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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.