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Neuropathology of the area postrema in sudden intrauterine and infant death syndromes related to tobacco smoke exposure

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ABSTRACT

The area postrema is a densely vascularized small protuberance at the inferoposterior limit of the fourth ventricle, outside of the blood–brain barrier. This structure, besides to induce emetic reflex in the presence of noxious chemical stimulation, has a multifunctional integrative capacity to send major and minor efferents to a variety of brain centers particularly involved in autonomic control of the cardiovascular and respiratory activities.

In this study we aimed to focus on the area postrema, which is so far little studied in humans, in a large sample of subjects aged from 25 gestational weeks to 10 postnatal months, who died of unknown (sudden unexplained perinatal and infant deaths) and known causes (controls). Besides we investigated a possible link between alterations of this structure, sudden unexplained fetal and infant deaths and maternal smoking.

By the application of morphological and immunohistochemical methods, we observed a significantly high incidence of alterations of the area postrema in fetal and infant victims of sudden death as compared with age-matched controls. These pathological findings, including hypoplasia, lack of vascularization, cystic formations and reactive gliosis, were related to maternal smoking. We hypothesize that components from maternal cigarette smoke, particularly in pregnancy, could affect neurons of the area postrema connected with specific nervous centers involved in the control of vital functions.

In conclusion, we suggest that the area postrema should be in depth examined particularly in victims of sudden fetal or infant death with smoker mothers.

1. Introduction

The area postrema (AP), already described by Retzius in (1986), is a bilateral structure located at the caudal end of the brain (hence its name) on either side of the medullary midline at the junction of the medulla with the spinal cord. It is one of the most highly vascularized regions in the mammalian brain with the unique access to the circulation due to presence of fenestrated capillaries.

Functionally, the AP for many years has been considered a chemoreceptor trigger zone able to induce the emetic reflex in the presence of noxious chemical stimulation (Borison and Brizzee, 1951; Borison and McCarthy, 1984; Carpenter and Briggs, 1986). Moreover, further studies disclosed additional roles for AP particularly in the autonomic regulation of many vital functions (Ferguson and Marcus, 1988; Bishop and Hay, 1993; Bongianni et al., 1998; Price et al., 2008).

Although the AP has been the subject of numerous experimental researches (Watson, 1985; Hasser and Bishop, 1990; Qian and Koon, 1998; Peuler et al., 1987), there is still little information about this structure in man. In a recent work on sudden intrauterine unexplained death syndrome (SIUDS) and sudden infant death syndrome (SIDS) (Lavezzi et al., 2010), we demonstrated a pathologic development of the ependyma, the lining of the cerebral ventricles that in caudal medulla is contiguous to AP. Therefore, the aim of the present study was to focus on the human AP and, in particular, to evaluate if the AP too shows morpho-functional alterations in victims of unexplained fetal and infant deaths.

Our research protocol, applied to a wide sample of victims aged from 25 gestational weeks to 10 months of life, included, in every case, the morphological examination of the AP in serial histological sections of caudal medulla oblongata and the immunohistochemical evaluation in this area of the astrocytic immunopositivity to highlight activated glial cells, indicative markers of neuronal responses to injuries.

2. Material and methods

In total, 54 brains were collected from 25 fresh stillbirths (25–40 gestational weeks, with a peak from 36 to 40 weeks) and 29 infants aged 1–10 months (mean age: 3 months).

Abbreviations: AP, area postrema; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; SIDS, sudden infant death syndrome; SIUDS, sudden intrauterine unexplained death syndrome.

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This was a selected set of cases sent to our Research Center in application of the 2006 guidelines stipulated by Italian law n. 31 "Regulations for Diagnostic Post Mortem Investigation in Victims of SIDS and Unexpected Fetal Death". This law decrees that all infants with suspected SIDS who died suddenly within the first year of age, as well as all fetuses who died after the 25th week of gestation without any apparent cause, must undergo an in-depth anatomo-pathological examination, particularly of the autonomic nervous system (Matturri et al., 2005, 2008).

Permission from the Ethics Committee was not required for this study as our Research Center is the national referral center for sudden unexplained fetal and infant deaths, in accordance with the above-mentioned Italian law n. 31.

After fixation in 10% phosphate-buffered formalin, samples from the brainstem and cerebellum, the main structures analyzed in our studies, were processed and embedded in paraffin.

Transverse serial sections of the midbrain, pons, medulla oblongata, and cerebellar hemispheres were obtained at intervals of 50–60 μm. At each level three of these sections were routinely stained for histological examination using hematoxylin–eosin, Klüver–Barrera and Bielchowsky's silver impregnation technique and additional sections were subjected to immunohistochemistry for the study of the gliosis by the GFAP (glial fibrillary acidic protein) method. The remaining sections were saved and stained as deemed necessary for further investigations.

The routine histological evaluation of the brainstem was focused on the locus coeruleus and the parabrachial/Kölliker-Fuse complex in the rostral pons/caudal mesencephalon, on the retrotrapezoid nucleus, the superior olivary complex and the facial/parafacial complex in the caudal pons; on the hypoglossus, the dorsal motor vagus, the tractus solitarius, the ambiguus, the pre-Bötzinger, the inferior olivary, the raphé and the arcuate nuclei in the medulla oblongata.

In the cerebellum, the cortex layers (external granular layer, molecular layer, Purkinje cell layer and internal granular layer) and the medullary deep nuclei (the dentate, the fastigial, the globose and the emboliform nuclei) were examined.

In 36 cases, after the in-depth anatomopathological examination the death remained totally unexplained. A diagnosis of SIUDS (sudden intrauterine unexplained death syndrome) was established for 16 fetuses, who died suddenly after the 25th gestational week before complete expulsion or retraction from the mother, and of SIDS (sudden infant death syndrome) for 20 infants who died within the first year of life. In the remaining 18 cases, 9 stillbirths, and 9 infant deaths, a precise cause of death was formulated at autopsy. These cases were considered as controls. Specific diagnoses among the fetal deaths included: chorioamnionitis (n = 6) and congenital heart disease (n = 3). The related infant death diagnoses in this group were: congenital heart disease (n = 3), severe bronchopneumonia (n = 2), myocarditis (n = 1), pulmonary dysplasia (n = 2), and mucopolysaccharidosis type I (n = 1).

For every case, a complete clinical history was collected. Additionally, mothers were asked to complete a questionnaire on their smoking habit, detailing the number of cigarettes smoked before, during and after pregnancy. Fifteen of the 36 SIDS/SIUDS mothers (42%) were active smokers before and during the pregnancy, smoking more than 3 cigarettes/day. The remaining 21 mothers (58%) admitted no history of cigarette smoking. Three of the 18 mothers in the control group (17%) reported a smoking habit, while the remaining 16 mothers (83%) were non smokers.

2.1. Immunohistochemical study

2.1.1. GFAP (glial fibrillary acidic protein) immunostaining

Sections were deparaffinized and washed in PBS. After blocking endogenous peroxidase with 3% H_2O_2, the slides were pretreated in a microwave-oven using a citrate solution (pH = 6). Then the sections were incubated overnight with primary monoclonal antibody NCL-GFAP-GA5 (anti GFAP, Novocastra, Newcastle Tyne, United Kingdom) at a dilution of 1:300. Immunohistochemical staining was performed with the peroxidase–antiperoxidase method and the avidin–biotin complex technique (ABC Kit, Vectastain, Vector Laboratories Inc., Burlingame, CA, U.S.A.). Diaminobenzidine (DAB, Vector Laboratories Inc., Burlingame, CA, U.S.A.) was used as chromogen substrate and counterstained with light hematoxylin. Negative controls of the same tissue were done using PBS instead of primary antibody.

2.2. Statistical analysis

Data obtained were tabulated and analyzed statistically for differences using chi-squared test, comparing pairs of groups, with a significance level established at $P<0.05$.

3. Results

The study of the AP was restricted in every case to a few serial sections in the lower medulla oblongata, given its limited extension. We defined the AP as a medullary structure lying at the base of the fourth ventricle on the dorsal surface of the medulla oblongata. Precisely, we observed two right and left masses attached to the inferior angle of the floor of the fourth ventricle below the obex, protruding into ventricular lumen (Fig. 1A). At the analysis of its morphology and histologic components in transversal plane, the AP appears in correspondence of the end of the ependymal epithelium like a loose gliovascular region, composed of astrocytes, collagen fibers, rare neurons, myelinated nerve fibers and scattered small capillaries surrounded by perivascular spaces (Fig. 1B). In the more caudal sections, the two structures link together along the median line in relation with the opening of the central canal of the spinal cord. Fig. 2 shows the determinant role of the AP in the formation of the central canal of the spinal cord.

3.1. Pathology of the AP in SIUDS/SIDS victims and controls

We observed a significantly high incidence of histological/immunohistochemical alterations of the AP in SIDS and SIUDS cases, as compared with age-matched controls. Overall, 24 of the 36 victims of sudden death (67%) and 3 of the 18 subjects belonging to control group (17%) showed AP modifications ($P<0.01$).

In 10 victims of SIDS (50% of SIDS and 28% of all sudden deaths) we found hypodevelopment of the AP. Precisely, in 6 cases a bilateral hypoplasia and in 3 cases a monolateral hypoplasia, involving the right portion, were diagnosed (Fig. 3A, B). In a further case a right monolateral hypoplasia of the AP was coupled with AP agenesis on the other side (Fig. 4). In 3 SIUDS and 5 SIDS victims the AP, with apparently normal morphology and development, showed absence of capillaries in its parenchyma (Fig. 5). On the contrary, 3 SIDS cases showed numerous large caliber capillaries anastomosed in a dense network (Fig. 6).

A peculiar finding was the presence in 14 (39%) of victims of sudden death (3 SIUDS/11 SIDS) and in 2 infants of the control group (11%) of cystic structures in and around the AP, frequently covered by ependymal cells and filled with a flocculent material. They were sometimes located in contact with the perivascular space of the AP capillaries (Fig. 7).

Immunohistochemistry showed a marked number of GFAP-immunopositive glial cells in the AP prevalently of SIDS cases (10 infant and 1 fetal sudden deaths). In 2 victims of the control group, all died of bronchopneumonia, some reactive glial cells were detectable.

Table 1 shows the distribution of the main AP defects in victims of sudden death and in controls; it must be remembered that individual victims may display any combination of these pathological findings. Frequently, above all in SIDS victims, the presence of cystic formations in AP was associated to GFAP immunopositivity.

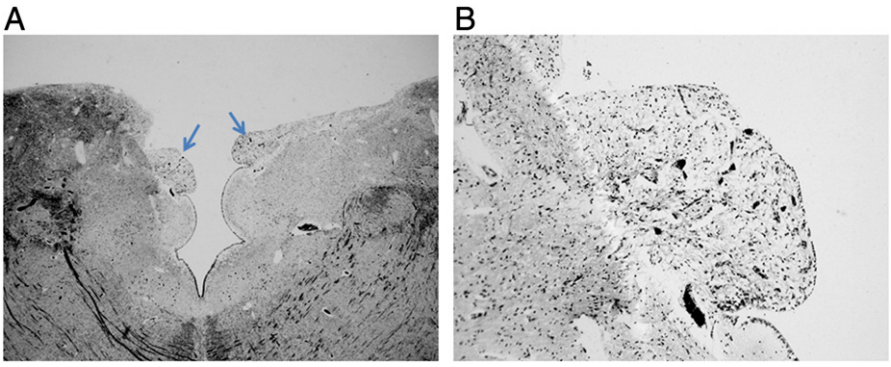

Fig. 1. Normal structure of the area postrema in an infant in the control group who died at 2 months. In A) the arrows show its bilateral localization on the floor of the IV ventricle protruding into the ventricular lumen. In B) the loose parenchyma with the gliovascular histologic components of the AP can be detected. Klüver–Barrera stain; magnification: A) 10×; B) 20×.

Altogether the AP alterations that we reported were significantly related to maternal smoking. In fact, 11 of the 15 victims of sudden death with a smoker mother (73%) showed developmental alterations of the AP. In the control group all the 3 cases with AP changes had a smoking mother, confirming the association between smoking absorption and AP developmental defects.

In most of both sudden fetal and infant death victims with developmental abnormalities of the AP, we also observed morphological alterations of different brainstem structures (namely hypoplasia/agenesis of the arcuate nucleus, pre-Bötzinger nucleus, serotonergic raphé nuclei, parafacial nucleus in the medulla oblongata/pons). The most frequent associations were between AP alterations and hypodevelopment of the serotonergic raphé nuclei and of the arcuate nucleus in SIDS and hypoplasia of the parafacial nucleus in SIUDS.

In the control group, hypoplasia of the arcuate nucleus was frequently observed.

4. Discussion

The area postrema (AP) is a circumventricular organ located in the dorsomedial medulla. The circumventricular organs are peculiar brain structures located in the walls of the third and fourth ventricles, characterized by a vascular network formed by special capillaries with a fenestrated endothelium that allow the passage of even large molecules (Ganong, 2000; Ferguson and Bains, 1996; McKinley et al., 2003). Thus, in these organs the nervous tissue and the blood are in direct communication. Moreover, the AP, that is the most distal of the circumventricular organs, is devoid of the blood–brain barrier and can act as a

Fig. 2. A), B) and C): image sequence of the formation of the spinal cord central canal in successive sections running toward the caudal pole of the medulla oblongata. Klüver–Barrera stain; magnification: 10×.

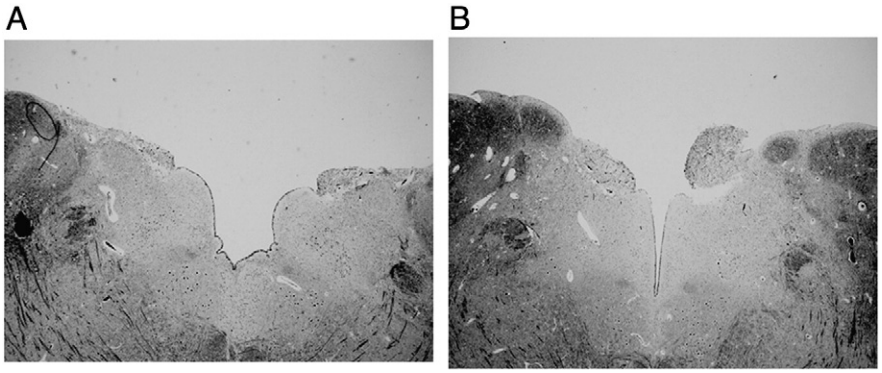

Fig. 3. Hypoplasia of the area postrema — A) bilateral hypoplasia in a SIDS victim aged 3 months; B) monolateral (right) hypoplasia in a 4 month-old SIDS case. Klüver–Barrera stain; magnification: 10×.

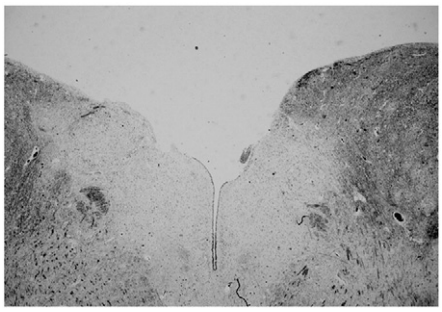

Fig. 4. Right hypoplasia and left agenesis of the area postrema in a victim of SIDS aged 2 months. Klüver–Barrera stain; magnification: 10×.

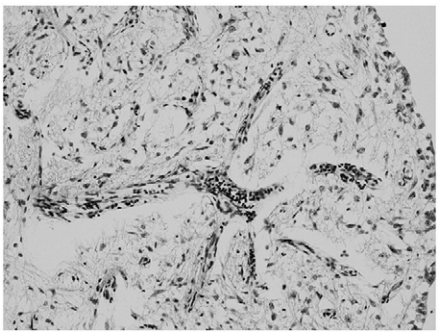

Fig. 6. Anastomosed capillaries in the area postrema of a victim of SIDS who died at 6 months. Klüver–Barrera stain; magnification: 20×.

sensor for chemical messengers circulating in the blood stream. In fact, through its vessels the blood substances can gain direct access to the inner neurons that function as chemosensors, converting the information into neural signals that are transmitted from the AP to the nearby nucleus tractus solitarius and the dorsolateral pontine neurons (lateral parabrachial and pre-locus coeruleus nuclei) (Van der Kooy and Koda, 1983; Shapiro and Miselis, 1985; Stein and Loewy, 2010a,b). In addition, the AP can discharge neurohormones into the blood stream, that exert functions such as selectively removing noxious substances, again thanks to the special capillary permeability. Also, the natural lack on the AP surface of ependymocytes, that form a protective barrier in the ventricular wall between the brain and the cerebrospinal fluid by means of tight junctions (Del Bigio, 1995; Lavezzi et al., 2010), contributes to facilitate these crossings.

The AP, commonly termed a "chemoreceptor trigger zone", has an integrative control capacity of a range of autonomic and visceral functions. In particular, the AP plays an important role in cardiovascular and respiratory regulation, as well as in the emetic reflex which controls the vomiting center situated in the reticular formation of the medulla (Watson, 1985; Borison and Brizzee, 1951; Borison and McCarthy, 1984; Carpenter and Briggs, 1986; Ferguson and Marcus, 1988; Hasser and Bishop, 1990; Bishop and Hay, 1993; Miller and Leslie, 1994; Qian and Koon, 1998; Peuler et al., 1987; Bongianni et al., 1998; Price et al., 2008). In addition, receptors for a variety of gastrointestinal-related peptide hormones are localized in the AP; these include amylin, cholecystokinin, ghrelin, and adiponectin. Finally, the AP may influence other visceral functions, including fluid balance and immune responses (Goehler et al., 2006).

Herein we have reported a wide spectrum of pathological alterations of the AP found in a large proportion of victims of unexplained fetal and infant deaths (67%). These changes included: hypoplasia, lack of vascularization, cystic formations and reactive gliosis.

Both AP hypoplasia and AP without capillaries can be interpreted as results of a delayed development. These alterations might prevent the discharge of neurotransmitters and/or the absorption of essential blood peptides and solutes, thus affecting vital functions.

It is difficult to interpret the significance of the presence of cystic neoformations in and around the AP. Cysts in the AP have already been described by Gotow and Hashimoto (1980) in rats. The authors stated that these formations arise from the ependyma and are related to the cerebrospinal fluid (CSF) and blood circulation, even if specific reactives injected into the CSF space failed to enter the cystic lumen.

Also in the present study the cysts were frequently covered by ependymal cells. Thus, in accordance with our previous observation of frequent invaginations of the ventricular ependyma penetrating into the subjacent brainstem parenchyma in SIUDS/SIDS victims (Lavezzi et al., 2010), we regarded these cystic structures as indicative of a heterotopic presence of ependymal alterations in the AP area.

The reactive GFAP-immunopositive astrocytes we found in the AP, particularly in SIDS victims, can be considered as a non-specific response to brain injuries. Hypoxic events, in particular, frequently induce the proliferation of activated astrocytes in specific brain regions that play an important role in the physiological control of breathing and arousal (Norenberg, 1994; Becker and Takashima, 1985).

Among the environmental stressors that can directly damage the AP, a key role could be attributed to components of cigarette smoke.

We suggest that many of the 4000 gases and substances that have been identified in tobacco smoke, particularly in pregnancy in case of maternal smoking, may come in direct contact with the AP neurons, through the AP vessel fenestrations, or permeation from the cerebrospinal fluid in cases with no development of capillaries. These neurons innervate specific nervous centers checking vital functions. These considerations are supported by the observation in our study of AP alterations, above all in SIUDS/SIDS victims with a smoker mother, like the developmental abnormalities of other nuclei and/or structures of the brainstem and cerebellum that we have reported

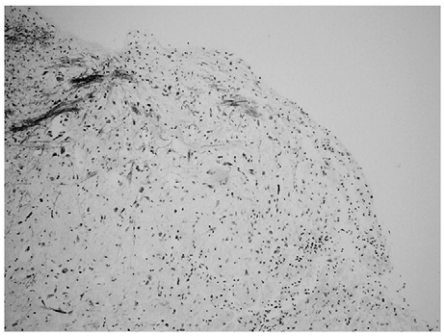

Fig. 5. Absence of capillaries in the area postrema of a 1 month-old victim of SIDS. Klüver–Barrera stain; magnification: 20×.

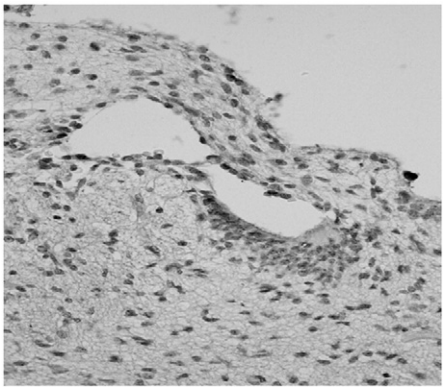

Fig. 7. Cystic formation in the area postrema partially carpeted by ependymal cells in a SIUDS victim aged 25 gestational weeks. Klüver–Barrera stain; magnification: 40×.

Table 1
Distribution of the main AP alterations in SIUDS/SIDS and controls. (Individual victims may display any combination of these alterations)

Area postrema alterations		SIUDS victims (n.16)	SIDS victims (n.20)		Controls (n.18)
Ipoplasia	Bilateral	–		6	–
	Monolateral	–	10	3	–
	Monol/agenesis	–		1	–
Capillary absence		3	5		–
Cysts		3	11		2
Reactive gliosis (GFAP immunohistochemistry)		1	10		2

in previous works to be significantly related to the absorption of cigarette smoke (Lavezzi et al., 2005a,b; Lavezzi et al., 2007).

It has been amply documented that smoking during pregnancy is associated with a number of adverse obstetric outcomes including spontaneous abortion, placenta previa, preterm birth, and low birth weight (Chelmow et al., 1996; Ananth et al., 1999; Bernstein et al., 2005; Fantuzzi et al., 2007). Besides, there is substantial evidence that after crossing the placenta the tobacco ingredients exert neurotoxic effects that impair the development of critical neuronal pathways in the developing brain (Pauly and Slotkin, 2008; Dwyer et al., 2009). Nicotine and above all its catabolite cotinine are also detectable in breast milk during lactation. In particular, during the first weeks of life of infants with smoker mothers who are exclusively breast-fed, urinary cotinine levels are significantly higher than in those who are only bottle-fed (Jordanov, 1990; Luck and Nau, 1985; 1987).

Fetal and neonatal exposure to cigarette smoke can also cause long-term consequences on the central nervous system in children and adolescents. Indeed, the long-term effects of tobacco exposure may be more profound at these early ages, since the neural circuitry is not yet completely mature and the inherent plasticity of the developing brain makes it particularly vulnerable to drug-induced alterations.

We have identified at least two key limitations to our study. First, the relatively small number of control cases showing both AP abnormalities and maternal history of smoking, to validate our findings and in particular the role of the tobacco smoke as potential factor triggering AP alterations.

Second, we have not considered more global environmental factors as potential contributors to pathogenetic mechanism of SIUDS and SIDS, such as air pollution. In fact many victims included in this study are from Lombardy, a highly polluted Italian region, in which the mean $PM_{2.5}$ and P_{10} levels are recognized to contribute in a substantial way to perinatal mortality. Moreover, the presence of astrocytosis in the setting of formation of cystic structures frequently observed in SIDS victims, suggests a multifactorial mechanism for the AP abnormalities.

5. Conclusions

Despite its small size and peculiar cytoarchitecture, combining a dense vasculature with a rarefied parenchyma and a fairly small number of cells, the location of the AP outside the blood–brain barrier, and its multifunctional integrative capacity to send major and minor efferents to a variety of brain centers involved in autonomic control, make this sensory circumventricular organ a vital player in many physiological functions, including cardiovascular and respiratory activities.

We believe that the AP is a very important area of the autonomic central nervous system and that a normal AP structure and functionality is crucial to defend a proper brain development. Hence, the alterations reported in this study may underline a defective AP activity as a brain monitor and integrator of the systemic autonomic state and consequently may play an important part in triggering sudden fetal and/or infant death.

Further and more in-depth analyses of the AP at autopsy in brainstems from perinatal death victims, also taking into account developmental aspects in conditions of tobacco exposure, will contribute to expand our knowledge of this important nervous structure and, more generally, to gain a better understanding of autonomic dysfunctions in many developmental brain disorders.

Conflict of interest

All authors declare that they have no conflicts of interest, financial or otherwise.

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