Neonatal loss of γ-aminobutyric acid pathway expression after human perinatal brain injury

SHENANDOAH ROBINSON, M.D., QING LI, M.D., ANNE DECHANT, M.S., AND MARK L. COHEN, M.D.

Departments of Neurosurgery and Neurosciences, Division of Neuropathology, University Hospitals of Cleveland, Case Research Institute, Case Western Reserve University School of Medicine, Cleveland, Ohio

Object. Perinatal brain injury leads to chronic neurological deficits in children. Damage to the premature brain produces white matter lesions (WMLs), but the impact on cortical development is less well defined. Gamma-aminobutyric acid (GABA)ergic neurons destined for the cerebral cortex migrate through the developing white matter and form the subplate during late gestation. The authors hypothesized that GABAergic neurons are vulnerable to perinatal systemic insults in premature infants, and that damage to these neurons contributes to impaired cortical development.

Methods. An immunohistochemical analysis involving markers for oligodendrocytes, GABAergic neurons, axons, and apoptosis was performed on a consecutive series of 15 human neonatal telencephalon samples obtained postmortem from infants born at 25 to 32 weeks of gestation. The tissue samples were divided into two groups based on the presence or absence of WMLs by performing routine histological analyses. The expression of GABAergic neurons was compared between the two groups by using age-matched samples. Two-tailed t-tests were used for statistical analyses.

Ten infants had WMLs and five did not. Significant losses of oligodendrocytes and axons and markedly increased apoptosis were appreciated in tissue samples from the infants with WMLs. Samples from infants with WMLs also showed significant losses of glutamic acid decarboxylase–67–positive cells and calretinin–positive cells, shorter neuropeptide Y–positive neurite lengths, and losses of cells expressing GABA_A1, GABA_R1, and N-acetylaspartate diethy lamide NR1 receptors when these factors were compared with those in samples from infants without WMLs (all p < 0.02).

Conclusions. In addition to oligodendrocyte loss, axonal disruption, and excess apoptosis, a significant loss of telencephalon GABAergic neuron expression was found in neonatal brains with WMLs, compared with neonates’ brains without WMLs. The loss of GABAergic subplate neurons in infants with WMLs may contribute to the pathogenesis of neurological deficits in children.

KEY WORDS • cerebral palsy • development • γ-aminobutyric acid • perinatal brain injury • white matter lesion • pediatric neurosurgery

Children who survive perinatal brain injury often later experience multiple chronic neurological deficits including cerebral palsy, epilepsy, cognitive delay, behavioral abnormalities, and neurosensory impairments. Premature infants born before 32 weeks of gestation are particularly vulnerable. Whereas neurosurgical interventions can markedly improve the quality of life for many of these children, current surgical options ablate (examples: selective dorsal rhizotomy and cortical resection for epilepsy) or saturate (examples: intrathecal baclofen pump and vagal nerve stimulator) rather than replenish the supply of neural cells. None of the current surgical interventions directly addresses cognitive delay, the most frequent and arguably most devastating sequela of perinatal brain injury. Neonatal interventions offer the potential to minimize the disruption of neurodevelopment that is caused by perinatal insults and to restore neurological function in these children. Use of these agents requires a better understanding of how neural cells are affected by early brain damage. For example, any attempt to restore damaged neural cell populations by inducing the formation of or transplanting replacement neural cells requires that the identity of the affected neuronal lineages be well defined. Previous studies have demonstrated that white matter oligodendrocytes are affected by perinatal insults, and that subsequent neuronal and axonal development is abnormal. Although many neurological deficits resulting from perinatal injury imply disruption of cortical development, little progress has been made in understanding the manner in which cortical neuron development has been affected. This study was undertaken...
to define whether human telencephalon neurons are vulnerable to perinatal brain damage.

White matter lesions with destruction of myelin-producing oligodendrocytes are a hallmark of perinatal brain injury associated with cerebral palsy.\textsuperscript{6,45,93} Periventricular leukomalacia was first described by Banker and Larroche\textsuperscript{2} as areas of focal coagulative necrosis in deep hemispheric white matter. Subsequently, Gilles and Murphy\textsuperscript{29} defined PTL as diffuse white matter gliosis with or without areas of focal necrosis. Fortunately, both premature infant death and severe cystic PVL now occur less frequently because of advances in obstetrics and neonatology, but a concomitant reduction in neurological morbidity for preterm infants has not occurred.\textsuperscript{30,68,92}

White matter damage defines the condition of infants with PVL or PTL. The extent of damage to the overlying cortex is less well characterized. Given that cortical gray matter volume increases fourfold between 30 and 40 weeks of gestation,\textsuperscript{94} cortical development is intimately associated with the development of the underlying white matter.\textsuperscript{45,64} and many of the deficits exhibited by children after perinatal damage imply cortical involvement, it is likely that the cerebral cortex is also damaged by the same perinatal insults that produce WMLs. Preterm infants have less cortical gray matter at term than infants born at term, and infants who suffer white matter damage have a disproportionately greater loss of cortical gray matter than unaffected preterm infants.\textsuperscript{39,40} Telencephalon undergoes maturation during the third trimester and the early neonatal period, during which these cells form and refine synapses to establish both local and distant circuits;\textsuperscript{64,66} these neurons would thus be expected to be vulnerable to injury in preterm neonates.

Multiple neuron populations in the telencephalon may be vulnerable to perinatal brain injury. Excitatory pyramidal neurons arise and migrate to the cerebral cortex primarily during the first and second trimesters.\textsuperscript{49} In the current study, we focus on the GABA pathway for the following reasons: 1) expression of the GABA pathway is maximal at 25 weeks of gestation in humans,\textsuperscript{1,2} a common time for premature birth injury; 2) GABA is the predominant telencephalon neurotransmitter during late gestation;\textsuperscript{24} and 3) GABA plays a crucial role in cortical development.\textsuperscript{14,75,95} During the third trimester, GABAergic neurons migrate through the developing white matter and subplate, a transient layer that lies deep with respect to the cortical plate.\textsuperscript{2,3,5,7,10} In humans, the subplate forms at 15 weeks, reaches maximal size at 26 to 29 weeks, and recedes during the first 6 months postnatally. When the subplate reaches its largest size, it is three-fold thicker than the cortical plate.\textsuperscript{46,47,73} Similar to developing white matter, the subplate is susceptible to damage from perinatal brain injury. Once GABAergic neurons arrive in the cortex, their synapses mature and form complex circuits with other neuronal systems during a prolonged postnatal period.\textsuperscript{14,75} In mice, disruption of GABAergic signaling during development produces phenotypes with epilepsy, cognitive delay, and behavioral problems similar to those described in children who have suffered perinatal brain injury.\textsuperscript{14,44,74,96} We propose that migratory GABAergic neurons are vulnerable to third trimester insults, resulting in altered GABA pathway expression during this critical developmental window.

In most premature infants brain damage begins in utero secondary to a variety of causes, including placental insufficiency and chorioamnionitis. In many of these infants WMLs become evident at birth or soon afterward.\textsuperscript{12} To investigate whether neonatal GABAergic neurons in the telencephalon are affected by perinatal brain insults, we undertook this survey of GABAergic neuron expression in neonates with and without WMLs. The presence of WMLs was determined using standard morphological criteria. Immunohistochemical analyses revealed oligodendrocyte loss, axon beading, excess apoptosis, and alterations in GABAergic expression in the cortex, subplate, and white matter. Our goal was to investigate changes in GABA pathway expression secondary to preterm birth and white matter injury so that we could avoid any confounding factors occurring during childhood. Not surprisingly, a comparison of age-matched specimens demonstrated that central white matter obtained from preterm infants with WMLs contained significantly fewer oligodendrocytes than specimens without WMLs. A significant loss of cells expressing markers of the GABA pathway was present in the cortex, subplate, and white matter in neonates with WMLs compared with those present in the same structures in infants without WMLs. These results suggest that perinatal brain injury resulting in WML affects GABAergic neuronal development in the telencephalon in humans. Given the role of GABAergic neurons in normal cortical development,\textsuperscript{14,54,75,80} the diminished cortical GABA pathway expression found in the present study in premature infants with WMLs suggests that a loss of migrating GABAergic neurons destined for the cortex may contribute significantly to the neurological morbidity children suffer after perinatal insults.

Materials and Methods

Human Tissue

Before commencement of the study, we obtained approval from our local institutional review board. A consecutive series of 15 infants was identified postmortem by conducting a search of the pathology registry. Inclusion criteria were live birth between 25 and 32 weeks of gestation within the calendar years 2001 through 2003. One infant was excluded because of severe hydranencephaly. Tissue specimens were procured from formalin-fixed brains after routine neuropathological analyses had been completed, and each specimen was assigned a coded number to protect confidentiality. A 5-mm-thick coronal tissue section at the level of the head of the caudate was removed from all infant cadavers; an additional coronal section of the parietal lobe was removed from five. A wedge of tissue extending from the superolateral ependymal surface to the cortical surface encompassing the white matter, subplate, and entire cortex was dissected and used for the analysis. Care was taken to obtain anatomically matched specimens from all infants. Tissue was sectioned on a vibratome (Leica VT1000S) for immunohistochemical analyses. Relevant clinical details were extracted from the pathology report and medical records. Standard definitions of gestational age and postconceptional age (gestation plus postnatal age) were used to denote the infant’s age at birth and death.\textsuperscript{26} The postmortem interval did not affect the quality of the tissue or any immunohistochemical reactions.

For the morphological analysis, coded Mayer hematoxylin-stained sections were reviewed by a neuropathologist (M.L.C.) who was blinded to all clinical details. White matter damage was graded as none (0), PTL with diffuse hypercellularity (+), or PVL with marked hypercellularity and necrosis (++). The subplate and cortex were also graded as no damage (0), moderate damage (+), or severe damage (++). Cortical damage was identified by the presence of a disturbed laminar architecture with nuclear pyknosis and/or karyorrhexis. This process was repeated after a 2-month interval to assess intraobserver variability. No discrepancies occurred.
Immunohistochemical Analysis

Sections (50 μm) of formalin-fixed tissue were cut and labeled in a manner previously described. Briefly, the tissue sections were sequentially incubated with 0.3% hydrogen peroxide, primary antibody (Table 1), and biotin-conjugated secondary antibody at appropriate dilutions. The biotin-conjugated secondary antibodies that we used were goat anti-mouse immunoglobulin G (dilution 1:200; ICN Radiochemicals, Irvine, CA) and goat anti-rabbit immunoglobulin G (dilution 1:200; Chemicon International, Temecula, CA). Tissue sections were then incubated with Vectastain (Vector Laboratories, Burlingame, CA). Peroxidase activity was visualized with the aid of diaminobenzidine (Sigma Chemical Co., St. Louis, MO). The GABA was synthesized by GAD, which exists in two isoforms (GAD-65 and GAD-67); GAD-67 was used in this study.

A small subset of oligodendroglial cells expresses GAD-67, and functional GABAα1 receptors exist on rat oligodendrocyte precursor cells. To determine whether alterations in the density of cells with GABA pathway expression in neonates with WMLs were a by-product of oligodendrocyte loss, we performed double-labeling with GABA pathway and oligodendrocyte antibodies. We cut cryostat sections and labeled them first with anti-O4 antibodies (antibodies used to detect the prooligodendrocyte marker), visualized them with diaminobenzidine, and then incubated the tissue sections with GAD-67 or GABA receptors by using appropriate biotinylated secondary antibodies visualized with Cy3 (dilution 1:1000; Jackson Immunologicals, West Grove, PA).

Quantitative differences between specimens removed from infants with WML and those without WML were compared using a two-tailed Student t-test. The nonparametric presence or absence of neurofilament disruption and beading or excess cortical caspase-3 labeling was compared by performing the chi-square test or the Fisher exact test. For double-labeling experiments (to determine whether cells labeled with GABAergic markers were oligodendrocytes), the number of O4+ oligodendrocytes, GABAergic labeled cells, and double-labeled cells were counted at a magnification of 20 in at least five fields in the white matter and subplate, and the proportions of double-labeled glial and GABAergic cells were calculated. To compare the morphological characteristics of NPY+ neurons, 50 randomly selected NPY+ subplate neurons for each infant were photographed at a magnification of 20. The length of the longest neurite was measured for each neuron, and the mean neurite length per cell was calculated for each age-matched group. Also, for each NPY+ neuron that was studied, the shape of the soma was scored as round or long, and the number of dendrites arising directly off the soma was counted. The proportion of cells with a round soma and the mean number of dendrites per cell were calculated for age-matched groups, with significance set at a probability value less than 0.05.

Results

Perinatal Insults in Premature Infants

Perinatal insults in premature human infants induce alterations in the cytoarchitecture and cellular composition of the telencephalon.

Perinatal brain injury often produces a characteristic pattern of white matter damage. For this study, anatomically matched coronal sections of the frontal lobe at the level of the head of the caudate were obtained postmortem from a consecutive series of infants who were between 25 and 32 weeks' gestation at birth. The infants' postconceptional ages at their deaths ranged from 25 to 38 weeks. A neuropathologist (M.L.C.) blinded to all other data scored the central white matter morphologically on hematoxylin-stained sections as normal (five specimens), PTL (eight specimens), or PVL (two specimens) on two separate occasions with no discrepancies (that is, no intraobserver error). The median survival time was less than 1 day for both infants with normal white matter and those with WMLs (range < 1 hour–10 weeks). Relevant clinical factors are presented in Table 2. Most infants had multiple medical problems, typical of preterm infants who expire during the neonatal period. In this study our focus is primarily on the pathology of white matter and its correlation with the expression of neurotransmitters and receptors associated with the GABA pathway. Because of the small sample size and the lack of standardization of inclusion of clinical data, further analyses of risk factors were not performed.

A comparison of the postmortem cytoarchitecture in hu-

### TABLE 1

**Primary antibodies used for the immunohistochemical analyses**

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<th>Dilution</th>
<th>Catalog No.</th>
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Neonatal GABA pathway after perinatal brain injury

human infant brain and correlation with pertinent recorded clinical factors*

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<th>PCA (wks)</th>
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<td></td>
<td>Placenta IU Sepsis Apgar Cause of Death</td>
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<td>31 + + + + +</td>
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<td>33 + + + + +</td>
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<td>38</td>
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<td>32</td>
<td>4 wks</td>
<td>37 + + + + +</td>
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* The clinical, morphological, and astrocyte immunohistochemical findings are presented for all specimens. Conventional terminology is used (see Engle, et al.), and gestational and postconceptional ages are not rounded (that is, an infant born at 28 weeks and 5 days is a 28-week-old infant). Abbreviations: An = analysis; Apgar = Apgar scores at 1, 5, and 10 minutes; card Ab = maternal cardiolipin antibodies; CSS = organ changes suggestive of chronic systemic stress; CWM = central white matter; CTX = cortex; DIC = disseminated intravascular coagulation; GA = gestational age at birth; G-neg sepsis = Gram-negative sepsis; HTN = hypertension; IHC = immunohistochemical; infection = intrauterine infection including funisitis, chorioamnionitis, or postnatal sepsis; IU = intrauterine; IUGR = intrauterine growth retardation; NEC = necrotizing enterocolitis; PCA = postconceptional age (gestation plus postnatal) age at death; preterm = infants in whom resuscitation failed after they had been born prematurely; PROM = premature rupture of membranes; Sub = subplate.
† For the morphological analysis: 0 = no damage; + = moderate damage; and ++ = severe damage; for the immunohistochemical analysis (GFAP immunolabeling): 0 = minimal gliosis; + = moderate gliosis; and ++ = severe gliosis.

Survival

Overlying cortex revealed similar changes, which suggested that neurofilament and oligodendroglial damage is a diffuse process. Together, these data indicate that insults that produce human WMLs also effect reproducible deleterious changes in the development of both white and gray matter.

Presence of Excess Apoptosis After Perinatal Brain Injury

To investigate whether the decreased cell density observed in brains with WMLs resulted from excessive programmed cell death, anti-cleaved caspase-3 immunolabeling was performed. Caspase-3 is the primary effector in the cascade of cysteiny1 aspartate–specific proteases that produce programmed cell death, and activated caspase-3 is present in the cytoplasm of cells undergoing apoptosis but not necrosis. Significantly more activated caspase-3–positive cells were found in the white matter, subplate, and cortex of specimens with WMLs than in age-matched controls with unaffected white matter (Fig. 1). In nine infants with WMLs there was a marked increase in caspase-3–positive cells in the subplate, whereas in none of the four controls was there excess subplate apoptosis. The difference in caspase-3 labeling in the cortex was less distinct. Whereas none (zero of four) of the infants with normal white matter had elevated cortical apoptosis, three (38%) of eight infants with WMLs had markedly elevated caspase-3 labeling in the cortex (p = 0.07, chi-square test). Together, these results suggest that morphological damage in the white matter correlates well with excess apoptosis. 

Man frontal lobes containing WMLs and age-matched frontal lobes lacking WMLs demonstrated consistent changes in both white and gray matter. In morphologically normal white matter (five specimens), hematoxylin staining revealed relatively uniform cellularity. An extensive pattern of fine neurofilament-immunoreactive axons, densely packed O4 astrocytes and O1 oligodendrocytes (Fig. 1), and minimal GFAP-reactive astrocytes (data not shown) were present. By contrast, white matter derived from brains with WMLs (10 specimens) displayed necrosis, numerous swollen and disrupted axons, and a marked decrease in the number of immature and mature oligodendrocytes in both central white matter and subplate regions (Fig. 1). More GFAP expression was present, consistent with gliosis typically observed in WMLs (data not shown). In summary, the morphological designation of relatively unaffected brain (control) or WMLs (PTL or PVL) correlated well with neurofilament and oligodendrocyte loss and GFAP-reactive astroglialosis.

Asphyxial changes in the central nervous system cytoarchitecture were not restricted to the white matter. Hematoxylin staining of the overlying cortex from neonates with WMLs revealed disruption of the normal laminar organization in the brain. This was accompanied by a significant loss of neurofilaments in the cortex in most (six [86%] of seven) infants with white matter damage, compared with the cortex in infants without white matter damage (three specimens; p < 0.002, Fisher exact test) (Fig. 1). In five infants, an evaluation of the parietal white matter and the
with the immunolabeling evidence of axon damage, oligodendrocyte loss, and increased apoptosis in the white matter. These results also support the hypothesis that perinatal brain damage that results in a WML is not limited to the central white matter, but also routinely affects the subplate and overlying cortex. Using these two groups (the control and WML groups), we examined the hypothesis that perinatal brain damage with WMLs affects the development of GABAergic neurons during the third trimester.

Perinatal Brain Injury Hinders GABA Expression in the Developing Subplate and White Matter

Because GABAergic neurons migrate through regions that have been affected by perinatal brain injury with WMLs, we propose that these GABAergic neurons are also damaged in brains with WMLs. We found GAD-67-immunolabeled cells in undamaged white matter and the subplate by 25 weeks of gestation. As development proceeded, the density of the GAD-67+ cells increased in both the white matter and subplate (Fig. 2). For example, GAD-67+ cells increased twofold from 26 or 27 weeks (28 ± 10 cells/mm²) to 29 or 30 weeks postconception (59 ± 15 cells/mm²) (p < 0.001). This increase suggests that GABAergic cells are migrating through the subplate at this time and are thus in a location potentially affected by perinatal brain damage.

A severe loss of GAD-67+ cells was found in the white matter and subplate from infants with WMLs (Fig. 2). In age-matched samples, a fourfold reduction in GAD-67+ cells was seen in damaged white matter compared with normal white matter. In the subplate GAD-67+ cell loss was more pronounced in relatively older infants (postconceptional age 28–38 weeks) than in younger infants (< 28 weeks). Markedly more apoptosis is present in WMLs and the overlying subplate and cortex in age-matched specimens (white matter [wm] > 28 weeks, p = 0.018; cortex ≤ 28 weeks, p = 0.03; all others p < 0.001).
the subplate and white matter followed perinatal brain injury with WMLs, suggesting that migrating GABAergic neurons bound for the cortex are damaged in neonates with WMLs.

Some O4+ proligodendrocytes have been reported to express GABA. To determine whether the loss of GAD-67 labeling found in infants with damaged white matter was primarily due to a loss of GAD-67+ oligodendrocytes, double-labeling studies were performed using oligodendroglial markers in white matter samples from three neonates. Ap-
proximately 1% of O4+ cells were double-labeled with GAD-67 antibodies. Conversely, approximately 5% of GAD-67+ cells were double-labeled with O4 antibodies. Thus, most of the reduction in GAD-67 expression in the subplate and white matter of infants with perinatal brain damage cannot be attributed solely to oligodendrocyte loss.

To determine whether other major neurotransmitter systems were affected by perinatal brain damage similar to GABA expression, glutamate expression was assayed by performing an immunohistochemical analysis for VGLUT1. The expression of VGLUT1 is found at excitatory synapses and gradually increases in the telencephalon as development progresses.\textsuperscript{15,24} In contrast to GAD-67 expression, VGLUT1 expression was found primarily in the cortex from 25 to 38 weeks postconception and not in the subplate or central white matter. No difference in the cortical expression of VGLUT1 was found between infants with PTL and those without WMLs. Diminished VGLUT1 expression was identified in the two infants with PVL, a finding consistent with the likelihood that these infants suffered more devastating injuries to the telencephalon (data not shown). From these initial results we can infer that glutamate expression in the telencephalon is relatively less vulnerable than GABA expression to the perinatal brain damage that produces PTL. Further studies are necessary to clarify whether the apparent discrepancy between neurotransmitter expression is due to the location and timing of development of different neuronal populations, the relative resilience of neuronal populations to injury, or other factors.

The GABAergic Neuronal Subtypes are Differentially Affected by Perinatal Brain Injury

The mature cerebral cortex contains three major nonoverlapping GABAergic interneuron subtypes: 1) those containing the calcium-binding proteins, parvalbumin or calretinin; 2) those containing the calcium-binding protein caretinin; and 3) those containing the neuropeptide somatostatin\textsuperscript{26} (Fig. 3). Parvalbumin, present in approximately 40% of cortical GABAergic neurons, is first observed in the white matter and cortex at approximately 38 weeks of gestation in humans.\textsuperscript{27} Similarly, calretinin, another calcium-binding protein, is not expressed until later in the developmental process.\textsuperscript{29} In our cases, neither parvalbumin nor calbindin was identified in the white matter, subplate, or cortex. Instead PVA+ and calbindin+ neurons were present in a few sections removed from older infants, including the outer edge of the basal ganglia, which provides an internal positive control confirming that the lack of PVA and calbindin immunolabeling in the white matter and cortex was not due to an ineffective immunolabeling technique.

Calretinin-containing neurons, an interneuron subset distinct from both the PVA and somatostatin neuronal subsets, comprises approximately 20% of cortical interneurons and is initially expressed in the telencephalon before 25 weeks of gestation.\textsuperscript{26} Somatostatin-containing neurons constitute most of the remaining 40% of cortical interneurons. Neuropeptide Y demonstrates significant overlap with somatostatin expression. Lack of NPY function has been implicated as a predisposing factor for seizures in studies of human and rodent epilepsy.\textsuperscript{109} In the current study, NPY immunolabeling was present in the white matter and cortex by 26 weeks of gestation, a finding consistent with previously published data.\textsuperscript{22} We therefore evaluated immunolabeling for calretinin and NPY in the telencephalon to determine how perinatal brain injury affects GABAergic neuronal subtypes.

In infants without WMLs, calretinin+ cell density decreased in the central white matter and subplate as development progressed, whereas cortical density remained relatively stable throughout the third trimester (Fig. 2). In infants 28 weeks of gestation or younger who had WMLs, the density of calretinin+ cells was decreased in the central white matter (101 ± 36 cells/mm\textsuperscript{2}) compared with infants without WMLs (185 ± 64 cells/mm\textsuperscript{2}) (p < 0.002), but no differences in these cell numbers were found in the subplate and cortex. Interestingly, calretinin+ cells were more susceptible to insults in older infants with WMLs, that is, those past 28 weeks of gestation, with a significantly decreased density of these cells within the central white matter, subplate, and midcortical layers (p < 0.0003, p < 0.002, and p < 0.0007, respectively), compared with age-matched infants without WMLs. Thus, the loss of calretinin+ cells contributes to the loss of GABAergic neuron expression after perinatal brain damage with WML.

The pattern and location of NPY+ neurons change rapidly throughout the third trimester, requiring that comparisons between samples with and without WMLs be made at specific gestational ages. The density of NPY+ neurons was not affected by perinatal damage at 26, 28, or 30 weeks of gestation, but the morphological characteristics of the neurons differed in age-matched specimens. To quantify these morphological changes, the shape of the soma, the number of soma dendrites, and the length of the longest neurite for each cell were measured in 50 randomly selected NPY+ neurons from the subplate of each infant. Alterations in the shape of the soma and the average number of dendrites per cell body did not demonstrate a clear pattern as development progressed from 26 to 30 weeks, and did not distinguish which infants had WMLs and which did not. In the control infant specimens, the average length of the leading
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The GABA receptors are slow-acting metabotropic receptors that signal via the Gₐα guanosine triphosphate-binding proteins. The GABAₐR1 subunit is developmentally regulated and found on neurons undergoing tangential migration. In infant brains without signs of WMLs, cells with detectable GABAₐR1 receptor expression were not apparent until 26 weeks of gestation. Although cells expressing GABAₐR1 receptors were only found in the subplate and not the white matter, cells with GABAₐR1 receptor expression were present primarily in the central white matter. Rare GABAₐR1 receptor+ cells were found in the subplate. In contrast to the decrease of cells in the subplate in which GABAₐR1 receptor expression was observed with maturation during the third trimester, more cells with GABAₐR1 receptor expression were present in the central white matter at 29 or 30 weeks (422 ± 103 cells/mm²) than at 26 or 27 weeks postconception (216 ± 46 cells/mm²). The density of cells with GABAₐR1 receptor expression also increased slightly in the cortex along with maturation in healthy infants. A marked loss of cells with GABAₐR1 receptor expression was observed after perinatal brain injury with WMLs in age-matched specimens. At 28 weeks or fewer of gestation, the density of cells with GABAₐR1 receptor expression in the white matter of infants with WMLs was fourfold lower (50 ± 28 cells/mm²) than it was in infants without WMLs (216 ± 46 cells/mm²) (p < 0.002). Similarly, in relatively older infants with WMLs (postconceptional age > 28 weeks), the GABAₐR1 receptor+ cell density was 40% less than that in controls (p < 0.001). A fourfold loss of cells with GABAₐR1 receptor expression in the cortex was seen throughout the third trimester in infants with WMLs compared with controls (Fig. 4). Double-labeling studies did not detect a significant overlap between O4+ oligodendrocytes and GABAₐR1 receptor expression. The loss of cells exhibiting GABAₐR1 or GABAₐR1 receptor subunit expression during the third trimester occurs after perinatal brain injury with WMLs.

To clarify whether the loss of GABA receptor expression in the telencephalon of infants with WMLs is representative of widespread neuronal loss in the subplate, or whether only GABAergic synapses are particularly vulnerable to perinatal brain injury, NMDA NR1 receptor expression was assayed. The NMDA NR1 receptors are present at excitatory synapses on both interneurons and excitatory neurons in all layers of the cortex. In this study cells with NMDA R1 expression were found in infants without WMLs in the upper subplate and cortex at 25 weeks postconception. By 28 weeks postconception, NMDA NR1 expression was distributed through the central white matter, subplate, and cortex (data not shown). Similar to GABAₐR1 receptors, a significant loss of expression of NMDA NR1 receptors was seen in the subplate in age-matched infants with WMLs (124 ± 23 cells/mm²) compared with infants without WMLs (90 ± 56 cells/mm²; p < 0.022). The approximately 30% decrease in NMDA NR1 receptor expression in the subplate in infants with WMLs was less dramatic than the fourfold loss in GABAₐR1 receptor expression. The density of NMDA NR1 labeling in the cortex did not differ between infants with or without WMLs. Together these receptor data suggest that the loss of GABA receptor expression in the subplate and white matter is representative of the loss of subcortical neurons in the telencephalon associated with WMLs that occurs during the developmental process.

Discussion

Significance of Perinatal Brain Damage With WMLs

The proportion of infants born prematurely in the US
continues to rise. Although preterm babies more frequently survive because of improvements in obstetrics and neonatology, there has been no decline in neurological morbidity. Among babies born with extremely low birth weights (<1000 g), normal neonatal ultrasonography findings, and no signs of WMLs, nearly one in ten will later experience cerebral palsy and at least a quarter will suffer cognitive delays, with a Bayley Mental Developmental Index lower than 70.

Understanding the pathogenesis of neurological morbidity as a prelude to the development of effective therapeutics is an urgent priority. On the basis of findings of previous pathological studies, we can infer that neuron development alters after perinatal brain injury. More recent studies in which high-quality magnetic resonance images have been obtained have shown that preterm birth induces a loss of cortical gray matter, and that this loss increases in infants who have WMLs. A better understanding of how perinatal brain injury with WMLs affects cortical development in humans is necessary to direct the development of effective interventions.

Oligodendrocyte Loss and Axonal Damage in WMLs

In this study, standard histopathological criteria were used to identify infants who had suffered perinatal brain injury resulting in WMLs. A consecutive series of telencephalon specimens from neonates born between 25 and 32 weeks of gestation was divided into those specimens containing WMLs and those not containing WMLs; histological examinations were performed to clarify the early impact of WMLs on cortical development. Fewer oligodendrocytes were found in age- and anatomically matched areas of the telencephalon from infants with WMLs than in infants without WMLs. These data are consistent with findings of other pathological studies of WMLs, and suggest that insults that produce human WMLs have a reproducible effect on the development of cells in the oligodendrocyte lineage within the white matter. Axon labeling in neonates without WMLs in this study was similar to that found in a recent study of axon development in normal infants. Axon disruption was present in both the white matter and cortex in...
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infants who had suffered perinatal brain damage, also similar to abnormalities found in earlier studies. Axon damage suggests that neuronal function may be affected by perinatal white matter damage.

Programmed Cell Death

As part of normal human development, excess neural cells arise and are selectively removed by programmed cell death (apoptosis). Previous human pathological studies have been performed to examine apoptosis in the cingulate gyrus of nonasphyxiated infants born at term, and in those with pontosubcortical neuronal necrosis; there have been no studies in which apoptosis associated with PTL or PVL has been evaluated in preterm infants. In the current study significantly more activated caspase-3 labeling was present in neonates with WMLs than in control samples, a finding indicative of excess apoptosis in the white matter, subplate, and cortex of these affected infants. A rodent model of pre- natal systemic hypoxia–ischemia and a piglet model of transient neonatal global hypoxia–ischemia have yielded similar results, with elevated apoptosis persisting well after the initial insult. These findings suggest that a potential window for intervention exists to replace neural cells lost through excess apoptosis, in the hopes of ameliorating or reversing the damage in infants with WMLs.

Loss of Expression of GABAergic Neuronal Marker in Infants With WMLs

It is likely that multiple neuronal lineages are affected by the perinatal brain injury that produces WMLs. We focused on GABAergic neurons migrating through the subplate to the cortex as a representative telencephalon population vulnerable to injury during the third trimester. In the current study, cells that displayed a positive reaction for the GABAergic neuronal marker GAD-67 were present in the white matter and subplate by 25 weeks of gestation in neonates without WMLs. We found a significant loss of GAD-67 expression in the white matter and subplate of neonates with WMLs compared with those without WMLs. Although few, if any, other studies have focused on the effect of prenatal insults on cortical development in humans, results of animal studies have demonstrated the detrimental effects of prenatal loss of GABAergic neurons on cortical development.

We found no difference in cortical glutamate neurons defined by VGLUT1 expression between infants with and without PTL. Further studies are necessary to clarify how perinatal brain damage affects the development of other neurotransmitter systems in the telencephalon.

Alterations in Expression Patterns for GABAergic Neuronal Subtypes

In only one previous study has expression of the GABAergic pathway been evaluated in human infants with WMLs. In older infants and children, Iai and colleagues found decreased parvalbumin+ neuronal density in 10 of 11 patients with severe PVL. Similar to our results, they also found that parvalbumin is not expressed in the human cortex and white matter until 38 weeks of gestation. Others have found rare parvalbumin+ neurons in the human occipital cortex after 26 weeks or 32 weeks. These authors may have detected parvalbumin+ neurons earlier because of regional differences in cortical development. Although calretinin and NPY expression were present during the developmental timeframe in this study, few investigators have examined calretinin expression in adult human brains or during the normal developmental process, and none has examined human calretinin expression as a function of perinatal brain damage. In the present study we demonstrated decreased calretinin expression in infants with perinatal brain damage.

In humans, NPY+ neurons migrate through the subplate during the developmental process and regress after birth along with the subplate; this indicates that they play a significant developmental role. The loss of NPY+ neurons has been associated with epilepsy, but NPY expression has not previously been examined after perinatal brain damage. In this study, the average length of the longest neurite was significantly less in infants with perinatal brain injury. The defective differentiation of NPY+ neurons and their synapses from other neurons may underlie the predisposition to epilepsy found in many premature infants. Given the role of GABAergic neuronal subtypes in the normal developmental process, the loss of expression of calretinin+ cells and alterations in NPY+ neurons observed in the current study suggest that alterations in GABAergic subtypes may contribute to impaired cortical function in children with WMLs.

Alterations in the Expression of the GABAergic Receptor Subtype

The expression of the GABA\(_\alpha\)1 receptor is developmentally regulated. Decreased expression has been found after systemic hypoxia in chicks. No previous studies of GABA\(_\alpha\)1 receptor expression in human neonates exist. A significant loss of GABA\(_\alpha\)1 receptor expression was found in premature infants after perinatal brain damage with WMLs. This loss of expression may reflect a loss of neurons, or decreased or delayed expression of this developmentally regulated subunit. The GABA\(_\alpha\) receptors are developmentally regulated and influence tangential migration; GABA\(_\alpha\) receptor inhibition impairs neuronal migration. The genetically induced loss of GABA\(_\alpha\)R1 receptor function predisposes mice to epilepsy and cognitive impairment; this is similar to outcomes found in children after perinatal brain damage associated with WMLs. A loss of cells with GABA\(_\alpha\)R1 receptor expression occurred in neonates who suffered perinatal brain damage with WMLs. The NMDA NR1 receptors are found at excitatory synapses throughout the cortex on both inhibitory interneurons and excitatory neurons in the mature central nervous system. There have been no prior studies in which NMDA NR1 receptor expression has been evaluated in human neonates. The loss of NMDA NR1 receptor expression was seen in the subplate, but this was much less pronounced than the loss of GABA receptor expression. No difference in NMDA NR1 expression was apparent in the cortex between infants with and without WMLs. Our results suggest that subplate receptor expression for both GABA and glutamate is adversely affected by perinatal brain damage with WMLs, and that affected synapses are likely to contribute to developmental alterations found in the cortex in preterm infants.

White Matter Lesions Affect Multiple Components of Neural Development

Perinatal brain damage resulting in WMLs is strongly as-
associated with cerebral palsy. Outcome studies of premature infants have repeatedly demonstrated that these children are also prone to chronic neurological deficits related to cortical function including cognitive delay, epilepsy, behavioral problems, and visual and auditory impairments. Previous studies of WMLs have focused on oligodendroglia. Pro-oligodendrocytes are particularly vulnerable to perinatal insults due to the timing and location of their development, as well as to their relatively impaired intrinsic mechanisms to tolerate oxidative stress. Axons coursing through the developing white matter are also prone to injury. Disruption of a third component of the developing telencephalon, subplate neurons, has been implicated in producing clinical symptoms associated with perinatal brain damage. Subplate neurons arise before formation of the cortical plate and subserve multiple functions in cortical development. A model of early (postnatal Day 2) hypoxia–ischemia in rats has documented the loss of subplate neurons.

In the current study, we have demonstrated involvement of a fourth component of the developing telencephalon in perinatal brain damage with WMLs: migrating GABAergic neurons. The GABAergic neurons that migrate tangentially through the white matter and subplate during the third trimester are in a region prone to damage in babies with WMLs. Because GABAergic neurons and GABA receptors profoundly influence the development and maturation of cortical synapses, the loss of these neurons may contribute to cortically based deficits observed in children with WMLs. The loss of GABAergic neurons may precipitate a cascade that also affects other neuronal populations including the glutamatergic, cholinergic, and serotonergic systems. In the future, investigators will examine the development of these systems in the human neonatal brain with WMLs. Interventions minimizing damage from WMLs need to be evaluated for their ability to influence the various neuronal and glial systems affected by perinatal insults. Complementary integrated strategies to address differential requirements of various neural components may be needed to effectively rectify disrupted neurodevelopment and, thereby, minimize neurological deficits in children with brain damage consequent to preterm birth.

Conclusions

The loss of GABAergic neurons in the human white matter, subplate and cortex after perinatal brain damage with WMLs is reported. Together, the loss of neurotransmitters (GABA and calretinin), impairment of NPY neurite growth, and loss of cells with GABAergic receptor expression suggest that GABAergic neurons are vulnerable to perinatal brain damage associated with WMLs. Future studies will address other neurotransmitter systems in greater depth. The combined insights from human and rodent studies will improve our understanding of perinatal brain damage and facilitate the development of effective interventions.

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Address reprint requests to: Shenandoah Robinson, M.D., Pediatric Neurosurgery, B501, Rainbow Babies and Children’s Hospital, 11100 Euclid Avenue, Cleveland, Ohio 44106, email: shenandoah.robinson@uhhs.com.