody, 1:50; Chemicon, Temecula, CA), mGluR1α (polyclonal rabbit, 1:100; Chemicon), mGluR2/3 (polyclonal rabbit, 1:100; Chemicon), mGluR5 (polyclonal rabbit, 1:100; Upstate Biotechnology, Lake Placid, NY), NMDA receptor 1 (GluN1, mouse clone 54.1; gift of Dr. J.H. Morrison, Mount Sinai, School of Medicine, New York, NY), AMPA (alpha-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor 1 (GluA1, polyclonal rabbit, 1:50; Upstate Biotechnology), AMPA receptor 2 (GluA2, mouse clone 6C4, gift of Dr. J.H. Morrison), IL-1β (goat polyclonal, sc-1250, 1:70; Santa Cruz Biotechnology, Santa Cruz, CA), caspase-3 (rabbit polyclonal, 1:100; Cell Signaling Technology, Danvers, MA), complement factors C1q and C3d (polyclonal; C1q, 1:100; C3d, 1:200; DAKO), phospho-S6 ribosomal protein (pS6, polyclonal rabbit, Ser235/236, 1:50; Cell Signaling Technology), and albumin (polyclonal rabbit, 1:20,000; DAKO).
Immunohistochemistry was carried out as previously described (22). Single-label immunocytochemistry was performed using the Powervision kit (Immunologic, Duiven, The Netherlands), with 3,3′-diaminobenzidine as chromogen. Sections were counterstained with hematoxylin.

All labeled tissue sections were evaluated by 2 independent observers who were blinded to the clinical data. Sections were evaluated for the presence or absence of various histopathologic parameters and specific immunoreactivity for the various markers. The intensities of GFAP, HLA-DR, iGluRs, mGluRs, IL-1β, C1q, C3d, AQP4, caspase-3, pS6, and albumin immunoreactivity were evaluated using semiquantitative scales ranging from 1 to 3 (1, rare; 2, moderate; 3, strong staining; frequency score). All areas of the stained specimen (hippocampal subfields CA1 to CA4) were examined; the scores represent the predominant cell staining intensity found in each area for each case. The frequencies of positive cells were also evaluated using a semiquantitative scale ranging from 1 to 3 (1, rare, <10%; 2, sparse, 11%–50%; 3, high, >50%; frequency score) to inform on the relative number of positive cells within the hippocampus. The overall total immunoreactivity score (IRS) represents the product of the 2 previously described scores (frequency scores × intensity scores = IRS) (23–25). The extent of the neuronal damage was semiquantitatively scored using a system that refers only to neuronal cell loss based on H&E and Nissl staining. For CA1, this was defined as follows: 1, no obvious neuronal loss (<30%); 2, moderate neuronal loss (30%–50%); and 3, severe neuronal loss (majority of neurons lost) (>50%). Note that the limitations of visual inspection alone indicate that the first detectable neuronal loss corresponds to approximately 30% cell loss in H&E stains shown by quantified neuronal density measurement.

Quantitative analysis was also performed by measuring hippocampal optical densities for HLA-DR, IL-1β, and C1q in the CA1. As previously described (26), sections were digitized using an Olympus microscope equipped with a DP-10 digital camera (Olympus, Tokyo, Japan). Images from consecutive nonoverlapping fields (20× magnification) were collected using image acquisition and analysis software (Phase 3 Image System integrated with Image Pro Plus; Media Cybernetics, Silver Spring, MD). The absolute pixel staining density and the background from fields lacking labeling were determined. A threshold value was determined before the beginning of the analysis and kept constant, and only pixels with an optical density above the threshold are taken into account in the calculation of the relative optical density ratio. The optical density ratio is calculated by determining the level of pixel staining density in labeled cells versus the pixel density of the noncellular background. An optical density ratio greater than 3 was used as a threshold to define immunopositivity for a given antibody, and comparisons were made between patients.

Statistical analyses were performed with SPSS for Windows (SPSS 20; SPSS, Inc., Chicago, IL). Continuous variables were described with median and ranges or mean and SEM; categoric variables were described with proportions and percentages. The 2-tailed Student t-test or the nonparametric Kruskal-Wallis test followed by Mann-Whitney U test was used to assess differences between the groups. Correlation between histologic/immunohistochemical data and clinical features (i.e. age at death, sex, type of delivery, HIE severity, persisting acidosis, presence of epileptiform activity, presence of multiorgan failure, cerebral ultrasound or MRI abnormalities) was assessed using the Spearman rank correlation test. A value of p < 0.05 was defined as statistically significant.

Full Lead EEG Analyses of Background and Seizure Activity

All of the available full lead digital EEGs were reevaluated and scored by a pediatric neurologist and a clinical neurophysiologist who were both blinded to the neuropathologic results and the clinical histories of the patients. The EEGs were scored for background pattern (A) and the extent of seizure activity (B) as follows:

For background pattern (A): 1, flat trace EEG (no electrical activity); 2, nearly flat trace EEG activity (<20 μV); 3, burst suppression pattern; 4, abnormal background pattern with increased level of asynchronicity; and 5, normal background pattern. For seizure activity (B): 1, no epileptiform activity; 2, paroxysmal epileptiform activity with a duration of 1 to 30 seconds; 3, paroxysmal epileptiform activity with a duration of more than 1 minute; 5, continuous epileptiform activity/status epilepticus. For all patients, the first and last recorded EEGs (if available) were analyzed.

MRI and Cerebral Ultrasound

Available MRI scans were reevaluated by 2 independent pediatric neuroradiologists who were blinded to the neuropathology, EEG results, and the clinical histories. Both conventional and diffusion-weighted images of the hippocampal region were reevaluated. They scored either altered or normal signal intensity. Cerebral ultrasound was reevaluated by a neonatologist unaware of the clinical history and (if present) MRI findings of the patient; they were scored for abnormalities in the cortex, basal ganglia, or hippocampal region and for the presence of cerebral edema.

RESULTS

Case Material and Clinical Features

A total of 287 newborns were admitted at the Neonatology Intensive Care Unit with a diagnosis of perinatal asphyxia of varying severity during the study period. A total of 44 patients died because of either severe multiorgan failure or withdrawal of intensive care treatment. Brain autopsy specimens were available for 16 of these 44 neonates (i.e. cases with informed consent for brain autopsy and the use of brain tissue for research purposes) (Table; Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A565). All neonates had perinatal asphyxia and were resuscitated at birth. In 10 of 16 cases, antenatal signs of fetal distress (i.e. maternally reported decreased fetal movements, fetal bradycardia on either antenatal auscultation by attending midwife or determined by cardiocography) were documented. In 4 of 16 cases, perinatal asphyxia was unexpected before delivery. Clinical data concerning the intensive care treatment and course were available for all cases. Full lead EEG results were available for 8 of 16 cases; cerebral ultrasound was
available in 14 of 16. On cerebral ultrasound, 8 of 14 patients demonstrated diffuse cerebral edema; and in 4 of 14 patients, there was obvious cortical infarction that was confirmed by postmortem examination. Cerebral MRI was performed as standard of care in asphyxiated patients from 2005 onward in our department. Magnetic resonance imaging was available for 7 of 16 cases. In these patients, MRI findings included areas with high signal intensities on T2W imaging and FLAIR with/without diffusion restriction in, for example, the thalamus, basal ganglia, internal capsule, and cerebral cortex (in the hippocampus in 2 of 7 patients). The more severe MRI findings were observed in infants with shorter survival. However, the number of cases with available MRI was too small to perform meaningful statistical comparisons with the histopathologic data.

Seizures

Ten of 16 patients were diagnosed as having epileptic seizures. Nine patients had clinical seizures; 8 of these patients underwent a full lead EEG. Four of these 8 showed epileptic activity on their EEGs. In 1 patient, EEG recording demonstrated paroxysmal epileptiform activity with duration of 30 to 60 seconds without clinical correlation. The seizures occurred within 24 hours after birth (range, 1–24 hours). These patients were all treated with at least 1 type of antiepileptic drug (mostly phenobarbital). A total of 5 patients needed more than 2 antiepileptic drugs to control clinical seizures, and midazolam and lidocaine were added in a stepwise manner. The presence of seizures was positively associated with older age at death ($r = 0.736; p < 0.001$).

Histologic and General Immunohistochemical Features

Variable (moderate to severe) neuronal cell loss was observed within the hippocampal pyramidal cell layer with prominent damage in CA1 (Figs. 1A, 2A). The hippocampal expression pattern and IRS for different glial and neuronal markers were evaluated, and correlations between immunostaining and different clinical variables (e.g. the presence of seizures) were assessed. Hippocampal damage was detected in all cases with moderate (44%) to severe (56%) neuronal cell loss. Neuronal cell loss was positively associated with older age at death ($r = 0.648; p < 0.007$), but we did not detect significant differences in the severity of neuronal cell loss between cases with and without seizures (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A566). The severity of hypoxia (Sarnat score) (Table; Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A565) within our cohort did not significantly affect the expression of neuronal or glial markers as well as the development of seizures. Few caspase-3 and pS6-positive neuronal cells were detected in the stratum pyramidale both in cases with and without seizures (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A567, parts E, F).

AQP4 Immunoreactivity

As previously reported in human brains (27–29), AQP4 immunoreactivity was detected in ependymal cells and particularly in astrocytic end feet at the pial surface and around blood vessels in all specimens. Low to moderate AQP4 immunoreactivity was detected around blood vessels and in the white matter in 8 of 16 cases (Fig. 1B), with a pattern similar to that observed in control hippocampi (i.e. adults and infants with sudden infant death syndrome). In the other 8 cases, we observed strong immunoreactivity in astrocytes within the hippocampus (Fig. 2B). The cases with strong AQP4 expression displayed prominent edematous cell changes throughout the hippocampi. We did not observe significant differences in the AQP4 IRSs between patients with and without seizures (Fig. 3B). There was a transient downregulation of AQP4 expression in the first days after birth that was followed by increased expression, reaching peak values at 6 days (Fig. 3C). In the 14 patients in which cerebral ultrasound was available, AQP4 IRS was positively correlated with the presence of diffuse cerebral edema ($r = 0.661; p < 0.01$).

Glutamate Receptor Immunoreactivity

We evaluated the expression pattern of both iGluRs (AMPA and NMDA receptors) and mGluRs (group I mGluRs, mGluR1 and mGluR5; and group II mGluRs, mGluR2/3). As previously reported, both iGluRs and mGluRs are highly expressed in the neonatal hippocampus (30, 31). The pattern of neuronal immunoreactivity was similar to that observed in control hippocampi (adults and sudden infant death syndrome victims, not shown). However, there was decreased expression in areas of neuronal cell loss (i.e. CA1, mGluR5; Fig. 2D). Differences in the expression patterns of mGluR5 and mGluR2/3 (the polyclonal Ab mGluR2/3 recognizes both subtypes of Group II mGluRs) were found in the hippocampi of patients with seizures; that is, there was strong immunoreactivity in astroglial cells (Figs. 2D, E; 4E, F). In contrast, we did not detect differences in the glial immunoreactivity pattern of the other receptor subtypes between cases with and without seizures (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A567, parts B-D; NMDA, not shown). Metabotropic glutamate receptor 5 and mGluR2/3 IRSs in glial cells were positively associated with older age at death (GluR5, $r = 0.765$, $p = 0.001$; mGluR2/3, $r = 0.681$, $p = 0.004$) and with the presence of seizures (mGluR5, $r = 0.902$, $p < 0.001$; mGluR2/3, $r = 0.608$, $p < 0.05$). There was also a positive correlation for both mGluR5 and mGluR2/3 IRSs in glial cells with GFAP (mGluR5, $r = 0.715$, $p < 0.001$; mGluR2/3, $r = 0.864$, $p < 0.01$) and between mGluR2/3 and neuronal cell loss ($r = 0.635$, $p < 0.01$).

GFAP and HLA-DR Immunoreactivity

In most of the cases, there was moderate to strong immunoreactivity detected for the astrocyte marker GFAP, but the GFAP IRS was significantly higher in cases with seizures compared with the IRS in cases without seizures (Fig. 4A). The microglia/macrophage marker HLA-DR immunoreactivity was detected in all cases, but higher IRSSs were detected in cases with seizures versus those without seizures (Figs. 1C, 2C, 3D). Glial fibrillary acidic protein and HLA-DR IRSSs were positively associated with older age at death (GFAP, $r = 0.776$; HLA-DR, $r = 0.776$; $p < 0.001$ for both) and with the presence of seizures (GFAP, $r = 0.595$; HLA-DR, $r = 0.436$; $p < 0.05$ for both). Positive correlation was also detected for both GFAP and HLA-DR IRS with the severity of
FIGURE 1. Histopathology of the hippocampus in a neonate without clinical seizures. (A) Hematoxylin and eosin staining (HE) shows relatively preserved stratum pyramidale (insert shows a high magnification of CA1 pyramidal neurons). (B) AQP4 immunostaining shows moderate astroglial expression, particularly around blood vessels within the white matter (insert). (C) HLA-DR immunostaining shows no detectable immunoreactivity (IR). (D) mGluR5 immunostaining shows neuronal/neuropil IR throughout the various hippocampal subfields. (E) mGluR2/3 immunostaining shows prominent neuronal/neuropil IR, particularly in the CA3 region and in the dentate gyrus (DG). IL-1β immunostaining (F) and C1q immunostaining (G) show no detectable IR in CA1. Hematoxylin counterstain (B–G). Scale bar in (G) refers to (A–E) = 400 μm; (F, G) 80 μm.
FIGURE 2. Histopathology of the hippocampus of a neonate with clinical seizures. (A) Hematoxylin and eosin staining (HE) shows loss of neurons within the stratum pyramidale (SP), particularly in CA1 (insert shows a high magnification of CA1). (B) AQP4 immunostaining shows strong astroglial expression throughout the different hippocampal subfields; insert shows a high magnification of AQP4-positive perivascular astrocytes. (C) HLA-DR immunostaining shows strong immunoreactivity (IR), particularly in CA1; insert shows a high magnification of HLA-DR-positive cells. (D) mGluR5 immunostaining shows reduced neuronal/neuropil IR expression in SP (CA1) but increased expression in astrocytes (insert). (E) mGluR2/3 immunostaining shows reduced neuronal/neuropil IR expression in SP but increased expression in astrocytes (insert). (F) Interleukin 1β (IL-1β) immunostaining shows increased expression in glial cells within the CA1; insert in (F) shows a high magnification of IL-1β-positive astrocytes. (G, H) C1q immunostaining shows increased expression in glial cells, particularly in CA1 (H); insert in (H) shows a high magnification of C1q-positive glial cells. (I, J) Albumin immunostaining shows positive perivascular astrocytes in CA1 (arrows). DG, dentate gyrus. Hematoxylin counterstain (B–H). Scale bar in (J) refers to (A–E, G) = 400 μm; (F) 80 μm; (H) 160 μm; (I) 70 μm; (J) 40 μm.
neuronal cell loss (GFAP, \( r = 0.813 \); HLA-DR, \( r = 0.805 \); \( p < 0.001 \) for both).

IL-1\( \alpha \) and Complement Component (C1q/C3d) Immunoreactivity

Variable expression of IL-1\( \alpha \), C1q, and C3d was observed within the cohort, including cases in which IL-1\( \alpha \) and C1q immunoreactivities were detected only in a few glial cells and cases with prominent expression (Fig. 4C, D; Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A567, part A). Cases with seizures displayed higher IRSs compared with those in cases without seizures for both IL-1\( \alpha \) and C1q (Figs. 1F, G; 2F-H; 3E, F; 4C, D). Interleukin 1\( \beta \), C1q, and C3d IRSs were positively associated with older age at death (IL-1\( \alpha \), \( r = 0.751 \), \( p < 0.001 \); C1q, \( r = 0.734 \), \( p < 0.001 \); C3d, \( r = 0.660 \), \( p = 0.005 \)), and IL-1\( \beta \) and C1q were positively associated with the presence of seizures (IL-1\( \beta \), \( r = 0.499 \); C1q, \( r = 0.559 \); \( p < 0.05 \) for both). Positive correlation was also detected for IL-1\( \beta \), C1q, and C3d IRSs with the severity of neuronal cell loss (IL-1\( \beta \), \( r = 0.717 \), \( p < 0.001 \); C1q, \( r = 0.704 \), \( p < 0.001 \); C3d, \( r = 0.657 \), \( p < 0.005 \)). No significant correlations were found between the IL-1\( \beta \) and C1q IRSs and other clinical variables, including the presence of underlying infection, which was detected in only 2 patients.

Albumin Immunoreactivity

Alterations in blood-brain barrier (BBB) permeability were detected using albumin immunocytochemistry. In all cases with seizures, albumin immunoreactivity was detected in perivascular astrocytes (Figs. 2I, J; 3A). Albumin IRS was positively associated with older age at death (\( r = 657 \), \( p < 0.005 \)) and with the presence of seizures (\( r = 0.775 \), \( p < 0.001 \)).

DISCUSSION

Clinical and experimental studies indicate that perinatal asphyxia results in functional compromise of the hippocampus (32, 33), even if structural damage cannot be detected by brain imaging techniques (21). In addition to acute neuronal injury, multiple subcellular changes may affect hippocampal function and contribute to the development of neonatal seizures and progression of the brain damage (7–9).

Immunocytochemical studies of postmortem tissue represent a valuable approach for studying neuronal and glial changes in the neonatal hippocampus after perinatal asphyxia. This study provides the first description of these complex changes involving glial cells.

Neuronal Cell Loss and Astroglial and Microglial Reactivity

Moderate to severe neuronal damage was observed within the hippocampal pyramidal cell layer in all cases. Determination of the exact time of onset of neuronal damage is difficult because multiple factors may influence the susceptibility to hypoxic damage at birth (34). For example, in the infants with signs of prenatal infection (one with severe bronchopneumonia but no seizures), there was severe neuronal damage.
In our cohort, neuronal damage was more severe in infants with longer survival. In addition, the presence of seizures was positively associated with older age. Thus, the effect of postnatal age on hippocampal pathology must also be taken into consideration. We did not observe differences in the severity of neuronal cell loss between cases with and without seizures, however. Although we cannot exclude the possibility that hypoxic/ischemic injury may have occurred in the hours before death, the time interval between the withdrawal of intensive care and actual death was always limited and equal between patients with and without seizures.

Because 2 different forms of cell death occur in the neonatal brain, that is, a primary form with early evidence of necrosis followed by a secondary phase of apoptotic cell death (9, 20, 35), we also evaluated the expression of the apoptosis marker caspase-3. The observed expression of caspase-3 within the hippocampus was modest even in cases with longer survival and seizures, indicating that apoptosis did not seem to play a major role in cell death in the time frame studied.

Previous experimental studies have shown that hypoxic/ischemic brain injury in neonatal rats induces GFAP mRNA and protein expression in different brain areas, including the hippocampus (35). We found that the expression of GFAP was higher in cases with seizures versus that in cases without seizures, supporting the role of reactive astrocytes in the pathophysiologic processes that underlie the development of post-asphyxia seizures (18, 36, 37).

Activation of microglia has been previously reported in both experimental and human studies after perinatal asphyxia (9, 20). In a recent study, evaluation of activated microglial cells/macrophages was used to detect hippocampal damage in term infants with neonatal encephalopathy after perinatal asphyxia (21). Here, we confirmed the presence of activated microglial cells/macrophages within the hippocampus and showed a positive correlation for both microglial and astroglial markers with the severity of neuronal cell death, as well as with the presence of seizures. Interestingly, large-scale analyses of gene expression profiles have demonstrated prominent up-regulation of genes related to microglial and astroglial activation in human conditions associated with seizures (18, 38).

**Activation of Inflammatory Pathways**

The activation of astrocytes and cells of the microglia/macrophage lineage was associated with increased expression of IL-1β and the complement components C1q and C3d within the hippocampus (particularly in CA1), where neuronal damage occurs. Induction of inflammatory pathways has been suggested to play an important role in the development of HIE. Although activation of inflammatory cells and their associated pathways may exert both beneficial and toxic effects, an uncontrolled inflammatory response becomes detrimental for tissue excitability and cell survival (9). In particular, enhanced expression of IL-1β has been demonstrated after hypoxia-ischemia in neonatal rats, and neuroprotection obtained with

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**FIGURE 4.** Evaluation of immunoreactivity in hippocampi of patients with and without seizures. (A-F) Immunoreactive scores (IRSs) for GFAP (A), HLA-DR (B), IL-1β (C), C1q (D), mGluR5-glia (E), and mGluR2/3-glia (F); IRSs in patients with seizures are significantly different (p < 0.05) from those in patients without seizures. Nonparametric Kruskal-Wallis test followed by Mann-Whitney U test was used to assess the differences between the groups.
IL-1 receptor antagonists in vivo supports the pathophysiologic role of IL-1β in HIE (16). Here, we confirm the up regulation of this cytokine in the neonatal human hippocampus with hypoxic injury, showing a positive correlation with the severity of neuronal cell loss, as well as with the presence of seizures. Interestingly, several studies strongly support a “pro”convulsant role of cerebral IL-1β in pathologic conditions (18, 19, 39).

Complement activation in the CNS is increasingly recognized to be associated with exacerbation and progression of various types of brain injury (40–42). Evidence of complement activation has been reported in the neonatal rodent brain after perinatal asphyxia (17, 43). Here, we confirm that activated microglia/macrophages represent an important local source of components of complement in neonatal human hippocampi with hypoxic injury. Moreover, we detected higher levels of C1q expression in cases with seizures compared with those in cases without seizures. According to data from previous experimental and human studies, this may support the role of complement activation in the destabilization of neuronal networks leading to increased susceptibility to seizure activity (18, 19, 44).

**AQP4 Immunoreactivity and Alterations in BBB Permeability**

We confirmed the previously described expression pattern of AQP4 in the neonatal hippocampus, with predominant expression in astrocytic end feet around blood vessels (29). In our cohort, cases with strong AQP4 expression displayed prominent edematous cell changes throughout the hippocampus, and in patients for whom cerebral ultrasound data were available, AQP4 expression was associated with the presence of diffuse cerebral edema. This should be interpreted with caution because of the low specificity of cerebral ultrasound results, however (45), and we did not have sufficient numbers of cases with available MRI data. The strong astroglial expression of AQP4 observed in neonatal hippocampi of a large number of cases suffering perinatal asphyxia supports the role of this protein in the control of brain water content in the neonatal brain (29).

The mechanisms underlying activation and regulation of AQP4 under physiologic and pathologic conditions are still a matter of debate (46). These mechanisms may include inflammation-mediated regulation (46, 47), as well as interaction with glutamate receptors, such as mGluR5 (48). Interestingly, both mGluR5 and inflammatory markers are upregulated in the hippocampus after neonatal HIE. Clinical epilepsy has been shown to be associated with an alteration of AQP4 expression pattern, and AQP4 may affect seizure severity and seizure threshold (46, 49). However, in our cohort, we did not detect differences in the expression pattern of AQP4 between patients with and without seizures. Future investigations in possibly larger cohorts (with more available MRI results) are necessary to address the relationship between AQP4 levels of expression and cerebral edema, as well as the effect of this regulation on seizure susceptibility.

In addition to AQP4 expression in perivascular astrocytes, we also observed perivascular parenchymal leakage of serum albumin with uptake in astrocytes, supporting the occurrence of alterations in BBB permeability within the neonatal hippocampus after perinatal asphyxia. The presence of albumin in astrocytes positively correlated with the presence of seizures. Interestingly, alterations in BBB permeability have recently been demonstrated in both human and experimental studies of temporal lobe epilepsy (50, 51), and serum proteins such as albumin have been shown to contribute to an increase in neuronal hyperexcitability (52, 53).

**mGluR5 and mGluR2/3 Upregulation in Astrocytes**

Several studies support involvement of glutamate-mediated excitotoxicity with a possible role for mGluRs (9, 12). We found evidence of upregulation of both mGluR5 and mGluR2/3 in astrocytes within the hippocampus after perinatal asphyxia. Although mGluRs are classically considered neuronal receptors, a growing body of evidence indicates that they may also critically regulate the function of glial cells (54, 55). In particular, mGluR3 and mGluR5 are the 2 predominant mGluR subtypes expressed in human astrocytes in vivo as well as in vitro, and they have been shown to regulate different cell functions, including glial cell proliferation and production and release of different proinflammatory cytokines (54, 56, 57). In addition, activation of group I and II mGluRs has been shown to contribute to regulate the extracellular levels of glutamate, regulating the expression of glial transporter proteins (56, 58). We observed that increased expression of glial mGluR5 and mGluR2/3 was positively associated with the presence of seizures. Similarly, upregulation of these receptor subtypes in astrocytes has been shown in different pathologic conditions associated with seizure development (54, 57, 59).

In conclusion, our findings confirm the complexity of the cascade of cellular and molecular changes occurring in human neonatal hippocampi after perinatal asphyxia. In particular, they support the role of glial cells with changes in the expression of AQP4 and glial mGluRs as well as activation of inflammatory pathways and alterations in BBB permeability. Future investigations in larger patient populations as well as in experimental models are necessary to address the possible contribution of these changes to progressive brain damage and/or seizure susceptibility after perinatal asphyxia. Expanding knowledge on the course of neuropahtologic events after perinatal asphyxia has important mechanistic and therapeutic implications that identify potential new mechanisms underlying HIE that may help be novel targets for pharmacologic intervention. The role of therapeutic hypothermia in neonatal intensive care treatment after perinatal asphyxia should be taken into account in the interpretation of future results.

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