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Neuron Deficit in the White Matter and Subplate in Periventricular Leukomalacia

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Abstract

Objective—The cellular basis of cognitive abnormalities in preterm infants with periventricular leukomalacia (PVL) is uncertain. One important possibility is that damage to white matter and subplate neurons which are critical to the formation of the cerebral cortex occurs in conjunction with oligodendrocyte and axonal injury in PVL. We tested the hypothesis that the overall density of neurons in the white matter and subplate region is significantly lower in PVL cases compared to non-PVL controls.

Methods—We used a computer-based method for the determination of the density of MAP2-immunolabeled neurons in the ventricular/subventricular region, periventricular white matter, central white matter, and subplate region in PVL cases and controls.

Results—There were five subtypes of subcortical neurons: granular, unipolar, bipolar, inverted pyramidal, and multipolar. The neuronal density of the granular neurons in each of the four regions was 54–80% lower ($p < 0.01$) in the PVL cases ($n=15$) compared to controls adjusted for age and postmortem interval ($n=10$). The overall densities of unipolar, bipolar, multipolar, and inverted pyramidal neurons did not differ significantly between the PVL cases and controls. No granular neurons expressed markers of neuronal and glial immaturity (Tuj1, doublecortin, or NG1).

Interpretation—These data suggest that quantitative deficits in susceptible granular neurons occur in the white matter distant from periventricular foci, including the subplate region, in PVL, and may contribute to abnormal cortical formation and cognitive dysfunction in preterm survivors.

Keywords

GABAergic neurons; doublecortin; hypoxia-ischemia; NG2; microtubule-associated protein 2; subplate

Introduction

Of the 63,000 very low birth weight infants born each year in the United States, 25–50% develop cognitive deficits^{1,2}. Periventricular leukomalacia (PVL) is the major substrate of such deficits in preterm survivors³. It is defined as periventricular foci of necrosis surrounded by diffuse astrogliosis and microglial activation in the adjacent cerebral white matter³. Cerebral ischemia/reperfusion compounded in certain instances by infection/inflammation is thought to be its major cause^{1,3}. During the peak time-frame of PVL, i.e., 24–34 weeks, neurons underlying the cerebral cortex are comprised of subplate neurons directly beneath Layer VI^{4–9} and late-migrating γ -aminobutyric acid (GABAergic) neurons in the white matter^{10–14}. During early development, subplate neurons play a critical role in the establishment of connections between the cortex and thalamus and cortical lamination^{4–9}. A subset of these subplate neurons persists into adulthood to modulate mature cortical processing^{8,9,15,16}. GABAergic interneurons in the cerebral cortex play a key role in cortical output and synaptic plasticity^{12,15–18}; the timing of GABAergic neuronal migrations through the white matter continues into the second half of gestation^{11,13}. In this report, we focused our attention upon the understudied neurons in the cerebral white matter and subplate region in human PVL because these neurons play a major role in the formation of the cerebral cortex, the key site involved in cognition, and subplate neuronal loss has been reported in a neonatal model of cerebral ischemia in rats⁴.

In this study, we refer to the subplate and white matter neurons collectively as “subcortical neurons”, recognizing that they have been variously labeled as subcortical, subplate, interstitial, extra-cortical, or outlying neurons^{15,16,18}. As a first step, we tested the hypothesis that the overall subcortical neuronal density (inclusive of all cellular phenotypes) is reduced in PVL cases compared to non-PVL controls adjusted for postconceptional age. We analyzed tissue sections with computer-based methods that spanned the complete distance from the ventricle to the pia on standard microscopic slides, thereby allowing assessment of the ventricular/subventricular region, periventricular and central white matter, and subplate region in one tissue section. We utilized the microtubule-associated protein 2 (MAP2), a cytoskeleton phosphoprotein marker of the neuronal phenotype¹⁸, to facilitate identification of all subcortical neurons. Because the dominate morphologic subtype of the MAP2-immunopositive neurons in the developing human cerebral white matter was granular in this study, and was also the main subtype affected in PVL, we characterized the granular phenotype in greater depth using double-labeling immunocytochemistry with immature neuronal and glial markers, i.e., NG2, a marker of predominantly glial lineage¹⁹, Tuj1, a marker of immature neurons²⁰, and doublecortin (DCX), a marker of postmitotic migrating neurons^{21–23}.

Material and Methods

Clinicopathologic Database

Tissue sections with cerebral cortex, underlying white matter, and ventricular zone were obtained from 25 cases and controls autopsied in the Departments of Pathology, Children’s Hospital Boston, MA, and Brigham and Women’s Hospital, Boston, MA, over the last 15 years, i.e., the modern era of neonatal intensive care. Parental permission for research was given according to the guidelines of the hospitals’ Institutional Review Boards. Periventricular leukomalacia was defined as: 1) necrotic foci in the periventricular and/or central white matter; and 2) diffuse astrogliosis and microglial activation in the surrounding white matter; controls did not demonstrate these white matter abnormalities, as in previous studies from our laboratory^{3,24,25}. Cases with known disorders of cortical maldevelopment and/or neuronal migration disturbances were excluded from analysis. The tissue was fixed in 4% paraformaldehyde, paraffin-embedded, and cut on a conventional microtome at 4 μ m.

Age was expressed as postconceptional age (PCA) in weeks (gestational age plus postnatal age). Blinded cell counts were not possible because of the qualitative features of PVL microscopically and the placement of the grids by design over the periventricular necrotic foci. Autopsy reports were reviewed for major clinical, systemic autopsy, and neuropathologic findings.

Immunocytochemistry with Antibodies to MAP2

We identified subcortical neurons in tissue sections with antibodies to MAP2. The antibody to MAP2 (dilution: 1:2000; Santa Cruz Biotechnology, Inc, sc-20172; Santa Cruz, Ca) was a rabbit polyclonal antibody raised against amino acids 1-300 at the N-terminus of MAP2 of human origin. Tissue sections were de-waxed and rehydrated through an ethanol gradient. Antigen retrieval was performed by boiling in citrate buffer (pH 6.0) in a microwave for 15 minutes. Slides were cooled for 30 minutes and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 minutes. Nonspecific immunostaining was reduced by applying a blocking solution (Phosphate buffered saline [PBS] 4% Normal Goat Serum [NGS]/0.1% Triton X-100) for 30 minutes. Sections were then incubated overnight at 4°C with primary antibodies diluted in PBS/NGS/Triton X-100 buffer. Antibody binding was detected using ABC kits and immunostaining visualized using 3, 3'-diaminobenzidine chromogen. Negative controls were determined with omission of the primary antibody.

Double-label Immunocytochemistry of MAP2 Granular Neurons

Phenotypic characterization of the MAP2 granular neurons was made with double label immunocytochemistry in a subset of the control group. For the NG2 and Tuj1 antibodies, assessments were made in tissues from 6–10 controls ranging in age from 19 to 41 gestational weeks. For the DCX antibody, they were made in tissues from 7 controls ranging from 35 to 42 postconceptional weeks²⁶. For double-labeling of MAP2 neurons with Tuj1, paraffin-embedded, paraformaldehyde-fixed tissue sections were incubated with rabbit polyclonal MAP2 (1:1000; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and monoclonal Tuj1 (1:10000, Covance, Emeryville, CA). Fluorescence was detected with Alexa Fluor goat anti-rabbit 594 and Alexa Fluor goat anti-mouse 488 (1:1000; Molecular Probes, Eugene, OR). For double-labeling of MAP2 neurons with DCX, sections were incubated with mouse monoclonal MAP2 (SMI 52, 1:800; Covance, Emeryville, CA) and rabbit polyclonal DCX (1:200, Abcam; Cambridge, MA). Fluorescence was detected with Alexa Fluor goat anti-rabbit 488 and Alexa Fluor goat anti-mouse 594 (1:1000; Molecular Probes, Eugene, OR). For double-labeling of MAP2 neurons with NG2, frozen tissue from the parieto-occipital lobe at the level of the atrium of the lateral ventricle (i.e., the standard frozen block in the laboratory's brain tissue bank) was sectioned at 30 µm, post-fixed for 20 minutes in 4% paraformaldehyde, and washed in PBS. The sections were then incubated with rabbit polyclonal MAP2 (1:1000; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and mouse monoclonal NG2 (1:400; Novus, Littleton, Co). Fluorescence was detected with Alexa Fluor goat anti-rabbit 594 and Alexa Fluor goat anti-mouse 488 (1:1000; Molecular Probes, Eugene, OR).

Determination of the Density of MAP2 Neurons

The density of subcortical neurons immunostained for MAP2 was determined from the ventricular surface to the border with cortical Layer VI in PVL cases and controls using computer-based methods with Neurolucida version 6.02.2 (Microbrightfield Inc, Williston, VT) (Fig. 1). Neuronal density in the ventricular/subventricular region, and periventricular and central white matter was determined by overlaying a standard grid across these regions in which each of three boxes delineated the white matter zone, i.e., Box 1—ventricular/subventricular zone; Box 2—periventricular white matter; and Box 3—central white matter (Fig. 1). The grid was comprised of 3 squares (each 2 × 2 mm²) which were placed over a

single tissue section oriented from the ventricle to the pia, and in the PVL cases, contained focal periventricular necrosis, as previously demonstrated by us²⁴. In both the PVL cases and controls, Box 1 abutted the ventricular surface and overlay the subventricular zone; Box 2 overlay the periventricular white matter, and Box 3 overlay the central white matter (Fig. 1). These subdivisions of the white matter correspond to sites routinely evaluated in diagnostic neuropathology. To assess the neuronal density directly beneath the cerebral cortex, i.e., in the subplate region and distant from any focally necrotic lesions, the white matter was outlined using Neurolucida at a uniform thickness of 750 microns (Fig. 1). Neuronal density was calculated by dividing the number of cells in the white matter grid or subplate region by the area of the box or subplate region, respectively, and expressed as neurons/mm².

Statistical analysis

We compared the distributions of the clinical variables between PVL cases and controls with the exact test for categorical variables and Wilcoxon rank sum test for continuous variables (Table 1). Analysis of covariance (ANCOVA) was used to examine the relationship of the continuous measures of subcortical neuronal density with case/control status after adjusting for postconceptional age and postmortem interval. Using ANCOVA, least square means (means values adjusted for postconceptional age and postmortem interval) for PVL cases and controls were compared. Cross-sectional age-related (developmental) changes in subcortical neuronal density of the controls were estimated using linear regression. A p -value<0.05 was considered significant. The immunocytochemical examination of the phenotype of the granule neurons involved light microscopic observations in tissue sections.

Results

Clinicopathologic Database

We analyzed subcortical neuronal density in the cerebral white matter and subplate in 15 PVL cases (34.7±4.6 postconceptional weeks) and 10 controls (31.6±6.6 postconceptional weeks) ($p=0.174$) (Table 1). The mean gestational age was 32.8±4.1 weeks in the PVL group and 30.1±5.9 weeks in the control group ($p=0.189$); the majority of PVL cases (80%, 12/15) and controls (90%, 9/10) were born prematurely (<37 gestational weeks) (Table 1). The primary causes of death in the PVL cases were: respiratory distress syndrome ($n=7$); congenital heart disease ($n=3$); primary skeletal disorders ($n=2$); congenital diaphragmatic hernia ($n=1$); inborn error of metabolism ($n=1$); and malformation of the great vein of Galen ($n=1$). The primary causes of death in the controls were: respiratory distress syndrome ($n=5$); congenital heart disease ($n=1$); hydrops fetalis due to placental chorioangiomas ($n=1$); hydrops fetalis due to parvovirus ($n=1$); primary pulmonary hypertension ($n=1$); and bronchiolitis ($n=1$). All of the PVL cases (100%, 15/15) and 90% (9/10) of the control cases required mechanical ventilation at some point in their hospital course ($p=0.417$). They underwent cardiopulmonary resuscitation with dopamine, epinephrine, and/or atropine. The standard of care for the PVL cases and controls receiving mechanical ventilation included fentanyl for sedation, and for the premature infants antenatal steroids for the induction of surfactant. There was no significant difference in the incidence of respiratory distress syndrome, congenital heart disease, necrotizing enterocolitis, or sepsis between the two groups (Table 1). The two PVL cases with seizures received anti-seizure medications, including phenobarbital. The median postmortem interval was not significantly different for the PVL cases and controls (14.0 versus 16.5 hours), respectively ($p=0.460$) (Table 1).

Developmental Profile of Subcortical Neurons in the Control Group

MAP2 immunostaining—We analyzed the spatial and temporal sequence of the subcortical neurons in the controls ($n=10$) to provide insight into normal development. The

postconceptional ages ranged from 23 to 43 weeks, i.e., the last half of gestation into the neonatal period. With MAP2 immunostaining, we identified five morphological subtypes of subcortical neurons in the white matter and subplate region. These subtypes were: 1) small, granular neurons with round nuclei, scant cytoplasm and no dendritic processes; 2) unipolar neurons with scant cytoplasm and one trailing process; 3) bipolar neurons with two processes aligned opposite to each other; 4) multipolar neurons with abundant cytoplasm and more than three dendritic processes; and 5) inverted pyramidal neurons with a triangular shape and orientation of the apical dendrite away from the cortex (Fig. 2). These five subtypes were differentially distributed throughout the regions sampled (Table 2), and their cytological features did not change over the time-frame analyzed. The most prevalent subtype of neurons over the entire time-frame analyzed was the granular neuron (Fig. 3). In all controls combined across ages, the granular neurons accounted for 64.8% of the total neuronal population in Boxes 1, 2, and 3 combined (Fig. 3), with similar results in the subplate region (data not shown). The unipolar neurons accounted for 30.8%; bipolar, 2.5%; inverted pyramidal, 1.6%, and multipolar, 0.2% (Fig. 3). The highest overall density of total neurons was in the subplate region (13.36 ± 2.41 cells/mm²) (Table 2). The highest cell density of the granular neurons was also in the subplate region (6.96 ± 0.94 cells/mm²); this density was approximately 2–7 times greater than that in the subventricular region (Box 1) and periventricular and central white matter (Boxes 2 and 3) (Table 2). While the granular, unipolar, and bipolar neurons were found throughout the white matter and in the subventricular region, the multipolar neurons were found only in the central white matter (Box 3) and subplate region, and the inverted pyramidal neurons, in the periventricular and central white matter (Boxes 2 and 3, respectively) and subplate region (Table 2).

Phenotypic characteristics of the MAP2-immunopositive granular neuron—

Labeling with NG2 and Tuj1 showed rare immunopositive cells in control cases with no colocalization with Map2 (data not shown). While DCX immunopositive cells were noted in control tissue, there was little colocalization of DCX with MAP2 (Fig 4).

Subcortical Neurons in PVL

There was a marginal increase in the brain weight adjusted by postconceptional age in the PVL cases (269 ± 89 grams) compared to controls (194 ± 120 grams) ($p=0.094$). The severity of white matter damage in the PVL cases varied in the degree of severity from grossly cystic to microscopic only. Eight of the 15 cases (53%) demonstrated macroscopic periventricular necrotic foci that were either cystic cavities measuring up to 2.2 cm in diameter or chalky-white foci, so-called “white spots”, measuring 2–4 mm in diameter. In the remaining 47% (7/15) of the PVL cases, the necrotic foci were microscopic, measuring less than 2 mm in diameter (Fig. 5). Among the PVL cases, the periventricular necrotic foci were located in the parietal lobe in 47% (7/15) of the cases, 40% (6/15) in the frontal lobe, and 13% (2/15) in the occipital lobe; none were located in the temporal lobe. The necrotic foci were present in Box 1 in 2/15 (13%) of PVL cases, Box 2 in 2/15 (13%), Box 3 in 1/15 (7%), Boxes 1 and 2 in 3/15 (20%), Boxes 2 and 3 in 2/15 (13%), and Boxes 1, 2, and 3 in 4/15 (27%). The subplate region was distant from these necrotic foci and did not contain necrotic foci in any PVL case. The density of all neuronal subtypes combined was significantly lower by 76–78% in the PVL cases ($n=15$, 34 ± 7 postconceptional weeks) compared to controls ($n=10$, 31.6 ± 6.6 postconceptional weeks) in the ventricular region (Box 1) ($p=0.025$), periventricular white matter (Box 2) ($p=0.009$), and central white matter (Box 3) ($p<0.001$) (Table 2). In addition, the total neuronal density tended to be lower in the PVL cases compared to controls (38%) in the subplate region ($p=0.123$) (Table 2). The change in total neuronal density was due mainly to a 54–80% reduction ($p<0.01$) in the granular density in all four regions sampled in the PVL cases compared to controls (Table 2). This change is exemplified in the central white matter (Box 3): PVL 0.51 ± 0.31 versus controls 3.02 ± 0.37

neurons/mm² ($p < 0.001$) (Table 2) (Fig. 6). The density of unipolar neurons was significantly lower in the PVL cases compared to controls in the central white matter region (Box 3) only (Table 2). The mean densities of bipolar, multipolar, and inverted pyramidal neurons, on the other hand, were not significantly different between the two groups in any region analyzed (Table 2). There was no significant difference in the proportion of each subtype between the PVL and control groups (data not shown). There was also no significant difference in the density of any neuronal subtype between the PVL lesions identified grossly at autopsy ($n=8$) compared to those in which the PVL lesions were detected only microscopically ($n=7$) (Table 3). There were no substantial differences in the overall neuron density or granular neuron density in the fronto-parieto-occipital regions sampled in either the PVL or control group (data not shown).

There was a marginally significant difference between PVL cases and controls in the incidence of hemorrhages in the ganglionic eminence which occurred in 33% (5/15) of the PVL cases compared to 0% (0/10) in the controls ($p=0.061$). Within the PVL group, however, there was no difference in the overall density of granular neurons in the subventricular region, periventricular or central white matter, or subplate region between cases with germinal matrix hemorrhages ($n=5$) compared to those without hemorrhages ($n=10$) (data not shown).

Discussion

The major finding of this study is a significant deficit of granular neurons in the ventricular/subventricular region, periventricular and central white matter, and subplate region in PVL. While the basis of the cognitive deficits in preterm survivors is certainly multifactorial and involves diverse neuronal/axonal/myelin abnormalities^{1, 3, 24, 25, 27, 28}, the deficit in granular subcortical neurons demonstrated here likely contributes due to their important roles in cortical development. Granular neurons embedded in the damaged white matter and subplate region in PVL are likely vulnerable to hypoxia-ischemia via the same mechanisms of toxicity to pre-myelinating oligodendrocytes, including free radical, glutamate, and cytokine injury^{1, 3, 24, 25, 27, 28}. A role of drug toxicity in the induction of neuronal cell death is likewise a consideration, given that certain drugs are known to experimentally trigger apoptosis, e.g., antenatal steroids for the induction of surfactant²⁹, fentanyl for sedation during mechanical ventilation³⁰ and anti-seizure medications³¹. Yet, both the PVL cases and controls were exposed to these agents, and only 2 of the 15 PVL cases were exposed to anti-seizure drugs, suggesting that they do not account, at least substantially, for the neuronal deficit in the PVL cases. Of note, subplate neurons in the human fetal brain express glutamate receptors which heighten risk for excitotoxicity³². Our observation that the density of subcortical neurons is low in regions with focally necrotic lesions is not unexpected as these lesions involve destruction of all cellular elements, representing core infarcts³. The deficit in the granular neurons distant from the focally necrotic lesions, i.e., in the subplate region, on the other hand, is of major interest since distant sites are in the penumbra of the core infarct². We also did not find an overall deficit in the unipolar, bipolar, multipolar, or inverted pyramidal subcortical neurons in PVL. Thus, the preferential damage to granular neurons, including distant from the necrotic foci, suggests that this particular subtype is exquisitely sensitive to hypoxia-ischemia.

The question arises, what is the molecular/neurotransmitter phenotype of the granular neurons in the white matter and subplate region in the second half of human gestation, i.e., the peak period of risk for PVL? In a separate report, we found that granular neurons in the subventricular/ventricular zone, white matter, and subplate region in early human life coexpress GAD67/65, consistent with a GABAergic phenotype of at least a subset of these neurons³³. Robinson et al reported a reduction in the density of GAD67-immunopositive

neurons and neurons expressing the GABA_A1 receptor in human perinatal white matter lesions (with and without focal necrosis) in 10 affected infants compared to 5 controls¹³, but the morphological phenotype of these GABAergic neurons is unclear. Approximately one-third of GABAergic neurons are known to arise from the ganglionic eminence to traverse the white matter to their final addresses in the cerebral cortex¹¹. In our study, 33% of the PVL cases had germinal matrix hemorrhages in the ganglionic eminence, raising the possibility that the reduction in neuronal density in the white matter in these PVL cases was accentuated by mechanical damage to the GABAergic neurons originating in this site. There was, however, no significant difference in the neuronal density in the white matter between PVL cases with and without ganglionic hemorrhages.

While MAP2-immunopositive neurons are also unipolar and bipolar in this study, the morphological features of active migration, they do not colocalize with the neuronal migration marker DCX. Nevertheless, these unipolar and bipolar MAP2-immunopositive neurons may express other markers of neuronal migration, e.g., LIS1³⁴ which we have not yet been able to successfully apply to our tissues. Of note, we did not find that the overall density of MAP2-immunopositive unipolar and/or bipolar neurons was reduced in the PVL cases compared to controls in this study. Our observation that MAP2-immunolabeled granular neurons do not express Tuj1, a marker of newly generated postmitotic neurons²⁰ suggest that they have differentiated beyond this early stage. We also considered the possibility that the MAP2-immunopositive granular neurons are NG2 cells, a class of progenitors which generate oligodendrocytes *in vivo* in immature and adult animals¹⁹, as well as in type-2 astrocytes in culture³⁵. Recent genetic fate mapping studies suggest that NG2 cells also give rise to neurons in both the adult piriform cortex^{36,37} and neonatal mouse forebrain³⁸. Our observation that MAP2-immunopositive granular cells do not colocalize with NG2 suggests that this latter possibility is not the case in our tissue, at least in the period of development examined.

In essence, we suggest that the putatively postmitotic MAP2-immunopositive granular neurons in the white matter and subplate region in this study are a relatively mature resident population in the last half of human gestation. In animal models, non-migrating GABAergic and non-GABAergic neurons in the white matter and subplate region are thought to function in local circuits at the stage when thalamocortical axons form transient synaptic contacts prior to innervating the cortical plate⁸. They may also form part of a permanent population that persists in the subplate and white matter into adulthood to influence mature cortical function^{8,9,15}. In a model of perinatal hypoxia-ischemia, subplate neurons identified by birth dating with BrdU undergo cell death⁴. Of note, there is no available immunomarker that specifically labels subplate neurons. Without a specific cellular marker or Golgi data indicating connectivity to the overlying cortex, we cannot be certain that the granular neurons deficient in PVL in this study are an integral part of the subplate proper.

A potential limitation of our study is the nature of the control population. The controls in this study are autopsied infants who were critically ill before death, and thus, not truly representative of normal living infants. We nevertheless found a specific difference between PVL and control brains in the density of granular neurons. A second limitation is that PVL may be a heterogeneous disorder with different mechanisms resulting in white matter damage. Nevertheless, the consensus is that PVL reflects mainly hypoxic-ischemic injury, and multiple disorders of cardiorespiratory function share this injury. Thus, the combination of different disease entities in the PVL group in our study appears reasonable. There also was no obvious correlation of a particular autopsy diagnosis and neuronal deficit in our cohort, although a larger sample size is needed for definitive correlations. A third potential limitation is the possibility that the decreased granular density demonstrated with MAP2 immunostaining reflects a loss of staining intensity, rather than of neurons, as its intensity

can decrease with hypoxia²⁵. In a previous study by us in which MAP2 was used to identify pyramidal neuronal counts for quantitation in the cerebral cortex overlying PVL, however, we did not find a loss of MAP2 immunostaining in the PVL cases²⁵.

In conclusion, this study provides direct evidence in the human brain of injury to a vulnerable subpopulation of neurons in the white matter and subplate region in PVL, i.e., the granular neurons. A critical next step is to determine if there is a specific loss of GABAergic neurons as a component of this population. Additional steps are to determine the other transmitter-specific (non-GABAergic) phenotypes of the granular neurons, and the relative contributions of injury to subplate neurons *per se*, as opposed to late migrating neurons coursing through the subplate region. Finally, the cause(s) of the granule neuronal deficit needs to be determined, including the roles for glutamate, free radical, cytokine, and drug toxicity. This study suggests new avenues for the elucidation of cognitive impairments in preterm survivors.

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Figure 1.

Strategy for the quantitation of subcortical neuronal density in PVL cases and controls, as demonstrated in a control case at 33 postconceptional weeks. A. We used a computer-based method (Neurolucida) for cell counting consisting of a grid over the ventricular/subventricular zone (Box 1), periventricular white matter (Box 2), central white matter (Box 3), and subplate region (defined “ribbon” of tissue directly beneath cortex). B. The corresponding histological section stained with hematoxylin-and-eosin illustrates the regions sampled, including the subplate region which is designated by a dotted line directly beneath the cortex. Abbreviations: CC, corpus callosum; CG, cingulate gyrus; vent, ventricle.

Figure 2.

Five subtypes of subcortical neurons based upon morphological criteria with MAP2 immunostaining at all ages studied, i.e., 23 to 43 postconceptional weeks. These subtypes are: granular (A), unipolar (B), bipolar (C), multipolar (D), and inverted pyramidal (E). All images are at x40. Scale bar = 25 microns

Figure 3.
The mean percentages of each morphological subtype of the total subcortical neuronal population of all controls combined. The main subtype is the granular neuron.

Figure 4.

Double-label immunocytochemistry with MAP2 to characterize the phenotype of the granular neurons. The neuronal migration marker DCX (green) is not expressed by the MAP2 neuron shown (red) and is rarely expressed by MAP2 neurons of any phenotype, including granular. We show the typical, non-granular, appearance of the DCX expressing cell with a small oval soma and a delicate thin trailing process.

Figure 5.

A. Focal necrosis is found in the periventricular region in a representative PVL case at 29 postconceptional week. Microcysts reflective of resolving foci of necrosis are present in the periventricular white matter [x10; scale bar = 200 microns] (B) surrounded by reactive gliosis [x40; scale bar = 25 microns] (C). Reactive astrocytes are indicated with arrows.

Figure 6.

The significantly lower granular density in PVL (white diamonds) exemplified in the central white matter (Box 3) in PVL cases (age- and postmortem interval-adjusted mean, 0.51 ± 0.31 neurons/mm²) compared to controls (black diamonds) (mean, 3.02 ± 0.37 neurons/mm²) ($p < 0.001$).

Table 1

Clinicopathologic information about the PVL cases and controls in the study.

Variable	Control	PVL	p-value*
N [†]	10	15	
Gestational age, weeks	30.1±5.9	32.8±4.1	0.189
Postnatal age, weeks	1.5±3.1	1.9±2.3	0.692
Postconceptional age, weeks	31.6±6.6	34.7±4.6	0.174
Apgar score at 1 min	3.3±2.0	4.1±3.0	0.725
Apgar score at 5 min	7.0±2.1	6.3±2.1	0.559
Male	7/10 (70%)	7/15 (47%)	0.414
Preterm (<37 weeks)	9/10 (90%)	12/15 (80%)	0.627
Congenital heart disease	0/10 (0%)	2/15 (13%)	0.500
Necrotizing enterocolitis	1/10 (10%)	1/15 (7%)	1.000
Sepsis	2/10 (20%)	6/15 (40%)	.0402
Mechanical ventilation	9/10 (90%)	15/15 (100%)	0.417
Seizures any time	0/10 (0%)	2/15 (13%)	0.500
Germinal matrix hemorrhage	0/10 (0%)	5/15 (33%)	0.061
Median postmortem interval, hrs,	16.5	14.0	
Interquartile range	11.0, 28.0	8.0, 24.0	0.460
Brain weight, grams	194±120	269±89	0.094

Mean±standard deviation or counts with percentage (%) unless otherwise noted.

* Exact test p-value for categorical variables; t-test for ages and brain weight, and Wilcoxon rank-sum test for Apgar scores and postmortem interval

[†] Sample sizes for Apgar scores are n=9 for PVL and n=6 for Controls; for mechanical ventilation, n=14 for PVL; for brain weight, n=9 for Controls.

Table 2

Neuronal density (neurons/mm²) in the ventricular/subventricular, periventricular and central white matter, and subplate region in PVL cases compared to controls adjusted for postconceptional age and postmortem interval (mean±standard error).

Site	Adjusted Mean ± Standard Error		p-value
	Control	PVL	
N	10	15	
Total Neurons (all subtypes combined)			
Box 1	2.55±0.62	0.57±0.50	0.025
Box 2	3.04±0.60	0.74±0.49	0.009
Box 3	5.25±0.75	1.08±0.63	<0.001
Subplate	13.46±2.38	8.29±1.93	0.115
Granular Neurons			
Box 1	1.29±0.28	0.26±0.22	0.010
Box 2	1.62±0.33	0.38±0.27	0.010
Box 3	3.02±0.37	0.51±0.31	<0.001
Subplate	6.96±0.94	3.22±0.77	0.007
Unipolar Neurons			
Box 1	1.15±0.47	0.30±0.38	0.183
Box 2	1.23±0.47	0.30±0.38	0.175
Box 3	1.97±0.46	0.38±0.38	0.018
Subplate	5.52±1.29	3.83±1.04	0.331
Bipolar Neurons			
Box 1	0.11±0.01	0.008±0.040	0.126
Box 2	0.17±0.08	0.04±0.07	0.251
Box 3	0.15±0.10	0.14±0.07	0.955
Subplate	0.87±0.23	0.77±0.19	0.745
Multipolar Neurons			
Box 1	Not present	Not present	-
Box 2	Not present	Not present	-
Box 3	0.056±0.035	-0.004±0.03	0.214
Subplate	0.010±0.047	0.063±0.038	0.494
Inverted Pyramidal Neurons			
Box 1	Not present	Not present	-
Box 2	0.025±0.016	0±0.013	0.252
Box 3	0.062±0.041	0.045±0.034	0.759
Subplate	0.26±0.11	0.36±0.09	0.494

Table 3

White matter neuron density (cells/mm²) in PVL group with microscopic periventricular necrotic lesions at autopsy (Micro PVL) compared to PVL group with visually obvious periventricular necrotic lesions (Gross PVL) at autopsy.

Neuronal Population	Micro PVL N=7	Gross PVL N=8	p-value
Total Subcortical	8.5±4.9	8.6±6.1	.877
Granular	3.4±1.4	3.4±3.3	.972
Unipolar	3.8±2.9	3.9±2.6	.907
Bipolar	0.7±0.7	0.9±0.7	.702
Inverted Pyramidal	0.5±0.4	0.3±0.3	.709
Multipolar	0±0	0.1±0.2	.219