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Developmental alterations of the respiratory human retrotrapezoid nucleus in sudden unexplained fetal and infant death

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ABSTRACT

The study aims were twofold: 1) identify the localization and the cytoarchitecture of the retrotrapezoid nucleus (RTN) in the human fetus and infant and 2) ascertain if the RTN, given its essential role in animal studies for the maintenance of breathing and chemoreception, showed abnormalities in victims of sudden perinatal and infant death (sudden intrauterine unexplained death/SIUD — and sudden infant death syndrome/SIDS). We examined SIDS and SIUD cases and Controls (n = 58) from 34 gestational weeks to 8 months of postnatal age by complete autopsy, in-depth autonomic nervous system histological examination, and immunohistochemical analysis of the *PHOX2B* gene, a transcriptional factor involved in Congenital Central Hypoventilation Syndrome that has been defined as a marker of rat RTN neurons.

We identified a group of PHOX2B-immunopositive neurons within the caudal pons, contiguous to the facial/parafacial complex, in 90% of Controls, likely the homologous human RTN (hRTN). We observed structural and/or *PHOX2B*-expression abnormalities of the hRTN in 71% of SIUD/SIDS cases *vs* 10% of Controls ($p<0.05$).

In conclusion we suggest that developmental abnormalities of the hRTN may seriously compromise chemoreception control, playing a critical role in the pathogenesis of both SIUD and SIDS.

1. Introduction

Efficient uptake and delivery of oxygen and removal of carbon dioxide are essential for sustaining life, and for normal growth and development in many species. Chemoreception is a fundamental mechanism to achieve these life-sustaining functions. Central chemoreceptors, distributed in specialized brainstem neurons, detect and regulate carbon dioxide (CO_2) and/or pH variations through a variety of responses. In so doing, these central chemoreceptors regulate extrauterine breathing (Nattie, 1995, 1998; Feldman et al., 2003; Nattie and Li, 2006) and are structurally and functionally intact, actively responding to chemical stimuli in the late-term fetus (Purves, 1981). The differences between prenatal and postnatal chemoreception appear to be primarily dependent on central inhibition in fetal life of the ventilatory responses to hypoxia and/or hypercapnia, with localization to the rostral pons in the parabrachial/Kölliker–Fuse complex (Walker, 1995; Lavezzi et al., 2004). The inhibitory effects of this neuronal complex on chemoreception are markedly reduced after birth. Thus, one might anticipate that chemoreception would function differently in the fetus than in the newborn, and that birth would be associated with an immediate abrupt change in the parabrachial/Kölliker–Fuse complex function. Consequently, it is essential that the chemoreceptor structure and function be mature in the late-term fetus — allowing for the rapid transition to coordinated breathing and respiratory control in postnatal extrauterine life.

Using different methodological approaches, experimental studies have identified the retrotrapezoid nucleus (RTN) as one of the main sites of central chemoreception and respiratory drive (Loeschcke, 1982; Smith et al., 1989). Smith et al. (1989), in particular, applied retrograde tracing methods to localize the RTN as a cluster of neurons at the ventral surface of the rostral medulla oblongata, just ventral to the facial nucleus, in a region long-suspected to mediate central chemoreception, and projecting to respiratory circuits in the ventrolateral medulla of the rat. Subsequently, numerous researchers have established the RTN as one of the more important regions mediating central chemoreception (Nattie et al., 1991; Nattie and Li, 1994; Onimaru and Homma, 2003). Specifically, acidification of the RTN stimulates the ventilatory activity and lesions or inhibition of RTN neurons produce a dramatic reduction of breathing consequent to diminished chemoreflexes in rats. Rodent and feline studies indicate that RTN neurons respond not only

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to carbon dioxide and acid–base status of extracellular fluid in the brain, but also to blood gas composition as detected by peripheral chemoreceptors, particularly by carotid O_2-sensitive chemoreceptors. Thus, the RTN appears to integrate central and peripheral chemoreceptor information, providing an important 'drive' to breath. Further, carotid body inputs reach CO_2-sensitive RTN neurons through a network including major respiratory neuronal groups of the brainstem in non-human species (Feldman et al., 2003; Guyenet et al., 2005; Takakura et al., 2006).

Neurophysiological and genetic evidence have also suggested that RTN neurons involved in the chemoreflex control in rats express the homeobox transcription factor *PHOX2B*, thereby representing a selective marker of the RTN (Stornetta et al., 2006; Kang et al., 2007; Takakura et al., 2008; Abbott et al., 2009; Dubreuil et al., 2009). In the animal, morphological identification of the RTN is supported by the immunohistochemical detection of PHOX2B in all its neurons, even if the immunoreactivity is not limited to this nucleus but detectable in scattered neurons of the facial region and in other nuclei (dorsal motor nucleus of the vagus, nucleus of the solitary tract, intermediate reticular nucleus) (Stornetta et al., 2006; Kang et al., 2007). In humans, studies on the RTN are very difficult. Nevertheless, Rudzinski and Kapur (2010) recently described PHOX2B-immunoreactivity in a small cohort (8 available cases among 17 samples) of fetus and infant "controls", describing a region at the pontomedullary junction ventral to the facial nucleus and lateral to the superior olivary nucleus as the potential human RTN. While an important study, it was limited by small cohort size, few term infants, variability in histological processing of the brainstems, and variable results by sample such that nearly half of the original cases lacked viable results.

The present study was designed to determine the possibility of tracing the anatomical boundaries and the cytoarchitecture of the human homologue of the animal RTN, with the main aid of PHOX2B immunohistochemistry. Given the long held theories of cardiorespiratory dysregulation or failure of automaticity of breathing in sudden infant death syndrome (SIDS) pathogenesis (Hunt, 1992; Thach, 2005), and the anticipated role of the RTN in the maintenance of normal breathing and chemoreception, we hypothesized that developmental abnormalities of the RTN may play a critical role in the etiology of sudden infant death syndrome (SIDS). Because of our prior publication indicating a potential relationship between SIDS and sudden fetal death (Sudden Intrauterine Unexplained death, SIUD) (Lavezzi et al., 2009), and despite the unclear role of chemoreception in fetal life (Purves, 1981, 1982; Teitel, 1996; Wood and Tong, 1999), we further hypothesize that the RTN may play a role in SIUD. In order to ascertain the precise localization and cytoarchitecture of the human RTN (hRTN) and its possible developmental alterations in both infant and fetal unexplained deaths, we examined serial brainstem histological sections in a cohort of SIDS and SIUD victims and controls aged from 34 gestational weeks to 8 months months of postnatal age.

2. Materials and methods

2.1. Study subjects

The study included three groups of Italian Caucasian infants: a SIDS cohort, a SIUD cohort, and a Control group (58 cases in all). Parents of all subjects (SIDS, SIUD) and controls provided written informed consent to both autopsy and genetic study, under protocols approved by the Milan University L. Rossi Research Center institutional review board. Half of these cases have been included in our previous publication (Lavezzi et al., 2009).

2.1.1. SIDS and SIUD victims

The SIDS cases included 22 infants (9 females, 13 males), aged from 1 to 8 postnatal months (median age: 3.5 months). The SIUD cases included 16 unexplained ante-partum deaths (7 females, 9 males), aged 34–40 gestational weeks (median age: 39 weeks). All cases were collected and diagnosed on the basis of the Italian law n.31/2006 "Regulations for Diagnostic Post Mortem Investigation in Victims of SIDS and Unexpected Fetal Death". This law requires that all infants suspected of SIDS, deceased suddenly within the first year of age, and all fetuses deceased after the 25th week of gestation without any apparent cause, must undergo in-depth anatomo-pathological examination, particularly of the autonomic nervous system (ANS).

2.1.2. Controls

This group included 20 suddenly deceased subjects: 11 infants (2 females, 9 males, aged from 2 to 8 postnatal months; median age: 3 months) and 9 fetuses (3 females, 6 males, aged 35 to 40 gestational weeks; median age: 37 weeks) in whom a complete autopsy and clinical history analysis established a precise cause of death. Specific diagnoses among the control infant deaths included the following: congenital heart disease (n = 4), severe bronchopneumonia (n = 3), myocarditis (n = 1), pulmonary artery dysplasia (n = 2), and mucopolysaccharidosis type I (n = 1). Specific diagnoses among the control fetal deaths included: necrotizing chorioamnionitis (n = 4), congenital heart disease (n = 4) and Potter's syndrome (n = 1).

2.2. Autopsy and tissue preparation protocols

All cases and controls were subjected to a complete autopsy, including examination of the placental disk, umbilical cord and membranes in fetal deaths. In all cases an in-depth histological examination of the ANS was made, according to the protocol routinely followed by the Lino Rossi Research Center of the Milan University (Matturri et al., 2005, 2008).

Specifically, after fixation in 10% phosphate-buffered formalin, the brainstem and cerebellum were processed and embedded in paraffin. The examination of the brainstem included the sampling of three specimens. The first specimen included the upper third of the pons and the adjacent portion of midbrain. The second extended from the upper third of the medulla oblongata to the portion adjacent to the pons. The third specimen relied on the obex as reference point, and extended 2–3 mm above it and below.

Transverse serial sections of the midbrain, pons and medulla oblongata were made in each of these three samples at intervals of 60 μm. For each level, six 4 μm sections were obtained, three of which were routinely stained for histological examination using hematoxylin-eosin, Klüver–Barrera and Bielschowsky's silver impregnation technique. The remaining sections were subjected to immunohistochemical study of *PHOX2B* gene and of tyrosine-hydroxylase enzyme. A morphometric study was applied to all the PHOX2B-immunostained sections to define the extension and the boundaries of the hRTN.

2.2.1. Routine histological evaluation

The routine histological evaluation of the brainstem was focused on the locus coeruleus, parafacial/facial complex, superior olivary complex, parabrachial/Kölliker–Fuse complex, rostral raphé nuclei in the pons/mesencephalon, and on the hypoglossus, the dorsal motor vagal, the tractus solitarius, the ambiguus, the inferior olivary complex, the caudal raphé, the arcuate nuclei and the pre-Bötzinger complex in the medulla oblongata. In addition, efforts were made to define the localization and the features of the hRTN. All the histological analyses were carried out by two independent and blinded observers and comparison among the results was performed to evaluate the inter-observer reproducibility.

2.2.2. Immunohistochemical study

Immunohistochemistry was performed on formalin fixed paraffin-embedded tissues.

2.2.2.1. PHOX2B immunohistochemistry. The selected sections, after deparaffinizing, were incubated with anti-PHOX2B antibody (1:750) over-night at +4 °C, following producer's instructions (courtesy of J.-F. Brunet, Ecole Normale Supérieure, Paris, France). Sections were then incubated with secondary antibody (Dako Real, cod.K5005, Dako cytomation, Glostrup, Denmark) following the Dako protocol. A red chromogen solution was used as enzyme substrate. Finally, each section was counter-stained in Mayer's Hematoxylin solution, dehydrated and coverslipped with Depex mounting medium.

Antibody characterization – The PHOX2B antibody was raised against the fourteen amino acid C-terminal sequence of the PHOX2B protein with an added N-terminal tyrosine (YFHRKPGPALKTNLF).

2.2.2.2. Control experiments[(*)]. To establish the specificity of the immunocytochemical methods and of the antibody here used, we applied two types of procedures:

a) 'No primary' control (to test the method specificity) — for 10 cases chosen at random, the primary antibody was not added, but the tissue samples were processed through all the subsequent steps, including the secondary antibody. Because no specific staining was observed, we interpreted that the secondary antibody was binding non-specifically to different sites on the tissue but not binding specifically to the primary antibody.
b) 'Preadsorption' (to test the antibody specificity) — the antibody was pre-incubated at its working dilution with the respective antigen. The reaction mixture was centrifuged, and the resulting supernatant (preadsorbed serum) was used in place of the primary antibody in 10 cases chosen at random. No staining resulted from incubation with the preadsorbed serum, so we interpret this to show the absence of any free antibody.

2.2.3. Tyrosine hydroxylase (TH) immunohistochemistry

The sections were rinsed three times in 0.1 M Trizma buffered saline (TBS) followed by a 48-h incubation at 4 °C with a 1/500 dilution of primary rabbit antiserum to TH (Novocastra Laboratories, Newcastle, UK). The dilutions were prepared with a solution of 1% normal goat serum (NGS) and 0.25% Triton X-100 in 0.1 M Tris-saline. This was followed by a 2.5-h incubation with biotinylated goat antirabbit immunoglobulin G (IgG) (Vector Laboratories, Burlingame, CA, USA) diluted 1/200 with 1% NGS in Tris-saline. After incubation for 2 h with the avidin–biotin complex diluted 1/100 with 1% NGS in Tris-saline (Vector), the sections were treated for 6 min with a 0.05% solution of 3,3′diamino-benzidine and 0.01% hydrogen peroxide, rinsed in phosphate buffer, mounted on gel-coated slides, cleared in xylene and coverslipped.

2.2.4. Morphometric study

The morphometric analysis was performed with an image analyzer (Image-Pro Plus — Media Cybernetics, Silver Spring, Maryland, USA) on immunostained serial sections through the rostrocaudal extent of the recognized hRTN. The measurements were calculated after plotting the boundary of the nucleus on each slide to be sampled, capturing the PHOX2B strongly-labeled neurons belonging to the hRTN. Diffusely distributed neurons beyond the defined conventional contours or weakly immunostained neurons were not included. The data were then digitized, coded, and stored in the computer for future analysis.

The following parameters were evaluated in each section: hRTN area (expressed in mm^2), neuronal density (expressed as number of neurons per mm^2), and neuronal size (cell body area, expressed in μm^2); data were recorded by right or left side.

For the three-dimensional reconstruction, a computer program developed by Voxblast (Vaytek, Inc. Fairfield, Iowa, USA) was used to digitize and align the hRTN anatomic boundary tracings in the serial sections and to obtain volumetric measurements (in mm^3). The total number of neurons along the whole length of the hRTN was also determined. All the morphometric data were expressed as mean values and standard deviation (mean ± SD).

2.3. PHOX2B testing of tissue from cases and controls

The PCR reactions were carried out using the GC-RICH System (Roche Molecular Biochemicals, Indianapolis, IN) in a final volume of 25 μl containing 50 ng genomic DNA, 0.2 mM dNTPs, 5 μl of 5X GC-RICH PCR reaction buffer, 2.5 μl of 5 M GC-RICH resolution solution, 0.4 μM of each primer, and 0.6 U of Taq DNA Polymerase mixture (28–30). The amplification was performed using an ABI 9800 thermal cycler (Applied Biosystems, CA) with an initial denaturation at 95 °C for 5 min followed by 10 cycles of denaturation at 95 °C for 1 min, annealing at 61 °C for 1 min, and extension at 72 °C for 45 s and 25 cycles of denaturation at 95 °C for 1 min, annealing at 59 °C for 1 min, and extension at 72 °C for 45 s Final extension was at 72 °C for 7 min. The PCR products were column-purified with a Microcom Ultracel YM-100 Purification Kit (Millipore Corporation, Bedford, MA) to remove reaction buffer and unincorporated primers, and visualized by running 2 μl of each sample on eGene (Qiagen, CA) to verify amplicon and size before sequencing. The PCR products were directly sequenced with the "Big Dye Terminator v1.1 Cycle Sequencing" kit (Applied Biosystems, CA) by the forward primer 5′ – CTT CAC CGT CTC TCC TTC C – 3′ and reverse primer 5′ – TAC CCG CTC GCC CAC TC – 3′, in an ABI Prism 3130 xl automatic sequencer (Applied Biosystems, CA) in both directions. The data were analyzed by Sequencing Analysis 5.1.1 and Autoassembler (Applied Biosystems, CA).

2.4. Statistical analysis

The statistical significance of direct comparison between the groups of victims and controls was determined using analysis of variance (ANOVA). Statistical calculations were carried out on a personal computer with SPSS statistical software (Chicago, IL). The selected threshold level for statistical significance was $p<0.05$.

3. Results

3.1. Routine histological evaluation of the brainstem and cerebellum (Table 1)

3.1.1. Controls

Alterations were observed at routine histological examination among 25% of controls (2 fetal and 3 infant deaths). These alterations include the following:

- hypoplasia of the arcuate nucleus in 25% of controls (2 fetal and 3 infant deaths);
- hypoplasia of raphé obscurus nucleus in 5% of controls (1 infant death);
- delayed cerebellar cortex maturation in 10% of controls (2 infant deaths).

(*) As proposed by Saper (2003), it is necessary to emphasize that the criteria of specificity in immunohistochemistry does not prove but only suggests that the revealed antigen is the antigen of interest. In addition, one needs to consider that when the material is subjected to histological procedures, as in the case of our study, discrepancies can occur in immunohistochemical analyses due to a potentially suboptimal fixation procedure. Moreover, as indicated by Saper, when the epitope is on a protein, it can change its configuration in formalin-fixed brain tissues. So, an antibody staining should always be considered to label "antigen-like" molecules rather than necessarily staining the intended antigen.

Table 1
Overall neuropathological brainstem and cerebellum findings in SIDS, SIUD and Control groups.

Study group	Arcuate nucleus hypoplasia	Pre-Bötzinger nucleus hypoplasia	Parafacial/facial complex hypoplasia	Raphé nuclei hypoplasia	Cerebellar cortex delayed maturation
	Number of cases (%)	Number of cases (%)	Number of cases (%)	Number of cases (%)	Number of cases (%)
SIDS cases n = 22	13 (59%)	7 (32%)		10 (45%)	7 (32%)
Infant controls n = 11	3 (27%)	–	–	1 (9%)	2 (18%)
SIUD cases n = 16	9 (56%)	4 (25%)	12 (75%)	5 (31%)	3 (19%)
Fetal controls n = 9	2 (22%)	–	–	–	–

Individual victims may display any combination of these pathological alterations.

These alterations were associated in 2 of the 3 infant deaths (one case: all three alterations co-occurred; one case: hypoplasia of the arcuate nucleus co-occurred with delayed cortex maturation of cerebellum).

3.1.2. SIDS and SIUD cases

Several alterations were observed at routine histological examination of the brainstem and cerebellum among 74% of SIDS and SIUD cases, including the highlighted hypodevelopment of select nuclei and structures described below:

Brainstem:

- hypoplasia/agenesis of arcuate nucleus in 58% of cases (13 SIDS; 9 SIUD). Hypoplasia was bilateral (concerning entire nucleus) in 6 SIDS and 4 SIUD victims; partial (generally confined to inferior 2/3 of the nucleus) in 4 SIDS and 1 SIUD; and unilateral (involving right portion) in 2 SIDS cases. Further, agenesis of the arcuate nucleus was identified in 1 SIDS and 4 SIUD cases;
- hypoplasia of pre-Bötzinger nucleus, with decreased neuronal number and/or dendritic hypodevelopment of the reticular formation, in 29% of cases (7 SIDS; 4 SIUD);
- hypoplasia of one or more nuclei of the raphé system (obscurus, pallidus, median, magnum, caudal linear raphé nuclei) in 39% of cases (10 SIDS; 5 SIUD);
- hypoplasia/agenesis of parabrachial/Kölliker–Fuse complex in 11% of cases (4 SIUD);
- hypoplasia of parafacial/facial complex, with decreased neuronal density and area, in 32% of cases (12 SIUD).

Cerebellum:

- immature structure of cerebellar cortex, uniformly comprised of small round cells without the usual four-layered shape, in 29% of cases (7 SIDS; 3 SIUD).

Frequently, above all in fetal deaths, two or more alterations were simultaneously present in the same case, such as hypoplasia of the arcuate, the pre-Bötzinger and parafacial nuclei.

Inter-observer reproducibility related to histological analyses revealed a Kappa Index = 0.85.

3.2. Immunohistochemical study to localize and define morphology of hRTN (Table 2)

3.2.1. Controls

Following immunohistochemical methodology in published rat studies (Stornetta et al., 2006; Kang et al., 2007; Takakura et al., 2008; Abbott et al., 2009; Dubreuil et al., 2009) with indication that the RTN lies mainly ventral to the facial nucleus, at the medullary surface, we identified a group of PHOX2B immunopositive neurons located deeper within parenchyma of the caudal pons, ventral and medial to the facial/parafacial complex* and lateral to the superior olivary complex** — potentially the hRTN — in 90% of the controls (18/20). Consistently, the hRTN neurons were non-catecholaminergic (TH–). Fig. 1 indicates a coronal section of the pons at the caudal level, with proposed hRTN localization (3 month-old control). Fig. 2 indicates the histological pattern of the proposed hRTN on a routinely stained section by Klüver–Barrera method (2 month-old control), consecutive to those submitted to immunohistochemical procedures.

3.3. Morphometric analysis of hRTN

3.3.1. Controls

The morphometric evaluation was performed on immunostained sections using the 90% of the PHOX2B-immunostained neurons remaining after the exclusion of neurons with undefined edges and indistinct nucleus and also the weakly immunostained neurons into the anticipated localization of the hRTN in serial sections of the caudal pons. We observed a mean transverse section area of 1.32 ± 0.46 mm^2, a mean neuronal density of 32 ± 6 and a mean neuronal cell body area of 268.55 ± 0.36 μm^2. The three-dimensional mapping of hRTN through the analyzed serial sections indicated a mean volume of 2.35 ± 0.46 mm^3 and a mean neuronal cell count within the rostral-caudal length of the hRTN of 980 ± 23 neurons.

3.4. Alterations of the hRTN

3.4.1. Controls

Hypoplasia of the hRTN with decreased number of PHOX2B immunopositive neurons was found in 2 fetuses (10%; both born dead at 37 gestational weeks with congenital heart disease).

3.4.2. SIDS and SIUD cases

The examination of the immunostained preparations in the 38 victims of the study who died without an explained cause (22 SIDS; 16 SIUD) showed in 71% of cases (27 cases; $p<0.05$) structural and/or PHOX2B expression abnormalities of the hRTN. Specifically, in 55% of SIDS (12 cases) and 50% of SIUD (8 cases) the number of neurons, characterized by a PHOX2B cytoplasmic immunoreactivity, and area of hRTN were markedly decreased (hypoplasia) (Fig. 3B). The mean morphometric parameters obtained in these cases with hRTN hypoplasia were: a mean transverse section area of 0.77 ± 1.11 mm^2, a mean neuronal density of 21 ± 3, and a mean neuronal cell body area of 185.08 ± 0.13 μm^2. The three-dimensional mapping of the hRTN through the analyzed serial sections indicated a mean volume of 1.19 ± 0.15 mm^3 and a mean neuronal cell count within the rostral-caudal length of the hRTN of 243 ± 24. These morphometric values were significantly lower by 30–60% than those of controls ($p<0.05$ for all comparisons).

The hRTN neuronal number was as expected in 11% of the sudden death cases (9% of SIDS, 2 cases; 13% of SIUD, 2 cases), but the neurons showed a cytoplasmic expression of PHOX2B immunostaining (Fig. 3C). Further, the hRTN was essentially absent (agenesis) in 19% of SIUD cases (3 victims) (Fig. 3D).

* Facial/parafacial complex — The facial/parafacial complex (F-PFc) has been by us defined as the link between the facial nucleus (FN) and the parafacial neuron group (parafacial nucleus — PFN) (Lavezzi and Matturri, 2008). The FN is seen in transversal sections of caudal pons at the source of the facial nerve. It appears as a roundish cluster of large polygonal neurons with peripheral Nissl substance, evident axons and dendrites. The PFN ventro-laterally surrounds the facial nucleus. Compared with the FN neurons, the parafacial neurons are similar in shape but more intensely stained in both hematoxylin-eosin and Klüver–Barrera preparations, due to an increased number of pigment granules in the cytoplasm. Frequently, the neurons of the two nuclei are inter-mixed.

** Superior olivary complex — The superior olivary complex (SOc) includes two main nuclei, the medial superior olivary nucleus (MSON) and the lateral superior olivary nucleus (LSON), located anterior and medial to the F/PFc.

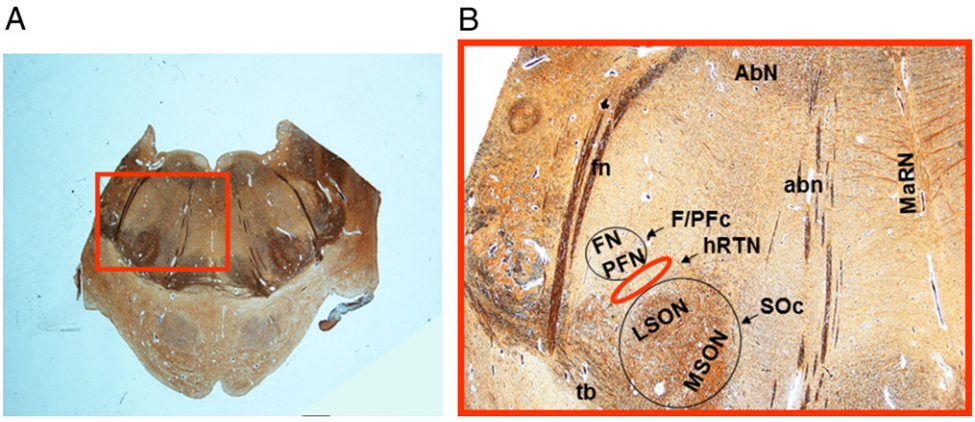

Fig. 1. Histological section of human caudal pons (3 month-old control) showing localization of the proposed human retrotrapezoid nucleus (hRTN), included between the facial/parafacial complex and the superior olivary complex . Boxed area in A is shown at greater magnification in B. Bielschowsky's silver impregnation technique. Magnification : A) 0.5×; B) 4×. *AbN — abducens nucleus; abn — abducens nerve; FN — facial nucleus; fn — facial nerve; F/PFc — facial/parafacial complex; LSON — lateral superior olivary nucleus; MSON — medial superior olivary nucleus; PFN — parafacial nucleus; MaRN — magnus raphé nucleus; hRTN — human retrotrapezoid nucleus; SOc — superior olivary complex; tb — trapezoid body.*

Collectively, a significantly greater proportion of hRTN alterations were observed in SIDS/SIUD victims compared with controls (71% *vs* 10%; $p<0.05$), with related brainstem and cerebellum alterations. SIUD cases with RTN alterations had associated parafacial nucleus hypoplasia/agenesis in 85% of cases (11/13 cases).

3.5. PHOX2B testing of tissue from cases and controls

DNA from SIDS and SIUD cases and controls had the normal *PHOX2B* 20/20 genotype; none had evidence of a non-polyalanine repeat expansion mutation. These results confirm that the tested cases/controls had no mutations compatible with Congenital Central Hypoventilation Syndrome (CCHS).

4. Discussion

In this study we propose a candidate region of the RTN, a neuronal center with an essential role in animal studies for the maintenance of breathing and chemoreception, in humans by using immunohistochemical detection of its selective marker, the transcriptional factor Phox2b (Kang et al., 2007; Takakura et al., 2008; Abbott et al., 2009; Dubreuil et al., 2009). Specifically we describe a cluster of PHOX2B-positive neurons in the brainstem of human victims who have died in intrauterine life or in the first year of life and suggest that this candidate region is the human RTN (hRTN).

In our study of SIUD and SIDS victims we identified altered development of the putative hRTN, either hypoplasia/agenesis, or a normal structure but population with immature neurons and defective expression of the transcription factor *PHOX2B*. We suggest that a yet unidentified *PHOX2B* mutation (or a mutation in the *PHOX2B* pathway/network) could be responsible for the hRTN abnormalities found in these immature and young victims. We infer that the majority of hRTN neurons with *PHOX2B* mutations fail to develop or degenerate, seriously compromising the central and peripheral chemoreception and thus leading to disordered respiratory and autonomic regulation and subsequent death. Stated differently, we propose that developmental abnormalities of the RTN may play a critical role in the etiology of both SIUD and SIDS.

Asserting a complete homology between this region of the human brainstem and the RTN described in animals presents some difficulties.

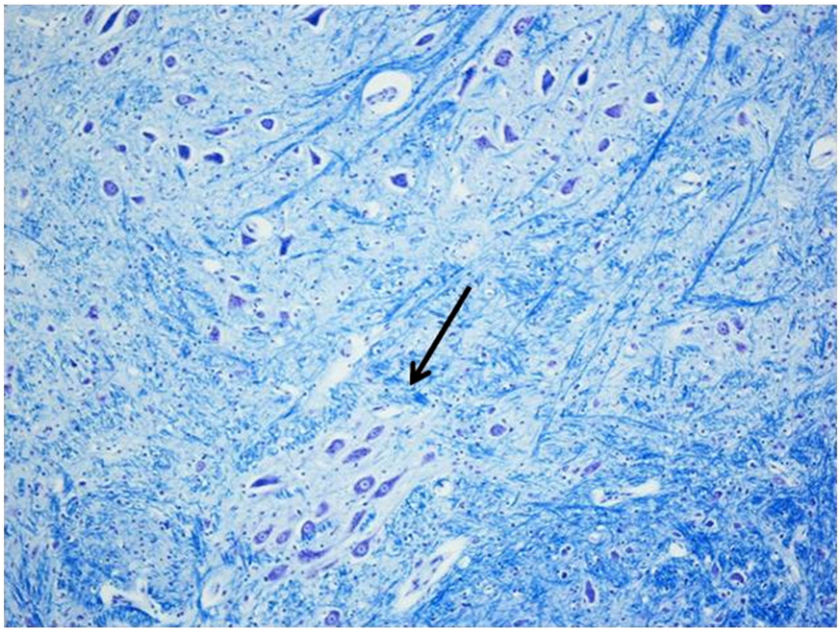

Fig. 2. Histological section of caudal pons (2 month-old control) showing a group of round/fusiform neurons, with recognizable axon and dendrites (see arrow), corresponding to the hRTN, ventral to the facial/parafacial complex. Klüver–Barrera stain. Magnification: 20×.

Table 2
Alterations of the human retrotrapezoid nucleus (hRTN) in sudden infant death syndrome (SIDS), sudden intrauterine deaths (SIUD) and control groups.

Alterations in human RTN (hRTN)				
Study group	Hypoplasia with altered *PHOX2B* expression	Agenesis	Normal structure with altered *PHOX2B* expression	Total number of subjects with alterations (%)
SIDS n = 22	12	–	2	14 (64%)
Infant controls n = 11	–	–	–	0 (0%)
SIUD n = 16	8	3	2	13 (81%)
Fetal controls n = 9	2	–	–	2 (22%)

When comparing SIDS *vs* infant controls, $p<0.01$.
When comparing SIUD *vs* fetus controls, $p<0.05$.

In fact the candidate region of the RTN is not located on the surface of the medulla as is the case in the cat/rodent brainstem, but deeper within the parenchyma of the caudal pons. This exclusive localization of the RTN in the caudal pons, as we have demonstrated through our immunohistochemical studies on serial brainstem sections, is also in contrast with the study of Rudzinski and Kapur in humans (2010). These authors, in fact, claim that their hRTN resides in a region that is precisely homologous to that of rats and is close to the ventral medulla surface, caudal to the trapezoid body, where it is located in other species.

We cannot exclude that, given the distinctive neuronal dispersion in the RTN, Rudzinsky and Kapur have examined the caudal lobe of the nucleus and we the rostral one. However, as supported by Niblock et al. (2005) in light of species differences, the complex brainstem has undergone significant evolutionarily changes due to its encephalization with massive development of the basis pontis in humans. In particular the facial nucleus, frequently used as an anatomical landmark given its distinctive appearance, in humans is located in the caudal pons, whereas in small mammals is located in the medulla. So we believe that the adjacent RTN could have followed the same evolutionary way change. To avoid confusion and to add clarity, however, it may be more precise for us to refer to our findings as the homologous human RTN (hRTN). Moreover, in the human few if any functional-anatomical experiments exist which would complement those which, in the rat and mouse, have demonstrated the significance of the RTN in providing a major source of central chemoreception. Nonetheless, the present study constitutes one of the first reports of a neurochemically homologous RTN in the human brainstem and the first to document distinct abnormalities of this nucleus in SIDS and SIUD cases as compared to controls.

A number of different approaches may serve to validate the correspondence of the hRTN with the RTN of other mammals. For example, the connections of the RTN in animals have been well characterized (Smith et al., 1989; Rosin et al., 2006) and it may be possible to examine the connections of the hRTN by applying the post-mortem axonal tracing method as reported by Zec and Kinney (2003). They examined human midgestational fetal specimens for the anatomic relationship between different brainstem nuclei using a bidirectional

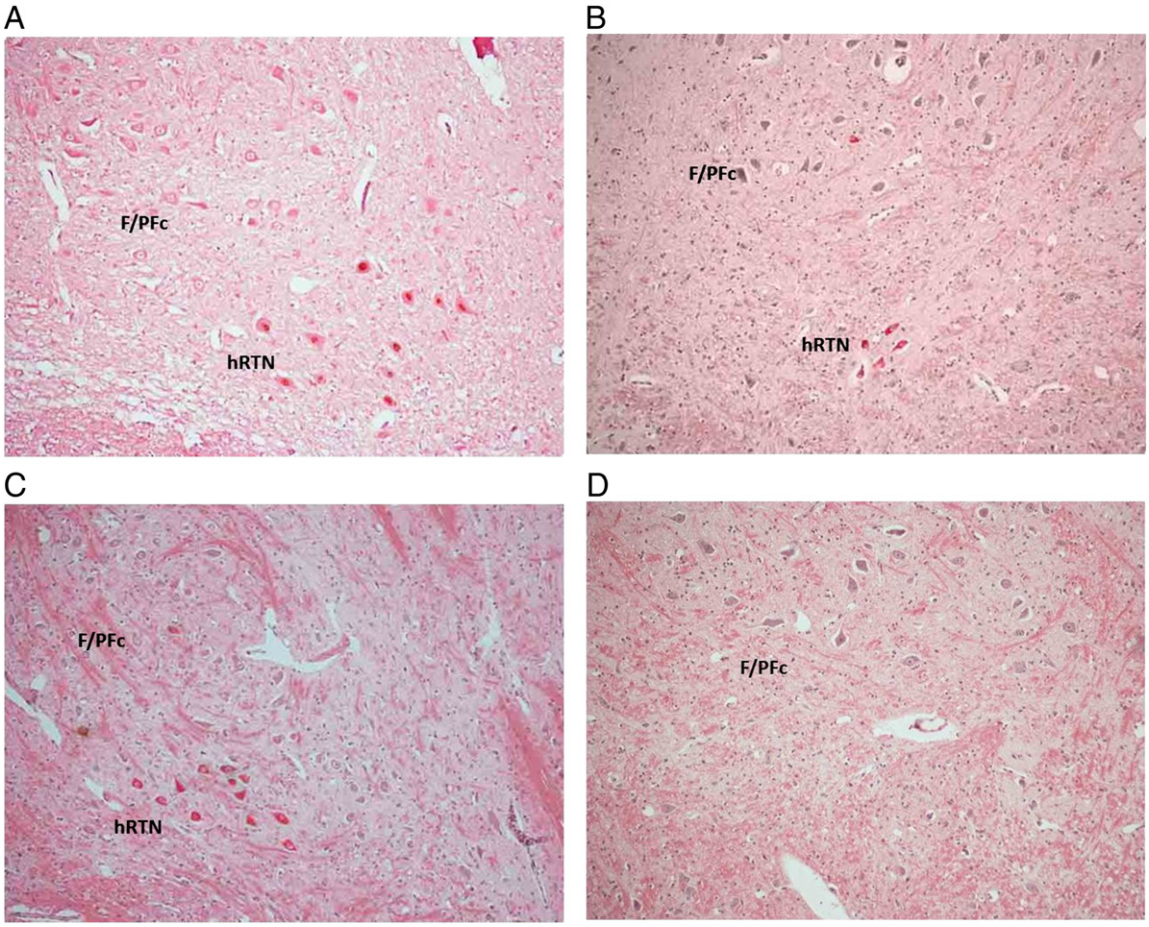

Fig. 3. *PHOX2B*-immunohistochemistry. Four examples of the main results obtained in histological sections at the same level of caudal pons. A. Normal structure of the identified human RTN with nuclear expression of PHOX2B (36 gestational week control). B. Decreased number of PHO2B immunostained neurons in the area of the hRTN (hRTN hypoplasia). The neurons show cytoplasmic PHOX2B immunopositivity. (1 month-old SIDS case). C. Normal number of neurons in the hRTN area with prevalent cytoplasmic *PHOX2B*-immunopositivity (38 gestational weeks SIUD case). D. Lack of neurons in the hRTN region (hRTN agenesis) (34 gestational weeks SIUD case). Phox2b immunohistochemistry. Magnification: 20×. *F/PFc — facial/parafacial complex; hRTN — human retrotrapezoid nucleus.*

lipophilic fluorescent tracer DiI that labels by diffusion the axons related to specific nuclei. They noted that a key limitation of the DiI technique is the need for application within 5.5 h of death. Because of Italian law precluding autopsy before 24 h of death, the DiI technique would only have applicability to therapeutic interruptions of pregnancy when an established post-mortem interval would not be imposed.

An alternative approach could potentially be found in the emerging tract-tracing methods applicable to the living human brain based on functional magnetic resonance imaging (fMRI). These include diffusion-tensor imaging of myelinated pathways connecting regions of the human brain (Kumar et al., 2008) and multivariate analysis of human brain interconnections based on time-dependant functional correlations discriminated with BOLD fMRI imaging of regional neuronal activation for differing functional contexts (Geha et al., 2008). In the fMRI study by Kumar et al. (2008), the constellation of connections disrupted in a small cohort of CCHS cases (albeit not consistently confirmed with *PHOX2B* mutations) included pontine regions that could conceivably encompass the hRTN. In the case of BOLD imaging, functional activation of human brainstem sites presumably related to breathing has already been demonstrated in experiments where CO_2 levels have been manipulated (Harper et al., 2005). Identification of a candidate region for the hRTN in the present study could allow this region to serve as the "seed" for future temporal correlation fMRI studies to confirm the connections of the parafacial PHOX2B neurons/putative hRTN in the human with the respiratory structures in the medulla as well as its correspondence with the constellation of connections characterizing the RTN of experimental animals.

The second objective of this study was to determine whether abnormalities in SIUD and SIDS cases are evident in the hRTN given the essential and critical role with respect to respiratory control of Phox2b RTN neurons in animal models, and the demonstrable role of *PHOX2B* mutations in disrupting breathing in humans (Weese-Mayer et al., 2003, 2009) and in mice (Abbott et al., 2009; Dubreuil et al., 2009). In rodents, the development of RTN neurons is uniquely sensitive to a *PHOX2B* mutation that causes a large decrease in the central chemoreflex and in breathing automaticity (Gallego and Dauger, 2008; Takakura et al., 2008). In the human, the paired like homeobox gene, *PHOX2B*, is the disease-defining gene for CCHS — an autosomal dominant genetic disorder characterized by alveolar hypoventilation in sleep and (in a subset of cases) wakefulness, altered response to hypoxemia and hypercarbia asleep and awake, and evidence of ANS dysregulation (Amiel et al., 2003; Weese-Mayer et al., 2008, 2009, 2010). Individuals with CCHS are heterozygous for a *PHOX2B* mutation, with approximately 90–92% of cases carrying a polyalanine repeat expansion mutation with 24–33 alanines on the affected allele (resulting genotypes of 20/24 to 20/33; normal genotype is 20/20) and the remaining 8–10% of cases expressing a non-polyalanine repeat expansion mutation (missense, nonsense, frameshift, or stop codon) in the *PHOX2B* gene. Though not yet studied in the human with CCHS, the knock-in mouse model for the *PHOX2B* 20/27 genotype (the normal 20 alanines on one allele and the expanded number of 27 alanines on the affected allele) has an 85% reduction of Phox2b-postive RTN neurons, with apparently no superficially evident anatomical defects elsewhere in the brainstem (Dubreuil et al., 2008, 2009). This RTN hypoplasia is presumed to result in the lack of carbon dioxide sensitivity. Furthermore, these *PHOX2B* 20/27 genotype knock-in mice cannot survive without artificial ventilation, suggesting that breathing and chemoreception are both entirely dependent on both structural and *PHOX2B* genetic integrity of RTN neurons. Though we are not suggesting that SIDS/SIUD are the result of CCHS-related *PHOX2B* mutations, we do suggest consideration that other mutations in the *PHOX2B* gene pathway/network might play a mechanistic role in SIDS/SIUD.

It is reasonable to speculate whether breathing alterations can lead to death during intrauterine life. It is known that the respiratory-related neuronal network is active before birth and controls respiratory-like rhythmic movements that are among the earliest detectable behavior of the mammalian fetus. Therefore, it becomes vital only after birth. Thus, an immature respiratory activity is demonstrable in prenatal stages, shortly after the onset of fetal movements. Consequently, the observed morpho-functional alterations of the hRTN in SIUD victims could determine defects of this occasional respiratory activity in prenatal life. Nevertheless, these breathing alterations would not be sufficient to justify fetal death. One possibility that we strongly support, is that this neuronal structure participates not only in respiratory control but also, more extensively, in the modulation of all the vital functions.

Another hypothesis could be, however, that in the last weeks of pregnancy, advancing towards the time of birth, a general check of all the essential functions for extra-uterine life occurs, particularly of the respiratory control. Sudden unexpected fetal death could therefore be ascribed to a selective process of natural suppression in the presence of developmental alterations of the breathing network, and particularly of the hRTN, given its fundamental role in postnatal life.

Despite these important findings, we have identified three key limitations to our study. First, there were a limited number of SIUD and SIDS subjects available for analysis. This is in part due to the fact that many healthcare structures do not observe the obligation, imposed by Italian law, to submit young suddenly deceased victims to autopsy. Study of a larger cohort than reported in this study has the potential to confirm our results and address other disparities regarding location of the hRTN in the human (*vs* animal). Second, we have not considered the relationship between hRTN alterations and maternal smoking that we have previously shown to be significantly associated with developmental nervous system alterations (Lavezzi et al., 2005, 2009). Third, the role of more global environmental factors such as air pollution were not considered. In fact many victims included in this study are from Lombardy, a highly polluted Italian region, in which the mean levels of atmosphere particulate matter both less than 2.5 μm in diameter ($PM_{2.5}$) and less than10 μm in diameter (PM_{10}) are recognized to contribute in a substantial way to perinatal mortality.

5. Conclusions

We believe that a normal hRTN structure and functionality is crucial to defend a proper brain development. Hence, the alterations of the hRTN reported in this study may underline a defective chemoreception and breathing control and consequently may play an important part in triggering sudden unexplained fetal and/or infant death.

Conflict of interest

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

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