Increased Expression of Tyrosine Hydroxylase in the Supraoptic Nucleus of the Human Neonate Under Hypoxic Conditions: A Potential Neuropathological Marker for Prolonged Perinatal Hypoxia

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INTRODUCTION

Magnocellular neurosecretory neurons in the hypothalamic paraventricular (PVN) and supraoptic (SON) nucleus synthesize the 2 major biologically active peptide hormones, vasopressin (VP) and oxytocin (OXY); these were originally purified and sequenced by Du Vigneaud et al (1, 2). In the rat hypothalamus, histochemical and in situ hybridization studies showed that, under experimental activation of the hypothalamic neurohypophyseal system (HNS), magnocellular neurons synthesize additional peptide and nonpeptide putative neurotransmitters that coexist with VP and OXY (3–6).

Tyrosine hydroxylase (TH), the first and rate-limiting enzyme in the catecholamine synthetic pathway (7), has been identified using immunohistochemistry (IHC) in human neurosecretory neurons in which it was found to colocalize with OXY (8, 9) and/or VP (10, 11). In the adult human hypothalamus, TH expression within the PVN and SON shows striking interindividual variability that depends on neuronal activation (12–14). In a large sample of adult subjects without known neurological, psychiatric, or endocrinological disease, many TH-immunoreactive (-IR) perikarya were observed specifically in subjects who experienced right-sided heart failure due to pulmonary hypertension, liver cirrhosis or dehydration, that is, somatic diseases that lead to increased production and secretion of VP due to a decrease in ‘effective’ blood volume or to osmotic stimulation (12, 13).

In the developing human diencephalon, TH expression is found in some neurons of the ventral hypothalamic anlage from the sixth gestational week onward (15, 16). In premature human neonates, only a very limited number of TH-IR neurons scattered within the PVN and SON has been detected, whereas in full-term infants, there was increased expression of TH (17) mainly within magnocellular VP-synthesizing neurons (10, 11). Because most of the full-term neonates had died of perinatal hypoxia, the question arose as to whether the increased expression of TH in the PVN and SON of full-term human neonates represents a primary developmental phenomenon (as in discrete neuronal populations during early embryonic stages [15, 16]) or reflects a secondary phenomenon...
related to hypoxia-induced activation of the VP systems (10, 11, 17). Inasmuch as the latter option could have diagnostic consequences (18), we investigated the expression of TH in neurosecretory neurons of the human neonate in relation to the age and the severity/duration of perinatal hypoxia estimated on the basis of clinical data and neuropathological assessment.

We focused on the dorsolateral supraoptic nucleus (dl-SON) because it is the main source of plasma VP and approximately 95% of its neurons in humans synthesize VP. Moreover, VP and OXY neurons in the dl-SON can be easily separated because VP-synthesizing neurons are localized in the central part, whereas OXY-synthesizing neurons are located in the dorsal boundaries of this nucleus (18). If the induction of TH in neurosecretory neurons of the human neonate depends on sustained fetal hypoxia, increased expression of TH in VP-synthesizing neurons could represent a neuropathological marker for prolonged perinatal hypoxia in autopsy material.

**MATERIAL AND METHODS**

**Patients and Tissues**

Formalin-fixed hypothalami of 15 autopsied infants (5 females and 10 males) were obtained from the Department of Pathology of the National Kapodistrian University of Athens, Greek Brain Bank (GBB, member of the BrainNet Europe), directed by Prof E. Patsouris. Most infants (13/15) were delivered by caesarian section, 8 were born prematurely before the 36th week of gestation, and 7 were delivered at or near term. The total corrected age (duration of pregnancy + postnatal age) ranged from 34 to 46.5 weeks. In view of the limitation of working with human autopsy samples and considering that all neonates who come to autopsy may sustain some degree of hypoxic insult, “controls” 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. The grading of the severity or duration of the hypoxic injury was based on established histopathological criteria (19–21), and 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 and the neuropathological grading are presented in Table 2.

**Immunohistochemistry**

Hypothalami were dissected from the brain, dehydrated in graded alcohol, and embedded in paraffin. Seven-micrometer-thick serial sections from throughout the whole hypothalamus were collected, and 1 section for every 100 was mounted on silane-coated slides and stained using IHC for neurophysin (NP) to determine the boundaries of the neurosecretory nuclei.

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

<table>
<thead>
<tr>
<th>Injury</th>
<th>Severity/Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gray matter injury</td>
<td>Severe/Abrupt</td>
</tr>
<tr>
<td>Topography of neuronal necrosis</td>
<td>Thalamus</td>
</tr>
<tr>
<td>Putamen</td>
<td>Thalamus</td>
</tr>
<tr>
<td>Putamen</td>
<td>Glial scar</td>
</tr>
</tbody>
</table>

**White Matter Injury**

<table>
<thead>
<tr>
<th>Histopathological findings</th>
<th>Acute</th>
<th>Subacute</th>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulation necrosis</td>
<td></td>
<td>Endothelial cell hyperplasia</td>
<td>Glial scar</td>
</tr>
<tr>
<td>Axonal swellings</td>
<td></td>
<td>Microglial proliferation</td>
<td>Caviation</td>
</tr>
<tr>
<td>Glial scar</td>
<td></td>
<td>Microcalcifications</td>
<td>Reactive gliosis</td>
</tr>
</tbody>
</table>

Consecutive sections (n = 4) at 3 central levels of the dl-SON (i.e. levels with the largest number of neurosecretory neurons per case), with a constant 700-μm distance between them were stained as follows. Section 1 was stained for NP using a polyclonal anti-NP serum (1:2000; DAKO, Carpinteria, CA) to delineate the neurosecretory nuclei. Section 2 was stained for TH using a polyclonal anti-TH serum (1:1000; Institut de Biotechnologie Jacques Boy, Reims, France) to estimate the number and distribution of the TH-IR neurons. Section 3 was stained for VP using a monoclonal anti-VP antibody (MAB III D7, 1:100; kindly donated by Dr Anna Hou-Yu, Columbia University, New York, NY) to count VP-synthesizing neurons. Section 4 was first stained for OXY using a monoclonal anti-OXY antibody (MAB 1-28, 1:100; also kindly donated by Dr Anna Hou-Yu) and counterstained with thionin to localize the OXY-synthesizing neurons and exclude them from counting. The specificity of the IHC reactions was assessed elsewhere (9, 10).

For detection of NP and TH, sections were rehydrated, washed in Tris-buffered saline (TBS; 0.05 M Tris, 0.15 mol/L NaCl, pH 7.6) for 2 × 10 minutes, and incubated in the primary antibody in Supermix (TBS with 0.25% gelatin, 0.5% Triton, pH 7.6) for 1 hour at room temperature (RT) and subsequently overnight at 4°C. Sections were then washed 2 × 10 minutes in TBS and incubated in the secondary polyclonal goat anti-rabbit antibody (AKON, 1:100; Netherlands Institute of Neuroscience) in Supermix for 1 hour at RT. Sections were then washed 2 × 10 minutes in TBS and incubated in peroxidase-antiperoxidase complex (1:1000; Netherlands Institute of Neuroscience) in Supermix for 1 hour at RT. After 2 × 10-minute washes in TBS and 15 minutes in 0.1 mol/L Tris-HCl buffer (pH 7.6), sections were incubated in 0.5 mg/mL DAB (Sigma, St Louis, MO) in Tris-HCl (pH 7.6) containing 0.02% nickel ammonium sulfate (DAB-Ni; Johnson Matthey Afla Products, London, UK) and 0.01% H2O2, then washed 2 × 10 minutes in water, dehydrated, and mounted in DPX (BDH, Poole, UK).
TABLE 2. Clinical and Pathological Data of Cases Studied, Neuropathological Grading of Perinatal Hypoxia and Estimate of Numbers of TH-IR/VP-Synthesizing Neurons in the Dorsolateral Supraoptic Nucleus

<table>
<thead>
<tr>
<th>GBB No</th>
<th>Age (wk, d, h) (Corrected Age, wk), Sex</th>
<th>Postmortem Delay (d)/Fixation Time (mo)</th>
<th>Body Weight (g)/Percentile</th>
<th>Brain Weight (g)</th>
<th>Head Perimeter/Percentile</th>
<th>Clinical and Pathological Data*/Medications</th>
<th>Hypoxia Grade</th>
<th>Density of TH-IR/VP Neurons in dl-SON</th>
</tr>
</thead>
<tbody>
<tr>
<td>2226/07</td>
<td>27 wk + 52 d (34 wk), F</td>
<td>&lt;1/2.5</td>
<td>1370/3</td>
<td>198</td>
<td>ND</td>
<td>RDS, BPD, septicemia, renal failure/</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Surfactant, antibiotics, inotropes, diuretics, sedatives, TPN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1149/04</td>
<td>35 wk + 17 h (35 wk), M</td>
<td>ND/2</td>
<td>3150/90</td>
<td>382</td>
<td>34.5/90</td>
<td>RDS, meconium aspiration/</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Antibiotics, sedatives</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1139/03</td>
<td>34 wk + 21 d (37 wk), M</td>
<td>3/8</td>
<td>2880/25-50</td>
<td>275</td>
<td>28.5/3</td>
<td>Down syndrome, congenital cyanotic heart defect, hypotension and bradycardia episodes/</td>
<td>3</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Antibiotics, inotropes, TPN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2807/07</td>
<td>37 wk + 0 h (37 wk), M</td>
<td>2/2.5</td>
<td>2445/10-25</td>
<td>444</td>
<td>32.5/25-30</td>
<td>Acute thrombosis of the umbilical vein</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>1705/05</td>
<td>37 wk + 8 d (38 wk), M</td>
<td>2/2</td>
<td>2600/10</td>
<td>345</td>
<td>32.5/10-25</td>
<td>Genetic thrombophilia, thrombosis of the descending aorta, thrombotic vasculitis, meconium aspiration, hypotension, hyperglycemia/</td>
<td>2</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Antibiotics, adenrenal, dopamine, inotropes, TPN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1965/06</td>
<td>39 wk + 2 h (39 wk), M</td>
<td>2/1</td>
<td>2744/10</td>
<td>337</td>
<td>34/50</td>
<td>Congenital cyanotic heart defect/</td>
<td>2</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adrenaline, bicarbonates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1836/06</td>
<td>35 wk + 29 d (39 wk), M</td>
<td>3/8</td>
<td>1950/3</td>
<td>310</td>
<td>31/3</td>
<td>Ivemark syndrome (asplenia – congenital cyanotic heart defect – heterotaxy)/Antibiotics, inotropes, adenrenal, TPN</td>
<td>2</td>
<td>+++</td>
</tr>
<tr>
<td>3907/07</td>
<td>39.5 wk + 2 h (39.5 wk), F</td>
<td>1.5/1</td>
<td>3255/50</td>
<td>380</td>
<td>35/50</td>
<td>Congenital infection, lung atelectasis/</td>
<td>1</td>
<td>+/−</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adrenaline, bicarbonates</td>
<td></td>
<td></td>
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<tr>
<td>2155/07</td>
<td>40 wk + 0 h (40 wk), F</td>
<td>3/1.7</td>
<td>3158/50</td>
<td>422</td>
<td>35/50</td>
<td>Intrapartum asphyxia, meconium aspiration</td>
<td>1</td>
<td>++</td>
</tr>
<tr>
<td>1593/05</td>
<td>41 wk + 1 d (41 wk), M</td>
<td>2/2</td>
<td>3120/10-25</td>
<td>380</td>
<td>33/3-10</td>
<td>Congenital cyanotic heart defect, congenital viral infection, meconium aspiration/Antibiotics, inotropes, adrenaline,</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>bicarbonates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2062/07</td>
<td>28 wk + 103 d (43 wk), M</td>
<td>0.7/4</td>
<td>2280/3</td>
<td>283</td>
<td>30.5/3</td>
<td>Cystic fibrosis, RDS, respiratory infection/Surfactant, antibiotics, inotropes, diuretics, sedatives, TPN</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td>1402/04</td>
<td>25 wk + 136 d (44 wk), M</td>
<td>3/8</td>
<td>3000/3</td>
<td>300</td>
<td>34/3</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>
<td>+++</td>
</tr>
<tr>
<td>1286/04</td>
<td>35 wk + 67 d (44.5 wk), M</td>
<td>3/12</td>
<td>3800/25</td>
<td>347</td>
<td>34.5/3</td>
<td>Placental insufficiency, respiratory infection, cholestasis, adenral hypoplasia/Antibiotics, anticonvulsants, sedatives, inotropes, adrenalin, TPN</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td>2325/07</td>
<td>39 wk + 49 d (46 wk), F</td>
<td>0.4/3.2</td>
<td>2890/3</td>
<td>413</td>
<td>33.5/3</td>
<td>RDS, genetic surfactant C deficiency/Surfactant, Antibiotics, sedatives, inotropes, TPN</td>
<td>3</td>
<td>++</td>
</tr>
<tr>
<td>1163/04</td>
<td>28 wk + 130 d (46.5 wk), F</td>
<td>0.4/0.5</td>
<td>3100/3</td>
<td>105</td>
<td>33/3</td>
<td>Sepsis, ischemic enterocolitis, renal – liver failure, nephromegaly with glomerular cysts/Surfactant, antibiotics, anticonvulsants, sedatives, inotropes, diuretics, TPN</td>
<td>3</td>
<td>+++</td>
</tr>
</tbody>
</table>

Relative differences in density of TH-IR/VP neurons: +/- = less than 5%, + = 5% to 25%, ++ = 25% to 50%, +++ = 50% to 75%, and ++++ = 75% to 100% of TH-IR/VP-synthesizing neurons.

*Excluding neuropathological findings.
BPD indicates bronchopulmonary dysplasia; d, days; dl-SON, dorsolateral supraoptic nucleus; F, female; GBB, Greek Brain Bank; h, hours of postnatal life; M, male; ND, not determined; RDS, respiratory distress syndrome; TH-IR/VP, tyrosine hydroxylase–immunoreactive/vasopressin-synthesizing; TPN, total parenteral nutrition; wk, weeks gestation.
For the visualization of VP and OXY, after incubating in primary antibodies, sections were washed in TBS 2 \times 10 \text{ minutes} and incubated in biotinylated antimouse IgG (H + L) (1:100, BA-9200; Vector Laboratories, Burlingame, CA) in Supermix for 1 hour at RT. After 2 \times 10\text{-minute} washes in TBS, sections were incubated with avidin-biotin complex (1:400, Vectastain ABC KIT, Vector Laboratories) in Supermix for 1 hour at RT and stained with DAB-Ni as previously described.

Because our previous double label IHC (10) and in situ hybridization (11) studies clearly showed that, in the human neonate, almost all TH-IR neurons are VP-synthesizing, the relative number of TH-IR/VP-synthesizing neurons was estimated semiquantitatively at 3 central levels across the rostrocaudal extent of the dl-SON on adjacent TH- or VP-stained sections of each subject by 3 independent observers (V.G., M.A.P., and M.T.P.), who did not know the source of the tissue samples. The cases were rated using a 1 to 4 scale as follows: +/− when less than 5% of the observed VP-synthesizing neurons at each level were TH-IR, and from + to ++++ when the percentage increased to 5% to 25%, 25% to 50%, 50% to 75%, and 75% to 100%, respectively.

For statistical analysis, the Spearman bivariate test was used to determine the correlation between the density of TH/VP-synthesizing neurons and the histopathological grading of hypoxic injury. The same statistical test was used to examine any correlation of the density of TH-IR neurons in dl-SON with total, prenatal and postnatal age, postmortem delay, fixation time, body and brain weight, head diameter, and percentile. \( p < 0.05 \) was considered significant.

**RESULTS**

Hypoxic injury was classified as grade 1 (clinically correlating to severe/acute hypoxia) in 4 cases, as grade 2 (subacute/prolonged hypoxia) in 3 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. In all cases, the IHC reactions for NP, VP, and OXY permitted the delineation of the rostrocaudal extension of dl-SON and the characterization of the peptidergic neurons according to their location and their VP- or OXY protein content (delineation of dl-SON, Fig. 1). OXY-IR neurons (stained black with DAB-Ni) were mainly located in the dorsal limits of the dl-SON and in the accessory (acc) SON; VP neurons (stained blue with thionin in the same section) occupied the central part of this nucleus (Figs. 1A, B).

In most cases, the intensity of NP staining between the VP and OXY parts of the dl-SON was different. Neuropeptide staining was intense in OXY neurons in the dorsal limits of the dl-SON but was lighter in VP neurons located at the center of this nucleus. This difference was more striking in grade 1 cases, for example, in GBB 1149/04 (Figs. 1, compare C with D), GBB 2807/07, GBB 3907/07, and GBB 2155/07. In grade 3 cases, the difference in the NP staining intensity between the OXY and VP part of the dl-SON was less remarkable, for example, in GBB 1286/04 (Fig. 1D). Immunohistochemical reaction for VP was evident in most of the cases studied, but in cases GBB 1836/06 and GBB 2155/07, VP staining in the dl-SON was very light (not shown).

There was a striking difference between grade 1 and grade 2 or grade 3 cases for TH staining. The semiquantitative estimate of the relative number of TH-IR/VP neurons among the cases studied is depicted in Table 2. Subjects with hypoxic injury grade 1 showed a limited number of lightly stained TH-IR/VP neurons throughout the rostrocaudal extent of the dl-SON (Fig. 1E). The expression of TH in VP-synthesizing neurons of dl-SON dramatically increased as perinatal hypoxia was prolonged. The largest number of TH-IR/VP-synthesizing neurons was observed in infants with hypoxic injury grade 2, with almost all VP neurons expressing TH in all studied cases. Most (usually up to 75%) VP-synthesizing neurons expressed TH in infants with hypoxic injury grade 3 (Fig. 1F).

The expression of TH in VP-synthesizing neurons was not related to the age of the subjects because even in the youngest neonate of our sample GBB 2226/07 (corrected age, 34 weeks) with hypoxic injury grade 3 there was an increased density of TH-IR neurons. By contrast, in the dl-SON of older infants (i.e. GBB 1149/04 [35 weeks], GBB 2807/07 [37 weeks], GBB 3907/07 [39.5 weeks], and GBB 2155/07 [40 weeks]) with hypoxia grade 1, only limited numbers of TH-IR neurons were found (Figs. 2A, B). Among subjects with similar ages, the size of TH-IR neurons appeared to increase with increasing grade of neuropathological injury. For example, case GBB 1139/03 (age 37 weeks, grade 3) contained larger TH-IR neurons (Fig. 2D) than those of case GBB 1705/05 (age 38 weeks, grade 2; Fig. 2C) and much larger than those of the older infant GBB 3907/07 (age 39.5 weeks, grade 1; Fig. 2A). No multinucleated neurons or neuronal swelling with neuronolysis was observed in the neurosecretory nuclei of the infants studied.

There was a strong positive correlation between the density of TH-IR neurons in the VP part of the dl-SON and hypoxia grade. Comparing grade 1 with grade 2 cases, Spearman \( r_s \) was 0.92 (\( p < 0.01 \)); the difference between grade 1 and grade 3 subjects was also significant (\( r_s = 0.84, p < 0.01 \)). There was no difference between grade 2 and 3, that is, both groups had subjects who had experienced prolonged perinatal hypoxia (\( r_s = 0.25 \)). Numbers of TH-IR neurons did not depend on total, prenatal or postnatal age, postmortem delay or fixation time of the tissue samples, body or brain weight, head perimeter, or percentile of the neonates. Head perimeter percentiles were inversely correlated to the degree of neuropathological injury (\( r_s = -0.6, p < 0.05 \)).

**DISCUSSION**

Our results indicate that the expression of TH in VP neurons of the human neonate does not represent a primary developmental phenomenon but reflects a secondary phenomenon related to the activation of VP systems due to the hypoxic conditions sustained by the infants. In subjects of approximately the same ages, increased TH expression was observed only in VP-synthesizing neurons of infants who experienced long durations of perinatal hypoxia. These VP neurons appeared activated as indicated by their increased neuronal and nuclear size, the generally accepted histological markers of activated neurosecretory neurons (22–25).
After the expulsion phase of labor in humans, VP levels in cord blood reached values that are in the highest range ever found in physiology (26–28). The VP levels in cord blood after normal delivery are much higher than after elective cesarean delivery not in labor, indicating that, during labor, there is active secretion of VP by the fetus, which is induced by stress, hypoxia, or rise in intracranial pressure (26, 27). This is believed to be an adaptive mechanism intended to redistribute...
cardiac output to vital organs such as the brain, heart, and adrenals (29). Although it is difficult to dissociate the effects of all types of stress sustained by the human fetus during labor, the observed correlation between the density of TH-IR/VP neurons and duration of perinatal hypoxia suggests that hypoxic insults are responsible for activity-related induction of TH in VP neurons in the human neonate. Other antemortem factors known to activate VP release (e.g. dehydration, subarachnoid hemorrhage, or congenital heart defects) that may lead to prolonged osmotic or nonosmotic stimulation of VP release (10, 12, 13) could have an additional role in activation of VP neurons as reported in experimental animals (30, 31) and therefore, in increased expression of TH. Indeed, in the present study, increased TH expression was observed in the group of

![Figure 1](image1.png)

**FIGURE 1.** Representative sections of the dorsolateral supraoptic nucleus (dl-SON), of case GBB 1149 (hypoxia grade 1) (A, C, E) and case GBB 1286 (hypoxia grade 3) (B, D, F). (A, B) Vasopressin (VP) neurons (blue) are located in the central part of the dl-SON; oxytocin (OXY) neurons (black) are located in the dorsal boundaries of the nucleus and in the accessory (acc) SON. (C, D) In (C), VP-synthesizing neurons in the center of the dl-SON are very lightly stained for neurophysin (NP); OXY-synthesizing neurons in the dorsal borders of dl-SON are intensely stained. In (D), this difference is less remarkable. (E, F) There are striking differences between the cases in numbers of TH-IR/VP-synthesizing neurons in the central part of the dl-SON. Only a few lightly stained TH-IR neurons are evident in (E), whereas in (F), most VP neurons in the central part of the dl-SON are intensely TH-immunoreactive. Magnification: 40×; bar = 150 μm.

![Figure 2](image2.png)

**FIGURE 2.** Tyrosine hydroxylase-immunoreactive (TH-IR) neurons in the vasopressin (VP) part of the dorsolateral supraoptic nucleus (dl-SON) of 4 subjects of different ages and with different neuropathological hypoxia grades. (A, B) A few lightly stained TH-IR neurons are evident in subjects GBB 3907/07 (A; aged 39.5 weeks) and GBB 1149/04 (B; aged 35 weeks), both with hypoxia grade 1. (C, D) Numerous intensely stained TH-IR neurons are evident in GBB 1705/05 (aged 38 weeks, hypoxia grade 2) (C) and GBB 1139/03 (aged 37 weeks, hypoxia grade 3) (D). The increased cellular and nuclear size of TH-IR neurons is not related to the age of the subjects but depends on the severity/duration of the sustained hypoxia. Tyrosine hydroxylase–IR perikarya are much larger in these cases than in the subjects who experienced severe but abrupt perinatal hypoxia (A, B). Magnification: 200×; bars = 50 μm.
neonates with hypoxic injury grade 2 in which all infants had congenital heart defect or hypotension that might have additionally activated VP neurons due to nonosmotic stimulation.

It has been reported that the PVN and SON only rarely exhibit hypoxic changes as revealed by conventional histological techniques (32). In agreement with this, no neuronal swelling or neuronolysis were observed in the neuroendocrine nuclei of the cases studied. Multinucleated VP neurons that have been associated with pulmonary pathology, including hypoxia in the adult (33), were not found. In the present study, we demonstrated qualitative changes in TH expression in the neurosecretory neurons of neonates due to perinatal hypoxia. Tyrosine hydroxylase is an oxygen-requiring enzyme that catalyzes the first and rate-limiting step in catecholamine synthesis; kinetic properties suggest that oxygen availability may limit brain catecholamine synthesis. However, TH loses its oxygen dependency during physical stress, indicating that substrate limitation can be overcome when neuronal needs are increased (34, 35). PC-12 pheochromocytoma cells cultured under hypoxic conditions showed increased TH activity that is attributed to increased TH protein synthesis (36) and hypoxia increases TH mRNA and TH protein in carotid body (34, 35). In neonates, TH protein levels (3, 5, 6, 45–49). Increased expression of TH protein in these neurons was combined with a parallel increase in VP mRNA but a decrease in VP protein cytoplasmic content due to increased release of this peptide from the neurohypophysis (3, 5, 47, 48). These data could explain the observed low cytoplasmic staining for VP, selectively in the VP part of dl-SON of the subjects studied, which could be due to acute increased demands for VP release to the periphery.

In the rat hypothalamus, under prolonged dehydration, TH mRNA synthesis was induced in parallel with the increased synthesis of VP mRNA, and the increased TH protein levels remained high in the cell body of the activated VP neurons even 2 days after rehydration (50). In adult humans, TH expression within magnocellular neurosecretory neurons was increased under clinical conditions leading to prolonged osmotic or nonosmotic stimulation of VP release and correlated with increased VP mRNA synthesis, as indicated by in situ hybridization and real-time PCR studies (13, 14). Hypoxia is a regulator of the 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) regulate the rate of TH gene transcription via their binding at specific cis-regulatory sequences located in the proximal 5′ region of the TH promoter (51). Hypoxia also regulates TH gene expression at the level of mRNA stability. A 27-bp-long pyrimidine-rich sequence within the 3′ untranslated region of the TH mRNA transcript, called the hypoxia-inducible protein binding sequence, binds protein factors in a hypoxia-inducible manner (52, 53).

Our observation that TH expression is related to the activation of VP neurons due to perinatal hypoxia supports our previous suggestion that TH in the human magnocellular neurons might have an active role in catecholamine synthesis (13, 14); therefore, it should not be considered to be an incidental ectopic expression of a catecholamine-synthesizing enzyme (49, 54). In the adult human hypothalamus, TH is indeed active in neurosecretory neurons because it is coexpressed with GTP cyclohydrolase, the first enzyme for synthesis of tetrahydropterin, the essential cofactor of TH (14). Because of the apparent limited expression of aromatic l-amino acid decarboxylase in these neurons, we suggested that under activation of the HNS, dopamine might be produced in human neurosecretory neurons, although the possibility that l-DOPA is the final product cannot be excluded (14). Dopamine could alternatively be synthesized at the level of the neurohypophysis by aromatic l-amino acid decarboxylase, which is known to be present in blood vessel walls (55, 56). Because dopamine can act as a VP-inhibiting factor in humans (57, 58), if it is indeed produced and secreted by the neurosecretory neurons under hypoxic conditions, this could have important clinical implications. Through massive inhibition of VP release, it might induce central diabetes insipidus, which has been reported to occur in patients who experienced severe hypoxic encephalopathy (59–61) or carbon monoxide poisoning (62, 63). The neurosecretory nuclei of the homozygous Brattleboro rat, a mutant deficient in VP synthesis that is used as an experimental model of central diabetes insipidus (64), contain a large number of magnocellular TH-IR neurons (45). An additional finding of our study was that head perimeter percentile was inversely correlated to the degree of neuropathological injury, apparently reflecting the role of severe/prolonged hypoxia in causing brain atrophy and microcephaly.

In summary, despite the limitations of an autopsy study and the complexity of the perinatal hypoxia events, our results indicate that the increased expression of TH in VP-synthesizing neurons of the human neonate (a) is observed selectively in the hypothalamus of neonates who experienced prolonged perinatal hypoxia; (b) is not related to the age, body weight/percentile, brain weight, or head perimeter of the subjects but depends on the severity/duration of the hypoxic injury (as determined by the neuropathological analysis); and (c) is found in VP-synthesizing neurosecretory neurons with increased cellular and nuclear size, that is, in neurons with histological evidence of activation. Tyrosine hydroxylase expression was observed to be very low or almost absent in the neurosecretory neurons of infants with acute only and limited neuropathological injury (grade 1). Tyrosine hydroxylase expression was observed to increase dramatically in association with prolonged or chronic hypoxic injury, either as a result of continuously sustained hypoxia or as the end effect of multiple recurrent hypoxic episodes. Taken together, the observations

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prompt us to propose the increased expression of TH in SON neurons of the human neonate as a potential neuropathological marker of prolonged perinatal hypoxia in autopsy material.

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