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Immunohistochemical demonstration of urocortin 1 in Edinger–Westphal nucleus of the human neonate: Colocalization with tyrosine hydroxylase under acute perinatal hypoxia

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HIGHLIGHTS

- In the human EW nucleus, UCN1 is expressed at least from 34 weeks of gestation.
- No UCN1-expression was found in SN and VTA of the human neonate.
- In EW, UCN1 expression was positively correlated to the age of the neonates.
- No correlation was found between UCN1 and neuropathological grade.
- UCN1 colocalized tyrosine hydroxylase in EW mainly under acute perinatal hypoxia.

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ABSTRACT

Perinatal hypoxia could cause long-term disturbances of the dopaminergic (DA) systems, leading to behavioral and/or neurological deficits later in life. Increased expression of tyrosine hydroxylase (TH) was shown in the substantia nigra (SN) and ventral tegmental area (VTA) of human neonates that suffered severe/acute perinatal hypoxic insults, but also in all neurons of the Edinger–Westphal nucleus (EW). Since EW, in humans, contains urocortin 1 (UCN1)/centrally projecting neurons (EWcp), we investigated: (a) the development of UCN1-positive neurons and the possible effect of neonatal hypoxic/ischemic encephalopathy on UCN1 expression and (b) the possible colocalization of UCN1 with TH in neonates with histological signs of acute hypoxic injury. Our results showed that in EWcp of the human neonate, UCN1-immunoreactivity was already evident from 34 weeks of gestation onwards at very low levels. No UCN1-immunoreactivity was found in neurons of SN or VTA. In EWcp, a positive correlation was found between UCN1 expression and the age of the neonates, but not with hypoxia neuropathological grade. UCN1 was colocalized with TH in most EWcp neurons. Since UCN1 in EWcp may play a significant role in stress adaptation and consequently in stress-related disorders, the role of catecholamine synthesis in this nucleus under acute hypoxic conditions must be further investigated.

1. Introduction

Perinatal hypoxic/ischemic injury remains a major cause of mortality and morbidity capable of generating permanent neurological and/or mental deficits later in life, such as supranuclear palsy, mental retardation, learning, language and memory disabilities [32], some types of parkinsonism in childhood [33] or Parkinson's disease later in life [4], attention deficit hyperactivity disorder [31] and schizophrenia [7]. Experimental animal models of perinatal hypoxia have shown that hypoxic/ischemic lesions can cause long-term disturbances of the central dopaminergic (DA) systems persisting into adulthood [6]. Our previous immunohistochemical

Abbreviations: ChAT, choline acetyltransferase; CRF, corticotropin-releasing factor; DA, dopaminergic; DAB, 3,3′-diaminobenzidine tetrahydrochloride; EW, Edinger–Westphal nucleus; EWcp, EW centrally projecting population; EWpg, EW preganglionic population; GBB, Greek Brain Bank; IR, immunoreactive; SEM, standard error of means; SN, substantia nigra; TH, tyrosine hydroxylase; UCN1, urocortin 1; VTA, ventral tegmental area.

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Table 1
Clinicopathological data in 15 infants with neonatal encephalopathy.

GBB no.	Age (w, d, h) (corrected age, w), sex	Body weight (g)/percentile	Brain weight (g)/head perimeter (cm)	Neuropathological grade
2226/07	27w + 52d (34w) F	1370/<3	198/ND	3
2807/07	37w + 0 h (37w) M	2445/10–25	444/32.5	1
2631/09	38w (38w) F	3970/>97	392/36.5	1
1705/05	37w + 8d (38w) M	2600/10	345/32.5	2
1965/06	39w + 2 h (39w) M	2744/10	337/34	2
2735/09	39w + 2d (39w) F	2960/10	313/32	1
1836/06	35w + 29d (39w) M	1950/<3	310/31	2
3907/07	39.5w + 2 h (39.5w) F	3255/50	380/35	1
1593/05	41w + 1d (41w) M	3120/10–25	380/33	3
1846/06	37w + 34d (42w) F	ND	635/40	1
2062/07	28w + 103d (43w) M	2280/<3	283/30.5	3
1402/04	25w + 136d (44w) M	3000/<3	300/34	3
1286/04	35w + 67d (44.5w) M	3800/25	347/34.5	3
2325/07	39w + 49d (46w) F	2890/<3	413/33.5	3
1163/04	28w + 130d (46.5w) F	3100/<3	105/33	3

Abbreviations: d, days of postnatal life; F, female; GBB, Greek Brain Bank; h, hours of postnatal life; M, male; ND, not determined; w, weeks of gestation.

study in human autopsy material [29] showed increased expression of tyrosine hydroxylase (TH, the first and rate limiting enzyme of catecholamine synthesis) in the well-known DA cell groups of the mesencephalon substantia nigra (SN) and ventral tegmental area (VTA), but also in almost all the neurons of the Edinger–Westphal nucleus (EW) of neonates who suffered acute perinatal hypoxia. In neonates who suffered prolonged perinatal hypoxia, however, a striking reduction or even absence of TH-immunoreactivity was observed. This was accompanied by decrease in neuronal size, indicating delayed or temporarily arrested development or even depicting early signs of degeneration in these DA cells [29].

The Edinger–Westphal nucleus was traditionally considered as a cholinergic, parasympathetic preganglionic structure associated with pupil constriction and lens accommodation [11,42]. Comparative studies, however, revealed that it is segregated into two fundamentally distinct neuronal subgroups: the "EW preganglionic population" (EWpg) that consists of neurons that are choline acetyltransferase (ChAT)-positive and project to the ciliary ganglion and the "EW centrally projecting population" (EWcp) that contains neurons that are ChAT-negative and abundantly express urocortin 1 (UCN1) and other neuropeptides [15,16,23,25,36]. UCN1 is a neuropeptide belonging to the corticotropin-releasing factor (CRF) family [39] and, as indicated in experimental animals, is expressed also in neurons of other brain areas including the lateral superior olive, SN, VTA and supraoptic nucleus of hypothalamus [30].

In humans, the EW nucleus, as delineated in Olszewski and Baxter's atlas [28], corresponds to the EWcp population that forms a cytoarchitecturally circumscribed entity lying immediately dorsal and medial to the oculomotor nucleus, and it contains mainly UCN1-positive neurons [15,16,18,36]. Since, in mice UCN1 in EWcp first appears on postnatal day P8 [9] and in rats EWcp neurons are involved in stress adaptation [23,43], we investigated immunohistochemically: (a) the development of UCN1-positive neurons and the possible effect of neonatal hypoxic/ischemic encephalopathy on UCN1 expression and (b) the possible colocalization of TH with UCN1 in human EWcp neurons in neonates with histological signs of acute perinatal hypoxia. A few TH-immunoreactive (IR) neurons have been observed in EWcp of both rodents [3,19] and humans [20,37,40], but no information is available about UCN1 synthesis in these neurons under normal or hypoxic conditions.

2. Materials and methods

2.1. Patients, tissues and histopathology

Formalin-fixed paraffin-embedded mesencephali of 15 autopsied infants with neonatal encephalopathy had been collected from the Greek Brain Bank (GBB, member of the BrainNet Europe) for the needs of our previous work [29]. The total corrected age of the neonates (duration of pregnancy + postnatal age) ranged from 34 to 46.5 weeks. Late intrauterine fetal deaths were excluded to avoid brain autolysis. The postmortem delay and the fixation time of the tissues used ranged from 0.4 to 4 days and from 0.5 to 12 months, respectively. Complete autopsies were performed after parental written consent for diagnostic and research purposes. The neuropathological evaluation and grading of neonatal hypoxic–ischemic encephalopathy (Table 1) were based on established criteria dependent on the pattern of gray and/or white matter lesions in specific brain regions, as previously described [12,29,34]. Three neuropathological grades were used: grade 1 compatible with severe/abrupt hypoxic–ischemic injury, grade 2 compatible with moderate/prolonged injury and grade 3 compatible with very severe/long duration injury [29].

2.2. Immunohistochemistry

For each case, two consecutive paraffin embedded 7-μm thick sections out of every 40 from the whole rostro-caudal extent of the mesencephalon were mounted on silane-coated slides and stained for UCN1 and TH, respectively. For the visualization of UCN1, sections were processed for an antigen retrieval procedure [29] and then incubated in a polyclonal UCN1 antibody (U4757; Sigma–Aldrich, Missouri, USA; 1:2000) for 1 h at room temperature and subsequently, overnight at 4 °C. The immunohistochemical reaction was visualized using an avidin-biotin peroxidase system and 3,3′-diaminobenzidine tetrahydrochloride (DAB), as a chromogen, containing nickel ammonium sulphate (Ni), as previously described [29]. For the detection of TH, sections were incubated in polyclonal anti-TH serum (Institut de Biotechnologie Jacques Boy, Reims, France, LOT 900210A; 1:1000) for 1 h at room temperature and then overnight at 4 °C. After washing, sections were incubated in secondary polyclonal goat anti-rabbit immunoglobulin/HRP antibody (P0448, DakoCytomation, Glostrup, Denmark; 1:100) and stained with DAB/Ni [29]. The specificity of the TH reaction in EW was checked both by Western blots and immunohistochemistry, as described in our previous study [29], while the specificity of the immunohistochemical reaction for UCN1 was checked by omitting the primary antibody from the incubation medium.

2.3. Morphometry and statistical analysis

The morphometric analysis was performed at three levels of EWcp: a central one at the level of the red and oculomotor