NEUROPATHOLOGY

Neuropathology 2011; **31**, 405–413 doi:10.1111/j.1440-1789.2010.01190.x

Original Article

Developmental alterations of the spinal trigeminal nucleus disclosed by substance P immunohistochemistry in fetal and infant sudden unexplained deaths

Anna M. Lavezzi, Riffat Mehboob and Luigi Matturri

"Lino Rossi" Research Center for the Study and Prevention of Unexpected Perinatal Death and SIDS, Department of Surgical, Reconstructive and Diagnostic Sciences, University of Milan, Milan, Italy

We investigated the immunohistochemical expression of substance P (SP) in the brainstems of 56 subjects aged from 17 gestational weeks to 10 post natal months, who died of unknown (sudden unexplained fetal deaths and SIDS) and known causes (controls). The goals of this study were: (i) to obtain basic information about the expression of SP during the first phases of human nervous system development; (ii) to evaluate whether there are alterations of this neuromodulator in victims of sudden death; and (iii) to verify any correlation with maternal cigarette smoking. Immunohistochemistry demonstrated SP immunoreactivity in the caudal trigeminal nucleus area, with a progressive increase in the density of SP-positive fibers of the corresponding tract during normal development from fetal life to the first post natal months. Delineation of the structure of the human trigeminal nucleus, little investigated so far, provided essential data on its morphologic and functional development. Instead, a negative or low SP expression was detectable in the fibers of this tract in a wide subset of SIDS victims and, conversely, a high SP-expression in a wide subset of sudden fetal deaths. We postulate, on the basis of these results, that SP has a functional importance in the early phases of central nervous system development and in the regulation of autonomic functions. In addition, the observation of a significant correlation between sudden unexplained death, altered SP staining and maternal smoking leads us to suggest a close relation between the absorption of cigarette smoke *in utero* and a decreased functional activity of the trigeminal nucleus, that can trigger sudden death of the fetus during pregnancy or of the infant in the first months of life.

Key words: neuropathology, SIDS, spinal trigeminal nucleus, substance P, sudden fetal death.

INTRODUCTION

The respiratory control system is influenced by classical neurotransmitters and by neuromodulators. These neuromodulators are neuroactive substances that can be secreted at a distance from their receptors and can have a non-synaptic transmission via the intercellular spaces. Neuromodulators currently considered to be natural participants in central respiratory control include dopamine, adenosine, endorphins, serotonin and substance P (SP).[1–4]

In particular, SP is a member of the tachykinin family of neuropeptides that, besides playing a role in the modulation of hemodynamic functions and neuronal pathways related to pain sensation,[5] is involved in the breathing acceleration process induced by conditions of hypoxemia.[6,7] SP expression can be highlighted, like that of many neurotransmitters, by a specific immunohistochemical method.

By immonohistochemistry, we have already demonstrated an abnormal distribution of different neurotransmitters involved in breathing control (namely somatostatin, serotonin, catecholamines),[8–10] both before and after birth, in the brainstem of sudden perinatal death and SIDS victims. We postulated that these alterations could affect the vital functions and/or induce fatal breathing in prenatal life,

Correspondence: Anna M. Lavezzi, MD, "Lino Rossi" Research Center, Department of Surgical, Reconstructive and Diagnostic Sciences, University of Milan, Via della Commenda, 19, 20122 Milan, Italy. Email: anna.lavezzi@unimi.it

Received 28 October 2010; revised 22 November 2010 and accepted 26 November 2010; published online 30 January 2011.

and conversely disrupt ventilation control in newborns, leading to irreversible apnea.

In the present study we aimed to widen our knowledge of these phenomena by evaluating the expression of SP in these victims. We investigated SP-immunoreactivity (SP-IR) in a total of 56 subjects aged from 17 gestational weeks to 10 post natal months, who had died of known or unknown causes. Besides obtaining basic information about the distribution of SP binding sites in the first phases of human nervous system development, we focused on evaluating the possible presence of SP alterations in cases of sudden perinatal and infant death, in addition to the morpho-functional disruptions of the autonomic nervous system we have already reported.[8–17]

Finally, our previous observations of a significantly increased incidence of morphological and functional alterations in the brainstems of stillborns and SIDS victims with smoker mothers, as compared with victims with non-smoker mothers,[9,14,15,18] prompted us to verify whether maternal cigarette smoking could also be related to SP abnormalities.

MATERIALS AND METHODS

A total of 56 brains were collected from 24 fresh fetuses (17–40 gestational weeks) and 32 infants aged 1–10 months.

This was a selected set of cases, all sent to the "L. Rossi" 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 in Italian regions within the first year of age, as well as all fetuses who died without any apparent cause, must undergo, after obtaining informed parental consent, an in-depth anatomo-pathological examination, particularly of the autonomic nervous system (ANS).[19,20]

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 death, in accordance with the above-mentioned Italian Law n.31.

Anatomo-pathological protocol for the examination of the ANS

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

Transverse serial sections of the midbrain, pons, medulla oblongata and cerebellum samples were made at intervals of 50–60 μm. For each level, serial 5 μm sections were obtained, two of which were routinely stained for histological examination using HE and KB and one was submitted to immunohistochemical study of SP. The remaining sections were saved and stained as deemed necessary for further investigations.

SP immunohistochemistry

Sections were deparaffinized and washed in PBS. After blocking endogenous peroxidase by using 3% H_2O_2, the slides were treated in a microwave-oven with citrate solution (pH = 6). After further PBS washing, sections were incubated overnight with primary rabbit polyclonal anti-substance P antibody (C191, DBA, Segrate, MI, Italy) at a dilution of 1:80. Immunohistochemical staining was performed using the peroxidase-antiperoxidase method with the avidin biotin complex technique (ABC-Peroxidase kit, Vectastain, Vector laboratories Inc., Burlingame, CA, USA) for 30 min. Diaminobenzidine (DAB, Vector Laboratories Inc.) was used as chromogen substrate, counterstained with light hematoxylin. Negative controls of the same tissue were obtained by using PBS instead of primary antibody.

An ocular micrometer applied to the microscope was used in order to optimize the evaluation of the thickening and the density of the SP-immunoreactive areas.

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 inferior olivary, the raphé, the arcuate nuclei and the pre-Bötzinger complex, 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 nucleus, the fastigial nucleus, the globose nucleus and the emboliform nucleus) were examined.

The examination of slides was performed blinded, with no prior knowledge of the cause of death, age or other clinicopathological information. Only after the histological and immunohistochemical assessment of the brainstem and cerebellum had been completed, were the findings matched with the corresponding records.

In 36 cases, after the in-depth anatomopathological examination the death remained totally unexplained. A diagnosis of SIUD (sudden intrauterine unexplained death) was established for 16 fetuses, who died 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 20 cases, nine

stillbirths, and 11 infant deaths, a precise cause of death was formulated at autopsy. These cases were used as controls. The related infant death diagnoses in this group were: congenital heart disease ($n = 5$), severe bronchopneumonia ($n = 2$), myocarditis ($n = 1$), pulmonary dysplasia ($n = 2$) and mucopolysaccharidosis type I ($n = 1$). Specific diagnoses among the fetal deaths included: chorioamnionitis ($n = 6$) and congenital heart disease ($n = 3$).

For every case, a complete clinical history was collected. Additionally, mothers were asked to complete a questionnaire on their smoking habits, detailing the number of cigarettes smoked before, during and after pregnancy. Twenty-two of the 36 SIUD/SIDS mothers (61%), 12 SIUD and 10 SIDS mothers, were active smokers before and during the pregnancy, smoking more than three cigarettes/day. The remaining 14 mothers (39%) admitted no history of cigarette smoking.

Four of the 20 mothers in the control group (20%), all mothers of an infant died of congenital heart disease, reported a smoking habit, while the remaining 16 mothers (80%) were non-smokers.

Statistical analysis

The association between immunohistochemical data, the main target of this study, and clinicopathological and maternal lifestyle data was evaluated by Cox regression analysis. Statistical significance was established at the $P < 0.05$ level using one-way analysis of variance followed by *t*-test or Student's *t*-test.

RESULTS

First, we concentrated on defining the localization of SP-IR, the target of this study, in the brainstem of fetal and infant control cases. SP positivity appeared mainly localized in fiber-structures, mostly consisting of dot-like varicosities and with densities ranging from low to very high. These SP-IR fibers generally surrounded non-IR neurons. A very few SP-immunoreactive perikarya were sometimes found.

Strong immunopositivity was constantly observable in the area of the trigeminal nucleus (TrN) in the dorsolateral part of the medulla and in the rostral spinal cord, both before and after birth.

Given the constant presence of SP-immunostaining in the TrN, with the aid of immunohistochemistry and following the indications exclusively provided by experimental studies in this field,[21–23] we first proceeded to elicit basic data on the morphological and functional developmental steps of this nucleus, that are so difficult to identify in histological sections of human brainstem due to its indefinite boundaries. Classical studies in rodents consider this nucleus as a neuronal complex, including throughout its extension, the "principal sensory nucleus" in the pons and the "spinal trigeminal nucleus" located in the medulla and rostral segments of the spinal cord. The spinal TrN is divided into three subnuclei, namely oral, interpolar and caudal, adjacent to the spinal trigeminal tract that appears, on transverse sections, to be layered. The spinal caudal subnucleus, in turn, includes a magnocellular core, composed of medium to large-sized cells with abundant Nissl substance.

Developmental cytoarchitecture of the human spinal TrN in control fetal and infant deaths

At the earliest observations (17th–18th gestational week) the TrN in the coronal sections of medulla oblongata and rostral spinal cord (corresponding to the "spinal TrN" in rodents) shows a low number of positive fibers at the level of the tract, surrounding undifferentiated small rounded neurons, with no defined outlines (Fig. 1).

This cytoarchitectonic feature persists in the following gestational weeks, with the only difference that the tract becomes increasingly thickened and stratified. The marginal immunonegative cells frequently appear medium-sized and pear-shaped or polygonal with sketchy axons and dendrites.

The most conspicuous positive SP-fiber plexus of the spinal TrN is found at birth and and in the first months

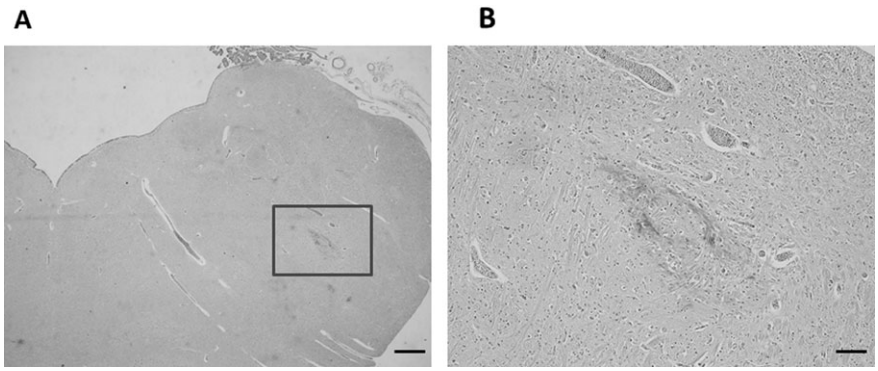

Fig. 1 (A) Photomicrograph of a transverse histological hemisection (dorsolateral portion) of the medulla oblongata of a control fetus that died at 18 gestational weeks of congenital heart disease, showing, in the box (magnified in B), the trigeminal nucleus with a poor number of positive fibers in the tract. Substance P immunostain. Magnification: (A) 10×; (B) 20×.

of life, with interstitial islands of immunonegative neurons embedded in bundles of the tract (Fig. 2). SP-immunoreactive fibers, that appear to be layered in transverse sections, form distinct pericellular arrays around the somata and dendrites of neurons. These interstitial cells show various edges: triangular, rounded, polygonal or star-shaped. They send out thin dendrites interconnecting the cells and reaching the plexus.

A magnocellular group consisting of large, lightly stained neurons with peripheral Nissl substance, either grouped or disseminated, is also found at these levels, corresponding to the magnocellular core of the caudal subnucleus in rodents (Fig. 3). We can identify the oral limit of the human spinal caudal subnucleus and the shift to the interpolar TrN at the point of disappearance of these neurons.

Overall, during development we observed a slight, progressive increase of the spinal TrN outlines, and in particular of the tract and neuronal cell body areas, whereas the neuronal density remained steady. In infant deaths, in addition to the TrN, the raphé complex and the reticular formation also displayed a variable density of SP-immunoreactivity.

Neuropathology of the spinal TrN in unexplained fetal death and SIDS

We observed, contrary to what was found in the controls, dense plexuses of SP immunoreactive fibers in the TrN area in 10 of the 16 SIUD victims (Fig. 4) and depletion or fewer reactive fibers in 11 of the 20 SIDS subjects (Fig. 5). In addition, in five of these 11 SIDS cases we diagnosed hypoplasia of the TrN, given the presence of rare interstitial neurons and the total absence of large cells in the magnocellular area of the caudal trigeminal subnucleus (Fig. 6).

Correlation with smoking exposure

A significant correlation was observed between an altered expression of SP in the TrN and a maternal smoking habit. In fact, 17 of the 21 victims of sudden death with TrN

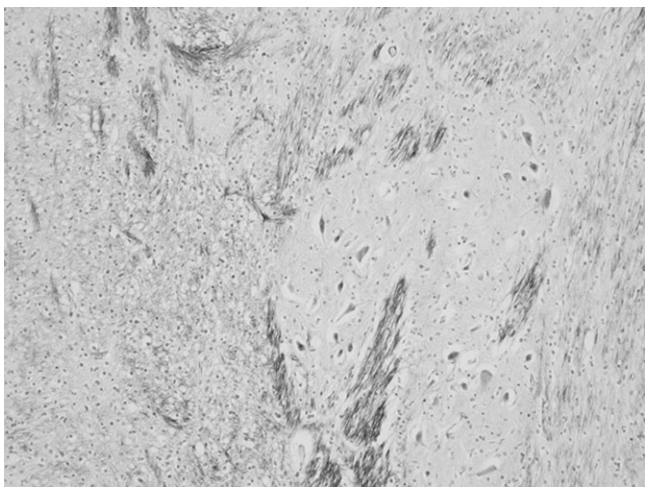

Fig. 2 Substance P immunoreactivity of fibers in the trigeminal nucleus, with an interstitial island of immunonegative neurons, in a control infant who died at 4 months of age of bronchopneumonia. Substance P immunostain. Magnification: 40×.

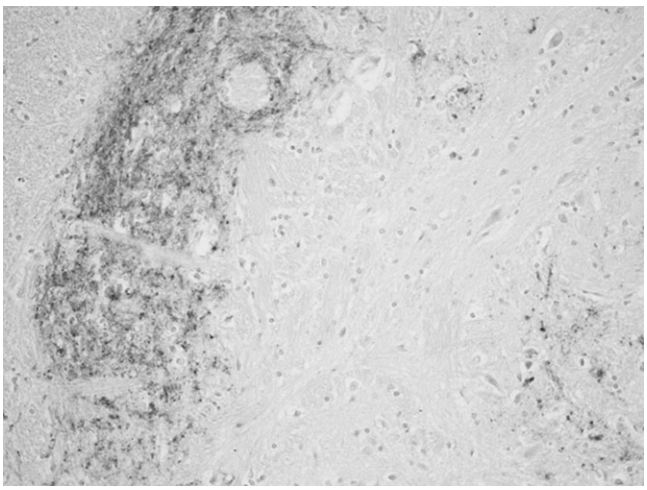

Fig. 4 Dense plexus of substance P-immunoreactive fibers in the trigeminal nucleus area in a sudden intrauterine unexplained death victim aged 32 gestational weeks. Substance P immunostain. Magnification: 20×.

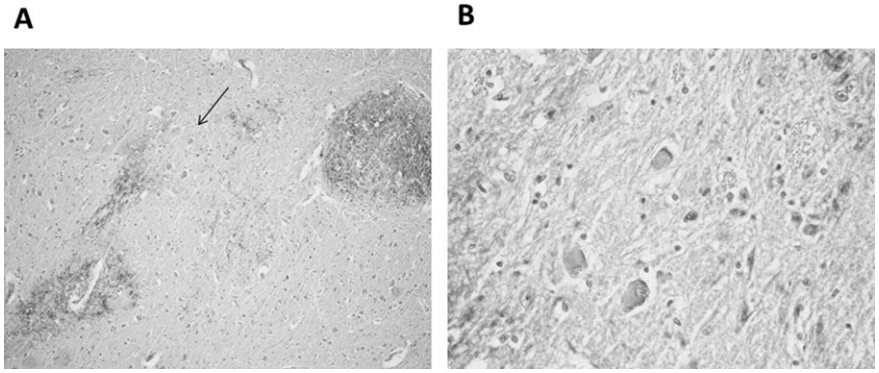

Fig. 3 (A) Magnocellular core (see arrow) of the spinal trigeminal nucleus in a control infant aged 2 months. (B) Higher magnification of the large neurons. Substance P immunostain. Magnification: (A) 20×; (B) 40×.

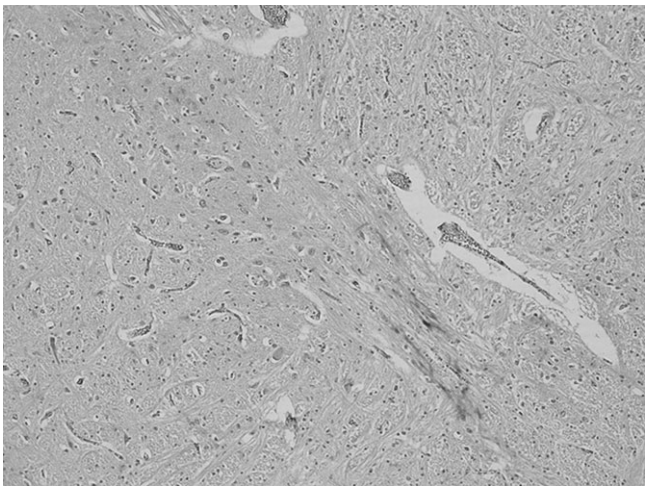

Fig. 5 Poor immunopositivity of fibers in the trigeminal nucleus area in a 3-month-old sudden infant death syndrome victim. Substance P immunostain. Magnification: 20×.

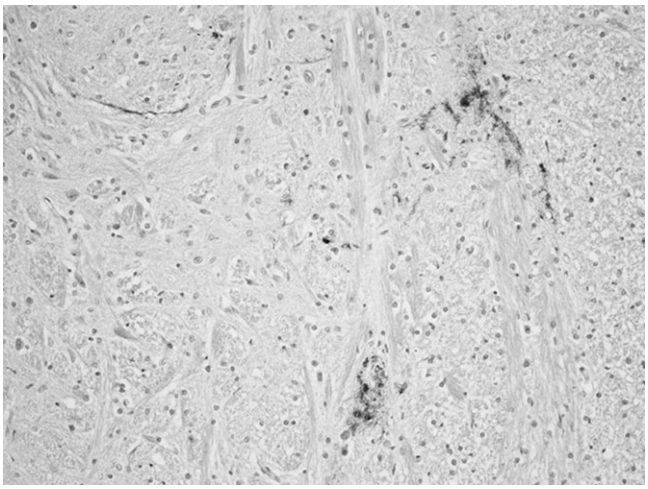

Fig. 6 Hypoplasia of the trigeminal nucleus (at the caudal level) with depleted immunopositive fibers, rare interstitial neurons and absence of large-sized cells. Substance P immunostain. Magnification: 20×.

neuropathology, precisely nine SIUD with increased SP expression and eight SIDS with decreased SP expression, had smoker mothers ($P < 0.05$).

No alteration of the SP expression was found in the four infant victims with smoking mothers belonging to the group of 20 controls.

Brainstem pathological results overall

Table 1 summarizes the neuropathological findings in the brainstem. Besides alterations of the spinal TrN, a subset of SIDS cases showed hypoplasia of the medullary arcuate nucleus or of the pre-Bötzinger complex, or different raphé nuclei. A subset of SIUD cases showed hypoplasia of the pre-Bötzinger complex or the parafacial nucleus or different raphé nuclei. A small subset of controls, both fetal and infant deaths, had hypoplasia of the arcuate nucleus and/or the raphé obscurus nucleus. Overall, a significantly greater proportion of neurological alterations was observed in SIDS-SIUD cases as compared with controls ($P < 0.01$). The most frequent association was between alterations of SP signalling in TrN and pre-Bötzinger complex hypoplasia. In fact, 17 of the 21 victims of sudden death with alterations of TrN SP-IR (including the five cases with TrN hypoplasia) showed hypoplasia/agenesis of the pre-Bötzinger complex ($P < 0.01$).

DISCUSSION

Topographical mapping of SP-immunoreactive structures in the human brain has already been reported by several authors, but there seems to be no consensus as to the localization of the SP binding sites.[24–26]

This study provides a detailed report of the distribution of SP-IR within the human fetal and infant brainstem. We detected SP positivity right from the early stages of ontogenesis, mainly in the dorsolateral part of the medulla

Table 1 Overall neuropathological brainstem findings in 16 SIUD, 20 SIDS, cases and 20 controls

Study group	Arcuate nucleus hypoplasia	Pre-Bötzinger complex hypoplasia	Parafacial nucleus hypoplasia	Raphé nuclei hypoplasia	SP alterations in trigeminal nucleus	
	Number of cases (%)	Number of cases (%)	Number of cases (%)	Number of cases (%)	Number of cases (%)	
					Increased expression	Decreased expression
SIUD $n = 16$	7 (44%)	8 (50%)	6 (37%)	4 (25%)	10 (65%)	–
Fetal controls $n = 9$	2 (22%)	–	–	1 (11%)	–	–
SIDS $n = 20$	9 (45%)	9 (45%)	–	7 (35%)	–	11[†] (55%)
Infant controls $n = 11$	2 (18%)	–	–	3 (17%)	–	–

[†] With neuronal depletion in five cases. SIDS, sudden infant death syndrome; SIUD, sudden intrauterine unexplained death.

oblongata and of the rostral spinal cord, exclusively in the area corresponding to the spinal trigeminal nucleus (TrN). These observations testify to the high specificity of the localization of SP in the human brain.

Only a few authors have focused on the TrN in man.[27,28] The structure of this nucleus, that includes a cellular component and a group of fibers (tract), is in fact difficult to identify, given its undefined boundaries. However, TrN recognition is facilitated by the expression of its major neurotransmitter, SP, in the tract. Thus, using immunohistochemistry, we first defined the normal localization and traced the cytoarchitectonic features of this nucleus, providing essential data on its morphologic and functional developmental dynamics in subjects who had died of a known etiology between the ages of 17 gestational weeks and the tenth month of life.

Comparative study of the fetus and newborn coronal sections of the medulla revealed that, while in fetuses the TrN was barely recognizable, due to a limited presence of fibers expressing SP, a clear structural organization of this nucleus with interstitial islands of neurons, embedded in a dense plexus of positive fibers in the tract, was evident in newborns.

The different SP densities, ranging from low in intrauterine life to very high in the first postnatal months, underline the existence of a precise functional program in the coordination of nervous system developmental steps. SP released by neurons of the TrN might thus be involved in critical developmental periods for brain plasticity and modulation of different autonomic vital functions.

Experimental studies in rodents have shown that the TrN is involved in a variety of rhythmic activities, such as suckling, mastication, swallowing and breathing.[29,30] Breathing, in particular, is an essential mammalian process and must be functional at birth.[31]

Humans usually breathe only through the nasal airway route, without using the oral airway route. However, oral breathing, along with jaw movements aimed at maintaining upper airway resistance, is required under loaded respiratory conditions, such as severe hypoxia.[32] Trigeminal respiratory activity plays a fundamental role in the network controlling oral breathing and a critical role in the regulation of upper airway patency. Several authors have reported that trigeminal motor activity is dramatically altered during respiratory disorders, such as sleep apnea.[33,34]

In the present study, as compared with the picture in control cases, we detected dense plexuses of SP immunoreactive fibers in the spinal TrN area in SIUD victims and, conversely, a depletion of SP-IR fibers in TrN in SIDS, frequently in a setting of hypoplasia of the whole nucleus.

Previously, while studying the brain expression of somatostatin, another neuropeptide involved in respiratory control, we similarly observed a different pattern of positivity of this neurotransmitter in the hypoglossal nucleus before and after birth in more than 50% of cases of sudden perinatal and infant death as compared to cases with a known etiology of death.[8]

The hypoglossus nucleus, even if it is not considered as a classic breathing center, like the TrN, contains motor neurons with respiratory-related rhythmic discharges. In particular, it controls the extrinsic muscles of the tongue, mainly the genioglossus that is important in maintaining a patent airway, especially during inspiration.[35,36]

We ascribe these opposite alterations of the TrN that we observed in fetal and infant deaths, to the same developmental neuronal defect. Under normal conditions, in fact, the functionality of this nucleus, given its involvement in respiratory activity, must be complete after birth but necessarily poor in prenatal life, as testified by the pattern of SP expression in TrN of control cases.

In fetal life the chemoreception, the main controlling mechanism of breathing, is normally well developed and potentially functioning.[37–39] However, its stimulatory effect on respiration is nullified by the dominant inhibitory input from nervous structures of the brainstem such as the pre-Bötzinger complex.[40] Only irregularly fetal breathing occurs, directed towards the release of tracheal fluid in the lung and consequently to promote alveolar expansion and lung development.[41]

The pre-Bötzinger complex has been physiologically defined as essential for the generation of respiratory rhythm immediately after delivery, but at the same time it represents an important breathing-inhibitor in fetal life.[42,43]

We maintain that in SIUD/SIDS cases with altered SP-signaling in the TrN, the inhibitory and excitatory effects exerted from the pre-Bötzinger complex before and after birth, respectively, are lost, allowing the triggering of fatal respiratory activity in fetuses and breathing drawback in newborns. This hypothesis is supported by the observation in this study of a significant association between morpho-functional alterations of the TrN and hypoplasia of the pre-Bötzinger complex.

This close connection was been pointed out also in experimental studies. In rat brainstem-sectioning experiments, Koizumi *et al.*[44] suggested that the origin of trigeminal respiratory activity is the pre-Bötzinger complex in the medulla. Their observations confirmed that trigeminal respiratory activity disappeared when the region corresponding to the pre-Bötzinger complex was completely removed. We also had proposed the essential role of the pre-Bötzinger complex for the generation of respiratory rhythm, as well as for the modulation of eupneic breathing, in a previous work.[16] In particular, we reported structural and/or functional developmental defects (hypoplasia with a decreased neuronal number and/or dendritic hypodevel-

opment of the reticular formation, abnormal neuronal morphology, immunonegativity of neurotransmitters and agenesis) of this nucleus in a high percentage of sudden fetal and infant deaths. Our next step will be to confirm, by SP immunohistochemistry in the recorded brainstem inclusions of these victims, the concomitant presence of TrN alterations.

Discordant findings have been described as regards SP-IR in the brainstem of SIDS victims, all obtained in a limited number of cases.[45–49] Only a few authors have observed immunopositivity in the TrN. In particular, Obonai *et al.*[45] and Ozawa *et al.*[46] reported an increased expression of SP in trigeminal fibers, as compared with age-matched controls. Conversely, in the study by Sawaguchi *et al.*[48] no significant correlation was found between the density of SP in the TrN and SIDS.

Our studies and previous works reported in the literature provide a greater insight into the cause of the neuronal abnormalities and of the pathogenic mechanism underlying perinatal sudden death, attributable to chronic hypoxia.[11,50–52] As reported above, severe hypoxia can trigger oral breathing by means of TrN activation.

A possible mechanism determining hypoxic crises may be maternal cigarette smoking in pregnancy.[18,53] In this study we observed a significant correlation between tobacco smoke exposure *in utero* and alterations of SP expression in the spinal TrN.

In cases of maternal smoking in pregnancy, carbon monoxide, a gaseous combustion product of nicotine, may readily cross by passive diffusion into the placenta, where it binds to hemoglobin. Consequently, carboxyhemoglobin, that is present in the fetal compartment at concentrations that are generally 15% higher than the maternal levels,[54] inhibits the release of oxygen into fetal tissues, causing hypoxia and a consequent delayed maturation of all the organs, especially of the brain.

Besides, nicotine is one of the few lipid-soluble substances that can spread beyond the blood-brain barrier by concentration gradient,[55,56] and act directly on the expression of genes that control the developing brain. Therefore, among the numerous compounds present in cigarette smoke, carbon monoxide and nicotine, as suggested by Gressness *et al.*,[57] could affect the fetal brain, via indirect and/or direct actions.

Our observations of TrN alterations in some victims with non-smoking mothers could be attributable to the fact that many women in pregnancy are exposed to nicotine through other people's smoking at home or in the work environment, that is, passive smoking. Furthermore, it should be considered that retrospective assessment of the maternal smoking habit, mainly performed after the fatal event, is sometimes unreliable.[58,59] This may be because smoking mothers are reluctant to admit to tobacco use, possibly due to feelings of guilt.

Moreover, other risk factors, such as air pollution, may contribute to cause defects in the nervous system. Since most of the victims in this study came from large industrialized cities, we can hypothesize that atmospheric pollution may also have contributed to an incorrect development of the TrN.

ACKNOWLEDGMENTS

The authors thank Mary Victoria Candace Pragnell, BA, for English language assistance and Dr Graziella Alfonsi for her excellent technical assistance.

CONFLICT OF INTEREST

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

FUNDING

No financial assistance was received to support this study.

REFERENCES

1. Moss IR, Inman JG. Neurochemicals and respiratory control during development. *J Appl Physiol* 1989; **67**: 1–13.
2. Moss IR, Denavit-Saubié M, Eldridge FL *et al*. Neuromodulators and transmitters in respiratory control. *Fed Proc* 1986; **45**: 2133–2147.
3. Hilaire G, Duron B. Maturation of the mammalian respiratory system. *Physiol Rev* 1999; **79**: 325–360.
4. Bonham AC. Neurotransmitters in the CNS control of breathing. *Respir Physiol* 1995; **101**: 219–230.
5. Niccol RA, Schenker C, Leeman SE. Substance P as a transmitter candidate. *Annu Rev Neurosci* 1980; **3**: 227–268.
6. Lagercrantz H, Srinivasan M, Yamamoto Y *et al*. Functional role of substance P for respiratory control during development. *Ann NY Acad Sci* 1991; **632**: 48–52.
7. Hedner T, Hedner J, Jonason T, Lundberg D. Evidence suggesting a role for substance P in central respiratory regulation in the rat. *Acta Physiol Scand* 1981; **112**: 487–489.
8. Lavezzi AM, Ottaviani G, Matturri L. Involvement of somatostatin in breathing control before and after birth, and in perinatal and infant sudden unexplained death. *Folia Neuropthol* 2004; **42**: 59–65.
9. Lavezzi AM, Ottaviani G, Mingrone R *et al*. Analysis of the human locus coeruleus in perinatal and infant

sudden unexplained death. Possible role of the cigarette smoking in the development of this nucleus. *Dev Brain Res* 2005; **154**: 71–80.
10. Lavezzi AM, Casale V, Oneda R *et al*. Sudden infant death syndrome and sudden intrauterine unexplained death: correlation between hypoplasia of raphé nuclei and serotonin transporter gene promoter polymorphism. *Pediatr Res* 2009; **66**: 22–27.
11. Matturri L, Minoli I, Lavezzi AM *et al*. Hypoplasia of meduallary arcuate nucleus in unexpected late fetal death (stillborn infants): a pathologic study. *Pediatrics* 2002; **109**: E43.
12. Matturri L, Lavezzi AM. Pathology of the central autonomic nervous system in stillbirth. *Open Ped Med J* 2007; **1**: 1–9.
13. Lavezzi AM, Ottaviani G, Ballabio GM *et al*. Preliminary study on the cytoarchitecture of the human parabrachial/Kölliker-Fuse complex with reference to sudden infant death syndrome and sudden intrauterine unexplained death. *Pediatr Dev Pathol* 2004; **7**: 171–179.
14. Lavezzi AM, Ottaviani G, Mauri M *et al*. Alterations of biological features of the cerebellum in sudden perinatal and infant death. *Curr Mol Med* 2006; **6**: 429–435.
15. Lavezzi AM, Ottaviani G, Mauri M *et al*. Biopathology of the olivocerebellar network in sudden unexplained perinatal and sudden infant death syndrome related to maternal cigarette smoking. *Neurol Res* 2007; **29**: 525–532.
16. Lavezzi AM, Matturri L. Functional neuroanatomy of the human pre-Bötzinger complex with particular reference to sudden unexplained perinatal and infant death. *Neuropathology* 2008; **28**: 10–16.
17. Lavezzi AM, Matturri L. Hypoplasia of the parafacial/facial complex: a very frequenty finding in sudden unexplained fetal death. *Open Neurosci J* 2008; **2**: 1–5.
18. Lavezzi AM, Ottaviani G *et al*. Adverse effects of prenatal tabacco smoke exposure on biological parameters of the developing brainstem. *Neurobiol Dis* 2005; **20**: 601–607.
19. Matturri L, Ottaviani G, Lavezzi AM. Techniques and criteria in pathologic and forensic-medical diagnostics of sudden unexpected infant and perinatal death. *Am J Clin Pathol* 2005; **124**: 259–268.
20. Matturri L, Ottaviani G, Lavezzi AM. Guidelines for neuropathologic diagnostics of perinatal unexpected loss and sudden infant death sindrome (SIDS). A technical protocol. *Virchows Arch* 2008; **452**: 19–25.
21. Luccarini P, Cadet R, Duale C *et al*. Effects of lesions in the trigreminal oralis and caudalis subnuclei on different orofacial nociceptive responses in the rat. *Brain Res* 1998; **803**: 79–85.
22. Phelan KD, Falls WM. The interstitial system of the spinal trigeminal tract in the rat: anatomical evidence for morphological and functional heterogeneity. *Somatosens Mot Res* 1989; **6**: 367–399.
23. Athanassiadis T, Westberg KG, Olsson KA *et al*. Physiological characterization, localization and synaptic inputs of bursting and nonbursting neurons in the trigeminal principal sensory nucleus of the rat. *Eur J Neurosci* 2005; **22**: 3099–3110.
24. Chigr F, Najiami M, Leduque P *et al*. Anatomical distribution of substance P-immunoreactive neurons in human brainstem during the first postnatal year. *Brain Res Bull* 1991; **26**: 515–523.
25. Jordan D, Kermadi I, Rambaud C *et al*. Regional distribution of substance P binding sites in the brainstem of the human newborn. *Brain Res* 1995; **695**: 117–124.
26. Del Fiacco M, Levanti MC. Topographical localization of substance P in the human post-mortem brainstem. An immunohistochemical study in the newborn and adult tissue. *Neuroscience* 1984; **12**: 591–611.
27. Rusu MC. The spinal trigeminal nucleus. Considerations on the structure of the nucleus caudalis. *Folia Morphol* 2004; **63**: 325–328.
28. Dallel R, Villanueva L, Woda A *et al*. Neurobiologie de la douleur trigeminale. *Medicine/Sciences* 2003; **19**: 67–74.
29. Goldberg LJ, Chandler SH. Central mechanisms of rhythmic trigeminal activity. In: Taylor A, ed. *Neurophysiology of Jaws and Teeth*. London: Macmillan, 1991; 268–321.
30. Lund JP, Kolta A, Westberg KG *et al*. Brainstem mechanisms underlying feeding behaviors. *Curr Opin Neurobiol* 1998; **8**: 718–724.
31. Feldman JL, Mitchell GS, Nattie EE. Breathing: rhythmicity, plasticity, chemosensitivity. *Annu Rev Neurosci* 2003; **26**: 239–266.
32. Bartlett DJ. Upper airway motor systems. In: Fishman AP, ed. *Handbook of Physiology. Section 3: The Respiratory System. Vol. II: Control of Breathing*. Bethesda, MD: American Physiological Society, 1986; 223–245.
33. Chandler SH, Chase MH, Nakamura Y. Intracellular analysis of synaptic mechanisms controlling trigeminal motoneuron activity during sleep and wakefulness. *J Neurophysiol* 1980; **44**: 359–371.
34. Chamberlin NL, Saper CB. A brainstem network mediating apneic reflexes in the rat. *J Neurosci* 1998; **18**: 6048–6056.
35. Withington-Wray DJ, Mifflin SW, Spyer KM. Intracellular analysis of respiratory-modulated hypoglossal motoneurons in the cat. *Neuroscience* 1988; **25**: 1041–1051.
36. Roda F, Gestreau C, Bianchi AL. Discharge patterns of hypoglossal motoneurons durino fictive breathing,

coughing and swallowing. *J Neurophysiol* 2002; **87**: 1703–1711.
37. Ballantyne D, Scheid P. Central chemosensitivity of respiration: a brief overview. *Respir Physiol* 2001; **129**: 5–15.
38. Bianchi AL, Denavit-Saubie M, Champagnat J. Central control of breathing in mammals: neuronal circuitry, membrane properties and neurotransmitters. *Physiol Rev* 1995; **75**: 1–45.
39. Blanco CE. Maturation of fetal breathing activity. *Biol Neonate* 1994; **65**: 182–188.
40. Coates EL, Li A, Nattie EE. Widespread sites of brainstem ventilatory chemoreceptors. *J Appl Physiol* 1993; **75**: 5–14.
41. Cosmi EV, Anceschi MM, Piazze JJ *et al*. Ultrasonographic patterns of fetal breathing movements in normal pregnancy. *Int J Gynaecol Obstet* 2003; **80**: 285–290.
42. Rekling JC, Feldman JL. PreBötzinger complex and pacemaker neurons: hypothesized site and kernel for respiratory rhythm generation. *Annu Rev Physiol* 1998; **60**: 385–405.
43. Smith JC, Ellenberger HH, Ballanyi K, Richter DW, Feldman JL. PreBötzinger complex: a brainstem region that may generate respiratory rhythm in mammals. *Science* 1991; **254**: 726–729.
44. Koizumi H, Nomura K, Yokota Y *et al*. Regulation of trigeminal respiratory motor activity in the brainstem. *J Dent Res* 2009; **88**: 1048–1053.
45. Obonai T, Takashima S, Becker LE *et al*. Relationship of substance P and gliosis in medulla oblongata in neonatal sudden infant death syndrome. *Pediatr Neurol* 1996; **15**: 189–192.
46. Ozawa Y, Takashima S. Developmental neurotransmitter pathology in the brainstem of sudden infant death syndrome: a review and sleep position. *Forensic Sci Int* 2002; **130** (Suppl): S53–S59.
47. Yamanouchi H, Takashima S, Becker LE. Correlation of astrogliosis and substance P immunoreactivity in the brainstem of victims of sudden infant death syndrome. *Neuropediatrics* 1993; **24**: 200–203.
48. Sawaguchi T, Ozawa Y, Patricia F *et al*. Substance P in the midbrains of SIDS victims and its correlation with sleep apnea. *Early Hum Dev* 2003; **75** (Suppl): S51–S59.
49. Takashima S, Mito T, Yamanouchi H. Developmental brain-stem pathology in sudden infant death syndrome. *Acta Paediatr Jpn* 1994; **36**: 317–320.
50. Lavezzi AM, Ottaviani G, Matturri L. Identification of neurons responding to hypoxia in sudden infant death syndrome. *Pathol Int* 2003; **53**: 769–777.
51. Kinney HC, Thach BT. The sudden infant death syndrome. *N Engl J Med* 2009; **361**: 795–805.
52. Hunt CE. The cardiorespiratory control hypothesis for sudden infant death syndrome. *Clin Perinatol* 1992; **19**: 757–771.
53. Wisborg K, Kesmodel U, Henriksen TB *et al*. Exposure to tobacco smoke in utero and the risk of stillbirth and death in the first year of life. *Am J Epidemiol* 2001; **154**: 322–327.
54. Lambers DS, Clark K. The maternal and fetal physiologic effects of nicotine. *Seminars Perinatol* 1996; **20**: 115–126.
55. Cutler RWP, Sperrel RB. Cerebrospinal fluid: a selective review. *Ann Neurol* 1982; **11**: 1–16.
56. Dawson H, Segal MB. *Physiology of CSF and Blood-Brain Barriers*. Londra: CRP Press, 1996.
57. Gressens P, Laudenbach V, Marret S. Mechanisms of action of tobacco smoke on the developing brain. *J Gynecol Obstet Biol Reprod (Paris)* 2003; **32** (1 Suppl): 30–32.
58. Heath AC, Knopik VS, Madden PA *et al*. Accuracy of mothers' retrospective reports of smoking during pregnancy: comparison with twin sister informant rating. *Twin Res* 2003; **6**: 297–230.
59. Walsh RA, Redman S, Adamson L. The accuracy of self-report of smoking status in pregnant women. *Addict Behav* 1996; **21**: 675–679.