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ORIGINAL ARTICLE

Ontogenesis of human cerebellar cortex and biopathological characterization in sudden unexplained fetal and infant death

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Abstract The aims of this study were to investigate in the human cerebellar cortex the structural and biological ontogenetic features, the possible presence of alterations in cases of sudden unexplained fetal and infant death, and the involvement of the maternal cigarette smoking in developmental abnormalities. We analyzed 52 brains of fetal and infant death victims, aged from the second gestational trimester to 12th postnatal month. In the cerebellar cortex we evaluated, besides the morphological aspects, the expression of several biomarkers implicated in proliferative processes (c-*fos*, proliferating cell nuclear antigen, and apoptosis) as well as the presence of the neurotransmitter somatostatin, which is strongly implicated in central nervous system differentiation, and of EN2 gene. The observed features of the cerebellar cortex, mainly confined to the transient external granular layer, were high proliferative activity and high expression of both somatostatin and EN2 gene in prenatal life and high apoptotic index after birth. In 41% of the sudden unexplained death victims, in the greater part with smoking mothers, we observed different biopathological alterations of the cerebellar cortex. Maternal smoking is increasingly being demonstrated to be one of the main contributors to developmental neurological alterations in the offspring.

Keywords Human cerebellar cortex · Sudden unexplained perinatal death · Sudden infant death syndrome · SIDS · Maternal cigarette smoking

Abbreviations

ED	explained death
gw	gestational week
PCNA	proliferating cell nuclear antigen
SIDS	sudden infant death syndrome
SIUD	sudden intrauterine unexplained death
SNUD	sudden neonatal unexplained death
SUD	sudden unexplained death

Introduction

The cerebellar cortex is one of the most studied areas of the brain. Its laminar pattern and rectilinear geometry have long been exploited to provide an important model for understanding the development and fiber connections of the central nervous system [2, 3, 30, 37, 53, 54].

Specific features of development are observed in the cerebellar cortex regarding the origin sites, the routes and patterns of migration, and the time frames of the two major neural cell population (granule cells and Purkinje cells).

In mammalians, the ventricular zone of the fourth ventricle produces in an early embryonic stage a small number of precursors of the Purkinje cells [4, 37]. They undergo terminal mitoses in this location and only in the postmitotic phase they migrate from the germinal zone into the cerebellar wall, probably following radial glial guides [17], where they

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aggregate to form an immature layer of 10–15 cells thick. During birth, the Purkinje cells form a row of large somata and start to extend elaborate dendritic arbor synapses.

The granule cells precursors arise later, toward the end of the embryonic period, from the dorsal portion of the metencephalon within a structure called the rhombic lip, from where the arcuate nucleus of the medulla oblongata also arises [37, 48, 56]. These cells reach the superficial zone of the cerebellum and organize themselves into a pluristratified layer, the external granular layer, which persists for different periods of time according to species but eventually becomes thinner and ultimately disappears.

Extensive proliferation of these cells during the first postnatal weeks gives rise to more than 100 million granule cells that migrate across the molecular layer and Purkinje cell layer to reach the internal granular layer, under the guidance of the radial glia [17].

To date, only few authors have studied the morphological and functional development of the cerebellar cortex in man [10, 47]. The results of such studies are prevalently based on the developmental patterns from experimental research in different animal species [10, 47]. The first aim of the present study was to investigate the structural and biological patterns of human cerebellar cortex development in a large group of fetal and infant death victims, aged from the 17th gestational week (gw) to 1 year of life. In particular, in the cerebellar cortex, besides the morphological aspects, we evaluated the expression of several biomarkers implicated in proliferative processes [c-*fos*, proliferating cell nuclear antigen (PCNA), and apoptosis], of somatostatin, a neurotransmitter strongly implicated in central nervous system differentiation [7, 44], and of EN2, a homeobox-engrailed gene that seems to be involved in the anatomic organization of structures derived from the rhombic lip, particularly the external granular layer of the cerebellar cortex [38, 39, 52].

Our second purpose was to evaluate whether morpho-functional disorders of the cerebellar cortex are present in cases of sudden and unexplained fetal and infant death, which we have previously demonstrated to be associated to specific alterations of the brainstem [6, 21, 22, 24, 31, 32].

Finally, the suggestion by some authors that the protracted development of the cerebellar cortex should make this structure particularly vulnerable to a broad spectrum of extrinsic environmental injuries [5, 14, 16] and the observation in our previous studies of a significantly increased incidence of structural and/or functional alterations of different brainstem nuclei in stillborns and sudden infant death syndrome (SIDS) victims with smoker mothers [23, 25, 27, 46], prompted us to determine whether maternal cigarette smoking could also be related to morphological and/or physiological developmental abnormalities of the cerebellar cortex.

Materials and methods

A total of 52 brains were collected from 21 fetuses (17–39 gw), 8 newborns who had died within the first month of life, and 23 infants aged 1–12 months.

Classification of cases

In 32 cases, after the in-depth necropsy examination, the result of death is totally unexplained (sudden unexplained death, SUD).

A case was classified as SIDS when an infant death, between 1 and 12 months, was sudden, completely unexpected, and unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene, and a review of the clinical history [34, 55].

A case was classified as sudden intrauterine unexplained death (SIUD) when a fetus died suddenly with no explained cause after the 25th gw, before complete expulsion or extraction of the fetus from the mother, resulting in a stillbirth for which there was no explanation despite postmortem examinations [20, 34].

Similarly, a case was classified as sudden neonatal unexplained death (SNUD) when a newborn suddenly died with no explained cause, from birth to the end of the first postnatal month of life [21].

A case was classified as explained death (ED) when a precise cause of death was documented by autopsy.

Accordingly, in this study, a diagnosis of SIUD was established for 12 fetuses, of SNUD for 5 newborns, and of SIDS for 15 infants. In the remaining 20 cases a precise cause of death was formulated at autopsy.

While taking the medical history, the mother was asked for information about any smoking habit before, during, and after pregnancy. Smoking habit was assigned to two categories (smokers vs nonsmokers). Overall, 21 out of the 52 mothers (40%) were smokers already before the onset of the pregnancy, and 31 were nonsmokers (60%).

The cases were examined in blinded fashion, without initial knowledge of the cause of death, age, or other clinicopathologic information. Only after the histologic and immunohistochemistry assessment of the cerebellum had been completed the pathologic findings were matched with the corresponding records.

Table 1 summarizes the case profiles in this study, with their relative diagnosis and the mother's smoking habit.

Autopsy and pathological techniques

The victims were subjected to a complete autopsy, including examination of the placental disk, umbilical cord, and membranes in fetuses. Every case was submitted to

Table 1 Case profiles of the study

Victims (*n*)	Age	Death diagnosis (*n*)	Maternal smoking habit	
			Smokers	Nonsmokers
Fetuses (21)	17–39 gw	Voluntary abortion (3)	1	2
		Necrotizing chorioamnionitis (2)	1	1
		Congenital heart diseases (3)	1	2
		Potter's syndrome (1)	–	1
		SIUD (12)	6	6
Newborns (8)	1–4 pd	Congenital heart diseases (3)	–	3
		SNUD (5)	2	3
Infants (23)	1–12 m	Pneumonia (3)	1	2
		Congenital heart diseases (4)	2	2
		Pericarditis (1)	–	1
		SIDS (15)	7	8

gw Gestational week, *pd* postnatal day, *m* month, *SIUD* sudden intrauterine unexplained death, *SNUD* sudden neonatal unexplained death, *SIDS* sudden infant death syndrome

accurate and specialized investigations including the study of the brainstem and the cardiac conduction system on serial sections, according to the protocol routinely followed by the Institute of Pathology, University of Milan [34, 36].

All infants, neonates, and fetuses selected for this study were submitted to a complete necropsy examination about 24 h after death. Multiple samples from all organs were immediately fixed in 10% phosphate-buffered formalin. After an average period of 2 weeks, the samples were processed and embedded in paraffin; 5-μm sections were stained with hematoxylin–eosin. After fixation, the brain was cut in coronal sections. On multiple samples of the various lobes, after fixation and processing, 5-μm sections were obtained and stained with hematoxylin–eosin and Klüver–Barrera. For the heart samples, 5-μm sections were stained with hematoxylin–eosin and trichromic Heidenhain (Azan). To examine the cardiac conduction system, two blocks of heart tissue were obtained for paraffin embedding and serial cutting. The first block contains the junction of superior vena cava and right atrium encompassing the entire area of the sinoatrial node. The second block contains the atrioventricular node, His bundle down to bifurcation, and bundle branches. All sections were cut serially at intervals of 40 μm (levels), as described previously [45].

In particular, the cerebellum, the target of this study, was firstly excised from the brainstem by cutting through the cerebellar peduncles. Then coronal sections of either cerebellar hemispheres were obtained through both the anterior (frontal) and the posterior (parietooccipital) cortices. Each sample included the full thickness of the ventricular wall. Care was taken to examine corresponding folia from different subjects whenever possible. For each case, two 5-μm sections were stained with hematoxylin–eosin and Klüver–Barrera. Additional sections were subjected to immunohistochemistry for the study of (1) the PCNA, (2) the c-*fos* gene, (3) the apoptosis, (4) the neurotransmitter somatostatin, (5) and the EN2 gene expression. The remaining sections were saved and stained as deemed necessary for further investigations.

Immunohistochemical methods

Apoptosis was detected by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) method, using deoxynucleotidyl transferase (0.3 U/ml) to incorporate digoxigenin-conjugated deoxyuridine (dUTP) into the ends of DNA fragments.

To analyze the immunoexpression of *PCNA*, c-*fos*, *EN2*, and *somatostatin*, we used specific primary antibodies after the application of the avidin-biotin-peroxidase method, in conformity with the conventional immunohistochemical procedures.

A detailed description of the immunohistochemical techniques applied in this study is available in our previous works [21, 22, 26, 27].

Immunohistochemistry evaluation All the immunostained sections were examined at light microscope separately in each layer (external granular layer, molecular layer, Purkinje cell layer, and internal granular layer), using a ×40 lens (high magnification). We quantified the scoring for each case on at least 15 random fields. Only the cells with intense brown immunostaining were considered to be positive.

We quantified the scoring for each case, as follows:

–	=	no positive cell
+	=	a number of positive cells ≤30% per unit area (moderate positivity)
++	=	a number of positive neurons >30% per unit area (strong positivity)

Statistical analysis

Statistical methods were employed to evaluate the association between maternal cigarette smoking habit and cerebellar cortex alterations in the two groups (SUD and sudden ED groups), using Fisher's exact test. The level of significance was set at $p<0.05$.

Results

Analysis of the morphological and immunohistochemical data obtained in this study demonstrated homogeneous, progressive developmental steps of cerebellar cortex maturation in most cases.

Morphological features

At the earliest observation (17–18 gw) the cerebellar cortex shows high, diffuse cellularity. Only the external granular layer is outlined, whereas the molecular layer, Purkinje cell layer, and internal granular layer are not identifiable or scarcely delineated (Fig. 1a).

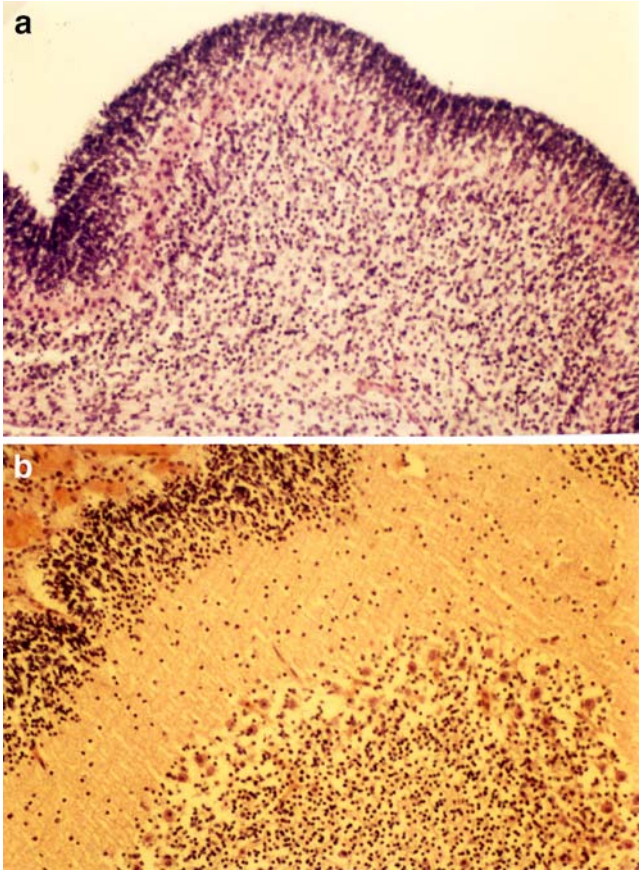

Fig. 1 Morphological aspects of the normal cerebellar cortex development. **a** Diffuse cellularity in the cerebellar cortex in a fetus aged 17 gw (hematoxylin–eosin, magnification ×20). **b** Four-layered structure of the cerebellar cortex in a 35 gw fetus. Note the dense layer of the Purkinje cells (hematoxylin–eosin, magnification ×20)

From the 20th to the 22nd week, besides a well-defined 10–12 cells thick pluristratified external granular layer, a dense row of small cells appears in correspondence of the Purkinje cell layer.

After 30 weeks, a four-layered structure is recognizable (Fig. 1b). The external granular layer is formed by six to eight rows of densely packed small round cells. Several mitoses can be detected in this layer. The molecular layer contains numerous cells resembling cells of the external granular layer, clearly in the migratory phase. The Purkinje cells form an ordered five to six cells thick layer of round immature larger neurons located between the molecular layer and the internal granular layer. This morphological pattern remains stable until the first days of life.

At 1 month after birth the external granular layer presents a superficial zone of small round cells and a substrate of horizontal bipolar neurons that are sometimes oriented centripetally toward the molecular layer.

From the second month of life the external granular layer preserves this cellular morphology but becomes progressively thinner. The molecular layer increases in thickness and shows lower cell density. The Purkinje cell layer is formed by numerous large cells with immature dendritic trees aligned in a single layer. Only in a few tracts are clusters of Purkinje cells still present. The internal granular layer is increased in thickness and neuronal density.

Between 5 and 7 months the external granular layer is constituted by three to four rows of prevalently elongated bipolar neurons and Purkinje cells show large mature somata, frequently polygonal in shape, with an evident axon and dendrites. They are now much more widely spaced and reduced in number.

At 10 months the external granular layer is a discontinuous single layer. In many areas the molecular layer represents the external surface.

Finally, by 12 months the external granular layer is totally absent. The cerebellar cortex shows the three-layered definitive structure (molecular layer, Purkinje cell layer, and internal granular layer).

Biological functional features

PCNA immunohistochemistry In fetuses, PCNA immunoreactivity is observed throughout the cerebellar cortex thickness from the 20th gw. Successively, positive cells are mainly localized in the external granular layer (++), being far less represented in the molecular layer and internal granular layer (+) and totally absent in the Purkinje cell layer. Within the internal granular layer, positive nuclei are concentrated at the boundaries of the Purkinje cell layer. This pattern of immunopositivity is still observed at birth,

but declines thereafter. At 2 months of life, rare PCNA-immunoreactive cells are detectable only in the upper part of the external granular layer.

c-fos immunohistochemistry From 17 to 25 gw, the vast majority of cells in the cerebellar cortex is c-*fos*-labeled (++). Then the positivity decreases and at 35 gw only rare c-*fos*-positive cells are visible, which are scattered in the external granular layer. By the last fetal period and in postnatal life c-*fos* expression is fully negative.

Apoptosis immunohistochemistry Immunostaining for apoptotic cells reveals only sporadic positive nuclei in the external granular layer in prenatal life after the 30th gw and in the first postnatal month. Between 2 and 4 months, the number of dying cells increases, confined to the outer part of the external granular layer (Fig. 2). This process becomes more marked during regression of the external granular layer, affecting the vast majority of its cells (++). Apoptotic neurons display intense staining by highly condensed chromatin and various nuclear fragmentation stages.

Somatostatin immunohistochemistry The somatostatin expression presents high levels in the Purkinje cell layer uring the prenatal period, reaching a maximum around the 28–30 gw (++), and starting to decline after birth at 1–2 months of life (+).

EN2 immunohistochemistry High expression of the EN2 gene is demonstrable in all the external granular layer cells from the 17th to 22nd gw (++). Successively, EN2 expression decreases up to the first days after birth (+), and is constantly negative in infants aged 1–12 months.

We found the above described cytoarchitectural and biological features in the vast majority of cases. Nevertheless, the cerebellar cortex of 16 cases (13 sudden unexpected deaths and 3 ED) showed different aspects (Table 2). In four cases diagnosed as SIDS, aged 4–6 months, and in two subjects who died at 3 months of hypertrophic cardiomyopathy, at histological examination, the external granular layer was exclusively constituted by small round cells, without any bipolar neurons in the inner rows. In two further SIDS cases, aged 3 and 4 months, respectively, the search for apoptotic cells was negative, particularly in the external granular layer. In another case of SIDS, aged 1 month, almost all the cells of the internal granular layer were immunostained for apoptosis (Fig. 3a).

An unexpected finding was the observation of programmed cell death of all the Purkinje cells in an infant who died of SIDS at 7 months (Fig. 3b,c).

In two fetuses who died suddenly and unexpectedly at 34 and 36 gw, respectively, EN2 gene expression was negative in the external granular layer. Somatostatin was also negative in the Purkinje cell layer of three stillborns, aged 32–36 gw (two dead of unknown cause and one dead of known causes). Finally, in a 2-day-old SNUD case, intense c-*fos* positivity was appreciable in all the layers, particularly in the Purkinje cell layer.

Overall, structural and biological alterations of the cerebellar cortex were present in 13 of the 32 SUD victims (41%) and in 3 of the 20 ED victims (15%) (Table 2).

Finally, we correlated the findings with the mother's smoking habit. A significant correlation was evident between maternal smoking, SUD, and anomalous features of the cerebellar cortex (Fig. 4). In fact, 12 among the 13 SUD and 2 of the 3 ED victims with cerebellar cortex alterations had smoker mothers (Table 3).

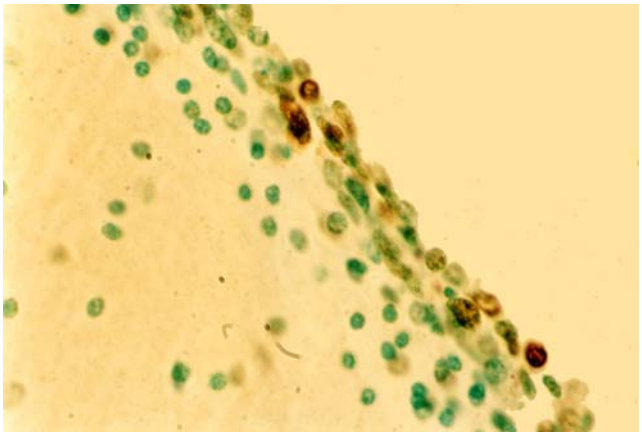

Fig. 2 Normal apoptotic aspects in the regressive process of the external granular layer in an infant deceased at 4 months (TUNEL method, magnification ×20)

Discussion

In this study, we have delineated the dynamic sequence of morphological and biological steps that occur in human cerebellar cortex development, from the second trimester of gestation to the first postnatal year.

Around the 17th–18th weeks of fetal life, the external granular layer is the only recognizable layer, with a thickness of 10–15 small cells. Successively, a pluristratified Purkinje cell layer is also identifiable and, after 30 weeks, a four-layered structure is evident (external granular layer, molecular layer, Purkinje cell layer, and internal granular layer).

In prenatal life proliferation is the predominant phase, testified by c-*fos* and PCNA positivity, which is at first widespread throughout the thickness of the cortex, and subsequently restricted to the external granular layer. Therefore, this layer contains at this stage undifferentiated small neurons, which progressively undergo proliferation and differentiation. In fact, c-*fos* and PCNA are known to

Table 2 Distribution of the cerebellar cortex alterations

Cerebellar cortex alterations	Fetuses		Cerebellar cortex alterations	Newborns		Cerebellar Cortex alterations	Infants	
	SIUD	ED		SNUD	ED		SIDS	ED
Negative EN2 in the external granular layer	2	–[a]	Positive c-*fos* in all layers	1	–[b]	Immaturity of the external granular layer	4	2
Negative somatostatin in the Purkinje cell layer	2	1				Negative apoptosis in the external granular layer	2	–[c]
						Positive apoptosis in the Purkinje cell layer	1	–[d]
						Positive apoptosis in the internal granular layer	1	–[e]

SIUD Sudden intrauterine unexplained death, *SNUD* sudden neonatal unexplained death, *SIDS* sudden infant death syndrome, *ED* explained death
[a] Presence of EN2-positive cells in the external granular layer (normal pattern)
[b] Absence of c-*fos*-positive cells in all cerebellar layers (normal pattern)
[c] Presence of apoptotic positive cells in the external granular layer (normal pattern)
[d] Absence of apoptotic positive cells in the Purkinje cell layer (normal pattern)
[e] Absence of apoptotic positive cells in the internal granular layer (normal pattern)

be markers of cell replication that regulate growth and maturation in a variety of tissues. First, the protooncogene c-*fos* synthesizes the corresponding DNA-binding protein as a signal for cell activation with mitogenic effect [11, 19]. Thus, it precedes the PCNA manifestation indicative of DNA duplication [40].

The biological features of cerebellar cortex development include positivity to the neurotransmitter somatostatin and the EN2 gene, observed to be more intensely expressed in the second and third gestational trimesters, respectively.

The somatostatin is a neuropeptide with a wide distribution in the central nervous system that controls various physiological processes [7, 44]. We have previously documented intense positivity of the somatostatin in brainstem nuclei involved in cardiorespiratory activity in stillbirths and its abrupt reduction in postnatal deaths [21, 22]. In the present study, we also observed high levels of somatostatin expression confined to the Purkinje cell layer during prenatal life and declining after birth. This finding diverges from the report by Laquerriere et al. [28]. These authors, with the use of different methods (precisely, by binding and autoradiographic studies on frozen human cerebellar samples), found from midgestation to the 15th postnatal month a high concentration of somatostatin receptors mainly in the granule cells of both the external and internal layers.

However, we believe that, independent of a specific localization, the somatostatin plays an important role, rather than in neurotransmitter activity, in the development and maturation of the cerebellar cortex, as of other structures of the central nervous system.

Moreover, the EN are homeobox-engrailed genes that were implicated in the development of the central nervous system. In particular, the EN2 gene seems to have an essential role in the anatomic organization of structures derived from the rhombic lip, in particular, of the arcuate nucleus in the ventral surface of the brainstem and of the external granular layer in the cerebellar cortex [10, 56].

We recently observed that the expression of the EN2 gene is very high in human arcuate nucleus neurons from the 17th to 22nd gw, which decreases thereafter up to the first day after birth and then disappears [26]. The same temporal pattern of EN2 manifestation was found in this study in the external granular layer neurons of the cerebellar cortex, confirming the same embryologic origin as that of the arcuate nucleus.

During the postnatal period, the external granular layer involutes leading to the definitive three-layered organization of the cerebellar cortex observed at 12 months of life. Many of the small undifferentiated neurons of the external granular layer migrate across the molecular and Purkinje cell layers, under the guidance of the radial glia, giving rise to the mature neurons of the internal granular layer. In addition, at this stage of development, a high number of pre- and postmitotic neurons of the external granular layer die upon activation of an apoptotic program [41, 57]. Therefore, the main biological observations in the cerebellar cortex after birth are (1) an abrupt decline of c-*fos*, PCNA, EN2, and somatostatin expression and (2) an increasing number of dying cells in the external granular layer, responsible for its progressive reduction in thickness.

In this study we observed several variations from the above delineated cytoarchitectural and biological steps of cerebellar cortex development, prevalently in cases of sudden death. Unexpected findings in SIDS victims were (1) immaturity of the external granular layer neurons observed in four cases and (2) anomalous apoptotic models in four cases. The anomalous apoptosis consisted of programmed death of all the Purkinje cells in an infant who died at 7 months and of the internal granular layer cells in an infant aged 1 month; lack of apoptotic expression in

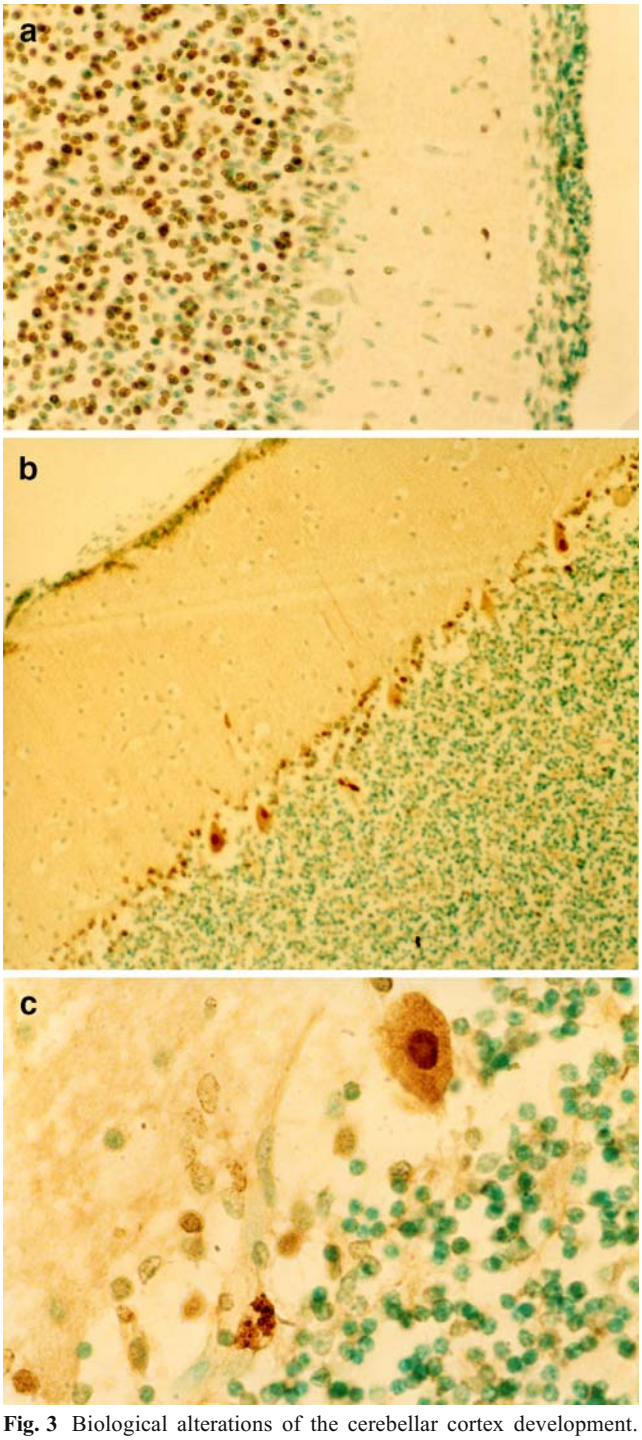

Fig. 3 Biological alterations of the cerebellar cortex development. **a** High apoptotic index of the internal granular layer in a SIDS victim aged 1 month (TUNEL method, magnification ×20). **b** High apoptotic index of the Purkinje cell layer in a SIDS victim aged 7 month (TUNEL method, magnification ×20). **c** Magnified detail (×100) of subpanel **b**: programmed cell death of a Purkinje cell and a Purkinje fragmented nucleus (TUNEL method)

the external granular layer could be the cause of death in two cases aged 2 and 4 months, respectively. In addition, we observed negative expression of somatostatin in three cases and of the EN2 gene in two cases of fetal death, and

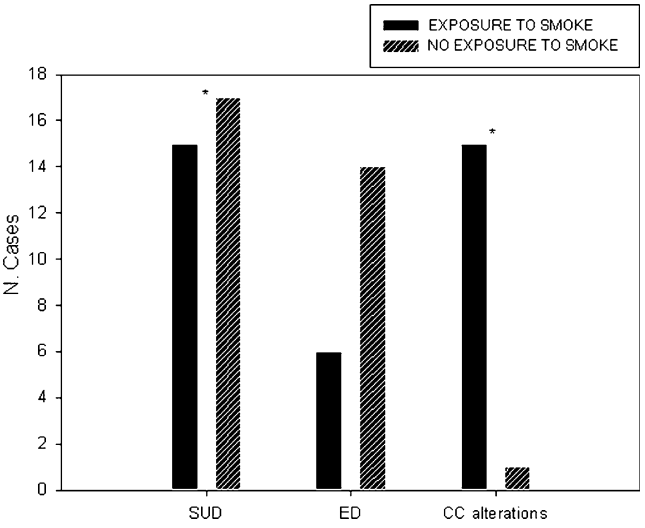

Fig. 4 Significant correlation between maternal smoking, *SUD* compared to *ED*, and anomalous features of the cerebellar cortex (*CC*) ($p<0.05$). *SUD*=Sudden Unexplained death; *ED*=Explained Death

intense c-*fos* positivity in a newborn who died 2 days after birth.

Dissimilar alterations were reported in literature in the cerebellar cortex of SIDS victims. One of the earliest papers showed a decreased number of the internal granular layer cells [15]. Further investigation indicated increased density of Purkinje cells among the youngest SIDS infants [42]. Instead, other authors observed a greater thickness and cell density of the external granular layer in SIDS cases than in age-related controls [9, 49].

We suggest that all these findings, including our own observations, are different manifestations of delayed or faulty maturation of the cerebellar cortex in sudden unexpected death victims. In stillbirths and infants, for which there was no explanation of death despite postmortem examination, we previously found morpho-functional developmental alterations of specific nuclei of the brainstem that are classically implicated in respiratory control (the parabrachial/Kölliker–Fuse complex and locus coeruleus in the pons, the dorsal motor vagal nerve, the tractus solitarius, the ambiguus, the hypoglossus,

Table 3 Distribution of neurodevelopmental defects of the cerebellar cortex in unexplained and ED in relation to maternal smoking habit

	Smoking mothers (n=21)		Non smoking mothers (n=31)	
	SUD	ED	SUD	ED
Cerebellar cortex alterations (n=16)	12	2	1	1
Normal cerebellar cortex structure (n=36)	3	4	16	13

SUD Sudden unexplained death, *ED* explained death

and the arcuate nuclei in the medulla oblongata) [6, 21, 22, 31, 32].

Thus, we believed that neuronal mechanisms responsible for compensatory responses to hypercapnia and/or hypoxia were associated to substantial functional changes of these nuclei.

Because the axons of the granule cells and Purkinje cells seem to have connections with dorsal and ventral medulla and with specific areas in the pons, as reported in experimental studies [18], we suggest that even the cerebellar cortex could be a putative neuronal region subserving ventilatory control.

In particular, the cerebellar cortex contributes to the control of muscle activity, working as a clock determining the time intervals between two successive contractions [12]. This activity should also extend to respiratory muscles that are implicated in the restoration of blood pressure and breathing rhythm in cases of hypoxia [58].

Moreover, severe hypoxic injuries may determine more harmful effects on the cerebellar cortex than on other central nervous system structures because the long duration of its development may increase its vulnerability to DNA-damaging conditions, including nicotine absorption.

In particular, smoke exposure reduces tissue oxygenation due to increased blood levels of carboxyhemoglobin. In fact, carbon monoxide, a gaseous combustion product of nicotine, is easily absorbed into the systemic circulation, crossing the placenta by passive diffusion in cases of maternal smoking in pregnancy [29]. It inhibits the release of oxygen, causing hypoxia especially in the most susceptible organs, namely, the brain and, to an even greater extent, the cerebellar cortex [43, 50, 51].

Animal studies have confirmed the presence of neurobehavioral deficits in the cerebellar cortex (a reduction in number of the Purkinje cells, high expression of glial fibrillary acidic protein, and changes in nicotinic receptors) associated to nicotine exposure [1, 8, 13].

We have previously studied the consequences of chronic exposure to tobacco smoke in utero on the morphological and functional development of different brainstem nuclei in perinatal and infant SUD [23, 25, 27, 46]. In particular, we have observed a significant correlation between maternal smoking during pregnancy and hypoplasia and negative expression of EN2 gene of the arcuate nucleus, hypoplasia and altered expression of the somatostatin in the hypoglossus nucleus, and decreased synthesis of catecholamines in the locus coeruleus.

We postulate that smoke can also exert adverse influences on the successful deployment of the genetic programs committed to the protracted process of pre- and postnatal cerebellar cortex development. The morphobiological defects of the cerebellar cortex layers observed in this study could have contributed to the failure of regulatory reactions to breathing challenges, leading to sudden death in vulnerable periods. Further studies on triggering factors and related functional mechanisms are needed to gain a better understanding of the complex pathophysiology of SUD. The results presented in this study may contribute to orient researches in this field.

In conclusion, this study provides suggestion that nicotine toxicity from maternal smoking is increasingly being demonstrated to be one of the main contributors to developmental neurological alterations in the offspring [23, 25, 27]. Therefore, all women should be warned that smoking places their children at serious risk of a variety of morphological, genetic, and functional abnormalities of the brain that can even lead to sudden, apparently unexplained death.

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