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Impaired orexin receptor expression in the Kölliker–Fuse nucleus in sudden infant death syndrome: possible involvement of this nucleus in arousal pathophysiology

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Objectives: As well known, the sudden infant death syndrome (SIDS) is characterized by the sudden death of a seemingly healthy infant during sleep, frequently resulted from a deficit in arousal phase. Awakening from sleep requires a fully developed and functioning neuronal respiratory network to modulate the ventilation as needed. The pontine Kölliker–Fuse nucleus (KFN) plays a pivotal role in breathing control, thanks to its interconnections with the widespread serotonin and noradrenaline neurons in the brainstem. Numerous studies to date have focused on the implication of orexin, a neuropeptide synthesized by neurons of the lateral hypothalamus, with major projections to the brainstem raphé nuclei and locus coeruleus, in arousal, a neurobiological process closely linked to breathing modifications. The aim of our research has been to demonstrate that also the KFN is a fundamental component of the orexin system, actively involved in arousal.

Methods: We have evaluated the expression and distribution of the orexin receptors (orexin-1 and orexin-2 receptors) particularly in the rostral pons, where the KFN is located, of 25 SIDS cases and 18 controls.

Results: An intense orexin-1 innervation around the KF neurons has been detected in almost all the controls and only in 20% of SIDS cases.

Discussion: On the basis of these results, we believe that: (1) the KFN plays a leading role not only in providing a regular breathing rhythm but also in the coordination of the sleep-to-wake transition; (2) a defective orexin expression in the KFN could prevent arousal, thus assuming a crucial importance in causing SIDS.

Keywords: Kölliker–Fuse nucleus, Orexin, Brainstem, Arousal, SIDS, Neuropathology

Abbreviations: ABC= avidin-biotin complex, BDNF= brain–derived neurotrophic factor, CPAP= continuous positive airway pressure, F/PFc= Facial/Parafacial complex, GC/MS= gas chromatography/mass spectrometry, GFAP= glial fibrillary acid protein, ILN= intermediolateral nucleus, KFN= Kölliker–Fuse nucleus, LC= locus coeruleus, NeuN= neuronal nuclear antigen, Ox= Orexin, OxR= Orexin receptor, pBN= pre-Bötzinger nucleus, RN= respiratory network, SIDS= sudden infant death syndrome, TSN= tractus solitarius nucleus.

Introduction

Sleep and wakefulness are two mutually exclusive behavioral states that are qualitatively and quantitatively easy to characterize, having well-defined electroencephalographic and electromyographic features.1,3

In mammals, sleep corresponds to a period of relative inactivity due to a reversible disconnection from the environment, generally divided into Rapid Eye Movement (REM) sleep, also called ‘paradoxical sleep’, and non-REM sleep (or slow-wave sleep).1,3 Wakefulness, on the contrary, is essentially a conscious state in which the subject can perceive and interact with the environment.4

The definition of arousal is more difficult: the term ‘arousal’ usually refers to an abrupt change in the brain activity, with a shift from sleep to the state of wakefulness, showing an increased motor activity, responsiveness to sensory inputs, enhanced emotional and cognitive processes.5,4 However, the neurobiological mechanisms related to arousal have not been fully disclosed.

It is well known that arousal can be a life-saving reflex since it protects against the adverse and potentially fatal effects of asphyxia during sleep.9 Evidences to date suggest that the arousal stimulus is proportional to the level
of breathing effort originated in a complex neuronal respiratory network (RN) responsible for ventilatory drive, that includes the Kölliker–Fuse nucleus (KFN), as main breathing coordinator, the Facial/Parafacial complex (F/ PFc) and the pre-Bötzing nucleus (pBN) in the brainstem, together with the intermediolateral nucleus (ILN) in the spinal cord.10–12

In 1998, two research teams13,14 independently demonstrated by immunohistochemistry that Orexin (Ox), a neuropeptide synthesized by neurons in the lateral hypothalamus, also called hypocretin, a critical factor in activating arousal. They identified two isoforms of orexin (OxA and OxB) with two types of receptors (OxR1 and OxR2). Moreover, they revealed their active involvement during arousal and wakefulness, and relative inactivity during REM and non-REM sleep.

Ox-signaling from hypothalamic neurons, through widely projecting efferents, provides excitatory stimulation to specific components of the central nervous system, the densest processes being at the noradrenergic neurons of the locus coeruleus (LC) and the serotonergic neurons of the raphé nuclei.15,16 Both noradrenaline and serotonin neurons have, in fact, chemosensitive properties contributing to the adaptation of breathing to changes of blood gas (O2, CO2) concentrations and pH during the sleep–wake cycle.17,18

A deficit in the arousal process from sleep in response to a life-threatening stressor has been indicated as pathogenic mechanism in the sudden infant death syndrome (SIDS), which occurs in most cases at awakening from sleep.19–21 A failure of the physiological hyperventilation that normally takes place in this sensitive hypoxic phase, through a rise in the amplitude and frequency of pulmonary movements to restore the normal concentration of plasmatic gas, has been proposed in this regard. Further experimental studies have shown that the pontine KFN plays an important role in respiratory modulation, according to the needs, through reciprocal afferent and efferent anatomical connectivity with other centers of the brainstem RN.24–26 Noteworthy among these respiratory centers is the tractus solitarius nucleus (TSN) in the medulla oblongata. Sarnat and Flores-Sarnat demonstrated that delayed formation of this nucleus (TSN) in the medulla oblongata, among these respiratory centers is the tractus solitarius nucleus (TSN) in the medulla oblongata. Sarnat and Flores-Sarnat demonstrated that delayed formation of this nucleus (TSN) in the medulla oblongata, together with the intermediolateral nucleus (ILN) in the spinal cord.10–12

In previous studies, performed in a very large number of fetuses and newborns, suddenly died, and age-matched controls, we have highlighted that the KFN acts as a breathing film-maker in human perinatal life, emphasizing its involvement in SIDS.26–28 In particular, we have reported that, although a normal cytoarchitecture of this nucleus is absolutely essential for eupneic breathing at birth, neurochemical alterations, such as an unusual immunopositivity of the brain-derived neurotrophic factor (BDNF) and a decreased expression of the neuronal nuclear antigen (NeuN) in the KFN neurons are very frequent findings in SIDS cases compared to controls.

Hunt et al. recently reported that Ox-immunoreactivity is significantly decreased in SIDS infants, with particular reference to the neurons of the tuberal hypothalamus and the fibers of the chemosensitive dorsal raphe nucleus and LC, compared to controls, supporting the hypothesis that impaired Ox-expression in hypothalamic neurons and their specific pontine projections are able to induce arousal dysfunction.29 Given that the KFN can exert a chemoreceptor control of breathing24 and that Ox can also explain an action on breathing modulation,30,31 the aim of the present study has been to evaluate whether the Ox-hypothalamic processes innervate this nucleus, too, in order to demonstrate its participation in arousal, and to highlight possible defects of Ox-expression in the KFN of SIDS victims.

To approach this issue, we have applied Ox-immunohistochemistry on brainstem histological sections, particularly from the rostral pons where the KFN is located, of infants who died in the first months of life for known and unknown causes (SIDS vs non-SIDS). This nucleus, and in particular its involvement in arousal, has been never investigated by the many authors who have published studies on Ox expression in the brain of both rats and humans.

Methods
In this study, we have investigated, by evaluating the expression and distribution of both the receptor proteins OxR1 and OxR2 in the rostral pons, where the KFN is located, 43 cases of infants who suddenly and unexpectedly died in the first months of life (survival time range 1 and 6 months).

Consent
Parents of all the infants included in the study provided written informed consent to autopsy and related researches; study approval was granted by the institutional review board of the Milan University (Lino Rossi Research Center).

After a thorough autopsy examination, according to the guidelines provided by the Italian law n.31/2006 “Regulations for Diagnostic Post Mortem Investigation in Victims of SIDS and Unexpected Fetal Death” (available at the website “http://users.unimi.it/centrolinorossi/en/guidelines.html”), 25 infant deaths were classified as “SIDS”, given the absence of any pathological findings. In the remaining 18 cases, a precise cause of death was formulated at autopsy. These cases, similar for gender, ethnicity, and age at the time of death to the SIDS victims, were regarded as ‘controls’.

For every case, a complete clinical history was collected. Additionally, mothers were asked to complete a questionnaire inquiring about a smoking habit. Furthermore, the guidelines of the Lino Rossi Research Center stipulate the removal of a lock of victims’ hair to search for xenobiotics and in particular cotinine, the main metabolite of nicotine, which has a long half-life, by gas chromatography/mass
spectrometry (GC/MS). Twenty of the 25 SIDS mothers (80%) resulted active smokers by their own admission, all claiming to have the smoking habit from before pregnancy, or after the nicotine-test. The remaining five mothers had no history of cigarette smoking, verified through the same analysis. Only two of the 18 mothers in the control group (11%) had a proven smoking habit. Information on cigarette smoking was collected also from fathers, with a positive match for 10 fathers in the SIDS group and three in the control group.

Table 1 summarizes the case profiles in the study.

**Neuropathological examination of the brainstem**

Briefly, the applied methodology, in accordance with the aforementioned guidelines, states that, after fixation in 10% phosphate-buffered formalin, the brainstem, where the main structures controlling the vital functions are located, must be processed and embedded in paraffin. Then three specimens are taken (Fig. 1): the first specimen (I) includes the upper third of the pons and the adjacent caudal portion of midbrain; the second specimen (II) is taken from the caudal portion of the pons; the third specimen (III) is from the medulla oblongata around the obex. Transverse serial 4-μm sections from the samples have been made at intervals of 30 μm, and processed according to the needs. For each level, two of these sections have been routinely stained for histological examination using the hematoxylin-eosin and Klüver-Barrera, while four sections have been treated for immunohistochemical detection of OxR1 and OxR2. In addition, the study protocol included the application of the immunohistochemistry for NeuN, a nuclear protein widely expressed in the mature postmitotic neurons, indicative of neuronal maturation, and of the glial fibrillary acid protein (GFAP), a marker of reactive gliosis in neurodegenerative processes.

**Histology**

The routine histological evaluation of the brainstem was performed on the LC, KFN, and the median raphe nucleus in the first rostral pontine specimen; on the F/PFc, superior olivary complex, retrorapheoid and magnus raphe nucleus in the second sample from the caudal pons, and on the hypoglossus, dorsal motor vagal, ambiguus, pBN, the

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### Table 1 Summary of the baseline data related to the infants included in the study

<table>
<thead>
<tr>
<th></th>
<th>SIDS (No. 25)</th>
<th>CONTROLS (No.18)*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in months (mean ± SD)</td>
<td>3.28 ± 1.3</td>
<td>3.5 ± 1.18</td>
<td>ns</td>
</tr>
<tr>
<td>Male/female (No.)</td>
<td>14/11</td>
<td>8/10</td>
<td>ns</td>
</tr>
<tr>
<td>Birth weight in grams (mean ± SD)</td>
<td>3236 ± 105</td>
<td>3400 ± 126</td>
<td>ns</td>
</tr>
<tr>
<td>Gestational age in days (mean ± SD)</td>
<td>266 ± 9</td>
<td>272 ± 5</td>
<td>ns</td>
</tr>
<tr>
<td>Preterm birth (No.)</td>
<td>2</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>Smoke absorption (No.)</td>
<td>18</td>
<td>2</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*Death diagnoses in controls were: congenital heart disease (No.8), myocarditis (No.2), severe bronchopneumonia (No.4), pulmonary dysplasia (No.3), pericarditis (No.1). ns = not significant (p ≥ 0.05).
inferior olivary, obscurus and pallidus raphé and arcuate nuclei and the TSN in the third medullary sample.

Neuropathology diagnostic criteria
All the nuclei and/or structures above listed were firstly deeply examined in specific histological sections, selected on the basis of over 20 of research activity in this field at the Lino Rossi Research Center. These sections are easily recognizable following specific landmarks: the superior cerebellar peduncle decussation in the rostral pons, the medial nucleus of the superior olivary complex in the caudal pons, and the dorsal accessory of the inferior olivary nucleus in the medulla oblongata.

The more frequent finding in sudden perinatal death is the hypoplasia of the main brain nuclei involved in the control of vital functions. When a given nucleus shows significant reduction in the number of neurons and/or a decreased area in the selected transversal histological sections, compared to the mean values obtained in a group of age-matched controls, previously collected and stored at the Lino Rossi Research Center, it has to be examined for all its extension in serial sections. A diagnosis of ‘hypoplasia’ can be formulated only when the hypodevelopment is confirmed at all levels.

Immunohistochemistry

Orexin receptors (OxR1 and OxR2) detection
The selected formalin-fixed paraffin-embedded brainstem tissue sections were deparaffinized and boiled for antigen retrieval by microwaving in citrate buffer (pH 6.0) at 600 W, 3 times for 5 min each, and finally cooled. Then, sections were incubated overnight at +4 °C in a wet chamber, using commercially available rabbit polyclonal antibodies Anti-Orexin Receptor 1 (ab83925, Abcam) and Anti-Orexin Receptor 2 (ab85899, Abcam), diluted 1:600 in phosphate-buffered saline (PBS; pH 7.4). A standard avidin-biotin complex (ABC) technique (Vectastain elite ABC KIT) was used with horseradish-peroxidase in dianiaminobenzidine substrate (HRP-DAB), to visualize and develop the antigen-antibody reaction. The antibody dilution at 1:1500 in PBS. Immunohistochemical staining was performed with the peroxidase-antiperoxidase method and the ABC technique (ABC Kit, Vectastain, Vector Laboratories Inc., Burlingame, CA, USA.). Diaminobenzidine (DAB, Vector Laboratories Inc., Burlingame, CA, USA) was used as chromogen substrate and counterstained with light hematoxylin.

NeuN detection
Sections from paraffin-embedded tissue blocks were stained using commercially supplied mouse monoclonal antibodies against the neuronal nuclear antigen NeuN (Chemicon International, MAB377). A standard ABC technique was used with peroxidase-diaminobenzidine to visualize and develop the antigen-antibody reaction. The antibody dilution at 1:1500 in PBS was used. Incubating solutions were boiled in 10 mM citric acid at pH 6.5, in a microwave oven, for 5 min at high power, then 5 min at 50% power, and finally cooled for 20 min. Sections were lightly counterstained with Mayer’s hematoxylin.

Negative controls for both GFAP and NeuN methods were done using PBS instead of primary antibody.
Quantification of the GFAP and NeuN immunohistochemical results

In the captured images, obtained as above indicated, only the cells with intense brown immunostaining were considered to be positive. We quantified the scoring for each case, similarly to the method for OxR evaluation, as follows:

- = no immunopositive cell per unit area
-/+ = weak immunopositive cells present in \( \leq 30\% \) per unit area
+ = strong immunopositive cells present in \( \leq 30\% \) per unit area
++ = weak immunopositive cells present in \( >30\% \) per unit area
+++ = strong immunopositive cells present in \( >30\% \) per unit area

The unit area was represented by square millimeter (mm\(^2\)).

Statistical analysis

Histological and immunohistochemical evaluations were done by groups of two independent and blinded pathologists. Comparison of results was performed, employing K Index (KI) to evaluate the inter-observer reproducibility. The Landis and Koch system\(^{34}\) for the K interpretation was used, where 0–0.2 is slight agreement, 0.21–0.40 indicates fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 strong or substantial agreement, and 0.81–1.00 indicates perfect agreement (a value of 1.0 being perfect agreement). The application of this method in the present study revealed an overall very satisfactory KI (0.88).

The statistical significance of direct comparisons between the groups of victims was determined by analysis of variance (ANOVA). Statistical calculations were carried out with SPSS (statistical package for social science) statistical software. Differences were regarded as statistically significant if the p value was \(<0.05\).

Results

Morphology of the KFN

We observed, in accordance with our previous reports focused on the KFN\(^{26–28}\) that the cytoarchitecture of this nucleus, extending longitudinally throughout the whole first brainstem sample (see Fig. 1) was well analyzable in the more cranial transverse sections of the pons, namely those bordering the caudal mesencephalon (Fig. 2A). At this level, the KFN appeared as a small population of large neurons located in the pontine tegmentum between the crossing of the superior cerebellar peduncles and the medial lemniscus (Fig. 2B). These neurons showed a distinct, eccentric nucleus with an evident nucleolus, and abundant cytoplasm with Nissl substance at the cell periphery. Intermixed with these large neurons, smaller cells (interneurons and astrocytes) were visible. This cytoarchitecture of the KFN was recognizable by the same features, in both SIDS and control infant cases.

Ox-Immunohistochemistry of the KFN

An indisputable substantial difference was observed comparing the appearance of the Ox-receptor proteins in the KF area of SIDS infants with non-SIDS controls (\( p < 0.01 \)). In fact, an intense Ox-innervation around the KF neurons, highlighted in particular by the presence of OxR1-immunoreactive fibers and varicosities in more than \( 30\% \) of the nucleus area (score ++ and +++), was detected in 17 of the 18 non-SIDS victims and in 5 of the 25 SIDS cases (Fig. 2C and D). Therefore, the OxR1-signaling was negative in one control newborn, died of severe bronchopneumonia, and 20 SIDS. Conversely, the OxR2 immunohistochemistry did not show in all cases of the study significant results. Only in two subjects of the control group a very weak OxR2-immunopositivity was observed in the KFN, whereas in all other cases (SIDS and non-SIDS) OxR2-immunoreactivity in this nucleus was absolutely absent.

NeuN and GFAP-Immunohistochemistry of the KFN

The antigen NeuN was well expressed in all the neurons of the KFN (score ++, ++++) in both SIDS and controls. No sign of immunopositive reactive astrogliosis was found in the KFN area of control cases, while a fair number of reactive astrocytes, characterized by high-level expression of GFAP (++ and ++++) in spongiform cell bodies and processes, were found nearby the large neurons of the KFN in about \( 20\% \) of SIDS (5/25).

Morphology of the other brainstem structures

The extensive morphological examination of the brainstem histological sections in the SIDS group revealed developmental alterations, prevalently hypoplasia of the arcuate nucleus (8 cases), serotonergic obscurus raphé nucleus (2 cases) and pBN (2 cases) in the medulla oblongata, hypoplasia of the magnus raphé nucleus (2 cases), and F/PF skilled by the presence of OxR1-immunoreactive fibers and varicosities in more than \( 30\% \) of the nucleus area (score ++ and +++), was detected in 17 of the 18 non-SIDS victims and in 5 of the 25 SIDS cases (Fig. 2C and D). Therefore, the OxR1-signaling was negative in one control newborn, died of severe bronchopneumonia, and 20 SIDS. Conversely, the OxR2 immunohistochemistry did not show in all cases of the study significant results. Only in two subjects of the control group a very weak OxR2-immunopositivity was observed in the KFN, whereas in all other cases (SIDS and non-SIDS) OxR2-immunoreactivity in this nucleus was absolutely absent.

Ox-Immunohistochemistry of the other brainstem structures

Ox1-immunoreactive fibers were fairly weakly widespread in the brainstem, with increased expression in the LC and raphé nuclei areas (score ++ and ++++), likewise in both SIDS and non-SIDS cases. Only poor immunopositivity related to OxR2 was found, especially in the ventral pars of the caudal pons (griseum pontis), in 2 SIDS and 3 controls.
significant immunopositive signs of reactive astrogliosis were generally found.

**NeuN and GFAP-Immunohistochemistry of the other brainstem structures**

Strong NeuN-immunoreactivity was present in almost all the examined neuronal cell population (score ++ and +++). The staining was present in both nuclei and cytoplasm, extending into the proximal parts of the processes. Only in 4 SIDS cases, the NeuN-labeling was significantly decreased above all in the nuclei of the caudal pons; no significant immunopositive signs of reactive astrogliosis were generally found.

**Correlation of OxR findings with smoke exposure**

High correlation was observed between OxR1 results and smoke absorption. In fact, all the 20 SIDS cases with lack of expression of OxR1 in neuronal processes of the KF...
Table 2  Brainstem neuropathological results related to the neuronal centers in 25 SIDS cases and 18 controls

<table>
<thead>
<tr>
<th>Brainstem structure</th>
<th>Orexin-1R expression</th>
<th>Cytoarchitecture</th>
<th>NeunN expression</th>
<th>GFAP expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pons</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnus raphé nucleus</td>
<td>+/++ (25)</td>
<td>++ (15)</td>
<td>Normal (23)</td>
<td>Normal (18)</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obscurus/pallidus raphé nuclei</td>
<td>–/++ (22)</td>
<td>–/++ (10)</td>
<td>Normal (23)</td>
<td>Normal (18)</td>
</tr>
<tr>
<td>Arcuate nucleus</td>
<td>+++ (3)</td>
<td>+++ (8)</td>
<td>Normal (17)</td>
<td>Hypoplasia (8)</td>
</tr>
</tbody>
</table>

The immunoexpression of Orexin receptors was estimated using a five-point scale: (−) absence of immunopositive fibers; (−/+) weak immunopositive fibers in ≤30% of the nucleus area; (+) strong immunopositive fibers in >30% of the nucleus area; (+++) strong immunopositive fibers in >30% of the nucleus area.

The immunoexpression of NeunN and GFAP was estimated using a five-point scale: (−) absence of immunopositive cells; (−/+ ) weak immunopositive cells in ≤30% per unit area; (+) strong immunopositive cells in >30% per unit area; (+++) strong immunopositive cells in >30% per unit area. The unit area corresponds to 1 mm².

*When comparing SIDS vs infant controls p < 0.01.

Neurons had a smoker mother, and 10 of these a smoker father, too. Furthermore, the only OxR-negative case in the control group, died of severe broncopneumonia, had a smoker mother. Noteworthy was also the simultaneous presence in 5 cases belonging to the 20 SIDS cases with OxR1 negativity in the KFN and smoking mother, of the medullary arcuate nucleus hypoplasia.

All findings are represented in Tables 2 and 3. Table 2 is a summary of the neuropathological observations in both SIDS and controls. In Table 3, the results related to the 25 SIDS victims are represented case by case.

Discussion

This study is the first to describe the expression of Ox-receptors in the pontine KFN during early human life, as well as OxR alterations in SIDS.

The KFN is a fundamental component of the RN with pivotal implications on breathing control, acting also as a central chemoreceptor with a different function pre- and post-natally.12,23,35 During intra-uterine life, in fact, the KFN inhibits the central and peripheral chemoreceptors, which are already fully developed and potentially functional, and therefore any respiratory reflex. Only occasional respiratory movements occur, aimed to promote lung development, coordinated by the ILN in the upper spinal cord. At birth, the KFN abruptly reduces its inhibitory function and becomes active as main neuronal center controlling the ventilatory activity, through extensive afferent and efferent connections with other brainstem respiratory-related structures, components of the RN. Moreover, always at birth, the F/PFc, located in the caudal pons, starts working under the stimulation of the KFN, so providing for the first inspiratory act. Indeed, its activity is called ‘pre-inspiratory’ since it is limited to activate the proper inspiratory nucleus in the medulla oblongata (i.e. the pBN), that is responsible for accomplishing the first and each postnatal breath.36

The importance of the KFN in the modulation of breathing in both pre- and post-natal life has been by us supported in numerous studies on the neuropathology of sudden unexplained fetal and infant death.26-28 We
essentially sustain, according to other authors,\textsuperscript{35,37,38} that the pulmonary activity is largely dependent on sensory inputs from the ILN in fetuses and from the F/PFc from birth, being both modulated by the KFN, which therefore represents the breathing film-maker. Its activity, changing from fetal to postnatal life thanks to a skillful interplay of activation and inactivation of its GABAergic inhibitory and glutamatergic excitatory neurons,\textsuperscript{35} is fundamental.

The KFN is able, in addition, to regulate the extra-uterine ventilation by responding to carbon dioxide (CO\textsubscript{2}) and/or pH variations in different ways.\textsuperscript{35,38} Whereas during normal breathing (eupnea), Pa CO\textsubscript{2} is maintained at physiological levels (normal range in humans 35–45 mmHg),\textsuperscript{39} in presence of a large increase of CO\textsubscript{2} due to an accidental airway block, severe bronchial disease, prone sleeping position, nicotine absorption, or other causes) the KFN strongly stimulates the sequence of RN nuclei to accelerate ventilation, thereby restoring normal gas values.

It is well known that the chemoreflexes are depressed during sleep and that an abrupt chemoreceptor stimulation, due to one of more of the aforementioned causes, immediately triggers the arousal process.\textsuperscript{39,40}

Numerous experimental studies performed during the last decades have been focused on the direct involvement of the neuropeptide Ox in CO\textsubscript{2}/pH sensitivity, particularly during the sleep-to-arousal transition.\textsuperscript{41–44} It has been widely demonstrated that Ox, which in general displays a low-frequency and even silent activity throughout the sleep periods, is promptly synthesized by specific secreting neurons in the lateral hypothalamus just before awakening; this evidence supports that Ox-signaling is necessary for regular arousal.\textsuperscript{45,46}

\begin{table}
\centering
\caption{Main data and neuropathological findings in the 25 SIDS cases}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
CASE & Sex & Postnatal age (months, weeks) & Orexin-1 immunoexpression in the KFN\textsuperscript{*} & Orexin-1 immunoexpression in other brainstem nuclei\textsuperscript{*} & Developmental alterations of the brainstem nuclei & Parental smoking \\
\hline
1 & M & 1,1 & (−) & (+) & magnus raphé nucleus hypoplasia & M \\
2 & F & 2 & (−) & (+) & arcuate nucleus hypoplasia & M+P \\
3 & F & 6 & (−) & (+) & obscurus raphé nucleus (+++) & M+P \\
4 & M & 4,3 & (−) & (+) & obscurus raphé nucleus hypoplasia & M+P \\
5 & M & 5 & (++) & (+) & arcuate nucleus hypoplasia & M \\
6 & M & 2,2 & (−) & (+) & magnus raphé nucleus hypoplasia & M \\
7 & F & 1,5 & (−) & (+) & arcuate nucleus hypoplasia & M \\
8 & F & 3 & (+++) & (+) & magnus raphé nucleus hypoplasia & M \\
9 & M & 3 & (+++) & arcuate nucleus hypoplasia & M \\
10 & F & 5 & (−) & arcuate nucleus hypoplasia & M+P \\
11 & M & 2 & (−) & arcuate nucleus hypoplasia & M+P \\
12 & M & 1,3 & (−) & arcuate nucleus hypoplasia & M+P \\
13 & M & 2,3 & (++) & arcuate nucleus hypoplasia & M+P \\
14 & M & 3,1 & (−) & arcuate nucleus hypoplasia & M+P \\
15 & F & 3 & (−) & arcuate nucleus hypoplasia & M+P \\
16 & F & 3 & (−) & arcuate nucleus hypoplasia & M+P \\
17 & M & 4,3 & (−) & arcuate nucleus hypoplasia & M+P \\
18 & M & 4 & (−) & arcuate nucleus hypoplasia & M+P \\
19 & M & 3,2 & (−) & arcuate nucleus hypoplasia & M+P \\
20 & F & 3 & (−) & arcuate nucleus hypoplasia & M+P \\
21 & F & 3,3 & (++) & arcuate nucleus hypoplasia & M+P \\
22 & F & 4 & (−) & arcuate nucleus hypoplasia & M+P \\
23 & M & 2,3 & (−) & arcuate nucleus hypoplasia & M+P \\
24 & F & 4 & (−) & arcuate nucleus hypoplasia & M+P \\
25 & M & 3 & (−) & arcuate nucleus hypoplasia & M+P \\
\hline
\end{tabular}
\footnote{The immunoexpression of Orexin was estimated using a five-point scale: (−) absence of immunopositive fibers; (−+/+) weak immunopositive fibers in ≤30% of the nucleus area; (+) strong immunopositive fibers in ≤30% of the nucleus area; (+++) weak immunopositive fibers in >30% of the nucleus area; (++++) strong immunopositive fibers in >30% of the nucleus area.}
\end{table}
The arousal process occurs thanks to the numerous scattered fiber projections from the hypothalamic neurons through the brain, with the densest synaptic terminals at the dorsolateral pons and rostral ventrolateral medulla. In particular, a substantial body of works performed on rats has demonstrated the prominent role of the noradrenergic LC in mediating the action of hypothalamic Ox-neurons on arousal.47–50

In humans, a direct involvement of Ox has been demonstrated in patients who suffer from sleep apnea, narcolepsy, Prader-Willy syndrome and other sleep-related disorders.51–54

Recently, Hunt et al. have described a decreased Ox immunoreactivity in the brain of infants who died of SIDS.29 These authors reported in particular that SIDS infants, compared with age-matched controls, show a significant decreased Ox expression in the neurons of the tuberolateral hypothalamus and in fibers of sleep-related nuclei of the pons, with the greatest reduction in the LC. No reference was made, either in this specific work or in other reports in literature, to the presence of Ox-immunopositive fibers in the human KFN; references to Ox signaling in this nucleus have been sometimes highlighted in rodents.24,55

More in detail, Gestreau et al.55 have reported that the distribution of Ox fibers in rats is mainly observed in the dorsal/ventrolateral medulla and on the dorsolateral pontine nuclei, including the KFN, without to mention the involvement of this nucleus in arousal.

It should be noted that in both the studies of Hunt et al.29 and Gestreau et al.55, no significant differences were observed between the expression of the two receptors in the immunoreactive fibers. Conversely, in our study we highlighted a prevalent Ox1R manifestation, above all in the KFN area, whereas the Ox2R showed no significant immunopositivity. These results anyway suggest a different functionality of the two receptors, according to the experimental study of Marcus et al.56 These authors have reported that the two orexin receptors, although both belonging to the family of G protein-coupled receptors, share an overall 64% sequence identity which implies a partially distinct distribution of expression through the brain. While the Ox1R has a much higher (100 to 1000-fold) affinity for OxA than for OxB, the Ox2R seems to have equal affinities for both neuropeptides. These distinctive features of the receptors allowed to hypothesize a sleep-specific role for the Ox1R and a more general role for Ox2R.

In our previous studies,26–28 we have provided detailed accounts of the cytoarchitecture and physiology of the human KFN in perinatal ages and on its primary role in the RN control. In addition, we reported the presence of hypoplasia, with few immature neurons, or agenesis of the KFN only in late fetal deaths (around 20%), never in newborns and infants. This means that a normal structure of the KFN is absolutely essential for breathing activity from birth and that its hypodevelopment results in uterine death.

In this work, focused on infant deaths, we have added new knowledge about the physiology of this important nucleus, showing the presence of Ox-positive fibers in a normally structured KFN, as expected, in almost all the control cases (17/18) but only in 5 of the 20 SIDS victims.

Since evidence to date suggests that the arousal stimulus is related to the level of inspiratory effort,56 we have good reason to believe, on the basis of the present results, that in addition to its pivotal role in coordinating ventilatory drive, the KFN plays an important part in promoting the rapid shift from sleep to wakefulness. In our opinion, the KFN is the first target of the hypothalamic neurons, it receives as soon as the arousal signal and it actively coordinates the other well-known Ox structures in promoting the sleep-to-waking transition. Thus, we argue that an impaired Ox-signaling limited to the KFN could have important implications in the pathogenesis of SIDS, undermining the normal respiratory rhythm, given the fundamental role of this nucleus as breathing filmmaker, also in the delicate awakening phase.

This hypothesis is supported by experimental studies on mice,57,58 showing the critical role of the parabrachial nucleus, a component of a neuronal complex in the dorsolateral pons where also the KFN is located,59 in arousal from sleep, particularly in conditions of asphyxia.

A failure of the normal sleep/wake behavior, that accounts for the final outcome in the majority of SIDS cases, could be primarily due to a decreased expression of the Ox-receptors in the KF neuronal fibers.

We are prone to believe, according to in our previous researches,60–63 that cigarette smoke absorption may have strongly influenced the OxR-related results, here observed. In fact, all the 20 SIDS infants with negative OxR expression in the KF area had a smoker mother, and, half of these, a smoker father, too.

A correlation between maternal smoking and hypoplasia of the arcuate nucleus has been also observed in five SIDS cases, all with negative expression of the OxR1 in the KFN. Kinney and her group have documented in many studies64–66 the key role exerted by the arcuate nucleus as integrative site for chemosensitivity, ventilation, autonomic function and arousal, together with the involvement of developmental abnormalities of this nucleus in SIDS.

Even if to date the pathological mechanisms of SIDS still remain unclear, both intra-uterine and post-birth exposure to cigarette smoke could affect the brainstem development and, above all, the Ox-expression, increasing the risk of a dysfunctional arousal threshold and, hence, of death during sleep.

The effects of nicotine on the expression of the orexin and its receptors have been previously demonstrated in animal models.67,68 Kane et al.68, in particular, have shown a large decrease in the affinity and number of Ox receptors in rat hypothalamus, due to continuous nicotine treatment, impairing the binding capacity of Ox to its receptors in brain. Our results do not prove the effective presence of
nicotine in the brainstem and its direct role in the KFN dysfunctions. However, the high incidence of smoking mothers associated to the positivity of the hair nicotine test lets us suppose the absorption of nicotine into the infant bloodstream and the easy crossing, given its high liposolubility, of the blood–brain barrier. Here, nicotine can exert its toxic effects on different neuronal structures, including the Ox receptors.

Future experimental studies will be needed to specifically examine how the Ox system can interact with the KFN during the arousal process, both in physiological conditions and under stress stimuli, such as cigarette smoke exposure.

At the conclusion of this study, we wondered how our research, besides contributing to understand the pathogenic mechanism of SIDS, could be applied in clinical care in the attempt to decrease the incidence of this syndrome. A hint arises from the awareness that the Ox-A can be detectable in the human bloodstream by radiomunouassay and that decreased plasmatic levels of this neuropetide have been found in patients with sleep severe disorders, as the obstructive sleep apnea syndrome (OSAS).69 Therefore, a low concentration of plasma Ox may be indicative of decreased Ox secretion from the specific hypothalamic neurons.

Thus, the detection of haematric Ox level below the normal values could represent a new biomarker, characterizing vulnerable infants with possible arousal dysfunction, susceptible to SIDS. The conduction of specific screening tests after birth could identify infants likely predisposed to failure of sleep-to-wake transition, and allows to take necessary measures to prevent SIDS, such as a treatment with nasal ‘continuous positive airway pressure (CPAP)’, an intervention of first choice in OSAS, very effective with nasal ‘continuous positive airway pressure (CPAP)’, necessary measures to prevent SIDS, such as a treatment to failure of sleep-to-wake transition, and allows to take necessary measures to prevent SIDS, such as a treatment with nasal ‘continuous positive airway pressure (CPAP)’, an intervention of first choice in OSAS, very effective in normalizing the sleep–wake cycle.70 The CPAP treatment is already applied at birth in premature infants with life-threatening anatomic and physiologic immaturities of the respiratory system to attenuate this pathophysiology.71

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Disclosure statement

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