The Effect of Perinatal Hypoxic/Ischemic Injury on Tyrosine Hydroxylase Expression in the Locus Coeruleus of the Human Neonate

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Key Words
Human locus coeruleus · Tyrosine hydroxylase · Noradrenaline · Perinatal hypoxia · Development of locus coeruleus · Fetal basis of adult disease · Anticonvulsant drugs · Depression · Alzheimer’s disease · Parkinson’s disease

Abstract
We have previously shown that perinatal hypoxic/ischemic injury (HII) may cause selective vulnerability of the mesencephalic dopaminergic neurons of human neonate. In the present study, we investigated the effect of perinatal HII on the noradrenergic neurons of the locus coeruleus (LC) of the same sample. We studied immunohistochemically the expression of tyrosine hydroxylase (TH, first limiting enzyme for catecholamine synthesis) in LC neurons of 15 autopsied infants (brains collected from the Greek Brain Bank) in relation to the neuropathological changes of acute or chronic HII of the neonatal brain. Our results showed that perinatal HII appears to affect the expression of TH and the size of LC neurons of the human neonate. In subjects with neuropathological lesions consistent with abrupt/severe HII, intense TH immunoreactivity was found in almost all neurons of the LC. In most of the neonates with neuropathological changes of prolonged or older injury, however, reduction in cell size and a decrease or absence of TH staining were observed in the LC. Intense TH immunoreactivity was found in the LC of 3 infants of the latter group, who interestingly had a longer survival time and had been treated with anticonvulsant drugs. Based on our observations and in view of experimental evidence indicating that the reduction of TH-immunoreactive neurons occurring in the LC after perinatal hypoxic insults persists into adulthood, we suggest that a dysregulation of monoaminergic neurotransmission in critical periods of brain development in humans is likely to predispose the survivors of perinatal HII, in combination with genetic susceptibility, to psychiatric and/or neurological disorders later in life.

Introduction
New insights into the etiopathogenesis of several neurological and/or psychiatric disorders come from the recently emerging theory of 'fetal basis of adult disease', which supports that environmental stressors encountered very early in life, i.e. in utero or perinatally, by interacting
with the genetic predisposition, could contribute to the development of certain diseases later in life [1, 2]. It is hypothesized that these early environmental factors can impact on the normal development of the brain, causing subtle or drastic changes on its structure and/or function, inducing a silent altered susceptible state. This initiative state, combined with multiple environmental insults occurring from gestation through older age, can eventually lead to the emergence of clinical disease manifestations [1–4].

Hypoxic/ischemic injury (HII) during the last trimester of gestation, birth or early neonatal period (perinatal period) remains a major cause of mortality and morbidity, capable of generating permanent neurological and/or mental deficits later in life, such as dyskinetic tetraplegic cerebral palsy, mental retardation, and learning, language and memory disabilities [5–7]. Several epidemiological studies have shown an association between obstetric and/or birth complications entailing hypoxia as the common underlying factor and the development of some types of parkinsonism in childhood [8] or Parkinson’s disease later in life [1], attention-deficit/hyperactivity disorder [9–11], depression [12], and schizophrenia [13–15].

In our previous studies concerning the effect of perinatal hypoxia/ischemia on specific brain areas in human autopsy material, we showed selective vulnerability of mesencephalic dopaminergic neurons after prolonged perinatal hypoxia [16]. We found a striking reduction or even absence of tyrosine hydroxylase (TH, the first and limiting enzyme of catecholamine synthesis) immunoreactivity in the dopaminergic neurons of the substantia nigra and ventral tegmental area, indicating possible early dysregulation of the dopaminergic neurotransmission under prolonged hypoxic conditions [16]. Experimental studies, however, have shown that perinatal hypoxia induces not only selective long-standing disturbances in the central dopaminergic systems of the mesencephalon [17, 18], but also in the noradrenergic and serotonergic systems of the brainstem that persist into adulthood [17–21]. The purpose of the present study was to immunohistochemically investigate the expression of TH in the noradrenergic locus coeruleus (LC) neurons of the human neonate in relation to the degree of neuropathological hypoxic/ischemic brain injury. TH – the immunohistochemical marker of catecholamine perikarya in the brain – is detectable in LC neurons very early in development, i.e. already from 5–6 weeks of gestation onwards [22–24], while at birth almost all the LC neurons appear to be TH immunoreactive (IR) [25], as occurs in adulthood [26, 27].

The pontine LC, located in the lateral aspect of the fourth ventricle, constitutes the principle source of noradrenaline in the central nervous system, innervating the entire cerebral cortex, as well as the hippocampus, amygdala, cerebellum cortex and spinal cord, and thus playing a distinct role in many functions, including arousal, stress and emotional memory responses, chemoreception, and modulation of brain microcirculation [28–35]. Age-related neurodegenerative Alzheimer’s and Parkinson’s disease, as well as affective depressive disorder are characterized by a significant loss of the noradrenergic LC neurons, possibly contributing to the manifestation of attention and cognitive deficits in these clinical entities [26, 28, 34–40].

The results of the present study provide important histological data on the effect of perinatal HII on LC neurons of the human neonate, adding some new insights into the puzzling etiopathology of severe adult noradrenaline-related neurological and/or psychiatric disorders.

**Material and Methods**

**Patients, Tissues and Histopathology**

Our material consisted of formalin-fixed brains of 15 autopsied infants with neuropathological changes of HII, obtained from the Greek Brain Bank (GBB; member of Brain-Net Europe, directed by Professor E. Patsouris). Part of this material was also used in our previous studies concerning the effect of perinatal hypoxia on the neurosecretory neurons of the hypothalamus [41, 42] and the dopaminergic neurons of the mesencephalon [16, 43]. Complete postmortem examination was carried out in all cases after parental written consent for diagnostic and research purposes. Late intrauterine fetal deaths were excluded to avoid brain autolysis.

Most infants (11/15) were delivered by emergency caesarean section; one was prematurely born before week 36 of gestation, while the remaining neonates were delivered at or near term. The total age of the neonates (duration of pregnancy + postnatal age) ranged from 25.5 to 46.5 weeks (table 1). All subjects were live-born, with the exception of cases GBB 2631/09, 3143/12 and GBB 3161/13, who were fresh stillbirths or intrapartum deaths.

The neuropathological evaluation of neonatal HII was based on established criteria dependent on the pattern of gray and/or white matter lesions (included in the spectrum of neuronal necrosis and periventricular leukomalacia changes) in specific brain regions [44, 45], summarized in table 2 and described in detail in our previous work [16]. Three neuropathological groups of HII were used: group 1 consistent with severe/acute HII; group 2 consistent with moderate/prolonged or older injury, and group 3 consistent with very severe/long duration or chronic HII. 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 as well as the neuropathological grading of the subjects studied are presented in table 1. The determination of brain atrophy was based on the presence of histological loss of gray matter (thinning or laminar necrosis of the cerebral cortex) or white matter (cavitation, periventricular leukomalacia with ventriculomegaly) or both.
Table 1. Clinical and pathological data of cases studied and neuropathological HII groups

<table>
<thead>
<tr>
<th>GBB No.</th>
<th>Age, w, d, h (total age, w)</th>
<th>Sex</th>
<th>Postmortem delay, days/fixation time, months</th>
<th>Body weight, g/percentile</th>
<th>Brain weight/expected1, g (atrophy)</th>
<th>Head perimeter, cm/percentile</th>
<th>Clinical and pathological data2/medications HII group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3143/12</td>
<td>25.5 w + 0 h (25.5 w)</td>
<td>M</td>
<td>2/9.8</td>
<td>811/50</td>
<td>121/118 (–)</td>
<td>24.0/50</td>
<td>stillborn: chondrodysplasia punctata 1</td>
</tr>
<tr>
<td>2807/07</td>
<td>37 w + 0 h (37 w)</td>
<td>M</td>
<td>2/2.5</td>
<td>2,445/7</td>
<td>444/339 (–)</td>
<td>32.5/25–30</td>
<td>acute thrombosis of the umbilical vein 1</td>
</tr>
<tr>
<td>2631/09</td>
<td>38 w + 0 h (38 w)</td>
<td>F</td>
<td>&lt;0.5/3</td>
<td>3,970/94</td>
<td>392/420 (–)</td>
<td>36.5/90–97</td>
<td>stillborn infant of diabetic mother – macrosomia, cardiomyopathy, organomegaly, pancreatic islet hyperplasia 1</td>
</tr>
<tr>
<td>1705/05</td>
<td>37 w + 8 d (38 w)</td>
<td>M</td>
<td>2/2</td>
<td>2,600/7</td>
<td>345/350 (G)</td>
<td>32.5/10–25</td>
<td>twin gestation, genetic thrombophilia, thrombosis of the descending aorta, septic cerebral thrombotic vasculitis, meconium aspiration, paleness, tachycardia, hypotension, body stiffness followed by hypotonia, acidosis, hyperglycemia/antibiotics, inotropes (dopamine, adrenaline), TPN 2</td>
</tr>
<tr>
<td>1965/06</td>
<td>39 w + 2 h (39 w)</td>
<td>M</td>
<td>2/1</td>
<td>2,744/7</td>
<td>337/362 (–)</td>
<td>34.0/50</td>
<td>congenital cyanotic heart defect, cyanosis, dyspnea, acidosis, heart failure/inotropes, adrenaline, bicarbonates 2</td>
</tr>
<tr>
<td>1836/06</td>
<td>35 w + 29 d (39 w)</td>
<td>M</td>
<td>3/8</td>
<td>1,950/&lt;3</td>
<td>310/281 (W)</td>
<td>31.0/&lt;3</td>
<td>congenital cyanotic heart defect and heterotaxy, cyanosis, dyspnea, arrhythmia, antibiotics, inotropes (adrenaline, bicarbonates), TPN 2</td>
</tr>
<tr>
<td>2735/09</td>
<td>39 w + 2 d (39.5 w)</td>
<td>F</td>
<td>0.3/6</td>
<td>2,960/16</td>
<td>313/380 (–)</td>
<td>32.0/3</td>
<td>fatty acid oxidation defect – liver steatosis, cardiomyopathy, pancreatic islet hyperplasia – vomiting, hypoglycemia, cardiac arrest 1</td>
</tr>
<tr>
<td>3907/07</td>
<td>39.5 w + 2 h (39.5 w)</td>
<td>F</td>
<td>1.5/1</td>
<td>3,255/37</td>
<td>380/395 (–)</td>
<td>35.0/50</td>
<td>lung atelectasis, acidosis/adrenaline, bicarbonates 1</td>
</tr>
<tr>
<td>3161/13</td>
<td>40 w + 0 h (40 w)</td>
<td>F</td>
<td>1/1.4</td>
<td>3,100/19</td>
<td>467/387 (–)</td>
<td>34.5/25–50</td>
<td>intrapartum death: umbilical cord prolapse, hypoxic placenta 1</td>
</tr>
<tr>
<td>1846/06</td>
<td>37 w + 34 d (42 w)</td>
<td>F</td>
<td>1/3</td>
<td>3,950/84</td>
<td>635/420 (–)</td>
<td>40.0/&gt;97</td>
<td>bronchopneumonia, reactive hepatitis – seizures, hypotonia/antibiotics, anticonvulsants 1</td>
</tr>
<tr>
<td>2062/07</td>
<td>28 w + 103 d (43 w)</td>
<td>M</td>
<td>0.7/4</td>
<td>2,280/83</td>
<td>283/308 (G+W)</td>
<td>30.5/3</td>
<td>cystic fibrosis, RDS, respiratory infection – irritability, hypotension, hypoxemia, acidosis/surfactant, antibiotics, corticosteroids, anticonvulsants, inotropes, diuretics, sedatives, TPN 3</td>
</tr>
<tr>
<td>1402/04</td>
<td>25 w + 136 d (44 w)</td>
<td>M</td>
<td>3/8</td>
<td>3,000/83</td>
<td>300/380 (G+W)</td>
<td>34.0/3</td>
<td>twin gestation, RDS, renal failure, congenital cystic renal hypoplasia, endocardial fibroelastosis, myocardial ischemia, brain hemorrhage, seizures, multiorgan failure/surfactant, antibiotics, corticosteroids, anticonvulsants, inotropes, diuretics, sedatives, TPN 3</td>
</tr>
<tr>
<td>1286/04</td>
<td>35 w + 67 d (44.5 w)</td>
<td>M</td>
<td>3/12</td>
<td>3,800/25</td>
<td>347/413 (G)</td>
<td>34.5/3</td>
<td>placental insufficiency, respiratory infection, cholestasis, adrenal hypoplasia – profound hypotonia, persistent seizures, absence of reflexes, cardiac arrest/antibiotics, anticonvulsants, sedatives, inotropes (adrenaline), TPN 3</td>
</tr>
<tr>
<td>2325/07</td>
<td>39 w + 49 d (46 w)</td>
<td>F</td>
<td>0.4/3.2</td>
<td>2,890/38</td>
<td>413/356 (G)</td>
<td>33.5/3</td>
<td>RDS, genetic surfactant C deficiency – ventilator dependence, pulmonary hypertension, dyspnea, hypoxemia, agitation, hypotonia/surfactant, antibiotics, sedatives, inotropes, TPN 3</td>
</tr>
<tr>
<td>1163/04</td>
<td>28 w + 130 d (46.5 w)</td>
<td>F</td>
<td>4/0.5</td>
<td>3,100/38</td>
<td>105/387 (G+W)</td>
<td>33.0/3</td>
<td>sepsis, necrotizing enterocolitis, renal-liver failure – seizures, multiorgan failure/surfactant, antibiotics, anticonvulsants, sedatives, inotropes, diuretics, TPN 3</td>
</tr>
</tbody>
</table>

Features in bold are consistent with neonatal hypoxic/ischemic encephalopathy. w = Weeks of gestation; h or d = hours or days of postnatal life; G = gray matter; W = white matter; – = no atrophy; RDS = respiratory distress syndrome; TPN = total parenteral nutrition. 1 Expected brain weight according to the body weight (data from the Women and Infant’s Hospital, Providence, R.I., USA) [45]. 2 Excluding neuropathological findings.
In view of the limitation of working with human autopsy samples and considering that all autopsied neonates who fulfilled the criteria of inclusion sustained some degree of hypoxic insult, true 'controls' deprived of any sign of histopathological hypoxic injury could not be included in the present study. Since we opted to exclude immature brains from our study, as well as mature brains with any signs of maceration due to intrauterine death, in our series of perinatal autopsies practically all the brains that fulfilled the criteria of inclusion were found to have, after detailed microscopic examination, morphological signs of HII, with at least focal neuronal pyknosis, designated as HII group 1. As positive controls, LC paraffin sections of 2 adult subjects were added from our archival collection, a 67-year-old female and 74-year-old male who died with rupture of aortic aneurysm and myocardial infarct, respectively (postmortem delay <24 h; fixation time: 1 month).

**Histology and Immunohistochemistry**

Tissue blocks of the pons at the level of the LC were dissected from the brains, dehydrated in graded alcohol and embedded in paraffin. Seven-micrometer-thick serial sections were collected from the whole rostrocaudal extent of the LC, and one section for every 50 was mounted on silane-coated slides and stained with cresyl violet/luxol fast blue, as previously described [16], to delineate the borders of the LC.

Two consecutive sections every 50, adjacent to those stained for cresyl violet/luxol fast blue, throughout the whole LC, were stained with toluidine to estimate the total number of LC neurons and respectively for TH to reveal the number, distribution and intensity of TH reaction in LC neurons. For toluidine staining, sections were immersed with toluidine to estimate the total number of LC neurons and respectively for TH to reveal the number, distribution and intensity of TH reaction in LC neurons. For toluidine staining, sections were immersed for 2 min in 0.1% toluidine solution (Sigma, 0.1 g toluidine blue in 100 ml alcohol 30%), washed in 50% ethanol, dehydrated in etha-

cresyl violet/luxol fast blue, as previously described [16], to delineate the borders of the LC.

**Table 2. HII groups based on neuropathological criteria**

<table>
<thead>
<tr>
<th>HII groups</th>
<th>Severity/duration/timing</th>
</tr>
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<tbody>
<tr>
<td>Gray matter injury</td>
<td>severe/abrupt, thalamus, basal ganglia</td>
</tr>
<tr>
<td>White matter injury</td>
<td>acute, coagulation necrosis, axonal swellings</td>
</tr>
</tbody>
</table>

The morphometric analysis of the optical density (OD) for TH was performed at the caudal levels of LC (see fig. 40 in Paxinos and Huang [46]), where the noradrenergic neurons are densely packed in a well-defined area of the ventromedial tip of the mesencephalic trigeminal tract. This was necessary in order to identify the boundaries of the nucleus since TH immunoreactivity was very low for some subjects. For all cases studied, at the selected anatomical level, images of all the noradrenergic neurons containing a nucleus and nucleolus were unilaterally captured (LC is bilaterally homogeneous) at a magnification of ×400, using a digital charge-coupled device color video-camera (SSC-C370P; Sony, Tokyo, Japan) connected to an optical microscope (BX50F-3; Olympus, Tokyo, Japan). The measurements of the OD for TH were done using the image analysis software Image-Pro Plus, version 4.5.1.29 (Media Cybernetics, Bethesda, Md., USA).

The morphometric analysis of the cellular and nuclear size of the noradrenergic neurons – used as markers of neuronal development – was performed on two sections stained for HE, one lying at the middle level of the LC (see fig. 44–45 from Paxinos and Huang [46]), which is considered representative of the whole rostrocaudal extent of the LC [47] and another at the caudal LC level (see fig. 40–41 from Paxinos and Huang [46]).

**Statistical Analysis**

The nonparametric Kruskal-Wallis test was performed to assess the effect of perinatal hypoxia and brain atrophy on the variables studied (i.e. OD for TH, cell and nucleus size). A Mann-Whitney U test was used to check possible differences between the neuropathological hypoxia groups, as well as sex differences. The correlation between the above variables and the neuropathological hypoxia grading with the total corrected (prenatal and postnatal) age, postmortem delay, fixation time, body and brain weights, head perimeter, and brain atrophy was investigated using a Spearman bivariate test. p < 0.05 was considered statistically significant. All tests were performed using SPSS, version 18.0.0 (SPSS, Chicago, Ill., USA).

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Results

Our results showed that perinatal HII affected the expression of TH and the size of LC noradrenaline neurons of the human neonate. Differences in both the number and size of TH-IR neurons and the intensity of the TH immunohistochemical reaction were detected in the entire rostrocaudal extent of the LC of each subject and statistically found not to be age or sex dependent.

In HII group 1 (consistent with severe/acute injury), intense or moderate TH staining was observed in almost all the neuronal bodies of the LC, as compared with the adjacent section stained for toluidine (fig. 1, compare a with b). In neonates of HII group 2 (moderate/more prolonged injury), a significant reduction in the intensity of the TH reaction was seen in several LC neurons (fig. 1, compare c with d), while some strongly TH-stained cells were also evident (fig. 1c). In HII group 3 (very severe/long duration or chronic HII), two subgroups were recognized showing opposite results: in neonates GBB 1286/04, 1163/04, and 1402/04, intense TH immunoreactivity was found in almost all LC neurons (fig. 1e), whereas in GBB 2062/07 and 2325/07, no TH reaction was demonstrated in the LC (fig. 1g). In the adjacent toluidine-stained sections, however, a large number of neurons was clearly evident in both subgroups (fig. 1f, h). Interestingly, the 3 cases of HII group 3 showing intense TH immunoreactivity were treated with the anticonvulsant drug phenobarbitone and had longer postnatal ages than the other 2 subjects of the same group.

These differences in TH expression were clearly observed under higher magnification, as shown in figure 2. In the brains of HII group 1, LC neurons appeared similar in size with a very intense TH immunoreactivity in both their neuronal body and processes. In HII group 2, the majority of neurons were lightly stained for TH, while some intensely stained TH-IR neurons and fibers were observed crossing the field (fig. 2b). In HII group 3, heterogeneity was revealed in both the number of TH-IR neurons and the intensity of the TH reaction among the subjects. For example, neonates GBB 1402/04 and GBB 2062/07 belong to the same HII group 3 and have a similar age (44 and 43 weeks, respectively), but present different immunohistochemical results for TH. In the former, the majority of LC neurons was intensely stained for TH (fig. 2c), while in the latter, only one intensely TH-stained cell was evident (fig. 2d). In the adult control, LC section TH immunoreactivity was observed in all neuronal perikarya, as well as in fibers crossing the field (fig. 2e, f).

In HE-stained sections, neurons in the LC of group 1 were uniformly large in size with some hyperchromatic nuclei (fig. 3a, b, arrows), while eosinophilic (degenerating) neurons were only occasionally seen. In HII group 2, small and large neurons were observed, the majority of which had a transparent nucleus (fig. 3c). In group 3, neurons significantly smaller in size were seen compared with those of group 1 cases (fig. 3, compare a with d). Pyknotic LC neurons were often observed in subjects of both groups 2 and 3 (fig. 3c, d, arrows).

The morphometric analysis of the OD for TH in the LC confirmed our qualitative observations analyzed above. The OD values for TH measured in group 2 cases were lower than those of group 1 neonates (fig. 4). In group 3, two subgroups were recognized: one (3 patients) was characterized by high OD values for TH, similar to the pattern seen in HII group 1, and a second subgroup (2 patients) with very low OD for TH – even lower than those measured in the LC of group 2. This variability did not permit our overall results for the OD of TH immunoreactivity to reach statistical significance.

The morphometric analysis of the nucleus and cell size in HE-stained sections revealed that the age or duration of HII can affect the nucleus and cell size of noradrenergic LC neurons (Kruskal-Wallis, p = 0.037, $\chi^2 = 6.595$; p = 0.045, $\chi^2 = 6.189$, respectively). Post hoc analysis using the Mann-Whitney U test showed statistical significant differences in the mean cell size between groups 1 and 3 (p = 0.030; fig. 5a), and also in the mean nucleus size between groups 1 and 3 (p = 0.018; fig. 5b). Similar statistical results were also revealed with Spearman’s test. Neuronal and nucleus size were negatively correlated with the neuropathological estimation of HII (p = –0.660, p = 0.007 and p = –0.675, p = 0.006, respectively), with the

Fig. 1. Two adjacent sections at the caudal level of the LC of cases GBB 2807/07 (37 weeks of age, HII group 1), GBB 1836/06 (39 weeks, HII group 2), GBB 1163/04 (46.5 weeks, HII group 3) and GBB 2062/07 (43 weeks, HII group 3) stained for TH and toluidine, respectively. The limits of the LC are marked with discontinuous line. TH-IR neurons are shown (a, c, e, g), while the same neurons are stained with toluidine in the adjacent section (b, d, f, h). In HII group 1 neonate GBB 2807/07, note the intense TH staining of all noradrenergic neurons (compare a with b). In HII group 2 neonate GBB 1836/06, the majority of neurons exhibit low TH immunoreactivity, while some strongly TH-stained neurons are also evident (e). In HII group 3 neonate GBB 1163/04, almost all of the LC neurons are positive for TH (e), while in GBB 2062/07 of the same HII group, only 2 cells express TH (g, arrows). A large number of neurons are clearly evident in the adjacent toluidine-stained section of both cases (f, h). Black asterisks show blood vessels in adjacent sections. 4V = Fourth ventricle. Scale bar = 150 μm.

(For figure see next page.)
Tyrosine Hydroxylase

2807/07, 37w, Hll group 1

Toluidine

1836/06, 39w, Hll group 2

1163/04, 46.5w, Hll group 3

2062/06, 43w, Hll group 3
Fig. 2. High magnification of TH-IR neurons in the LC of neonates GBB 3907/07 (a, HII group 1), 1965/06 (b, HII group 2), 1402/04 (c, HII group 3) and 2062/07 (d, HII group 3). a Note the very intense TH immunoreactivity in all neurons of GBB 3907/07. b In neonate GBB 1965/06, a reduction of TH staining is observed in the majority of the LC neurons, although some intensely TH-stained neurons are clearly evident. c, d Although the neonates GBB 1402/04 and 2062/07 belong to the same grade 3 group and are of similar age, differences in both the number and the intensity of the TH-IR neurons are found in the LC. In neonate GBB 1402/04, the majority of neurons are intensely stained for TH (c), whereas in neonate GBB 2062/07, only one cell expresses TH immunoreactivity (d). e In the adult control case P2/06 all of the LC neurons were TH-IR. f In higher magnification, note the intense staining for TH, as well as the brown (color in the online version only) neuromelanin granules, a pigment characteristic of catecholamine neurons in the human adult, not present in neonates. BV = Blood vessel. Scale bars = 50 μm for a–d, f and 100 μm for e.
lowest values being measured in cases of HII group 3. A positive correlation was also shown between neuronal size and the brain weight of neonates (p = 0.030, ρ = 0.561), but not with their age. The OD for TH as well as the cellular and nuclear size of the noradrenergic neurons of LC were not found to correlate with sex, postmortem delay or fixation time of the tissue samples, body weight, head perimeter and percentiles.

Our statistical analysis also revealed a negative correlation between the neuropathological scoring of perinatal HII and brain atrophy (p < 0.0001, ρ = 0.859). The cell and nucleus size of the noradrenergic neurons were also found to be negatively correlated with brain atrophy (p = 0.029, ρ = −0.563 and p = 0.026, ρ = −0.570, respectively), i.e. decreased neuronal and nucleus sizes in the LC were noted in neonatal brains with extensive atrophy.

Discussion

In the present study, we showed differences in the size and number, as well as in the intensity of TH immunoreactivity of LC neurons between neonatal brains with lesions of acute or more prolonged and chronic HII. Our statistical analysis showed that these differences were not related to neonatal age, postmortem delay or fixation time of tissues. Intense TH immunoreactivity in almost all the LC neurons was already observed in the youngest neonate of our sample (aged 25.5 weeks) who belonged to the severe/acute HII group 1. Immunohistochemical studies in human fetal tissues using antibodies against TH and/or dopamine-β-hydroxylase showed that noradrenergic neurons appear in the dorsal pons as early as gestational weeks 5–6 [22–24, 48], while at birth almost all LC neurons are TH-IR [25], as occurs in the adult [26, 27].
The presence of TH immunoreactivity in the adult LC was confirmed by the two adult brain specimens used as positive controls.

In the present study, very intense TH immunoreactivity was observed in almost all of the LC neurons of individuals with neuropathological changes of severe/acute perinatal hypoxic damage (group 1), which could be attributed to the activation of the LC neurons. Experimental studies in the fetal and newborn sheep showed activation of the LC in response to acute perinatal hypoxia, as assessed by c-fos immunoreactivity [49, 50]. Among different central chemosensitive nuclei, the LC has the largest percentage (>80%) of neurons excited by high CO₂/H⁺ [30]. This percentage and magnitude of their response are highest in young neonates, as reported in experimental animals [31]. Under normal conditions, TH mRNA levels are normally upregulated in the LC of mice during postnatal age, reaching their highest values 24 h after birth, and thereafter, stabilizing at lower levels [51]. Exposure of 1-day-old mice to acute hypoxia causes a further increase of TH mRNA levels in the LC compared to normoxic pups [51]. 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 [52, 53] and TH mRNA stability [54].

Reduction in the expression of TH was found in LC neurons in the majority of neonates with neuropathological lesions of more prolonged or older hypoxic damage that could not be attributed to loss of LC cells since a large number of neurons were revealed in the adjacent sections stained with toluidine. Interestingly, Chen et al. [55] have shown that perinatal asphyxia can cause long-term changes in the number of TH-IR neurons in the LC of rats. In that study, asphyxia was induced in pups on the last day of gestation using the model of delayed caesarean section introduced by Bjelke et al. [56], in which
the entire uterus including the fetuses was taken out and placed in a water bath for different time intervals, ranging from 15–16 to 21–22 min. In rats subjected to moderate perinatal hypoxia (15–16 min), which is not lethal for the animals, reduction (∼25%) in the number of TH-IR neurons has been observed in the LC at the 4th postnatal week. By increasing the duration of asphyxia, no further decrease in the number of TH-IR neurons was reported, although the survival rate of the pups gradually decreased. The above experiments showed that even after moderate perinatal hypoxia, a long-term reduction in the number of TH-IR neurons is induced in the LC observed later in life [55].

Similar results have been found in 3-week-old rats exposed on the 3rd postnatal day to hypoxia/ischemia [20] induced by unilateral ligation of the carotid artery, an animal model of perinatal hypoxia in humans [57, 58]. In addition to the loss of TH-IR neurons in LC ipsilateral to the ligation, Buller et al. [20] have further reported reduction of the cerebral hemisphere size, as well as decreased dopamine-β-hydroxylase immunoreactivity in the forebrain to the ligated hemisphere, indicating long-term alterations of the volume and the noradrenergic innervation of the cortex after perinatal hypoxia/ischemia. The mechanisms leading to this reduction in TH immunoreactivity in LC neurons after perinatal hypoxia are not currently known. The brainstem does not seem to undergo ischemia at least in animal models that involve ligation of the carotid artery because the brainstem lies outside this vascular network. On the contrary, an increase in its blood flow has been observed during hypoxic/ischemic episodes [59]. Therefore, lesions caused in the brainstem may evolve via secondary retrograde injury mechanisms, like deprivation of trophic factors from the target areas, or other factors, such as inflammation, excitotoxicity and/or loss of synaptic inputs that could contribute to degeneration of neural connections [for review, see 20].

In our sample, discordant results were seen among neonates of HII group 3, with old or chronic hypoxic damage. In 2 out of 5 subjects, low TH immunoreactivity was observed, while in the remaining 3 cases, intense TH staining was revealed in the LC, similar to the pattern of HII group 1 subjects with acute lesions. It is of note that such differences in the number and intensity of TH-IR perikarya among subjects of the same HII group were not evident in the other brain areas containing TH-IR neurons studied in our previous investigations in both the mesencephalon [16] and the hypothalamus of the same series of neonates [41, 42]. Notably, in our previous study the magnocellular neurosecretory neurons in the hypo-

thalamus appeared to induce TH expression under prolonged or chronic hypoxic damage in all the cases of group 3 [42]. The discordance in TH staining of LC neurons within HII group 3 is of unclear significance, in view also of the small subgroups of patients that do not allow safe conclusions to be drawn. The observed differences in LC may reflect interindividual variability, or the influence of factors, other than hypoxia. Another possible explanation could be the repetition of acute hypoxic insults on a background of chronic brain injury in the group 3 patients that showed intense TH immunoreactivity.

Another reason could be the recovery of TH expression in the LC of these 3 infants after prolonged perinatal hypoxia. Shiraishi et al. [60] reported that in rats exposed on the 7th postnatal day (which corresponds to a full-term human neonate) to hypoxia/ischemia, the number of TH-IR neurons in the LC transiently decreased 2 days after the hypoxic episode and normalized 1 week later, implying recovery of TH expression in the LC noradrenergic system after perinatal hypoxia/ischemia.

In accordance with the above hypothesis, it is important to note that these 3 infants had longer postnatal ages (mean age: 111 days) than the other two neonates of the same group (mean age: 76 days), and were medicated with the anticonvulsant drug phenobarbitone (for prevention or due to emergence of seizures – a frequent outcome of hypoxic/ischemic encephalopathy [61]). The combination of a longer postnatal survival time with the administration of anticonvulsants might have positive effects on the recovery of TH expression in the LC. Antiepileptic drugs can modify gene expression, including TH [62] and neurotrophic/neuroprotective proteins, via the regulation of specific transcriptional factors and/or through epigenetic modifications [63–69]. The antiepileptic drugs carisbamate and lamotrigine increase the levels of brain-derived neurotrophic factor (BDNF) in the frontal cortex and hippocampus, and subchronic lamotrigine treatment restores the stress-induced down-regulation of BDNF expression in these brain areas [70, 71]. Since the frontal cortex and hippocampus constitute important target areas of the LC noradrenergic neurons, the increased expression of neurotrophic factors in these brain areas after anticonvulsant treatment could be protective for LC neurons, a hypothesis that should be further investigated.

Dysregulation of the brainstem monoaminergic systems with interindividual variation of TH expression in the LC has been reported in sudden infant death syndrome (SIDS) [72]. Reduction and/or loss of TH staining in the LC has been reported by Lavezzi et al. [25, 47], while...
no differences in TH immunoreactivity in SIDS have been found by other authors [73, 74]. In all neonates of our series with changes of more prolonged or older HII, viable LC neurons appeared significantly smaller in size (compared to those of acute HII group 1), possibly indicating atrophy or delayed or temporarily arrested development of the human noradrenergic neurons that could render them more plastic later in life [75, 76]. The observed reduction in cell size, as well as in TH expression, appears to be functional and unrelated to factors such as the postmortem delay or fixation time of the tissues since in the hypothalamus of the same subjects, a dramatic increase in the neuronal size and induction of TH expression was observed likely due to activation of neurosecretory neurons after prolonged perinatal hypoxia [41, 42]. Chronic hypoxia causes an adaptive reduction in metabolism [77] and an increase in the number of α2-somatodendritic inhibitory autoreceptors in the LC [78], events that may contribute to the observed reduction in cell size, as well as to the reduction in TH staining in LC neurons of neonates with changes of prolonged, older or chronic HII. In the elderly, when a gradual reduction in brain blood flow emerges [79, 80], smaller TH-IR neurons have been described in the LC [26].

In summary, despite the inevitable heterogeneity of our sample with regard to the gestational/postnatal age, underlying congenital conditions and medical interventions, and despite the difficulties in dissociating the effect of all varieties of stress sustained by the human fetus during gestation and labor, we provide evidence that prolonged or older perinatal HII may cause a reduction in TH immunoreactivity and size of noradrenaline LC neurons, indicating atrophy or delayed or temporarily arrested development of this system in the human neonate. Moreover, in some neonates with prolonged, older or chronic HII lesions, we observed intense TH immunoreactivity in the LC, suggesting that longer survival time alone or in combination with anticonvulsant treatment may have a neuroprotective role to LC neurons. Since the LC may be involved in the arousal of the newborn and the first breaths after birth [81], decreased LC activity – caused by severe perinatal hypoxic/ischemic events – might be the underlying cause for the reduced alertness observed in severe hypoxic neonates [82]. In view also of experimental evidence that perinatal hypoxia causes a reduction of noradrenaline LC [20] as well as serotonergic raphe neurons [21, 83] that persists into adulthood, we suggest that early dysregulation of monoaminergic neurotransmission in critical periods of brain development in humans, in combination with genetic susceptibility, may predispose the survivors of perinatal hypoxia to psychiatric and/or neurological disorders, such as major depression, Parkinson’s disease and Alzheimer’s disease, later in life [1, 12, 84].

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Disclosure Statement

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References


