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Original Article

Functional neuroanatomy of the human pre-Bötzinger complex with particular reference to sudden unexplained perinatal and infant death

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The authors are the first to identify in man the pre-Bötzinger complex, a structure of the brainstem critical for respiratory rhythmogenesis, previously investigated only in rats. The evaluation of the neurokinin 1 receptors and somatostatin immunoreactivity in a total of 63 brains from 25 fetuses, nine newborns and 29 infants, allowed to delineate the anatomic structure and the boundaries of this human neural center in a restricted area of the ventrolateral medulla at the obex level, ventral to the semicompact ambiguus nucleus. The neurons of the pre-Bötzinger complex were roundish in fetuses before 30 gestational weeks and lengthened after birth, embedded in a dendritic system belonging to the reticular formation. Besides, structural and/or functional alterations of the pre-Bötzinger complex were present in a high percentage of sudden deaths (47%), prevalent in late fetal deaths. In particular, different developmental defects (hypoplasia with a decreased neuronal number and/or dendritic hypodevelopment of the reticular formation, abnormal neuronal morphology, immunonegativity of neurotransmitters, and agenesis) were found. The authors suggest that the pre-Bötzinger complex contains a variety of neurons not only involved in respiratory rhythm generation, but more extensively, essential to the control of all vital functions. Sudden death and in particular sudden unexpected fetal death could therefore be ascribed to a selective process when developmental alterations of the pre-Bötzinger complex arise.

Key words: neuropathology, pre-Bötzinger complex, SIDS, sudden fetal death, vital functions.

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INTRODUCTION

This study is the first to identify the anatomic localization, the cytoarchitecture and the functional features of the pre-Bötzinger complex (pBc) in humans. In fact, a review of the literature shows that up to now this structure has been described only in experimental studies on rats. The pBc has been physiologically defined as a group of neurons of the ventrolateral medulla that are essential for the generation of the respiratory rhythm, as well as for the modulation of eupneic breathing.[1–4] This statement is mainly based on the observation that removal of a restricted area of the medulla containing the pBc abolishes breathing rhythm generation in neonatal rats, and results in ataxic breathing during wakefulness in adult rats. Immunoreactivity for neurokinin 1 receptors (NK1R) and somatostatin (SS) has been applied in further experimental works to delineate the precise anatomic structure and localization of the pBc.[5–7]

In the present study, we sought to evaluate whether these neurotransmitters and their receptors might be markers of the pBc also in man, and thus to trace its anatomical boundaries as well as its physiological properties. Besides, we aimed to determine whether this important nervous center, given its essential role in the respiratory rhythm-generating circuit, shows morpho-functional alterations in sudden unexplained perinatal and infant deaths, like the alterations in nuclei and/or structures of the brainstem and cerebellum, checking vital functions, that we have previously demonstrated in these pathologies.[8–14]

MATERIALS AND METHODS

A total of 63 brains were collected from 25 fresh fetuses (17–39 gestational weeks), nine newborns who died within the first month of life and 29 infants aged 1–12 months.

After parental consent, the subjects underwent a complete autopsy, including examination of the placental disk,

umbilical cord and membranes in fetuses and in all cases an in-depth histological examination of the autonomic nervous system was made, according to the protocol routinely followed by the Institute of Pathology, University of Milan.[15,16]

Permission from the ethic committee was not required for this study as the Institute of Pathology of the Milan University is the referral national center for sudden unexpected and unexplained infant and perinatal death, according to Italian Law n. 31/2006 "Regulations for Diagnostic Post Mortem Investigation in Victims of Sudden Infant Death Syndrome (SIDS) and Unexpected Fetal Death".

In particular, after fixation in 10% phosphate-buffered formalin, the brainstem, the target of this study, was processed and embedded in paraffin. Transverse serial sections were made at intervals of 30 µm. For each level, 12 5 µm sections were obtained, three of which were routinely stained for histological examination using HE, Klüver-Barrera and Gless stains. Additional sections at each level were subjected to immunohistochemistry for the examination of the NK1R and SS immunoreactive neurons. The remaining sections were saved and stained as deemed necessary for further investigations.

NK1R immunohistochemistry

Tissue sections were washed in 0.01 mol/L phosphate-buffered saline (PBS) at pH 7.4 for 5 min and then rinsed in 3% hydrogen peroxide in PBS for 15 min in order to inactivate endogenous peroxidase. After peroxide treatment, tissue sections were washed three times for 5 min each in PBS and then incubated in 10% normal fetal calf serum (Novocastlab, Newcastle, UK) in PBS for 20 min, followed by an overnight incubation at 4°C with polyclonal guinea pig anti-NK1R antibody (Chemicon, Temecula, CA, US), diluted 1:500 in PBS.

After three washes in PBS, sections were incubated with secondary antibody (biotinylated goat antirabbit IgG – Novocastlab) for 30 min diluted 1:300 in PBS. An ABC peroxidase procedure (ABC Elite kit – Vector Laboratories, Toronto, Ontario, Canada) with diaminobenzidine was used to visualize NK1R staining.

Somatostatin (SS) immunohistochemistry

Lyophilized rabbit serum diluted in PBS (Novocastlab) was used in this study. This antiserum recognizes the N-terminal part of SS-28, composed of 28 amino acids. SS immunoreactivity was visualized by the peroxidase-antiperoxidase method. In order to neutralize endogenous peroxidase, sections were pretreated with a solution of 0.3% hydrogenase peroxide for 20 min. After rinsing in buffer, sections were exposed for 48 h to the specific primary antiserum diluted 1:150 at 25°C. After 10 min in buffer, the site of antigen-antibody reaction was revealed with antirabbit immunoglobulin followed by peroxidase-antiperoxidase complex. The unlabeled antiserum was placed on the sections for 2 h, diluted 1:200 at room temperature. The sections were then exposed to peroxidase-antiperoxidase complex for 2 h at a dilution of 1:200. The sites of peroxidase activity were visualized with 0.3% hydrogen peroxide in buffer containing 0.04% diaminobenzidine tetrahydrochloride and 0.5 g nickel ammonium sulfate. Slides were rinsed, dehydrated, mounted and examined by light microscopy.

In 32 cases, after the indepth autopsy examination, the death remained totally unexplained (SUD).

In particular, a case was classified as sudden infant death syndrome (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.[17]

A case was classified as sudden intrauterine unexplained death (SIUD) when a fetus died suddenly with no explained cause after the 22nd gestational week, before complete expulsion or retraction of the fetus from the mother, resulting in a stillbirth for which there was no explanation despite postmortem examinations.

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

Accordingly, in this study, a diagnosis of SIUD was established for 12 fetuses, of SNUD for five newborns and of SIDS for 15 infants. In the remaining 31 cases a precise cause of death was formulated at autopsy. Therefore, these 31 cases were regarded as control cases. Table 1 summarizes the case profiles in this study, with their relative diagnoses.

Table 1 Case profiles of the study

Subjects (*n*)	Age	Death diagnosis (*n*)
Fetuses (25)	17–39 gw	Abortion (3)
		Necrotizing chorioamnionitis (4)
		Congenital heart diseases (5)
		Potter's syndrome (1)
		SIUD (12)
Newborns (9)	1–4 pd	Congenital heart diseases (4)
		SNUD (5)
Infants (29)	1–12 m	Pneumonia (6)
		Congenital heart diseases (7)
		Pericarditis (1)
		SIDS (15)

gw, gestational weeks; pd, postnatal days; m, months old; SIUD, sudden intrauterine unexplained death; SIDS, sudden infant death syndrome; SNUD, sudden neonatal unexplained death.

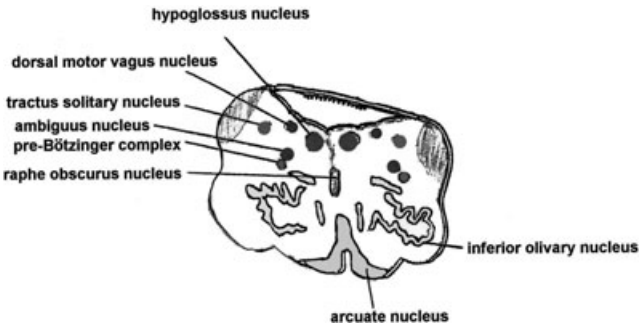

Fig. 1 Schematic representation of a transverse section of the medulla oblongata (at the obex level) showing the localization of the principal nuclei including the pre-Bötzinger complex.

RESULTS

Histological examination performed on serial sections of the 63 brainstems demonstrated, in most of the cases, particularly in all the victims with a known etiology of death, a normal morphology of the principal nuclei throughout their extension.

Nevertheless, developmental abnormalities of the medullary arcuate nucleus were found in 13 of the 32 sudden death victims (5 SIUD, 1 SNUD and 7 SIDS) (41%) and in two of the 31 control subjects who died of other causes (6%).

Examination of NK1R and SS immunohistochemical expression in brainstem sections of the control cases allowed us to recognize and to circumscribe the boundaries of the pBc in man.

In fact, a high immunopositivity was present in a group of neurons located in a restricted area of the reticular formation in the ventrolateral medulla, ventral to the semicompact division and caudal to the compact division of the ambiguus nucleus (Fig. 1).

Specifically, high NK1R immuno-expression levels were present mainly along the inner surface of the plasma membrane of both cell bodies and contiguous processes of some pBc neurons, outlining their neuronal surfaces (Fig. 2). In particular, primary prominent immunopositive long dendrites were visible in this area, frequently oriented in the dorsoventral axis. A few background particles were occasionally seen, scattered randomly, bearing no apparent relationship to NK1R-ir neurons.

SS-immunolabeling contributed to a definition of the anatomical localization of the pBc only in infant deaths. In fact, before birth, intense SS positivity was constantly seen in almost all the brainstem nuclei. Instead, in the postnatal deaths, we observed a moderate number of positive cell bodies limited to the tractus solitary nucleus (precisely to the ventrolateral subnucleus) and to the same area defined as pBc by NK1R immunostaining (Fig. 3).

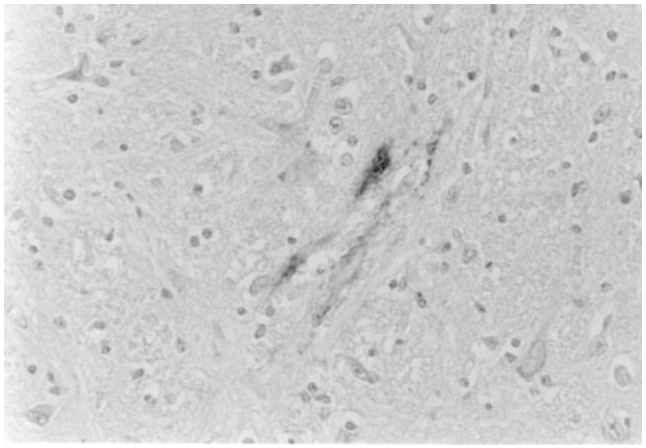

Fig. 2 NK1R-immunoreactivity of neuronal somata and processes in the pre-Bötzinger complex area. Magnification 40×.

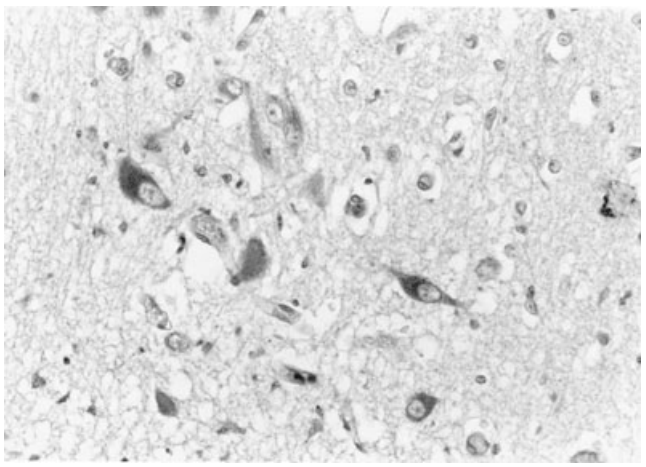

Fig. 3 Somatostatin-immunoreactive neurons in the pre-Bötzinger complex area. Magnification 40×.

Immunoreactive neurons in the pBc were roundish in fetuses before 30 gestational weeks, and had an oval, fusiform, pyramidal or irregular shape in late fetal deaths and after birth, with a vesicular nucleus, loose chromatin and a clearly identifiable nucleolus. The neurons were embedded in a complex radial dendritic tree, belonging to the reticular formation. The extension of the pBc, defined through the examination of serial immunostained sections, was very limited, just rostral to the obex, aligned and with a similar length to that of the more easily recognizable dorsal accessory of the inferior olivary nucleus. Therefore, it was not difficult to identify, even in routinely stained sections with HE and/or Klüver-Barrera staining, the localization of the pBc, adjacent to, and lying at the back of the dorsal accessory olivary nucleus (Fig. 4).

After the definition of the anatomical localization and boundaries of the pBc in the control cases, we examined

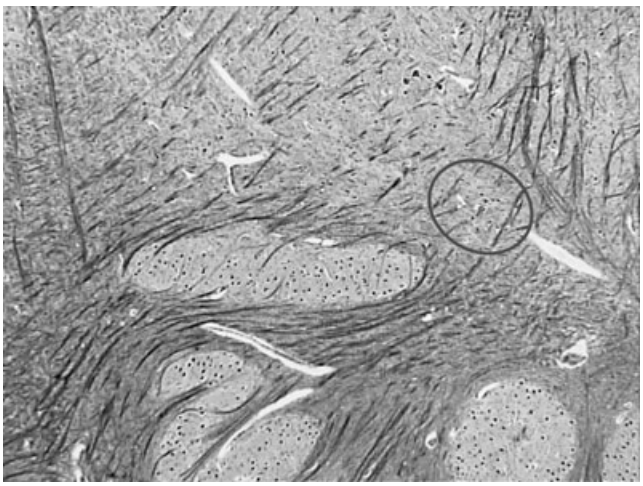

Fig. 4 Photomicrograph of a partial histological section of medulla oblongata showing the localization of the pre-Bötzinger complex (in the circled area) ventral to the ambiguous nucleus and at the back of the dorsal accessory olivary nucleus. Klüver-Barrera stain. Magnification 20×.

this neuronal center in the 32 study cases without a precise cause of death (12 SIUD, 5 SNUD and 15 SIDS).

Hypoplasia of the pBc, with a decreased number of neuronal bodies and/or dendritic fibers, was diagnosed in 13 cases (8 SIUD, 2 SNUD and 3 SIDS). Sometimes, a relatively normal neuronal density was associated to a lack of dendrites (Fig. 5). Depletion of the processes was particularly evident in Gless stained sections compared with the corresponding sections of control cases (Fig. 6). Neuronal cell bodies of hypoplasic pBc were sometimes similar in shape to control neurons or, more frequently, they were smaller and very lengthened with a flattened nucleus, compact chromatin and a poorly evident nucleolus (Fig. 7). In pBc hypoplasic areas the immunopreparations were negative for Nk1R and SS expression. Only rare NK1R immunopositive fibers could be seen. In two additional SIUD cases the pBc was not identifiable in all the serial sections. Therefore, we defined this absence as agenesis of the pBc.

Altogether the hypoplasia of the pBc resulted in association with the hypoplasia of the arcuate nucleus in five cases (3 SIUD and 2 SIDS victims).

DISCUSSION

Respiration is a highly integrated process that involves a complex interplay between the brain, brainstem, cerebellum, spinal cord, cranial and spinal nerves, diaphragm, intercostal muscles, laryngeal and pharyngeal structures, lungs and vasculature.[18,19] Central chemoreceptors also contribute to the ventilatory output in response to normal or abnormal mixtures of gases and hydrogen ion concentrations in the blood. These chemosensitive neurons are widespread in the central nervous system, including multiple areas within the brainstem, especially the raphe nuclei, locus coeruleus, tractus solitarii nucleus, parafacial nucleus and arcuate nucleus.[20–22]

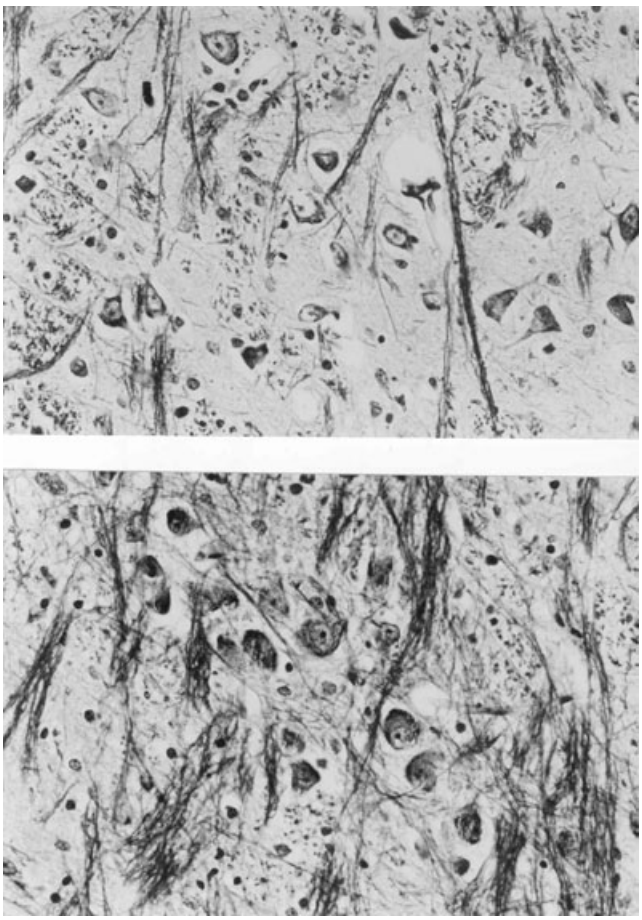

Fig. 5 Upper image: Hypoplasia of the pre-Bötzinger complex with a decreased number of dendrites belonging to the reticular formation, in a case of sudden intrauterine unexplained death (36 gestational weeks).Lower image: Normal pre-Bötzinger complex configuration in an age-matched control case. Klüver-Barrera stain. Magnification 40×.

Experimental studies both *in vivo* and *in vitro* suggest that this intricate network is driven by a single site of the brainstem, which is critical for respiratory rhythmogenesis, named the pre-Bötzinger complex (pBc). Besides, a central role of the pBc in the modulation of eupneic breathing in adult life has been proposed and has gained wide acceptance.[1–4]

The anatomic structure and the localization of the pBc were not known until recently, when the evaluation of NK1R (neurokinin 1 receptors)-immunoreactivity allowed a precise delineation of its boundaries in the ventrolateral medulla oblongata of rats, in a restricted area ventral to the semicompact ambiguus nucleus.[5–7] Besides, Stornetta *et al.*[7]

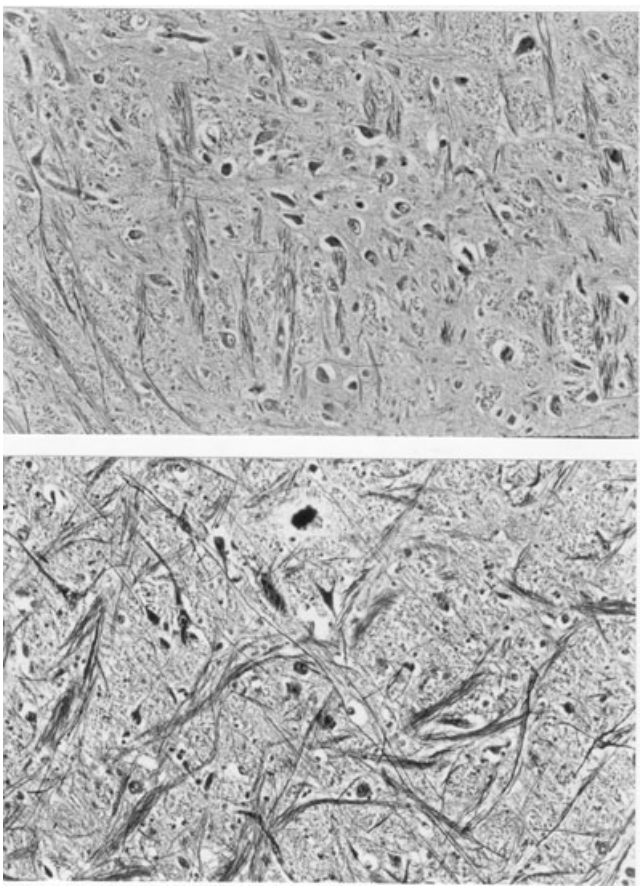

Fig. 6 Upper image: Evident depletion of dendritic fibers in the pre-Bötzinger complex in a case of sudden intrauterine unexplained death (35 gestational weeks). Lower image: Normal dendritic configuration in the pre-Bötzinger complex in an age-matched control case. Gless stain. Magnification 40×.

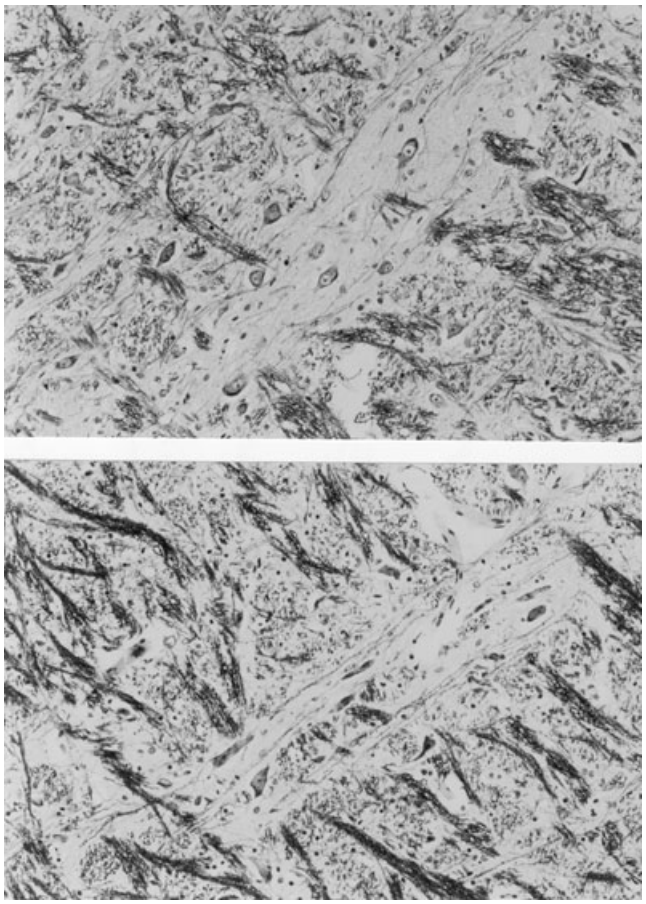

Fig. 7 Upper image: Hypoplasia of the pre-Bötzinger complex with lack of dendrites and normal neuronal cell bodies, in a case of sudden intrauterine unexplained death (38 gestational weeks). Lower image: Hypoplasia of the pre-Bötzinger complex with lack of dendrites and flattened neuronal cell bodies, in a case of sudden intrauterine unexplained death (36 gestational weeks). Klüver-Barrera stain. Magnification 40×.

suggested that somatostatin (SS)-immunolabeling may also be a marker of neurons in the pBc.

Thus, the aim of the present study was to use NK1R and SS immunoneurochemistry to identify the pBc anatomic structure in humans, never previously investigated.

Our observations provided evidence that the pBc region in man corresponds anatomically and functionally to the structure defined in experimental studies. In fact, in coronal sections of brainstem we observed NK1R-immunoreactive neurons and processes in a limited area between the semicompact region of the ambiguus nucleus and the dorsal accessory of the inferior olivary nucleus.

Some SS-immunolabeled neurons were also detectable in the same location of the brainstem in infants.

The presence of immunopositive neurons also suggests the involvement of the pBc in breathing in man. In fact, the natural ligand for the NK1R is substance P, a peptide neuromodulator with strong stimulatory effects on respiratory system output.[23,24] Similarly, the SS, that represents an important breathing inhibitor in fetal life, becomes important for the physiological control of respiration immediately after delivery.[9,25]

This study also allowed us to define the main morphological features of the pBc neurons, that are roundish in fetuses before 30 gestational weeks, and subsequently become lengthened, embedded in a dendritic system belonging to the reticular formation.

The reticular formation is an important neural structure with no distinct cytoarchitectural boundaries, extending from the spinal cord junction with the medulla, through the pons, to terminate at the border with the caudal-most intralaminar thalamus. It is subdivided into a bewildering array of fields and nuclei, with anatomical and functional connections, including the pBc.[26–28] This neuronal circuit seems to be involved in many functions, including cardiorespiratory control.

Nevertheless, despite its supposedly important features and its large area extension, the reticular formation has received no sufficient, modern structural and functional evaluation.

We observed structural and/or functional alterations of the pBc in a high percentage of sudden deaths (47%), prevalent in late fetal deaths. In particular, different developmental defects (hypoplasia with a decreased neuronal number and/or dendritic hypodevelopment of the reticular formation, abnormal neuronal morphology, immunonegativity of neurotransmitters, and agenesis) were found.

Although a functional respiratory network is necessary for survival at birth, respiratory-like movements are detectable before birth not only in experimental animals[29] but also in humans.[30,31] Thus, an immature neuronal respiratory network, checked by the pBc, is usually active at low frequency in prenatal stages, shortly after the onset of fetal movements. Hypoplasia/agenesis of the pBc, observed in this study in 83% of the SIUD victims, could determine defects of this occasional respiratory activity in prenatal life. Nevertheless, these would not be sufficient to justify the fetal death.

On the other hand, we observed only some pBc neurons but not all, labeled with SS and/or NK1R. One possibility is that single unlabeled neurons may serve functions other than respiration. In support of this hypothesis there is the work by Standish *et al.*[32] In experimental studies these authors have demonstrated the presence in the pBc also of adrenergic cells, cardiovagal motoneurons and γ-aminobutyric-acidergic (GABAergic) interneurons involved in the baroreflex. Therefore, we believe that the pBc is a neuronal center of the reticular formation that contains a variety of neurons and is not only involved in respiratory rhythm generation but, more extensively, is essential to the control of all vital functions.

Advancing towards the time of birth, very likely a general check of all the essential functions for extrauterine life occurs. Sudden unexpected fetal death could therefore be ascribed to a selective process when developmental alterations of the pBc arise.

ACKNOWLEDGMENTS

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