NEW APPROACHES TO THE PATHOGENESIS OF SUDDEN INTRAUTERINE UNEXPLAINED DEATH AND SUDDEN INFANT DEATH SYNDROME

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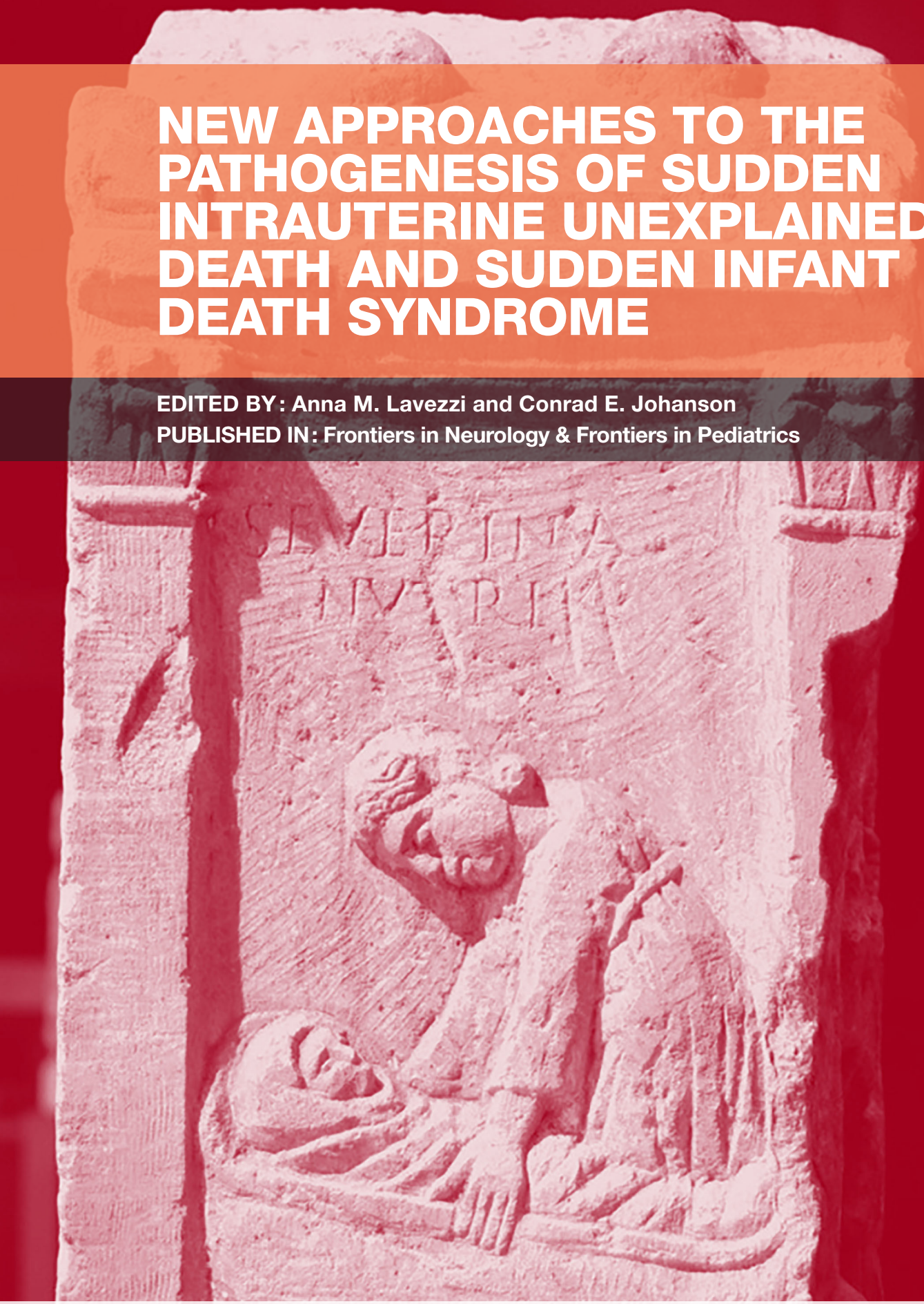

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NEW APPROACHES TO THE PATHOGENESIS OF SUDDEN INTRAUTERINE UNEXPLAINED DEATH AND SUDDEN INFANT DEATH SYNDROME

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Severina Nutrix

Roman tomb epigraph commemorating a possible victim of "crib death" dedicated to the mythical Severina Nutrix (3rd century A.D.; Köln, Römisch-Germanisches Museum, inv. 122,1)

Sudden Infant Death Syndrome (SIDS) is the leading cause of death among infants in the first year of age. The more known definition of SIDS is the sudden unexpected death of an infant less than 1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history.

Despite the success of the "Back to Sleep" campaigns to reduce the risks introduced worldwide, the frequency of SIDS (striking one infant every 750-1,000 live births) has not significantly declined in the last years. Sudden Intrauterine Unexplained Death Syndrome (SIUDS), referring to fetuses that die unexpectedly, particularly in the last weeks of gestation, without any cause even after a complete autopsy, including examination of the placental disk, umbilical cord and fetal membranes, has a six-eightfold greater incidence than that of SIDS.

Even if the pathogenetic mechanism of these deaths has not yet been determined, the

neuropathology seems to be a consistent substrate in both SIUDS and SIDS. Subtle common developmental abnormalities of brainstem nuclei checking the vital functions have been highlighted, frequently related to environmental risk factors, such as cigarette smoke, air and water pollution, pesticides, food contamination, etc. Exogenous toxic factors can in fact interact in complex ways with the genetic constitution of the infant leading to polymorphisms and/or mutations of specific genes (as polymorphisms of the serotonin transporter gene 5-HTT, the regulator of the synaptic serotonin concentration, and of the PHOX2B, the key gene in the Congenital Central Hypoventilation Syndrome). These interactions can directly injure the development of the autonomic nervous system, frequently resulting in hypoplasia of the vital brainstem centers, and consequently in sudden death.

It is very important to continue studying these syndromes and in particular identify all possible congenital alterations and their correlation with the exposure to environmental risk factors, in order to reduce their incidence and mitigate the surrounding social concern.

The goal of this research topic is to propose new approaches to explain the pathogenesis of both SIUDS and SIDS and consequently new prevention strategies to decrease the incidence of these unexpected and very devastating events for families.

Expert authors in the Topic field are encouraged to submit original research articles aimed to widen the current knowledge on the pathological substrates of these deaths, also considering the correlations with possible risk factors. Submissions of hypotheses, opinions and commentaries are also welcome. This Research Topic would lead to development of targeted risk-lowering strategies to reduce the incidence of both SIUDS and SIDS. Furthermore, the adoption of appropriate preventive measures could also lead to improve the quality of life in adults, promoting active and healthy aging.

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EDITORIAL
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Editorial: New Approaches to the Pathogenesis of Sudden Intrauterine Unexplained Death and Sudden Infant Death Syndrome

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Keywords: sudden infant death syndrome, fetal death, neuropathology, hypotheses, guidelines

Editorial on the Research Topic

New Approaches to the Pathogenesis of Sudden Intrauterine Unexplained Death and Sudden Infant Death Syndrome

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Sudden Infant Death Syndrome (SIDS) is the leading cause of death among infants in the first year of age in the developed world. The better known definition of SIDS is the sudden unexpected death of an infant less than 1 year of age, with onset of the fatal episode apparently occurring during sleep, which remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history (1). Despite the success of the "Back to Sleep" campaigns to reduce the risks encountered worldwide, the frequency of SIDS (striking one infant in every 750–1,000 live births) has not significantly declined in recent years (2).

Sudden Intrauterine Unexplained Death, referring to fetuses that die unexpectedly, particularly in the last weeks of gestation without any cause even after a complete autopsy, including examination of the placental disk, umbilical cord, and membranes, has a sixfold to eightfold greater incidence than that of SIDS (3). This death should be considered as a syndrome and referred to with the acronym "SIUDS," "Sudden Intrauterine Unexplained Death Syndrome," like "SIDS" (4). This definition is based on the observation that several conditions correlated with each other and occurring together may contribute to fetal death.

At this moment, the pathogenetic mechanism of these deaths has not yet been determined, even if the neuropathology seems to be a consistent substrate in both SIDS and SIUDS (5, 6). Subtle common developmental abnormalities of brainstem nuclei checking the vital functions have been highlighted, frequently related to environmental risk factors, such as cigarette smoke, air and water pollution, food contamination, etc. Exogenous toxic factors can also interact in complex ways with the infant genetic constitution, leading to polymorphisms and/or mutations of specific genes (such as polymorphisms of the serotonin transporter gene 5-HTT, the regulator of the synaptic serotonin concentration, and of the PHOX2B, the key gene in the Congenital Central Hypoventilation Syndrome) (7).

It is very important to stimulate research in this field to identify all the pathologic aspects and their correlation with exposure to environmental risk factors, in order to reduce the incidence of both SIUDS and SIDS, and mitigate the surrounding social concerns.

Therefore, we have launched this special Research Topic to collect innovative, still not yet considered approaches highlighting new possible pathological substrates, useful for planning specific

prevention strategies to decrease the incidence of these unexpected and very devastating events for families and clinicians. Furthermore, the adoption of these preventive measures could also improve the quality of life in adults, promoting active and healthy aging.

In order to summarize the contributions, we have grouped the articles in two main sections.

(1) Original hypotheses

New original interpretations of the pathogenetic mechanism leading to SIDS are proposed, assigning important causative roles to different issues. They are:

(a) *SIDS–critical diaphragm failure (CDF) hypothesis*. The basic premise of this hypothesis is that the diaphragm is a vital organ that must continuously generate adequate force to maintain ventilation. Consequently, a CDF, caused by different combinations of factors, can be the terminal event leading to SIDS (Siren).

(b) *Microbiome–Gut–Brain axis hypothesis*. This hypothesis is based on the consideration that the infant's gut microbiome, with its metabolites, is able to stimulate the afferent gut vagal endings and consequently to modulate the brainstem 5-HT level (the so-called "microbiome–gut–brain axis"). A decreased release of serotonin, concomitant with critical periods of gut flora development and infant vulnerability, could play a significant role in SIDS (Praveen and Praveen).

(c) *"Wear and Tear" hypothesis*. This hypothesis proposes that SIDS is the result of cumulative painful, stressful, or traumatic exposures that begin *in utero* and, after birth, lead to allostatic overload with consequent impairment of the vital regulatory systems (Elhaik).

(d) *Acute respiratory infection and anemia hypothesis*. Through the use of probability models, an association between an occult prodromal respiratory infection and unmeasured asymptomatic anemia, is proposed as a possible cause of SIDS. The maternal iron-deficiency anemia *in utero*, in particular, plays a causative role in severe physiological anemia associated to delayed neurological development *ex utero* (Mage et al.).

(e) *Fetal reflex awake hypothesis*. During the first months of life, in hypoxic conditions, a particularly vulnerable infant can unexpectedly awaken an ancestral fetal behavior aimed to suspend respiration to save energy, as happens *in utero*, when breathing is not an essential activity. This mechanism represents a protective reflex in the womb but rapidly leads to a fatal outcome in postnatal life (Lavezzi).

(2) Guidelines

(a) Indispensable procedures for the examination of the young victims are recommended, above an in-depth histopathological analysis of the cardiac conduction system and autonomic nervous system performed by specialized pathologists (Alfonsi and Crippa; Ottaviani).

(b) The neuropathological study must be focused particularly to specific nuclei and structures of the brainstem checking the vital functions and whose development is frequently compromised in SIUDS and SIDS (Mehboob et al.).

(c) Care, in the context of the neuropathological examination, should be given even to the cerebellar cortex, with particular attention to Purkinje cells. Developmental loss of these cells, in fact, seems to be involved in reduced compensatory response to the increased body burden of CO_2 (Calton et al.).

(d) Application of immunohistochemical techniques is advocated to highlight the specific involvement of the catecholamine and trigeminal systems in SIDS. Alterations of the immunoreactivity for tyrosine hydroxylase in the dorsal vagal nucleus and ventrolateral reticular formation in the medulla oblongata, and for the neuromodulator Substance P and its receptor Neurokinin 1 in the spinal trigeminal nucleus, may lead to disturbed autonomic regulation and/or respiratory control, so providing a possible explanation of SIDS (Hayashi and Sakuma; Mehboob).

(e) Among the *risk factors* for sudden perinatal deaths, worthy of note is the proposal to systematically search, by means of toxicological analysis of brain samples concomitant with the autopsy, the endocrine disruptors (EDs), a wide group of organic chemicals able to persist in the environment for a long time due to their degradation resistance (Roncati et al.). EDs are able to interfere with the endocrine system, so affecting important biological processes, especially when exposure occurs during early life stages (8).

Our hope, in conclusion, is that all these contributions can be useful to expand the current knowledge on SIUDS and SIDS, thus allowing the broadening of the diagnostic criteria and preventive strategies.

As editors of this Research Topic, we would like to express our sincere gratitude to all of the authors who accepted the invitation to participate and for their significant efforts for identifying very interesting approaches to explain the pathogenesis of these pathologies.

We would like also to thank the reviewers, for their significant comments, useful for the improvement of the submitted articles. Finally, we thank Frontiers and, in particular, its Editorial Office for their competent and essential support.

AUTHOR CONTRIBUTIONS

AL and CJ are the editors of the research topic "New approaches to the pathogenesis of sudden intrauterine unexplained death and sudden infant death syndrome." Together they wrote the editorial that summarizes all the contents of the twelve contributions.

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PERSPECTIVE
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SIDS–CDF Hypothesis Revisited: Cause vs. Contributing Factors

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The sudden infant death syndrome (SIDS)–critical diaphragm failure (CDF) hypothesis was first published by Siren and Siren in 2011 (1). Since its publication, the hypothesis has continued to generate interest and several colleagues have contributed perspectives and insights to it (2–5). The basic premise of the hypothesis is that the diaphragm is a vital organ that must continuously generate adequate force to maintain ventilation, and that CDF is a terminal event and the cause of death in SIDS. I have argued in two follow-up articles that all SIDS factors either increase the workload of the respiratory muscles, the diaphragm being the primary muscle affected, or reduce its force generating capacity (6, 7). The SIDS–CDF hypothesis posits that SIDS has many contributing factors but only one cause, namely, the failure of the vital respiratory pump. There are several known SIDS factors, such as the prone sleeping position, non-lethal infections, deep sleep, gestational prematurity, low birth weight, cigarette smoke, male gender, and altitude, but of these, some such as the prone sleeping position more significantly both impact diaphragm function and correlate with SIDS. However, SIDS cases are multifactorial and as such can be caused by different combinations of factors. An infection combined with a prone sleeping position and elevated room temperature could lead to SIDS, whereas in other circumstances, low birth weight, cigarette smoke, prone sleeping position, and altitude could result in CDF and SIDS. The SIDS–CDF hypothesis also posits that SIDS does not have a congenital or genetic origin, and that efforts to identify significant genetic anomalies in SIDS victims are unlikely to be successful (8–11).

Keywords: SIDS, diaphragm, respiratory failure, hyperthermia, magnesium deficiency

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This short review has two purposes. The first is to examine two SIDS risk factors in the context of the SIDS–CDF hypothesis that have not been addressed in previous articles, namely, magnesium deficiency and hyperthermia. Second, this review is an attempt to raise awareness of the research community to the possible role of the vital respiratory muscles in etiology of the syndrome. As I reiterated in a recent article, the possibility that SIDS is caused by critical weakness of the diaphragm has been largely ignored. It is telling that as of November 2016, out of approximately 11,000 SIDS articles in PubMed, only 50 articles contained the search words "SIDS and diaphragm," and only a few of those actually address the role of the vital respiratory muscle in SIDS. At the end of the commentary, I will suggest avenues for future research.

In 1972, Joan Caddell advanced a hypothesis in *The Lancet* arguing that magnesium deficiency is the cause of death in SIDS (12). Between 1972 and 2001, Caddell and others attempted to provide experimental evidence between magnesium deficiency and SIDS, but the hypothesis remains neither proven nor disproven (13). Systemic magnesium levels are notoriously difficult to measure accurately, and studies on magnesium deficiency in SIDS victims are inconclusive (14, 15). A causal mechanism

was never established, although magnesium deficiency shock and compromised thermoregulation were proposed as possible culprits (16, 17). However, Caddell's hypothesis prompted several interesting studies. She asserted that ethnic groups with low SIDS rates (at or below 1.2 per 1,000 live births) have rich dietary sources of magnesium, while those with SIDS rates exceeding 5.0 typically have magnesium poor diets (17), and while the evidence for this is circumstantial, there are two other population level studies that warrant our closer attention. Following the publication of Caddell's hypothesis, Swift and Emery suggested that "the best way to test Caddell's hypothesis would be to attempt a correlation of the incidence of unexpected death to areas where there is a deprivation of magnesium in the water-supply" (18). Two studies, conducted some 30 years apart in USA and Taiwan, do exactly that. Despite the different population base and geography, the studies reach strikingly similar conclusions about the relationship between magnesium in municipal drinking water and the incidence of SIDS.

The first study was published in *The Lancet* in 1973 and was based on data from the California State Department of Public Health that provided ranges of magnesium and calcium concentrations in county water supplies. The authors concluded that "the median maximum magnesium concentration is lower in counties with higher rates of S.U.D. [sudden unexpected infant death]." The authors note that the study has several limitations such as the counties having large ranges for magnesium and the strong negative correlation of magnesium and calcium concentrations to overall infant mortality (19). By itself, the study provides interesting but insufficient data to suggest that magnesium levels in municipal water affect SIDS rates.

However, a similar, but far more robust, study was conducted in Taiwan in 2005, which reached similar conclusions. The study by Chiu and colleagues used data from the Taiwan Water Supply Corporation and mapped all SIDS death (501 cases) from 1988 to 1997 to controls who died from other causes (20). The mean magnesium concentration in municipal water was 9.69 mg/l for SIDS cases and 11.46 for controls. The authors note: "the group with the highest magnesium levels (>14.1 mg/l) had an OR [odds ratio] which remained significantly less than 1.0 (0.70, 95% CI = 0.51–0.97). In addition, there was a significant trend toward a decreased SIDS risk with increasing magnesium levels in drinking water (X^2 for linear trend = 12.83, $p < 0.05$)." The authors conclude that there seems to be a significant protective effect of magnesium intake from drinking water against SIDS. They further observe that: "the fact that a significantly protective effect of magnesium intake *via* drinking water was found in the group with the highest levels of intake suggested that only subjects with magnesium intake *via* drinking water above a certain level receive a beneficial effect on their risk of SIDS." The authors also address the question of how the relatively small intake on magnesium from drinking water can significantly affect the amount of magnesium in the body and point to research on magnesium absorption from drinking water that support this hypothesis (21). Any study of this nature has limitations, but due to the sophisticated health care and administrative system in Taiwan and the rigorous categorization of causes of death, the authors argue that these have been appropriately mitigated. The same research group has established correlations between magnesium levels in municipal water and the occurrence of various types of cancers, hypertension, and diabetes. No causal mechanism for the role of magnesium in SIDS is offered.

The studies by Chiu and his team and the earlier work by Godwin and Brown provide enough robust data to warrant an explanation, and I will address how the SIDS–CDF hypothesis would explain the data. The hypothesis posits that SIDS is caused by factors that either increase the respiratory workload of the diaphragm or decrease its force generating capacity. It is well known that magnesium deficiency can cause significant muscle weakness in humans (22, 23), and as Caddell points out, "reduced muscle power is a major clinical finding in magnesium deficient children" (24). Dhingra and colleagues showed a direct correlation between hypomagnesemia and respiratory muscle function in humans (25), and Stendig-Lindberg and colleagues demonstrated that maximum isometric voluntary contraction force was significantly weaker in hypomagnesaemic subjects (26, 27). These and other recent studies (28) show that magnesium deficiency directly and significantly impacts muscle strength in general and respiratory muscle strength specifically. As Matias and colleagues explain, magnesium deficiency has a significant effect on muscle performance, probably due to the key role of magnesium in energetic metabolism, transmembrane transport, muscle contraction, and on the cellular level on Na-K ATPase, Na–K–Cl co-transport, K channels, charge screening, and permeability effects on membranes (29). In this context, it is important to note that in terms of contractile properties and fatigue, the diaphragm behaves like other skeletal muscles (30).

At least two studies show that SIDS victims and near-miss infants have abnormalities in muscle tone. In 1976, the results from a large study containing 1,553 infants and 12 unexpected deaths were published. The study used the Graham–Rosenblith Scales behavioral examination to measure muscle strength and co-ordination. Of the 12 likely SIDS cases, all but 1 had no unusual findings on muscle tonicity. The infants displayed marked head lag, poor tonicity, moderate trembling or shaking, arching, and hyperactivity (31). The authors associated this with central nervous system involvement, but it is useful to consider other possible explanations. As Flink notes, important symptoms of magnesium deficiency are "neuromuscular hyperactivity including tremor, myoclonic jerks [and] convulsions" (32). Similarly, Wong and Teh reported on 13 cases of convulsions or tremors in children (aged 1 day to 14 months) with hypomagnesemia. The authors note that on recovery, either spontaneously or after administration of magnesium, serum magnesium returned to normal levels, and that it seems likely that hypomagnesemia caused the tremors or convulsions (33). Another SIDS study to consider in this context is the systematic neurologic examination of 41 near-miss infants, 7 normal siblings of SIDS victims, and 21 normal control infants conducted by the Sudden Death Research Project at Stanford University School of Medicine. The study found consistent abnormalities of muscle tone in the near-miss infants who were under 3 months of age (34).

The evidence discussed above helps put the municipal water/magnesium studies in context and suggests that SIDS victims and near-miss infants have compromised muscle function (whether or not associated with hypomagnesemia). This fact by itself is significant in the context of the SIDS–CDF hypothesis.

Magnesium is a central element with multiple roles in human biology, and as Baaij and colleagues point out, "magnesium is an essential ion for health and it plays an important role in the physiological function of the brain, heart, and skeletal muscles" (35). In light of the existing evidence, it would be presumptuous to discuss its potential role in SIDS but tentatively. Still, the two population-level studies and the clinical results from SIDS and near-miss infants provide intriguing data on the potential role of magnesium and muscle weakness in the etiology of SIDS. The critical role of magnesium in muscle function is well established, and compelling data suggest that SIDS victims have abnormal muscle tonicity. Combined with the existing evidence regarding the possible role of respiratory muscles in SIDS, it seems obvious that the role of the diaphragm in SIDS should be rigorously investigated.

Hyperthermia is the other factor that I would like to discuss in the context of the SIDS–CDF hypothesis. Dallas first suggested that overheating might contribute to SIDS in 1974 but presented no evidence (36). Several authors have since argued that hyperthermia is significantly associated with SIDS (37–39). In 1984, Stanton reported that of the 34 SIDS victims: "19 babies were unusually hot or sweating when found dead; 14 died in an unusually warm environment; 17 had evidence of a terminal infective illness; and 24 were excessively clothed or overwrapped … In 6 of 15 babies (40%) whose rectal temperature was recorded after death, the temperature was above 37°C, the highest being 42°C" (40). In 1990, Fleming and colleagues reported that, "overheating and the prone position are *independently* associated with an increased risk of sudden unexpected infant death, particularly in infants aged more than 70 days" (41), and *The Lancet* published an editorial titled *Prone, Hot and Dead* that discussed link between hyperthermia and SIDS the same year. The editors note the pathological findings are non-specific and that no mode of action has been established. Interestingly, they also highlight that researchers have observed that: "environmental temperature of healthy infants increased respiratory movement, a finding that suggests an effect on the respiratory control system." The editorial suggests that a possible mechanism is: "[hyperthermia] together with an additional factor—for example, a viral infection—is the stimulus for a further increase in metabolic rate, with subsequent loss of respiratory control. This view accords with several other observations that symptoms of illness, especially of respiratory infection, are present in many infants who die from SIDS." (42).

However, while there is robust evidence linking hyperthermia to SIDS, no causal mechanism has been established. The elevated temperatures observed in SIDS victims are regularly observed in infants who do not succumb to the syndrome, and importantly, many SIDS victims show no evidence of hyperthermia. Recent research found no significant expression of heat-shock proteins (HSP27 and HSP70), thereby suggesting that hyperthermia is not a primary causal factor in SIDS (43). As with many SIDS risk factors, hyperthermia is a paradox. There is compelling epidemiological and population data to suggest that at least in some cases, hyperthermia is a significant risk factor for SIDS, but at the same time, it is highly unlikely that hyperthermia is the cause of SIDS.

To explain this paradox, it is useful to recall the observation by *The Lancet* editors regarding how higher environmental temperature increases the respiratory movement in healthy infants. Indeed, it has been known since 1905 that hyperthermia increases the ventilatory workload (44). More recently, Maskrey observed that: "high body temperature causes an increased ventilation (chiefly through increased frequency of breathing) *via* a descending drive from thermoreceptors and thermoregulatory integrating centers in the hypothalamus" (45). In a comprehensive recent review, Tsuji and colleagues again show that hyperthermia can induce hyperventilation in humans (46). The authors point out that: "during passive heating at rest, elevation of body core temperature leads to increased ventilation independently of metabolic factors, resulting in a reduction of arterial CO_2 pressure (hypercapnia)." The critical threshold for the initiation of hyperventilation for passive heating at rest is when body core temperature reaches a critical threshold of ~38.5°C. As discussed in the beginning of this review, the SIDS–CDF hypothesis argues that any factor that either increases the respiratory workload or decreases the force generating capacity of the respiratory muscles can contribute to SIDS. Hyperthermia is known to increase the respiratory workload, and combined with other factors such as non-lethal infections and the prone sleeping position, it could, in some circumstances, push the diaphragm over its endurance threshold.

I have reviewed evidence suggesting that magnesium deficiency and hyperthermia are contributing, but not causal, factors in SIDS, and that by themselves, neither magnesium deficiency nor hyperthermia lead to CDF. As noted earlier, both magnesium deficiency and hyperthermia affect multiple physiological functions, and we must be cautious in drawing conclusions about their role in SIDS. Suffice it to say that the SIDS–CDF hypothesis offers a parsimonious causal mechanism for the large body of data on magnesium deficiency, hyperthermia, and SIDS.

A central challenge of SIDS research is that is it very difficult to design experiments that mimic the syndrome. The underlying reason is that experiments by their very nature seek to isolate variables and SIDS is multifactorial. No single factor is "the cause" of SIDS, but many factors can contribute to respiratory muscle failure, often in different configurations. We know that severe respiratory muscle weakness can lead to death in adults; it remains to be determined if SIDS victims succumb to respiratory muscle weakness. To test the SIDS–CDF hypothesis, experiments should focus on the diaphragm and its vulnerabilities, and we should seek to develop experimental protocols to determine if induced and progressive respiratory muscle weakness in experimental animals replicates the pathophysical outcomes of SIDS. We should also seek identify possible molecular markers from the diaphragms of adults who have died of respiratory muscle failure and should actively leverage the extensive research by Supinski and Callahan into the molecular pathways of infection induced

respiratory muscle weakness (47, 48). The respiratory pump is as vital as the heart, but very little experimental research has been conducted on the possible role of the diaphragm in SIDS. The purpose of this article has been, in part, to inspire that research.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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HYPOTHESIS AND THEORY
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Microbiome–Gut–Brain Axis: A Pathway for Improving Brainstem Serotonin Homeostasis and Successful Autoresuscitation in SIDS—A Novel Hypothesis

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Sudden infant death syndrome (SIDS) continues to be a major public health issue. Following its major decline since the "Back to Sleep" campaign, the incidence of SIDS has plateaued, with an annual incidence of about 1,500 SIDS-related deaths in the United States and thousands more throughout the world. The etiology of SIDS, the major cause of postneonatal mortality in the western world, is still poorly understood. Although sleeping in prone position is a major risk factor, SIDS continues to occur even in the supine sleeping position. The triple-risk model of Filiano and Kinney emphasizes the interaction between a susceptible infant during a critical developmental period and stressor/s in the pathogenesis of SIDS. Recent evidence ranges from dysregulated autonomic control to findings of altered neurochemistry, especially the serotonergic system that plays an important role in brainstem cardiorespiratory/thermoregulatory centers. Brainstem serotonin (5-HT) and tryptophan hydroxylase-2 (TPH-2) levels have been shown to be lower in SIDS, supporting the evidence that defects in the medullary serotonergic system play a significant role in SIDS. Pathogenic bacteria and their enterotoxins have been associated with SIDS, although no direct evidence has been established. We present a new hypothesis that the infant's gut microbiome, and/or its metabolites, by its direct effects on the gut enterochromaffin cells, stimulates the afferent gut vagal endings by releasing serotonin (paracrine effect), optimizing autoresuscitation by modulating brainstem 5-HT levels through the microbiome–gut–brain axis, thus playing a significant role in SIDS during the critical period of gut flora development and vulnerability to SIDS. The shared similarities between various risk factors for SIDS and their relationship with the infant gut microbiome support our hypothesis. Comprehensive gut-microbiome studies are required to test our hypothesis.

Keywords: 5-HT, SIDS, gut–brain axis, gut flora, autoresuscitation

INTRODUCTION

Sudden infant death syndrome (SIDS) is defined as a sudden unexplained death in the first year of life in a previously healthy infant, where the cause of death remains unidentified despite thorough investigations including a complete autopsy, death scene investigation, and review of clinical history (1). SIDS is a major cause of postneonatal infant mortality in the western world. In the United States, ~1,500 infants died of SIDS in 2013 alone, despite the steady reduction (1994–2000) in such deaths since the "Back to Sleep" campaign. The incidence of SIDS has remained fairly constant in the last decade, while the rate of other causes of ill-defined, unspecified, and sudden unexpected infant deaths has increased (1, 2). Some infant deaths, which would have been classified as SIDS in the past, are now being classified as resulting from suffocation and asphyxia. The significant reduction in SIDS rate in the past 20 years may be related to increasing diagnoses of other causes of death (1). Japan and the Netherlands have the lowest SIDS rates, at 0.09 and 0.1 per 1,000 live births, respectively, whereas New Zealand has the highest reported SIDS rate (0.8 per 1,000 live births) (3–6). The United States and UK have SIDS rates of 0.57 and 0.41 per 1,000 live births, respectively (7, 8). Prone sleeping position, a significant SIDS risk factor, cannot be easily associated with the other epidemiological risk factors related to SIDS (9).

Current Hypotheses for SIDS

Sudden infant death syndrome is a condition without a widely accepted singular pathological mechanism.

(1) *Triple-risk model*: this model proposes that SIDS occurs when external stressors simultaneously act upon on a susceptible infant with a vulnerable homeostatic system during a critical developmental period (10).
(2) *Failed autoresuscitation*: animal studies have shown that cardiorespiratory, sleep, and arousal mechanisms are abnormal following exposure to risk factors associated with SIDS or in infants who later succumb to SIDS (11, 12). Although the exact cause of SIDS is unknown, immaturity of brain stem autonomic cardiorespiratory/thermoregulatory control and failure of autoresuscitation during sleep are significant determinants of survival (11, 12). A leading SIDS hypothesis states that a structural/neurochemical brainstem abnormality results in failure of autoresuscitation following exposure to a stressor during a critical developmental period (13, 14). SIDS vulnerability is specific to failed autoresuscitation from an adverse autonomic event (AAE). The initial self-initiated gasp during such an event is dependent of optimal serotonin homeostasis in the brain, which is undermined in SIDS. Imbalance in serotonin homeostasis alters sleep rhythm, thus increasing the chances of AAE (15).
(3) *Medullary serotonergic network deficiency*: SIDS is associated with multiple serotonergic defects including serotonin deficiency (16–19). It has been associated with reduced serotonin in the ventral medulla, pointing to a brainstem-based autonomic dysfunction affecting sleep/arousal/cardiorespiratory reflexes (20–23). Gene polymorphisms related to serotonergic autonomic system may play a role in SIDS (24). In a recent study in neonatal rodents, loss of brain stem 5HT may explain the cardiovascular collapse during apparent severe hypoxic event in some SIDS cases (25). Recent neuropathology studies in SIDS implicate defective neurotransmitter function in the medullary arcuate nucleus, receptor immaturity of the "respiratory center" nucleus tractus solitarius (NTS), and defective function of the serotonergic raphé nuclei of the ponto-medullary ventral median septum and other brainstem serotonergic neurons (26). Abnormalities of the dorsal motor nucleus of the vagus (DMNV) have been associated with SIDS (27). In a significant number of SIDS infants, cerebellar dentate nucleus lesions may represent a developmental susceptibility leading to autonomic cardiorespiratory/arousal dysfunction and sleep-related death when exposed to homeostatic stressors (28). Cummings et al. report that, in addition to respiratory and cardiac dysfunction in normoxemic conditions, neonatal mice with reduced (by 60–70%) brainstem serotonergic neurons from early embryogenesis onward (Pet-$1^{-/-}$) have major defects in autoresuscitation, a life-preserving process utilized by neonatal mammals in severely hypoxic conditions (29–33).
(4) *Neurotransmitters*: neurotransmitter systems (e.g., cholinergic and GABA-ergic) have been shown to be involved in SIDS (34, 35). Reduced muscarinic cholinergic binding in the medullary arcuate nucleus (involved in cardiorespiratory control) has been shown to occur in SIDS (34). GABA neurons in the medulla help regulate homeostasis through interactions with the medullary serotonergic system (35). Significant decrease in GABA A receptor binding was found in the medullary serotonergic system in SIDS cases associated with 5-HT defects (35).

NEW HYPOTHESIS

We propose a new hypothesis that the infant gut microbiome plays an important role in SIDS during the period critical to both gut flora maturation/development and vulnerability to SIDS, by modulating brainstem serotonergic system through the bidirectional microbiome–gut–brain axis, thus tilting the balance in favor of successful autoresuscitation during a sleep-related AAE. The components of our hypothesis, though individually and separately studied in the past, have never been put together as the structure of a SIDS hypothesis. The factors protective against as well as the risk factors of SIDS show some compelling circumstantial evidence of their effects on gut microbiome leading to beneficial and dysbiotic infant gut flora, respectively, with corresponding effects on brainstem serotonergic system. The plausibility of such an SIDS hypothesis would open up a new paradigm for preventative and therapeutic approaches in SIDS. Our hypothesis is the only one till date, which connects the protective/risk factors of SIDS with infant gut flora, their effect *via* microbiome–gut–brain axis on brainstem serotonergic system, and subsequent successful autoresuscitation (**Figure 1**).

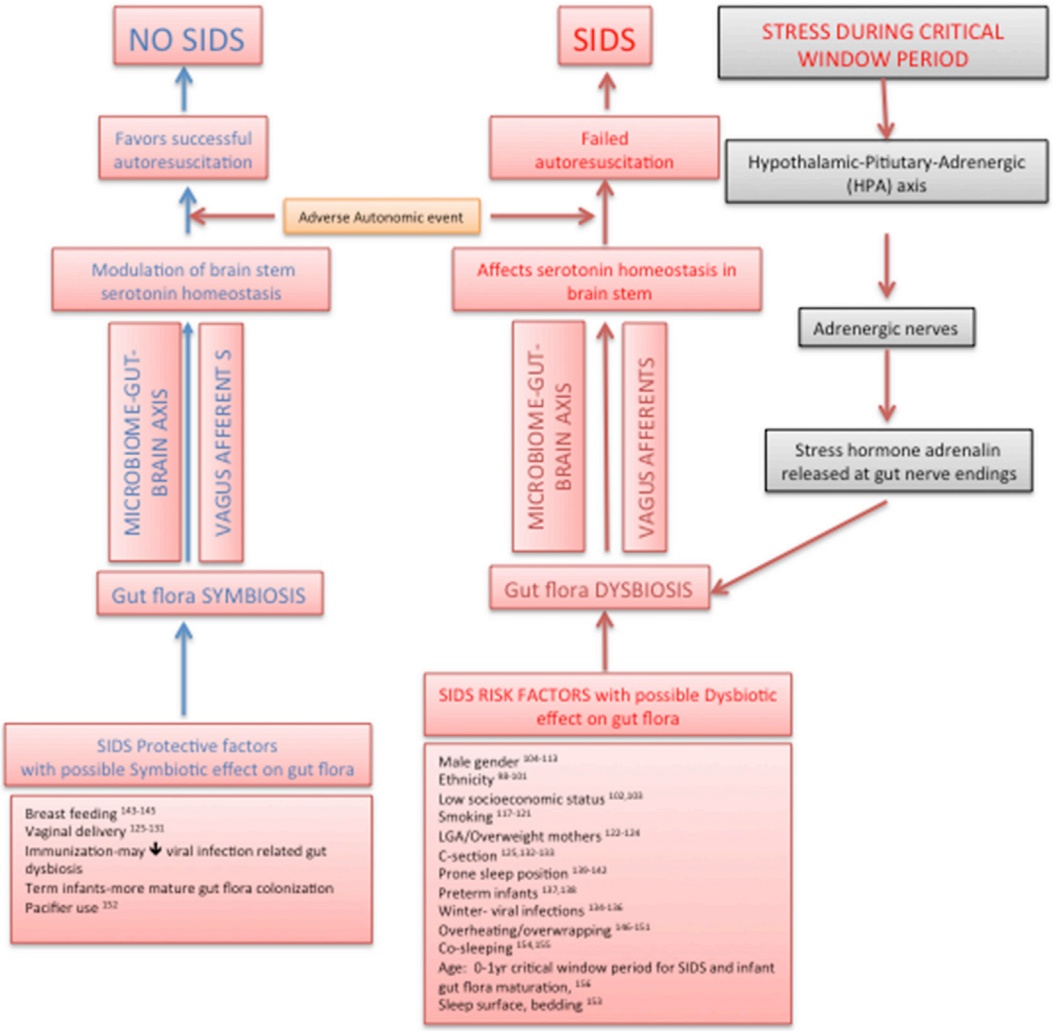

FIGURE 1 | New sudden infant death syndrome hypothesis pathway: effect on infant gut flora on brainstem serotonin homeostasis and autoresuscitation *via* microbiome–gut–brain axis.

Microbiome–Gut–Brain Axis

The human adult gut has about 10^{14} microorganisms, 10-fold the human cells and 150-fold the amount of human DNA (36, 37). The human gut microbiome comprises more than 1,000 species, predominantly obligate anaerobes, and includes viruses, protozoa, archaea, and fungi (36–39). Gastrointestinal homeostasis has a significant role in the human general health and well-being (39–41). The concept of the brain–gut axis involves the complex bidirectional homeostatic neuronal communication through the vagus nerve that exists between the central nervous system (CNS) and the enteric nervous system (ENS) (42). Current research studies the mechanism of such communication along this axis and its relationship to normal homeostasis and disease states (42–48). The basic skeleton of the microbiome–gut–brain axis includes gut microbiome, the CNS, neuroendocrine and neuroimmune systems, ENS, sympathetic, and parasympathetic arms of the autonomic nervous system (49). The gut accounts for 95% of the body's serotonin content. The detailed structure, integration, and functioning of the various components of the above axis have been reviewed extensively elsewhere (45).

Gut microbial colonization also plays a major role in the postnatal development of the endocrine and immune systems, which in turn support CNS function, particularly the developing serotonergic system (41, 42, 50–52). Neurotransmitters, neurohormones, and receptors are ubiquitous in nature, e.g., catecholamines corticotrophin, somatostatin, and GABA derived from bacteria (53–56). Evolutionally speaking, bacteria preceded humans in developing neurotransmitters and recognizing them (57–59). The ontogeny of neurochemicals in mammals has been postulated to arise as a consequence of bacterial lateral gene transfer (60). Thus, the gut microbiota might have played an important role in the evolution of neurodevelopment (61).

Gut Vagal Afferents and the Medullary Serotonergic System

Sudden infant death syndrome is associated with multiple serotonergic defects including serotonin deficiency and DMV abnormalities (15–18, 62). We briefly review the vagal afferents, brainstem respiratory neurons, and the medullary serotonergic system.

Vagal afferents outnumber vagal efferents by 10:1, which are sensitive to the paracrine effects of the enterochromaffin cells (ECC), relay through nodose and dorsal root ganglia before synapsing with second-order neurons in the spinal cord, which in turn project into the brainstem. The brainstem has major respiratory neurons concentrated into three recognizable groups comprised of four major nuclei. These include the following: (1) dorsal respiratory group (DRG) centered in the NTS; (2) ventral respiratory group that encompasses the nucleus ambiguous and the nucleus retroambigualis; (3) pre-Botzinger complex (pre-BotC) which contains putative pacemaker neurons; and (4) BotC located in and near the nucleus retrofacialis. The DRG neurons through the phrenic neurons in the cervical spinal cord control the diaphragm.

The medullary serotonergic system projects to brainstem cardiorespiratory nuclei (including the DMNV), cerebellum, and spinal cord, thus modulating cardiorespiratory protective reflexes, central chemoprotective reflexes, arousal/sleep cycles, thermoregulatory reflexes, and maintenance of upper airway patency (63).

Vagal afferents affect respiratory control as shown by altered respiratory pattern after stimulation of visceral vagal afferents in guinea pigs which died within a few hours of bilateral vagotomy; their frequency of breathing significantly decreased within minutes of the procedure (64, 65). Serotonin may regulate developmental brainstem neuronal apoptosis with its pro- or antiapoptotic effects as a result of the receptor sub-family activated (66). Animal studies have shown that the highest density of 5-HT3 receptors are found within the afferent vagal fibers of dorsal vagal complex (67, 68) and vagotomy was found to significantly reduce receptor density (69–71). Stimulation of the NTS 5-HT3 receptors leads to elevation of blood pressure and inhibition of the chemoreceptor-mediated bradycardia and the Bezold–Jarisch reflexes. As an example of sensory neural plasticity, recent rat studies have shown that glucose in the intestinal tract probably induces serotonin release from neuroendocrine cells, which activates 5HT3 on vagal afferent terminals and transmitted centrally (72–79).

Gut Microbiome Affects the Brainstem

There is emerging evidence from animal and clinical studies on the role of gut microbiome in CNS signaling.

Animal Studies

Evidence from rodent studies indicates that the gut microbiome can affect neural development, chemistry, and behaviors, e.g., emotion, pain perception, and stress responses. As rodent gut colonization pattern is similar to humans, they are subjects of choice for gut microbiome studies. CNS tryptophan concentrations are dependent on peripheral content, which suggests that gut flora might play a part in regulating peripheral and central serotonin synthesis (44, 80, 81). TPH2 is responsible for the synthesis of serotonin in brainstem raphe nuclei, which is the origin of most central serotonergic projections (82). Probiotics have been shown to modulate serotonin—a critical central neurotransmitter through multiple strain-specific mechanisms (83). Lyte et al. proposed a "delivery system" by which gut flora can communicate neurochemical messages to the brain. Gut bacteria produce and react to the same neurotransmitters (e.g., serotonin, norepinephrine, dopamine, and GABA) that play a role centrally in modulation of mood (84). Animal studies studying effects of probiotics on CNS function have been extensively and systematically reviewed elsewhere (85). In addition, we have listed few rodent studies looking at the role of pathogenic bacteria and vagus on CNS neurochemistry and behavior (52, 86–92) (**Table 1**).

Clinical Studies

Emerging evidence from clinical studies in autism indicates a relationship between gut flora and cognitive function. Researchers have reported gut flora dysbiosis with increases in *Clostridium* spp. in autism (93). A probiotic mixture of *Lactobacillus helveticus* and *Bifidobacterium longum* for a month has been reported to decrease anxiety and depression in healthy human (94). Other adult human clinical studies looking at probiotic effects on neurobehavior have been systematically reviewed elsewhere (85).

Brain Affects Gut Microbiome

Stress induces gut permeability, which allows bacteria/bacterial antigen translocation across the epithelial barrier, thereby activating immune response and resulting in changes in the gut microbiome characteristics (95). Psychological stressors have been reported to modulate infant gut microbiome (47). Prenatal stressors have been reported to cause dysbiosis by decreasing gut Bifidobacteria and Lactobacilli in infant rhesus monkeys (96). In rodent studies, the stress of maternal separation significantly decreased stool lactobacilli on the third day, which returned to baseline by day 7 following separation (97). Stressors acting on an at-risk infant during the critical window period could affect the favorable nature of infant gut flora and consequently affect the brainstem neurotransmitters through bidirectional communication and/or gut barrier function locally.

Based on the evidence from the experimental and clinical studies discussed above, we propose that an optimal (diversity, complexity, and colony counts) gut flora interacts with ECC and modulates (possibly by its serotonin and other paracrine effects) through the afferent vagal endings to the brain stem medullary serotonergic cardiorespiratory centers in infants at risk for SIDS. Recent research in microbiome–gut–brain axis supports role of probiotics to modulate central brain neurochemistry, thus opening up a site for therapeutic targeting for central brain disorders.

Shared Risk Factors for SIDS and Gut Dysbiosis

In the following section, we report how each of the protective as well as risk factors for SIDS seems to offer evidence of promoting symbiotic (favorable) and dysbiotic gut (non-favorable) flora,

TABLE 1 | Animal studies showing effect of gut microbiome/probiotics on the central nervous system (CNS).

Reference	Study characteristics
1. Sudo et al. (86)	Participants: mice study, *in vivo*. Germ-free (GF) at 9 weeks of age Intervention: stress protocol Controls: specific pathogen-free (SPF) BALBc mice, gnotobiotic mice Primary outcome: plasma ACTH, corticosterone levels, fecal microflora analysis, plasma cytokine assays Conclusion: plasma ACTH and corticosterone responses of GF mice were more susceptible to stress than those of SPF mice. Gut flora regulates the development of the HPA stress response
2. Bravo et al. (87)	Participants: adult male BALB/c mice, *in vivo* ($n = 36$) Intervention: *Lactobacillus rhamnosus* 10^9 cfu gavaged for 28 days Control: control broth Type of probiotic: *L. rhamnosus* (JB-1) Primary outcome: corticosterone level, behavioral analysis, GABA B1b mRNA expression in hippocampus, amygdala, and locus coeruleus Conclusion: *L. rhamnosus* supplementation reduced corticosterone response to stress and modulated the GABAergic system in mice. Vagotomized mice did not show the neurochemical effects of this bacterium
3. Desbonnet et al. (88)	Participants: adult Sprauge-Dawley rats ($n = 20$) Intervention: *Bifidobacterium infantis* 35624 gavaged for 14 days ($n = 12$) Controls: $n = 8$ Type of probiotic: *B. infantis* 35624 Primary outcome: corticosterone level, tryptophan and IFN-g, TNF-alpha and IL-6, brain monoamines analysis Conclusion: attenuation of pro-inflammatory immune responses and the elevation of the serotonergic precursor, tryptophan, in probiotic-treated group
4. Alenina et al. (89)	Participants: *Tph2*-deficient (*Tph2*$^{-/-}$) mice, *in vivo* study Intervention: gene targeting leading to mice with absent TPH2, $n = 4$ Type of probiotic: none Controls: $n = 6$ Primary outcome: serotonin in the brain of Tph2$^{-/-}$ mice Conclusion: the lack of central serotonin in these mice leads to impaired early postnatal growth and altered autonomic control of sleep, thermoregulation, and cardiorespiratory reflexes
5. Lyte et al. (84)	Participants: 9-week-old CF-1 male mice, *in vivo* study Intervention: in an animal model of IBD, infection with *Citrobacter rodentium*, to determine whether the infection could lead to anxiety-like behavior Controls: saline Type of probiotic: none Primary outcome: tested for anxiety-like behavior measurement, immune cytokine analysis, and colon for histological analysis Conclusion: *C. rodentium* infection could induce anxiety-like symptoms that are likely mediated *via* vagus
6. Gareau et al. (90)	Participants: mouse *in vivo* study Intervention: behavior was assessed following infection with the non-invasive enteric pathogen, *C. rodentium* in both C57BL/6 mice and GF Swiss-Webster mice Primary outcome: whether daily treatment with probiotics normalized behavior was assessed Conclusion: memory dysfunction occurred in infected mice exposed to acute stress, while in the GF setting, memory was altered at baseline
7. McVey Neufeld et al. (91)	Participants: mouse *ex vivo* study Intervention: segments of jejunum from 8- to 12-week old GF, SPF, and CONV-GF mice dissected to expose myenteric plexus. Intracellular recordings by impaling cells with sharp microelectrodes Type of probiotic: none Primary outcome: action potential shapes, firing thresholds, the number of APs fired at 2× threshold, and passive membrane characteristics were measured Conclusion: commensal intestinal microbiota are essential for normal excitability of gut sensory neurons. When the vagus nerve is severed, effects of gut bacteria on brain biochemistry, stress response, and behavior disappear
8. Heijtz et al. (92)	Participants: mouse *in vivo* study GF versus SPF mice with normal microbiological gut flora Intervention: motor activity and anxiety-like behavior measured Conclusion: unstressed GF mice were more active and willing to explore exposed areas of a maze than mice that had normal gut microbiota. Transplanting normal gut bacteria into the GF mice erased those behavioral differences only in early life, suggesting that there is a critical window for gut bacteria to establish normal patterns of behavior
9. Clarke et al. (52)	Participants: male GF animals compared with conventionally colonized control animals Intervention: measurement of 5-HT in hippocampus Male GF animals have a sex-specific significant elevation in hippocampal 5-HT and 5-HIAA compared with conventionally colonized control animals. Concentrations of tryptophan, the precursor of serotonin, are increased in the plasma of male GF animals, suggesting a humoral route through which the microbiota can influence CNS serotonergic neurotransmission Conclusion: microbiome–gut–brain axis in early life modulate hippocampal serotonin levels in a gender-dependent manner

respectively, during the critical window when both SIDS tends to occur and early infant gut colonization is being established.

Demographic Factors

(1) *Ethnicity*: studies in indigenous populations have reported a higher SIDS rate compared to the non-indigenous groups within the same countries (98). These differences may reflect differences in maternal smoking, which could affect frequency and density of colonization of infants by potentially pathogenic bacteria and induction/control of inflammatory responses (98). Maternal cigarette smoking and/or alcohol consumption may contribute to abnormal fetal medullary

5-HT development in Native American SIDS infants (99). A recent study has reported diet-related differences in gut flora composition between African-Americans and native Africans. African-Americans had higher levels of 7-α-dehydroxylating bacteria and lower levels of *Lactobacillus plantarum* (which produce methane and is protective against dysbiosis) (100, 101).

(2) *Low socioeconomic status*: SIDS has been associated with lower socioeconomic groups (102). Fecal lactobacilli numbers have been related to socioeconomic status (103). Gut flora differences related to diet, smoking status, and access to health services could be a proxy for lower socioeconomic status.

(3) *Gender*: SIDS shows a male preponderance. Animal studies have shown gender differences in the regulation of serotonergic system (104, 105). Estrogen has been implicated in the modulation of hippocampal serotoninergic system (106, 107). Gender influences gut microbiome (108–111) through unclear mechanisms (110) including hormone–microbe interactions (111, 112) and gender-specific immune responses (113).

(4) *Genetic control*: genes regulating serotonergic network, brain function and development, and cardiac function play an important role in SIDS (114). Studying the role of genetics on gut microbiome is important in understanding the pathogenesis of bacterial diseases (115, 116).

Prenatal Risk Factors

(1) *Maternal smoking*: SIDS is five times more common in infants born to mothers who smoked during pregnancy and three times more common in those exposed postnatally to smoking (117, 118). Cigarette smoke exposure and prone sleep position is associated with decreased 5HT1A receptors in the DMNV of SIDS infants (119). A reduction in 5HT1A receptors has been reported in the DMNV of piglets subjected to intermittent hypercapnic hypoxia and nicotine exposure (120). A recent study showed that cessation of smoking improved gut microbial diversity (121). Smoking may play a role in SIDS through its effects on infant gut flora and brainstem serotonin homeostasis.

(2) *Being overweight*: overweight infants and mothers have a higher risk of SIDS (122). Obese human adults had less *Bacteroides* and more *Firmicutes* in their gut flora compared with lean controls (123). A recent review looked at maternal obesity-related pro-inflammatory state and its effect on maternal and *in utero* fetal gut microbiome and development (124).

(3) *Delivery route*: infants delivered by cesarean section have an increased risk of SIDS than those born by vaginal route (125). The mode of delivery has a significant effect on newborn gut flora development (126–128). The gut flora in infants born by cesarean may be altered till 6 months following delivery (129). Prolonged duration of labor during vaginal birth increases the chances of isolation of viable microbes from the stomach and mouth of the infant (130, 131). In addition to exposure to maternal flora, infants born by cesarean section acquire gut flora from their exposure to the immediate environment (132). Aseptic precautions in obstetrics and neonatal units may result in dysbiosis of the infant gut microbiome (133).

Postnatal Risks

(1) *Season*: SIDS is more common during winter months (134). There is an association of a viral infection in the days preceding SIDS (135). Stressors such as viral infections during winter may cause dysbiosis in infants (136). Such dysbiosis could play a role in successful autoresuscitation *via* microbiome–gut–brainstem pathway.

(2) *Low birth weight*: the rate of SIDS is higher in low birth weight infants (137). This may be related to the gut colonization patterns of very low birth weight (VLBW) infants compared with normal weight infants. In an elegant study, the initial gut colonization by *Enterobacteria* and *Streptococci* was similar in both VLBW and full-term infants; however, both microorganisms predominated for a longer period of time and the establishment of *Bifidobacterium*, *Bacteroides*, *Clostridium*, and *Lactobacillus* was delayed in VLBW infants (138).

(3) *Prone sleep position*: prone sleeping position has been the most important risk factor associated with SIDS (139). In addition to decreased arousal response related to prone sleeping, body temperature seems to be slightly elevated in prone infants (140, 141). Prone sleep position has been associated with *Staphylococcus aureus* gut colonization in SIDS. The increased risk of ingestion/inhalation of bacteria contaminating the sleeping surface during prone position, with resultant gut dysbiosis, could account for the increased risk of SIDS in such infants (22).

(4) *Breastfeeding*: breastfeeding has been shown to be protective against SIDS (142, 143). Breast milk oligosaccharides when fermented by gut flora to form fatty acids results in modulation of infant gut flora. Breast-fed infants show predominant proliferation of *Bifidobacteria* and *Lactobacilli*, whereas formula-fed infants show more *Enterococci* and *Enterobacteria* in their gut flora. In addition, infants who are breast-fed exclusively have been reported to have better sleep arousal patterns than formula-fed infants (144).

(5) *Elevated or reduced room temperature*: overheating of infants has been reported with an elevated risk of SIDS (2, 145). Animal studies have showed that the presence of certain gut flora elevates body temperature of mice and rats. Conn et al. demonstrated that Gram-positive organisms are a major source of the stimulatory effect of gut flora on normal body temperature in mice (146). Body temperature has been shown to have effects on the intestinal flora of hibernating squirrels (147, 148). Oral antibiotics have been shown to reduce nighttime body temperature in rabbits as a result of their effect on their native intestinal flora (149). These studies may help in understanding whether the increased body temperature as a risk factor for SIDS could be a result of aberrant gut flora or vice versa. Elevated body temperature associated with

prone sleep position may also play a role in affecting gut flora composition (140, 141).

(6) *Pacifier use*: pacifier sucking has been shown to be strongly associated with the oral colonization of salivary lactobacilli (150). Thus, pacifier use might play a role in favorable oral and subsequently gut flora in infants.

(7) *Sleep surface, bedding, and stuffed toys*: apart from mechanical suffocation and overheating issues, these may act as fomites contributing to the infant gut flora. Sherburn et al. showed that simulated infant head movements and mattress-related factors affect aerial release of bacteria from beds (151).

(8) *Co-sleeping*: recent meta-analyses showed that bed sharing during sleep increases the risk of SIDS, which is further increased when combined with parental smoking, maternal alcohol consumption, and/or drug use (152). The results of a Swedish study suggest that parental skin *S. aureus* establish readily in the infant's gut, perhaps due to poor competition from other gut bacteria (153). The possible role of acquiring abnormal gut flora from parents/caregivers skin/gut flora by prolonged close contact during bed sharing needs to be investigated further.

(9) *Infant's age*: SIDS incidence peaks around 2–4 months of the infant's age, and subsequently decreases by 1 year. Infant gut flora develops through a period of instability in the early months of infancy and reaches more mature adult-like microbiome by 1 year of age, the time by which SIDS disappears. Another condition, not fully explained, affecting the infant during a typical window period is infant colic, in which aberrant gut flora has been recently shown to play a role, amenable to probiotics. Infantile colic is associated with a greater extent with near-miss SIDS infants than among control infants, thereby hypothesizing that colic might play a role as a protective arousal mechanism in such infants (154). From infection/immunity standpoint, this is the time when maternal antibodies reach their nadir making infants more susceptible to infections, including from indigenous pathogenic gut flora. Introduction of supplementary foods around 6 months of age leads to more gut microbial diversity.

(10) *Gestation at birth*: prematurity is associated with a fourfold increased risk of SIDS (137) as well as a dysbiotic intestinal flora, and impaired gut mucosal barrier function and permeability (155–161). *Lactobacillus GG* has been shown to decrease the frequency of *Escherichia coli* K1A translocation in a neonatal rabbit model (162, 163). Extremely preterm newborns (<28 weeks) have a 5- to 10-fold higher incidence of microbial infections than term newborn (164). The preterm neonatal gut colonization is different from that in the healthy, full-term infant gut. Preterm neonates requiring intensive care are colonized by organisms such as Bifidobacteria only gradually and in a delayed fashion. Schwiertz et al. reported similar bacterial colonization patterns in preterm infants in contrast to breast-fed, full-term infants. Bacterial colonization has been observed to be similar in different preterm neonates irrespective of birth weight, feeding regime, and antibiotic therapy. The initial colonization of the newborn GI tract is highly dependent on the environment, and cross-transmission of bacteria is a serious problem in the hospital (165).

(11) *Small for gestational age (SGA)*: it has been hypothesized that SGA infants may have a higher incidence of SIDS as a result of fetal hypoxia-induced decrease in brain serotonergic receptors (16, 166–169).

CONCLUSION

We have provided a new SIDS hypothesis whereby the right composition of gut flora in the early critical stage of infant development could possibly optimize or modulate serotonin homeostasis in the serotonergic cardiorespiratory/thermoregulatory brain stem nuclei by a direct communication *via* the vagal afferents as part of the microbiome–gut–brain axis. This may tip the balance in favor of a successful autoresuscitation response to an AAE during sleep. Investigating the role of infant microbiome using newer culture-independent techniques as well as the developmental physiology and neuropathology associated with SIDS may provide more specific strategies than those available currently to define the at risk population. As Hippocrates once stated "All diseases begin in the gut," research on the gut flora in at risk infants would open new avenues for identifying potential biomarkers and strategies for prevention (e.g., maternal and/or early postnatal probiotic/synbiotic supplementation, diet changes) of SIDS (170).

AUTHOR CONTRIBUTIONS

VP was involved in concept and manuscript preparation. SP was involved in editing of the final manuscript.

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HYPOTHESIS AND THEORY
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A "Wear and Tear" Hypothesis to Explain Sudden Infant Death Syndrome

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Sudden infant death syndrome (SIDS) is the leading cause of death among USA infants under 1 year of age accounting for ~2,700 deaths per year. Although formally SIDS dates back at least 2,000 years and was even mentioned in the Hebrew Bible (Kings 3:19), its etiology remains unexplained prompting the CDC to initiate a sudden unexpected infant death case registry in 2010. Due to their total dependence, the ability of the infant to allostatically regulate stressors and stress responses shaped by genetic and environmental factors is severely constrained. We propose that SIDS is the result of cumulative painful, stressful, or traumatic exposures that begin *in utero* and tax neonatal regulatory systems incompatible with allostasis. We also identify several putative biochemical mechanisms involved in SIDS. We argue that the important characteristics of SIDS, namely male predominance (60:40), the significantly different SIDS rate among USA Hispanics (80% lower) compared to whites, 50% of cases occurring between 7.6 and 17.6 weeks after birth with only 10% after 24.7 weeks, and seasonal variation with most cases occurring during winter, are all associated with common environmental stressors, such as neonatal circumcision and seasonal illnesses. We predict that neonatal circumcision is associated with hypersensitivity to pain and decreased heart rate variability, which increase the risk for SIDS. We also predict that neonatal male circumcision will account for the SIDS gender bias and that groups that practice high male circumcision rates, such as USA whites, will have higher SIDS rates compared to groups with lower circumcision rates. SIDS rates will also be higher in USA states where Medicaid covers circumcision and lower among people that do not practice neonatal circumcision and/or cannot afford to pay for circumcision. We last predict that winter-born premature infants who are circumcised will be at higher risk of SIDS compared to infants who experienced fewer nociceptive exposures. All these predictions are testable experimentally using animal models or cohort studies in humans. Our hypothesis provides new insights into novel risk factors for SIDS that can reduce its risk by modifying current infant care practices to reduce nociceptive exposures.

Keywords: sudden infant death syndrome, allostatic load, neonatal circumcision, trauma, pain, stress

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BACKGROUND

The Etiology of Sudden Infant Death Syndrome

Sudden infant death syndrome (SIDS) (9ICD 798.0; 10ICD R95), "crib death," or "cot death" was first coined in 1953 and by 2004 was defined as: "the sudden unexpected death of an infant under 1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained

after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history" (1). SIDS classification spurred the development of diverse approaches to determine the cause of death in order to exclude deaths due to accidental or non-accidental injuries, suffocation and strangulation, or medical causes (2, 3). Despite the publication of ~11,000 SIDS-related articles (4), of which over 100 SIDS studies appearing in *Medical Hypotheses*, biomarkers are still unavailable (5) and SIDS remains the leading cause of death for infants between 1 month and 1 year in western countries (accounting for ~2,700 deaths per year in 2010 in USA) (6). In developed countries, the 2005 SIDS rates (birth to 1 year) ranged from 0.16 (Japan) to 0.54 (United States) per 1000 live births (7). Using non-Hispanic whites as a reference point (1), SIDS rates are 1.41 per 1000 live births in non-Hispanic blacks and 0.46 in Hispanics (8). In the absence of a proven intervention even if prospective identification was feasible, SIDS is one of the most frequent worries for parents.

Although prone sleeping campaigns, such as "Back to Sleep" reduced SIDS and postneonatal mortality rates in the 1990s (9), their effect was uneven in different countries (10). Subsequent to 1999, the plateau, or slower decline in SIDS rates, has been associated with a diagnostic shift from classification of SIDS deaths (11, 12) to a concurrent increase in rates of other categories of sleep associated sudden and unexpected infant deaths attributed to accidental suffocation and strangulation in bed or "unknown" causes (13), questioning the magnitude of the actual decrease in SIDS deaths (14, 15). While no confirmed SIDS biomarkers exist [e.g., Ref. (16, 17)] nor it is possible to differentiate accidental asphyxia from SIDS (18), a number of modifiable risk factors have been associated with SIDS, including prone sleep position, parental smoking during and after pregnancy, alcohol consumption by caregivers, overheating, preterm infants, infant head covering by soft bedding, bed sharing, and upper respiratory tract infection (19–21). Decreasing in these risk factors was effective in reducing the mortality rates over the past two decades (22), though not necessarily SIDS, which remains distinct from known mortalities and its main characteristics – male predominance (60:40 male:female USA ratio), significantly lower SIDS rates in USA Hispanics compared with whites, infants aged 2–4 months being at greatest risk of SIDS with most SIDS-related deaths occurring by 6 months, and seasonal variation with most cases occurring during winter (8, 23) – remain largely unexplained. Here, we propose an allostasis-based model to explain SIDS and argue that it explains SIDS's main characteristics (**Table 1**).

The Allostasis Model

The allostasis model assigns a central role to the brain as the organ of stress and adaptation in enabling efficient regulation of the internal milieu (24). Facing low- or high-level toxic and pathologic stressors, the brain attempts to adapt via neuroendocrine and autonomic signals and through the synaptic plasticity facilitated by multiple epigenetic mechanisms during early development (25–27). The cumulative effect of stressors on the brain and body for either potentially protective or pathologic responses is termed *allostatic load* or *overload*, respectively (27). An allostatic overload occurs when allostatic responses that allow neurophysiologic stress systems to function at critical periods of the developing nervous system are maladaptive for future environmental stressors. The physiological cost of increased allostatic load has also been dubbed a "wear and tear" process (28) and encompasses both the prenatal and postnatal periods (29).

We postulate that while low-level stress can stimulate adaptation, prolonged and repetitive iatrogenic stressful, painful, or traumatic experiences during prenatal, perinatal, neonatal, and postneonatal development constitute allostatic overload and are risk factors for SIDS. Due to their total dependence, the infant's ability to allostatically regulate exposure to stressors is severely constrained (30), which increases their vulnerability to disease and premature death (26) (**Figures 1** and **2**). Due to their difficulties in maintaining homeostasis and inability to escape/avoid iatrogenic or non-medically nociceptive exposure, infants are vulnerable to toxic stress with preterm infants being the most vulnerable (31).

Stress Experienced *in utero*

Infants begin to experience various stresses *in utero* due to maternal smoking or drug abuse (19), caffeine consumption (32), maternal inflammation, and traumatic injuries that account toward allostatic load. Interestingly, several authors have shown sex-dependent changes in pain responsiveness at maturity in animals having experienced repeated neonatal pain *in utero* and infancy. For example, Mueller and Bale (33) showed that stress experienced early in pregnancy created maladaptive behavioral stress responsivity, anhedonia, and an increased sensitivity to selective serotonin reuptake inhibitor treatment. Provided the lower expression of DNMT1, the enzyme responsible for methylation maintenance in male placentas compared to females, the authors reasoned that females are able to circumvent the effects of stress by strengthening the maintenance of methylation during

TABLE 1 | The main characteristic of SIDS explained by the allostatic load hypothesis.

SIDS characteristic	Allostatic load hypothesis
Male predominance (60:40)	Females are more resilient to nociceptive stimuli than males, which reduces their allostasis compared to males. Male circumcision further increases male allostasis.
SIDS rate in the USA vary between Hispanics and whites	Circumcision rates among Hispanics are much lower than in non-Hispanic white.
Mortality peaks between 2 and 4 months	Infants lose the protection of maternally acquired antibodies at 2–4 months of age. The wound healing procedure from circumcision may increase susceptibility to infection peaks during the same period. Preterm infants have also decreased heart rate variability at that period (corrected age).
Seasonal variation with most cases occurring during winter	Waning of maternal antibody levels and/or low levels of acquired immunity followed by recent infection and inflammation during a developmental period in the infant increase the allostatic load.

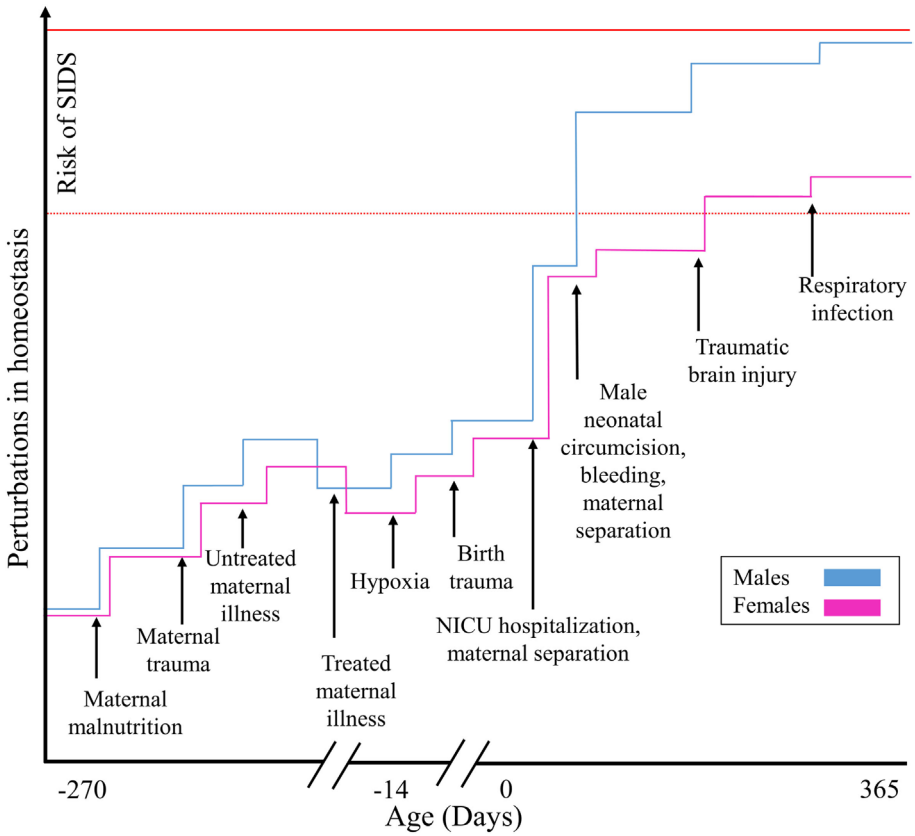

FIGURE 1 | Illustrating how SIDS is explained by the allostatic load model for males and females. Cumulative stressful, painful, or traumatic stimuli contribute additively toward an increased risk of SIDS.

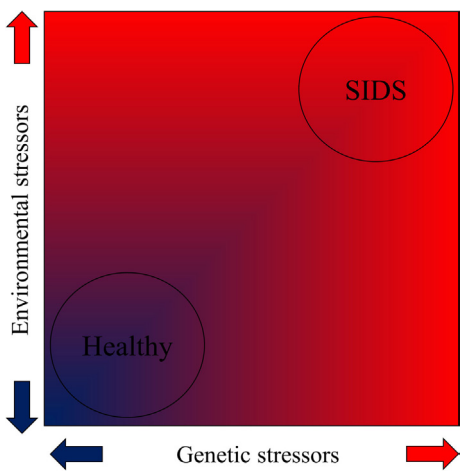

FIGURE 2 | The contribution of genetic and environmental stressors toward SIDS according to the allostatic load model as demonstrated by a heat map (red indicates high stress).

perturbations. Page et al. (34) reported a higher mechanical sensitivity in male rats in response to neonatal-paw-needle-stick, whereas females showed increased sensitivity to inflammatory stimuli. Such female "protective effects" may be attributed to hypothalamic–pituitary–adrenal (HPA) axis hormones known to be elevated in females both at baseline and in response to stress (35). Indeed, Paterson et al. (36) reported that SIDS cases had a significantly larger deficiency in serotonin 5-HT_{1A} receptors compared with controls [$\mu = 53.9$ ($\sigma = 19.8$)] and that male SIDS cases had significantly lower receptor counts [16.2 (4.8)] compared with female SIDS cases [29.6 (16.5)].

Preterm Births

It is known that the postnatal age for SIDS and other deaths of unknown causes decreases as gestational age (GA) at birth progresses. What remains unknown is why preterm infants remain at increased risk for sudden infant death despite the dramatic drop in rates since the 1990s and whether the increased risk across all categories of sudden infant death suggest a common mechanism (37). We speculate that preterm infants are at an increased risk due to their immature organ systems and the multiple stressors they experience during hospitalization and later on.

Preterm infants (24–32 weeks), physiologically unprepared for the stress outside the protective intrauterine environment, are hospitalized for lengthy periods where they are exposed to multiple stressors, including extended exposure to light, noise, acute and chronic illness, maternal separation, invasive procedures,

handling, multiple medications, endotracheal intubation, repeated blood tests, insertion of peripheral lines, and surgery (38, 39). Preterm infants who experienced at least 40 days of intensive care have increased brain neuronal responses to noxious stimuli compared to healthy newborns at the same age (40). The cumulative effect of these early repetitive pain/adverse experiences on perinatal brain plasticity contributes to the observed neurodevelopmental and behavioral abnormalities associated with early life stress and allostasis (40–42).

Preterm infants are also at higher risk of hypoxia than term infants. Following hypoxia, infants maintain adequate tissue oxygenation through perfusion by prompting an increase in heart rate to maintain cardiac output. The heart is relatively insensitive to hypoxia in infancy, as opposed to hypercapnia, which rises rapidly during asphyxia and leads to adrenal and catecholamine release that increases heart rate and blood pressure (43). Infants who succumb to life-threatening cyanosis/asphyxia have been observed to breathe vigorously/gasp but without an increase in heart rate, suggesting an abnormal/impaired tachycardia response to hypercapnia despite ventilatory efforts (43). An impaired baroreflex sensitivity can result in a failure to control perfusion pressure. Very preterm infants have impaired and delayed maturation of baroreflex sensitivity (44) and decreased heart rate variability at peak SIDS age of 2–3 months (corrected age) consistent with delayed maturation of parasympathetic innervation regardless of sleeping position (45). Cerebral autoregulation is also impaired in preterm infants with decreased cerebral oxygenation in the prone position and increased variability in cerebral oxygenation with head up tilt (orthostatic challenge) indicating greater risk of cerebral hypoxia and immature cerebrovascular control in preterm infants over the first 6 months (46).

Non-Urgent Pediatric Surgeries

Concerns that pediatric surgeries requiring general anesthesia are toxic stressors that elicit long-term deficits in cognitive and learning behavior have been the subject of recent reviews [e.g., Ref. (47, 48)]. The 2014 Food and Drug Administration (FDA) Science Board reviewing anesthetic neurotoxicity stated that the data "are sufficient to conclude that adverse effects noted in juvenile animals are reasonably expected to occur in developing humans" (49). The FDA Board recommended avoiding non-urgent surgical procedures in children younger than 3 years of age (48). In addition to general anesthesia, the risk of local anesthetic neurotoxicity, due to lack of adequate studies of safety and effectiveness in the developing infant, has recently been highlighted (50).

The surgery itself may elicit neural injury in neonates (51). The significantly increased risk of death or neurodevelopmental impairment in very low birth weight newborns following major or minor surgery that does not require a general anesthetic (52) is consistent with nociceptive exposure, pain, and overall neurotoxic risk (47, 53, 54). The American Academy of Pediatrics (AAP) and the Canadian Pediatric Society (CPS) have recognized the neurotoxic risk of pain in their joint policy statement urging the avoidance, prevention, and possible elimination of pain even during routine minor procedures to protect the developing brain (55). Therefore, the safest course of action is avoidance of non-medical and non-urgent surgeries, as recommended by the FDA Science Board, as well as avoidance of iatrogenic procedural pain, as recommended by AAP–CPS, to protect the developing brain in infants. While pediatric surgeries are typically rare and cannot account for the high rates of SIDS, particularly if the cause of death is ascribed to the surgery, we suspect that a specific type of voluntary painful surgery concealed under the cloak of routine hospital practices account for the high volume of SIDS rates.

Neonatal Circumcision

Circumcision is one of the most common elective surgical procedures in the world and is performed primarily on males (56). Female circumcision is practiced in nearly 30 African countries, some Southeast Asian and Middle Eastern countries, and in immigrant communities in Europe and North America (57). Despite its relevancy, female neonatal circumcision will not be discussed here since in most western countries it is illegal and thereby under-reported and we lack SIDS data for the remaining countries. In North America, ~1.2 million male infants are circumcised every year (58) often within the first 2 days of life (59). Although not requiring general anesthesia, circumcision is an intensively painful procedure requiring adequate analgesia (60). Circumcision is associated with intra-operative and postoperative risks, including bleeding, shock, sepsis, circulatory shock, and hemorrhage (61–63) that can result in death (63, 64).

Infant deaths following religious neonatal circumcision have been known for at least two millennia (65). Talmud (the central text of Rabbinic Judaism) sages ruled in the first centuries A.D. that mothers with two children who have died following the surgery should receive an exemption from circumcising their infants. During the nineteenth century, developments in medical knowledge on one hand and the rise of Jewish "Enlightenment" on the other hand, brought many Jews to reject the authority of the Talmud and with that the practice of circumcision. A new wave of accusations toward Jewish circumcisers (mohels) and rabbis of infant deaths following circumcision soon appeared and prompted community leaders to appeal to the governing authorities to forbid this practice – efforts that were countered by rabbis' threats to ban the admission of uncircumcised Jewish children from Jewish schools. The fierce arguments about the necessity of the procedure last to this day and led many Jews to opt their infants out of the procedure, including Theodor Herzl, one of the fathers of modern political Zionism (66). In the UK, Gairdner (67) estimated an annual rate of 16 per 100,000 circumcision-associated deaths for boys under 1-year old in a study that influenced the British government to exclude circumcision coverage from the National Health Service. Remarkably, the SIDS rates in the UK (0.38 per 1000) are much lower than in the USA (0.55 per 1000) (10) where most male infants are circumcised (58). Moreover, most of the deaths in the USA occur in non-Hispanic blacks (83% higher death rate compared with non-Hispanic whites). SIDS rates were 44% lower for Hispanics compared with non-Hispanic whites (68). Interestingly the circumcision rates among Hispanics are about half that of the two other groups (69).

Circumcision contributes to the rise in allostatic load and increased risk for SIDS through multiple conduits. Circumcision produces crush and incisional injuries during amputation,

resulting in damage to normal prepuce tissue, the associated nerves, and blood vessels. Wound healing manifested by hyperaemia and swelling at day 7 postoperative is observed in 70% of infants with minimally retractile prepuces seen in infants circumcised before 1 year of age with subsequent bacterial carriage of skin commensals (70). Circumcised males have increased pain responses to childhood immunization 4–6 months post-surgery (71, 72) consistent with central sensitization (73). The abnormal development of sensory pathways in the developing nervous system elicited by the pain during critical postnatal periods is manifested in later life following nociceptive reexposure by abnormal sensory thresholds and pain responses that are not restricted to the original site of postnatal trauma (74–76). Neonatal nociceptive exposure induces long-term hypoalgesia or hyperalgesia depending on the nature and timing of the trauma (54, 77) and is consistent with surgery and pain adversely impacting neurodevelopment independent of anesthetic (76).

Post-circumcision, tactile hypersensitivity increases due to post-surgical and -traumatic mechanisms that contribute toward allostasis and the risk of SIDS. This is evident by the increase in toll-like receptor 4 (78) associated with post-circumcision wound healing, which is also observed in post-surgical tactile hypersensitivity in males and dependent on testosterone (79). Following peripheral nerve injury, the purinergic receptors in the spinal cord microglial cells release BDNF (79) and mitogen-activated protein kinase p38 (80) that contribute to neuropathic pain and tactile hypersensitivity. Due to their testosterone dependency, they are seen only in males (79). The testosterone surge occurring during the first 2- to 4-month period may increase susceptibility to the initial stages of infection and is consistent with the peak in SIDS mortality (81).

Male neonates subjected to circumcision can experience severe cardiorespiratory pain responses, including cyanosis, apnea, increased heart rate (82), and increased pitch (fundamental frequency) of cry (as high as 800–2000 Hz) associated with decreased heart rate variability, i.e., decreased vagotonia (83–85), a likely risk factor for SIDS. Other circumcision sequelae of sufficient severity to require emergency room evaluation or hospital admission and contribute toward allostasis include infection, urinary retention, inflammatory redness and swelling ascribed to healing (86, 87), and amputation/necrosis of the glans (88). Behavioral abnormalities, such as eating disturbance and disturbed sleep, are also the consequence of pain exposure (89).

Postoperative circumcision pain of ample severity to require analgesia is expected for about 10 days for healing with incomplete wound healing past day 14 seen in up to 6% of infants depends on the device used to amputate the foreskin (88), which is also associated with various adverse events (56, 90). The overall complication rate for circumcision ranges from 0.2 to 10% with many USA physicians performing the procedure without formal training, being unaware of contraindications, and incapable of handling post-op complications (56, 91, 92). Lower complication rates for early and late adverse events have been attributed to underreporting with late adverse events mistakenly not attributed to circumcision (92, 93). Consequently, the low number ascribed to circumcision as the cause of death (63) may be underreported and erroneously attributed to other causes, such as sepsis (94) or SIDS.

One mechanism by which circumcision may elicit SIDS concerns the inhibition of nerves involved in nociception processing that produces prolonged apnea while impairing cortical arousal. Neonatal surgery that traumatizes peripheral nerves with associated tactile hypersensitivity followed by a subsequent surgery later in development can increase spinal cord microglia signaling and elicit persistent hyperalgesia (80). It can also produce post-surgical hyperalgesia that subsequently alters postnatal development of the rostral rostroventral medulla (RVM), which controls the excitability of spinal neurons by spinally projecting neurons from the nucleus paragigantocellularis lateralis (PGCL) and the nucleus raphe magnus. Alterations in the RVM result in a descending inhibition of spinal reflex excitability on nociception (95). Inhibition of RVM neurons was shown to limit the duration of the laryngeal chemoreflex and produce prolonged apnea that contributes toward SIDS, particularly when combined with stimuli that inhibit respiration (96). In SIDS, norepinephrine, which depresses respiration, is increased in the PGCL and serotonin 5-HT_{1A} receptor that mediates nociceptive stimuli in the brainstem (97) and decreased in the raphe nuclei and the arcuate nuclei (98). The reduction in 5-HT_{1A} receptors observed in the brainstem of SIDS infants prompts the hypothesis that SIDS is caused by a brainstem abnormality that impairs the ability to generate protective responses to life-threatening challenges (99, 100). This hypothesis, however, does not explain why SIDS peaks at 2–4 months, rather than in an earlier GA (101). Orexin is another important regulator of both pain and sleep dysfunction. Orexin knockout mice presented greater degree of hyperalgesia induced by peripheral inflammation and less stress-induced analgesia than wild-type mice (102). In the rostral ventrolateral medulla and PGCL, orexin receptors are expressed in sympathoexcitatory bulbospinal neurons (103). A significantly decreased orexin immunoreactivity in the hypothalamus and pontine nuclei was observed in SIDS infants (104).

Another mechanism that can explain the SIDS toll following circumcision is the loss of ~1–2 ounces (oz) of blood out of a total of ~11 oz that a 3,000 gram male newborn has (105), the equivalent of ~1–2 blood donations in an adult. Excessive bleeding is highly common in circumcision with reports range from 0.1 to 35% (91, 106) in neonates. However, even moderate bleeding puts the infant as risk, and, being an inherent part of the procedure, it is not reported as a complication. Blood loss of 2–2.5 oz, ~15% of the total blood volume at birth, is sufficient to cause hypovolemia and death. Since a large fraction of newborns (26%), particularly premature infants, weigh much less than 3,000 grams (107), a smaller amount of blood loss may trigger hypovolemic shock. Therefore, when bleeding an infant of low birth weight or GA, the effect may be pathological resulting in a reduced blood pressure that has been associated with obstructive sleep apnea (OSA), a condition where the walls of the throat relax and narrow during sleep, interrupting normal breathing (108). It is, therefore, not surprising that most of the deaths following circumcision in high-income countries were due to bleeding (63). While it is accepted that failure of neural

mechanisms causing arousal from sleep may play a role in at least some SIDS cases [e.g., Ref. (109)], it is unclear what causes the initial failure of the respiratory control (110). Comparing the breathing characteristics of 40 infants who eventually died of SIDS with 607 healthy controls, Kato and colleagues reported that SIDS infants have a greater proportion of obstructive and mixed apneic episodes than the control group (111). Although the frequency of these episodes decreased with age, the decrease was smaller in the SIDS infants than in the controls, in support of either immature or impaired respiratory control. Looking at the data by gender, however, shows that only boys exhibit a difference in apnea frequency in support of an impaired respiratory control (111), perhaps due to circumcision.

To date, circumcision in the USA, despite being the most common pediatric surgery, has not been subjected to the same systematic scientific scrutiny looking at immediate and delayed adverse effects, including pain [e.g., Ref. (112)], nor has circumcision status been included as part of a thorough SIDS investigation/registry or analyses [e.g., Ref. (2)] in spite of the male predominance of both neonatal circumcision and SIDS. However, based on assessment of risk of harms versus benefit, despite the latter including decreased risk of urinary tract infection (113), the Royal Australasian College of Physicians, the British Medical Association, the Canadian Paediatric Society (87), and several west European medical societies have recommended against routine neonatal circumcision (114), arguing that the benefits of circumcision to children are minimal, non-existent, or outweighed by the risks, and that circumcision is thereby unwarranted. The AAP's recommendation in favor of this routine (115) has been widely criticized [e.g., Ref. (116)].

Skin-Breaking Procedures

Skin-breaking procedures are a large and diverse group of insults that contribute toward allostasis. Infants subjected to repetitive heel lances, the most common skin-breaking procedure in neonates, have increased pain responses to subsequent skin cleansing and venipuncture (117). Heel lances in newborns elicit nociceptive-specific EEG brain activity associated with reflex withdrawal dependent on the stimulus intensity in the absence of clinical pain, which is difficult to measure (118). Brain imaging of newborn infants demonstrates increased sensitivity to nociceptive stimuli with greater amplitude and duration of reflex withdrawal compared to adults (119). Goksan and colleagues (2015) reported the activation of 18 out of 20 brain areas (including anterior cingulate cortex, bilateral thalamus, all divisions of insular cortex, and primary somatosensory cortex) in infants in response to nociceptive stimuli. Seven-day-old newborns do not have activation of the amygdala, orbitofrontal cortex, or anterior division of the insular cortex, suggesting that reward value/anticipation of future outcomes/emotional significance may not be attached to the nociceptive stimuli at this early postnatal stage of development, although activation of anterior cingulate cortex may suggest perceived unpleasantness (119). Non-specific neuronal bursting activity in response to heel lances is seen on EEG in preterm infants (<35 weeks of GA) while after 35–37 GA somatosensory potentials discriminate touch from nociceptive exposure (120). Exhibiting cortical activation following heel sticks is also associated with increase in heart rate and clinical pain behaviors (117), suggesting that preterm infants have at least the same increased pain response as neonates, with some studies suggesting a higher one (40).

The number of repetitive skin-breaking pain procedures (e.g., heel lance, intramuscular injection, tape removal, and intramuscular injection) in preterm infants (24–32 weeks) without neurodevelopmental, sensorimotor, or severe brain injury is significantly associated with decreased subcortical gray matter and maturation of white matter at term-equivalent age (40 weeks) (121). Overall, it is well established that intense and repetitive pain in early childhood is associated with negative life outcome (31) that contributes toward allostasis and thereby growing risk of SIDS.

Inflammation, Birth Order, and Seasonality

Waning of maternal antibody levels and/or low levels of acquired immunity followed by recent infection and inflammation during a developmental period in the infant may result in a dysregulated inflammatory response, a risk factor for SIDS (122, 123). Approximately one-half of SIDS victims have a slight upper airway infection before death (124) with males reported to have an excess of infant mortality from respiratory deaths as well as SIDS (125). The risk of SIDS also increases with the number of siblings, otherwise known as "birth order" (124), another known SIDS risk factor. Mage and Donner (23) proposed that the higher risk of SIDS with increasing birth order could be due to greater probability of exposure to respiratory viral infection via contact with a sibling. This assumption is reasonable, but it does not explain the excess in male mortality.

The association between respiratory illnesses and seasonality is also elusive. In some countries, the winter seasonal predominance of SIDS has declined or disappeared when the prevalence of infants sleeping in the prone position has decreased, in support of an interaction between sleeping position and factors more common during colder months (e.g., overheating and infection) (19). However, in other countries, SIDS was shown to have a small seasonality component, suggesting that seasonality is a risk factor (23), though it also does not explain why males are at higher risk.

We speculate that the winter seasonal predominance of SIDS is due to the increase in respiratory illnesses among household members that are in contact with the infant (126), particularly older siblings, that contributes toward the infant's allostatic load. Infants lose the protection of maternally acquired antibodies at 2–4 months of age (127), when they become susceptible to upper airway infections. At that time, even otherwise benign upper respiratory infections can augment the laryngeal cough reflex and produce prolonged apnea, an important risk factor for SIDS. This risk may be elevated among infants with an impaired immune system (128) and further elevated among circumcised infants struggling to regulate their allostatic exposures to the recent and new stressors (26) (**Figure 1**).

The Nightly SIDS Cascade

Compared with other infants, those who subsequently succumb to SIDS have higher heart rates, reduced heart rate variations, abnormal QT intervals, increased baseline heart rates, and bradycardias preceding apnea or during ventilatory effort (129). In this section, we highlight some of the critical events in the nightly SIDS cascade.

Depending on the GA, nearly 90% of preterm infants experience intermittent hypoxia or recurrent apneas (130) attributable to central apnea, with obstructed breaths appearing during periods of prolonged central apnea (131). OSAs have been associated with postoperative pain (132, 133), increased norepinephrine and epinephrine levels (134), higher blood pressure (135), and upper airway hypotonia during rapid eye movement (REM) sleep due to decreased genioglossus activity (136). Repetitive apneas have also been associated with decreased arterial oxygen saturation and decreased cerebral oxygenation, which would have gone undiagnosed and thereby contribute toward the SIDS diagnosis (101). Such repetitive apneas may also contribute to higher cerebral oxidative stress, increased sympathetic activity, and hypoxic loss of neurons that alter or become less efficient in adulthood (137). These events are more frequent in the supine position and when the tonsils are swollen which accompany a number of upper respiratory tract infections. Unsurprisingly, infants succumbing to SIDS, who have been monitored, experienced significantly more frequent episodes of obstructive and mixed sleep apnea, especially males (111) during REM sleep (138). SIDS infants experience fewer cortical arousals during REM and non-REM (NREM) sleep, more subcortical activations in REM sleep of longer durations in both REM and NREM sleep, more frequent subcortical activations in the first part of night, and fewer cortical arousals in the early AM hours (138). Polygraphic changes, considered pathological, recorded in the brainstem of SIDS infants include apoptosis, hypoplasia, and gliosis (138).

Unusual breathing patterns and decreased heart rate variability may thereby indicate at-risk infants. Breathing patterns influence cardiovascular response (131, 139) with decreased heart rate variability in the neonate attributable in part to increased sympathetic activity and/or decreased parasympathetic activity with gradually increasing parasympathetic activity over the first year of life (140–142). Infants normally spend about 70% of a 24 h period asleep, and those who subsequently succumb to SIDS demonstrate a decreased heart rate variability due to increased sympathetic activity and/or decreased parasympathetic activity in all sleep phases (active/quiet sleep) (129, 140). Sympathetic activation/catecholamine release in response to hypoxia/hypercapnia is mediated by carotid body glomus cells acutely (143) and by peripheral chemoreceptor organs [e.g., adrenal medulla and oxygen sensing pulmonary neuroendocrine cells (PNEC)] (144). Lungs from infants dying of SIDS have demonstrated hyperplasia of PNEC with reduced myelination of its vagal afferents impairing oxygen sensing (145) as well as increased airway (146) and thick pulmonary arteries in males (147).

Channelopathies can induce cardiac arrhythmias, such as abnormal QT syndromes, catecholaminergic ventricular tachycardia, and prolonged QT interval associated with intracellular acidosis (148). We note that SIDS diagnosis may not be given to infants that suffer lethal arrhythmias or diagnosed with channelopathies, however channelopathies can inflict sudden unexpected death during infancy via a lethal ventricular arrhythmia without leaving a trace of structural evidence detectable during postmortem examination, in which case they are candidate suspects in the etiology of SIDS (149). Increasing QT interval is noted during early development up to four postnatal months followed by decreasing ECG's QT and PR intervals during childhood (150). Prolonged QT has been long associated with SIDS (151) and is a risk factor for ventricular arryhthmia with 50% of infants succumbing to SIDS observed to have prolonged QT interval during the first week of life (150). Hypoxia interferes with hemichannel function and maturation of the cardiac conduction system, which increases the risk of arrhythmogenic death (150). In this respect, mutations in genes associated with development of the cardiac conduction system (152) and cardiomyopathy (153) have been implied in SIDS, although the extent to which they contribute to SIDS remains under debate. While reports have estimated that up to 15% of SIDS were related to specific genetic variants [e.g., Ref. (154)], a recent study that sequenced the full exons of 64 genes associated with sudden death in the largest known cohort (351) of infant and young sudden death decedents reported that less than 4% of unexpected deaths were associated with a pathogenic genetic variant (155). These results suggest that many pathogenic variants involved in SIDS are unknown or, most likely, that pathogenic variants play a minute role is SIDS.

The Significance of the Allostatic Load Model for SIDS

Sudden infant death syndrome occurs when an infant dies suddenly, unexpectedly, and without a cause identified through a forensic autopsy or death-scene investigation. We speculate that SIDS is caused by prolonged and repetitive iatrogenic stressful, painful, or traumatic experiences during critical development stages that constitute allostatic overload (156). Over the past years, allostatic load models were proposed to explain several leading medical conditions, including mental health disorders (157, 158), preterm birth (159), and chronic stress (160).

While the infant's first environment is typically romanticized as peaceful, painless, hygienic, safe, and harmless, in practicality it may be anything but that. Already in the uterus, the fetus may be exposed to maternal substance use (e.g., smoking and drug use) associated with SIDS (19, 161). During a prolonged hospitalization in the Neonatal Intensive Care Unit that follows a preterm birth, infants may be exposed to extended and repeated pain, which thier unstable and immature physiological systems are unable to offset and will potentially render them more vulnerable to the effects of repeated invasive procedures (38). Neonatal circumcision typically involves maternal separation, pain, bleeding, and shock and, like any operation, puts the infant at risks of hemorrhage and sepsis even when anesthetic is used (67). The long-term consequences of circumcision include, among else, greater pain response to routine immunizations within the few months past birth (72). During winter time, the infant is at risk of infection and illnesses that grows with the number of household members, particularly older children (126), which explains why

an elevated immune response is one of the hallmarks of SIDS (123, 128). Other common stressors may include birth trauma, birth injury, traumatic injury, life-threatening event, inadequate nutrition, heel lances, prolonged institutionalization, skin breaks, and air pollution – all contribute to the build-up of toxic allostatic load.

Our model represents a major departure from previous models, such as the "three interrelated causal spheres of influence model" that requires two out of three factors to act simultaneously (subclinical tissue damage, deficiency in postnatal development of reflexes and responses, and environmental factors) (162), or the more popular "triple-risk model," which advocates that the combined effect of three factors (vulnerable infant, critical development period, and environmental stressors) causes SIDS (163). Our model posits that any infant may succumb to SIDS when the combined and cumulative effect of the environmental stressors has exceeded their tolerance level shaped by their unique genetic and environmental factors (**Figure 1**).

TESTING THE HYPOTHESIS

Our hypothesis makes several testable predictions.

Neonatal Circumcision is a Risk Factor for SIDS

Double-blinded case–control human studies aiming to test our hypothesis are unfeasible due to ethical consideration and the difficulties in matching cases and controls (19). Fortunately, the prepuce has been well conserved throughout mammalian evolution (164), which attests to its functional importance, and allows carrying out animal studies. Our hypothesis can be tested by circumcising the prepuce of mammalian animal models and measuring whether an excess of SIDS is observed among cases when compared with untreated controls. Curiously, none of the studies purporting the "benefits" of neonatal circumcision has ever been demonstrated using animal models, which are the only viable means to carry out double-blinded case–control studies assessing the short- and long-term health impacts of circumcision. In humans, we can expect higher SIDS rates in Anglophone countries that adopted male neonatal circumcision in the nineteenth century, compared to Iberio-American that traditionally have opposed circumcision (66). We can also expect a higher incidence of SIDS in USA states where Medicaid, the most common health insurance, covers circumcision, compares to states where this procedure is not covered by Medicaid after accounting for culture and socioeconomic status. The data for such study can be obtained from the CDC's SIDS registry (165). Finally, we can compare the circumcision status of SIDS victims versus healthy controls, obtained through autopsies and questionnaires, respectively. New genetic tools, such as Case-control matcher (http://www.elhaik-lab.group.shef.ac.uk/ElhaikLab/index.php), based on biogeographic ancestry tools [e.g., Ref. (166)], can be instrumental in optimizing case–control matches by identifying individuals that have similar population structure and genetic background and minimizing the bias studies due to population stratification.

Male Neonatal Circumcision Accounts for a Large Fraction of the Gender Bias in SIDS

We speculate that the male bias in SIDS observed in western countries may be due to both natural protections that render females more resilient to nociceptive stimuli and legal-cultural ones that protect females from circumcision in these countries. The weights of these two factors are unknown, yet we expect the gender deviations from even proportions in SIDS to be correlated with circumcision rates. Consequently, large male bias is expected in societies that practice neonatal circumcision whereas smaller bias is expected in societies that circumcise both males and females or avoid it altogether.

Circumcised Premature Infants Are at High Risk

We predict that circumcised premature infants would be at higher risk for SIDS compared with intact preterm infants. This can be tested by an analysis of hospital records after properly matching cases with controls (19).

Additional complications that should be considered when testing these predictions in humans include misclassification of SIDS to other categories, inconsistent reports of SIDS over time in certain countries due to changes in definitions, inconsistent reports of circumcision (167), and the absence of legislation requiring an autopsy or thorough death-scene investigation.

IMPLICATIONS OF THE HYPOTHESIS

Our hypothesis denotes that while some infants are genetically more vulnerable to the effects of allostatic load, all infants living in a stress fraught environment may be at risk of SIDS. If proven, this hypothesis will generate a paradigm shift in our understanding of neonates and pain toward focusing on factors that contribute toward allostasis.

The implication of our hypothesis, which explains many of the findings already reported in the literature, is that environmental risks should be mapped and eliminated or mitigated to reduce cases of infant deaths. Many of these implications can be put to use immediately, such as applying pain management techniques to infants that experience repetitive pain, eliminating neonatal circumcisions when possible, and postponing non-medical circumcisions to later ages. We note that postponing the circumcision does not alleviate individuals from associated complications. Studies of female circumcision among adolescents consistently report major physical, psychological, and sociological complications following the surgery (168, 169).

Although at the time being identifying infants at higher risk for SIDS is impossible, our hypothesis predicts that they would be highly sensitive to pain which can be estimated, for example, from the pitch and tone of their cry following negative stimulus (170). Such approach requires developing a standardized method that yields a small percentage of false positives. Progress should also be made in screening for genetic variants that increase the risk for SIDS.

In summary, SIDS is a complex, multifactorial syndrome in which continued research is needed to fully understand the relevant interactions between genetic and environmental risk factors that affect causation. We introduced an allostatic load model to explain SIDS and argued that it explains the main characteristic of this syndrome (**Table 1**). We also proposed how to test the hypothesis and offered guidelines on how to reduce the risk of SIDS.

AUTHOR CONTRIBUTIONS

EE developed the hypothesis and wrote the paper.

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An Acute Respiratory Infection of a Physiologically Anemic Infant is a More Likely Cause of SIDS than Neurological Prematurity

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Introduction: The cause of the sudden infant death syndrome (SIDS) is perhaps the oldest of unsolved mysteries of medicine, possibly dating back to Exodus in Biblical times when Egyptian children died in their sleep as if from a plague. It occurs when infants die unexpectedly with no sufficient cause of death found in a forensic autopsy, including death scene investigation and review of medical history. That SIDS is an X-linked recessive death from infectious respiratory disease of a physiologically anemic infant and not a simple anomalous cardiac or neurological condition is an extraordinary claim that requires extraordinary evidence. If it were by a simple cause, it would have already been solved, with over 11,000 papers on SIDS listed now in PubMed. Our aim is to use mathematical models of SIDS to explain: (1) its 50% excess male death rate; (2) its 4-parameter lognormal distribution of ages at death; (3) its winter maxima and summer minima; and (4) its increasing rate with live-birth order.

Methods: From extensive SIDS vital statistics data and published epidemiologic studies, we developed probability models to explain the mathematical behavior of SIDS meeting the four constraints mentioned above. We, then, compare these SIDS properties to infant death from acute respiratory infection (ARI), and infant death from encephalopathy, unspecified (EU).

Results: Comparisons show that SIDS are congruent with ARI and are not consistent with EU and that these probability models not only fit the SIDS data but they also predict and fit the male fraction of *all* infant and child mortality from birth through the first 5 years of their life.

Conclusion: SIDS are not rejected as an X-linked disease involving ARI and are not explained by a triple risk model that has been commonly accepted by the SIDS medical community, as implicating a neurological causation process in a subset of SIDS.

Keywords: SIDS, X-linkage, 4-parameter lognormal distribution, live-birth order, acute respiratory infections, physiologcal anemia

Abbreviations: ARI, acute respiratory infection; ASSB, accidental suffocation strangulation in bed, CDC, Centers for Disease Control and Prevention; CFM, cohabiting family members; EU, encephalopathy, unspecified; HWE, Hardy–Weinberg equilibrium; Hb, hemoglobin; ICD, international classification of diseases; LBO, live-birth order; RSV, respiratory syncytial virus; SIDS, sudden infant death syndrome; SUID sudden unexpected infant death; TRM, triple risk model; UNK, unknown.

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BACKGROUND

The sudden unexpected death of an apparently healthy and well-nurtured infant or young child during sleep – that in modern times remains unexplained after forensic autopsy, medical history review, and death scene investigation – is a phenomenon that has appeared throughout human history. It only became known as the sudden infant death syndrome (SIDS) in 1969. In Exodus (11:4–6), the Bible records a plague of such deaths in Egypt that were given a religious supernatural explanation. The history of SIDS is replete with hundreds of theories for its explanation. They range from overlaying of the infant by the mother falling asleep while nursing, suffocation from head covering, thymic asthma from a large thymus occluding the trachea, cardiac failure from long QT syndrome, to neurological deficits of the serotonergic system of the brain stem (1). Until now, even with new and advanced diagnostics, modern medical science has still been unable to discern the SIDS cause or discover a common identifying internal factor other than the defining absence of any apparent and sufficient cause of death. The aim of our paper is to show through mathematical modeling that an occult acute respiratory infection (ARI) plays a major role in the succession of events that lead a child to die suddenly and unexpectedly, without any explanation.

"Any viable hypothesis for the cause of SIDS must account for its characteristic age distribution" (2). A left-censored 4-parameter lognormal (a.k.a. Johnson S_B) age distribution fits these age data and predicts that SIDS is negligible at birth, rises to a maximum rate between 2 and 3 months of completed life, and goes to 0 at or about 3.5 years (3). Note that the limitation of SIDS to ages under 1 year in recent years is a 'legal fiction' for research purposes only (4). We reason that SIDS must involve age-varying risk factors that are necessary but insufficient-alone to cause SIDS, including some that are not measured at autopsy, and, collectively, they create that age distribution.

For example, physiological anemia is a natural phenomenon that occurs when fetal hemoglobin (HbF) disappears faster than it is replaced by adult hemoglobin (HbA). Hemoglobin (Hb) is not measured at autopsy because of hemostatic gravitational settling of red blood cells leading to lividity and also because it is a natural phenomenon that is compensated for by infants increasing heart rate to maintain oxygen throughput to the brain (5, 6). For term infants at birth, the mean Hb is about 16.5 g/dl with a SD σ of about 2 g/dl. The mean Hb falls to its nadir of 10.5 g/dl with $\sigma = 1.5$ g/dl at or about 8 weeks, the age at which SIDS has its peak rate. However, there is no margin of safety for those 2% of the infants with the lowest total Hb ($<-2\sigma$). We note the high Hb at birth can explain the absence of SIDS in the first days of life when most other causes of infant death from neurological immaturity have their highest rates.

Whereas traditional medicine repeatedly autopsies SIDS over and over again, expecting to find its cause, we took an engineering approach and looked to the numerical structure of SIDS vital statistics data for an insight. We propose that probability models of risk factors show that: (1) an X-linkage may create the 50% male excess SIDS rate (7, 8); (2) the observed same 4-parameter lognormal age distribution for both males-and-females and prone-and-supine sleepers, is predicted by Cramér's Theorem (3, 9, 10) (NB: because total of all SIDS ages have a normal transform distribution, any subsets of SIDS must also have the same normal transform distribution); (3) SIDS and ARI have the same seasonal pattern (11); and (4) the increasing SIDS rate with live-birth order (LBO) is related to increased probability of ARI brought home to the infant by family members (12, 13). The X-linkage model can then predict the 5/9 male fraction of *all* infant mortality for equal numbers of males and females at risk (14–16).

Given that an infant put to bed to sleep is found dead in exactly the same circumstances as for the immediately preceding sleep period that was survived, one has to ask, "*why was that night different from any other night*" to cause the infant to die in just that interval? The SIDS' parents or other caregivers have no premonition that their infant is at immediate risk of imminent death, so the precipitating fatal event in SIDS must occur suddenly without warning, or they would have sought prompt medical help. We propose it is an occult prodromal respiratory infection that fulminates lethally in the infant (12) with unmeasured asymptomatic physiological anemia (total Hb $< -2\sigma$), autopsied without blood or lung viral or bacterial cultures (17), or with such testing leading to a culture-negative sepsis (12, 18). "Apart from the problems resulting from post mortem effects, culture, immunofluorescence, and ELISA tests are known to give significant false negative (FN) rates" (19). An hypothesized recessive $q = 2/3$ X-linkage could allow acute anoxic encephalopathy, perhaps with apnea, to occur in possibly immature neurons, or a deficient number of respiratory control neurons of the brain stem, and the infant never awakens. If the complimentary dominant X-linked allele with frequency $p = 1 - q = 1/3$ could provide for an enzyme that would allow the respiratory control neurons to switch from aerobic to anaerobic oxidation, the infant could survive the transient anoxia. A recent study to identify this possibility could not identify such a gene locus involved, perhaps because the Illumina platform used only covered an estimated 90–95% of the X-chromosome (20, 21). We explain below why autosomal–androgen interactions are unlikely to play a role (14).

The current SIDS literature, as exemplified by papers in this very Frontiers topic, still considers a published "Triple Risk Model" (TRM) as possible for a subset of SIDS (22), even though, there is no known, common internal marker of susceptibility, no common external factor of risk, and no common restriction of SIDS ages to a distinct sub-period of SIDS susceptibility – because a single equation covers the entire age range from birth to 3.5 years (3). Such a TRM with congenital neurologic immaturity and underdevelopment of the serotonergic neurological systems would have maximal danger at or immediately after the birth as do other congenital anomalies, such as encephalopathy, unspecified (EU), whereas SIDS has a minimal rate there (23). Conversely, our proposed model with the effects of maternal iron-deficiency anemia *in utero*, delaying neurological development (24, 25), and leading to severe physiological anemia *ex utero* (5, 6) has the anemia, not the neurological deficits, playing a causative role. This anemia has the infants presenting with their maximal Hb at birth that may explain this unique property of minimal neonatal SIDS and the neurological underdevelopment observed in a

subset of SIDS (22). That is, the same maternal iron-deficiency anemia may cause both developmental delays in the infant's monoaminergic systems [including serotonin (5-HT) transporters] and the infant's relatively low postnatal Hb – leading to a fatal cerebral anoxia, so their correlation may be mistaken for the causation of SIDS.

We, now, discuss the four factors cited above (gender, age, seasonality, infectivity) that can explain SIDS and, then, we predict the total male fraction of *all* infant mortality that support our probability models for the cause of SIDS.

Gender and the 50% Male Excess of SIDS

As stated by Naeye et al. (7), "The general disadvantage of male infants has long been recognized. The biologic difference *must* originate in the genetic differences between the sexes and those genetic differences are the consequence of disparity in the number of the X chromosomes … This gives the female options for variability not open to the male." **Table 1** shows the male fraction of SIDS and other respiratory infant deaths and diseases that all seem to fluctuate about a value of 0.612 for the male fraction of SIDS. We know of no mechanism other than a recessive X-linkage in Hardy–Weinberg equilibrium (HWE) that can cause such a constant excessive male fraction of infant mortality. Whereas there may be autosomal–androgen interactions that can lead to a male excess for conditions, such as cleft lip and male pattern baldness, we have shown that the same 50% male excess occurs monthly throughout the first year of life, while testosterone rises and falls in the months after birth to aid in the descent of the testes into the scrotum (14).

It is interesting to note that Guntheroth (1) in a table of the most important epidemiologic facts on SIDS did not include the constant male fraction of 0.61, as shown in **Table 1** (33). We proposed (8) that this male fraction of 0.61 could be caused by a non-protective X-linked recessive allele with frequency $q = 2/3$ and a protective dominant corresponding X-linked allele with frequency $p = 1/3$. The XY male would be at risk with frequency $q = 2/3$ and the XX female would be at risk with frequency $q^2 = 4/9$, giving the male a 50% excess risk for equal numbers of males and females at risk (3 males:2 females). However, there is a male live-birth excess rate of order 5% that has a slight variation from country to country and from year to year within a country, so that there would be 3.15 males:2 females, giving a male fraction of order $3.15/(3.15 + 2) = 0.6117$. Therefore, some of the variance in the male fractions of **Table 1** may be due to those fluctuations at or about the nominal male live-birth excess of 5%. We also noted that Carpenter and Gardner (28) reported in their Table 1, for England and Wales 1965–1976, that all infant deaths from non-respiratory anomalies were 5,653 males and 5,369 females, a 5.3% male excess, similar to the nominal 5% male live-birth excess. This should have indicated to those looking for autosomal variants related to SIDS that androgen interactions were also unlikely.

The 4-Parameter Lognormal Distribution of the SIDS Ages

The most unique characteristic of SIDS is its 4-parameter lognormal age distribution (a.k.a. Johnson S_B) that must be explained by any theory for the cause of SIDS (2, 3). The equation we developed for SIDS is as follows: $y = \text{Log}\,[(m - [-0.31])/(41.2 - m)] = \mu + \sigma z$, where m is age in months, μ is the value of y at the median point, σ is the SD of y, and z is a standard normal deviate (Note, the negative third parameter −0.31 requires the distribution to

TABLE 1 | Male fractions of SIDS and other respiratory diseases showing the same infant male fractions of order 0.61 (26).

Authors	Diseases	Male mortality	Female mortality	Male fraction
CDC (27); U.S.; 1968–2014	Suffocation by inhalation of food or foreign object <5 years	8,940	6,070	0.596
Carpenter and Gardner (28); England and Wales; 1965–1976	Sudden respiratory death (70% SIDS) – at home	11,212	7,443	0.601
Carpenter and Gardner (28); England and Wales; 1965–1976	Respiratory deaths not sudden – in hospital	2,375	1,564	0.603
Naeye et al. (7); U.S.	Total neonatal <72 h (less antenatal aspiration identified by squamous cells in terminal airspaces)	1,009	660	0.604
Fard et al. (29); Hannover	SIDS	163	104	0.610
Mage and Donner (8); Global	SIDS (36 data sets)	41,238	26,140	0.612
Carpenter et al. (30); Europe, NZ	SIDS	1,466	898	0.613
Gupta et al. (31); Scotland; 1982–1990	Bronchiolitis: hospital discharge diagnoses	6,127	3,881	0.614
Wilkinson and Skuza (32); Australia; 1981–2000	SIDS	4,402	2,752	0.615
CDC (27); U.S.; 1968–2014	Respiratory distress syndrome	98,328	61,790	0.619
Gupta et al. (31); Scotland; 1982–1990	SIDS	751	460	0.620
Total	All the above	176,011	110,962	0.613

TABLE 2 | The age distribution of SIDS – multiple studies pooled together (34).

Age m[a]	1	2	3	4	5	6	7	8	9	10	11	12	13–41	N
y	−1.487	−1.230	−1.060	−0.936	−0.834	−0.747	−0.670	−0.602	−0.539	−0.481	−0.427	−0.375		
n	3,550	10,496	12,354	10,036	6,436	4,046	2,811	2,007	1,430	940	683	511	1,110[b]	56,410

[a]*Months are defined differently by different authors as 1,461 days/48 months is not an integer.*

[b]*Estimated by semi-logarithmic extrapolation of numbers at 4 – 12 to 41 months.*

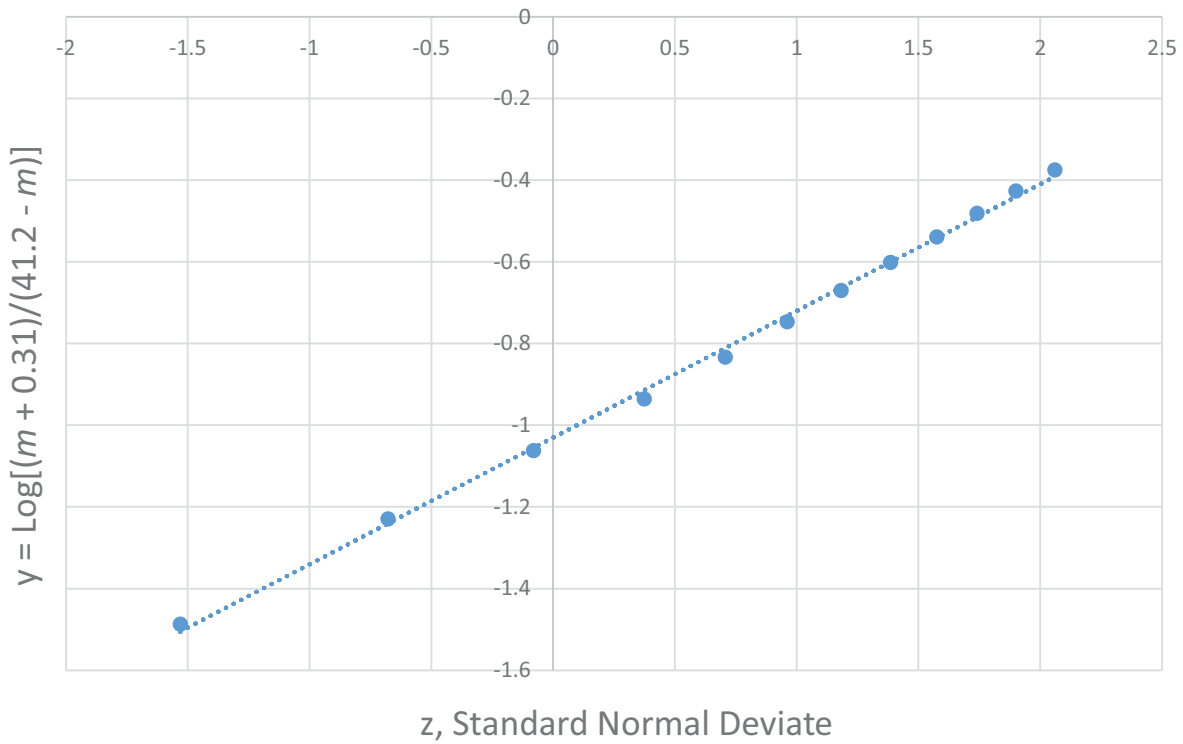

FIGURE 1 | 4-parameter lognormal distribution of SIDS ages (34). $y = 0.31\,z - 1.03$.

be censored at $m = 0$). **Table 2** shows the age distribution data for multiple SIDS studies combined (34) and **Figure 1** is the frequency distribution of y vs. z described by a 4-parameter lognormal distribution ($y = 0.31\,z - 1.03$).

To explain the generation of this observed 4-parameter lognormal distribution as required for any correct explanation of SIDS (2), we developed a Venn diagram (**Figure 2**) for a quadruple risk model of SIDS (35). The four probability factors involved with SIDS discussed in this paper explain the age and gender distributions invariant with different sleep position and subsets of SIDS found with and without neurological prematurity (Pn) and respiratory infection (Pi). It is proposed that a prone infant is susceptible to SIDS anywhere in the intersection between the genetic (Pg) and anemia-related apnea (Pa) factors with *either* Pn *or* Pi, but a supine sleeping infant is only susceptible to SIDS if it is in the intersection of all four factors (Pa, Pg, Pi, *and* Pn). This is easily explained mathematically from our model as follows: let there be two causal-risk factors, one with probability increasing with age in months (m) as $\mathrm{Pi} = 0.31/(41.2 - m)$ and the other with probability decreasing with age as $\mathrm{Pn} = 0.31/(m + 0.31)$. For supine sleep, let the infant require both simultaneously with the probability equal to their product as $\mathrm{PiPn} = 0.1/[(41.2 - m)(m + 0.31)]$. For prone sleep, let the infant only require one of them, which will have the probability approximately equal to their sum Pi + Pn, as $0.31[1/(41.2 - m) + 1/(m + 0.31)]$. However, this sum can be rewritten as equal to $0.31\,[(41.2 - m) + (m + 0.31)]/$

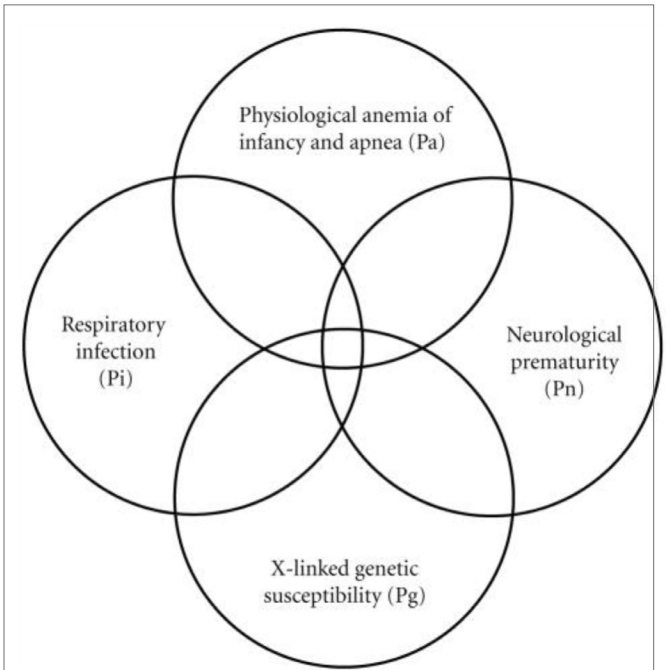

FIGURE 2 | Venn diagram explaining the generation of the 4-parameter lognormal age distribution of SIDS satisfying Cramér's Theorem (3, 9, 10, 35).

$[(41.2 - m)\ (m + 0.31)] = 12.9/[(41.2 - m)(m + 0.31)]$ that has the same form as for supine sleep, varying only by the constants 0.1 and 12.9.

Seasonal Pattern of SIDS

Sudden infant death syndrome has a known seasonal pattern, with maximal rate during the cold winter and minimal rate during the warm summer months found in Europe and North America. CDC (27) reports the monthly numbers of SIDS, ARI, and EU for 1999–2014. We sum the numbers reported by calendar month (28–31 days) and adjust these totals to a fixed month length of 1,461 days/48 months = 30.44 days, by multiplying each total by 30.44/days per month. **Table 3** shows the ICD-10 numbers for sudden unexpected infant deaths {SUID = [SIDS (R95), UNK (R99), accidental suffocation strangulation in bed (ASSB) (W75)]}, ARI (J00-J26), and EU (G93.4). **Figure 3** shows that SUID and ARI have similar sinusoidal variation, but EU does not.

Seasonality in SUID/SIDS is not a function of ambient temperature variation between winter and summer. This was shown by Mage (11) looking at semi-tropical Hawaii, which has negligible temperature variation throughout the year. However, it does have seasonal variation of SIDS that was attributed to the tourist influx during the year from the U.S. and Japan where there is ARI seasonal fluctuation that is transmitted to the Hawaiian population. The South American country Colombia is also semi-tropical with wide changes in elevation, but no cold winter. There, the respiratory infection peak occurs during the rainy season that matches their SUID peak (36).

Respiratory Infection and SIDS

Infants are normally and routinely placed to sleep by their parents in a habitual customary manner. In the case of SIDS, the parents or other caregivers have no premonition that the infant is at imminent risk of dying. The baby's clothing, sleep position, bed and bedding, and other items, such as pacifier use, room temperature, and feeding pattern will be very like the infant's normal pattern in the immediately previous sleep period. One needs to ask "*what then could have changed between the immediately previous sleeping conditions and the final sleep from which the infant never awoke*?" We propose that virtually the only likely thing that could have changed, which is capable of causing a sudden death, is the rapid fulmination of an occult prodromal

TABLE 3 | Seasonal variation of SUID, ARI, and EU, U.S. 1999–2014 (27).

Disease	January	February	March	April	May	June	July	August	September	October	November	December
SUID	5,247	5,280	5,061	5,096	5,012	4,730	4,547	4,647	4,891	5,161	5,308	5,476
ARI	636	710	568	393	340	272	240	231	284	296	372	509
EU	121	111	130	114	131	109	115	141	125	135	119	123

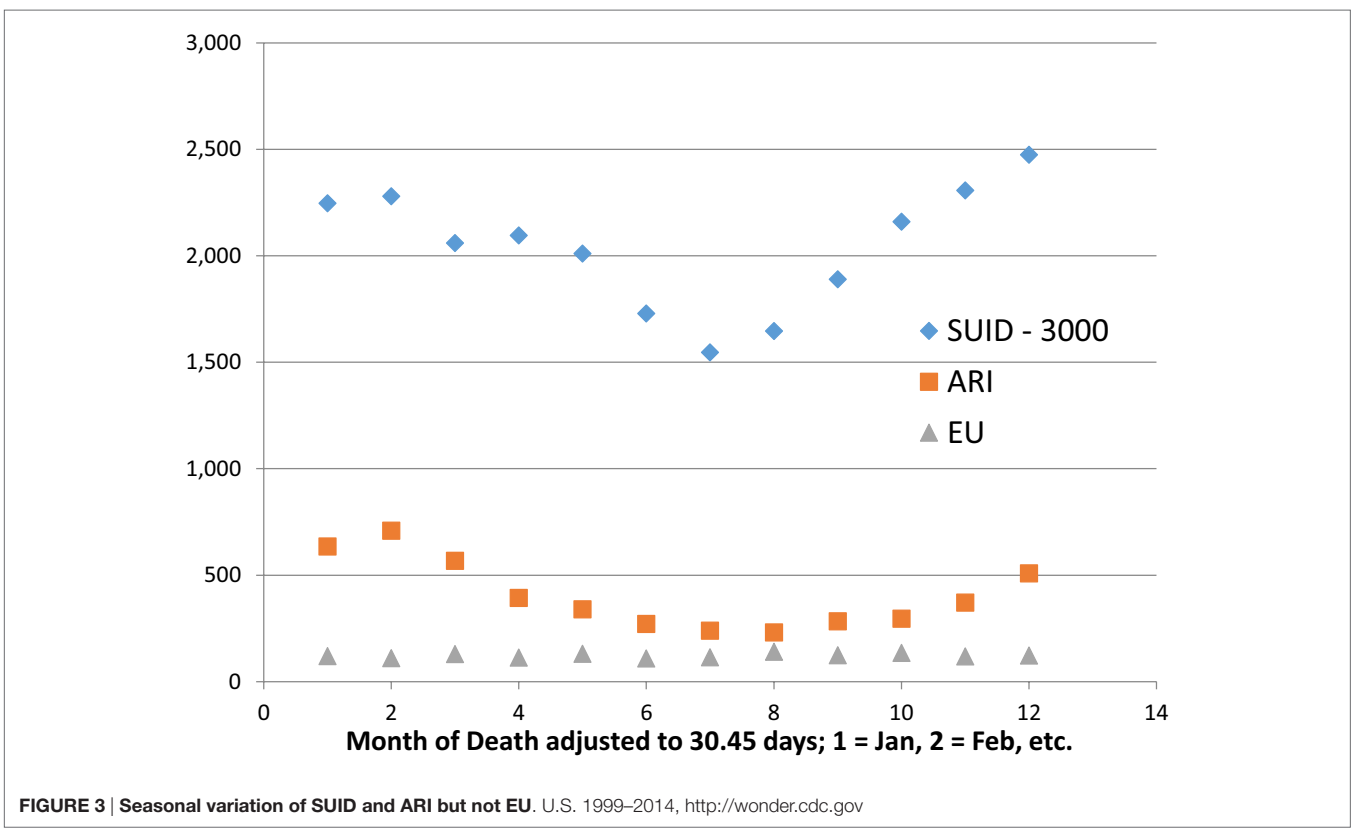

FIGURE 3 | Seasonal variation of SUID and ARI but not EU. U.S. 1999–2014, http://wonder.cdc.gov

respiratory infection that was invisible to the person placing the infant to sleep (12).

Infants who catch a respiratory infection must get it from contact with a carrier of that communicable infection, which is most likely a cohabiting family member (CFM) (37). For infants of a given LBO, we assume that they live with two parents and all LBO – one older siblings, so that CFM = LBO + 1. **Table 4** shows combined global data from four studies from the U.S. (28, 38), Europe (30), and Colombia (39) of SUID. SUID was defined by CDC (27) for ICD-10 as R-95 SIDS, R-99 Unknown cause or SIDS with an incomplete forensic investigation (UNK) and W-75 ASSB – with possible suffocation from prone sleep position. As readily seen from the next to last column, the rate of total SUID increases, monotonically, with LBO and our estimate of CFM.

TABLE 4 | Four combined studies of global SUID = SIDS + UNK + ASSB (13).

Live-birth order (CFM)	SIDS + UNK + ASSB	Infants at risk	SUID rate per 1,000	SUID model per 1,000 = 3.60*(1–0.9^{CFM})
0 (0)	0	0	0	0
1 (2)	27,945	38,759,660	0.7210	0.6843
2 (3)	30,037	30,465,292	0.9859	0.9760
3 (4)	18,288	15,418,084	1.1861	1.2386
4 (5)	9,539	6,467,509	[a]1.4749	[a]1.4749
5 (6)	3,327	1,989,679	1.6721	1.6876
6 (7)	2,818	1,520,300	1.8536	1.8790

[a]*Model fit by matching to this datum point.*

We noted the increase of SUID rate as LBO/CFM increases and have developed the following probability model to express the concave shape of the relationship: let P equal the average probability of a family member *not* being a carrier of a communicable respiratory infection, the probability that all CFM are non-infective will then be equal to P^{CFM}, and the probability that the infant will be exposed to *at least one* such CFM will be equal to $1 - P^{CFM}$. Our model fit to these data is that $P = 0.9$ and the SUID rate per 1,000 at risk with a given LBO, shown in the last column of **Table 4**, is as follows: rate per 1,000 = $3.60*(1 - 0.9^{CFM})$. **Figure 4** shows the goodness of fit of this model to these data. Note how the model goes to the origin (0 SUID for a virtual cloned infant that has 0 CFM – as in Aldous Huxley's *Brave New World*), smoothly without any discontinuity. Therefore, all SUID appear to be related to a possible source of an ARI.

Table 5 shows the U.S. infant mortality rate from both Upper and Lower ARIs from 1995–2013 (27). The corresponding codes are ICD-10, J00-J06, J20-J22, and ICD-9 460-466. As for

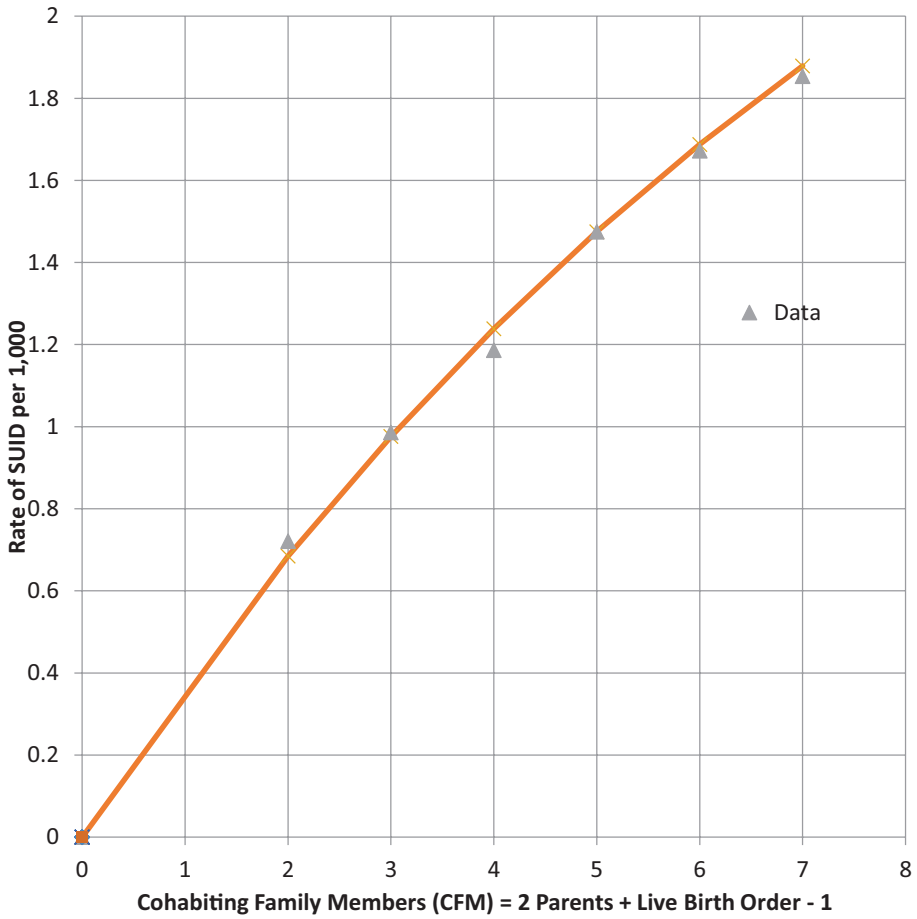

FIGURE 4 | SUID rate per 1,000 increasing with family size. (CFM) Global Rate = $3.60*(1 - 0.9^{CFM})$.

SIDS, the ARI mortality rate increases with LBO in **Figure 5** and with a similar mathematical relationship for prediction as from **Figure 4**. This supports our finding that SIDS appears to have a causal relationship to the initial fulmination of an occult prodromal ARI that may cause neuronal death in the physiologically susceptible infants (12). We note again that many U.S. medical examiners do not culture lung exudate of potential SIDS cases because "of a perceived lack of testing utility" (17), and that, in many cases, the cultures are negative in spite of other evidence of severe sepsis (18, 19). Indeed, Farber wrote, in some such cases, "no growth was obtained from the blood stream although gross and histological changes in these cases were identical with those in which a positive culture was found." (12).

In comparison to SIDS and ARI which have increasing risks of mortality with increasing family size as potential carriers of an ARI, infant mortality from neurological cell death in infants with underdeveloped brain structure at birth, from EU is independent of the infant's family size or LBO. **Table 6** shows U.S. EU data on this cause (27).

Figure 6 shows that the family size of the infant dying from neurological underdevelopment and immaturity at birth, with a diagnosis of EU, has no consistent relation to the rate of EU.

TABLE 5 | U.S. 1995–2013 (http://wonder.cdc.gov) acute upper and lower respiratory infection mortality (ICD-10, J00-J06, J20-J22, and ICD-9 460-466) (27).

Live-birth order (CFM)	ARI J00-J06, J20-J22 ICD-9 460-466	Infants at risk	Rate per 100,000	Model rate per 100,000 = $6.36*(1–0.9^{CFM})$
0 (0)	0	0	0	0
1 (2)	280	30,740,193	0.9108	1.2084
2 (3)	387	24,528,771	1.5777	1.7235
3 (4)	265	12,707,378	2.0854	2.1872
4 (5)	139	5,081,597	2.7353	2.6044
5 (6)	58	1,890,067	3.0686	2.9800
6 (7)	50	1,470,973	3.3991	3.3180

In addition, whereas EU occurs predominantly (60%) in the first 4 weeks after birth, SIDS spares the neonate (3, 21). Thus, it is unlikely that neurological deficiencies from fetal underdevelopment of serotonergic brain cells in SIDS can be responsible for a large subset of SIDS. The reader should note that Cramér's theorem requires that all subsets of SUID, such as SIDS, must have the same normal transform age distribution as SIDS that would also argue against a neurological subset (9).

Figures 7 and **8** show that the age distribution of hospital discharges for bronchiolitis and SIDS deaths, respectively, in Scotland 1982–1990, have the same lognormal form, as well as the same male fractions (SIDS 0.612 and bronchiolitis 0.614). Note that SIDS in **Figure 8** have virtually the same slope and intercept as SIDS in **Figure 1**. Gupta et al. (31) came to the conclusion that "the two conditions do not appear to be closely related" by a chi-square test and analyses of their autocorrelation structures. They attributed the bronchiolitis hospitalization cases to the ubiquitous respiratory syncytial virus (RSV) to which virtually all infants are exposed by the end of the second year of life (37). However, there are several reasons why the statistical comparison between SIDS and RSV hospitalization may show no significant relation if one did exist:

All statistical testing assumes that measurements are made without error, but SIDS has false positives (FP) and FN. Thus, Recorded SIDS (RS) = Actual SIDS (AS) + False Positives (FP) − False Negatives (FN). In addition, not all ARI are caused by RSV (12, 37). Therefore, SIDS should have been compared by Gupta et al. to all infant hospitalization discharges for upper and lower ARI, not just those for bronchiolitis.

PREDICTION OF MALE FRACTION OF *ALL* TOTAL INFANT MORTALITY FROM THE X-LINKAGE MODEL FOR SIDS

All natural infant deaths occur either from cardiac failure (heart stops beating first) or respiratory failure (breathing stops first),

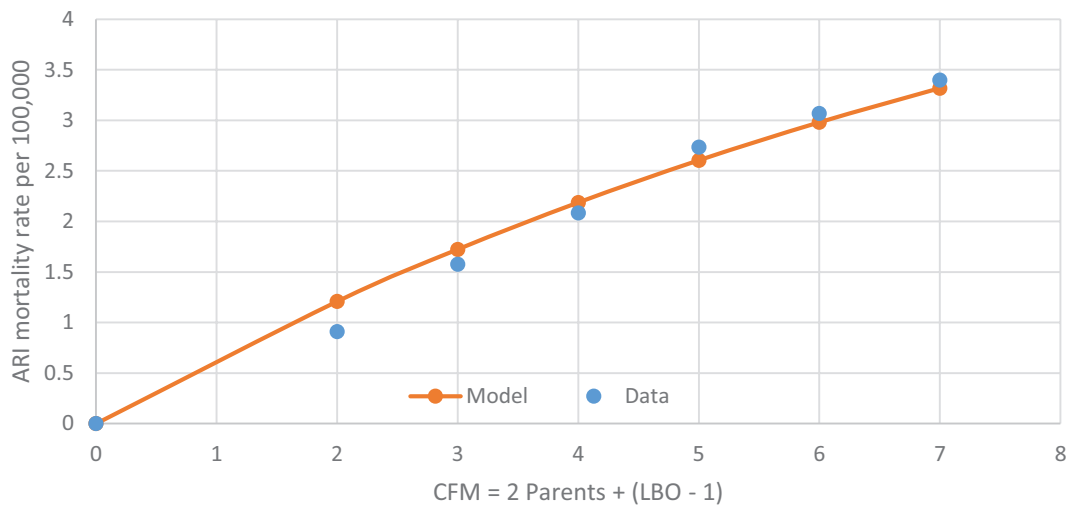

FIGURE 5 | U.S. 1995–2013 ARI rate/100,000 increasing with CFM as a respiratory infection vector, with rate = $6.36*(1 - 0.9^{CFM})$.

neglecting trauma cases, such as fall from great height when both stop simultaneously. As described above, all infant mortality from respiratory causes has an apparent 50% X-linkage male excess rate for equal numbers of XY males and XX females at risk (we neglect chromosomal abnormalities, such as XXY male and XXX female, and assume no infanticides). As also shown, there is no male excess (0%) for cardiac failures, which include congenital anomalies related to genetic variants on the 22 autosomes, which males and females have with equal probabilities, assuming HWE (40, 41). That is, for every two females dying from respiratory causes, three males will die, and for every two females dying of cardiac causes, two males will die.

We, then, assumed that female infants are equally likely to die from cardiac failure as respiratory failure (14, 15). If so, then for every two females dying from respiratory failure there will be three males, and two males and two females dying from cardiac causes. Thus, for equal numbers of males and females at risk there will be five males dying for every four females dying from all natural causes, predicting a 5/9 = 0.55555 male fraction for *all* infant mortality. Given that there is a nominal 5% male excess live-birth rate, 5.25 males will die for every 4 female infants dying from all causes, predicting a male fraction of 5.25/9.25 = 21/37 = 0.567567. We assume that infants under 5 years neither play independently of adult supervision nor display the male hyperactivity that increases their death rates from accidents and trauma later as they age. We, therefore extend our analysis in **Table 7** up to 5 years where data are available as in the U.S.

We chose to report the male fractions without correcting for the male excess at risk fluctuations from 5% (16). For example, dividing total deaths by total births in the same periods would be misleading because, as for the U.S., some children below 5 years dying in 1968 would have been born in the previous 5 years and some born in 2014 would die in the next 5 years.

TABLE 6 | U.S. 1995–2013; brain cell death, encephalopathy, unspecified, in infants with neurological underdevelopment at birth (http://wonder.cdc.gov) ICD-10 G93.4, ICD-9 348.3 (27).

Live-birth order (CFM)	Encephalopathy, unspecified, ICD-10 G93.4, ICD-9 348.3	Infants at risk	Rate per 10,000	No appropriate model for EU
1 (2)	200	30,740,193	0.0651	–
2 (3)	152	24,528,771	0.0620	–
3 (4)	67	12,707,378	0.0527	–
4 (5)	29	5,081,597	0.0571	–
5 (6)	12	1,890,067	0.0631	–
6 (7)	13	1,470,973	0.0884	–

SUMMARY

The results shown above become visible when large sample sizes are created by pooling observations from different data sets. We propose that, because SIDS has no objective finding, the diagnoses are subjective, and different pathologists reviewing the same slides and findings will assign different causes of death (43). Consequently, FP and FN SIDS are likely not randomly created by a given pathologist so their case studies will have either a positive or negative bias. When independent SIDS data sets are pooled

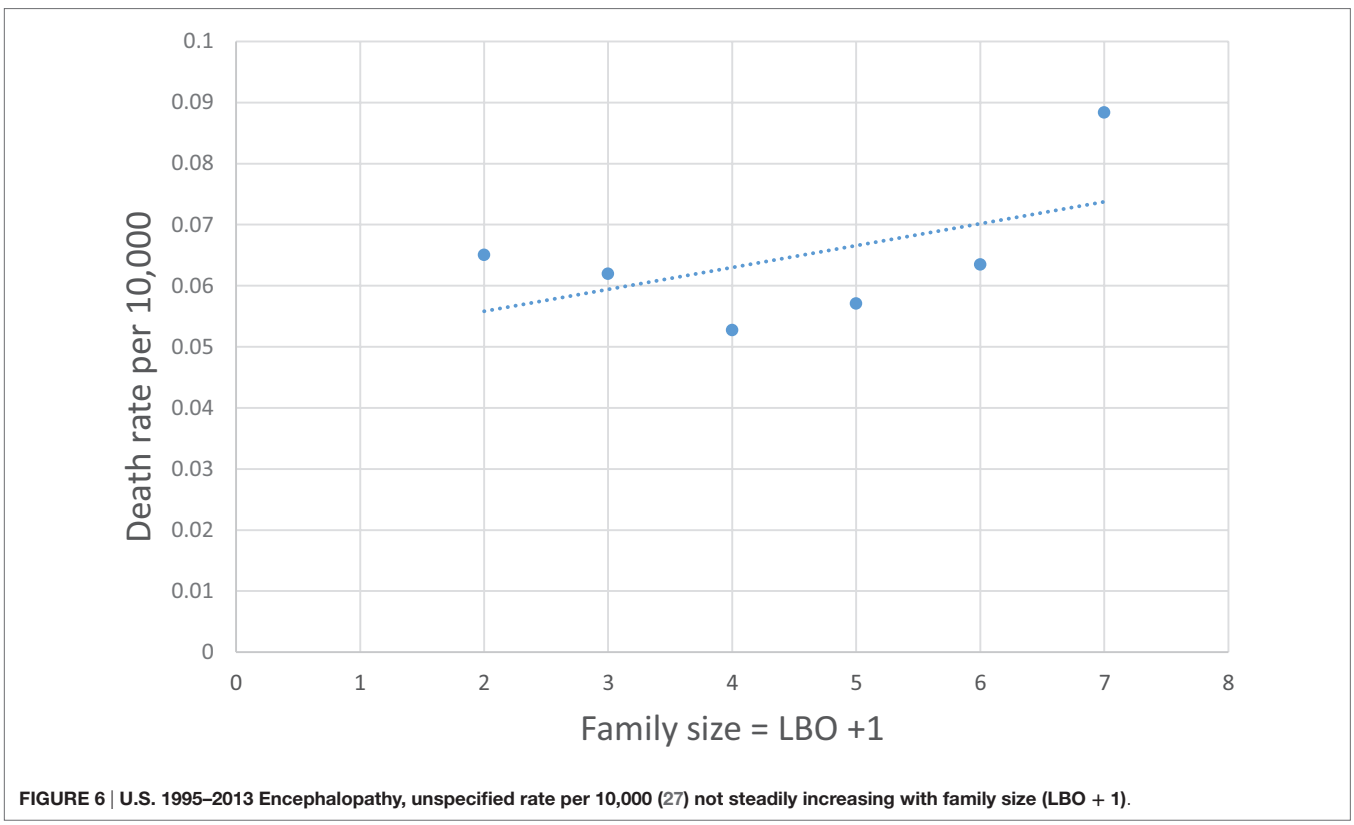

FIGURE 6 | U.S. 1995–2013 Encephalopathy, unspecified rate per 10,000 (27) not steadily increasing with family size (LBO + 1).

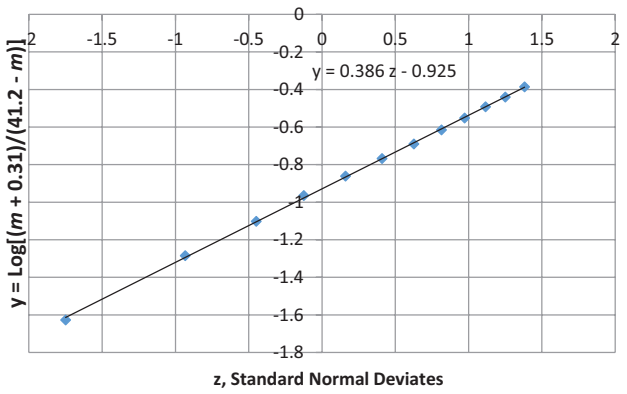

FIGURE 7 | Age distribution of bronchiolitis hospital admissions in Scotland, 1982–1990 (31).

together, the FP and FN will tend to cancel out and, by the law of large numbers, the mean value of the errors will approach 0 as the total number of observations becomes large. In the pooled data sets, we have analyzed, the numbers of observations are very large totaling from tens of thousands up to a few million, so the means of the data sets closely approach the means of the underlying distributions.

As shown here, and in our other papers cited, the following mathematical relationships that SIDS display must be explained by any proposed cause for SIDS:

(1) The constant SIDS 50% male excess rate compared to the female rate for equal numbers of infants at risk. Given the nominal 5% excess male birth rate, this leads to the observed male fraction of 0.612 (8);
(2) The SIDS left-censored 4-parameter lognormal (Johnson S_B) age distribution that has 3rd and 4th parameters of

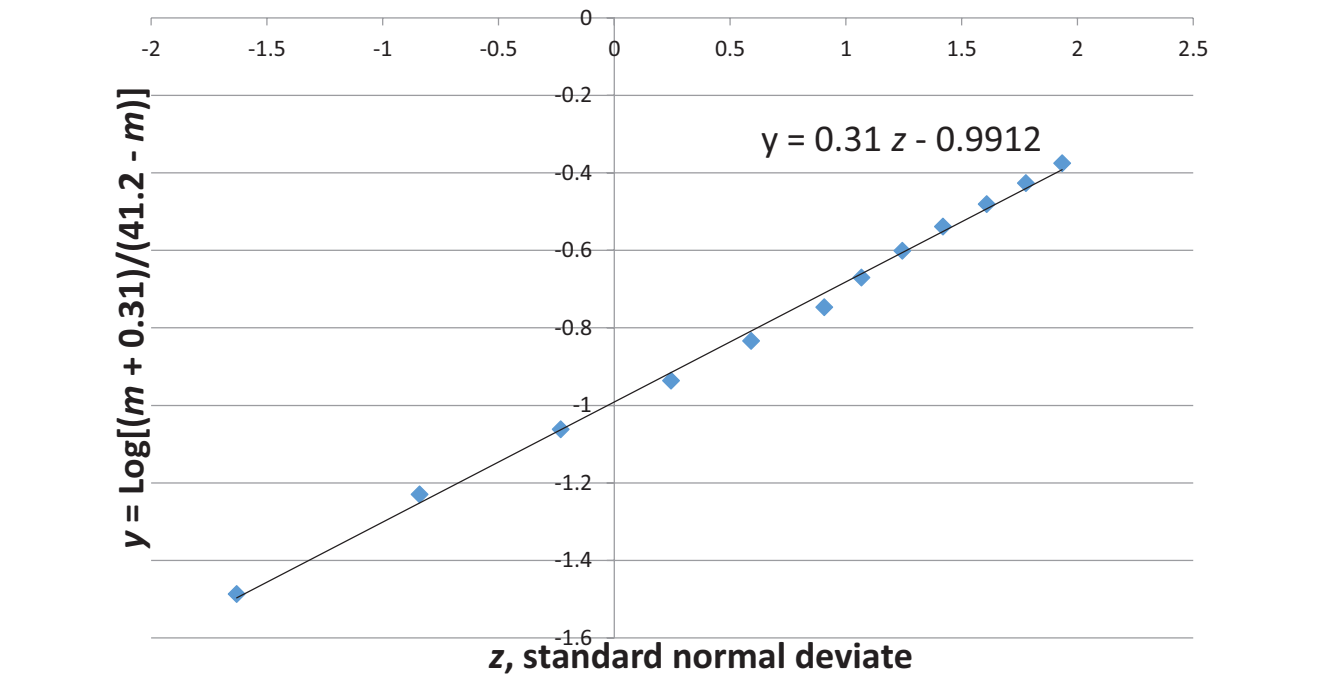

FIGURE 8 | SIDS in Scotland, 1982–1990 (31) y = Log[(m + 0.31)/(41.2 − m)] vs. z, standard normal deviate.

TABLE 7 | Total male and female infant deaths and male % excess at risk for the U.S. (27), 13 countries pooled together (16) Argentina (42), Colombia (39), Norway (43), and England and Wales (28).

Country	Years	Ages	Male excess, at risk, %	Total male mortality	Total female mortality	Male fraction
U.S.	1968–2014	<5 years	4.62	1,221,981	932,096	0.5673
13 countries	Various	<5 years	5.11	294,827	223,004	0.5694
England and Wales	1969–1976	<1 year	5.99	22,965	17,502	0.5675
Argentina	1980–2012	<1 year	5.28	263,680	203,802	0.5640
Colombia	1979–2012	<1 year	5.51	261,676	200,296	0.5664
Norway	1967–1988	<1 year	5.44	3,103	2,344	0.5697

Predicted male fraction for 5% male excess at risk is 0.5676 and 0.5699 for 6%.

order −0.31 and 41.2 months, respectively, with median of approximately −1.0 and approximate SD of 0.30;
(3) Maximum SIDS rate in winter months and minimum rate in summer months (10);
(4) The increased risk of SIDS with the infant's increasing numbers of older siblings, proportional to the factor $(1 - 0.9^{CFM})$, where CFM = 2 parents + (LBO − 1) siblings (13, 14);
(5) The SIDS X-linkage model predicts the 5/9 male fraction of *all* infant mortality for equal numbers of males and females at risk (13, 14).

To the best of our knowledge, no other cause of SIDS has been proposed that meets these five essential conditions that are necessary, but insufficient, to prove that they are the cause of SIDS. For proof of its causation, the predicted missing X-linked $p = 1/3$ dominant allele that is protective of neuronal cell death by acute anoxic encephalopathy by enabling the infant to shift from aerobic to anaerobic oxidation must be identified (4, 14). This may be complicated because of the likely presence of FP SIDS in the study cohorts, where non-SIDS cardiac causes of death or cases of infanticide may have been missed. In addition, our model requires that the SIDS infant be in the lowest percentiles ($<-2\sigma$) of Hb from the natural physiological anemia that minimizes for all infants between 2 and 3 months of age (5, 6). However, due to the gravitational settling of the red blood cells during hemostasis leading to lividity, an accurate blood Hb cannot be measured. If all infants in a birth cohort had their blood Hb measured at birth, then, perhaps, the lowest Hb infants could be identified as the susceptible cohort and, if so, treated to increase their Hb (44).

CONCLUSION

We propose that the most physiologic-anemic infants can be identified by measuring Hb at birth. Then, if an enzyme coded for by the putative protective dominant X-linked allele (that passes through the blood–brain barrier) can be identified and given to the unprotected infant, it may be possible to reduce infant mortality significantly, by reducing the numbers of infants dying from SIDS and all other respiratory causes (16).

AUTHOR CONTRIBUTIONS

DM prepared the first draft and developed probability models to fit the age and family data. ED provided the genetic model, discussed the first draft with DM and made revisions, and approved the final draft. AJ provided and discussed the vital statistics for Argentina, reviewed the first draft and commented on the medical aspects, and approved the final draft. ML provided and discussed the vital statistics for Colombia, reviewed the first draft, and commented on the medical aspects and approved the final draft.

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A new theory to explain the underlying pathogenetic mechanism of sudden infant death syndrome

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The author, on the basis of numerous studies on the neuropathology of SIDS, performed on a very wide set of cases, first highlights the neuronal centers of the human brainstem involved in breathing control in perinatal life, with the pontine Kölliker–Fuse nucleus (KFN) as main coordinator. What emerges from this analysis is that the prenatal respiratory movements differ from those post-natally in two respects: (1) they are episodic, only aimed at the lung development and (2) they are abolished by hypoxia, not being of vital importance *in utero*, mainly to limit the consumption of oxygen. Then, as this fetal inhibitory reflex represents an important defense expedient, the author proposes a new original interpretation of the pathogenetic mechanism leading to SIDS. Infants, in a critical moment of the autonomic control development, in hypoxic conditions could awaken the reflex left over from fetal life and arrest breathing, as he did in similar situations in prenatal life, rather than promote the hyperventilation usually occurring to restore the normal concentration of oxygen. This behaviour obviously leads to a fatal outcome. This hypothesis is supported by immunohistochemical results showing in high percentage of SIDS victims, and not in age-matched infant controls, neurochemical alterations of the Kölliker–Fuse neurons, potentially indicative of their inactivation. The new explanation of SIDS blames a sort of auto-inhibition of the KFN functionality, wrongly arisen with the same protective purpose to preserve the life *in utero*, as trigger of the sudden infant death.

Keywords: SIDS, pathogenesis, brainstem, respiratory network, fetal breathing, Kölliker–Fuse nucleus, BDNF, NeuN

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INTRODUCTION

Most Reliable Definition and Hypothesis on SIDS Pathogenesis

The best known definition of SIDS is "the sudden unexpected death of an infant <1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history" (1). The causes are still unknown, although several even conflicting hypotheses of the underlying mechanisms of SIDS have been proposed (2). The most reliable seems to be the "triple risk hypothesis," which predicts that fetal brain development of infants who subsequently succumb to SIDS is abnormal, leaving them unable to respond appropriately to stressors during a vulnerable period of the autonomic control (3). Consistent with this assumption, many studies have reported a high incidence of morphological abnormalities and biochemical defects of neurotransmission, particularly serotonergic, in the brainstem of SIDS victims compared

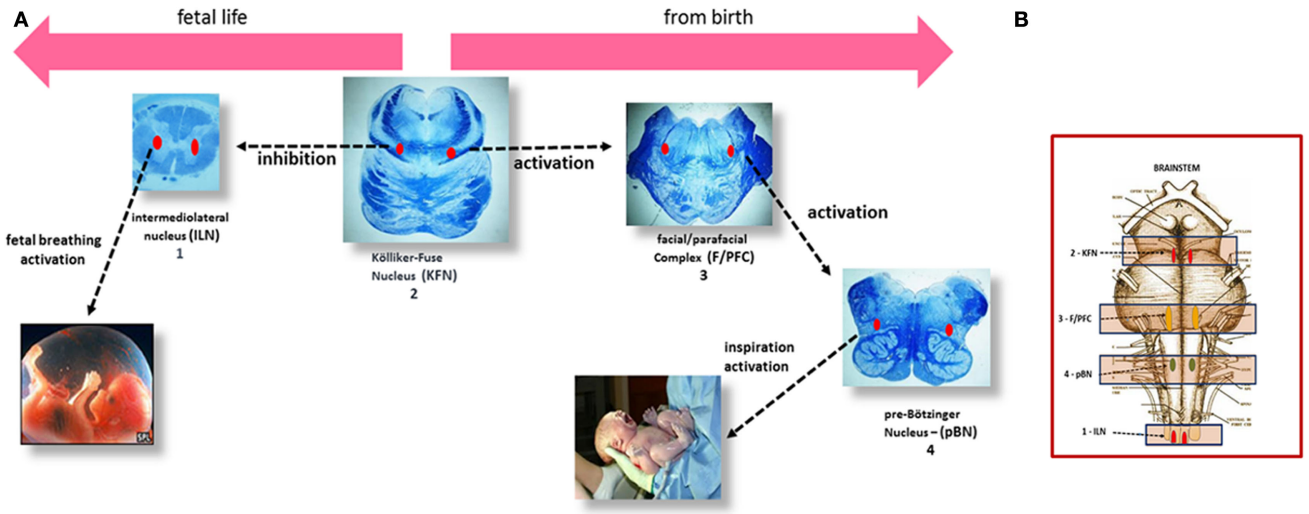

FIGURE 1 | (A) Left to right, the steps of the respiratory control in human perinatal life. (1) In the human fetus, episodic respiratory activity aimed at promoting lung development is generated by the intermediolateral nucleus (ILN) in the upper spinal cord. (2) At the same time, during intrauterine life, the Kölliker–Fuse nucleus (KFN), located in the rostral pons, plays an important function by inhibiting the response of central and peripheral chemoreceptors and therefore any respiratory reflex, while allowing the occasional breathing activity headed by the ILN. (3) The facial/parafacial complex (F/PFC), in the caudal pons, starts working at birth, under the stimulation of the KFN, which drastically changes its function, giving rise to the first inspiratory act. The activity of the F/PFC is called "pre-inspiratory" because it is limited to activating, in its turn, the proper inspiratory nucleus in the medulla oblongata: the pre-Bötzinger nucleus (pBN) (4), responsible for starting postnatal breathing. **(B)** Brainstem schematic representation showing the localization of the RN components.

with control infants dying of other causes (4, 5). This brain region includes the main nuclei and structures that coordinate the vital activities, such as cardiovascular function and breathing, before and after birth.

A Rightful Observation

But if the placenta is the effective source of oxygen in fetal life, what is the significance of the respiratory activity in prenatal life? The answer seems to be that the fetus must train so as to be ready to put the lungs to use, once outside the womb. At the time of birth, he will take a few moments to dilate the lungs and begin to breathe. If he fails, survival is threatened. So, *in utero* intermittent breathing movements occur with the main purpose of adequately developing the respiratory system, essential as soon as postnatal life begins (6). The full functionality of the lungs and respiratory muscles acquired will allow the autonomous ventilatory activity that the newborn needs to survive to start up (7).

Breathing Behavior Before and After Birth in Hypoxic Conditions

A very interesting phenomenon was observed by low-voltage cortical electrical tests in experimental studies on sheep fetuses (8, 9), and also by ultrasound real-time scanning in human fetuses (10): if the amount of oxygen through the placenta decreases (for whatever reason), the fetus immediately suspends pulmonary movements. Precisely, when the partial pressure of oxygen falls below 16–18 mmHg, respiratory activity *in utero* stops, not being a vital function, but only to limit the consumption of oxygen. This is a defense mechanism of the fetus, a way to save energy, because oxygen must go first and foremost to the brain and heart to ensure life.

On the contrary, hypoxia induces hyperventilation in newborns, mainly in the arousal phase from sleep, through a rise in the amplitude and frequency of pulmonary movements, to restore the normal concentration of plasmatic gas and above all of oxygen (11).

A New Plausible Hypothesis on SIDS Pathogenesis

And it is at this point, on the basis of the above considerations, that I propose a new perspective that could provide a physiological explanation of SIDS. Infants, in a critical moment of the autonomic control development, could awaken a conditioned reflex left over from fetal life: after birth, a situation of lack of oxygen (due to a prone sleep position, nicotine absorption, or any other reason) could induce a vulnerable baby to arrest breathing, he did in similar situations in prenatal life, and, therefore, die. Very probably, being used to being oxygenated by the placenta for 9 months, this ancient instinctive survival behavior could remain registered in the brain, but becoming fatal after birth.

How could the brainstem centers checking the respiratory function be involved in this intrinsic devastating reflex leading to SIDS?

The Brainstem Respiratory Network

Previous studies, performed at the "Lino Rossi" Research Center of the Milan University, have identified specific nuclei and structures designated to control the breathing, hitherto highlighted only in experimental animals. Given, obviously, the impossibility of performing experiments in humans, the homologous nuclei were identified on the basis of morphological criteria of similarity with regard to the location, the cytoarchitecture, and

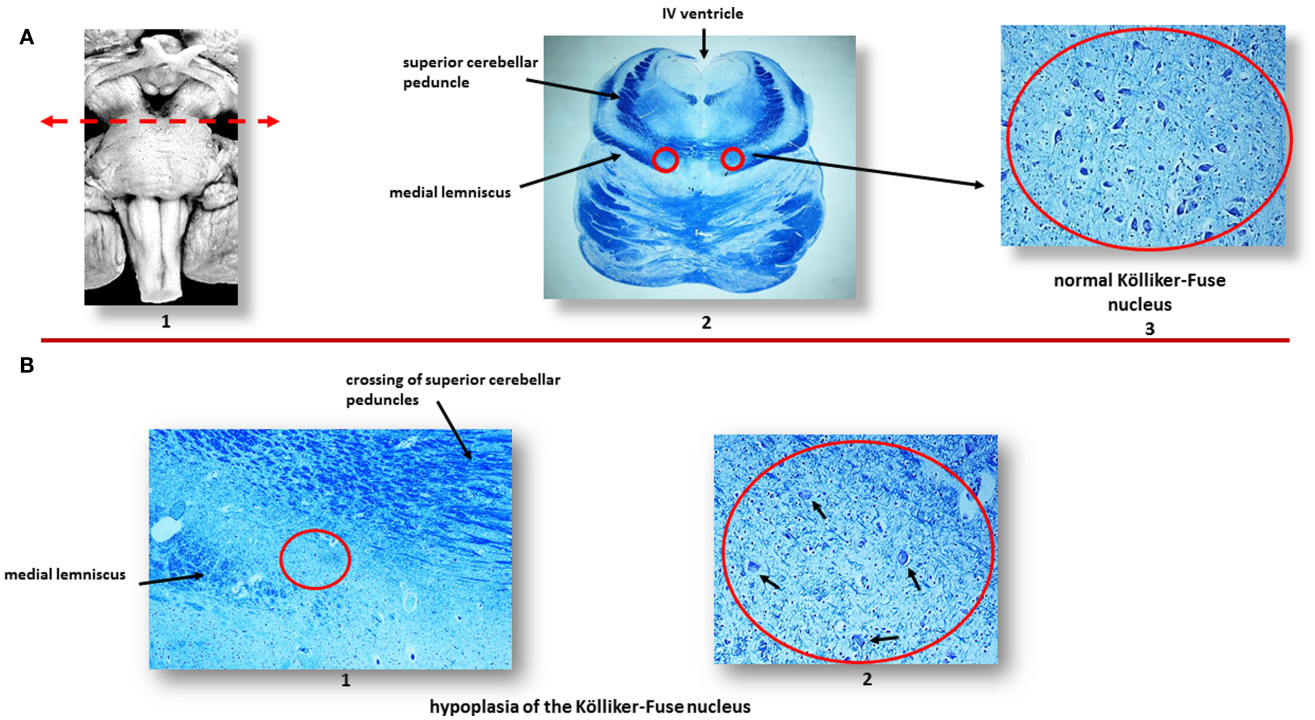

FIGURE 2 | (A) Normal Kölliker–Fuse nucleus (KFN) in a control newborn (3-month-old). (1) Brainstem schematic showing the optimal level to examine the KFN (see arrow), though this nucleus is longitudinally extended from the rostral pons to the lower portion of the mesencephalon. (2) Histological transverse section of rostral pons with the indication of the KFN location, between the crossing of the superior cerebellar peduncles and the medial lemniscus. (3) Magnification of the encircled area in (2): the KFN shows its cytoarchitecture consisting of a numerous group of large neurons with distinct, eccentric nucleus, evident nucleolus, and abundant cytoplasm with Nissl substance located at cell periphery. Intermixed with these large neurons, smaller cells (interneurons and astrocytes) are visible. **(B)** Hypoplasia of the KFN in a SIUDS case (39 gestational weeks). The encircled area in (1), included between the crossing of the superior cerebellar peduncles and the medial lemniscus, is represented at higher magnification in (2). In this area, only rare suffering KF neurons are visible (see arrows). Klüver–Barrera staining. Magnification: **(A)** (2): 0.5×; **(A)** (3) 20×; **(B)** (1) 10×; **(B)** (2) 20×.

number of neurons and applying, when possible, immunohistochemical methods to highlight the same neurotransmitters and receptors recognized as specific for several structures, above all in rats. Through this original methodology, the Kölliker–Fuse nucleus (KFN), the facial/parafacial complex (F/PFC), the pre-Bötzinger (pBN) nucleus in the pons/medulla oblongata, and the intermediolateral nucleus (ILN) in the spinal cord were defined (12–15). These nervous centers are linked together via interneuronal synapses in a "respiratory network" (RN), and can modulate one another. I propose a scheme (**Figure 1**) to illustrate the human breathing control mechanism in perinatal life, indicating the more representative brainstem histological sections where the RN structures are well analyzable, depicted in a chronologically functional sequence, as explained in the legend.

Role of the Kölliker–Fuse Nucleus in the RN

Essentially, the pulmonary activity is largely dependent on sensory inputs from the ILN in prenatal life and from the F/PFC from birth, being both modulated by the KFN, which therefore represents the breathing filmmaker. 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 (16), is fundamental. In hypoxic conditions, a normal fully functional KFN abolishes the rhythmic activity of the ILN in fetuses and greatly accelerates the ventilatory function of the F/PFc in newborns, with the same aim of safeguarding life.

This chief central function exerted by the KFN is supported by experimental studies in fetal lambs performed by brainstem transactions at various levels (17). Results demonstrated the existence of a locus in the rostral lateral pons whose integrity is essential for the depression of breathing during hypoxia *in utero*. The authors suggested that this structure, even if not well-identified anatomically in the sheep brainstem, could correspond to the KFN, previously defined as the core of the "pneuomotaxic center" (18).

Histological and Immunohistochemical Personal Findings in SIDS

The new etiopathogenic interpretation of SIDS here presented is validated by the findings obtained over many years of personal studies on sudden intrauterine unexplained death syndrome (SIUDS) and SIDS. The "Lino Rossi" Research Center of the Milan University has, in fact, collected a large number of sudden fetal and infant death cases, in application of the Italian Law 31/2006 "*Regulations for diagnostic post mortem investigation in victims of the SIDS and unexpected fetal death*" (19). This law stipulates that all infants who died suddenly in Italian regions within the first year of life and fetuses that die after the 25th week of gestation without any apparent cause, must be rapidly

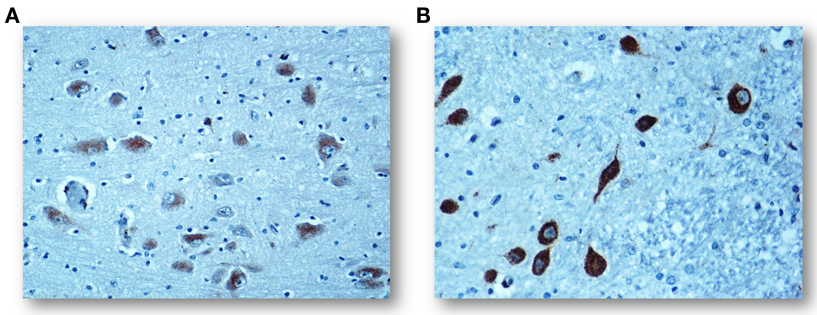

FIGURE 3 | (A) Regular negative/weakly immunopositive BDNF expression in the cytoplasm of the KF neurons in a control infant (3-month-old). **(B)** Intense immunopositivity of the KF neurons with dark brown cytoplasmatic staining in a SIDS case, died at 4 months of life. BDNF immunostaining. Magnification: 20×.

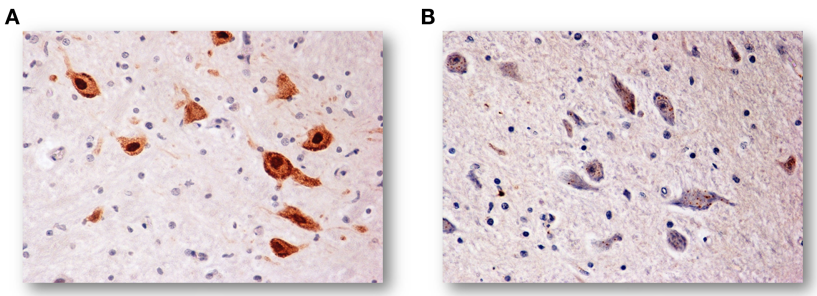

FIGURE 4 | (A) NeuN-immunoreactive neurons of the KFN in an infant of the control group (2-month-old). The staining is particularly strong in the neuronal nucleus but also the cytoplasm and the proximal part of the processes are immunoreactive, though to a lesser intensity. **(B)** NeuN-immunonegative KF neurons in an age-matched infant died of SIDS. NeuN immunostaining. Magnification: 20×.

submitted, after obtaining informed parental consent, to in-depth anatomopathological examination, particularly of the autonomic nervous system, with the components of the RN as the main object of study.

Permission from the Ethics Committee was not required for this study as the "Lino Rossi" Research Center is the national referral center for the sudden unexplained fetal and infant deaths, in accordance with the above mentioned Law 31/2006.

In a large number of SIUDS (up to now no. 95 cases, aged from 25 to 40 gestational weeks), SIDS (no. 150 cases, 1- to 11-month-old), and age-matched controls (no. 35 fetuses and 30 infants), I found developmental hypoplasia of the KFN only in late fetal unexplained deaths (20%), never in newborns and infants (**Figure 2**) (20). This means that a normal structure of this nucleus is absolutely essential for vital breathing from birth. However, in a high percentage of SIDS cases (no.105, 70%), and not in infant of the control group, despite a normal morphological cytoarchitecture of the KFN, neurochemical alterations, such as an unusual immunopositivity of the brain-derived neurotrophic factor (BDNF) and a decreased expression of the neuronal nuclear antigen (NeuN) were highlighted in the KF neurons (**Figures 3** and **4**) (21, 22).

The BDNF is a member of the neurotrophin class of growth factors required for the normal development and maturation of specific brainstem centers involved in respiratory control, and therefore expressed in both inhibitory and excitatory neurons of the KFN during the biphasic breathing modulation in intrauterine life (23, 24). After birth, the BDNF is unexpressed in the KFN in human control newborns, while the positivity, as observed in SIDS cases, seems to hinder any ventilatory activity.

By contrast, the NeuN is a protein expressed in post-mitotic functional neurons (25). Then, the specific immunohistochemical method can be applied in neuropathological studies to highlight the physiological status of the neurons (26). While intense NeuN expression is shown by healthy neurons, a considerably reduced NeuN immunopositivity in postnatal life can be indicative of a degeneration of differentiated neurons.

Correlation of Findings with Nicotine Absorption

In general, morphological and functional brainstem abnormalities resulted significantly related to severe injuries, such as hypoxia. Indeed, the majority of mothers, and often also fathers, of infants with altered KFN development were found to be smokers, either by their own admission, or based on positivity of the cotinine test in the hair of the victims. In case of maternal smoking in pregnancy, carbon monoxide, a gaseous combustion product of nicotine, easily crosses the placental barrier by passive diffusion, and binds to fetal hemoglobin, so giving rise to carboxyhemoglobin (COHb). The same chemical bond occurs when an

infant inhales considerable amounts of environmental smoke. COHb is not able to release oxygen into tissues, leading to a general hypoxic status (27). Besides, nicotine is one of the few lipid-soluble substances that can go beyond the blood–brain barrier and induce specific molecular alterations in the DNA, RNA, and antigenic proteins of the nervous cells (28, 29).

CONCLUSION

Based on these observations, I propose that during the first months of life, when a predisposed subject is particularly vulnerable, hypoxia can unexpectedly switch on again the ancestral fetal behavior designed to suspend respiration, being this as a non-essential activity, through a functional degeneration of the KF neurons and then a depression of the central respiratory control. This is a protective reflex in the womb but a rapidly fatal device in postnatal life.

Clearly, further studies are required, specifically designed to address this exciting theory that offers consistent assumptions to explain the pathogenetic mechanism occurring in a substantial group of SIDS.

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Updates on the Methodological Approaches for Carrying Out an In-Depth Study of the Cardiac Conduction System and the Autonomic Nervous System of Victims of Sudden Unexplained Fetal and Infant Death

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This article contains a set of protocols for histopathological techniques that can be used for carrying out in-depth studies of cases of sudden infant death syndrome and sudden intrauterine unexplained fetal death syndrome. In order to enable researchers to advance hypotheses regarding the causes of the unexpected death of infants and fetuses, the authors propose three innovative and accurate methodologies for studying the cardiac conduction system, the peripheral cardiac nervous system, and the central autonomic nervous system. Over the years, these protocols have been developed, modified, and improved on a vast number of cases which has enabled pathologists to carry out the microscopic analyses of the structures which regulate life, in order to highlight all the possible morphological substrates of pathophysiological mechanisms that may underlie these syndromes.

In memory of our research professor Lino Rossi (1923–2004).

Keywords: SIDS, SIUDS, cardiac conduction system, brainstem, technical protocol

INTRODUCTION

In 1987, the first case of sudden infant death syndrome (SIDS) was delivered to the Institute of Pathology, University of Milan, Italy, as an object of study.

Since then, our researchers have written a large number of scientific articles and books, and held conferences and training courses on SIDS and sudden intrauterine unexpected fetal death syndrome (SIUDS) (1, 2).

Most of their research has been based on histological slides analysis, carried out with histopathological techniques that were intentionally and progressively designed to study the reasons behind the sudden deaths of the young victims.

The closure of the Institute of Pathology, the foundation of the "Lino Rossi" Research Center for the study and the prevention of unexpected perinatal death and SIDS, University of Milan, as well as the enactment of Italian law No. 31 of February 2, 2006 have led us to examine a considerable

number of cases, including control cases, in order to monitor the protocols continually.

MATERIALS AND METHODS

The diagnostic protocol for carrying out anatomic–pathological and forensic–medical investigations on SIDS and SIUDS victims during the last 3 months of pregnancy includes three investigation procedures:

1. The anatomic–pathological investigation.
2. The molecular genetics investigation.
3. The toxicological investigation.

All the technical protocols used for this study strictly concern anatomic–pathological investigation (3, 4).

For each case, it is essential to have family medical records, documents stating the place of death (in the event of SIDS), the informed consent of the victim's parents, as well as the anatomical samples required for carrying out the investigations.

Downloadable forms are available online: http://users.unimi.it/centrolinorossi/en/protocollo_diagnostico.html.

The anatomical samples required specifically for the study are:

- the thoracic block, composed of both lungs, larynx, trachea, esophagus, thymus, the whole heart, within the pericardial sac, and part of the diaphragm;
- encephalon;
- thoracic spinal medulla.

According to Title X° of Italian D. Lgs 81/08, "Behavioural Rules in Biological Laboratories," the anatomical samples must be immersed in 10% neutral-buffered formalin solution, placed in a hermetic plastic container, in order to avoid operator risks, and marked with patient identifiable information and the medical record number of the hospital in which the case originated.

When a case is accepted, the doctor checks both the data and anatomical samples provided and dictates a description of the organs to the technician.

After full fixation, the doctor dissects the organs: he cuts standard samples, that will be processed and treated according to routine histological techniques (5–8), as well as samples for carrying out specialized diagnostic analyses and research procedures (9–12).

Peculiar Specimens to Be Taken

The specimens (13–15) excised specifically for studying the cardiac conduction system (CCS) are:

- sinatrial (SA) node;
- atrioventricular (AV) system.

The specimens (16) required for studying the autonomic cardiorespiratory nervous system are:

- mediastinal plexuses (coronary, intertruncal);
- intercarotid receptors;
- cervical sympathetic ganglia (stellate ganglion);
- thoracic spinal medulla (T1–T5);
- samples of brainstem.

In the event of SIUDS, fetal adnexa may be observed, both in anatomical samples and on histological slides.

The specimens excised for fetal adnexa are:

- one roll of free amniochorionic membrane (two rolls, if infection is suspected);
- three segments of umbilical cord;
- the area below the umbilical cord;
- the main ramifications of amniochorionic vessels;
- a macroscopically undamaged cotyledon from the third central placental disk;
- all areas that the anatomic-pathologist considers to be of interest;
- all changes in caliber and shape of the amniochorionic and funicle vessels (17).

As for routine samples, these samples are processed with an automatic processor.

STEPWISE PROCEDURES

The Basic Histological Techniques

There are fundamental steps that a technician should take in order to obtain optimal outcomes (18).

The basic steps are as follows.

1. *Fixation*: this step is required to prevent postmortem changes such as autolysis and putrefaction. For organs obtained from an anatomy theater, the most used fixative is 10% neutral-buffered formalin solution (in phosphate buffer), commercially produced, due to the risks involved at maximum concentration (H311, H331, H351, H371, H314, and H317).
2. *The pathologist's sampling methods*: according to an established protocol, it is essential to cut the organs into thin (4 mm thick) slices, so that the fixative will penetrate the tissue within a reasonably short time.
3. *Post-fixation*: after sampling, it is essential to change the old formalin with new formalin.
4. *Processing*: i.e., the anatomic sample undergoes a sequence of graduated transformations, to replace the water contained in the tissue with hardener medium, in order to give support and solidity to the sample itself. Various histopathological methods can be applied for this purpose. In a routine histological technique, this step is carried out by an automated routine tissue processor, which is able to process samples automatically in time periods that range from 2 to 17 h, in relation to the size of the samples (the bigger the sample, the longer the processing time). At the same time, it is possible to process biopsies (minimum size 1 mm) and operating samples (3 mm maximum thickness), individually contained in small closed biocassettes.
5. *Embedding*: i.e., to make the sample into a geometrical shape, with a possible support (ring or cassette), which can be firmly fixed to the specimen holder of the microtome. Embedding is always carried out with the aid of a device, the complete embedding center, which dispenses hot paraffin

(56–58°C) and subsequently cools the block, placed into standard base mold.

6. *Cutting with the microtome*: the final sample is cut into 3 μm thick sections (1 μm = 1/1000 mm). The microtomes most widely used in diagnostic anatomic pathology laboratories are rotary, manual, or automatic microtomes that are very precise as they can cut extremely thin sections, while the most suitable microtome for the protocols described in this article is a steady and heavy sledge microtome, with spacers on the knife block, for cutting wider than standard blocks, as some of the blocks are over 4 cm wide (standard thickness is 0.3–0.5 cm).
7. *Extension of the sections*: the thin sections cut with the microtome are immersed in a flotation bath, containing water heated to 37°C, to aid extension, and they are then placed on port-object slides, countermarked with the case number and the number of the specimen. The slides are then placed into a 37°C oven, overnight, to improve the adherence of the section to port-object slides [it is also advisable to add chemical adhesive [FIX-ON, Kaltek (PD), code 0872] to the water in the flotation bath]. The drying time of a histological slide can be shortened by placing the slide in oven at 60°C, for 30–60 min.
8. *Staining*: the sections obtained must undergo "staining" procedures for viewing them under a microscope. The various staining procedures are histological staining (to see the morphology of the sample), histochemical staining (to see particular components of various tissues), and immunohistochemical staining (when a specific cytoplasmic and/or nuclear component of the tissue or the cell must be branded). In routine procedures, technicians generally use histostainers and immune-histostainers which have numerous colorations and reactions, but not all. There are also automatic devices for mounting slides, instead of technicians.

As a matter of routine, it is essential to standardize the samples as much as possible and use several types of scientific equipment in order to speed up the setup time of the histological slides. A histological/histopathological slide can be ready in about 2 days.

Technical Protocol

The peculiarity of this technical research, for studying the CCS and the autonomic central and peripheral nervous system, is that the tissue components are not macroscopically visible, and they have a tridimensional development, therefore, in order to pinpoint and study them, it is essential to cut the blocks with the microtome for most or all of the block of the research.

This is known as the "seriation" technique, which is carried out using a microtome.

As you can see in **Figure 1**, there are two "seriation" techniques:

1. Seriation at prearranged intervals: i.e., when the technician discards anatomic matter between one level and next, which is the method used for the CCS.
2. Continual seriation: i.e., the technician slices extremely thin sections (only a few microns thick) one after the other, without prearranged intervals, which is the method used for the central and peripheral nervous system.

At the frosted-end of the port-object slide, the technician writes the case number, the sample number, the level number, and the sequence of the section in the level.

Technical Protocol for Studying the Cardiac Conduction System

The tissue block sampling method for studying the CCS was developed by Professor Lino Rossi in 1955 (AV system) and in 1958 (SA). The technical procedure is described in the appendix of his book "Arrhytmologic Pathology of Sudden Cardiac Death" (19).

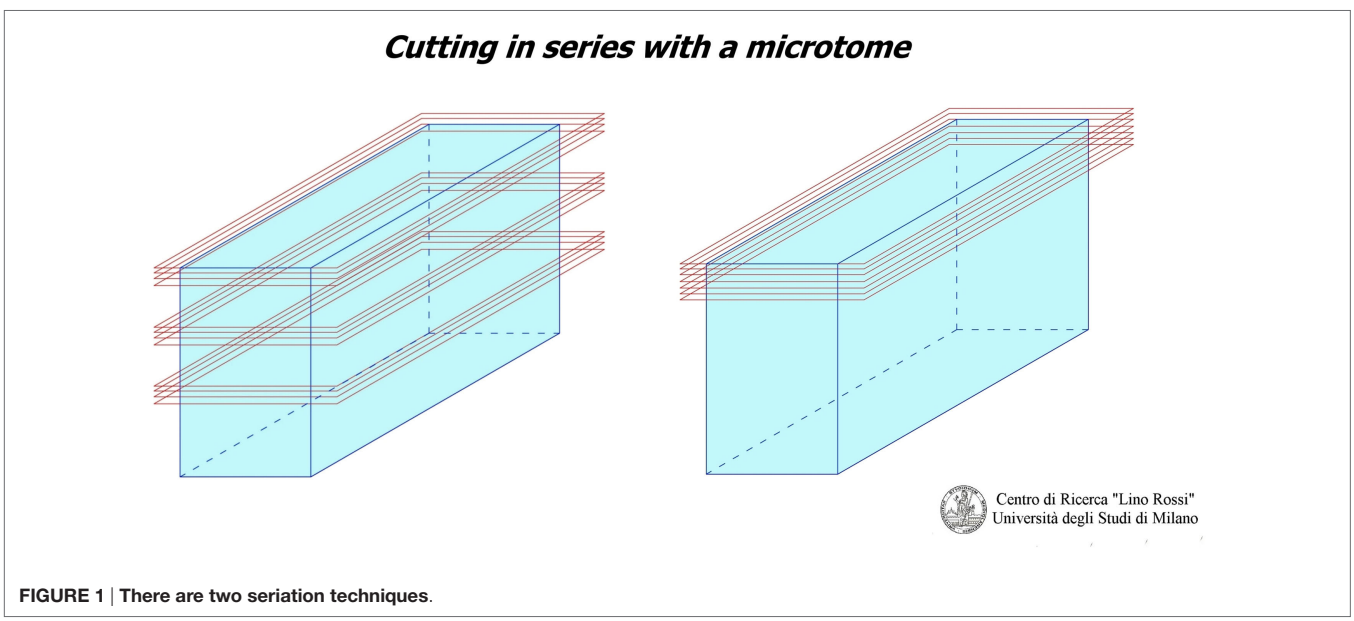

FIGURE 1 | **There are two seriation techniques**.

Professor Lino Rossi left his technical methodology to the authors who "have correctly interpreted his expectations" in over 20 years of work experience and are capable of speaking and writing on these topics (20).

The first block, SA, "includes the sinoatrial node, its atrial approaches," the terminal crest, and the sinoatrial node ganglial plexus (21).

The second block, AV, includes the AV system and its atrial approaches, as shown in **Figure 2**.

The two blocks, such as SA and AV, will be partially reduced by excising redundant portions (papillary muscles, chordae tendinae, etc.), which will be post-fixed in 10% phosphate-buffered formalin.

With the aim of reducing the number of histological sections, some researchers cut these two blocks into five to six large, parallel slices (22).

The large slices are then processed and embedded by hand, or by means of automatic processors.

The slices of interest can be cut in series with the microtome.

This method is definitely faster even if less accurate than the method developed by Professor Rossi as anatomical material can be lost and the spatial orientation of the structures of interest can be hard to define.

Once fixation is complete (3–14 days according to specimen volume): in order to simplify this process, it has been summarized as follows.

All of the following steps are performed manually, because these samples are very long and wider than routine samples.

The samples must be rinsed under running water for approximately 20 min in order to remove the formalin. The tissue is transferred to 95% ethanol for 24 h and immersed in pure 1,4-dioxane (diethylene dioxide) four times for 12 h each treatment, for dehydration and clarification.

The samples are then partially impregnated in 1/3 dioxane-2/3 paraffin (fusion point 56–58°C) and then totally impregnated in pure paraffin (fusion point 56–58°C), in oven (**Figure 3**).

Professor Lino Rossi made a wise decision by using dioxane as it is both a dehydrator and a paraffin solvent and is therefore essential for this conduction system technique: on the other hand, if the routine ethylic dehydration method is used, the material tends to harden, resulting in breakages during microtome sectioning.

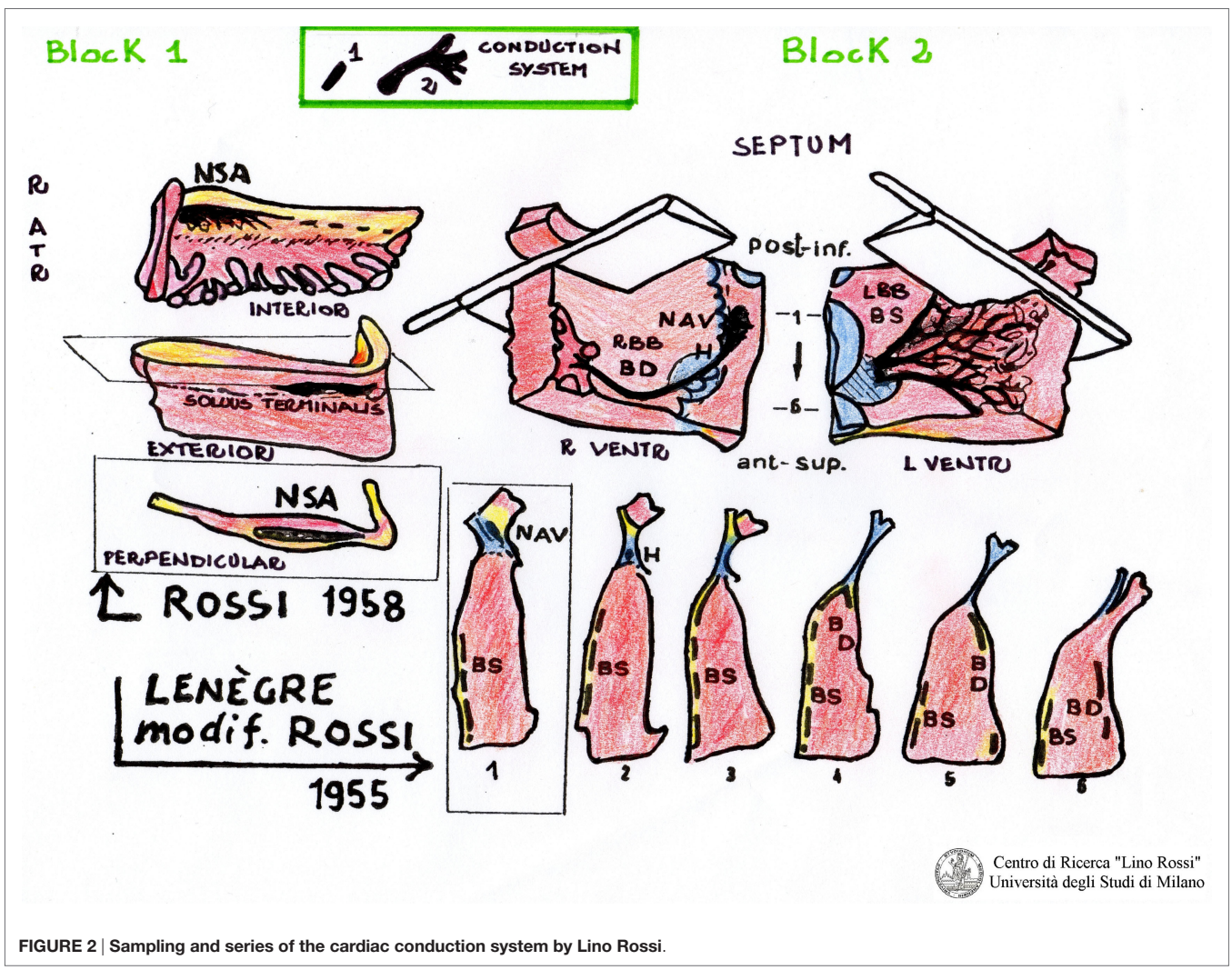

FIGURE 2 | Sampling and series of the cardiac conduction system by Lino Rossi.

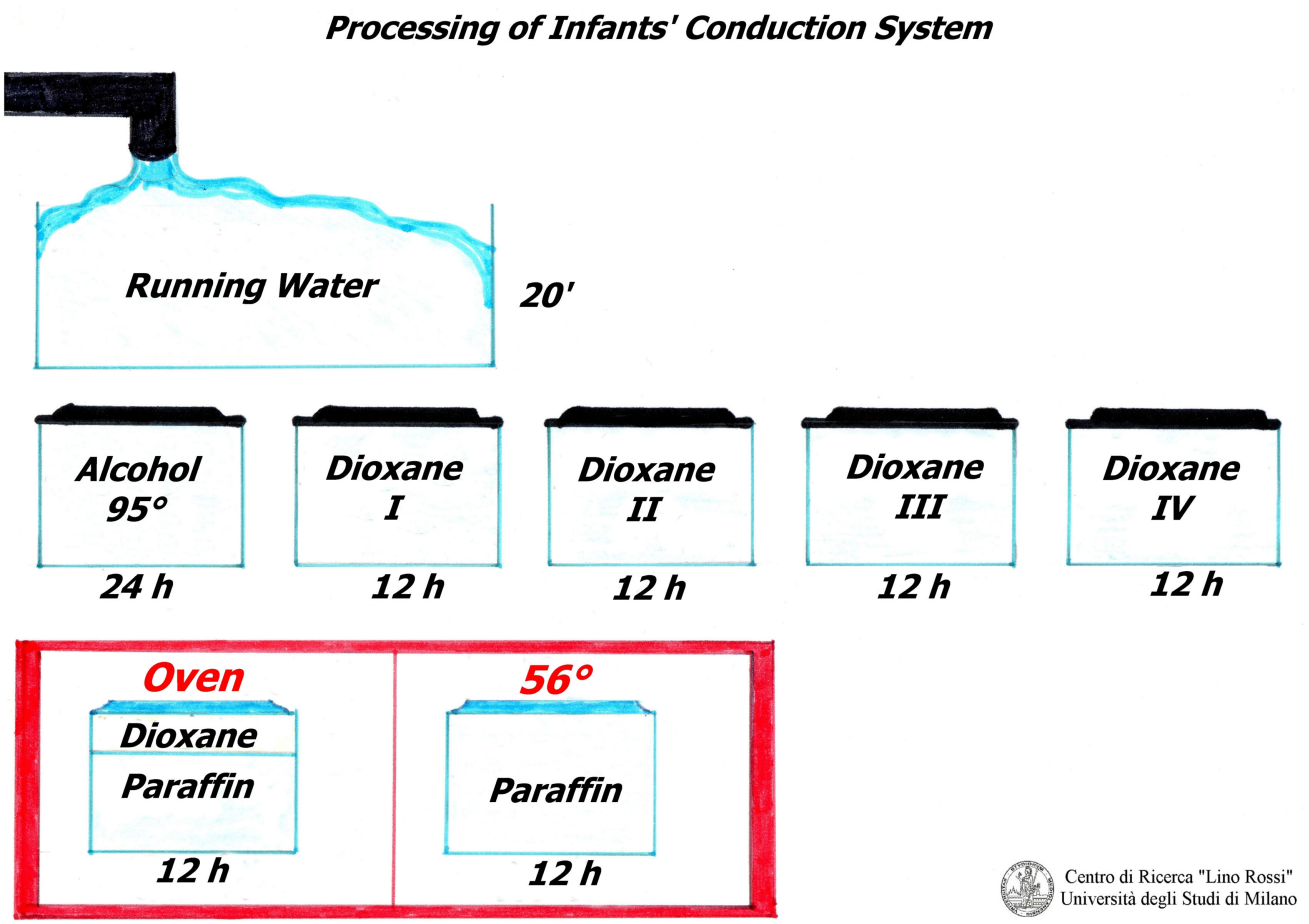

FIGURE 3 | **Processing of the cardiac conduction system**.

In 1931, Graupner and Weissberger introduced successfully dioxane; the only drawback being that it costs approximately three times more than ethanol.

Following the dehydration process, tissue embedding in paraffin is carried out with metal molds (Leuckhart's "L" pieces), if the specimens are oversized.

The same technique is used for new-born babies and fetuses, although at times it is difficult to excise the SA and AV blocks in fetuses of low gestational age.

A whole fetal heart can be embedded with this protocol, taking care to extend the process phases.

The inclusion must be carefully oriented.

For the SA node, it is essential that the pectinates muscles point upwards, as shown in **Figure 4**.

For the AVS, the aortic semilunar valves should be placed opposite the point of incidence of the cut.

The blocks must be stored in a refrigerator.

As shown in **Figure 5**, the surface paraffin is previously removed from each inclusion, and molded, around the specimen with a scalpel in order to facilitate cutting with a microtome and to improve its distension in warm water.

It is advisable to make the inclusion fairly high up, so that it can be firmly fastened into the holder of the microtome; wood or plastic supports should be avoided as they tend to come off.

As already mentioned, the serial section is essential for investigating conduction system as it allows for a three-dimensional reconstruction of the AV tissue formation under study.

Due to the dimension of the specimen, it is advisable to carry out intercalated section every 20–40 μm interval, thus collecting three 8 μm sections at individual level; it would be rather time-consuming to carry out a global serial section of the entire specimen on a routine basis in addition to the work and material (**Figure 6**).

Following the microscopic control of the slides, one can decide whether to terminate the analysis. In this case, the residual inclusion could be stored for further control.

The serial sections can only be carried out with normal manual sledge microtomes, adapted for tall specimens.

The sections obtained are then distended in 37–40°C warm water, in a flotation bath, containing a chemical adhesive.

The sections for histochemical analysis are collected on clean frosted-end slides, while the sections for immunohistochemistry

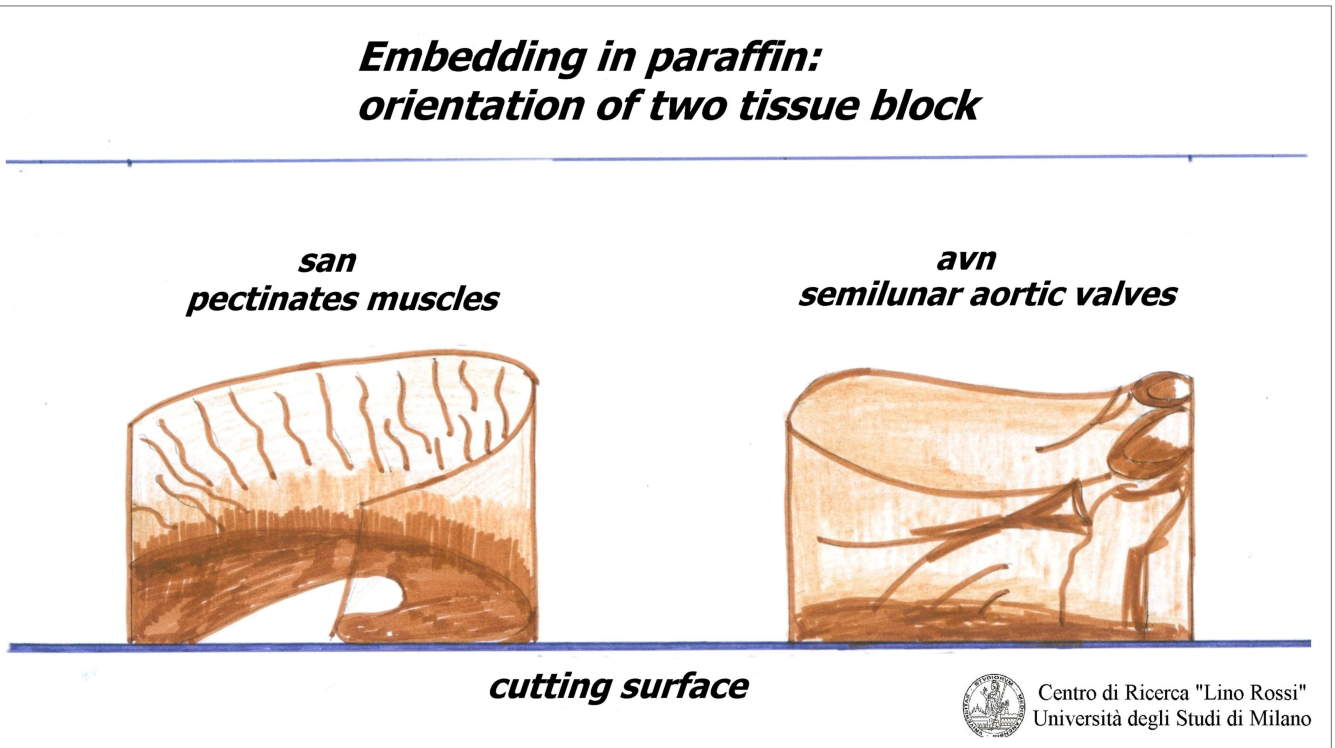

FIGURE 4 | **Embedding of SA and AV**.

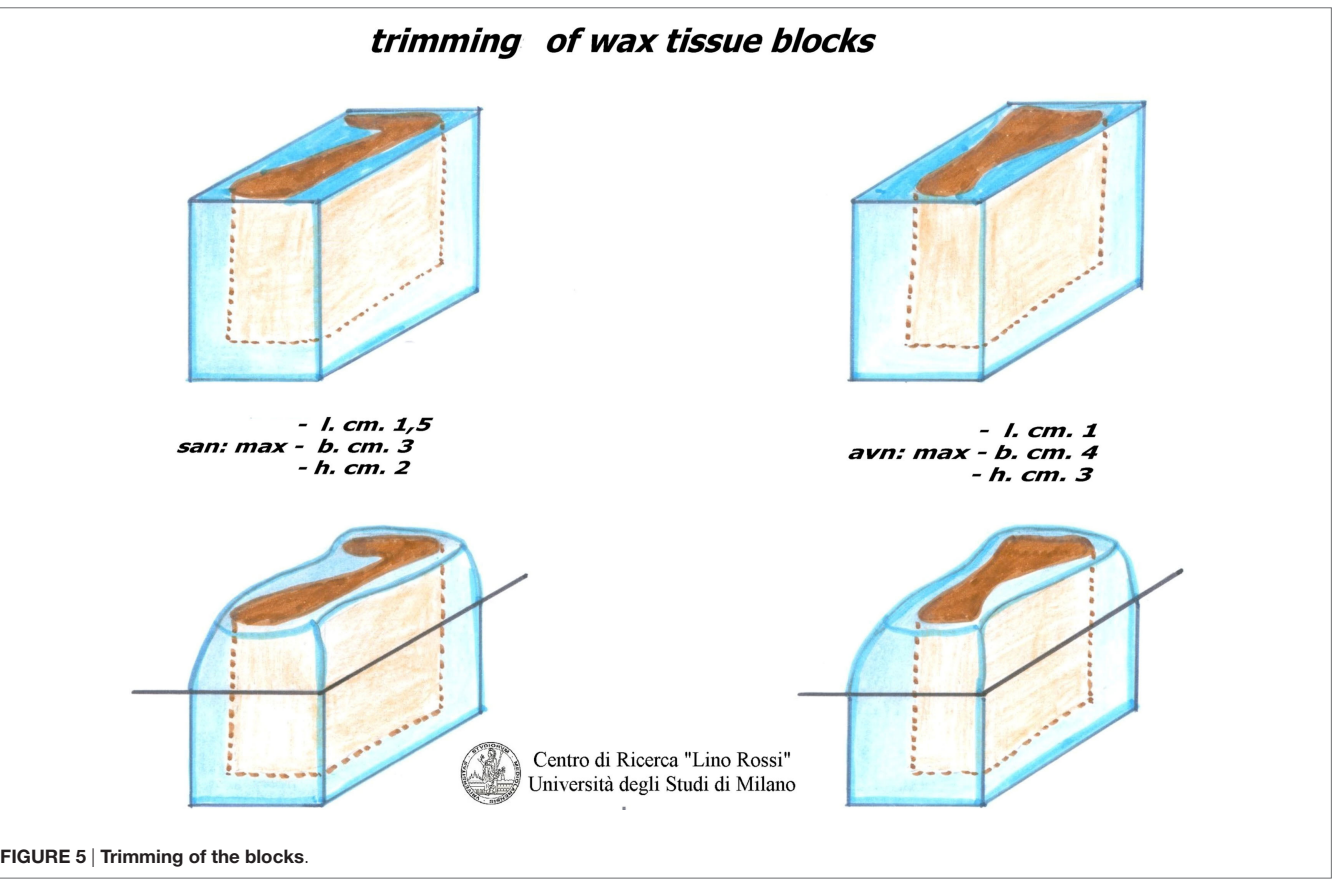

FIGURE 5 | **Trimming of the blocks**.

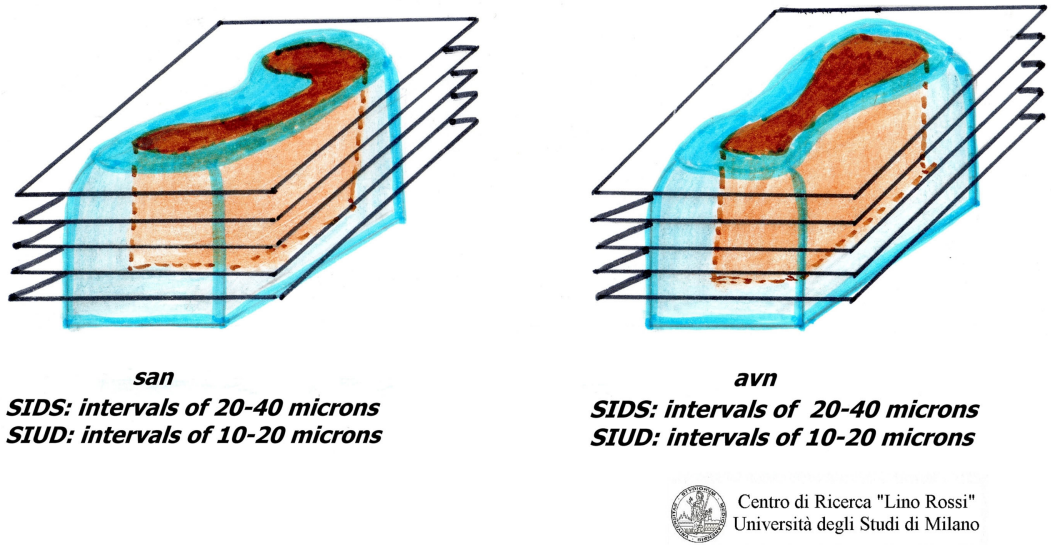

FIGURE 6 | Cutting of the blocks.

are placed on slides pretreated with 3-aminopropyltriethoxysilano (silanized) (Sigma-Aldrich, code A 3648).

Both types of slides must be marked with the case number, the inclusion number and the level sequence number, with "silanized" or "unsilanized" indicated on the slide which is, then placed in an oven heated to 37°C and left overnight.

The slides, to be stained for apoptosis (Apoptosis detection kit, Chemicon International, Temecula, CA, USA, code S 7101), must be put back into the oven heated to 60°C for 4 h in order to improve the adherence of the finely cut tissue into the glass slide, which is of great importance.

It is not necessary to stain all of the level slides (for SA 70-100, for AV 100-130) in order to prescreen. It is sufficient to select specimens every five levels and stain them using the hematoxylin–eosin (H&E) and Heidenhain staining method for connective tissue (AZAN) alternately (**Figure 7**).

Hematoxylin–eosin is probably the most widely used histomorphological staining procedure. This staining method is able to demonstrate an enormous number of different tissue structures, including conduction system structures: the hematoxylin stains the cell nuclei blue, and outlines the nuclear details, while eosin stains cell cytoplasms and connective tissue fibers various shades of pink and orange.

The stains are made in our laboratory: Mayer's hematoxylin, an alum hematoxylin, chemically ripened with sodium iodate, and eosin 0.5%, both in aqueous solutions.

The second, Heidenhain's AZAN (1916), is a variant of Mallory's method. It is a trichromic staining procedure for connective and muscular tissues. This technique stains nuclei and erythrocytes red, muscles orange–red, and collagen various shades of blue.

The term AZAN is derived from the first syllables of AZocarmin G (Color Index 50085, Sigma-Aldrich, code A-1091) and ANilinblue water soluble (Color Index 42755, VWR International, code ACRO 229661000)-Orange G (Color Index 16230, Carlo Erba Reagents, code no. 13), which are the names of the principal dyes used.

In order to obtain good results, one should overstain the myofibril conducting muscle with azocarmin, preheated in oven at 55–56°C, and differentiate quickly with anilin oil, thus blocking it with diluted acetic acid and then mordanting with phosphotungstic acid before staining in anilinblue-orange G (for a combination) (23).

Heidenhain's trichrome clearly differentiates the common myocardium, which is colored with orange G in bright red, from the specific myocardial which appear pale, with the same dye, due to the presence of fewer contractile fibers, while the connective tissue assumes an anilinblue staining that is a more or less intense bright blue (**Figure 8**).

As for H&E, all the staining solutions of AZAN are made in the laboratory.

The blank slides are archived for carrying out follow ups of the investigations.

Depending on the abnormalities observed, it is possible to use blank slides for additional stainings.

The histochemical stainings, Periodic Acid Schiff (PAS) and Alcian Blue 8GX (Color Index 74240, Sigma-Aldrich, code 05500) at pH 2.5, are used to point out the cartilaginoid hypermetaplasia of the central fibrous body of the heart of infants who died of SIDS.

Alcian blue is also useful to highlight mast cells in tissue: cytoplasm deep azure with red nucleus in a light blue background.

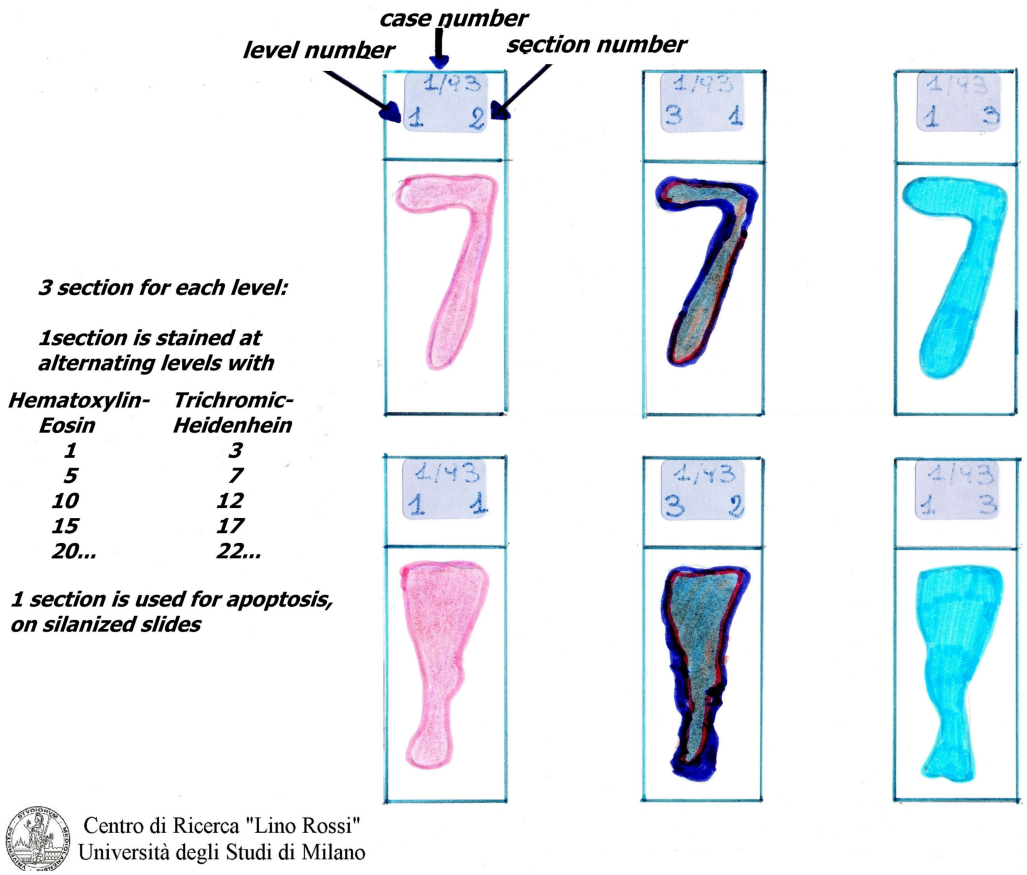

FIGURE 7 | **Routine staining of the CCS**.

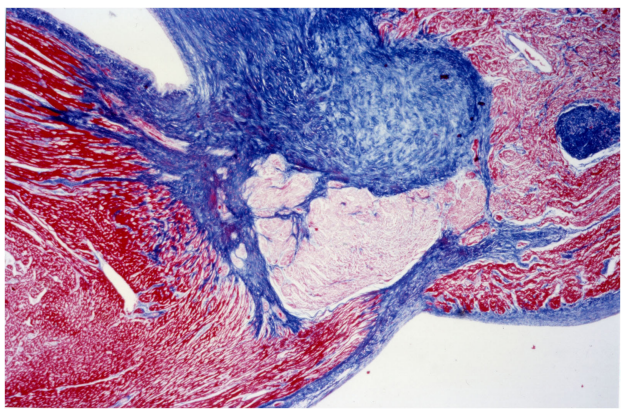

FIGURE 8 | **AV system, His bundle: staining difference between common myocardium and specific myocardium, AZAN, 4×**.

According to Frank B. Mallory, following the modified method of Pathology Institute of American Armed Forces (A.F.I.P.), satisfactory results are obtained with Phosphotungstic Acid Hematoxylin (P.T.A.H.) instead of, or in association with AZAN.

This staining is suitable for the central nervous system and the heart. The results of the staining are nuclei, fibrin, fibrils of neuroglia and the myoglia, the fibroglia and the contractile elements of striated myocardial and skeletal muscle blue; collagen, reticulum, elastin, cartilage and bone matrix are yellowish to brownish red (**Figure 9**).

Weigert's method is suitable for staining elastic fibers black or deep brown, on a yellow-red background according to Van Gieson staining for gross connective and muscle tissue, yet it is seldom used as it is a contrast-staining and generally fades away after two or three years.

The Resorcin–Fuchsin method provides satisfactory results for elastic fibers (1898).

Good results were obtained with the "slow Eosin" (very diluted, a couple of drops in a glass of water, approximately 0.1%) for the differential staining of leukocytes in eosinophilic myocarditis.

The silanized blank sections may be suitable for immunohistochemistry such as proliferating cell nuclear antigen (PCNA)

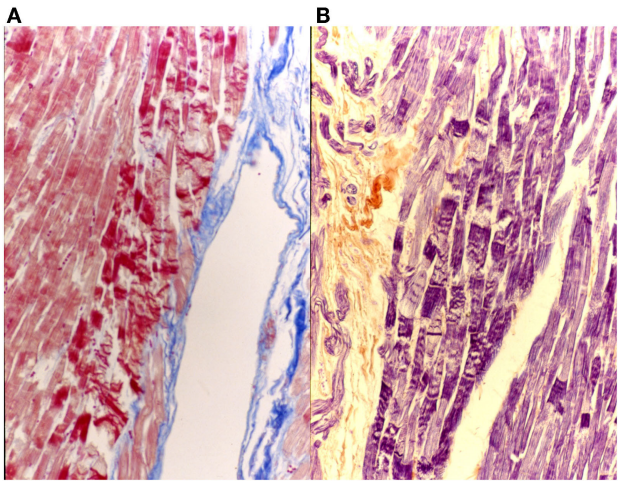

FIGURE 9 | Myocardial tissue suffering hyperacute ischemia (initial contraction bands). On the left **(A)**, AZAN 40×; on the right **(B)**, P.T.A.H 40×.

(Novocastra Reagents – Leica, code NCL-L.PCNA), Apoptosis (TUNEL assay), S 100A1 (Dako, code Z0311), c-Fos (Santa Cruz Biotechnology, code S52), Sarcomeric Actin, clone Alpha-Sr-1 (Dako, code no. M 0874) (24, 25).

Technical Protocol for Studying the Peripheral Cardiac Nervous System

As far as the peripheral cardiopulmonary nervous system is concerned, adequately countersigned, mediastinic plexuses, intercarotid receptors, and stellate ganglia are fixed in 10% neutral-buffered formalin.

For infants and fetuses, it is advisable to carry out sampling on previously fixed material.

The sampling of medistinic plexuses consists in removing the fibroadipose tissue between the pulmonary artery and the aorta and between the aortic arch and the hilum of the lungs (intertruncal plexuses). The coronary plexus is in the roots of the coronary artery. In infant and in fetus sampling is limited.

The carotid body is located near the carotid bifurcation, beside the internal carotid, in the vicinity of the carotid sinus. The carotid body is a differentiated paragangliar organelle, while the carotid sinus is an anatomic area of the middle tunica of the artery, in which many nerve endings are present.

Sympathetic cervical superior ganglion is located near the intercarotid block. The middle sympathetic cervical ganglion is difficult to find, while the inferior sympathetic cervical ganglion is joined to the first thoracic ganglia to form the stellate ganglia, near the branch of the vertebral subclavian artery.

Samples should be thoroughly rinsed with running water immediately after fixation with 10% neutral-buffered formalin.

Regarding the peripheral cardiac nervous system, the brainstem and thoracic spinal cord, the dehydration of these tissues differs to that observed for the conduction system study. The ablated fragments have a soft consistency (fibro adipose tissue) therefore it is advisable to use a suitable dehydration to harden up the specimens. For this purpose, ethylic alcohol was used at increasing concentrations (70%, 95% in two changes, 100% in two changes), followed by xylene, in two changes, and embedding into two changes of pure paraffin at fusion point 56–58°C, in the oven.

The procedure used for the brainstem can also be used for these samples (as shown in **Figure 12**, below), but the samples are kept in the solvents for less time (1 h and half instead of 3 h). The second change in alcohol 95% is overnight, also for the second change of xylene.

It is advisable to make these changes manually since the dimension of the specimens may vary.

Embedding is then carried out following a precise protocol.

As shown in **Figure 10**, the plexuses must be embedded by pressing the fibroadipose tissue onto the cutting surface in order to visualize the maximal surface of each section; in this way, we are more likely to be able to see the microscopic nervous receptors.

The ganglia can be embedded whole, or can be subdivided according to length and analyzed together.

Only a flat embedding of the carotid bifurcation, right and left separately, provides a good view of specimens, since the carotid glomus is located between the two carotid branches, a little above the bifurcation, as the sinus is part of the internal carotid root.

The embedding of plexuses, ganglia, and bifurcations must be carried out with embedding rings, which are more stable than disposable cassettes, because these specimens are small in the fetuses and infants.

After cooling in a refrigerator, the upper paraffin layer should be taken off the blocks obtained and scalpel-trimmed in order to reduce the peripheral paraffin surplus surrounding them, thus facilitating section distension during microtome cutting.

The blocks must be cut into series, to allow for three-dimensional reconstruction and/or to increase the likelihood of finding the neuro-receptor, since these structures have numerous individual variants.

Conventionally, the serial sections at plexus, ganglia, and carotid bifurcation are six sections for each level, the individual sections are 5 μm thick (two for H.E. staining and four blanks), without intervals. The specimens should be cut whole.

Which is the best histologic staining technique?

The H&E as a screening, for everything.

An argyrophil silver technique, such as the Grimelius silver method, can be useful for demonstrating the granulations in the receptorial cells of the small ganglions present in the plexuses, the glomera and the ganglia.

These granulations are able to link with silver alogenures, which must be reduced with a reducing reagent, by transforming silver salt into metallic silver with a solution containing hydroquinone. The results show dark black granulations on a yellow gold background.

The electivity of Grimelius method is obtained by pretreating silver salts in a 60°C solution, followed by a short 45°C

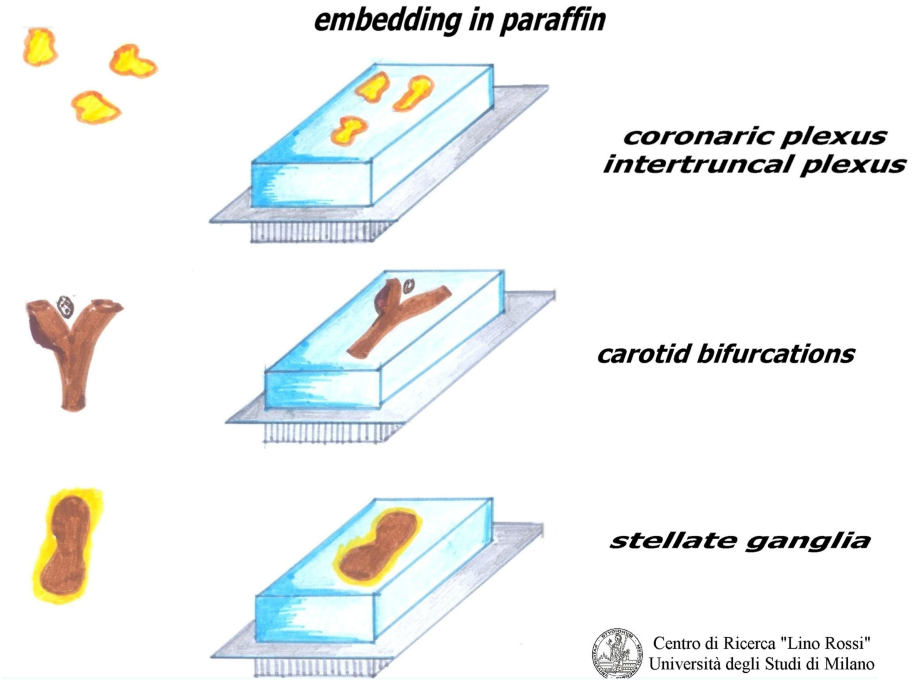

FIGURE 10 | **Embedding peripheric cardiac nervous system**.

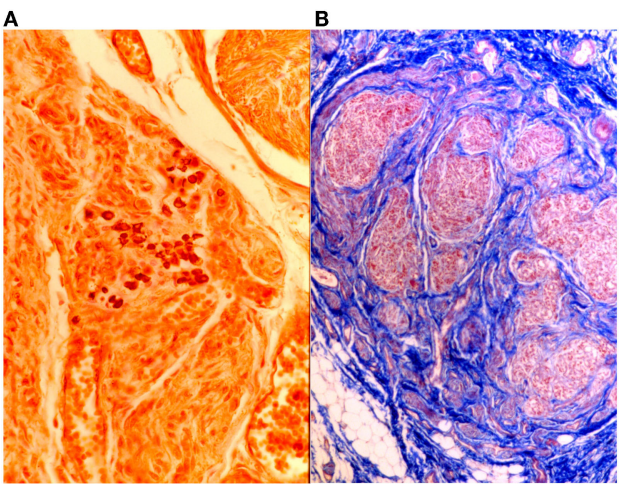

FIGURE 11 | **On the left (A), intertruncal plexus with chief cells rich in cytoplasmic argyrophilic neurotransmitter granules, Grimelius, 40×; on the right (B), carotid body in AZAN, 25×**.

re-impregnation which provides a further metal deposition, thus making the final slide more visible.

Heidenhain's AZAN can also be used to highlight glomic neuroreceptors (**Figure 11**).

Over the last few years, few studies have been carried out on peripheral cardiac receptors due to the continual in-depth studies of the neuronal centers "of the human brainstem, involved in breathing control in perinatal life" (26).

Technical Protocol for Studying the Brainstem and the Thoracic Spinal Cord

The sampling of the brainstem foresees the following four sections:

1. Third lower midbrain – third upper of pons (embedded from pons).
2. Intermediate pons (not to be seriated) (embedded from caudal surface).
3. Third lower pons – third upper brainstem (embedded from pons).
4. Obex (embedded cranial surface).

Figure 12 shows the manual processing of these samples.

After fixation in 10% neutral-buffered formalin, samples must be rinsed under running water to remove formalin.

The work sequence is alcohol 70% for 30 min, alcohol 95% I° for 2–3 h, alcohol 95% II° overnight, alcohol 100% I° 2–4 h, alcohol 100% II° 2–4 h, xylene I° 2–4 h, xylene II° overnight, paraffin I° 2–4 h, paraffin II° 2–4 h, in the oven at 56–58°C.

As the specimens of the brain stem are fairly large, it is advisable to use metal molds as for the conduction system.

The first block is embedded from the pons; the second block is embedded from the caudal surface; however, it is not cut serially if not required; the third block is embedded from the pons; the last block is embedded from the cranial surface (**Figure 13**).

In this way, all the samples are embedded, as it is essential to include all of the anatomical structures.

The samples are embedded with the aim of achieving the control of vital functions before the main structures.

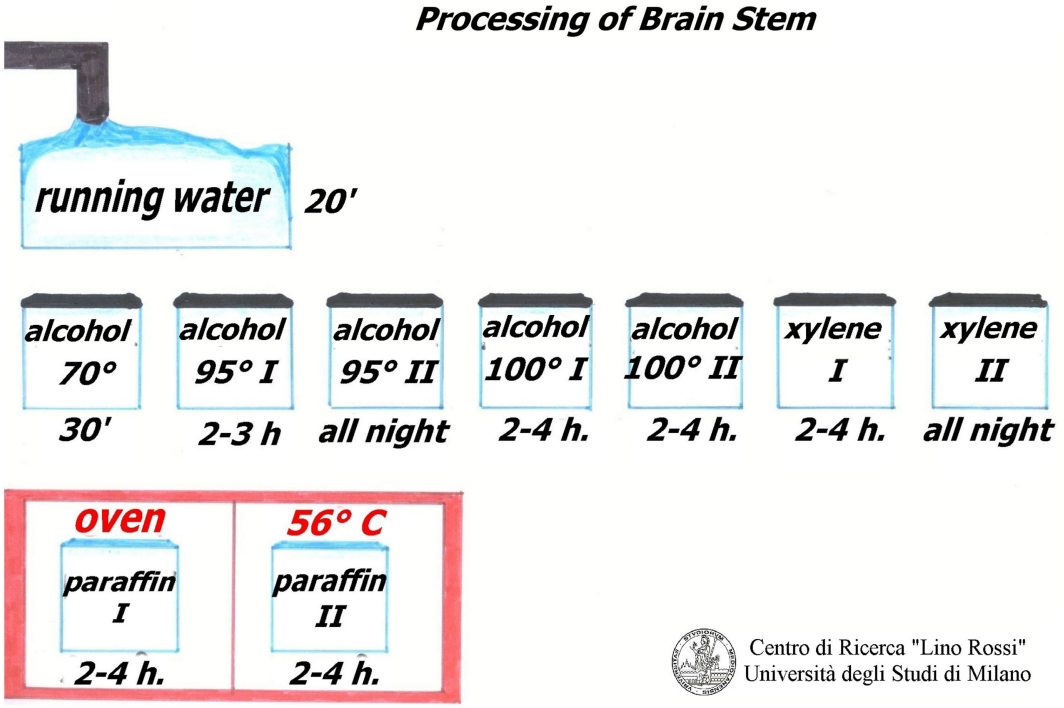

FIGURE 12 | **Processing brainstem and thoracic spinal cord**.

The thoracic spinal cord is excised from the tract between T1 and T5.

It is cut in slices, which are processed together with the brainstem, but with shorter processing times.

The slices of the thoracic spinal cord are put between two sponge pads for biopsy that are then placed in mega-cassettes, in order to avoid movement.

The slices are embedded in the same way, in the correct orientation, for an accurate reconstruction (**Figure 14**).

This procedure helps to reduce the number of slides.

The cutting protocol for the brainstem is as follows: first, the block is cut for DNA extraction; the sections are prepared with a blade cleaned with xylene in order to avoid contamination, while the technician, wearing gloves, cuts six 5 µm thick sections and places them in well-sealed sterile test tubes.

The brainstem serial section is carried out without intermediate intervals, apart from those required for adjusting the surface after necessary interruption.

First, all the blocks are cut for 15 levels each. Following a preliminary examination, it is possible to cut them again, until all the nervous centers have been sliced.

Each level requires 12 6 µm thick sections (4 normal blank and 8 on silanized slides). All of them must be carefully marked and placed in an oven heated to 37°C overnight.

The following day the H.E. sections are screened, one for each level, subsequently further methods will be decided.

This protocol can also be used for the thoracic spinal cord, but only on three levels, and material ranging between 200 and 300 µm in size is discarded from one level and the next.

Klüver-Barrera (KB) (1953) is the most suitable staining for the brainstem. It is carry out with Luxol Fast Blue MBS (Color Index Solvent Blue 38, Sigma-Aldrich, code S3382) which is generally used for staining myelin fibers; the dye is classed as a solvent (oil-soluble) dye, which links components such as lecytin and sphingo-myelin, i.e., phospholipidic myelin substances.

The Luxol counterstain is provided by the Cresyl Violet (Sigma-Aldrich, code C5042), which enables us to obtain a clear image of the nuclear chromatin.

Klüver-Barrera was used for taking morphometric measurements at each suitable level (27).

For highlighting tigroid substance and ribonucleic acid, the best results are obtained with the Cresyl Violet method.

Another popular staining technique is the silver impregnation, Bielschowsky's method, which is used for demonstrating neurons, axons and dendrites by staining them black. First, the sections are impregnated with silver nitrate salts and the deposit is intensified by adding a solution containing silver nitrate and formalin as a reducing substance. Tone with gold chloride and fix with sodium thiosulphate, as the final step.

Another of our methods is the silver impregnation by Gless-Marsland, which is particularly suitable for the brain stem, which stains the neuronal bodies and the neurofibres black or brown, on a lighter bronze-brown background (**Figure 15**).

As already mentioned, Mallory's P.T.A.H. (see above) can be used to stain the glia satisfactorily.

Other staining materials are Lillie for the neuromelanin pigment (28) and Perl's Prussian blue for iron in oxygen-transporting heme mechanism (29).

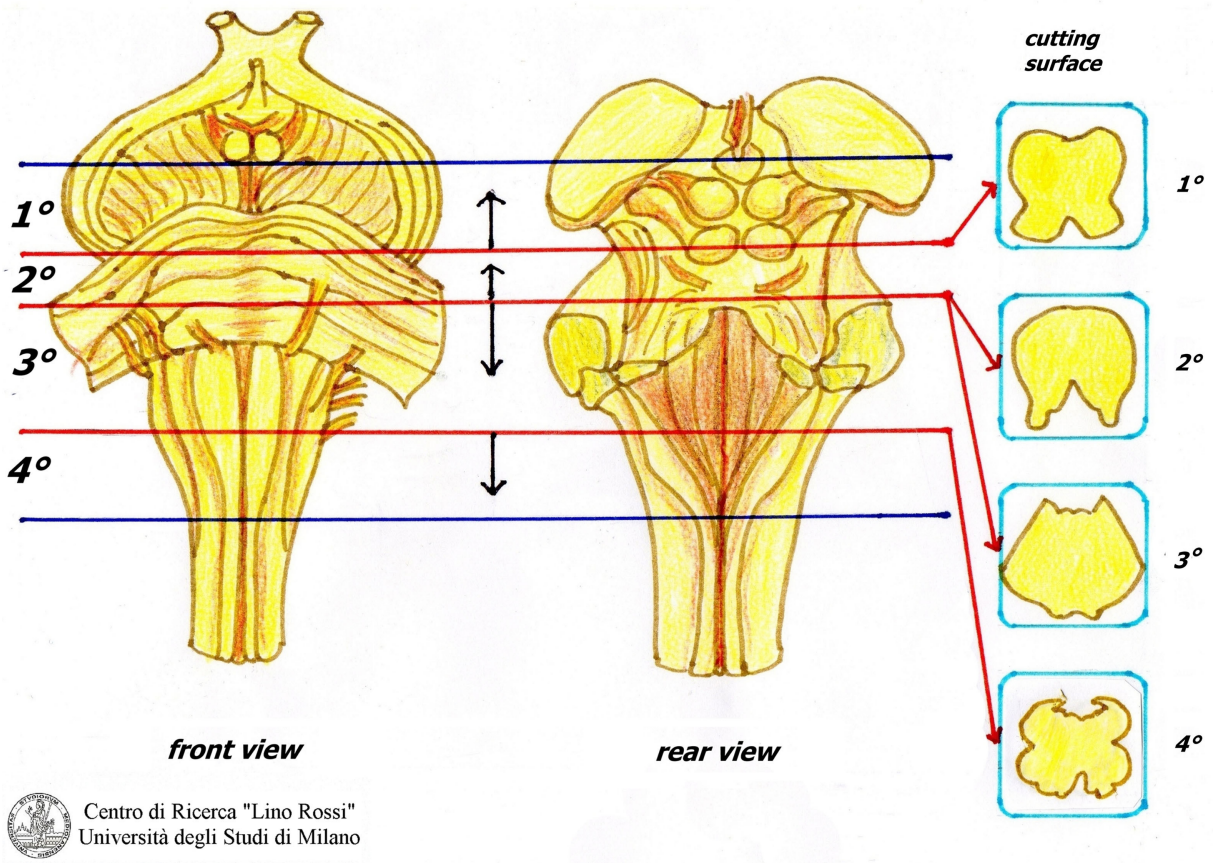

FIGURE 13 | Orientation of brainstem samples.

While for apoptosis, the blank slides must be kept in an oven heated to 60°C for 4 h prior to performing the reaction.

Substances, such as somatostatin (Abcam, ab108456), substance P antibody [DBA, Segrate (MI), Italy, SP, C 191], and tyrosine-hydroxylase (Abcam, ab75875), PHOX2B (H-20) gene (Santa Cruz Biotechnology, SC 13224), and NeuN antibodies (Millipore, S 7100), are used for some of the immune-histochemical reactions (30–32).

Most of the staining solutions are made from basic commercial substances within the laboratory, and the blank slides proposed, correctly labeled and archived, are available upon request for carrying out further staining using appropriate histo- and/or immunochemical techniques.

EXPECTED RESULTS

The regulations of Mortuary Police; paragraph of statute 8, 9, 10 (Italian Law 2 December 1975, no. 644) foresees that "no dead body … may be subjected to autopsy … earlier than 24 hours from the time of death" (33).

This means that the body degenerative events occur as autolysis, self-digestion, and putrefaction by microorganisms.

However, the autopsy is carried out in time, and the chemical fixation of the samples is performed immediately, only slight damage is caused by degenerative phenomena.

Following the technical protocols described in this paper, it should ensure an excellent outcome of the histological slides.

The best way of preserving anatomical structures is through timely and prolonged fixation, manual processing with variable times according to the size of the samples, thickness of sections, and hand-made stainings.

Unfortunately, the damage caused by lack of fixation is irreparable, as in the case of those fetuses who die in the womb.

Inside the heart, large blood clots develop within the cardiac chambers. As soon as possible, before sampling, it would be advisable to open the heart by cutting the tip and cleaning it out.

Lysis phenomena, partial or total degeneration, and bacterial infiltration, up to complete loss of morphological details can occur very severely in the brain, as well as other organs.

These histological slides show the complete loss of morphology and affinities of the dyes, the immune-histochemical reactions are negative and/or diffuse.

Brain liquefaction may occur leading to incomplete diagnosis.

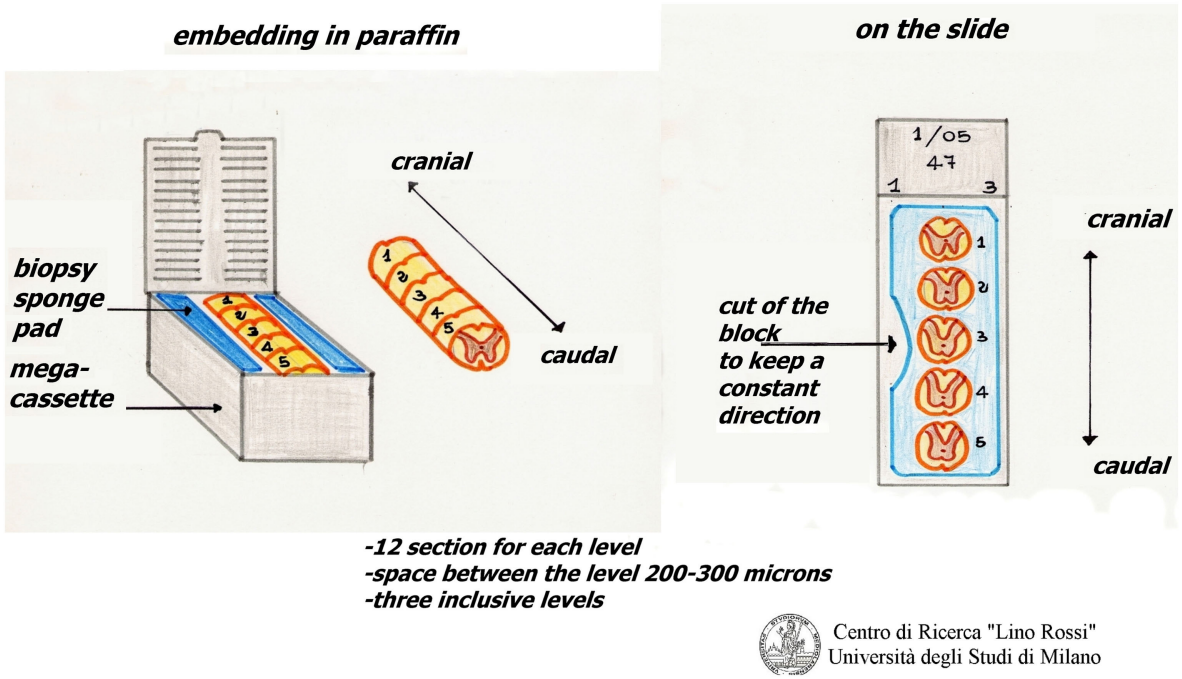

FIGURE 14 | Embedding of thoracic spinal cord.

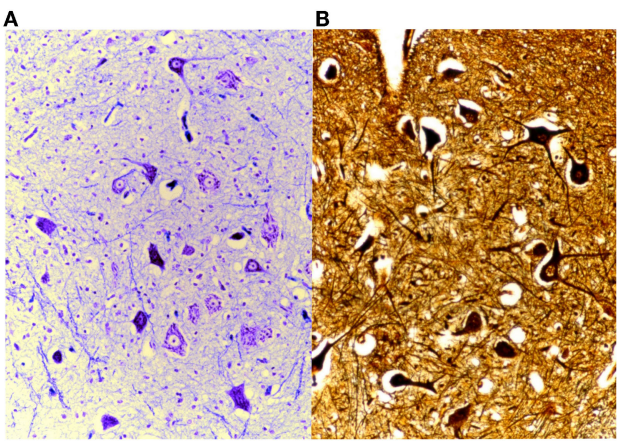

FIGURE 15 | On the left (A), brain stained with Klüver-Barrera, 25× and, on the right (B), Gless-Marsland, 25×.

CONCLUSION

In the event of SIDS or SIUDS, a large number of slides are required in order to carry out an accurate pathological investigation.

A complete case requires approximately 2100 slides of which 488 stained and 1612 blanks, while 1272 slides are require for an incomplete case, including 274 stained.

Approximately 3–4 weeks are generally required to prepare each case.

A large number of slides are required for diagnosis and research: this means that these protocols enable us to carry out in-depth studies on anatomical structures.

The slides are performed before a case is found to be of interest.

The technique is simple but lengthy in the preliminary phase.

For this purpose, a laboratory and specialized technicians are required for manual processing, which differs according to the type of sample. Considerable knowledge is required for this task as well as automated routine techniques.

Using stains made in the laboratory helps to lower costs but it foresees collective and individual protection.

The technician has to seek and love the "perfect slide."

AUTHOR CONTRIBUTIONS

GA wrote the text of the article and MC contributed to the graphic, drawing the figures. All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Defining Sudden Infant Death and Sudden Intrauterine Unexpected Death Syndromes with Regard to Anatomo-Pathological Examination

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Crib death, or sudden infant death syndrome (SIDS), is the most frequent form of death in the first year of life, striking one baby in every 1,700–2,000. Yet, despite advances in maternal–infant care, sudden intrauterine unexplained/unexpected death syndrome (SIUDS) has a sixfold to eightfold greater incidence than that of SIDS. Frequent congenital abnormalities, likely morphological substrates for SIDS–SIUDS, were detected, mainly represented by alterations of the cardiac conduction system, such as accessory pathways and abnormal resorptive degeneration, and hypoplasia/agenesis of the vital brainstem structures. On the basis of these considerations, the new common definition of the SIDS–SIUDS complex is "The sudden death of a fetus after the 25th gestational week or infant under one year of age which is unexpected by history and remains unexplained after a thorough case investigation, including examination of the death scene, performance of a general autopsy and examination of the fetal adnexa". Therefore, given that the general autopsy does not disclose any cause of death, a more in-depth histopathological analysis of the cardiac conduction system and autonomic nervous system by specialized pathologists is necessary.

Keywords: sudden infant death syndrome, sudden intrauterine unexpected death syndrome, anatomo-pathological examination definition, cardiac conduction system, brainstem, autopsy

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INTRODUCTION

Sudden infant death syndrome (SIDS), or crib death, is the most frequent form of death during the first year of life. The sudden unexpected death of a baby in the crib and, even worse, in the mother's womb is surely one of the most heartbreaking tragedies for the family, the pathologists, clinicians, and epidemiologists, as well as the general public, given that the affected individuals, before the lethal event, were regarded as healthy.

According to the Centers of Disease Control and Prevention (CDC) (1), SIDS has a death rate of 0.42 per 1,000 births, striking one baby in every 1,700–2,000. In developed countries, one in 100–200 pregnancies ends in stillbirth, which has a sixfold to eightfold greater incidence than that of SIDS and remains completely unexpected in 40–80% of cases, occurring in pregnancies that had seemed problem-free. Unexpected stillbirth has a sixfold to eightfold greater incidence than that of SIDS. The frequency of sudden intrauterine unexplained/unexpected death syndrome (SIUDS),

which has ranged from 5 to 12‰ in the last 25 years, has not declined significantly despite modern advances in maternal–infant care (2–4).

The consequences among families are devastating, with high social cost, considering the unexpected loss of many potentially productive individuals. Despite the increasing general interest and number of published works, SIDS and SIUDS represent a great enigma of modern medicine whose etiology remains uncertain.

According to the most recent report from the American Heart Association (5), congenital heart diseases occur in about 0.8% of full-term live births. The true incidence of congenital heart diseases could be significantly higher than the reported one, given that cardiac anomalies occur 10 times more frequently in stillborn and premature infants than in full-term infants. Anatomic cardiac anomalies and channelopathies are important considerations for congenital heart disease inclusion, being characterized by common congenital histopathological substrates (6).

Even if SIUDS and SIDS are generally considered diagnoses of exclusion, subtle developmental abnormalities of the cardiac conduction system and brainstem have been highlighted. Risk factors of SIDS and SIUDS include exposure to maternal smoking, air and water pollution, food contamination, agricultural and household pesticides, etc., that, starting *in utero*, act as causative and triggering factors in vulnerable infants with developmental abnormalities in the cardiac conduction system and/or autonomic nervous system. Specific environmental risk factors can interact in complex ways with the genetic constitution leading to polymorphisms and/or mutations of specific genes. At least four categories of genes are involved in the pathogenesis of SIDS and SIUDS: genes for ion channel proteins involved in cardiac channelopathies, mainly the sodium channel gene (*SCN5A*/*LQT3*) and the potassium channel genes (*KCNQ*, *KCNH12*, *KCNE2*); genes regulating the brainstem functions, such as serotonin transporter (*5-HTT*), the regulator of the synaptic serotonergic receptor binding in the brainstem, *PHOX2B*, the major gene involved in congenital central hypoventilation syndrome (CCHS), and *En*-2, involved in the development of the autonomic nervous system; genes regulating inflammation and infections, such polymorphisms in complement C4 and interleukins IL-6 and IL-10; genes regulating energy production, hypoglycemia, and thermal regulation, such as the medium-chain acyl CoA dehydrogenase (*MCAD*) genes (2, 7, 8).

The objective that this study wishes to achieve is to introduce a new definition for SIDS and SIUDS, based on the anatomopathological findings from the investigation of a wide number of cases, with regard to the common pathogenesis of SIDS–SIUDS. This increased knowledge would untimely lead to the development of targeted risk-lowering strategies to reduce the incidence of these deadly forms of death.

DISCUSSION

Over the years, increasing interest has been focused on the possible developmental anomalies of the cardiac conduction and autonomic nervous systems involved in the pathogenesis of SIDS and SIUDS. This interest has led to investigations of a wide number of fetuses and infants from the 25th gestational week through the first year of postnatal life (2, 9).

Retrospective research cases are currently represented by over 140 SIDS, 120 SIUDS victims, and 60 age-matched controls, since the Italian national authority (10) decreed that the regional and national cases of supposed SIDS or SIUDS were to be converged and investigated at the "Lino Rossi" Research Center of the University of Milan. All cases were accurately studied through analysis of clinical data, of the risk factors, and particularly *postmortem* through the histopathological investigation of the cardiac conduction system and of the autonomic nervous system, both central and peripheral (11). SIDS and SIUDS share developmental abnormalities of the cardiac conduction system, such as defective or exaggerated resorptive degeneration, atrioventricular dispersion or septation, conductive accessory pathways, cartilaginous meta/hyperplasia of the central fibrous body, and dualism of the atrioventricular junction, as morphological substrates for cardiac arrhythmias. SIDS and SIUDS also share developmental abnormalities of the vital brainstem structures, such as hypoplasia, agenesis or neuronal immaturity of the arcuate, hypoglossus, pre-Bötzinger, cochlear, locus coeruleus, or Kölliker-Fuse nuclei. Therefore, the need to perform an in-depth study of the structures of the cardiac conduction and of the autonomic nervous systems, modulating the respiratory, cardiovascular, upper-digestive, and arousal activities, is self-evident.

Genetic mutations underlying cardiac arrhythmias have been focused on the long-QT syndrome and have been detected in no more than 10% of SIDS and of unexplained intrauterine fetal deaths (12, 13). The genetic studies of increasing interest involve the application of advanced analytical approaches, including whole exome sequencing to cases of sudden unexpected death in neonates and infants (14, 15).

The new perspective of the SIDS and SIUDS definition includes the detection of common congenital anomalies of the cardiac conduction and autonomic nervous systems, similar in both SIDS and SIUDS victims, which indicate a continuity between these two deadly forms of death that can, therefore, be regarded as the so-called SIDS–SIUDS complex.

Sudden Infant Death Syndrome

Currently, the CDC (16) describe SIDS as a subset of sudden unexpected infant death (SUID), defined as the "death of an infant less than one year of age that occurs suddenly and unexpectedly, and whose cause of death is not immediately obvious before investigation". SUID combines the following three forms of death: SIDS, death by unknown cause, and accidental suffocation, and strangulation in bed (ASSB) (17).

In 1970, Beckwith (18) published the first definition of SIDS introduced at the second international conference held in Seattle, WA, USA, in 1969, as follows: "The sudden death of any infant or young child, which is unexpected by history, and in which a thorough postmortem examination fails to demonstrate an adequate cause for death". This definition does not refer to specific age or to any common features and SIDS was only a syndrome of exclusion.

In 1991, Willinger et al. (19) published the following new definition of SIDS according to the National Institute of Child

Health and Human Development (NICHD) panel convened in 1989: "The sudden death of an infant under one year of age, which remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene, and review of the clinical history". SIDS becomes a diagnosis made by pathologists, based primarily on the finding of a negative autopsy. This definition has been largely adopted all over the world for over 20 years.

In 2002, Matturri et al. (20), at the 7th International Conference on SIDS, proposed that the definition of SIDS as "the sudden death of an infant under one year of age which remains unexplained after a thorough case investigation, including the performance of a complete autopsy, examination of the death scene, and review of the clinical history" should be modified by adding, at the end, the following: "a complete autopsy with an in-depth histopathologic analysis of the cardiorespiratory innervation and specialized myocardium, performed only by an experienced, reliable pathologist".

In 2004, Krous et al. (21) defined SIDS as "The sudden unexpected death of an infant less than one year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history".

This latest definition of SIDS that describes SIDS as unexplained after a complete autopsy is, hereby, under review as the findings in the cardiac conduction and autonomic nervous systems detected in SIDS can be morphological substrates for the sudden unexpected death.

In 2016, Goldstein et al. (22) reported that there is no consensus on the use of the term SIDS, as external factors, such as prone sleep position or bed sharing, may at times explain the cause of death as positional asphyxia or accidental suffocation.

Sudden Intrauterine Unexplained Death Syndrome

In 2001, Frøen et al. (23) defined sudden intrauterine unexplained death as the "Intrauterine death before the onset of labor of a fetus at ≥22 completed weeks of gestation or with ≥500 g body mass, which is unexpected by history and in which a thorough autopsy of the fetus, together with gross and histologic examination of the umbilical cord, placenta, and membranes, fails to demonstrate an adequate cause of death".

In 2002, Matturri et al. (24) reported the first anatomopathological evidence of hypoplasia or agenesis of the arcuate nucleus in unexpected stillborns, in a similar manner than that detected in SIDS victims. Since then, additional anatomopathological studies confirmed similar findings in the autonomic nervous and cardiac conduction systems in both SIDS and SIUDS victims (2, 9, 11).

In 2006, the Italian Law no. 31 (10), "Regulations for diagnostic postmortem investigation in victims of SIDS and unexpected fetal death", was introduced, imposing common rules for the referral of cases and postmortem procedures equally in victims of SIDS and unexpected sudden fetal death starting from the 25th gestational week.

In 2009, the then US Senator Barack Obama (25) introduced the Preventing Stillbirth and SUID Act, which enhances public health activities related to understanding and preventing unexplained stillbirth and SUID.

In 2014, Matturri et al. (26) defined sudden intrauterine unexplained death syndrome (SIUDS) as "The sudden death during pregnancy that remains unexplained after an in-depth autopsy including examination of the placental disk, umbilical cord and membranes, detailed pregnancy history analysis and molecular and microbiological investigations".

Sudden intrauterine unexplained/unexpected death syndrome, or unexpected stillbirth, is herein defined as "The late fetal death before the complete expulsion or removal of the fetus from the mother ≥25 weeks of gestation which is unexpected by history and is unexplained after review of the maternal clinical history and the performance of a general autopsy of the fetus, including examination of the placental disk, umbilical cord and membranes, and microbiological and genetic investigations".

Sudden Unexpected Infant and Perinatal Death

The acronym "SPUD" for sudden unexpected infant and perinatal death, which itself includes SIUDS and sudden neonatal unexpected death (SNUD) (2).

Perinatal mortality refers to death around the time of delivery and includes both fetal deaths (at least 20 weeks of gestation) and neonatal (early infant) deaths (27). Pathologically, SIDS and SIUDS can be included in the extended domain of perinatal-infant pathology, as a continuity of cardiac conduction system and autonomic nervous system findings have been detected without a clear separation between sudden unexpected perinatal and infant death. The in-depth postmortem examination is equally mandatory in every case of sudden unexpected infant and perinatal death (SPUD), which itself includes SIUDS and SNUD.

Sudden Neonatal Unexpected Death

Sudden infant death syndrome's pathology includes an extended domain of neonatal pathology, particularly if within the diagnosis of SIDS one wishes to enclose the acronym "SNUD" for sudden neonatal unexpected death, not definitely separable from the unifying concept of syndrome (2).

SIDS–SIUDS Complex

The SIDS–SIUDS complex describes a multifactorial pathology likely consisting of congenital anomalies of the cardiac conduction system and of the autonomic nervous system, mainly cardiorespiratory, of the first upper-digestive pathways, and arousal, detected in both infants and fetuses dying suddenly and unexpectedly (2). Both, SIDS and SIUDS, could be attributed to multiple causes and characterized by the unifying etiological concept of "syndrome", adopted to describe the SIDS–SIUDS complex as a frequent form of death in fetuses and infants.

Commonalities have been reported among cases of unexplained stillbirth and SIDS, i.e., dysfunction in limbic forebrain, hippocampus, and brainstem circuits, designated as the central homeostatic network and the triple risk model (22, 28, 29).

On the basis of these considerations, the herein presented new common definition of the SIDS–SIUDS complex is "The sudden death of a fetus after the 25th gestational week or infant under one year of age which is unexpected by history and remains unexplained after a thorough case investigation, including examination of the death scene, performance of a general autopsy and examination of the fetal adnexa". Therefore, given that the general autopsy does not disclose any cause of death, a more in-depth histopathological analysis of the cardiac conduction system and autonomic nervous system by specialized pathologists is necessary.

SIDS and SIUDS Gray Zone

In 1995, Gregerson et al. (30), in the Nordic SIDS study, described the SIDS borderline cases in which pre-existing congenital disorders or clinical symptoms and/or postmortem findings are not severe enough to explain the cause of death.

In 2001, Rognum (31) described the gray zone as a group of "in between" cases in which there are pathological findings, either morphologic or microbiologic, or information from the history or the circumstances of death, that are significant, but most likely not sufficient to explain death. This gray zone may also include neglect, abuse, and even murder.

In 2004, Krous et al. (21) in their definition of SIDS, did not mention a SIDS gray zone, and described as "unclassified sudden infant deaths" those cases that do not meet the criteria for a diagnosis of SIDS with equivocal alternative diagnoses of natural or unnatural conditions. They anticipated that their definitions of SIDS would have been modified to accommodate new understandings of SIDS and sudden infant death.

In 2007, Matturri et al. (32) described nine fetuses that died suddenly and unexpectedly with concomitant abnormalities of the fetal adnexa, classified as SIUD gray zone. These cases presented with cardiac conduction and brainstem lesions in association with choriamnionitis (seven cases), abnormally short umbilical cord (one case), and placental infection by parvovirus (one case), which alone might not have accounted for the sudden death.

Sudden infant death syndrome and SIUDS are classified as borderline or gray zone when the sudden unexpected death is concomitant to a coexistent disease, such as mild to moderate bronchus-pneumonic infection or chorionamnionitis, itself not enough to cause death, acting as a triggering phenomenon in a vulnerable infant or fetus (2, 9, 28, 33).

In the SIDS/SIUDS gray zone the infants/fetuses are vulnerable due to pre-existing nervous, autonomic, or cardiac conduction alterations, such as hypoplasia of the arcuate nucleus or a Mahaim fiber, which are plausible bases for the diagnosis of SIDS/SIUDS, and with the lethal event being triggered by the concomitant pathology.

Following these considerations, the SIDS/SIUDS gray zone is now defined as: "The infant/late fetal death ≥25 weeks of gestation which is unexpected by history and is unexplained after review of the clinical history and the performance of a general autopsy which includes examination of the fetal adnexa, as well as microbiological, and genetic investigations, which occurs with another event, acting as a triggering phenomenon itself not enough to cause death, in a vulnerable infant/fetus".

CONCLUDING REMARKS

Congenital abnormalities, likely morphological substrates for SIDS and SIUDS, were frequently detected, mainly represented by alterations of the cardiac conduction system, such as accessory pathways and abnormal resorptive degeneration, and hypoplasia/agenesis of the vital brainstem structures (2, 9, 11, 24).

Current SIDS–SIUDS research is attempting to integrate the anatomo-pathological findings with the genetic and environmental substrates. Any case of suspected SIDS or SIUDS should be submitted to an in-depth postmortem examination, particularly focused on the investigation of the cardiac conduction system and brainstem on serial sections, along with toxicological–environmental and genetic investigations.

AUTHOR CONTRIBUTIONS

GO has primary responsability for development of the concept and design, writing, drafting, and revising the manuscript.

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REVIEW
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Towards Better Understanding of the Pathogenesis of Neuronal Respiratory Network in Sudden Perinatal Death

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Sudden perinatal death that includes the victims of sudden infant death syndrome, sudden intrauterine death syndrome, and stillbirth are heartbreaking events in the life of parents. Most of the studies about sudden perinatal death were reported from Italy, highlighting two main etiological factors: prone sleeping position and smoking. Other probable contributory factors are prematurity, male gender, lack of breastfeeding, respiratory tract infections, use of pacifiers, infant botulism, extensive use of pesticides and insecticides, etc. However, extensive studies across the world are required to establish the role of these factors in a different subset of populations. Previous studies confirmed the widely accepted hypothesis that neuropathology of the brainstem is one of the main cause of sudden perinatal death. This study is an effort to summarize the neuropathological evaluation of the brainstems and their association to sudden perinatal death. Brainstem nuclei in vulnerable infants undergo certain changes that may alter the sleep arousal cycle, cardiorespiratory control, and ultimately culminate in death. This review focuses on the roles of different brainstem nuclei, their pathologies, and the established facts in this regard in terms of it's link to such deaths. This study will also help to understand the role of brainstem nuclei in controlling the cardiorespiratory cycles in sudden perinatal death and may provide a better understanding to resolve the mystery of these deaths in future. It is also found that a global initiative to deal with perinatal death is required to facilitate the diagnosis and prevention in developed and as well as developing countries.

Keywords: sudden infant death, sudden fetal death, sudden perinatal death, sudden intrauterine death, stillbirth, neuropathology

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INTRODUCTION

Sudden perinatal mortalities include sudden fetal death or Sudden Intrauterine Death Syndrome (SIUDS), stillbirths, and Sudden Infant Death Syndrome (SIDS) due to some unknown reason. Stillbirth is death of a fetus after 20 weeks of gestation, weighing 350–1,000 g (1). The annual global incidence of stillbirths is 2.7 billion, with 15–35% more deaths in developing countries, which is very alarming (2, 3). SIDS also termed as "Crib death" or "Cot death" is defined as the sudden and inexplicable death of an apparently healthy newborn or infant who dies before the first birthday and reason remains a mystery even after a complete autopsy or thorough investigation (4). To find out the

exact cause of death in SIDS or SIUDS, victims are a major diagnostic challenge. SIUDS are broadly categorized as accidental and non-accidental mortalities (5). It was found that victims of sudden perinatal deaths usually belong to economically poor family and incidence is high in winter, during midnight and weekends (6–8). Many other risk factors were also observed in the SIDS victims such as male gender (9), ethnicity (10), and deformational plagiocephaly (11). Some maternal factors reported were maternal age (12), obesity (13), and smoking during pregnancy (14), whereas environmental factors were prone sleeping position (15), soft bedding, over heating (16), lack of breastfeeding (17), and higher latitudes (18). More recently, some new theories have been proposed, and it was highlighted that infant gut microbiome may modulate the brainstem serotonergic system and may serve as a new possible risk factor for causing SIDS (19). Latest theories like SIDS-critical diaphragm failure hypothesis suggest that the critical diaphragm failure during pregnancy may end up in SIDS by cessation of breathing (15), whereas substance P–neurokinin 1 hypothesis suggests a possible involvement of this tachykinin peptide in sudden perinatal deaths by modulating the cardiorespiratory control (20).

The causes of these unexplained deaths can be environmental, genetic or congenital, etc. So far, the most accepted hypothesis to define SIDS is triple-risk model of Filiano and Kinney (21), in which the infants exposed to external stress, and have some intrinsic vulnerability will be at higher risk of having neurological and developmental abnormalities that can result in SIDS (22). The National Institute of Child Health and Development SIDS Strategic Plan 2000 states that "SIDS is a developmental disorder. It's origins are during fetal development" (23). Subtle hippocampus abnormalities, seizures, malfunctioning in central nervous system mechanisms, abnormalities in neurotransmitter secretions, and in the nuclei of brainstem cells are also suggested as causes of SIDS (24).

NEUROPATHOLOGY OF SUDDEN PERINATAL DEATH

Neuropathology deals with the diseases of the nervous system tissue, either through small surgical biopsies or whole-body autopsies. Neuropathological studies include anatomy, pathology, neurology, and neurosurgery (4). In this study, the main focus is on summarizing the neuropathological anomalies of sudden fetal deaths, stillbirths, and infant mortalities due to alterations in neurotransmitter's release and nuclei of brainstem neuronal centers. The human cerebellar cortex development involves rapid transformations, thickness, as well as the reorganization of cortical layers in the fetal and early postnatal stages (25). Any change due to mutations, epigenetic and environmental factors such as smoking, hypoxia, pesticide exposure, and infection can result in neuropathological conditions. Even though current studies are unable to pin point the causes but brainstem abnormalities that are responsible for respiration and responses to asphyxia, especially in the sleep and arousal, are thought to be the probable causes (26, 27). Defects in brainstem neural circuits involved in cardiorespiratory regulation may be one of the leading causes of SIDS (28).

BRAINSTEM CONTROL OF RESPIRATION DURING THE TRANSITION FROM WATER TO AIR BREATHING

Breathing rhythm in fetus begins at the 10th week of gestation (29) which changes from irregular to a regular pattern at the time of birth by unknown mechanisms. In the neonatal period, a regular respiratory rhythm (RR) and cardiorespiratory coupling is controlled by neuronal centers in the brainstem (30). These RRs are controlled by several pathways in the neuronal networks, e.g., pre-Botzinger complex and the Kölliker–Fuse as well as some cortical and cerebellar networks (31). These pathways are also involved in involuntary functions, sleep–awake cycle, and upper respiratory tract reflexes. It is found that brain-derived neurotrophic factor (BDNF) is involved in steady rhythm generation.

In response to stress such as hypoxia, these networks are able to reconfigure, to generate multiple breathing patterns, and to facilitate autoresuscitation. There are vital changes in caudal serotonergic (5-HT) system at the end of the fetal period and the start of the neonatal period that are regulated by neuronal networks. Serotonin (5-HT) receptor binding is gradually decreased as the gestation progresses.

Instability in the early control of breathing is proportional to frequency of apnea in infants. Brief apneic spells are common within the first few minutes after birth, later on more prolonged episodes of apnea are observed. These apneic episodes (breath holding) are associated with prematurity, laryngeal chemoreflex activity or bradycardia, and loss of muscle tone ("near-miss SIDS" or apparent life-threatening events) (32). Episodic apnea and bradycardia have been observed in the infants who died of SIDS (33). Vulnerable infants with immature neuronal centers are unable to face the life-threatening challenges such as hypoxia and hypercapnia during sleep, which may lead to imbalances in serotonergic networks (34). Consequently, abnormality in specific brainstem neuronal networks have been observed in SIDS that cause failure of these reflex responses to arousal.

Pontine Kölliker–Fuse Nucleus (KFN)

Studies established the role of pontine KFN (**Figure 1**; **Table 1**) in breathing control; it is interconnected with the prevalent serotonin and noradrenaline neurons in the brainstem. It is suggested that orexin has a strong effect on the brainstem raphe nuclei (RN) and locus coeruleus, in arousal from sleep (35). The neurobiological functions of these stem cell nuclei are closely linked to the breathing modifications (36). As KFN is a main component of the orexin system that is involved in arousal, KFN was observed to play a key role in providing a breathing rhythm and coordination of sleep-to-wake transition. Any defect in orexin expression in KFN is responsible for prevention of arousal and can be a crucial factor in causing SIDS (36).

Experimental studies indicate that the neurotrophin BDNF has a vital role in the central respiratory network development to sustain life. In the prenatal and postnatal breathing circuit, pontine KFN is a fundamental component (54). BDNF pathway dysfunctions may possibly distort the normal KFN development

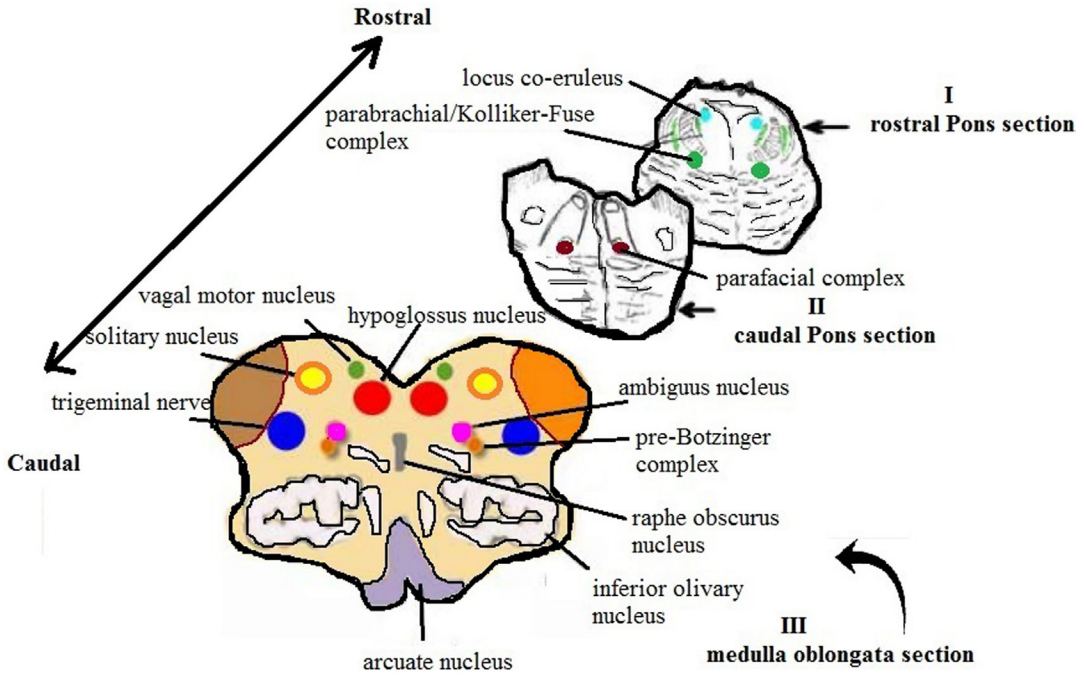

FIGURE 1 | Schematic representation of the main histological sections obtained from the brainstem for the anatomopathological examination [modified image adapted from Matturri et al. (31)].

in SIUDS and SIDS victims by interfering with the breathing control. Alterations in the BDNF expression in KFN have been observed in many respiratory diseases in human such as the Rett's and the congenital central hypoventilation syndromes (37).

Inferior Colliculus Nucleus

Developmental defects of hearing pathways involve defects in the specific brainstem centers, specifically in the cochlear, vestibular, superior olivary, and inferior olivary complex (**Figure 1**). Significantly, more alterations were observed in cytoarchitecture of auditory and respiratory networks of SIDS cases as compared to controls in one study (26). The inferior colliculus has a vital role in the processing of acoustic information. It is believed that neuromodulator serotonin concentration can be a factor in sudden unexplained fetal and infant death syndromes. Weak serotonin positivity was observed in a study conducted on brainstems of SIDS and SIUDS victims, indicative of functional abnormality of inferior colliculus. Hypoplasia or anomalies in the associated structures, e.g., RN and the superior olivary complex was also observed in the fetus of smoking mothers (**Table 1**). A role of inferior colliculus in breathing apart from hearing was also suggested (38).

Locus Coeruleus Complex

Locus coeruleus complex (**Figure 1**; **Table 1**) is a part of the brainstem in pons mainly responsible for the physiological responses to conditions of stress and panic. It is the main region that produces norepinephrine (noradrenaline), tyrosine hydroxylase, and neuromelanin (NM) (55). A strong correlation between defects in noradrenaline system, low levels of NM, hypoplasia, along with a high neuronal death rate, were found mainly in the locus coeruleus complex of fetal and infant sudden death victims (44). Studies have shown that locus coeruleus complex is involved in vital activities related to the brain interconnections and behavioral adjustments, including coordination of the sleep–wake cycle and control of the cardiorespiratory functions (56).

Superior Olivary Complex

The superior olivary complex (**Figure 1**; **Table 1**) is a group of brainstem nuclei that have multiple roles in hearing and is involved in ascending and descending auditory pathways (57). Irregular cytoarchitectural patterns like hypoplasia/agenesis, immature hypercellularity, and dysgenesis of contiguous structures involved in breathing circuit in medial superior olivary nucleus were reported in a study, and it was proposed that this nucleus had influence on all the vital activities along with hearing (45).

Retrotrapezoid Nucleus (RTN)

The RTN is part of caudal pons and comprises cluster of glutamatergic and non-aminergic neurons that are responsible for the homeodomain transcription factor Phox2b (a transcriptional factor involved in congenital central hypoventilation syndrome) expression (58). Immunohistochemical expression of Phox2b neurons inside the caudal pons points out the developmental abnormalities of the human RTN (**Table 1**). It may acutely affect the chemoreception control, thus, performing a vital part in the pathogenesis of SIUDS and SIDS (46).

Spinal Trigeminal Nucleus (STrN)

The STrN (**Figure 2**; **Table 1**) is part of medulla, and it transmits information related to pain and temperature in the orofacial

TABLE 1 | Summary of studies on brainstem nuclei along with their physiological and pathological roles (+ indicate increase and – is decrease in expression).

Nucleus	Brain area	Role of nucleus	Neurotransmitter	Expression	Alteration in function	Possible cause	Reference
KFN	Rostral PONS, brainstem	Arousal/sleep breathing control in perinatal life, synaptic plasticity	OR, BDNF	–	Fetal inhibitory reflex arrest breathing, deranged normal KFN development, and loss of breathing control	Hypoxic conditions, smoking	(36, 37)
ICN	Mesencephalon	Acoustic processing	5-HT	–	Dysgenesis of RN, superior ON, ICN	Nicotinic absorption, smoking	(26, 38)
Nucleolus	Brainstem	Ribosomal synthesis	AgNOR	–	PC degeneration, disturbed cardiac cycle	Nicotinic absorption, smoking	(39)
AP	Fourth ventricle	Controls vomiting	–	–	AP lesion	Insecticide	(40)
LC, KFN, CAN, RN, pre-BotC, PF/FC	Cerebral cortex	Breathing control, sleep–awake cycle	α7-NAcR	+	Hypoplasia of all nuclei	Smoking, insecticide	(14, 41)
POD	Cerebellar Purkinje	RR	α7-NAcR		Alterations of POD network	Smoking	(42)
NN	Brainstem	Mitotic cycle	NeuN	–	Cell death increased, neuronal immaturity	Smoking	(43)
LC	Brainstem	Sleep–wake cycle, control of CRS	TK, NM, TH	–	NM, hypoplasia, neuronal death, alterations of noradrenaline system, low neuromelanin, neuronal death	Smoking	(44)
SOC	Brainstem	Acoustic information	–	–	Hypoplasia of ON, RTN, FN, hypercellularity, dysgenesis of structures related to RR, alterations in auditory, and respiratory network	Smoking	(26, 45)
RTN	Caudal pons	Breathing, chemoreception	PHOX2B	–	Developmental abnormalities in RTN	Smoking	(46)
AP	Brainstem, fourth ventricle choroid plexus	Autonomic control of cardiac and respiratory activity	–	–	Lack of vascularization, hypoplasia, cystic formations, reactive gliosis	Smoking	(47)
STrN	Brainstem	Pain, thermofluctuations, RR	SP	–+	Pre-BtzC, RN, and AN hypoplasia	Smoking	(48)
IMN	Brainstem	Breathing activity	–	–	Hypoplasia, neuronal immaturity	Smoking	(49)
G-Mt	Brainstem	Modulation of spinal cord motor activity	–	–	Hypoplasia, apoptosis	Smoking	(50)
HGN	Brainstem	Swallowing, chewing, vocalization, inspiration	SM	+	Hypoplasia, hyperplasia, no interneurons	Smoking	(51)
RN	Brainstem	Sleep–wake cycle	5-HTT	–	Hypoplasia	Smoking	(52)
Pre-BotC	Medulla	RR	NK1R, SM	–	Hypoplasia, low neuronal no., dendritic hypodevelopment	Smoking	(53)

OR, orexin receptor; BDNF, brain-derived neurotrophic factor; 5-HT, serotonin; AP, area prostrema; RTN, retrotrapezoid nucleus; ON, olivary nucleus; LC, locus coerulius; STrN, spinal trigeminal nucleus; KFN, Kölliker–Fuse nucleus; ICN, inferior collicus nucleus; RN, raphe nucleus; AN, arcuate nucleus; PF/FC, parafacial/facial complex; pre-BotC, pre-Bötzinger; IMN, intermediolateral nucleus; G-Mt, Guillain–Mollaret triangle (dentato-rubro-olivary network); HGN, hypoglossal nucleus; α7-NacR, α7-nicotinic acetylcholine receptor; TK, tyrosine kinase; NM, neuromelanin; CRS, cardiorespiratory system; RR, respiratory rhythm; TH, tyrosine hydroxylase; NN, nucleus of neurons; POD, Purkinje-olivo-dentate network; SM, somatostatin; 5-HTT, serotonin transporter; NK1R, neurokinin 1 receptor.

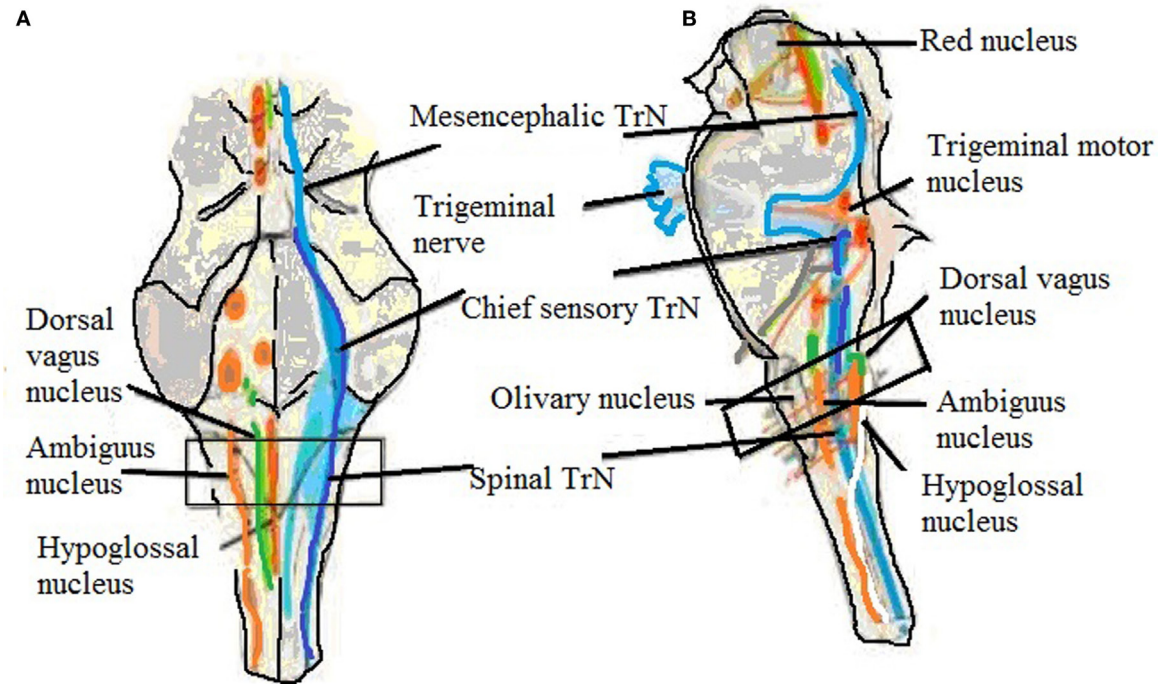

FIGURE 2 | Brainstem showing trigeminal nucleus and also shows the level of sampling. **(A)** Ventral view and **(B)** side view showing trigeminal nerve, mesencephalic, chief sensory, and spinal trigeminal nucleus (60).

region. The cranial nerves transmit pain stimuli from peripheral regions to the STrN (59). A reduced SP expression levels in the fibers of STrN in SIDS victims and higher levels in SIUDS victims were observed (20, 48).

Intermediolateral Nucleus (IMN)

In the brain, the sympathetic preganglionic neurons reside in the IMN that is a part of spinal cord. These are groups of columnar cells organized longitudinally, in the gray matter of the lateral horn. These cells are present between the first thoracic spinal region and the third lumbar region (61). Experimental studies have demonstrated the role of IMN in the breathing activities and development of a spinal cord–brainstem network (62). In SIDS, IMN fails to mature progressively; it's neurons do not transform from a round to a polygonal shape with extended axons and drastically decrease in number. In unexplained fetal and infant death victims, hypodevelopment of IMN such as neuronal immaturity in a normal structure, hypoplasia, and agenesis was seen (49) (**Table 1**).

Guillain–Mollaret Triangle (G-Mt) (Dentato-Rubro-Olivary Network)

The G-Mt (**Figure 3**; **Table 1**) has three parts: the ipsilateral red nucleus, the inferior olive, and the contralateral dentate nucleus in the midbrain, medulla, and cerebellum to form dentato-rubro-olivary pathway (63). G-Mt is known to be involved in the pathogenetic mechanisms of the palatal myoclonus, in SIDS and SIUDS. A significant increase of lesions of these three nuclei were found in SIDS victims (50).

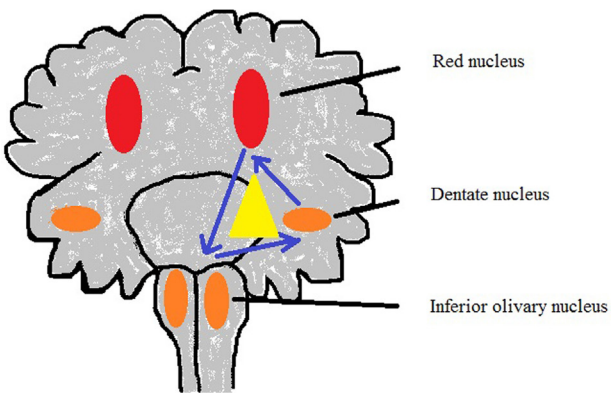

FIGURE 3 | Sketch of Guillain–Mollaret triangle derived from Lavezzi et al. (50).

Medullary Hypoglossal Nucleus (HGN)

The hypoglossal nerve is a motor nerve that controls extrinsic and intrinsic muscles of the tongue. It arises from the HGN (**Figure 1**; **Table 1**) in the brain stem and controls swallowing, chewing, vocalization, and inspiration (64). HGN anomalies such as hypo/hyperplasia, somatostatin positivity, and absence of interneurons were evident in SIDS cases (51). Unlike to the trigeminal nucleus, HGN is not considered as a main respiratory regulatory center, yet, it contains motoneurons with respiratory-related rhythmical discharges. Primarily, HGN controls the genioglossus, an extrinsic muscle of the tongue, which plays a significant role in regulating a patent airway during inspiration (65, 66).

Raphe Nuclei

The RN (**Figure 1**; **Table 1**) are medial part of the reticular formation that forms a crest of cells in the center and in the medial portion of the brainstem (67). In a study, cytoarchitecture and the localization of human RN in the brainstem were done to analyze the association of raphe nucleus pathology and serotonin transporter gene (5-HTT) polymorphisms. It was also suggested that SIUDS should not be viewed separately from SIDS, due to potentially shared neuropathological and genetic grounds (52).

Pre-Bötzinger Complex (Pre-BötC)

In the ventrolateral medulla of the brainstem, a cluster of interneurons is present known as pre-BötC (**Figure 1**; **Table 1**). It is believed that it has a vital role in the generation of RR in humans (68). Neuropathology of the pre-BötC, altered neurokinin 1 receptors, and somatostatin expression were observed in a subset of SIDS and SIUDS victims as compared to the controls. Hypoplasia with a low neuronal number with dendritic hypodevelopment, defective neuronal morphology, immunonegativity of neurotransmitters, and agenesis was sighted. These abnormalities are directly linked with the neonatal deaths and still births (53).

In most of these studies, an association has been found with maternal smoking. Nicotine is one of the few lipid-soluble substances that are able to go beyond the blood–brain barrier (69) 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 could affect the fetal brain through indirect or direct action (70). As there are not many studies conducted on SIDS and SIUDS worldwide and it is multifactorial, so we cannot conclude concretely, that only smoking is the main etiological factor. Recently, some studies have been done on the role of pesticides and insecticides in these sudden deaths and an association has been observed (41, 71). Most of these studies were conducted mainly in Italy, so there is a need to explore the risk factors in other parts of the world too, e.g., Southeast Asia where infant mortality rate is very high and population is exposed to extra risk factors like consumption of banned insecticides like DDT among others. Moreover, there is no epidemiological data available regarding SIDS and SIUDS in these regions.

CONCLUSION

Neuropathology in brainstems of SIDS and SIUDS victims are summarized in this study. It is found that several alterations in the brain centers possibly lead to sudden deaths. This updated effort will help in better diagnosis and identification of such cases. Moreover, an association with maternal smoking has been observed in the reported studies. It is noticed that sufficient data to establish all causative factors is not available. So, there is a need to study other dimensions to find out the etiological factors in different populations and different regions of the world. There is an urgent need to expand these studies in other regions of the world, particularly in South East Asia where health-care facilities are very poor and banned agricultural pesticides are still in use.

AUTHOR CONTRIBUTIONS

RM conceived the idea, planned the review manuscript, made some figures, helped in writing the manuscript, and finalized it. MK gathered already existing literature in the field, made some figures, and helped in writing some parts of manuscript. NA made the table and helped in writing the manuscript. FA finalized the manuscript. All the authors have revised, checked, and approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fneur.2017.00320/full#supplementary-material.

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The Cerebellum and SIDS: Disordered Breathing in a Mouse Model of Developmental Cerebellar Purkinje Cell Loss during Recovery from Hypercarbia

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The cerebellum assists coordination of somatomotor, respiratory, and autonomic actions. Purkinje cell alterations or loss appear in sudden infant death and sudden death in epilepsy victims, possibly contributing to the fatal event. We evaluated breathing patterns in 12 wild-type (WT) and *Lurcher* mutant mice with 100% developmental cerebellar Purkinje cell loss under baseline (room air), and recovery from hypercapnia, a concern in sudden death events. Six mutant and six WT mice were exposed to 4-min blocks of increasing CO_2 (2, 4, 6, and 8%), separated by 4-min recovery intervals in room air. Breath-by-breath patterns, including depth of breathing and end-expiratory pause (EEP) durations during recovery, were recorded. No baseline genotypic differences emerged. However, during recovery, EEP durations significantly lengthened in mutants, compared to WT mice, following the relatively low levels of CO_2 exposure. Additionally, mutant mice exhibited signs of post-sigh disordered breathing during recovery following each exposure. Developmental cerebellar Purkinje cell loss significantly affects compensatory breathing patterns following mild CO_2 exposure, possibly by inhibiting recovery from elevated CO_2. These data implicate cerebellar Purkinje cells in the ability to recover from hypercarbia, suggesting that neuropathologic changes or loss of these cells contribute to inadequate ventilatory recovery to increased environmental CO_2. Multiple disorders, including sudden infant death syndrome (SIDS) and sudden unexpected death in epilepsy (SUDEP), appear to involve both cardiorespiratory failure and loss or injury to cerebellar Purkinje cells; the findings support the concept that such neuropathology may precede and exert a prominent role in these fatal events.

Keywords: cerebellum, Purkinje cells, disordered breathing, sudden death, SIDS, SUDC, SUDEP

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INTRODUCTION

Sudden unexpected death occurs in several syndromes, with mechanisms underlying the fatal event not yet understood. Sudden infant death syndrome (SIDS) is one such syndrome; the condition occurs in infants <1 year of age, often during sleep, and with victims succumbing suddenly with major causes excluded even after autopsy and a thorough investigation (1, 2). Sudden death in epilepsy

(SUDEP), appears in adults as well as older children, often occurs during sleep, but leaves few signs of mechanisms operating to induce a fatal event. A defect in breathing, or deficient responses to a ventilatory challenge is suspected in both SIDS and SUDEP. Although an inability to respond to severe, transient hypotension is a strong possibility (3, 4), the circumstances surrounding both SIDS and SUDEP conditions suggest an unknown brain abnormality that results in an inability to appropriately compensate for, or recover from, exposure to an intrinsic or exogenous stressor (5). Here, we evaluate the role of the cerebellum in mediating the consequences of exposure to one exogenous stressor, increased levels of environmental carbon dioxide (CO_2; hypercapnia).

The focus on high CO_2 arises from the circumstances normally associated with SIDS or postictal cessation of breathing in epilepsy. SIDS and SUDEP victims are often found deceased in bed, in the prone position, and sometimes in soft bedding, focusing attention on exposure to elevated CO_2 concentrations associated with these conditions during sleep (6, 7). Thus, SIDS likely results from an inability to respond appropriately with respiratory and cardiovascular patterns to accommodate such a breathing stressor. Similarly, postictal respiratory failure in SUDEP would elevate CO_2 levels, imposing a breathing challenge that brain structures may not be able to manage.

Structures within the cerebellum play a significant role in responding to extreme respiratory or blood pressure challenge (8–11). Developmental neuropathology of the cerebellum, as well as surgical or other insult to this structure, is accompanied by various forms of disordered breathing, including apnea, obstructive apnea, and hypoventilation (12–16). Such disordered breathing induces substantial increases in markers for CO_2 (17), which, if not compensated, could place the subject at risk. Cerebellar neuropathology appears in SIDS victims, with a higher incidence of delayed cerebellar cortex maturation (18), and increased Purkinje cell apoptosis than controls (19). Cerebellar injury is very common in patients with epilepsy, likely induced either by excitotoxic injury or as a consequence of long-term antiepileptic medication. Additionally, in SIDS, transcriptional nucleolar activity is significantly reduced in cerebellar Purkinje cells relative to controls, suggesting hypofunction of the Purkinje cells in these victims. The nature of mechanisms underlying these pathologies or whether they underlie failure is unknown (20).

Cerebellar Purkinje cells provide the sole output of the cerebellar cortex, and innervate the deep cerebellar nuclei which, in turn, send efferents to multiple areas of the brainstem involved in respiratory and autonomic functions (21, 22). One pair of nuclei, the fastigial nuclei (FN), is responsive to chemical respiratory challenges, with cell body lesions of these nuclei significantly attenuating compensatory respiratory responses to increased levels of environmental CO_2. However, such lesions have little effect on relaxed, rhythmic breathing (10, 23). Additionally, *Lurcher* mutant mice (*Lc*/+) with global, developmental cerebellar Purkinje cell loss show reduced respiratory responses following exposure to increased environmental CO_2 (24), an outcome interpreted as a reduction in sensitivity to increased blood CO_2 levels (25–27).

Exposure to increased CO_2 in the environment is commonly encountered in everyday life, resulting in robust physiological responses, even with small increases in CO_2 (26). The phenotypic response to rising CO_2 levels is to increase minute ventilation by adjusting the depth of breathing [tidal volume (TV)], the frequency of breathing, or both (25–27). Recovery from ventilatory challenges results in a gradual return to baseline minute ventilation as blood levels of CO_2 decrease and blood levels of oxygen (O_2) increase (26). Disordered breathing patterns during recovery from environmental stressors indicate respiratory distress and can include periods of abnormally large TVs (sighs) coupled with apneic-like periods of extended pauses between breaths [end-expiratory pause (EEP)]. Large TVs and extended EEPs limit the ability to reduce increased blood levels of CO_2 by reducing the rate of gas exchange in the lungs (28). As disordered breathing patterns would reduce the ability to appropriately respond to, or recover from, exposure to exogenous stressors including hypercapnia or hypoxia, it has been suggested that such respiratory dysregulation could increase vulnerability to SIDS (29–31).

To determine if loss of cerebellar Purkinje cells results in distressed breathing following exposure to increased environmental CO_2, we used whole body plethysmography (WBP) to investigate the hypothesis that *Lurcher* mutants heterozygous for the *Lurcher* spontaneous mutation (*Grid2*^{Lc}), would exhibit disordered breathing patterns during recovery from hypercapnia, in comparison to wild-type (WT) controls. *Lurcher* mice were selected because we had previously observed that deficits in breath frequency appeared during recovery, following exposure to low levels of CO_2 (24). In the current study, we specifically targeted two components of breath frequency, including TV and EEP periods. These dependent variables were selected because of their importance in modulating increased blood levels of CO_2 by affecting the rate of gas exchange in the lungs.

MATERIALS AND METHODS

Animals

Mice were bred and housed in the Animal Care Facility of the Department of Psychology at the University of Memphis. They were maintained in a temperature-controlled environment (21 ± 1°C) on a 12:12 light–dark cycle (lights on at 0700 hours) and given free access to food and water. Original *Lurcher* (#001046; Mouse Genome Identifier #: 1857337) breeders were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All experiments were conducted with strict adherence to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The protocol was approved by the University of Memphis Institutional Animal Care and Use Committee.

The breeding of *Lurcher* mice entailed filial pairing of a non-ataxic female WT (B6CBACa *A*$^{w\text{-}J}$/*A*-*Grid2*$^{+}$) with a mutant ataxic male heterozygous for the *Lurcher* spontaneous mutation (B6CBACa *A*$^{w\text{-}J}$/*A*-*Grid2*^{Lc}). This breeding strategy produced litters that are composed of both heterozygous *Lc*/+ and WT mice. The *Lc*/+ mouse is phenotypically distinguishable from WT littermates as early as postnatal day 12 (PND 12) with cerebellar signs, including an ataxic gait, which permits non-invasive differentiation of *Lc*/+ mice from their non-ataxic WT littermates (32, 33).

Animals were weaned at PND 25 ± 4 days, and sibling housed in groups of three to five in ventilated polystyrene cages. In order to avoid litter effects, six *Lc*/+ and six WT littermates were selected randomly, each pair from different litters and mating cages. All mice were PND 60 at testing onset with a mean weight of 20.05 g (SD = 1.79 g). The subjects consisted of 12 male mice. Male mice were chosen because the incidence of sudden death and cerebellar neuropathology is much larger in males than in females (1, 34).

Whole Body Plethysmography

Experimental data were collected during light hours using a WBP system (Emka Technologies, Falls Church, VA, USA), as previously described (24). Briefly, a transducer was mounted to the WBP device, which converted pressure differentials in the chamber caused by respiration into electrical signals that were then transmitted to, and interpreted by, the software. Data were collected for each individual breath with sampling rates set at least twice the typical eupneic breathing levels observed in mice: typical TV <30 mL, typical expired volume <30 mL, and typical breath frequency <500/bpm (35, 36), to account for changes in breathing due to the response to, and recovery from hypercapnia. Pressure differentials that met all of these requirements, as interpreted by the software, were counted as individual breaths and added to the data output. Differentials that did not meet all of these parameters were flagged as movement artifacts and not included in the data file for analysis.

Procedure

Mice were weighed prior to placement in the WBP chamber. The experimental room temperature (22 ± 3°C) and humidity (20 ± 5%) were monitored daily to ensure stability throughout the study. An in-house program coupled to EMKA software was used to assess the subjects' respiratory responses at baseline (normal room air: 21% O_2, 0% CO_2, and 79% N_2), under conditions of increased CO_2 (2, 4, 6, and 8%), and during recovery from each CO_2 challenge in normal room air. Each test day began with a 10-min habituation period, followed by a 4-min exposure to baseline (room air) period. Under all conditions, air in the WBP chamber was removed continuously (0.8–1.0 L/m), to prevent accumulation of excessive CO_2 due to exhalation.

Baseline

After the 10-min habituation period, the dependent variables TV and EEP were continuously recorded, while the mice were exposed to room air for a total of 4 min (21% O_2, 0% CO_2, and 79% N).

Hypercapnia and Recovery

The entire hypercapnia program was 52 min in duration and consisted of an initial baseline (4 min) measured as described above, followed by four sequential challenges where CO_2 content was progressively increased from 2, 4, 6, and 8% (21% O_2, N_2 on balance). Each of the four CO_2 challenges consisted of a 2-min chamber fill period (the approximate time required for the WBP to fully achieve the desired gas percentages), followed by a 4-min exposure. To assess the recovery responses to multiple hypercapnic challenges, the program returned to room air following each challenge (again, including a 2-min chamber refill period and a 4-min recovery period). At termination of the final CO_2 exposure (8%), and return to room air, the mouse was removed from the chamber.

Variables and Data Analysis

To maintain sample equality among all animals on the measure of breath frequency, the dependent variables, TVs and EEPs, were recorded for the first 510 breaths from each animal during baseline and during recovery from each of the CO_2 challenge conditions. Because TV and EEP are each components of breath frequency, the breath samples from each animal were made equal at 510 breaths based on the animal with the lowest breaths per minute. Analyses were only conducted on individual breaths to specifically determine if genotypic differences in TVs and EEP durations contributed to previously observed deficits in overall breath frequency during recovery from increased levels of CO_2.

Animal Weights

An independent samples *t*-test was performed with genotype (*Lc*/+ and WT) as the independent variable and weights as the dependent variable to determine whether there was a need to include weight as a covariate, since lung function is tightly correlated with body size (37).

Baseline

Baseline data were analyzed using repeated measures analysis of variance (RMANOVA), with genotype (*Lc*/+ and WT) as the between-subjects factor and with the first 510 breaths as the within-subjects factors. Thus, the omnibus analysis was a 2 (Genotype) × 510 (Breaths) mixed design.

Hypercapnia Recovery

The recovery conditions were analyzed using an omnibus RMANOVA with genotype (*Lc*/+ and WT) again serving as the between-subjects factor. Within-subjects factors included four levels of recovery from CO_2 exposure (2, 4, 6, and 8% CO_2), and from each recovery period, the first 510 breaths to accurately track TVs and moment-to-moment changes in EEP. Therefore, the omnibus analysis became a 2 (Genotype) × 4 (Recovery from CO_2 exposure) × 510 (Breaths) mixed design. Depending on the results of the omnibus RMANOVAs, additional simple-effects tests were used to analyze interaction effects.

RESULTS

Animals

An independent samples *t*-test revealed no significant difference between the body weights of the two genotypes, $t(10) = -0.497$, $p = 0.630$.

Baseline

The omnibus RMANOVA revealed the depth of breathing (TV) in the two groups was equivalent during normal room air

[*Lc*/+ $M = 0.158$ mL, WT $M = 0.170$ ms, SEM 0.013 mL; Group, $F(1, 10) = 0.879$, $p = 0.371$]. **Figure 1A** shows the mean TVs of the two groups for the first 510 breaths. Note that breaths have been averaged into groups of 10 for clarity of presentation [Breath, $F(509, 5090) = 0.970$, $p = 0.670$; Group × Breaths, $F(509, 5090) = 0.900$, $p = 0.940$].

A second omnibus RMANOVA further revealed the two genotypes had equivalent EEP durations during exposure to room air [*Lc*/+ $M = 11.819$ ms, WT $M = 11.922$ ms, SEM 1.233 ms; Group, $F(1, 10) = 0.007$, $p = 0.935$]. As shown in **Figure 1B**, the two groups maintained equivalent EEP intervals throughout the continuum of 510 breaths and across individual breaths [Breath, $F(509, 5090) = 0.891$, $p = 0.957$; Group × Breaths, $F(509, 5090) = 1.011$, $p = 0.427$].

Hypercapnia Recovery

Figure 2A shows changes in average TV during recovery following each exposure to CO_2. Changes in TV were comparable in the two genotypes. Mice in both groups showed a typical increase in TV following exposure to increasing percentages of CO_2, indicating increased work to reduce the body burden of CO_2 [2, 4, 6, and 8% CO_2; Recovery, $F(3, 30) = 3.765$, $p = 0.021$]. Additionally, when considered across the 510 breaths within each recovery condition, both genotypes showed a gradual decline in TV, likely corresponding to gradual reductions in body levels of CO_2 with the return to room air [data not shown; Breaths, $F(509, 5090) = 1.420$, $p < 0.001$].

As shown in **Figure 2B**, when comparing recovery conditions, EEPs between the two genotypes differed significantly [Recovery × Group, $F(3, 30) = 4.044$, $p = 0.016$]. Simple-effects tests revealed that *Lc*/+ mice had significantly longer average EEP periods than WT mice during recovery from 2 and 4% CO_2 [2% Group, $F(1, 10) = 6.295$, $p = 0.031$; 4% Group, $F(1, 10) = 6.457$, $p = 0.029$]. Although genotypic differences only approached significance following exposure to 6% CO_2 [Group, $F(1, 10) = 3.128$, $p = 0.107$], there was an interval of approximately 100 breaths when *Lc*/+ mice had significantly longer EEPs than WT controls during recovery from 6% CO_2 [data not shown; Group × Breaths, $F(509, 5090) = 1.114$, $p = 0.046$].

Figure 3, graphically illustrates the pattern of individual breaths from a representative mutant and WT mouse at the midpoint of each 4-min recovery period following successive CO_2 exposures. Each panel of this figure includes the entire data form, over 10-s containing approximately 40 breaths. Visually, the two genotypes differed greatly in their breathing patterns. During the recovery periods following CO_2 exposure, the *Lc*/+ mice exhibited a distinctly abnormal respiratory pattern, visually consistent with previous reports of post-sigh apnea in mice (38). Thus, the *Lc*/+ mouse had abnormally deep breaths (>100% of the average TV over the previous 10-s), which were followed by multiple breaths consisting of atypically long EEPs (>100% of the average over the previous 10-s). The WT mice, however, did not exhibit this pattern of disordered breathing, maintaining a more uniform pattern, including TVs and EEPs that did not differ in volume or length.

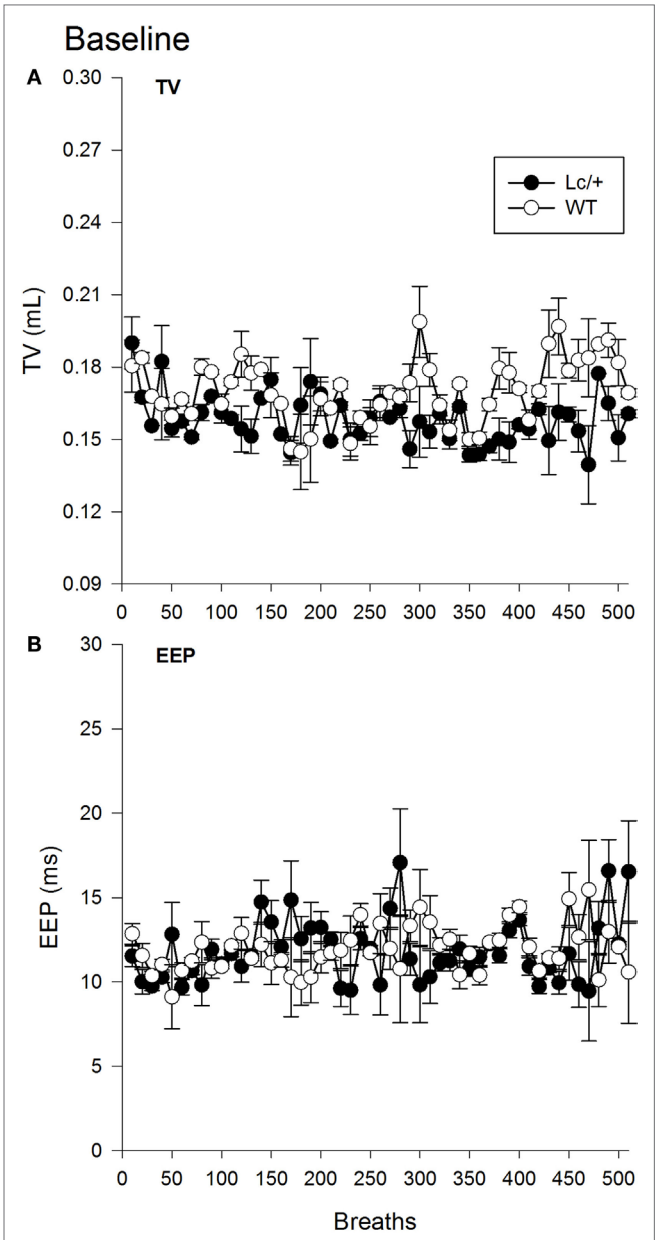

FIGURE 1 | Baseline tidal volume (A) and end-expiratory pause (B) data for 510 breaths at normal room air in *Lurcher* mutant (*Lc*/+) and wild-type (WT) control mice. Breaths have been averaged into groups of 10 for clarity of presentation. Values represent mean ± SEM.

DISCUSSION

Lc/+ mice and their WT littermates showed similar patterns of respiration when breathing room air at baseline. This finding further substantiates earlier results, indicating that cerebellar Purkinje cell loss has little effect on components of eupneic breathing (11, 12, 24).

The main outcomes of this study are that developmental loss of cerebellar Purkinje cells significantly reduced the ability of mice to compensate for the increased body burden of CO_2 as indicated by

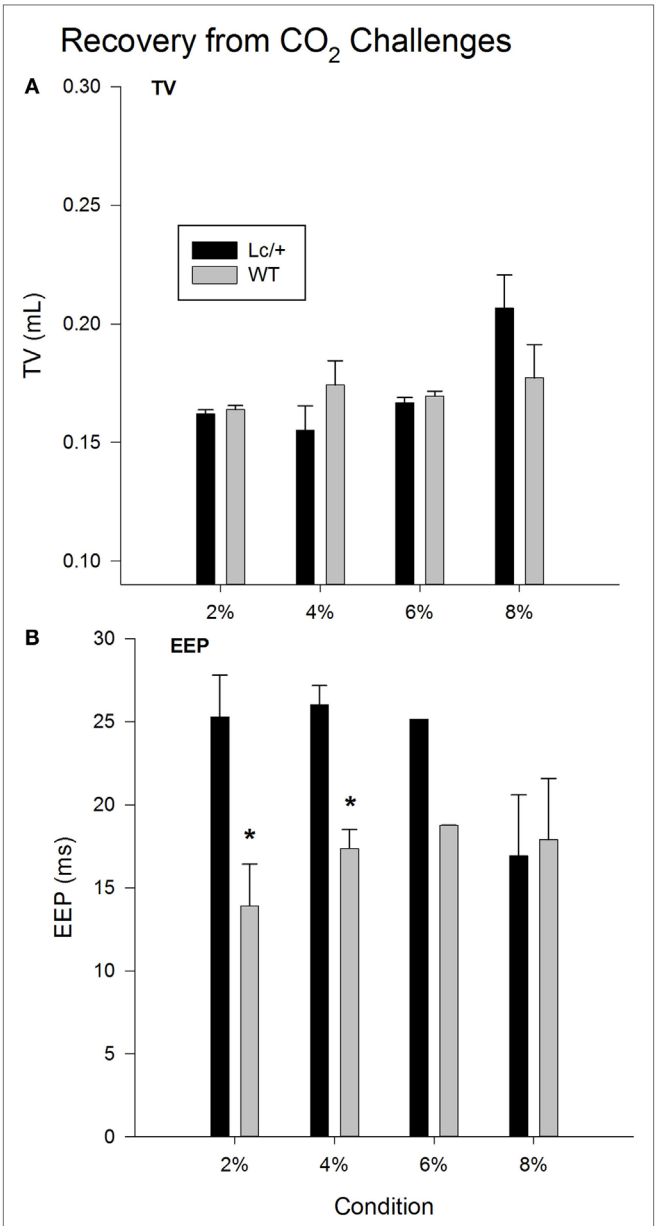

FIGURE 2 | Recovery tidal volume (A) and end-expiratory pause (B) data, averaged across 510 breaths at room air (21% O_2, 79% N_2, and 0% CO_2), following each CO_2 exposure period in *Lurcher* mutant (*Lc*/+) and wildtype (WT) mice. Values represent mean ± SEM. Asterisks indicate significant differences found between genotypes.

extended EEP periods following exposure to relatively low levels of hypercapnia. This is important because reduced compensatory responses to the increased body burden of CO_2 would result in prolonged hypoxemia, which itself could facilitate the cardiorespiratory failure that likely precedes sudden death events (28, 30). A similar pattern of disordered breathing has been documented in a report of sudden onset gasping and apnea in nine infants who subsequently died from SIDS (39). Collectively this preclinical and clinical evidence indicates the importance of evaluating breathing patterns and their potential contribution to impending fatal events. That *Lc*/+ mutants breathing approximated WT levels at 8% further suggests that the threshold for detection for rising levels of CO_2 in the mutants is much higher than that observed in control mice. This putative deficit in sensitivity to low levels of CO_2 likely results in a reduced ability to compensate for rising blood levels of CO_2 and the reduced ability to recover homeostatic ventilatory patterns. Since the cardiovascular system is closely integrated with breathing, disordered cardiovascular patterns, including cerebral blood flow, blood pressure, and heart rate, likely simultaneously occur (25–27). This possibility deserves further investigation.

Mechanisms potentially responsible for the patterns of disordered breathing observed in *Lc*/+ mice are unlikely to include peripheral muscular deficits, as *Lc*/+ mice show more fatigue-resistant diaphragm musculature than WT mice (40). Relatedly, a previous examination of the diaphragm in near-miss sudden death infants also revealed more fatigue-resistant diaphragm muscles compared to controls (41).

A more likely mechanism for the deficits in the recovery patterns of *Lc*/+ mice involves the absence of Purkinje cell input to the FN. The FN are predominately innervated by Purkinje cells of the cerebellum, especially those originating from the vermis, and serve significant roles in cardiorespiratory compensatory responses to multiple challenges, including hypercapnia and hypoxia (8, 10, 42–44). The FN are highly responsive to blood CO_2 changes, and to a lesser degree, O_2 changes, and modulate respiration during challenging conditions, but not during normal, rhythmic breathing. Cell body lesions of this structure attenuated responses to increased levels of environmental CO_2, but had no effect on breathing patterns in room air conditions (10, 14, 23). Structurally, following the loss of 100% of cerebellar Purkinje cells, *Lc*/+ mice exhibit a nearly 60% reduction in FN size, making it reasonable to conclude that this loss of volume could reduce FN-mediated chemosensitivity (45, 46). Such an inability to appropriately detect and respond to increasing CO_2 levels could increase the risk of a fatal outcome in chemosensitive-related extreme challenges, such as those expected in conditions such as SIDS or postictal periods in epilepsy patients.

Deficits in serotonergic neural systems may also play a role in the current findings. Cerebral serotonin [5-hydroxytryptamine (5-HT)] has been repeatedly implicated as integral in respiratory modulation, and pathophysiological changes in the number or distribution of central 5-HT-containing neurons may play a role in disordered breathing associated with sudden unexplained death (28, 47, 48). *Lc*/+ mice, in comparison to controls, show cerebellar patterns of disordered 5-HT that include a redistribution of 5-HT levels, resulting in increased levels in the deep cerebellar nuclei, including the FN, as well as increased serotonin binding in these nuclei (49, 50). In normal mice, an extensive 5-HT projection to Purkinje cells, originating from medullary and pontine respiratory areas exists (51, 52). As *Lc*/+ mutants lack Purkinje cells, the increased 5-HT projection to the deep cerebellar nuclei possibly represents a reprograming of this serotonergic projection from the Purkinje cells to the deep cerebellar nuclei. This speculative reprograming, in turn, may provide an additional contribution to the loss of sensitivity to low levels of CO_2. Thus, the differences

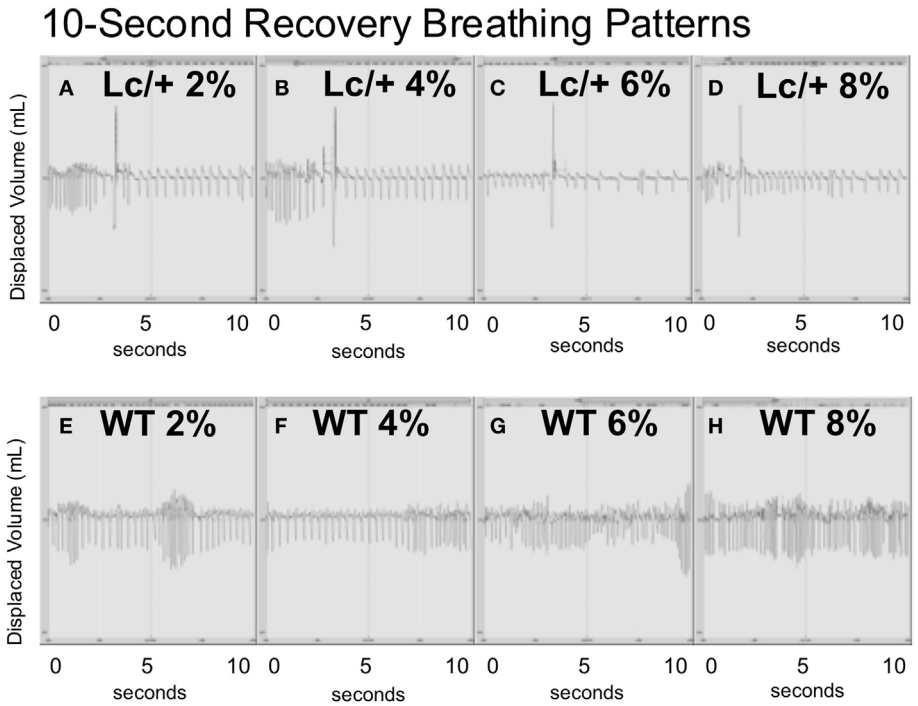

FIGURE 3 | All data including breaths during 10-s intervals from the middle point of each recovery period (at room air) following exposure to successive CO_2 challenges (2, 4, 6, and 8%) in an *Lc*/+ mouse (A–D), and a WT control (E–H). Data are centered on a zero line on the *y*-axis with inhalations falling below the zero line, and exhalations rising above the zero line. The patterns depicted with the above pair were consistent across all *Lc*/+ mice and wildtype controls.

observed in recovery patterns between *Lc*/+ and WT mice could result from neurochemical events that unfold relative to the developmental Purkinje cell loss in *Lc*/+ mice and further suggest an integral role of the cerebellum in respiratory modulation.

Other disorders also exude the characteristic traits of cerebellar neuropathology, including Purkinje cell loss, shrinkage of deep cerebellar nuclei, serotonergic disruptions, and disordered breathing patterns accompanying unexpected fatalities. Such signs are frequently found in epilepsy. People with epilepsy are roughly 24 times more likely to die unexpectedly than people without epilepsy (53), and ventilatory failure is a major suspect in the fatal event. As in SIDS, SUDEP is an exclusionary diagnosis, and SUDEP victims are typically found in the prone position in bed, which supports the possibility that death has occurred during sleep (54, 55). Postmortem examination of the brains of patients with chronic epilepsy and SUDEP victims consistently reveals cerebellar neuropathology, including cerebellar atrophy and Purkinje cell loss (56–58). Examination of the cerebellum of SUDEP victims revealed significantly higher amounts of Bergmann's gliosis and folial atrophy compared to age- and sex-matched controls (57). Additionally, decreased cerebellar Purkinje cell densities in either the anterior or posterior lobe appear in SUDEP victims relative to controls (56). Antiepileptic drugs (AEDs), specifically phenytoin (Dilantin), exacerbate cerebellar atrophy and Purkinje cell loss found in patients with epilepsy, and cause cerebellar neuronal damage in both humans and animal models (56, 59–63). Additionally, AEDs, including phenytoin and benzodiazepines, such as clonazepam are associated with an increased incidence of sleep-disordered breathing (64).

Sleep-disordered breathing frequently presents comorbidly with epilepsy (64, 65) and even without treatment with AEDs; patients report excessive daytime sleepiness, which can be an indicator of undiagnosed sleep apnea (66–72). The relevance of these findings for the current study is that sleep-disordered breathing, even without evidence of epilepsy or AEDs, induces significant cerebellar injury (73), and such injury can further intensify disturbed respiratory patterning.

Sudden unexplained death in childhood (SUDC), another exclusionary fatal event, occurs in children >1 year of age (74). It has been speculated that the mechanisms underlying sudden death in SIDS, SUDEP, and SUDC may be closely related (75). Evidence for such a link includes the finding that SUDC victims were significantly more likely to die unexpectedly when one or more febrile seizures were experienced (76). Additionally, an inverse relationship between SIDS and SUDC cases has recently been reported, suggesting that children who die of SUDC may have escaped the window of SIDS only to fail to respond appropriately to a subsequent exogenous stressor (77). As cerebellar neuropathology is often reported in sudden death syndromes, such as SIDS and SUDEP (18, 19, 56–58), and because the cerebellum, especially the Purkinje cells, are particularly vulnerable to hypoxic insult (78), this raises the suspicion that such pathology may be present in the brains of SUDC victims. This possibility,

however, has yet to be investigated as research on SUDC is still rudimentary.

Human clinical studies have yielded inconsistent support for the notion that cerebellar neuropathology is involved in sudden death events. For example, in 19 SIDS cases and 12 age-related controls, immunohistochemical staining of the cerebellar vermis revealed evidence of delayed maturation of the cerebellar cortex (18). Further, histological examination at autopsy for 35 cases of unexplained death and 20 controls revealed cerebellar cortex alterations including delayed maturation and apoptosis of the cerebellar Purkinje cells in the sudden unexplained death victims but not the controls (19). Conversely, two separate examinations of the left and right hemispheres of 12 SIDS victims and age- and sex-matched controls revealed no difference between the groups in cerebellar Purkinje cell density (79), Purkinje cell layer volume, or Purkinje cell number (80). However, as cerebellar Purkinje cells are especially vulnerable to even short periods of intermittent hypoxia (78), in the aforementioned studies, the use of controls who had died from conditions involving hypoxia (e.g., suffocation, carbon monoxide intoxication, and heart defect) leaves open the possibility that differences in Purkinje cell number between the two groups were mitigated by circumstances surrounding death. Further, these two studies did not examine the cerebellar vermis – the area of the cerebellum where the Purkinje cells that predominately innervate the FN arise from (81). As the FN is one area responsible for modulating chemosensitive responsivity in situations such as hypoxia (which are believed to occur just prior to unexpected death), it would be of value to include examination of the cerebellar vermis in future studies of sudden unexpected death victims including SIDS.

Collectively, it appears that developmental damage to the cerebellum may play an important role in the development of, or vulnerability to serious, and perhaps life-threatening breathing patterns in several patient populations. Multiple mechanisms may underlie cerebellar Purkinje cell loss, including genetic endowment, excitotoxic injury due to excessive neural activation in epilepsy, treatment with AEDs, hypoxic injury, and surgical insult. Despite various sources of damage to Purkinje cells, the present findings suggest that a potential for impaired breathing patterns exists, including a reduced ability to recover from mild hypercapnia, as demonstrated by a heightened incidence of apneic episodes (i.e., increased EEPs). Several patient populations at risk for sudden death exhibit a combination of cerebellar neuropathology and sleep-disordered breathing, a relationship that suggests that cerebellar injury may contribute to inappropriate responses following hypercapnic exposure that may lead to seriously compromised breathing. The interactions among cerebellar Purkinje cells, their output to autonomic- and respiratory-mediating FN, and integration with other neurotransmitter systems, such as serotonin, that regulate breathing patterns merits continued investigation along with the possibility of sex differences similar to those reported in the human population in sudden death events.

AUTHOR CONTRIBUTIONS

MC designed the software program and oversaw data acquisition. JH was integral in data acquisition and provided revision input. RH, DG, and GM conceived this work. GM oversaw data acquisition, and performed analyses and interpretation. GM and MC cleaned data, analyzed and interpreted data, and drafted the manuscript and revisions. RH and DG provided substantial revision guidance. All authors were involved in the final approval of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Immunohistochemical Analysis of Brainstem Lesions in the Autopsy Cases with Severe Motor and Intellectual Disabilities Showing Sudden Unexplained Death

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It is known that patients with severe motor and intellectual disabilities (SMID) showed sudden unexplained death (SUD), in which autopsy failed to identify causes of death. Although the involvement of brainstem dysfunction is speculated, the detailed neuropathological analysis still remains to be performed. In order to clarify pathogenesis, we investigated the brainstem functions in autopsy cases of SMID showing SUD. We immunohistochemically examined expressions of tyrosine hydroxylase, tryptophan hydroxylase, substance P, methionine-enkephalin, and c-fos in the serial sections of the midbrain, pons, and medulla oblongata in eight SUD cases and seven controls, having neither unexplained death nor pathological changes in the brain. Expressions of tyrosine hydroxylase and tryptophan hydroxylase were reduced in two of eight cases, and those of substance P and/or methionine-enkephalin were augmented in the pons and medulla oblongata in seven of eight cases, including the aforementioned two cases, when compared with those in controls. The hypoglossal nucleus and/or the dorsal vagal nucleus demonstrated increased neuronal immunoreactivity for c-fos in seven of eight cases, although there was no neuronal loss or gliosis in both the nuclei. Controls rarely showed immunoreactivity for c-fos in the medulla oblongata. These data suggest the possible involvement of brainstem dysfunction in SUD in patients with SMID, and consecutive neurophysiological evaluation of brainstem functions, such as all-night polysomnography and blink reflex, may be useful for the prevention of SUD, because some parameters in the neurophysiological examination are known to be related to the brainstem catecholamine neurons and the spinal tract nucleus of trigeminal nerve.

Keywords: severe motor and intellectual disabilities, sudden unexplained death, brainstem, immunohistochemistry, catecholamine, substance P, c-fos

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INTRODUCTION

Severe motor and intellectual disabilities (SMID) describe a heterogeneous group of disorders with severe physical disabilities and profound mental retardation (1, 2). Patients with SMID are frequently complicated with various neurological disorders and respiratory problems, such as upper airway obstruction, aspiration, and central apnea (3–5), in addition to circulatory disturbances. In patients with SMID, it is known that sudden unexplained death (SUD) accounts for 5% of cause of death (6), and definite causes are not identified even after autopsy in some cases. The involvement of brainstem

dysfunction is speculated in sudden infant death syndrome (SIDS) (7–9), and disturbances of brainstem catecholamine neurons were involved in sudden death in developmental brain disorders, such as SIDS and Fukuyama-type congenital muscular dystrophy (FCMD) (10, 11). The brainstem expression of substance P was augmented in the spinal tract nucleus of trigeminal nerve in SIDS victims and cases of FCMD showing SUD (10, 12). In addition, the altered expression of proto-oncogene c-fos, a marker of early neuronal activation subsequent to noxious stimulation, was observed in sudden death in neurological disorders (12–14). Herein, we performed the comprehensive immunohistochemistry on the brainstem lesions in autopsy cases, in order to clarify the pathogenesis of SUD in patients with SMID, and discussed the possibility of monitoring SUD using neurophysiological examination.

PATIENTS AND METHODS

The clinical subjects comprised eight cases of SMID showing SUD (the SUD cases) between 1968 and 2001 at Tokyo Metropolitan Fuchu Medical Center for the Disabled and Tokyo Metropolitan Neurological Hospital and seven controls, aged from 7 to 55 years, showing neither SUD nor pathological changes in the central nervous system (**Table 1**). The family of each subject provided informed consent to the detailed neuropathological analysis. The brains were fixed in a buffered formalin solution, and each formalin-fixed brain was cut coronary, embedded in paraffin. We examined the cerebral cortex and white matters, basal ganglia, thalamus, cerebellum, brainstem, and spinal cord by routine histochemical staining and failed to find neither neuronal loss nor gliosis in each region. Considering the possibility of functional lesions in the brainstem, which have been pointed out in SIDS and/or FCMD, we performed immunohistochemistry in the brainstem sections subsequently. For immunohistochemical staining of the brainstem, sections with thickness of 5 μm were serially cut in the upper and lower parts of the midbrain, and the upper, middle, and lower parts of the pons, and medulla oblongata. They were deparaffinized, quenched with 1% hydrogen peroxide, and treated after microwave antigen retrieval with the following antibodies: mouse monoclonal antibodies to glial fibrillary acidic protein (GFAP, Dako, Glostrup, Denmark), tyrosine hydroxylase (Affinity Bioreagents, Inc., Golden, CO, USA), and tryptophan hydroxylase (Oncogen Research Product, Cambridge, MA, USA), in addition to rabbit polyclonal antibodies to substance P (Zymed Laboratories, Foster City, CA, USA), methionine-enkephalin (Chemikon International, Inc., Temecula, CA, USA), and c-fos (Santa Cruz Biotechnology, CA, USA) at the following concentrations: 1:50 (GFAP, substance P), 1:100 (tryptophan hydroxylase), 1:400 (tyrosine hydroxylase), 1:1000 (methionine-enkephalin, c-fos). Antibody binding was visualized by means of the avidin–biotin–immunoperoxidase complex method (Nichirei, Tokyo, Japan) following the manufacturer's protocol. No staining was confirmed in sections in the absence of either antibody. In immunohistochemistry for tyrosine hydroxylase and tryptophan hydroxylase, counts of immunoreactive neurons were performed by the manual-labeling of appropriate cells with nuclei in two serial sections, and the mean value were calculated in each brainstem region (**Table 2**). The mean and SD of averages in the controls and SUD cases was analyzed with *t*-test for the comparison, and reported *p* values were shown in **Table 2**. This study was approved by the Ethical Committee in Tokyo Metropolitan Institute of Medical Science.

RESULTS

A mild increase of the goblet cells was found in the SUD cases (**Table 1**), but that may be related to recurrent subtle aspiration, and did not lead to the diagnosis of bronchopneumonia. Neither

TABLE 1 | Summary of clinical and pathological findings.

No.	Age (year)/sex	Brain disorders	Cause of death	Resuscitation time (h)	Postmortem time (h)	Brain weight (g)	Increase of goblet cells
Controls							
1	7/male	(–)	Sepsis		nd	nd	(–)
2	9/male	(–)	Sepsis		nd	nd	(–)
3	10/male	(–)	Leukemia		4	nd	(–)
4	22/male	(–)	DIC		nd	nd	(–)
5	29/male	(–)	Heart failure		nd	nd	(–)
6	38/male	(–)	Leukemia		nd	nd	(–)
7	55/male	(–)	Pulmonary cancer		5	1400	(–)
Sudden unexplained death							
1	6/male	Kernicterus		2	3	1331	1+
2	9/male	Schizencephaly		10	2	499	1+
3	13/male	Perinatal HIE		3	2	635	1+
4	20/male	Perinatal HIE		0.5	3	580	1+
5	23/female	Micro syndrome		1	7.5	260	(–)
6	29/female	Kernicterus		0.5	2	1437	1+
7	36/male	Postnatal HIE		2	6	578	1+
8	47/male	Encephalopathy		3	4	1348	(–)

The degree of goblet cell change was graded by visual inspection as absence (–) or presence 1+. DIC, disseminated intravascular coagulation; HIE, hypoxic ischemic encephalopathy; nd, not determined.

TABLE 2 | Summary of immunohistochemistry for tyrosine hydroxylase and tryptophan hydroxylase.

No.	Tyrosine hydroxylase					Tryptophan hydroxylase	
	Periaqueductal gray matter	Locus ceruleus		Dorsal vagal nucleus		Superior central nucleus	Medullary raphe nucleus
		Left	Right	Left	Right		
Controls							
1	28	124.5	124.5	17.5	17	nd	34.5
2	19	88.5	91	6.5	7	170.5	49.5
3	10	nd	nd	10	12.5	nd	35.5
4	13.5	144.5	132.5	11	13.5	165	37
5	35	124	151	10.5	11.5	52	31
6	26.5	147	127	12	10	94.5	12.5
7	23.5	116.5	51	9	9.5	141	20.5
mean ± SD	22 ± 9	124 ± 21	113 ± 36	11 ± 3	12 ± 3	125 ± 50	32 ± 12
Sudden unexplained death							
1	34	94	88.5	11.5	13	164	59
2	7.5	**23**	**24.5**	**0**	**0**	110	28
3	28	136.5	136.5	7	8	80	13
4	35.5	98	114	10	13	67.5	56.5
5	29	137.5	138	12	14.5	119	13
6	5.5	**17**	**15.5**	**0**	**0**	53.5	**4.5**
7	25.5	76	73.5	7.5	11	55	47.5
8	17	85	79.5	19.5	18	93	16
mean ± SD	23 ± 12	83 ± 45	84 ± 46	8 ± 6	10 ± 7	93 ± 37	30 ± 22
p value (*t*-test)	0.92	0.06	0.23	0.38	0.51	0.22	0.85

*The number of tyrosine hydroxylase- and tryptophan hydroxylase-immunoreactive neurons in the each brainstem region is expressed. The mean of averages in controls and the SUD cases was analyzed with t-test for the comparison, and reported p values were shown in **Table 2**. Hatched labeling with bold characters denotes a reduction in the number of neurons less than the mean minus 2SD of average in controls.*
nd, not determined.

neuronal loss nor increase of astrocytes immunoreactive for GFAP was observed in the brainstem regions examined in the SUD cases, in comparison with sections in controls. Neurons and neuronal processes immunoreactive for tyrosine hydroxylase were observed in the periaqueductal gray matter in the midbrain, locus ceruleus, and dorsal vagal nucleus in controls (**Table 2**; **Figure 1A**). Neurons immunoreactive for tryptophan hydroxylase were found in the superior central nucleus and raphe nucleus in the medulla oblongata in controls (**Table 2**). There was no significant difference in the number of neurons immunoreactive for either tyrosine hydroxylase or tryptophan hydroxylase between controls and the SUD cases. However, the SUD cases 2 and 6 exhibited a reduction in the number of neurons immunoreactive for tyrosine and/or tryptophan hydroxylase less than the mean minus 2SD of average in the controls (**Table 2**; **Figure 1B**). There was a variation in the degree of immunoreactivity for substance P and methionine-enkephalin in the neuropil and neuronal fibers in the substantia nigra in both controls and the SUD cases. Immunoreactivity for substance P was augmented in seven of eight SUD cases in the spinal tract nucleus of trigeminal nerve, in comparison with that in controls (**Table 3**; **Figure 2**). Four of the aforementioned seven SUD cases showed the increased immunoreactivity for substance P in the dorsal vagal nucleus. Moreover, the four of seven SUD cases with the augmented immunoreactivity for substance P also demonstrated increased immunoreactivity for methionine-enkephalin in the spinal tract nucleus of trigeminal nerve (**Table 3**). There were a few neurons immunoreactive for c-fos in the periaqueductal gray matter, pontine tegmentum, and posterior funicular nucleus in both controls and the SUD cases (**Table 4**). In the hypoglossal nucleus, neurons immunoreactive for c-fos were found in six of eight SUD cases, but not in controls (**Figures 3A,B**). In addition, five of the aforementioned SUD cases and the SUD case 1 showed neurons immunoreactive for c-fos in the dorsal vagal nucleus, in which those were observed only in the control 4 (**Figures 3C,D**).

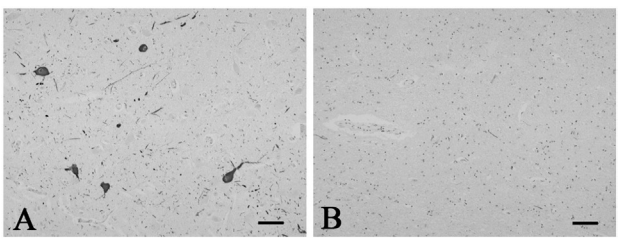

FIGURE 1 | Representative photographs in immunohistochemistry for tyrosine hydroxylase. Immunoreactivity for tyrosine hydroxylase was observed in the neurons and neuronal processes in the periaqueductal gray matter in the control 5 **(A)**. The SUD case 6 showed a reduction in neurons and processes immunoreactive for tyrosine hydroxylase in the same area **(B)**. Bars = 80 μm.

DISCUSSION

In the SIDS victims, fine morphological changes have been noted in the brainstem respiratory network, including Kölliker–Fuse

TABLE 3 | Summary of immunohistochemistry for substance P and methionine-enkephalin.

No.	Substance P			Methionine-enkephalin		
	Substantia nigra	Dorsal vagal nucleus	Spinal tract nucleus of trigeminal nerve	Substantia nigra	Dorsal vagal nucleus	Spinal tract nucleus of trigeminal nerve
Controls						
1	2+	1+	1+	1+	(–)	(–)
2	nd	1+	1+	nd	1+	(–)
3	2+	1+	1+	1+	1+	(–)
4	1+	1+	1+	1+	1+	1+
5	2+	1+	1+	2+	1+	1+
6	1+	1+	1+	1+	1+	1+
7	2+	1+	1+	2+	1+	1+
Sudden unexplained death						
1	2+	1+	1+	2+	1+	(–)
2	2+	1+	**2+**	1+	1+	(–)
3	2+	**2+**	**2+**	1+	1+	1+
4	1+	**2+**	**2+**	1+	1+	1+
5	2+	**2+**	**2+**	2+	1+	**2+**
6	2+	1+	**2+**	1+	1+	**2+**
7	1+	1+	**2+**	1+	1+	**2+**
8	2+	**2+**	**2+**	2+	1+	**2+**

The degree of immunoreactivity of neuronal processes was graded as follows: absence as (–); a few as 1+; and many as 2+. Hatched labeling with bold characters denotes an augmentation of immunoreactivity compared with that in controls.
nd, not determined.

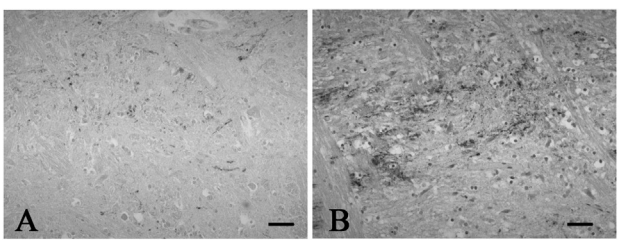

FIGURE 2 | Representative photographs in immunohistochemistry for substance P. Immunoreactivity for substance P was observed in a few neuronal processes in the spinal tract nucleus of trigeminal nerve in the control 1 **(A)**, while that was found in many neuronal processes in the SUD case 2 **(B)**. Bars = 40 μm.

TABLE 4 | Summary of immunohistochemistry for c-fos.

No.	Periaqueductal gray matter	Pontine tegmentum	Posterior funicilar nucleus	Hypoglossal nucleus	Dorsal vagal nucleus
Controls					
1	1+	(–)	(–)	(–)	(–)
2	(–)	1+	(–)	(–)	(–)
3	1+	1+	(–)	(–)	(–)
4	1+	(–)	1+	(–)	1+
5	(–)	(–)	(–)	(–)	(–)
6	1+	1+	(–)	(–)	(–)
7	1+	1+	(–)	(–)	(–)
Sudden unexplained death					
1	1+	(–)	(–)	(–)	1+
2	(–)	(–)	(–)	(–)	(–)
3	1+	(–)	(–)	1+	1+
4	(–)	(–)	(–)	1+	1+
5	(–)	(–)	(–)	1+	1+
6	(–)	(–)	(–)	1+	(–)
7	(–)	(–)	1+	1+	1+
8	1+	(–)	(–)	1+	1+

The absence and presence of neurons immunoreactive for c-fos are denoted as (–) and 1+.

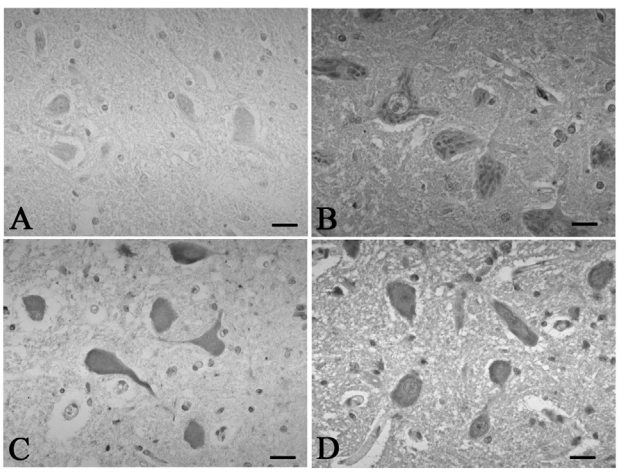

FIGURE 3 | Representative photographs in immunohistochemistry for c-Fos. In the hypoglossal nucleus, there were no neurons immunoreactive for c-fos in the control 5 **(A)**, which was found in the SUD cases 5 **(B)**. Neurons immunoreactive for c-fos were observed in the dorsal vagal nucleus in both the control 4 **(C)** and SUD case 4 **(D)**. Bars = 20 μm.

nucleus (7–9), although autopsy failed to reveal coarse lesions in the brain. Similarly, the disturbances in the autonomic nervous system, fluctuating muscle tone with involuntary movements and/or hypersensitivity to environmental stimuli, may be involved in SUD in patients with neurological disorders (12), but the detailed pathogenesis still remains to be investigated. We previously reported the selective disturbance in the brainstem expressions of tyrosine hydroxylase, tryptophan hydroxylase, and methionine-enkephalin in autopsy cases of West and Lennox–Gastaut syndromes (15). In this analysis, the expressions of neurotransmitters, substance P, methionine-enkephalin, and c-fos were examined in serial brainstem sections in the SUD cases, focusing on the brainstem dysfunction. The SUD cases 2 and 6 seemed to show disturbances in catecholamine and serotonin neurons (**Table 2**). In addition, seven of the SUD cases including the cases 2 and 6 showed increased immunoreactivity for substance P and/or methionine-enkephalin in the spinal tract nucleus of trigeminal nerve (**Table 3**). Seven of the eight SUD cases demonstrated neurons immunoreactive for c-fos in the hypoglossal nucleus and/or dorsal vagal nucleus (**Table 4**). These immunohistochemical abnormalities lacked both obvious neuronal loss and gliosis, suggesting the possible disturbances of brainstem function.

Sudden infant death syndrome victims were reported to show reduced immunoreactivity for tyrosine hydroxylase in

the dorsal vagal nucleus and ventrolateral reticular formation in the medulla oblongata (10). Sudden death sometimes occurs in patients with FCMD, in which the neurons immunoreactive for tyrosine hydroxylase were reduced in the reticular formation of medulla oblongata, dorsal vagal nucleus, and solitary tract nucleus (11, 12). Tyrosine hydroxylase is a rate-limiting enzyme in catecholamine biosynthesis, and the reduced expression of tyrosine hydroxylase in the brainstem are assumed to reflect the dysfunction of catecholamine neuron system, which can lead to disturbed autonomic regulation and/or neural respiratory control. The similar pathological processes can be speculated in the SUD cases 2 and 6 in this analysis. The medullary serotonin neurons, which are also involved in the autonomic and respiratory homeostasis, showed increased cell density with reduced binding receptor sites in SIDS victims (16). The SUD case 6 only showed a reduction of medullary serotonin neurons, and the lesion of serotonin neurons in the SUD cases seems to be less predominant and different from that in SIDS. Increased immunoreactivity for substance P associated with proliferation of astrocytes was found in the pontine reticular formation and the medullary spinal trigeminal nucleus in the SIDS victims (10). Inasmuch as substance P increases central inspiratory activity and can mediate the central nervous response to hypoxia, it is speculated that the augmented expression of substance P in the SIDS victims may be related to the influence of hypoxia. The augmented immunoreactivity for substance P was also observed in the spinal tract nucleus of trigeminal nerve and dorsal vagal nucleus in the SUD cases, but astrocytes immunoreactive for GFAP were not increased. In our previous study, the immunoreactivity for methionine-enkephalin, but not substance P, was reduced in the absence of neuronal loss and gliosis in the spinal tract nucleus of trigeminal nerve in the autopsy cases of West and Lennox–Gastaut syndromes (15). Although the exact mechanism of difference in the disturbed expressions of substance P and methionine-enkephalin between intractable epilepsy and SUD is not clear, it is suggested that the impairments of trigeminal system can be involved. C-fos is the first gene activated by noxious signals, particularly in the presence of hypoxia, and useful to demonstrate the stimulus-related local neuronal activation (13, 14). Neurons immunoreactive for c-fos were increased in the dorsal vagal nucleus in SIDS victims (13), and those were observed in the hypoglossal nucleus and/or the dorsal vagal nucleus in the SUD cases in this analysis. Since the hypoglossal nucleus and dorsal vagal nucleus are known to be associated with neuronal cardiopulmonary regulation (17), the increase of neurons immunoreactive for c-fos in SIDS victims and the SUD cases may reflect a certain response to hypoxic brain insults.

It is controversial whether the immunohistochemical changes in this analysis are causes or results of SUD; however, the data suggest the possible involvement of brainstem dysfunction in SUD. Patients with SMID occasionally demonstrate abnormalities in both the sleep parameters in all-night polysomnography and the R2 components in blink reflex, which are related to the brainstem catecholamine neurons and the spinal tract nucleus of trigeminal nerve, respectively, both of which demonstrated changes in the SUD cases of SMID in this analysis (18). We believe that routine neurophysiological evaluation of brainstem functions, including all-night polysomnography and blink reflex, will give us a clue to prevent SUD in patients with SMID. Furthermore, we will try to quantify the protein expressions by Western blot analysis in the frozen specimen of autopsy brains in the future.

CONCLUSION

Sudden unexplained death remains to be one of the important causes of death in patients with SMID, although the exact pathomechanisms are not clarified fully. The data in this analysis suggest the possible involvement of brainstem dysfunction in SUD, and the detailed neurophysiological evaluation of brainstem functions is recommended in patients with SMID.

AUTHOR CONTRIBUTIONS

Prof. MH planned this analysis and conducted all processes, such as selection of subjects, immunohistochemical staining, quantitative assessment of each specimen, taking photographs, and writing the manuscript. Prof. HS financially supported this analysis, supervised all processes, and discussed the context of manuscript.

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REVIEW
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Substance P/Neurokinin 1 and Trigeminal System: A Possible Link to the Pathogenesis in Sudden Perinatal Deaths

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Sudden demise of a healthy fetus or a neonate is a very tragic episode in the life of parents. These deaths have been a mystery since ages but still remain unexplained. This review proposes the involvement of trigeminal nerve, neurotransmitter substance P (SP), and its receptor neurokinin 1 (NK-1R) in regulation of cardiorespiratory control in fetuses and newborns. Anomalies and immaturity of neuroregulatory systems such as trigeminal system in medulla oblongata of brainstem may provide a possible mechanism of sudden perinatal deaths. Vulnerable infants are born with respiratory center immaturity which in combination with any stressor such as cold, hypoxia, and smoking may lead to cessation of breathing and ventilatory response. SP/NK-1R may be involved in regulating the ventilatory control in neonates while it is decreased in fetal and adult life in humans, and any alterations from these may lead to irreversible sleep apnea and fatal breathing, ultimately sudden death. This review summarizes the studies performed to highlight the expression of SP or NK-1R in sudden perinatal deaths and proposes the involvement of trigeminal ganglion along with its nerve and SP/NK-1R expression alteration as one of the possible pathophysiological underlying mechanism. However, further studies are required to explore the role of SP, NK-1R, and trigeminal system in the pathogenesis of sudden infant deaths, sudden intrauterine deaths, stillbirths, and sudden deaths later in human life.

Keywords: sudden infant deaths, sudden perinatal death, substance P, neurokinin 1 receptor, trigeminal nerve

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INTRODUCTION

Sudden perinatal deaths comprise of stillbirths, fetal [sudden unexplained intrauterine deaths (SUID)], and infant death [less than 1 year of age, sudden infant deaths (SIDS)] that is spontaneous and mysterious. These cases remain unresolved even after complete autopsy, medical examination, and thorough investigation of death scene (1). Theories on the possible causes of SIDS are more than 100. It is widely accepted that SIDS is a combination of multifactors that occur during the period of increased vulnerability and may cause the fatal outcome in some infant (2). Bergman et al. also suggested that SIDS is not caused by "single characteristic that ordains an infant for death," but depends on an "interaction of risk factors with variable probabilities" (3). Pathophysiology of SIDS remains unexplained (2).

Brainstem anomalies as a possible cause of sudden perinatal death is mostly accepted hypothesis as suggested by Filiano and Kinney (4) in their famous triple risk model. These neuropathologies lead to vulnerable fetus or newborn who becomes unable to respond to any kind of stressor and dies suddenly (5). All the factors whether maternal, infant, environmental, or genetic interfere with

the cardiorespiratory control leading to final common pathway (death) (6). Mechanisms underlying SIDS appear to originate in fetal period of development resulting in neural damage and affect breathing or blood pressure during sleep later on (5). Neuromodulators like somatostatin, serotonins (7), substance P (SP), etc. regulate the breathing control activities (8–10). The focus of current review is to highlight the SP and its receptor NK-1R in neuronal respiratory control system during critical ontogenetic periods of human brain development. Our research group for the first time in 2011 (11) suggested the involvement of SP in the pathogenesis of SUID.

SUBSTANCE P

Substance P is the prototype and first discovered tachykinin. It is a neurotransmitter of the afferent sensory nervous system (12). It is a small peptide hormone consisting of 11 amino acids belonging to tachykinin family (TK) (13). It is the most abundant TK peptide and neurotransmitter in CNS of mammals (14). It has been implicated in various physiological and pathophysiological processes (15) and found in many central and peripheral neural pathways. SP is released from fifth cranial nerve, the trigeminal, which is part of trigeminal system that is explained below.

TRIGEMINAL SYSTEM

Trigeminal system is highly established and well-studied system in mammals and birds (16). SP immunoreactivity (SP-IR) has been observed in trigeminal (17) and dorsal root ganglia (DRG) (18). Main feature of this system is the presence of two distinct primary afferent neuronal groups: trigeminal ganglion (TG) and mesencephalic trigeminal nucleus (MTN). Cell bodies of these primary afferent neurons are present in TG (19), and few lie in MTN. MTN is involved mainly in proprioception (mainly orofacial musculature) (20). TG dorsomedial part is involved in nociception, thermoreception, and proprioception while its ventrolateral part is involved in mechanoreception (21). Signals from the trigeminal system are transmitted by second order neurons in brainstem to different regions of CNS pain centers (22). The processes in the middle of ganglion end up on various groups of second order neurons, which convey their signals to the somatosensory cortex *via* thalamus (23) (**Figure 1**).

TRIGEMINAL GANGLION

Trigeminal ganglion is accumulation of pseudounipolar neurons (24) and consists of neurons and their fibers. TG is a cranial analog of DRG in PNS (21). Activation of TG nerves plays a central role in most forms of orofacial pain (25). Many neurotransmitters and their receptors are localized in different subpopulations of TG neurons (21). TG neurons supply innervations mostly to the mechanoreceptors, thermoreceptors, and nociceptors in orofacial region (16). Glial cells also known as satellite cells (26) completely enclose the neuronal somata of TG neurons, and thus they have no synaptic contacts (27).

Based on their appearance, ganglion cells are classified as follows: large light (A) and small dark (B) cells (28). Large light A cells produce thick myelinated fibers while the thin C fibers (both myelinated and unmyelinated) are originated from small dark B cells (29). Two primary afferent neuron subpopulations are noticed in TG: small- and medium-sized neurons with small somata, including glutamate, somatostatin, SP, neurokinin A, CGRP, cholecystokinin, vasoactive intestinal peptide, and galanin, and larger sized neurons that are relatively less and include neuropeptide Y and peptide 19. Presence of SP in small diameter primary afferent fibers and in nociceptive centers of brain gives us an idea of its nociceptive role (30).

TRIGEMINAL NERVE (TrN)

Trigeminal ganglion provides somatosensory innervations of face and oral cavity through TrN (31) (**Figure 2**). It has three branches: V1 and V2 are purely sensory while V3 has both sensory and motor functions. V1 innervates forehead, upper eyelid, cornea, conjunctiva, mucosa of frontal ethmoid and sphenoid sinuses, and dorsum of nose. V2 innervates upper lip, lateral portions of nose, parts of oral cavity, mucosa of nasal cavity, maxillary sinus, upper jaw, and roof of mouth and upper dental arch while V3 innervates lower lip, chin, cheek, lower teeth, gingival, mucosa of lower jaw, floor of mouth, and anterior two-thirds of tongue (25).

NEUROKININ RECEPTORS

Tachykinin receptors also known as neurokinins (NK) are G protein-coupled receptors, localized in the nucleus of the solitary

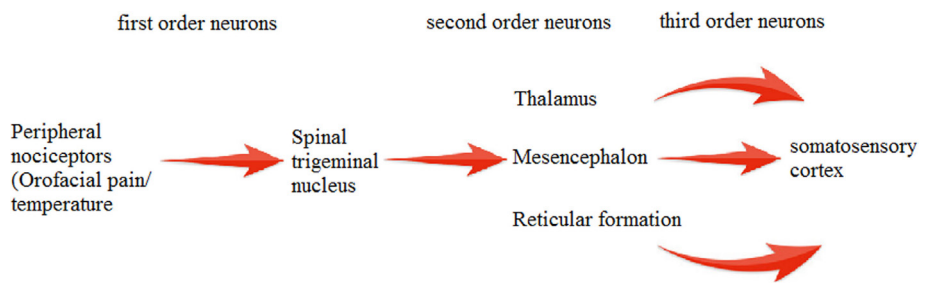

FIGURE 1 | Processing of nociceptive stimulus within brain.

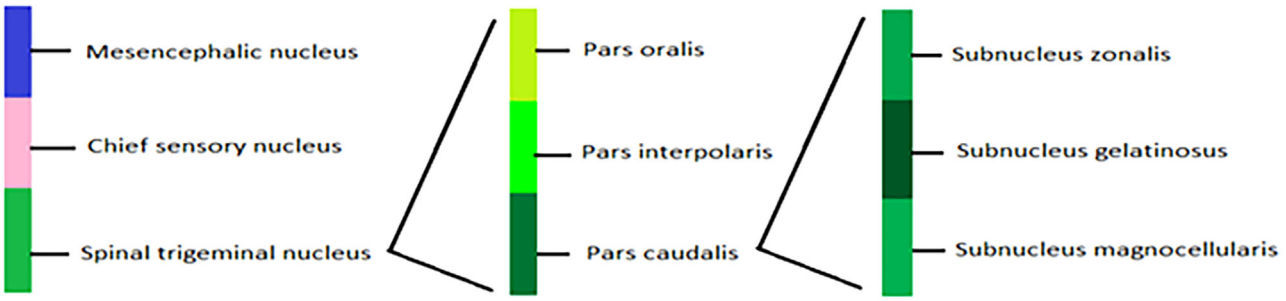

FIGURE 2 | **Graphical representation of trigeminal nerve nucleus**.

tract, which is known to be involved in the rhythmic control (32). There are three NK receptors (1R-3R). Functional activities of SP are initiated after binding to the neurokinin 1 (NK-1R), which is a transmembrane protein (33). Upon binding, a chain of signaling events is activated by the internalization of SP–NK-1R complex (34), which stimulates the second messenger phospholipase C resulting in the production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (35). Calcium is released intracellularly as a result of stimulation of endoplasmic reticulum by IP3, and protein kinase C is activated by DAG (36).

These neurokinin receptors are present within the cardiorespiratory regulatory control centers and in the phrenic nucleus, which controls the diaphragm and mediates the respiratory responses to SP. Prototype TK, SP, was reported to stimulate the respiratory rhythm in wild-type mice, in the *in vitro* brainstem–spinal cord preparation but not in the NK-1R knockout (NK-1R−/−) mice (37). The study of *in vitro* brainstem preparations revealed that NK-Rs have a vital role in the regulation of respiratory control and lung burst activity during the development of bullfrog from tadpole to adult stage (38). Role of SP has been implicated in the development of plasticity of respiratory system and the regulation of respiratory rhythm. A functional SP-ergic system is necessary for the generation of sufficient ventilatory responses to hypoxia in newborn mice and during early maturation (39). Under increasing hypoxia, SP manifests as natural anti-hypoxant and is not only involved in nociception mechanisms but also in brain adaptation to oxygen deficiency (40). SP-ergic system was found to be more active in regulating the respiratory responses during the early postnatal period in neonatal rat brainstem–spinal cord preparation (41) and medullary slice preparations of newborn mice (42). But surprisingly, SP was not found to control ventilatory rhythm generation in fetal rats, and it was hypothesized that, may be, SP does not modulate the generation of respiratory responses before birth and affects the phrenic motoneurons only after birth (43).

ROLE OF SP/NK-1R IN REGULATION OF RESPIRATORY RHYTHM

Neuromodulator SP causes dilation of vessels, contraction of smooth muscles in the respiratory system, increased action potential in neurons, an increase in vascular flow, and production of saliva (44). In humans, NK-1R is involved in causing bronchoconstriction (45). NK-1Rs mediate increases in secretion of mucous glands in human trachea (46). SP-IR was observed to be potentiated in a study conducted on the bronchoalveolar lavage fluid (47) and sputum samples (48) from asthmatic patients. NK-1R mRNA expression was also higher in asthmatic lung tissue when compared to non-asthmatic (49). Elevated levels of SP and PPT-A mRNA were observed in the nodose ganglia of ovalbumin-sensitized guinea pigs (50) with increased neurogenic inflammation and bronchoconstriction produced by NK-1R (51). It suggests that SP/NK-1R and neurogenic immunoreactivity are critical for the progression of airway hyperresponsiveness (AHR) (52), and NK-1R antagonists attenuated the AHR and plasma extravasation in animal models *in vivo* (51). The underlying mechanism in causing AHR may be the airway inflammation and interaction of SP and CGRP (calcitonin gene-related peptide) (53). Mice deficient in NK-1Rs show reduced IgG-mediated lung injury and neutrophil infiltration as compared to the control group (54). SP is present in bronchopulmonary C fibers (PCFs) and defend the lungs against injury from inhaled agents by a CNS reflex consisting of apnea, cough, bronchoconstriction, hypotension, bradycardia (55), secretion from seromucous glands, release of mediators (including prostaglandins and NO) from the airway epithelium (56), and bronchorelaxation (57). SP synthesis in vagal airway C fibers may be enhanced in pathological conditions such as allergic asthma and chronic bronchitis and may be responsible for some of the associated respiratory symptoms stated above (55). SP produces bronchoconstriction and lung resistance in cynomolgus monkeys (58) and sheep (59) *via* NK-1 receptors, and this effect is more pronounced when they are given by the intravenous route.

EVIDENCE OF INVOLVEMENT OF SPINAL TRIGEMINAL NUCLEUS IN RESPIRATORY RHYTHMIC CONTROL

Few earlier studies with small sample size have been performed in relation to SP expression in brainstems of SIDS (60–63). Our group (11) defined localization, morphology, and functional aspects of TrN in different developmental stages of human brain

development for the first time in cases of sudden perinatal death victims. This study revealed that fetuses in control group had no well-defined TrN as there was either weak or no SP expression, while in medulla oblongata sections from control infants, a well-defined TrN with recognizable tract was observed. While SP expression was depleted in SIDS victim brainstems showing TrN hypoplasia and enhanced in SUID victims. Density of SP varied from very low in fetuses to very high in infants, which points out to the functional requirement of TrN in postnatal period of human life. TrN development is enhanced in late developmental stages of brain, which was identified by enhanced SP expression. Recently, trigeminocardiac reflex has been suggested as an underlying mechanism for the pathogenesis of SIDS (7).

I have also reported in a previous computational study that SP/NK-1R has small protein interaction network, its gene is singleton, providing a possibility that it may be involved in some extremely crucial activities of human developmental phases, and any perturbation in terms of mutations in the gene can lead to lethal outcomes including sudden death (64). Similarly, in another study, I along with my colleagues observed higher expression of SP in oral cancer patients from Pakistani population. This expression was directly proportional to the grade of cancer and poor prognosis. This study also confirms the involvement of SP in nociceptive stimuli in orofacial region (65). In our previous study (11), we detected SP expression in medulla oblongata and spinal cord from early stages of human brainstem development, exclusively in the spinal TrN. This phenomenon exhibits that SP is highly specific and localized in human brain. Very few studies focus on the TrN in humans (66, 67). As spinal trigeminal nucleus and its tract have no definite boundaries, it is difficult to identify it. Nevertheless, it can be recognized by labeling it with SP, which is its main neurotransmitter (11). In experimental murine studies, TrN was observed to be involved in rhythmic autonomic behaviors including breathing (68, 69). These studies also suggested an essential role of TrN in controlling respiratory rhythm, and its activity was observed to be altered in respiratory disorders (70, 71).

Observations from a recent study by Hayashi and Sakuma (72) are also concordant with our observations and findings that SP expression was elevated in seven out of eight victims of sudden unexplained deaths. A previous study also suggested that SP containing cells in human TG is variable in different age groups; 24% in neonates while 17% in adults (73). Study of Hayashi and Sakuma (72) shows expression of SP in brainstem of children and adults as compared to our study, which reported SP expression in SIDS and SUID. If we summarize the findings of both studies and compare them with our study also, we reach to conclusion that SP expression in brainstems is negligible in fetus, enhanced in neonates while decreased in children and adults in controls. While if *vice versa* it leads to sudden deaths (**Figure 3**).

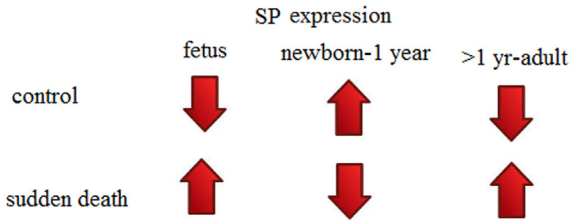

FIGURE 3 | Substance P (SP) expression theory (in human brainstems) to explain sudden perinatal deaths.

CONCLUSION

Studies suggest an important role of SP/NK-1R and trigeminal system during the critical neurodevelopmental periods in which brain is preparing to deal with vital autonomic functions such as breathing, required for the start of new life after birth. Trigeminal nucleus hypoplasia and variations in expression of SP in sudden perinatal death victims as compared to controls provide a possibility of revealing one of the underlying mechanisms in its pathophysiology.

AUTHOR CONTRIBUTIONS

RM has designed, planned, written, and contributed to this review manuscript.

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Negative Role of the Environmental Endocrine Disruptors in the Human Neurodevelopment

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The endocrine disruptors (EDs) are able to influence the endocrine system, mimicking or antagonizing hormonal molecules. They are bio-persistent for their degradation resistance in the environment. Our research group has investigated by gas chromatography–mass spectrometry (GC–MS) the EDs presence in 35 brain samples, coming from 27 cases of sudden intrauterine unexplained death syndrome (SIUDS) and 8 cases of sudden infant death syndrome (SIDS), collected by centralization in the last year (2015). More in detail, a mixture of 25 EDs has been subjected to analytical procedure, following standard protocols. Among the target analytes, some organochlorine pesticides, that is α-chlordane, γ-chlordane, heptachlor, *p*,*p*-DDE, *p*,*p*-DDT, and the two most commonly used organophosphorus pesticides (OPPs), chlorpyrifos and chlorfenvinfos, have been found in seven and three samples, respectively. The analytical procedure used to detect the presence of environmental EDs in cortex samples has been successfully implemented on SIUDS and SIDS victims. The environmental EDs have been found to be able to overcome the placental barrier, reaching also the basal ganglia assigned to the control of the vital functions. This finding, related to the OPPs bio-persistence, implies a conceptual redefinition of the fetal–placental and fetal blood–brain barriers: not real safety barriers but simply time-deferral mechanisms of absorption.

Keywords: endocrine disruptors, gas chromatography–mass spectrometry, stillbirth, sudden intrauterine unexplained death syndrome, sudden infant death syndrome, pesticides, neurodevelopment

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INTRODUCTION

According to the definition of the *United Nations Environment Programme* and of the *World Health Organization*, the endocrine disruptors (EDs) are exogenous substances that alter the function(s) of the endocrine system and, consequently, cause adverse effects in an intact organism (1). They can be found in pesticides, metals, additives or contaminants of food, deep and superficial waters, and personal care products (1). A growing role of EDs has been ascertained in many diseases and particular attention has been recently focused on maternal, fetal, and childhood exposure (2). In fact, EDs have the capacity to interfere with the tissue and organ development and the related functions (3, 4). For example, the exposure to EDs has been associated with female reproductive dysfunctions (endometriosis, polycystic ovary syndrome, and infertility) and breast cancer risk or progression (5–9). The members of the *Endocrine Society* have established that exist scientific

evidences for the association of EDs exposure to the following conditions: (I) obesity and diabetes; (II) dysfunction of female reproduction; (III) dysfunction of male reproduction; (IV) hormone-sensitive female cancers; (V) prostate diseases; (VI) thyroid dysfunctions; and (VII) diseases of neurodevelopment and neuroendocrine systems (10). Our attention has been focused to search environmental EDs in brain samples of sudden intrauterine death syndrome (SIUDS) and sudden infant death syndrome (SIDS) victims, coming from agriculture areas of the Northeast Italy, in whom a complete autopsy and a detailed analysis of the clinical history have ruled out any other rare and possible cause of death (11–15).

MATERIALS AND METHODS

We have analyzed 35 cases of sudden perinatal death, that is 27 SIUDS (age 25–41 gestational weeks) and 8 SIDS (age 2 h–6.5 months), coming from the Northeast Italy, Autonomus Province of Trento included, collected by centralization in the last year (2015). Significant samples of cerebral cortex of the victims have been sent to LC-MS Laboratory of the University of Urbino for gas chromatography–mass spectrometry (GC–MS) investigation. All the samples have been frozen at −20°C until analysis, which has been performed according to our previously published method (16). Briefly, each defrosted brain sample has been weighed (approximately 0.5 g) and homogenized with 2 mL of *n*-hexane to obtain a dense rich supernatant. The homogenized tissue has been transferred into a solid phase extraction (SPE) cartridge containing 500 mg of C18 sorbent, in order to retain most of the matrix impurities and to release the compounds of interest with hexane. The SPE cartridge has been conditioned with 4 mL of *n*-hexane, before purification step, and it has been washed with 1 mL of *n*-hexane, followed by 1 mL of dichloromethane after elution step. The extraction method has been developed and validated in terms of accuracy, precision, limit of quantification (LOQ), limit of detection (LOD), and linearity. Nine isotopically labeled internal standards (ISTDs) have been used for method validation. The validated method has been subsequently applied to human tissues from SIUDS and SIDS victims.

Chemicals and Materials

A mixture of 20 organochlorine compounds (EPA CLP mix), chlorpyrifos, chlorfenvinfos, captan, boscalid, and bisphenol A have been purchased from Sigma-Aldrich (Milan, Italy). All solvents used (*n*-hexane and dichloromethane) have been supplied by Merck (Suprasolv 99% purity, Merck, Darmstadt, Germany). Stock solutions have been prepared in *n*-hexane at 100 μg/mL concentration. A standard mixture containing all compounds (25 specific EDs and 9 ISTDs) has been prepared by appropriate dilution and stored at 4°C in the dark. SPE cartridges HyperSep-C18 (500 mg/6 mL) have been purchased from Thermo Scientific (Bellefonte, PA, USA).

Instrumentations

The analyses on the extracted samples have been performed by an Agilent Technologies gas chromatograph 6890N, equipped with a single quadrupole mass spectrometer 5975C TAD/MS, working in electron ionization. All brain tissues have been subjected to analytical procedure in order to determine the level of the 25 selected compounds. The chromatographic separation has been carried out using an HP-5MS (Agilent J&W GC columns, Folsom, CA, USA), i.d. 30.0 m × 0.25 mm, containing 5% phenyl-methylsiloxane, with a phase thickness of 0.25 μm. As carrier gas, helium at 1 mL/min (constant flow) has been adopted. The GC oven temperatures have been programmed as follows: 80°C held for 1 min, ramped at 30°C/min to 180°C, ramped at 3°C/min to 225°C, held for 4 min, ramped at 20°C/min to 300°C, and held for 4.08 min (total acquisition time: 25 min). Splitless sample injection of 1 μL at 250°C has been selected. The transfer line and ion source temperature have been kept at 290 and 300°C, respectively.

RESULTS

Among the target analytes, organochlorine pesticides (OCPs) and organophosphorus pesticides (OPPs) have been detected in part-per-billion (ppb) in 7 and 3 out of 35 cortex samples coming from SIUDS (7 cases) and SIDS (3 cases) victims, respectively. The following OCPs and OPPs have been found: α-chlordane, γ-chlordane, heptachlor, *p*,*p*′-dichlorodiphenyldichloroethylene (DDE), *p*,*p*′-dichlorodiphenyltrichloroethane (DDT), chlorfenvinfos, and chlorpyrifos. More in detail, heptachlor, DDE, and DDT have been detected in three separate cases, while chlorfenvinfos and chlorpyrifos in association with α-chlordane or γ-chlordane. Today, chlorfenvinfos and chlorpyrifos are the two most commonly used organophosphate pesticides for pest control in intensive agricultural areas, such as the Northeast Italy is. These non-persistent compounds, also called contemporary-use pesticides, are currently available in place of organochlorine compounds, banned since 1980s. However, the detection of DDE and DDT confirms their degradation resistance over the years, and their extensive use has exposed the population to insidious environmental administrations. Fetuses, newborns, and pregnant women appear to be the most vulnerable subjects to these exposures.

DISCUSSION

The detection of some EDs in 10 positive samples out of 35 fetal and neonatal brain tissues confirms the possibility of these chemicals to pass from mother to fetus, overcoming the fetal–placental and fetal blood–brain barriers, not real safety barriers but simply time-deferral mechanisms of absorption, and to be collected in brain tissue (2–4). Here, they can give origin to impairment of receptorial expression, such as orexin, and to development alterations, especially of the basal ganglia, the major controllers of basic vital functions (17, 18). This is consistent with further literature data, which report the presence of these selected pollutants in other human tissues (19, 20). Moreover, by our preliminary data (not shown), they seem to be able to interfere with circulating mitochondrial DNA (mtDNA), causing mitochondrial dysfunction in SIUDS and SIDS. It is already known that during normal pregnancy mtDNA level significantly decreases in different trimesters (21). In case of

intrauterine growth restricted pregnancy, placental and blood mtDNA content has been found significantly increased, if compared to normal pregnancies, probably for a compensatory action (22). On the other hand, circulating mtDNA content has been significantly found decreased in gestational diabetes mellitus (23) and in HELLP syndrome (24), indicating a reduction of the mitochondrial activity. For all these reasons, environmental EDs should be systematically searched during autopsy in order to establish a possible correlation with SIUDS and SIDS events. Moreover, it is interesting to remark that seven brain samples have shown the presence in ppb levels of several OCPs and three samples have disclosed the presence of two OPPs, chlorpyrifos and chlorfenvinfos, the two most common pesticides used in apples cultivation. These findings are in accordance with the environmental diffusion of contaminants in intensive agricultural areas represented by the Northeast Italy (25). The *Italian National Institute for Environmental Protection and Research* (ISPRA) has published a detailed report on the presence of pesticides in surface and ground water in the period 2013–2014 (26). The aim of this national investigation has been to provide reliable information on the water quality in relation to these substances. The report has been the outcome of a complex activity, also involving the *Regional Agencies for Environmental Protection* (ARPA) and the *Provincial Agencies for Environmental Protection* (APPA). The data collected by these institutions have concerned with recovery, frequency, and distribution of pesticides on the whole Italian territory. Moreover, the measured concentrations have been compared with the legal threshold fixed by the European and National legislation: the Standard of Environmental Quality for the surface water (dir. acque 2008/105/CE; D.Lgs 152/2006). In the Northeast Italy, the Autonomous Province of Trento has provided 70 monitoring stations: 764 samples have been collected and 64,283 measurements have been performed. Thanks to this extensive analysis, 102 substances have been researched, and 33 of them have been detected in 18–23% samples of surface waters. Among these, boscalid, dimetomorf, fluopicolide, and chlorpyrifos have been resulted the chemicals most frequently found. Unsurprisingly, chlorpyrifos is one of the EDs detected also in our examined brain samples. In conclusion, for the first time in literature, our research group has successfully demonstrated the presence of pesticides at ppb levels in cortex samples of SIUDS and SIDS victims; it is not ethically possible to compare the obtained results with brain samples coming from healthy subjects, and, consequently, it will not be possible to draw up threshold values for the future. However, even though the contaminants have been detected at ppb levels, their presence can be considered significant due to their certified high degree of toxicity and to fetal/infant known vulnerability.

AUTHOR CONTRIBUTIONS

LR: study design and supervision; VT: data acquisition; and TP: drafting of the manuscript.

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