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The Mesencephalic Periaqueductal Gray, a Further Structure Involved in Breathing Failure Underlying Sudden Infant Death Syndrome

Anna M. Lavezzi[1] **and Riffat Mehboob**[1,2]

Abstract

The aim of this study was to investigate the involvement of the periaqueductal gray (PAG), an area of gray matter surrounding the cerebral aqueduct of Sylvius, in the pathogenetic mechanism of SIDS, a syndrome frequently ascribed to arousal failure from sleep. We reconsidered the same samples of brainstem, more precisely midbrain specimens, taken from a large series of sudden infant deaths, namely 46 cases aged from 1 to about 7 months, among which 26 SIDS and 20 controls, in which we already highlighted significant developmental alterations of the substantia nigra, another mesencephalic structure with a critical role in breath and awakening regulation. Specific histological and immunohistochemical methods were applied to examine the PAG cytoarchitecture and the expression of the tyrosine hydroxylase, a marker of catecholaminergic neurons. Hypoplasia of the PAG subnucleus medialis was observed in 65% of SIDS but never in controls; tyrosine hydroxylase expression was significantly higher in controls than in SIDS. A significant correlation was found between these findings and those related to the substantia nigra, demonstrating a link between these neuronal centers and the brainstem respiratory network and a common involvement in the sleep-arousal phase failure leading to SIDS.

Keywords

neuropathology, periaqueductal gray, brainstem, sudden infant death syndrome, tyrosine hydroxylase, dopamine

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Introduction

The periaqueductal gray (PAG) is an area of gray matter surrounding the cerebral aqueduct of Sylvius, the structure of the midbrain that connects the third to the fourth ventricle, and extending from the level of the posterior commissure down to the level of the locus coeruleus. Researchers have identified in experimental studies a columnar organization of the PAG, consisting of the dorsomedial, dorsolateral, lateral, and ventrolateral columns even if with no clear cytoarchitectonic boundaries between them (Bandler & Shipley, 1994; Beitz, 1985; Carrive, 1993; Holstege, 1991). PAG has been recognized as an "analgesia center" for half a century as it plays a role in suppressing pain (Basbaum & Fields, 1978; Baskin et al., 1986; Reynolds, 1969; Roizen et al., 1985).

However, considering that the PAG, despite being a small area, is densely connected to many regions of the brain with output and input transport of neurotransmitters, recognizing to it only a role in pain control is a limited perspective. In fact, a range of additional and different functions of PAG besides the analgesia, and in particular heart rate, blood pressure modulation, production of vocalization, anxiety, and control of bladder contraction was increasingly demonstrated (Holstege, 1989; Lovick, 2000; Rossi et al., 1994; Sitsapesan et al., 2013; Subramanian et al., 2020; Zare et al., 2019). The PAG potential as an integrative neural structure for breathing, particularly involved in the perception of respiratory troubles and breathlessness, has been also highlighted (Faull et al.,

[1]"Lino Rossi" Research Center for the study and prevention of unexpected perinatal death and SIDS, Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy
[2]Faculty of Allied Health Sciences, University of Lahore, Lahore, Pakistan

Corresponding Author:
Anna Maria Lavezzi "Lino Rossi" Research Center for the study and prevention of unexpected perinatal death and SIDS, Department of Biomedical, Surgical and Dental Sciences, University of Milan.
E-mail: anna.lavezzi@unimi.it

2016; Faull et al., 2019; Subramanian et al., 2008; Subramanian & Holstege, 2010).

Given that SIDS has been recognized as a consequence of breathing control disorders, mainly in awakening from sleep (Hunt, 1989; Kahn et al., 2003; Kato et al., 2003), the aim of this study was to verify whether even alterations of the PAG could contribute to death in this syndrome. Then, for this purpose, we reconsidered a large series of SIDS, in which we already highlighted significant morphological and functional alterations of the substantia nigra pars compacta (SNpc), another mesencephalic structure that is involved in the breath and the sleep-wake cycle regulation (Lavezzi et al., 2020). Then, we set out to focus the present study on the PAG, a region that has been quite neglected or not examined in depth in the previous research, correlating the results with the previous ones related to the SN, with the hope to offer a contribution to the current knowledge on the pathogenetic mechanism of SIDS.

Methods

We investigated the cytoarchitecture of the PAG and the expression of tyrosine hydroxylase (TH), the enzyme involved in the catecholamine synthesis, in its neurons in a cohort of 46 victims of sudden infant death, 26 males and 20 females, aged between 4 and 30 postnatal weeks (approximately 1–7 months), and precisely in the same series of cases in which we previously reported the frequent presence of morphological and functional alterations of the substantia nigra (Lavezzi et al., 2020).

These cases were sent to the Lino Rossi Research Center after a routine post mortem examination that did not lead to a death diagnosis, for the deep analysis of the nervous system, in accordance with the guidelines provided by the Italian law n.31/2006 "*Regulations for Diagnostic Post Mortem Investigation in Victims of sudden Infant Death Syndrome (SIDS) and Unexpected Fetal Death*" (Available at: https://www.linorossi.center/guidelines).

Ethics Approval

The Institutional review board approval was not required for this study since it complies with the requirements of the aforementioned law. Furthermore, the Lino Rossi Research Center of Milan University is the national referral center for its application. Anyway, the parents of all the infants included in the study provided written informed consent to autopsy, related research and publication of the results.

After extensive examination, which led to the exclusion of cardiac, neurologic, respiratory, infectious, metabolic and genetic conditions, 26 infant deaths were classified as "SIDS", due to the absence of any pathological finding. As for genetic tests, our protocol provides for the taking of specific samples from the brains of small victims before proceeding with the usual histological methods, for the search for possible gene variants predisposing to sudden unexpected infant death. In particular, we analyze the PHOX2B gene, to exclude the congenital central hypoventilation syndrome, the promoter region of the serotonin transporter protein (5-HTT) gene, since the presence of the "L" allele represents a predisposing factor for sudden infant deaths, and, among the inborn errors of metabolism, when possible, the medium-chain acyl-coenzyme A dehydrogenase (MCAD), a gene which catalyses the first step in the β-oxidation of fatty acids, given that the enzyme deficit resulting from its mutation can lead to sudden fatal hypoglycemia. Most of the SIDS (24/26 cases) were found dead in their sleep. A precise cause of death was formulated at autopsy for the remaining 20 cases. As they shared certain sociodemographic characteristics (gender, ethnicity and age) at the time of death with the SIDS victims, they were used as "controls". Related death diagnoses were: congenital heart disease (9 cases), severe bronchopneumonia (4 cases), pulmonary dysplasia (3 cases), myocarditis (2 cases), malaria (1 case), and pericarditis (n.1 case).

The neuropathological protocol essentially consisted of the morphological examination of the brainstem, where the main centers controlling the vital functions are located. The related procedures are described in depth in some of our previous articles (Lavezzi et al., 2016; Lavezzi et al., 2019; Lavezzi et al., 2019). In addition to the structures routinely considered, here we analyzed the PAG in mesencephalic serial sections from the superior to the inferior colliculus, with a special focus on the cytoarchitecture and on the expression of the tyrosine hydroxylase (TH), a marker of catecholaminergic neurons. All the observations were performed with a Nikon Eclipse E800 light microscope (Nikon Corporation, Tokyo, Japan) and the images of interest were captured using a Nikon Coolpix 8400 digital camera attached to the microscope.

Tyrosine Hydroxylase (TH) Immunohistochemistry of the PAG Neurons

For the immunohistochemical study of the neuronal catecholaminergic population in the area surrounding the third ventricle, appropriate midbrain sections were incubated with rabbit anti-TH primary antibody diluted in phosphate buffered saline (PBS) and reacted overnight at 4°C. Biotin conjugated secondary antibody incubation (1:200 *cat #S-1000 Vector Laboratories*) was performed for 30 min at room temperature. After several washes in PBS, antibody complex was localized using the ABC system (Vectastain ABC Elite kit *cat #PK6101, Vector Laboratories*) followed by 3,3′-diaminobenzidine reaction. The sections were then counterstained with Mayer-hematoxylin for nuclei and coverslipped after dehydration in ascending concentrations of ethanol and cleared in xylene. To evaluate the specificity of the immunohistochemical method, negative controls were performed by pre-absorbing the primary antibody with a relative antigen excess (100 μg mL^{-1}) and incubating the complex with the sections in the specific step, or by replacing

the primary antibody with PBS in the incubation. In these procedures staining always failed to occur.

Statistical Methods. All the histological and immunohistochemical findings were analyzed by two independent blinded pathologists. The evaluations obtained by each observer in relation to the different parameters were reported, case by case, in a table. Then, once the mean values were calculated, they were compared by using the K Index (KI) in order to evaluate the inter-observer reproducibility. The Landis and Koch (Landis & Koch, 1977) method for the K coefficient interpretation was then used (0 to 0.2 = slight agreement; 0.21 to 0.40 = fair agreement, 0.41 to 0.60 = moderate agreement; 0.61 to 0.80 = strong or substantial agreement; 0.81 to 0.99 = 1.00 = very strong or almost perfect agreement; 1.0 = perfect agreement). A very satisfactory KI value (=0.87) was obtained in this study. Distributions of continuous variables were analyzed by the one-way ANOVA test. The categorical data have been expressed as number and percentages. Statistical calculations were carried out using SPSS software version 20.0 (SPSS Inc.Chicago, IL). A p-value <0.05 was considered statistically significant.

Results

Neuropathological Findings Related to the PAG

Cytoarchitecture. The histological examination of the PAG was performed on specific serial sections of midbrain stained with Klüver-Barrera. Scattered neurons in the gray matter surrounding the aqueduct of Sylvius, immediately adjacent to a thin stratum gliosum, were clearly visible. However, it was not possible to identify the columnar organization reported in experimental studies, i.e. the dorsomedial, dorsolateral, lateral, and ventrolateral PAG columns (Bandler & Shipley, 1994; Beitz, 1985; Carrive, 1993; Holstege, 1991). In most cases we identified only two main groups of neurons which envelops the dorsal and ventrolateral parts of the aqueduct with no clear cytoarchitectonic boundaries and variation in cell type and density: 1) a posterior dense mantle of small sized, round or fusiform cells, defined by us, in accordance with to the Atlas of Olszewski and Baxter (Olszewski & Baxter, 2014), "PAG subnucleus dorsalis" (PAGsd), and bilaterally 2) a wide cluster of larger elongated, more scattered darkly stained neurons with long processes in the ventrolateral area named, according to the same criteria, "PAG subnucleus medialis" (PAGsm) (Figure 1).

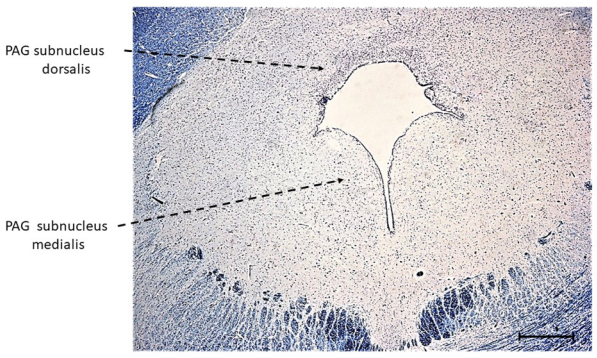

Figure 1. Photomicrographs of a transverse section of midbrain, at the level of inferior colliculus with the indication of the two subnuclei of the human periaqueductal gray (PAG): the subnucleus dorsalis and the subnucleus medialis. Klüver-Barrera stain; scale Bar: 200 μm.

In all SIDS cases, as in controls, the PAGsd presented the same morphological cytoarchitecture characterized by a marked accumulation of small rounded cells, among which occasional elongated neurons were recognized (Figure 2). On the contrary, a substantial difference was found with regard to the PAGsm. In fact, while many large sized neurons were visible in this subnucleus in all the control cases and in 9 SIDS (35%) (Figure 3A), an hypoplasia with clear reduction in the cell number, was observed in the remaining 17 SIDS (65%), all died in the sleep phase (Figure 3B).

TH-immunohistochemistry. In the control group and in 5 SIDS (19%) a widespread TH-immunopositivity was highlighted in a high percentage of the PAGsm neurons (around 50%) (Figure 4A and B), indicative of dopamine-containing cells, given that most neurons within the ventrolateral pars of the PAG are recognized as dopaminergic (Meyer et al., 2009). Contrastingly, a greater loss of TH immunoexpression in the PAGsm neurons was observed in the remaining 21 SIDS victims (81%), all found dead in their sleep (5 of which with PAGsm normal structure) (Figure 4C). No immunopositive cell was present in the PAGd.

Table 1 and Figure 5 summarize the cytoarchitectural and immunohistochemical results.

Correlation Between PAG and SNpc Findings

The above results were compared with those of our previous study on the same case series (Lavezzi et al., 2020), among which the most important was the hypoplasia of the substantia nigra, mainly affecting its pars compacta (SNpc) and characterized by a remarkable reduction of neuron number, found in 18 of the 26 SIDS cases. A strong correlation between SNpc hypoplasia and that of the PAGsm was highlighted. In fact, 15 of the 17 cases with hypoplasia of the PAGsm belonged to the SIDS group with SNpc hypoplasia. Similarly, all 21 cases with negative or poor PAG TH-immunoexpression presented $\leq$ 10% of TH-immunopositive neurons in the SNpc.

Discussion

Previously, in many studies we have highlighted that SIDS, as well as unexpected fetal death, are often a consequence of

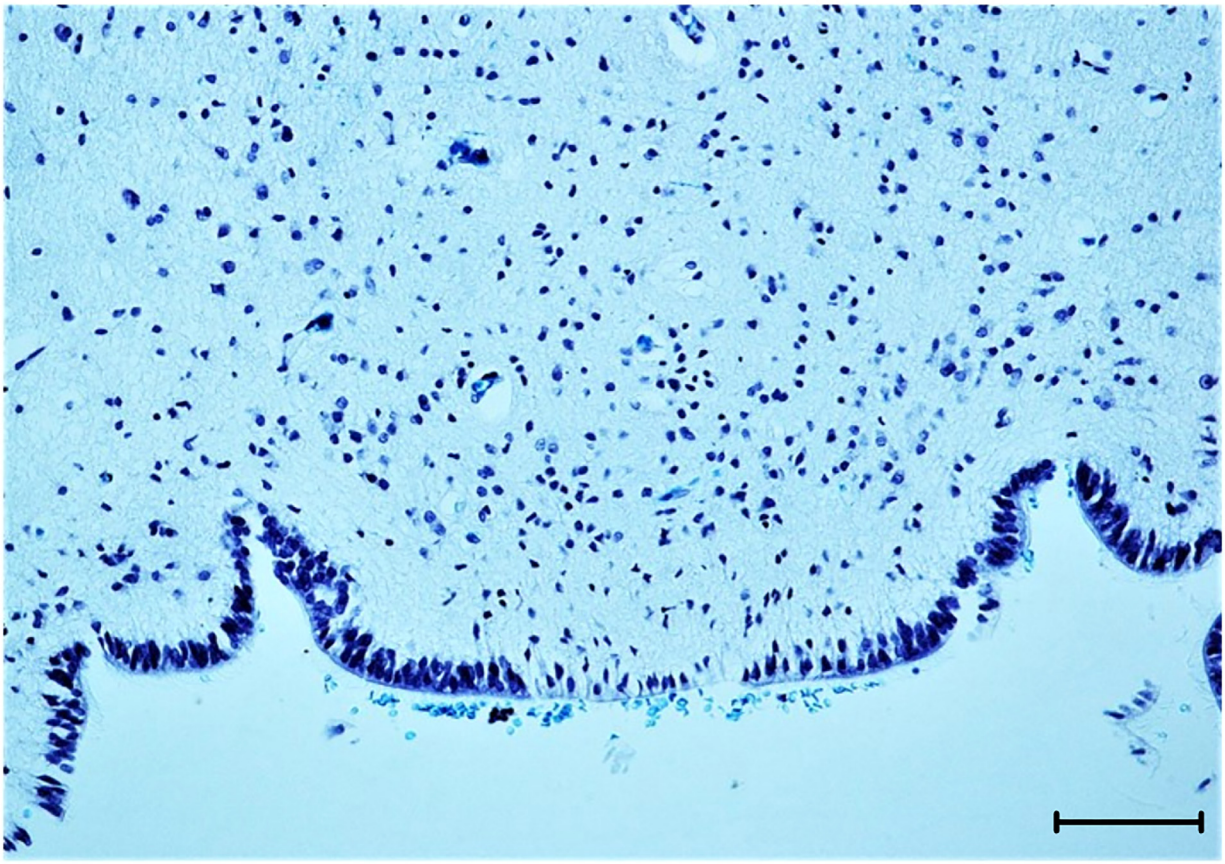

Figure 2. Periaqueductal gray subnucleus dorsalis (PAGsd), characterized by a wide and dense cluster of small sized, round or fusiform cells, located back to the Sylvius aqueduct. Klüver-Barrera stain; scale Bar: 50 μm.

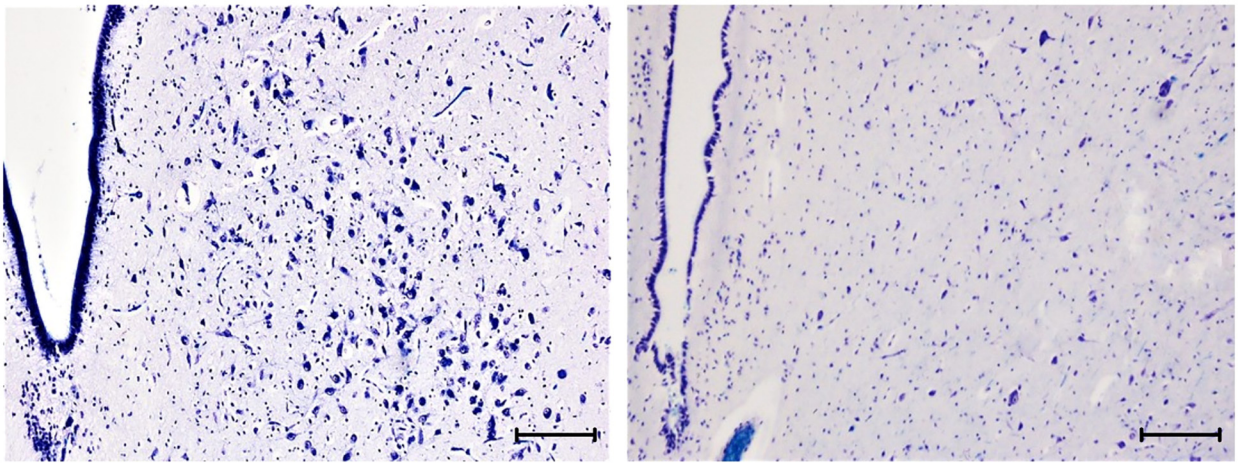

Figure 3. Periaqueductal gray subnucleus medialis (PAGsm). A) large elongated, darkly stained neurons with long processes in the ventrolateral area surrounding the Sylvius aqueduct (control case). B) rare neurons in the same area (SIDS case). Klüver-Barrera stain; scale Bar: 50 μm.

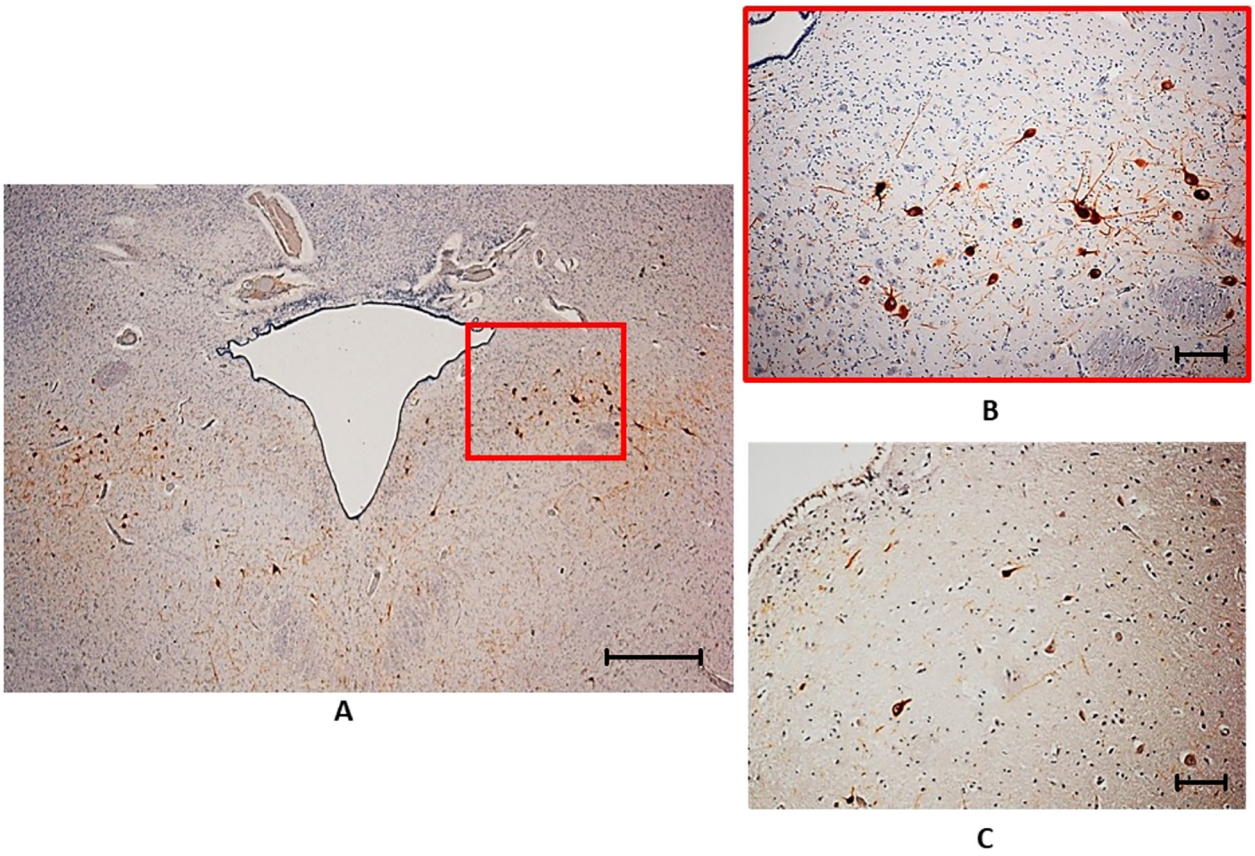

Figure 4. Tyrosine hydroxylase (TH) immunohistochemistry. (A) TH-immunopositivity in the PAGsm in a control case. The boxed area is shown at higher magnification in (B); here intensely stained neuronal bodies and processes are clearly visible. (C) Rare presence of weakly immunostained neurons in the PAGsm of a SIDS case. TH immunostaining; scale Bar: (A) 200 µm, (B) and (C) 50 µm.

developmental morphological and functional alterations of brainstem nerve centers prevalently located in the pons and medulla oblongata that are responsible for breathing control (Lavezzi et al., 2004; Lavezzi et al., 2014; Lavezzi et al., 2016; Lavezzi & Matturri, 2008a; Lavezzi & Matturri, 2008b). Recently we have focused our attention on a higher ventilatory monitoring center located in the midbrain, i.e., the substantia nigra (SN) and notably its pars compacta (SNpc) (Lavezzi et al., 2020). Here, in an expanded research on the same case series, we reached a further important step in understanding the SIDS pathogenesis related to another mesencephalic structure and precisely the PAG, a specific portion of the ventricular gray matter surrounding the Sylvius aqueduct. We have not been able to identify the plural PAG columnar structure recognized in experimental studies (Bandler & Shipley, 1994; Beitz, 1985; Carrive, 1993; Holstege, 1991) but only two main topographically distinct groups of neurons, the PAGsd and the PAGsm. Moreover, it is known that, as reported by Faull (Faull et al., 2019), "distinguishing sub-structures identified in animals in the human PAG remains a challenge". Care must be taken, in fact, when trying to transfer the results obtained from experimental studies into human brain research. While animal models allow a detailed investigation of the neuroanatomy and functions of brain structures, investigations in the human field are limited to non-exhaustive neuroimaging methodologies that cannot even identify direct neuronal connections. Another difference between our results and those reported in studies on animal models is that, while in mice the PAGd influences the ventilatory responses to hypoxia (Hayward et al., 2003; Lopes

Table 1. PAGsm Morphological and Immunohistochemical Findings in SIDS and Controls.

		PAGsm-histology	PAGsm TH immunohistochemical expression	
			normal	decreased
SIDS*	Normal	9 (35%)	4 (44%)	5 (56%)
	Hypoplasia	17 (65%)*	1 (6%)	16 (94%)*
Controls	Normal	20 (100%)	20 (100%)	0 (0%)
	Hypoplasia	0 (0%)	0 (0%)	0 (0%)

The categorical data are expressed as numbers and percentages
*Significance of SIDS values related to those of the Control group: $p < 0.01$
PAGsm = periaqueductal gray subnucleus medialis

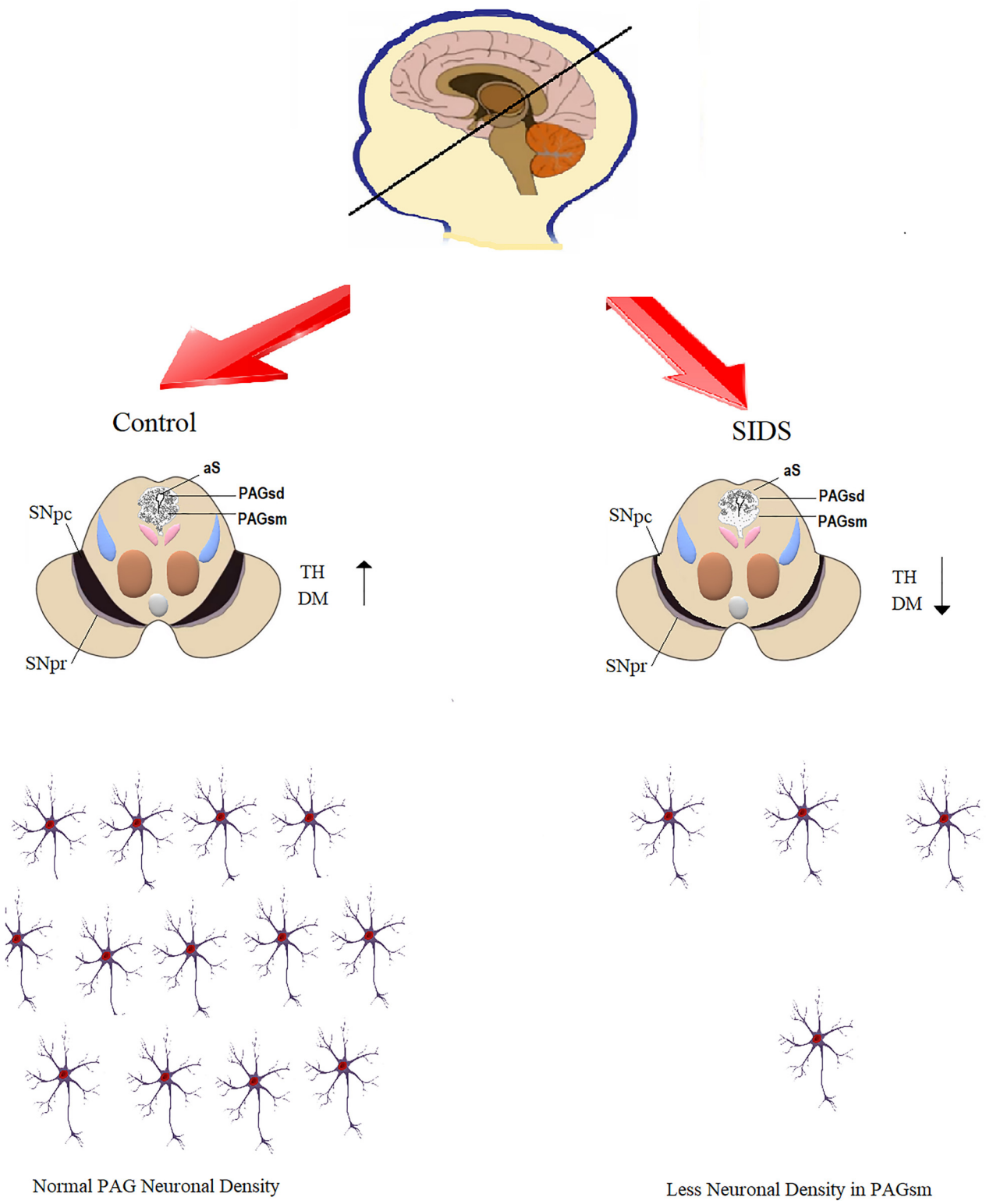

Figure 5. Schematic representation of all the results obtained in this study showing that i the neuron number and the TH expression in the PAG are decreased in SIDS cases compared to controls. aS = aqueduct of Sylvius; DM = dopamine; PAGsd = periaqueductal grey subnucleus dorsalis; PAGsm = periaqueductal grey subnucleus medialis; SNpc = substantia nigra pars compacta; SNpr = substantia negra pars reticulata; TH = tyrosine hydroxylase.

et al., 2014; Zhang et al., 2009), in humans the modifications of breathing in adverse conditions are modulated by the PAGsm.

In support of our interpretation, however, Carrive already in 1993 demonstrated how the stimulation of the ventral part of the lateral PAG generates tachypnea (Carrive, 1993).

Furthermore, the significant association observed in the present study between the PAGsm and SNpc hypoplasia previously detected (Lavezzi et al., 2020) (15 of the 17 cases with hypoplasia of the PAGsm, in fact, also presented SNpc hypoplasia) leads us to believe that the two mesencephalic structures are connected to each other and joined through synaptic descending ways to lower brainstem nuclei of the respiratory network. This view finds support in the experimental study by Lima et al. on animal models (Lima et al., 2018), showing evidence of direct projection from SNpc to PAG and, from here, to the medullary retrotrapezoid nucleus, a group of glutamatergic neurons expressing the transcription factor PHOX2B and involved in activation of breathing (Moreira et al., 2021; Takakura et al., 2014). Interconnections between the PAG and other respiratory nuclei of the medulla oblongata (as retroambiguus and tractus solitarius nuclei) have also been reported in further studies on rats (Huang et al., 2000; Klop et al., 2002; Subramanian, 2013; Subramanian & Holstege, 2013). The PAG even receives descending projections from the superior colliculi, the frontal cortex and hypothalamus (Beitz, 1982; Faull & Pattinson, 2017; George Zaki Ghali, 2020; Ryan & Waldrop, 1995), indicating the existence of a larger network involved in breathing modulation. We believe, in agreement with Subramanian and Holstege (Subramanian & Holstege, 2014), that the PAG is the mediator of this network, able to modify the action of its components also according to the afferent information from pulmonary stretch receptors and peripheral carotid body chemoreceptors, in order to survive threatening events. If there is a structural and/or functional developmental alteration, the PAG could therefore no longer be able to coordinate the respiratory responses to stressful events and then lead to breathing arrest.

The loss of neurons in the PAGsm here observed in the great part of SIDS (65%), all found dead while sleeping, can therefore provide valuable insight into the involvement of this subnucleus in the pathogenetic mechanism of this syndrome that, as it is well known, mostly occurs during arousal from sleep (Hunt, 1989; Kahn et al., 2003; Kato et al., 2003). Our assumption is validated by the paper of Benarroch (Benarroch, 2012) stating that the PAG contributes to REM sleep and arousal control. In addition, the decreased TH-immunopositivity found in 81% of SIDS (precisely in 21 of the 24 cases who died during sleep), reinforces this assertion given that dopamine, one of the main catecholamines produced by the TH enzyme here detected in the PAGsm, regulates the sleep-wake cycle (Dzirasa et al., 2006; Monti & Monti, 2007). Then, we can conclude that defective development of the PAG, and more specifically of its medialis subnucleus, can lead to respiratory failure particularly in the passage from sleep to waking, resulting in premature sudden death. Further research, also focused on other higher brain centers and correlating the results with risk factors, will lead to a full understanding of the main causes of SIDS. In this regard, we must point out a limitation of our study since we have not defined the relationship between findings and environmental toxic factors, which seem to be increasingly involved in the defective development of vital nerve centers of the human brain. We are currently addressing our interest in this field by using a Field Emission Gun Environmental Scanning Electron Microscope in order to highlight the presence of atmospheric pollutants in the brain. Preliminary results are promising since, albeit in a few cases of SIDS, we are showing the presence in the brainstem parenchyma of micro- and nano- sized foreign bodies, in particular nanomaterials that are widely used in biomedicine, biotechnology and environmental industry (Bundschuh et al., 2018).

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Author Contributions

AML and RM together designed the study, developed the methods and wrote the manuscript.

ORCID iDs

Anna M. Lavezzi https://orcid.org/0000-0002-0377-5245
Riffat Mehboob https://orcid.org/0000-0001-8965-1587

Supplemental material

Supplemental material for this article is available online.

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Abbreviations

ABC	avidin-biotin complex
PAG	periaqueductal gray
PAGsd	PAG subnucleus dorsalis
PAGsm	PAG subnucleus medialis
PBS	Phosphate buffered saline
TH	tyrosine hydroxylase
SIDS	sudden infant death syndrome
SN	substantia nigra
SNpc	substantia nigra pars compacta.