Vulnerability of the Mesencephalic Dopaminergic Neurons of the Human Neonate to Prolonged Perinatal Hypoxia: An Immunohistochemical Study of Tyrosine Hydroxylase Expression in Autopsy Material

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INTRODUCTION

The “fetal basis of adult disease” is a recently emerging theory that gives a distinct perspective on possible causes of several neurological and psychiatric diseases that have onsets later in life. The basis of this theory is that early environmental stressors, in utero or perinatally, by interacting with genetic vulnerability, can establish sensitivity to certain diseases through the induction of a permanent “silent toxic” state that is characterized by increased susceptibility and higher risk of cell death. This initiative state, combined with multiple environmental insults occurring from gestation through older age, can eventually lead to the emergence of clinical disease manifestations (1).

Perinatal hypoxic/ischemic injury remains a major cause of mortality and morbidity that can generate permanent neurological and/or mental deficits later in life (2, 3). A range of childhood disabilities has been attributed to perinatal hypoxic ischemia, the strongest evidence being for dyskinetic tetraplegic cerebral palsy (4). An association was reported between perinatal exposures to environmental factors, such as toxic substances or hypoxic conditions, and the development of some types of parkinsonism (hypokinetic/parkinsonoid syndrome) in childhood (5) or Parkinson disease later in life (1), as well as of delayed-onset progressive movement disorders (6). Careful follow-up studies showed that childhood survivors of perinatal hypoxia are at risk for cognitive deficits (7), even in the absence of functional motor disorders (4). Hypoxia during labor, birth, or neonatal period is a major environmental risk factor for the development of later onset mental retardation, learning, language and memory disabilities (4), attention deficit hyperactivity disorder (ADHD) (8–13), and schizophrenia (14–25).

Experimental models of perinatal hypoxia showed that hypoxic/ischemic lesions can cause long-term damage to the CNS, leading to neurological and/or behavioral deficits later in development. Hypoxia to the fetus, a consequence common to many birth complications in humans, resulted in selective long-term disturbances of the rat central dopaminergic (DA) systems that persist in adulthood (26). Most importantly, birth insults interact with stress in adulthood to alter DA function, indicating a possible implication of such events in the development of later onset dopamine-related mental disorders such...
as schizophrenia (15). Dysregulation of DA neurotransmission plays a critical role in the pathophysiology of several neurological and psychiatric disorders, notably Parkinson disease (27–31), ADHD (9, 32), and schizophrenia (15, 33–38).

Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in dopamine synthesis (39). We previously reported the induction of TH in the neurosecretory nuclei of neonates who experienced prolonged hypoxic insult; therefore, we regard it as a neuropathological marker of prolonged perinatal hypoxia in autopsy material (40). Tyrosine hydroxylase is an oxygen-requiring enzyme, and kinetic properties suggest that oxygen availability may limit the synthesis of catecholamines in the brain (41). Tyrosine hydroxylase loses its oxygen dependency during physical stress, indicating that substrate limitation can be overcome when the neuronal needs for neurotransmitter are increased (41, 42). Hypoxia is a major regulator of TH gene expression. Several transcription factors that are activated by hypoxic conditions (e.g., hypoxia-inducible factor, activator protein 1, and cAMP-responsive element–binding protein) can regulate both the rate of TH gene expression (43) and TH mRNA stability (44).

To assess effects of perinatal hypoxia on the mesencephalic DA neurons in human autopsy material, we investigated TH expression in the substantia nigra (SN) and ventral tegmental area (VTA) of the human neonate in relation to the age and severity/duration of sustained hypoxic injury. Portions of the autopsy material were used in our previous study of the expression of TH in the hypothalamic DA neurons of the human neonate (40).

MATERIALS AND METHODS

Patients and Tissues

Formalin-fixed mesencephali of 18 autopsied infants (8 females and 10 males) were prospectively collected from the Department of Pathology of the National Kapodistrian University of Athens, Greek Brain Bank (GBB, member of the BrainNet Europe), directed by Professor E. Patsouris. Most infants (13 of 18) were delivered by cesarean section, 8 were born prematurely before the 36th week of gestation, and 9 were delivered at or near term. The total corrected age (duration of pregnancy + postnatal age) ranged from 32.5 to 46.5 weeks. To exclude neuronal immaturity we studied tissues from “mature” brains of the late third trimester of gestation onward; immature brains from pregnancy terminations legally induced before week 26 were excluded from the study. Dysplastic brains were also excluded. Late intrauterine fetal deaths were excluded to avoid brain autolysis. All neonates were born alive except case GBB 2631/09, who died intrapartum. In view of the limitation of working with human autopsy samples and considering that all autopsied neonates who filled the criteria of inclusion sustained some degree of hypoxic insult, true “controls” deprived of any sign of neuropathological hypoxic injury could not be included in this study. Complete autopsies were performed in all cases after parental written consent for diagnostic and research purposes.

Histopathology

The neuropathological evaluation of neonatal hypoxic-ischemic encephalopathy was based on the presence of gray and/or white matter lesions (periventricular leukomalacia and neuronal necrosis), infarcts, and hemorrhage. Neuronal necrosis was evaluated in the frontal and occipital cortex, basal ganglia, thalami, and brainstem on the basis of conventional morphological criteria, that is, cellular pyknosis or cytologic swelling with tigroidis in mature neurons, karyorrhexis, and focal, laminar, or diffuse loss of neurons in the cortical layers (45). The grading of the severity or duration of the hypoxic injury was based on established histopathological criteria, taking into consideration the regional aspects of neuronal necrosis (45–47). Accordingly, 3 grades of severity were used (Table 1). When multiple lesions coexisted (combinations of gray and white matter injury or multiple lesions of differing ages or severity), the highest score observed was assigned to the case. Clinical and pathological data, including the neuropathological grading and the presence of atrophy in gray (G) or white (W) matter as well as the sex, prenatal and postnatal ages, body and brain weights, head perimeter, and percentile of each case, are presented in Table 2.

Histology and Immunohistochemistry

The mesencephali were dissected from the brain, dehydrated in graded alcohol, and embedded in paraffin. Seven-micrometer-thick serial sections throughout the whole mesencephalon were collected, and 1 section of every 50 was mounted on silane-coated slides and stained with cresyl violet (CV)–Luxol fast blue (LFB) to delineate the main mesencephalic nuclei, that is, SN and VTA. Sections were

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**TABLE 1. Grading of Duration/Severity of Hypoxia Based on Neuropathological Criteria**

<table>
<thead>
<tr>
<th>Injury</th>
<th>Severe/abrupt</th>
<th>Moderate/prolonged</th>
<th>Very severe/long duration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Topography of neuronal necrosis</strong></td>
<td></td>
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<tr>
<td>Gray matter injury</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Thalamus</td>
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<tr>
<td>Basal ganglia</td>
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<tr>
<td><strong>White matter injury</strong></td>
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<td></td>
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<tr>
<td>Acute</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Coagulation necrosis</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Axonal swellings</td>
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<td></td>
<td></td>
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<tr>
<td>Histopathological findings</td>
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<td></td>
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<tr>
<td>Axonal swellings</td>
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<td></td>
<td></td>
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<tr>
<td>Coagulation necrosis</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Endothelial cell hyperplasia</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Microglial proliferation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axial calcifications</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Reactive gliosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Neuropathological grading of lesions</strong></td>
<td>Grade 1</td>
<td>Grade 2</td>
<td>Grade 3</td>
</tr>
</tbody>
</table>

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TABLE 2. Clinical and Pathological Data of Cases Studied and Neuropathological Grading of Perinatal Hypoxia

<table>
<thead>
<tr>
<th>GBB No.</th>
<th>Age (weeks, days, hours) (Corrected Age, weeks), Sex</th>
<th>Postmortem Delay (days)/Fixation Time (months)</th>
<th>Body Weight (g)/Percentile</th>
<th>Brain Weight (g)/Atrophy</th>
<th>Head Perimeter (cm)/Percentile</th>
<th>Clinical and Pathological Data*/Medications</th>
<th>Hypoxia Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>2426/08</td>
<td>32.5 weeks + 3 hours (32.5 weeks), F</td>
<td>4/2.5</td>
<td>2,032/90 -97</td>
<td>219/-</td>
<td>31.097</td>
<td>Bronchopneumonia, persistent fetal circulation, cholestasis</td>
<td>2</td>
</tr>
<tr>
<td>2226/07</td>
<td>27 weeks + 52 days (34 weeks), F</td>
<td>&lt;1/2.5</td>
<td>1,370/3</td>
<td>198/G + W</td>
<td>ND</td>
<td>RDS, BPD, sepsisemia, renal failure/surfactant, antibiotics, inotropes, diuretics, sedatives, TPN</td>
<td>3</td>
</tr>
<tr>
<td>1139/03</td>
<td>34 weeks + 21 days (37.5 weeks), M</td>
<td>3/8</td>
<td>2,880/25-50</td>
<td>275/-</td>
<td>28.5/-3</td>
<td>Down syndrome, congenital cyanotic heart defect, hypotension, and bradycardia episodes/antibiotics, inotropes, TPN</td>
<td>3</td>
</tr>
<tr>
<td>2807/07</td>
<td>37 weeks + 0 hours (37 weeks), M</td>
<td>2/2.5</td>
<td>2,445/10-25</td>
<td>444/-</td>
<td>32.5/5-30</td>
<td>Acute thrombosis of the umbilical vein</td>
<td>1</td>
</tr>
<tr>
<td>2631/09</td>
<td>38 weeks (38 weeks), F</td>
<td>&lt;0.5/3</td>
<td>3,970/90-97</td>
<td>392/-</td>
<td>36.590-97</td>
<td>Intrapartum death, infant of diabetic mother-Cardiomyopathy, hepatosplenomegaly, pancreatic islet hyperplasia</td>
<td>1</td>
</tr>
<tr>
<td>1705/05</td>
<td>37 weeks + 8 days (38 weeks), M</td>
<td>2/2</td>
<td>2,600/10</td>
<td>345/G</td>
<td>32.5/10-25</td>
<td>Genetic thrombophilia, thrombosis of the descending aorta, thrombotic vasculitis, meconium aspiration, hypotension, hyperglycemia/antibiotics, adrenaline, dopamine, inotropes, TPN</td>
<td>2</td>
</tr>
<tr>
<td>1138/03</td>
<td>38 weeks + 7 days (39 weeks), M</td>
<td>4/ND</td>
<td>ND</td>
<td>310/G</td>
<td>32.0-3-10</td>
<td>Congenital cyanotic heart defect, infection</td>
<td>2</td>
</tr>
<tr>
<td>1965/03</td>
<td>39 weeks + 2 hours (39 weeks), M</td>
<td>2/1</td>
<td>2,744/10</td>
<td>337/-</td>
<td>34.0/50</td>
<td>Congenital cyanotic heart defect/adrenaline, bicarbonates</td>
<td>2</td>
</tr>
<tr>
<td>2735/09</td>
<td>39 weeks + 2 days (39 weeks), F</td>
<td>0.3/6</td>
<td>2,960/10</td>
<td>313/-</td>
<td>32.0/3</td>
<td>Fatty acid oxidation defect-Liver steatosis, cardiomyopathy, pancreatic islet hyperplasia</td>
<td>1</td>
</tr>
<tr>
<td>1836/06</td>
<td>35 weeks + 29 days (39 weeks), M</td>
<td>3/8</td>
<td>1,950/3</td>
<td>310/W</td>
<td>31.0/3</td>
<td>Ivemark syndrome (asplenia-congenital cyanotic heart defect-heterotaxy)/antibiotics, inotropes, adrenaline, TPN</td>
<td>2</td>
</tr>
<tr>
<td>3907/07</td>
<td>39.5 weeks + 2 hours (39.5 weeks), M</td>
<td>1.5/1</td>
<td>3,255/50</td>
<td>380/-</td>
<td>35.0/50</td>
<td>Congenital infection, lung atelectasis/adrenaline, bicarbonates</td>
<td>1</td>
</tr>
<tr>
<td>1593/05</td>
<td>41 weeks + 1 day (41 weeks), M</td>
<td>2/2</td>
<td>3,120/10-25</td>
<td>380/G</td>
<td>33.0/3-10</td>
<td>Congenital cyanotic heart defect, congenital viral infection, meconium aspiration/antibiotics, inotropes, adrenaline</td>
<td>3</td>
</tr>
<tr>
<td>1846/06</td>
<td>37 weeks + 34 days (42 weeks), F</td>
<td>1/3</td>
<td>ND</td>
<td>65/-</td>
<td>40.0/-97</td>
<td>Bronchopneumonia, reactive hepatitis</td>
<td>1</td>
</tr>
<tr>
<td>2062/07</td>
<td>28 weeks + 103 days (43 weeks), M</td>
<td>0.7/4</td>
<td>2,280/3</td>
<td>283/G + W</td>
<td>30.5/-3</td>
<td>Cystic fibrosis, RDS, respiratory infection/surfactant, antibiotics, inotropes, diuretics, sedatives, TPN</td>
<td>3</td>
</tr>
<tr>
<td>1402/04</td>
<td>25 weeks + 136 days (44 weeks), M</td>
<td>3/8</td>
<td>3,000/3</td>
<td>300/G + W</td>
<td>34.0/3</td>
<td>RDS, renal failure, congenital cystic renal hypoplasia, endocardial fibroelastosis, myocardial ischemia/surfactant, antibiotics, corticosteroids, anticonvulsants, inotropes, diuretics, sedatives, TPN</td>
<td>3</td>
</tr>
<tr>
<td>1286/04</td>
<td>35 weeks + 67 days (44.5 weeks), M</td>
<td>3/12</td>
<td>3,800/25</td>
<td>347/G</td>
<td>34.5/3</td>
<td>Placental insufficiency, respiratory infection, cholestasis, adrenal hypoplasia/antibiotics, anticonvulsants, sedatives, inotropes, adrenaline, TPN</td>
<td>3</td>
</tr>
<tr>
<td>2325/07</td>
<td>39 weeks + 49 days (46 weeks), F</td>
<td>0.4/3.2</td>
<td>2,890/-3</td>
<td>413/G</td>
<td>33.5/-3</td>
<td>RDS, genetic surfactant C deficiency/surfactant, antibiotics, sedatives, inotropes, TPN</td>
<td>3</td>
</tr>
<tr>
<td>1163/04</td>
<td>28 weeks + 130 days (46.5 weeks), F</td>
<td>4/0.5</td>
<td>3,100/-3</td>
<td>105/G + W</td>
<td>33.0/-3</td>
<td>Sepsis, ischemic enterocolitis, renal–liver failure, nephromegaly with glomerular cysts/surfactant, antibiotics, anticonvulsants, sedatives, inotropes, diuretics, TPN</td>
<td>3</td>
</tr>
</tbody>
</table>

*Excluding neuropathological findings.

BPD, bronchopulmonary dysplasia; F, female; G, gray matter; GBB, Greek Brain Bank; hours, hours of postnatal life; M, male; W, white matter; -, no atrophy; ND, not determined; RDS, respiratory distress syndrome; TPN, total parenteral nutrition; weeks, weeks of gestation.
deparaffinized in xylene (2 × 10 minutes), washed in 100% (5 minutes) and 95% ethanol (2 × 5 minutes), and stained for 2 hours at 60°C in 0.1% LFB (S-3382; Sigma, St. Louis, MO) (0.1 g LFB in 100 mL 95% ethanol and 0.5 mL 10% acetic acid). Sections were rinsed in 95% ethanol to remove the excess dye and in distilled water; they were then dipped in 0.05% lithium carbonate (0.05 g LiCO₃ in 100 mL distilled water) and 70% ethanol. Slides were then washed in distilled water and stained for 6 minutes at 60°C in 0.1% CV (Fluka, Buchs, Switzerland; 0.1 g CV in 100 mL distilled water and 15 drops of 10% acetic acid). Sections were washed in 95% ethanol (2 × 3 minutes), dehydrated in 100% ethanol (2 × 3 minutes), rinsed in xylene (2 × 10 minutes), and mounted in DPX mountant medium (BDH, Poole, UK).

One section every 100, adjacent to the CV/LFB-stained section, at the whole rostrocaudal extension of the mesencephalon was immunostained for TH with the classical peroxidase-antiperoxidase (PAP) technique, as previously described (40), using a polyclonal anti-TH serum (LOT 900210A, 1:1000; Institut de Biotechnologie Jacques Boy, Reims, France) and diaminobenzidine intensified by nickel ammonium sulfate (DAB-Ni) as a chromogen to reveal the number and distribution of TH-immunoreactive (IR) neurons and estimate the intensity of TH. This antibody was generated using as an immunogen pure active TH, isolated from rat pheochromocytoma tumors. The specificity of the TH immunohistochemical reaction on the sections was routinely checked by either processing the sections with TH antibody adsorbed with pure TH enzyme (kindly provided by Professor J. Thibault, Paris, and Dr. Y. Tillet, Tours, France) or by omitting it.

Because TH reaction revealed with the classical PAP technique was very low for some subjects, a second set of sections was stained with the avidin-biotin peroxidase antiperoxidase complex (ABC) method using biotinylated anti-rabbit IgG (H + L) (1:100, BA-1000; Vector Laboratories, Burlingame, CA) in Supermix for 1 hour at room temperature (RT) and ABC complex (1:400, Vectastain ABC Kit, Vector Laboratories) in Supermix for 1 hour at RT and washed and stained with DAB-Ni. Because even after staining with ABC complex the intensity of staining remained very low for some cases, we also applied antigen retrieval before staining with the classical PAP technique (48). Sections were bathed in 1% zinc sulfate and incubated for 2 × 5 minutes at 400 W (with a 1-minute break between the 2 incubation times) in microwaves. Sections were then cooled for 25 minutes at RT, washed, and incubated in TBS with 5% milk for 45 minutes at RT. After washing, sections were incubated in 10% normal goat serum (S-1000; Vector Laboratories) and then in the primary TH antiserum and stained with DAB-Ni.

Western Blot Analysis

To test the specificity of the anti-TH antibody, we performed Western blot analysis using human adrenal medulla as a control tissue rich in TH. For that purpose, human postmortem adrenal medulla tissue was minced and directly homogenized by sonication in a solution containing 8 mol/L urea, 0.5% sodium dodecyl sulfate, 1 mmol/L sodium orthovanadate, and a cocktail of protease inhibitors, as described (49). Insoluble material was removed by centrifuga-

![FIGURE 1. Diagrammatic representation of the mesencephalon at the level used for morphometric analysis showing the substantia nigra (SN) and ventral tegmental area (VTA) at the level of red (RN) and oculomotor (n III) nucleus where the fibers of the III cranial nerve first appear. Note the clear delineation of paragnial nucleus (PN) (part of the VTA) at this level and the localization of the measuring fields (in black) in a central area of SN pars compacta and in the center of PN for both sides of the mesencephalon. Black dots depict the distribution of tyrosine hydroxylase-immunoreactive (TH-IR) perikarya, as observed in the hypoxia grade 1 group. CP, cerebral peduncle; CTT, central tegmental tract; ML, medial lemniscus; n III, oculomotor nerve nucleus; PAG, periaqueductal gray; PBN, parabrachial nucleus; RN, red nucleus.](http://jnen.oxfordjournals.org/)

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PN, providing a better delineation of these DA cell areas. Images of selected measuring fields in a standardized 238 × 317-μm area in SN and PN (Fig. 1, in black) were captured using a digital CCD color video camera (SSC-C370P; SONY, Tokyo, Japan) connected to an optical microscope (BX50F-3; Olympus, Tokyo, Japan). The image analysis program “Image-Pro Plus” (v4.5.1.29; Media Cybernetics, Bethesda, MD) was used to measure the optical density (OD) of TH reaction in 15 cells containing nucleus and nucleolus from each area at a magnification of 200×. The cell and nuclear sizes and the nucleus/cytoplasm (N/C) ratio were studied in the adjacent CV/LFB-stained sections by measuring 25 neurons from each area at a magnification of 400×. Cases 1593/05, 1846/06, and 2325/07 were excluded from the morphometric analysis of the PN because the tissue specimens of these subjects did not permit the clear delineation of this nucleus.

**Statistical Analysis**

All the variables studied (i.e. OD for TH reaction, cell and nuclear sizes, and N/C ratios in SN and PN) showed normal distribution. Because the sample sizes were small and

![Figure 2](http://jnen.oxfordjournals.org/)

**FIGURE 2.** Two adjacent sections of the mesencephalon of 3 cases with different grades of hypoxia: GBB 3907/07 (hypoxia 1), GBB 1836/06 (hypoxia 2), and GBB 1402/04 (hypoxia 3) immunostained for tyrosine hydroxylase (TH) and with Luxol fast blue/cresyl violet (CV/LFB), respectively. (A, D, G) TH-immunoreactive (IR) neurons in the substantia nigra (SN) pars compacta. (B, E, H) The same SN neurons stained with CV/LFB. (C, F, I) TH-IR neurons in the paranigral nucleus (PN). Note the intense TH staining of both SN and PN neurons in hypoxia grade 1 case GBB 3907/07 (A, C). In hypoxia grade 2 case GBB 1836/06, TH-IR is almost absent in SN neurons, even after antigen retrieval (D); the neurons are clearly evident in (E). Note the absence of TH immunoreactivity in the PN (F) in the same case. In hypoxia 3 case GBB 1402/04, no TH immunoreactivity is evident in SN neurons (G), whereas some TH-IR perikarya are observed in the PN (I). Also note that the cell size of SN neurons does not correlate with the total age of the neonates, that is, SN neurons of GBB 3907/07 (total age, 39.5 weeks; hypoxia grade 1) seem much larger than those of GBB 1402/04 (total age, 44 weeks; hypoxia grade 3) (B vs H). In the PN, the cell size correlates with the total age of the neonates (C vs I). Black arrows and asterisks show cells or blood vessels, used as landmarks for the identification of SN neurons. Scale bar = 50 μm.

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The variances between groups were not similar, the nonparametric Kruskal-Wallis (KW) test was performed to assess the effect of perinatal hypoxia on the variables studied. Mann-Whitney U test was used to check possible sex differences.

The correlation between the above variables and hypoxia grade with the total (prenatal and postnatal) age, postmortem delay, fixation time, body and brain weights, head perimeter, and percentile was investigated using Spearman bivariate test;

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**Figure 1:**

- **A1:** CV/LFB staining showing Hypoxia grade 1.
- **A2:** TH staining showing Hypoxia grade 1.
- **B1:** CV/LFB staining showing Hypoxia grade 1.
- **B2:** TH staining showing Hypoxia grade 1.
- **C1:** CV/LFB staining showing Hypoxia grade 3.
- **C2:** TH staining showing Hypoxia grade 3.

**GBB 2807/07 Hypoxia grade 1**

**GBB 2807/07 Hypoxia grade 1**

**GBB 2062/07 Hypoxia grade 3**

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RESULTS

All autopsied neonates included in this study sustained some degree of hypoxic insult and had neuropathologic evidence of hypoxic injury. Gross neuropathological findings compatible with hypoxic-ischemic encephalopathy included brain edema, vascular congestion, hemorrhage, infarction, ventricular distention, posthemorrhagic hydrocephaly, microgyria, atrophy, periventricular white chalky spots of degeneration, cavitations, and end-stage multicystic encephalopathy. The microscopic neuropathological lesions that were compatible with hypoxic-ischemic encephalopathy and were selectively used for the hypoxia grading included the following: gray matter lesions in the frontal cortex, basal ganglia, brainstem, and cerebellum were seen as focal, laminar-cortical, or diffuse neuronal necrosis, with or without gliosis, lipid-laden macrophages, neuronophagia, and neuronal mineralization. Atrophy of the cortical gyri was seen as a sequel of diffuse neuronal necrosis (Table 2). White matter lesions covered the temporal range of periventricular leukomalacia and included focal acute coagulation necrosis, globoid bodies, endothelial hyperplasia, microglial proliferation, microcalcifications, astrocytic gliosis, glial scarring, and cavitations (Table 1).

Hypoxic injury was classified as grade 1 (clinically correlating to severe/acute hypoxia) in 5 cases, as grade 2 (subacute/prolonged hypoxia) in 5 cases, and as grade 3 (very prolonged/chronic hypoxia) in 8 cases (Table 2). A combination of white and gray matter scars or a combination of chronic and acute injury was often seen in the hypoxia grade 3 group. The average total age of the neonates in each hypoxia group studied was 39.1 ± 0.84, 37.5 ± 1.26, and 42 ± 1.57 weeks, respectively. No statistically significant differences were found between hypoxia groups and the total age of the neonates (KW, $\chi^2 = 4.016$, $p = 0.134$).

A striking difference in the expression of TH in the mesencephalic DA nuclei was observed between subjects of hypoxia grade 1 and hypoxia grade 2 and 3 groups. This difference was evident in the entire rostrocaudal extension of the mesencephalon studied by TH immunohistochemistry. In hypoxia grade 1 group, intense TH staining was observed in all the DA neuronal bodies and their processes of both SN and PN (Fig. 2A, C). Intense TH reaction was also observed in neurons of the parabrachial (part of the VTA) and, surprisingly, in almost all the neurons of the Edinger-Westphal (EW) nucleus located above and medially of the n III, according to the atlas of the human brainstem of Olszewski and Baxter (52) (Fig. 3A2). Numerous TH-IR perikarya with intense reaction were also observed around the aqueduct, as well as scattered in the midline and the mesencephalic reticular formation area, as diagrammatically depicted in Figure 1. Tyrosine hydroxylase-IR neurons of EW were strictly localized within the boundaries of this nucleus (Fig. 3A2) and appeared smaller than TH-IR perikarya scattered in the midline (Fig. 3A2, B1, arrows) or reticular formation area. Tyrosine hydroxylase-immunoreactive neurons were not observable in any of the above nuclei (including EW) in sections stained with the adsorbed TH antibody (Fig. 4A1 vs A2) or by omitting the primary antibody from the incubation medium.

In subjects of the hypoxia grade 2 group, there was a dramatic loss of TH immunoreactivity that was evident even by the naked eye. Microscopically, TH staining was absent from the entire mesencephalon. No TH-IR neurons were evident in either SN or PN neurons with the classical PAP technique, whereas neurons were clearly evident in the adjacent CV/LFB-stained sections (Fig. 2E). Even after antigen retrieval, no TH reaction was observed (Fig. 2D). In subjects of hypoxia grade 3 group, TH immunoreactivity was also almost absent from SN neurons (Fig. 2G); a light reaction was observed in PN neurons of some cases (Fig. 2I). No reaction for TH was revealed either in neurons of the parabrachial nucleus or EW (Fig. 3C2) or in the midline and reticular formation area in subjects of both hypoxia grade 2 and 3. Some neurons were positive for TH in all the above areas only in GBB 2226/07 (a preterm 34-week-old neonate/hypoxia grade 3).

Western blot analysis showed that the TH antibody recognized a single band for purified TH at the expected Mr of approximately 56 kDa (Fig. 4B, TH lanes). In the human adrenal medulla samples, this band was slightly higher at approximately 59 kDa, whereas a second fainter band corresponding to a molecular weight of approximately 55 kDa (adrenal medulla samples, this band was slightly higher at approximately 59 kDa) was detected. Whereas the lower band may represent a degradation product of the 59-kDa species, it is highly possible that this antibody detected TH protein products of 2 different mRNA of the 4 distinct TH mRNA variants that, unlike all other mammals, humans express; these products of alternatively spliced mRNA have predicted molecular weight ranging from 55 to 60 kDa (53, 54). Moreover, densitometry analysis verified that this antibody allows quantitative measurements of expression, as we established linearity of signal in concentrations ranging from nanograms (purified TH) to picograms (adrenal medulla tissue).

Morphometric analysis of the OD for TH staining (based on the classical PAP technique for all cases) revealed that TH immunoreactivity was significantly decreased by prolonged perinatal hypoxia in both SN and PN neurons.
The OD of TH reaction in SN of hypoxia 2 and 3 groups was much lower than that of hypoxia 1 group (Fig. 5). Similar changes were observed in the PN (Fig. 5). The lowest values of TH OD were found in SN and PN of the hypoxia grade 2 group. In both SN and VTA, there was no correlation between TH OD and the total age of the neonates (Spearman, \( r_s = -0.079, p = 0.756; r_s = -0.090, p = 0.749 \), respectively). Because of the very low TH immunoreactivity of SN and PN neurons in hypoxia 2 and 3 groups, quantification of TH-expressing neurons was not possible. Reduced TH immunoreactivity was evident in neonates that had been treated with different combinations of drugs and in cases without reported medication (GBB 2426/08 and GBB 1138/03); thus, our results cannot be attributed to effects of drugs.

Morphometric analysis of cell and nuclear sizes in SN and PN (measured in the adjacent CV/LFB-stained sections) revealed a significant effect of hypoxia grade only on cell size.
were inversely correlated to the degree of neuropathological injury (Spearman, \( r_s = -0.664, p = 0.003; r_s = -0.514, p = 0.029 \), respectively).

**DISCUSSION**

Taking into consideration the limitation of this study caused by the lack of hypoxia grade 0 pure controls, the observed pattern of TH immunoreactivity in the subgroups of hypoxia grading prompted us to assume that DA neurons of the mesencephalon of the human neonate are vulnerable to prolonged perinatal hypoxia. Massive reduction of TH immunoreactivity was observed in the major DA areas of the brain, SN, and VTA (A9 and A10 cell groups [55]), indicating inhibition of dopamine synthesis in these neurons under prolonged hypoxic insult. This effect might be specific for the DA neurons of the mesencephalon because, in the hypothalamus of the same cases, there was increased TH expression in neurosecretory neurons of neonates who experienced prolonged perinatal hypoxia (i.e. in the subjects without TH immunoreactivity in SN and VTA observed in the present study) [40]. Moreover, our preliminary results on the noradrenergic neurons of locus coeruleus of the same sample indicate that locus coeruleus neurons are not equally affected by prolonged perinatal hypoxia, that is, some of the cases without TH immunoreactivity in SN/VTA neurons in the present study exhibit intense TH immunohistochemical reaction in locus coeruleus neurons (unpublished results). Given the above observations and our statistical testing, the reduced expression of TH in SN/VTA does not seem to be related to the degree of postmortem delay and tissue degradation.

**FIGURE 5.** The means of the optical densities (ODs) of tyrosine hydroxylase (TH) reaction for each hypoxia grade group. In the substantia nigra (SN), the mean ± SEM values in hypoxia grade 1 group is much higher (−2.197 ± 0.032) versus that in hypoxia grade 2 and 3 groups (−2.307 ± 0.002 and −2.268 ± 0.018, respectively). In the paraventricular nucleus (PN), the means OD of TH staining in the hypoxia grade 1 group is also higher (−2.168 ± 0.014) than that observed in hypoxia 2 and 3 groups (−2.297 ± 0.008 and −2.233 ± 0.016, respectively). Note that the lowest OD value for both areas occurs in the hypoxia 2 group.

**FIGURE 6.** The mean values of cell sizes in substantia nigra (SN) and paraventricular nucleus (PN) for each hypoxia group, as assessed in cresyl violet/Luxol fast blue–stained sections. The means ± SEM of cell size in hypoxia 1, 2, and 3 groups are 351 ± 26.8, 231 ± 23.8, and 249 ± 15.2, respectively, in the SN and 216 ± 26.9, 208 ± 18.1, and 215 ± 21.9, respectively, in the PN. Note the greater cell size of SN neurons in the hypoxia grade 1 group versus the hypoxia grade 2 and 3 groups. In addition, note, in contrast to the SN, the cell size of PN neurons does not change with hypoxia grade.
The findings also cannot be attributed to the developmental process because they were observed in cases of different total (prenatal + postnatal) ages specifically when the sustained hypoxia was prolonged. According to Verney (51), TH-IR neurons in the developing SN and VTA generate during the 5 to 7 postovulatory weeks in the ventricular zone of the floor plate and reach the overall adult distribution pattern in the fourth month of gestation. In 20- to 24-week-old human fetuses, widespread TH-IR innervation of the frontal cortex comparable to that observed in the adult was also shown (51). In our sample, the neonates ranged from 32.5 to 46.5 weeks and, therefore, we would expect an almost “mature” DA system in all subjects. This was indeed the case only for the subjects of hypoxia grade 1 group, where intensely stained TH-IR neurons were observed. This was not only in the SN and VTA but also, as expected, scattered in the midline and reticular formation area. Surprisingly, however, TH was also expressed in almost all the neurons of the EW nucleus in hypoxia grade 1 cases. Some TH-IR neurons in this nucleus have been reported in the mesencephalon of the human fetus as a part of the VTA (51) but are not mentioned in the adult (56). The EW nucleus was originally described as the location of cholinergic preganglionic neurons of the ciliary ganglion (57, 58), but it is now considered as containing neurons with different chemistry and function in different species (59). The EW nucleus in humans (as identified in the Olszewski and Baxter atlas [52]) contains urocortin 1–positive/centrally projecting neurons that belong to the nonpreganglionic part (60, 61). Urocortin 1 neurons in this nucleus are involved in adaptation to stress and consequently in stress-related disorders such as anxiety and major depression and the use of drugs of abuse (59). The increased expression of TH in neurons of the nonpreganglionic/centrally projecting part of EW selectively in hypoxia grade 1 group of our sample could, therefore, represent a catecholamine-dependent adaptation of the human neonate to acute hypoxic stress.

Perinatal hypoxia/ischemia triggers a cascade of biochemical events that result in neuronal injury. Unilateral hypoxic/ischemic injury induced by carotid ligation in the neonatal rat results in a persistent into adulthood increase in the density of TH-IR fibers in both striatum and nucleus accumbens, accompanied by shrinkage of the striatum and the overlying cortex and ventricular enlargement only on the injured side (62). Moreover, the observed elevated homovanillinc acid levels were attributed to a massive release of dopamine from nerve terminals, accompanied by injury to the ipsilateral hemisphere (63). Massive dopamine release (64–67) in striatal dialysates, as well as in mesencephalic cell cultures (68), was observed by exposure to various degrees of hypoxia. Increased low-affinity dopamine uptake was shown related to the severity of hypoxia, considered as protective for the striatum by mediating a faster removal of dopamine, which could be neurotoxic (65). If a similar dramatic increase in dopamine synthesis and release occurs also in the human neonate during the initial phase of severe perinatal hypoxia, the observed reduction of TH immunoreactivity in our sample under prolonged hypoxia might be attributed to feedback inhibition of TH by the dopamine end product (69, 70). Brief ischemia causes long-term depression in SN that could be protective by reducing excitotoxic injury to dopamine neurons (71).

A variety of in vitro and in vivo studies have demonstrated that dopamine in high concentrations is a toxic molecule with genotoxic potential (72) and may contribute to neurodegenerative disorders, such as Parkinson disease and ischemia-induced striatal damage (73–75). Direct dopamine injection into the striatum induces selective toxicity to DA terminals (76) and neuronal apoptosis (77, 78), whereas SN lesions or dopamine depletion prevents ischemic damage in the rat (79, 80). Dopamine metabolism produces DA quinone reactive oxygen species that contribute to cell damage through caspase-3 activation (81), inhibition of mitochondrial respiration (82, 83), and DNA damage (72, 75). Neuroprotection requires intact adenosine triphosphate levels and adenosine triphosphate metabolism to keep dopamine compartmentalized inside synaptic terminals and to maintain the normal reuptake process (73–75). However, during hypoxia, tissue energy stores are depleted, leading to failure of dopamine reuptake and storage, thus making dopamine excess dangerous for striatal neurons, especially under the excitotoxic effect of glutamate (66, 79).

The observed dramatic reduction of TH immunoreactivity in the major DA areas of the human neonate after prolonged hypoxia is in agreement with experimental data indicating that perinatal hypoxia causes duration-dependant changes in the number of the DA cell bodies in SN and VTA of the mesencephalon, as well as in DA neurotransmission in their target areas, that is, basal ganglia and prefrontal cortex/mesolimbic regions. According to these data, an increased number of DA neurons was observed in SN and VTA of the rat after a short perinatal hypoxic insult, possibly reflecting proliferation of DA neurons or reduction in cell death during development (84, 85). When perinatal hypoxic conditions lasted for longer periods (leading to almost 100% mortality of the pups), there were reduced numbers of DA neurons detected in both SN and VTA of the surviving pups, however, and, most importantly, this loss of DA neurons was sustained in adult life (26, 85). The reduced number of dopaminergic SN neurons in the adult was attributed to “pathological” apoptosis caused by a diminution in developmental target-derived trophic support from the striatum (86). As suggested by Young et al (87), a first excitotoxic wave destroys neurons that normally provide synaptic inputs or synaptic targets for neurons that die in the second apoptotic wave, as those described in the adult SN after experimental perinatal hypoxia (26, 86, 88). Analysis of gene expression in humans provided strong evidence for a reduction in neurotrophic support and alterations in axon guidance cues in early stages of cellular stress events, ultimately leading to dopaminergic SN cell death in Parkinson disease (89).

The SN and VTA contain most of the dopaminergic neurons in the brain and are heavily involved in the control of motor and cognitive behaviors. Their dysregulation underlies many neurologic and neuropsychiatric disorders, notably Parkinson disease (5, 27, 28, 30, 31) and schizophrenia (33–38, 90, 91), 2 severe disorders of possibly neurodevelopmental origin (1, 92). Dopamine is a key neurotransmitter...
in the brain involved in reinforcement learning, reward seeking, hippocampal plasticity, working memory, addiction, behavioral drive, and incentive motivation (56). Functional evidence of dopaminergic damage cannot be obtained at the neonatal age either from electrophysiologic or clinical criteria, these being obscured by the clinical signs of severe neonatal encephalopathy. Nevertheless, the early dysregulation of TH expression reported in this study in postmortem human material provides the histologic background to the hypothesis that perinatal hypoxia may contribute to dopamine-related neuropsychiatric disorders later in life.

Interestingly, we found that the size of SN neurons of neonates who experienced prolonged hypoxia was significantly smaller than those of neonates who died after acute hypoxic insults. Most of these neurons were smaller but not pyknotic, an observation indicating a delayed or temporarily arrested development of the human DA neurons under prolonged perinatal hypoxic conditions. Smaller sizes of SN neurons were also reported in adult offspring born after ligation of the uterine artery in pregnant rats, an experimental model of intraterine growth retardation (93). Delayed maturation of DA neurons and decreased expression of basic fibroblast growth factor (a major neurotrophic and neuroprotective factor of midbrain DA neurons) is observed in the rat mesencephalon after perinatal anoxia (94). In relation to the above, it is important to notice the structural abnormality of SN in children with ADHD detected by transcranial sonography, indicating abnormal maturation of SN in this disease (95). Delayed maturation might indirectly render these neurons more plastic in later life and contribute to enhanced responsiveness of the midbrain DA system to stress and amphetamine after birth hypoxia (96–98). Whether the significant decrease in size and the reduction of TH immunoreactivity of SN neurons in the neonates that experienced prolonged perinatal hypoxia depict an early sign of possible degeneration of mesencephalic DA neurons later in life remain to be investigated. If indeed this is the case, the supposed blockage of dopamine synthesis through inhibition of TH (observed in our study) could be protective for the striatum because activation of DA systems caused by perinatal hypoxia could enhance the initial excitotoxic insult (99, 100) induced by massive accumulation of extracellular glutamate through an additional massive release of dopamine.

Future investigations should also address whether premature neonates react in a different way to perinatal hypoxia than mature ones, as shown for mature and immature mesencephalic cell cultures (101). In hypoxia grade 3 group of our sample, TH expression was present in some mesencephalic DA neurons of the infant GBB 2226/07, a premature neonate born at 27 weeks of gestation, with the lowest brain and body weight (percentile, <3), indicating partially preserved DA synthesis in this immature brain.

Persistent changes in DA receptors were evident after experimental perinatal hypoxic/ischemic injury, indicating an early-onset dysregulation of the DA system that may play an important role in the development of motor and/or cognitive disturbances later in adulthood (85, 102–104). Widhalm (105), as reported in 5), in 1985, described extrapyramidal problems, termed “hypokinetic/parkinsonoid syndrome,” in children who had experienced overt problems early in life, such as asphyxia during delivery. The syndrome was reversible when i-DOPA was introduced, indicating that mechanisms are available that compensate damage to SN, the identification of which would be crucial for the understanding of adult idiopathic Parkinson disease.

Most importantly, perinatal hypoxia, as reported in experimental animals, increased the sensitivity of mesolimbic DA neurons to repeated stress during adulthood, supporting the idea that birth complications may contribute to the pathophysiology of psychiatric disorders, in particular, those that involve central DA dysfunction, such as schizophrenia (96, 106) and ADHD (9, 32, 107, 108).

In summary, our study indicates that the human neonatal mesencephalic neurons are vulnerable to severe perinatal hypoxic/ischemic insults. Massive reduction of TH immunoreactivity was observed in all the mesencephalic DA neurons of the human neonate when the duration of the hypoxia was prolonged, as estimated by neuropathological criteria. Based on experimental evidence and our results, we suggest that a massive increase in dopamine synthesis and release may occur at the early stages of perinatal hypoxia, followed by a feedback blockage of dopamine synthesis in SN and VTA through inhibition of the first and limiting enzyme TH. Early dysregulation of DA neurotransmission in the human neonate, depending on the severity and duration of perinatal hypoxia, could predispose the infant survivors, in combination with a possible genetic predisposition, to dopamine-related neurological and/or cognitive deficits later in life, supporting the “fetal basis of adult disease” hypothesis.

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