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I battiti del cervello e i respiri del cuore: una teoria unificatrice del controllo neuro- cardio-respiratorio nelle morti improvvise ed inaspettate dell'infante e dell'adulto

The brain beating and heart breathing: a unifying theory of the neuro- cardiac- respiratory control in infant and adult sudden unexpected deaths

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“Nothing! nothing! nothing! nothing! nothing!...”

Contents

Summary

Background: Sudden Infant Death Syndrome (SIDS) is characterized by the death of an infant that cannot be explained, despite a systematic case examination, including death scene investigation, autopsy and review of the clinical history. Nowadays, Sudden Unexpected Infant Death (SUID) is a wide-ranging concept used to describe any sudden and unexpected death, whether explained or unexplained, including SIDS, which occurs during the first year of life. Several differing and sometimes contradictory hypotheses of the underlying mechanisms of SIDS have been proposed. The most reliable seems to be the "triple risk hypothesis". Based on this theory, unexpected infant deaths might arise as a consequence of the combination of three factors coming together: a vulnerable infant, a vulnerable phase of development and a final insult occurring in this window of vulnerability. Recently, a unified neuropathological theory contributes to describing SIDS. According to this, serotonergic neurons play a crucial homeostatic function in the cardiorespiratory brainstem centres. A high incidence of morphological abnormalities and biochemical defects of serotoninergic neurotransmission have been reported in the brainstem of SIDS victims. This brain region includes the main nuclei and structures that coordinate the vital activities, such as cardiovascular function and breathing, perinatal and after birth. Nevertheless, evidence suggests likely genomic complexity and a degree of overlap among SIDS, Sudden Intrauterine Death (SIUD), Sudden Cardiac Death (SCD) and Sudden Unexpected Death in Epilepsy (SUDEP). In SUDEP, which has clinical parallels with SIDS, alterations to medullary serotoninergic neural populations and autonomic dysregulation have been shown too.

Molecular profiling of SUDEP cases and the investigation of genetic models have directed to the identification of putative SUDEP genes of which most are ion channel active along the neurocardiac, neuroautonomic, and neurorespiratory pathway. Concurrently, anomalous time- activation, transcription or regional expression of candidate neuro-cardiac-respiratory genes implicated for SUDEP, could be similarly involved in other unexpected sudden deaths. A small but significant proportion of infants who die suddenly and unexpectedly have been

shown on postmortem genetic testing to have Developmental Serotonopathies, Cardiac Channelopathies and Autonomic Nervous System Dysregulation, with considerable implications for surviving and future family members. This has led to the demonstration that neuro-cardiac genes are expressed in both tissues (brain and heart) and recently in the respiratory system.

Aim: Despite their decreasing incidence, SIDS and SUDEP are still important causes of death. There are many nuclei in the cardio and respiratory centres of the brain involved in unexpected and sudden deaths. Cardiac, sympathetic, and respiratory motor activities can be viewed as a unified rhythm controlled by brainstem neural circuits for effective and efficient gas exchange. We aim to describe abnormalities in these nuclei, in part because robust molecular or functional examination of these nuclei has not been carefully performed. We intend to perform detailed functional mapping of these brainstem nuclei. Specifically, the cardiorespiratory and cardioventilatory coupling can be understood as a unified vital rhythm controlled by brainstem neural circuits. By cardiorespiratory coupling, we mean the Respiratory Sinus Arrhythmia (RSA) that is characterized by a heart rate (HR) increasing during inspiration and an HR decrease during expiration. Conversely, Cardioventilatory coupling (CVC) is considered the influence of heartbeats and arterial pulse pressure on respiration with the tendency for the next inspiration to start at a preferred latency after the last heartbeat in expiration. We hypothesized that these two reflex systems are not separate, but constitute an integrated network. We defined this last concept as "unifying theory". By studying all the maps of the cardiorespiratory nuclei of the Literature, we integrated this concept into a reworking map of brainstem nuclei that could also explain the gasping and blocking cardiorespiratory of sudden deaths. The theory of a unique, unifying cardiorespiratory network, it has been recently demonstrated in some cases of arrhythmia, in some cases of SUDEP with striking systolic hypotensive changes and in some cases of SIDS too.

Material and Methods: We investigated articles, reviews indexed in PubMed describing putative neuro-cardiac-respiratory genes and cardiorespiratory, and cardioventilatory coupling theories. Specifically, we evaluated cardiorespiratory brainstem nuclei and whole brains of fetal, infant and adult autopsies respectively

to detect congenital errors in the cerebral development or malformations, but also to identify the “normal” or “dysplastic” brainstem centres.

Results: Based on the Literature, we identified a brain-heart gene mapping and a scheme of cardiorespiratory brainstem nuclei network. Contemporary, we collected a large pool of fetal brain malformations and cardiorespiratory nuclei dysgenesis both in infants both in adult sudden deaths. We found dysgenesia, agenesia and hypoplasia of brainstem nuclei associated with SIDS cases, compared with post-mortem infant control cases. However, the arcuate nucleus showed insignificant inter-variations regarding adults autoptic cases.

Discussion: Many intrinsic and extrinsic factors increase fetal, perinatal, infant, and adult sudden death susceptibility. The final common pathway for SIDS and SUDEP involves a failure to arouse and autoresuscitate in response to environmental challenge. The different risk factors, among these a prone position, can directly alter the function of cardiorespiratory nuclei and impair the ability of this network to coordinate cardiorespiratory–cardioventilatory coupling.

Conclusions: Neuropathological analysis of the infant brainstem and neuro-cardiac-respiratory gene mapping represents a good tool to infer on the final events of SIDS and SUDEP, although nothing it is clear regarding the role of adult cardiorespiratory centres. An integrated study of postmortem neuropathology and molecular autopsies could help to understand the network of this beating-breathing-thinking unit.

Original papers

1. "In"

This thesis is based on the following papers:

1. "From fix to fit into the autoptic human brains" 2018, European Journal of Histochemistry Vol 62 (3). doi: 10.4081/ejh.2018.2944

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2. "Tullio Terni (1888-1946): the life of a neurocardioanatomist with a tragic epilogue" 2018, International Journal of Cardiology submission

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3. "Neuropathology of early Sudden Infant Death Syndrome – Hypoplasia of the pontine Kölliker-Fuse Nucleus: a marker of unexpected collapse during skin care" 2018, American Journal of Perinatology doi.1055/s-0038-1669398

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4. "Variability of the Medullary Arcuate Nucleus in adult humans" 2018, Brain and Behavior accepted

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5. "Unexpected in utero exposure to psychotropic medications"
   New Horizons in Clinical Case Reports 1, August 2017 pp 1–28, Abstract of Oral Presentation http://dx.doi.org/10.1016/j.nhccr.2017.06.142

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6. "The brain beating and the heart breathing. Cardiorespiratory nuclei analysis and brainstem mapping in sudden infant death"
   Clinical Neuropathology, Vol. 36 – No. 3/2017 (p. 135) Joint meeting 53rd Congress of the Italian Association of Neuropathology and Clinical Neurobiology (AINPeNC) 43rd Congress of the Italian Association for Research on Brain Aging (AIRIC), Abstract of Oral Presentation

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7. Descriptive epidemiology on Sudden Unexpected infant death (SUID) and Sudden Infant Death Syndrome (SIDS) of Veneto Regional Center: Neuropathological evidences. PROCEEDINGS 7° Congresso Triennale di Anatomia Patologica SIAPEC-IAP 2016. Pathologica (2016) Vol 108 (4) p. 276-277, Abstract of Oral Presentation

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8. EP01.11: "Congenital cytomegalovirus infection: an interesting case of clinical, molecular and pathologic correlations"
   Ultrasound Obstet Gynecol (2016) Suppl 1:273. doi: 10.1002/uog.16812

   Visentin S[1], Fedrigo M[2], Talenti G[3], Gussetti N[4], Forner G[4], Mardari R[3], **Paradiso B**[2], Angelini A[2], Cosmi E[1]

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1. Review: "The past, present and future challenges in epilepsy-related and sudden deaths and biobanking"
   Neuropathol Appl Neurobiol. 2018 Feb;44 (1):32-55. doi: 10.1111/nan.12453

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2. "Towards Better Understanding of the Pathogenesis of Neuronal Respiratory Network in Sudden Perinatal Death"
   Front Neurol. 2017 Jul 6;8:320. doi: 10.3389/fneur.2017.00320

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2. "Out"

Original papers not included in this thesis but published during the three years of PhD course:

1. "Multinodular and vacuolating neuronal tumors in epilepsy: dysplasia or neoplasia?" Brain Pathol. 2018 Mar; 28(2):155-171. doi: 10.1111/bpa.12555. Epub 2017 Sep 19

   Thom M[1], Liu J[1], Bongaarts A[2], Reinten RJ[2], **Paradiso B**[1,3], Jäger HR[4], Reeves C[1], Somani A[1], An S[1], Marsdon D[1], McEvoy A[5], Miserocchi A[5], Thorne L[5], Newman F[6], Bucur S[6], Honavar M[7], Jacques T[8], Aronica E[2,9].

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[9]Stichting Epilepsie Instellingen Nederland (SEIN), Heemstede, The Netherlands.

2. "Personalized Needles for Microinjections in the Rodent Brain"
   J Vis Exp. 2018 Jan 24;(131). doi: 10.3791/55751

   Paolone G[1], Falcicchia C[1], Verlengia G[2], Barbieri M[1], Binaschi A[1], Paliotto F[1], **Paradiso B**[1], Soukupova M[1], Zucchini S[2], Simonato M[2].

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3. "Multinodular and vacuolating neuronal tumours in epilepsy: dysplasia or neoplasia?"
   In: (Proceedings) (2017) 118th Meeting of the British-Neuropathological-Society (pp. 19-20). WILEY, Abstract of Oral Presentation

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4. P19: "BRAF V600E mutations in glioneuronal tumours in epilepsy" Neuropathology and Applied Neurobiology (2015), 41 (Suppl. 1), 30–58 [Epub ahead of print], Abstract of Oral Presentation

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5. “Eponyms and Nazi Era” Piero Martinello, Piero Casentini (and others, **Beatrice Paradiso**) book- catalog in submission to Atelier Editions

Introduction

1. The control of autonomic functions of Brainstem

1.1 Overview of Brainstem Anatomy: a look into the stalk of Life

The brain is developed from the anterior end of the neural tube, which at four weeks becomes expanded into 3 vesicles that demarcate the territory for cerebral hemispheres and brainstem. These constitute the 3 primary cerebral vesicles and correspond, respectively, to the future forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon). The anterior part of the forebrain, including the rudiments of the cerebral hemispheres, is called telencephalon, and its posterior portion is termed diencephalon. Simultaneously, the rhombencephalon is segmented into 2 structures: the anterior one, named metencephalon, consisting of the pons, cerebellum, and the intermediate part of the 4th ventricle; and the myelencephalon, which comprises the medulla oblongata and the lower part of the 4th ventricle. The brainstem is composed of 4 ectodermal primary structures: the diencephalon, mesencephalon, pons, and medulla oblongata. Its role is to connect the cerebral hemispheres with the medulla and the cerebellum and is responsible for basic vital functions, such as breathing, heartbeat blood pressure, control of consciousness, and sleep. The gray matter of the brainstem is found in clumps and clusters of neurons throughout the brainstem to form the cranial nerve nuclei, the reticular formation, and pontine nuclei. The white matter consists of fiber tracts (axons of neuronal cells) running down from the cerebral cortex—important for voluntary motor function—and up from peripheral nerves and the spinal cord—where somatosensory pathways travel—to the highest parts of the brain. The internal organization of brainstem, is complex and is ordered in 3 laminae (tectum, tegmentum, and basis), which extend its entire length (Fig. 1). The motor pathways, corticobulbar and corticospinal tracts, pass down through the basis, which is the ventral lamina and is located at the extremely anterior part. In the medulla this lamina corresponds to the pyramids (Fig. 2). The pons basis it is largest because of the presence of numerous nuclei (pontine nuclei), which receive numerous fibers that project to

cerebellum, forming the pontocerebellar pathway (Fig. 3). In the midbrain the pes cerebri form the basis (Fig. 4). The cranial nerve nuclei are settled into the middle layer (the tegmentum), just in front of the 4th ventricle and are placed, from medial to lateral, on the basis of their function: somatic motor, visceral motor, visceral sensory, and somatic sensory. All the somatosensory tracts run upward to the thalamus crossing the tegmentum in front of the cranial nerve nuclei. The tegmentum is divided in 2 layers: the dorsal contains all the somato-motor and general sensory cranial nerve nuclei. The ventral contains the supplementary motor nuclei: the substantia nigra and red nucleus (midbrain) and the inferior olivary nucleus (medulla), which are located close to the basis. The running tracts (sensory tracts or lemniscus) cross the rest of the tegmentum and they are surrounded by the reticular formation.

The tectum, formed by the quadrigeminal plate and the medullary velum, contains no cranial nuclei, no tracts and no reticular formation.

Thus, the gray matter of the brainstem (nuclei) lies close to the central canal (Sylvian aqueduct and 4th ventricle), whereas the white matter is in the middle of the tegmentum, with the exception of the pyramidal pathway, which runs anteriorly [1].

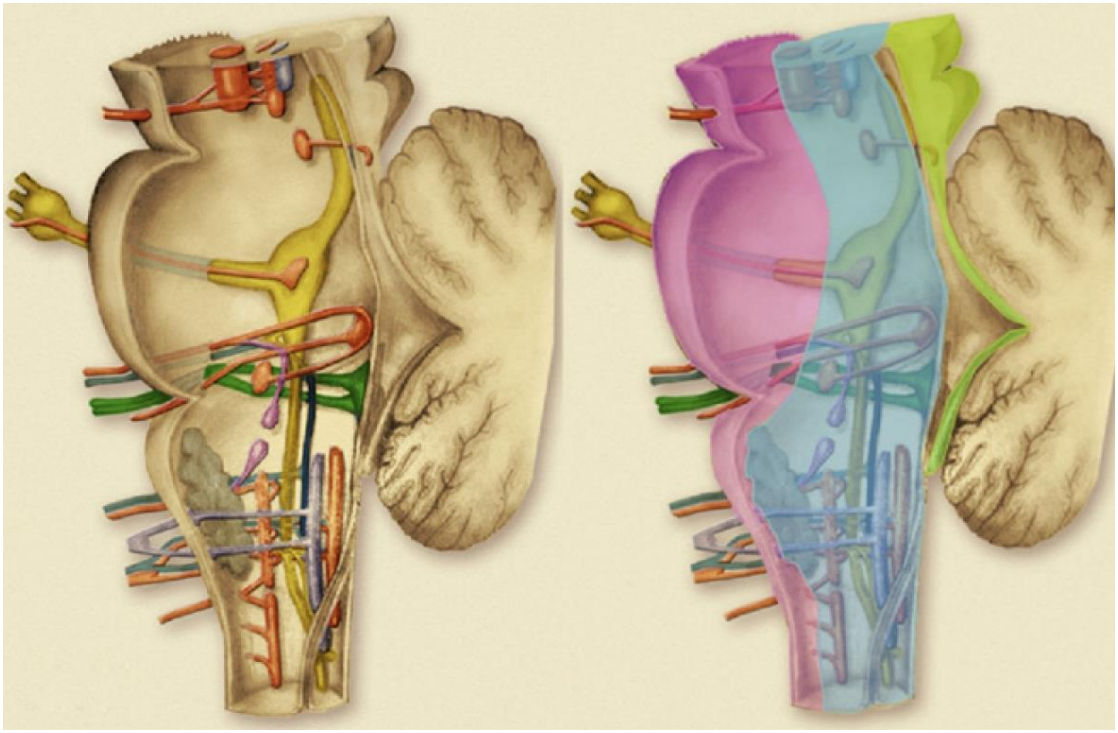


Figure 1 *Laminae of the brainstem. Pink: basis; blue: tegmentum; green: tectum [1]*

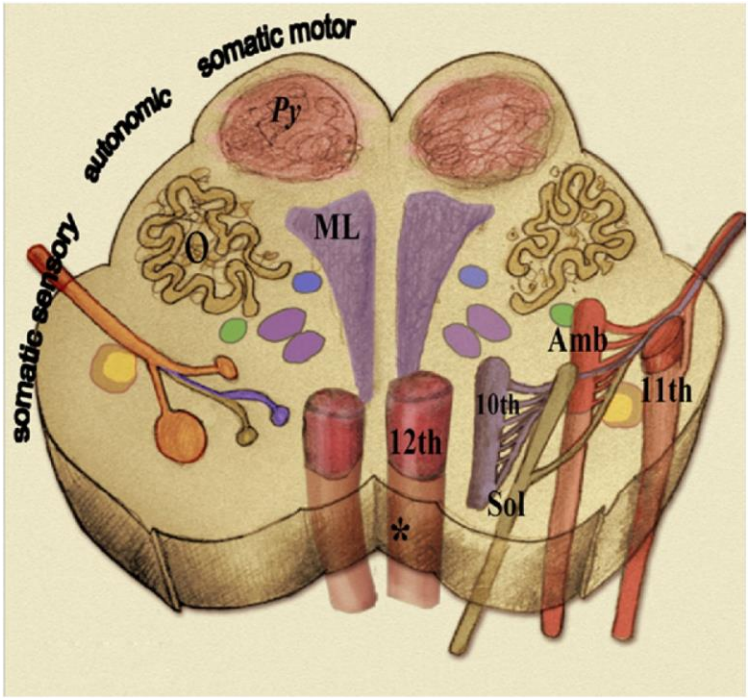


Figure 2 *Axial drawing of the medulla oblongata showing the tegmentum (yellow area) where the lower cranial nerve nuclei are located. At the midline is the 12th (hypoglossal) nerve nuclus, which occupies the somatic motor area. Lateral to this is the visceral motor and visceral sensory (autonomic) territory where the motor nucleus of the 10th (vagus) nerve are located, as well as the solitary tract (Sol) and the nucleus ambiguus (Amb). Further, laterally, the spinal extension of the sensory nucleus of the 5th nerve and the spinotrigeminal pathway (thickarrow) are visualized. Hypoglossal eminence (asterisk); O, olive; Py, pyramid; ML, medial lemniscus [1]*

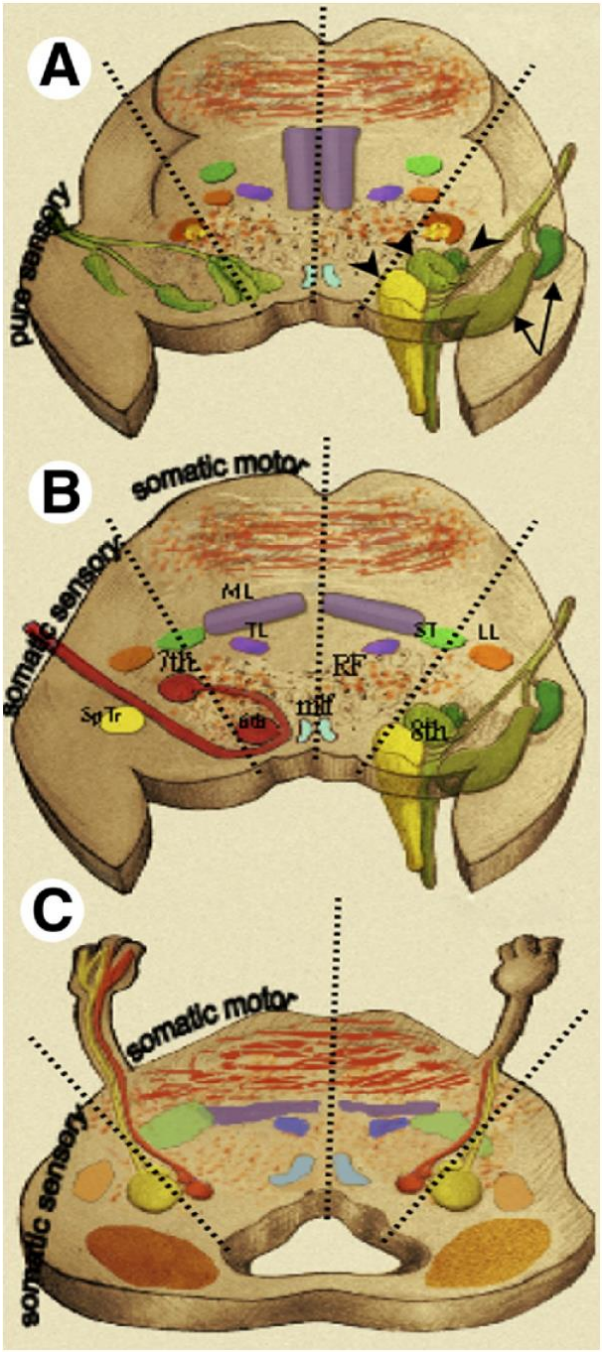


Figure 3 *Axial drawings showing the pons and corresponding 5th, 6th, 7th, and 8th cranial nerve nuclei. (A) The 8th cranial nerve nuclei are located at the lower level, at the acoustic area, a zone dorsal and lateral adjacent to the medial cerebellar peduncles. The vestibulocochlear nucleus is composed of the cochlear (arrows) part and the acoustic part (arrowheads). (B) at the medium level we can see the 6th and the 7th nerve nuclei. The 6th is located medial and dorsal and the 7th is more anterior and lateral. The axons of the 7th nucleus travel backward and*

pass around the 6th cranial nerve; this produces the facial colliculus (an eminence on the floor of the 4th ventricle). (C) Cranial segment through the trigeminal nuclei. The sensory nucleus is larger than the motor and is located as well as the 7th cranial nerve nucleus in the autonomic space (visceral sensory and visceral motor). ML, medial lemniscus; TL, trigeminal lemniscus; LL, lateral lemniscus; ST, spinothalamic tract; mlf, medial longitudinal fasciculus; SpTr, spinptrigeminal tract; RF, reticular formation [1]

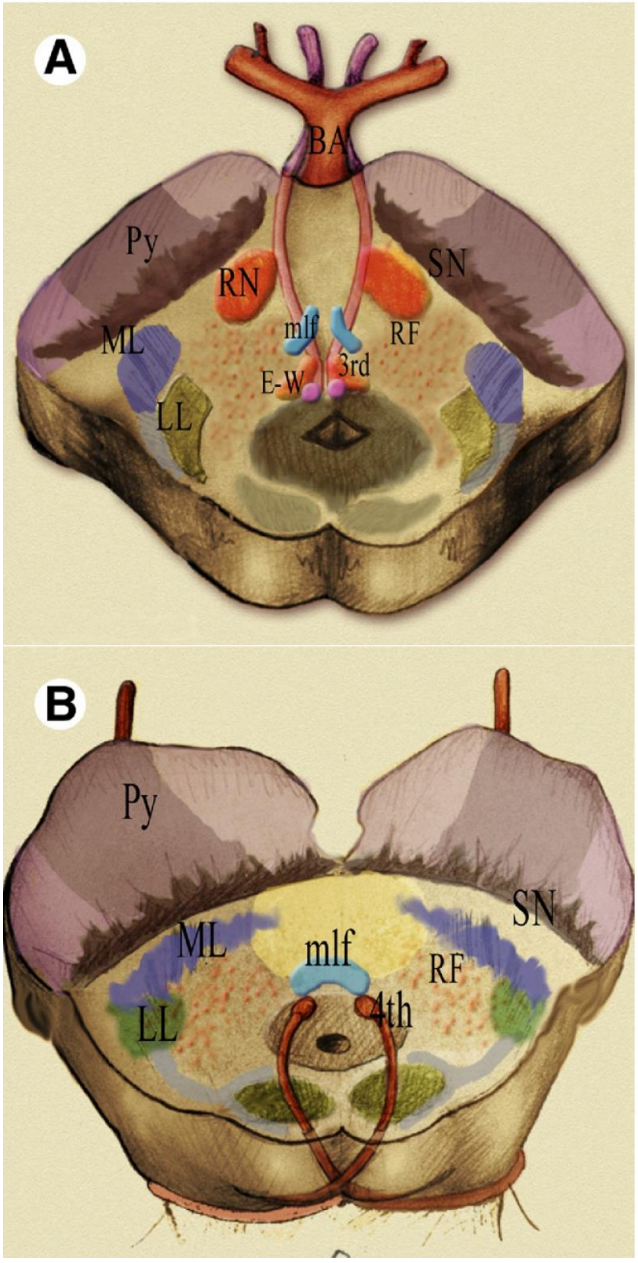


Figure 4 *Schematic drawings though the midbrain. (A) superior colliculi level and (B) inferior colliculi level. BA, basilar artery; Py, pyramidal tracts; RN, red nucleus; mlf, medial longitudinal fasciculus; ML, medial lemniscus; LL, lateral lemniscus; 3rd, oculomotor nerve nucleus; E-W, Edinger–Westphal nucleus; 4^{th}, trochlear nucleus; RF, reticular formation [1]*

1.2 Central Autonomic Control

Central control of the sympathetic and parasympathetic outputs involves several interconnected neuraxis areas; this central autonomic network has a critical role in regulation of visceral function, homeostasis, by adaptation to internal or external stimules. The central autonomic network is systematized in four interconnected hierarchical levels: spinal, bulbopontine, pontomesencephalic and forebrain levels (Fig. 5, 6) [2]. The spinal level mediates segmental sympathetic or sacral parasympathetic reflexes and is involved in stimulus-specific patterned responses, which are influenced by the other levels. The bulbopontine (lower brainstem) level is ingagged in reflex control of circulation, respiration, gastrointestinal function, and micturition. The pain modulation and integrated behavioral responses to stress are regulated by pontomesencephalic (upper brainstem) level. The hypothalamus, which has homeostatic and endocrine function, the anterior limbic circuit, including the insula, anterior cingulate cortex, and amygdala, which are involved in integration of bodily sensation with emotional and goal-related autonomic responses, constitute the forebrain level.

The insular cortex is the primary interoceptive cortex and integrates visceral, pain and temperature sensations. The dorsal insula has a viscerotropic organization and receives inputs from gustatory, visceral, muscle, and skin receptors via the thalamus. The dorsal insula projects to the right anterior insula, which, via its connections with neocortical association and limbic areas, integrates these interoceptive inputs with emotional and cognitive processing to convey the conscious experience of bodily sensation. The insula is also a visceromotor area controlling both the sympathetic and parasympathetic outputs, primarily via a relay in the lateral hypothalamus.

The anterior cingulate cortex is interconnected with the anterior insula and is distinguished into ventral (affective and "default mode network") and dorsal (cognitive and "frontoparietal attention networks") regions. The ventral anterior cingulate cortex comprises subcallosal and precallosal portions that have extensive connections with the insula, prefrontal cortex, amygdala, hypothalamus, and brainstem. Via these projections, the anterior cingulate cortex controls sympathetic and parasympathetic functions.

The amygdala connotes of affective or emotional significance to incoming sensory information and has multiple downstream neuronal stations that participate in the autonomic and neuroendocrine response to stress. The central nucleus of the amygdala (CeA), both directly and via the bed nucleus of the stria terminalis, has a major role in integration of the stress responses, particularly fear responses, via its wide spread connections with the hypothalamus and brainstem, mostly the periaqueductal gray and the medullary reticular formation.

The hypothalamus is a visceromotor pattern generator that initiates specific patterns of autonomic and endocrine responses according to the stimulus, such as hypoglycemia, changes in blood temperature or osmolarity, or external stressors. The preoptic-hypothalamic area is functionally subdivided into three regions, periventricular, medial, and lateral: The periventricular zone includes the suprachiasmatic nucleus (the circadian pacemaker), and several areas involved in neuroendocrine control via the pituitary gland. The medial zone includes the medial preoptic area, paraventricular nucleus (PVN) and dorsomedial nucleus (DMH), which are involved in thermoregulation, osmoregulation, and stress responses. The lateral zone includes nuclei that control arousal, sleep and motivated behavior. The main autonomic outputs of the hypothalamus originate from the PVN, DMH, and lateral hypothalamic area. The PVN contains different neuronal populations that are differentially activated during stress responses, among these there are magnocellular neurons that release arginine-vasopressin (AVP) to the general circulation; neurons that release corticotropin-releasing hormone and activate the adrenocortical axis, and neurons that project to autonomic nuclei of the brainstem and spinal cord. Via these outputs, the PVN modulates stress responses, food and sodium intake, glucose metabolism, and cardiovascular, renal, gastrointestinal, and respiratory functions. The DMH participates in stress responses, thermoregulation and cardiovascular control. Hypocretin/orexin neurons of the posterior lateral hypothalamus regulate arousal, feeding and reward driven behaviors.

The brainstem areas involved in autonomic output include the periaqueductal gray matter of the midbrain (PAG), the parabrachial nucleus (PBN) and different

medullary regions, including the NTS, ventrolateral reticular formation of the medulla, and medullary raphe (Fig. 5) [3].

The PAG has an integrated autonomic role between the forebrain and the lower brainstem and responds to stress, pain modulation, and somatic adaptive functions. It is characterized by different longitudinal columns that, via their different spinal, brainstem, and cortical connections, participate in cardiovascular responses associated with pain modulation, coordination of the micturition reflex and control of respiration.

The PBN is a major relay centre that receives converging visceral, nociceptive and thermoreceptive inputs from the spinal cord and carries this information to the hypothalamus, amygdala, and thalamus. The PBN, in particular, is an important centre participating in the control of respiratory, cardiovascular, and gastrointestinal functions.

The NTS is the first relay station of taste and visceral afferent information and includes several subnuclei with a viscerotropic organization. The rostral portion of the NTS receives taste inputs; the intermediate portion receives gastrointestinal afferents; and the caudal portion receives baroreceptor, cardiac, chemoreceptor, and pulmonary afferents. The NTS relays this information, either directly or via the PBN, to rostral brainstem and forebrain areas. Therefore, the NTS is the first central relay for all medullary reflexes controlling cardiovascular function (baroreflex and cardiac reflexes), respiration (carotid chemoreflex and pulmonary mechanoreflexes), and gastrointestinal motility.

The rostral ventrolateral medulla (RVLM), containing the C1 group of epinephrine-containing neurons, is a crucial region for regulation of arterial blood pressure. The sympathoexcitatory RVLM neurons collect and integrate a large variety of inputs from the brainstem and forebrain, in particular hypothalamus, including the PVN. Glutamatergic neurons of the RVLM project directly and provide tonic excitation to sympathetic preganglionic neurons controlling cardiac output and total peripheral resistance. The RVLM mediates all reflexes controlling arterial blood pressure, including the baroreflex, cardiopulmonary reflexes, and chemoreflexes. These include inhibitory signals from baroreceptor-

sensitive neurons of the NTS and mediated via inhibitory gamma-aminobutyric acid (GABA) ergic neurons of the caudal ventrolateral medulla.

The caudal ventrolateral medulla contains GABAergic neurons that maintain a tonic inhibitory control on the RVLM and relay the inhibitory inputs from the NTS mediating the sympathoinhibitory component of the arterial baroreflex. Data of Electrical stimulations indicate that the caudal medulla also contains pressor regions. The caudal ventrolateral medulla also contains the A1 group of neurons that supply noradrenergic innervation to the hypothalamus and are a component of a reflex pathway that triggers AVP release in response to hypovolemia or hypotension.

The rostral ventromedial medulla including the caudal raphe nuclei, has an important role in thermoregulation, pain modulation, and control of automatic ventilation. One group of medullary raphe neurons initiate sympathetic responses to cold via input to preganglionic sympathetic neurons that activate skin vasoconstriction and non-shivering thermogenesis in the brown adipose tissue.

The sympathetic output is critical for preservation of arterial pressure, thermoregulation, and redistribution of regional blood flow during stress and exercise. The sympathetic output originates from sympathetic preganglionic neurons placed in the thoracolumbar spinal cord at the T1 to -L2 segments, primarily in the intermediolateral cell column. These neurons are organized into functionally separate units that innervate selective subpopulations of sympathetic ganglion neurons and receive distinct segmental afferent inputs triggering segmental somato- and viscerosympathetic reflexes. Different preganglionic sympathetic units are recruited in an integrated mode by premotor neurons in the brainstem and hypothalamus to initiate different patterns of responses to specific internal or external stressors, such as postural changes, exercise, hypoglycemia, dehydration, exposure to heat or cold, or stress. The main sources of premotor sympathetic innervation are the RVLM, medullary raphe, A5 noradrenergic group of the pons PVN, and lateral hypothalamic area.

In contrast to the sympathetic system, which affects on multiple effectors, the parasympathetic system mediates reflexes activated in an organ-specific fashion. The vagal output originates from preganglionic neurons located in the dorsal

motor nucleus of the vagus (DMV) and in the ventrolateral portion of the nucleus ambiguus (NAmb) in the medulla. The DMV contains most of the vagal preganglionic parasympathetic neurons that are organized in a viscerotropic fashion and innervate the local ganglia in the respiratory tract, enteric nervous system (ENS), pancreas and liver. The DMV receives inputs from the NTS and mediates all vago-vagal reflexes controling gastrointestinal motility and secretion. Vagal preganglionic neurons located in the ventrolateral portion of the NAmb provide the primary control of the heart via the cardiac ganglia. These cardiovagal outputs inhibit the automatism of the sinoatrial node exerting a beat-to-beat control of the heart rate. As we will see in particular dedicated chapter 1.3, the cardiovagal NAmb neurons are activated by the NTS during the baroreflex and are inhibited during inspiration. In the same time, interactions between the heart and the brain are vital for maintaining homeostasis and survival in an ever-changing environment.

The sacral preganglionic output originates in neurons located in the lateral gray matter at the S2-S4 segments of the sacral spinal cord. These neurons are critical for normal micturition, defecation, and sexual organs; this involves their coordinated interactions with both lumbar sympathetic neurons located at T12-L2 levels and somatic motor neurons of the Onuf nucleus at the S2-S4 levels innervating the external urinary sphincter and pelvic floor.

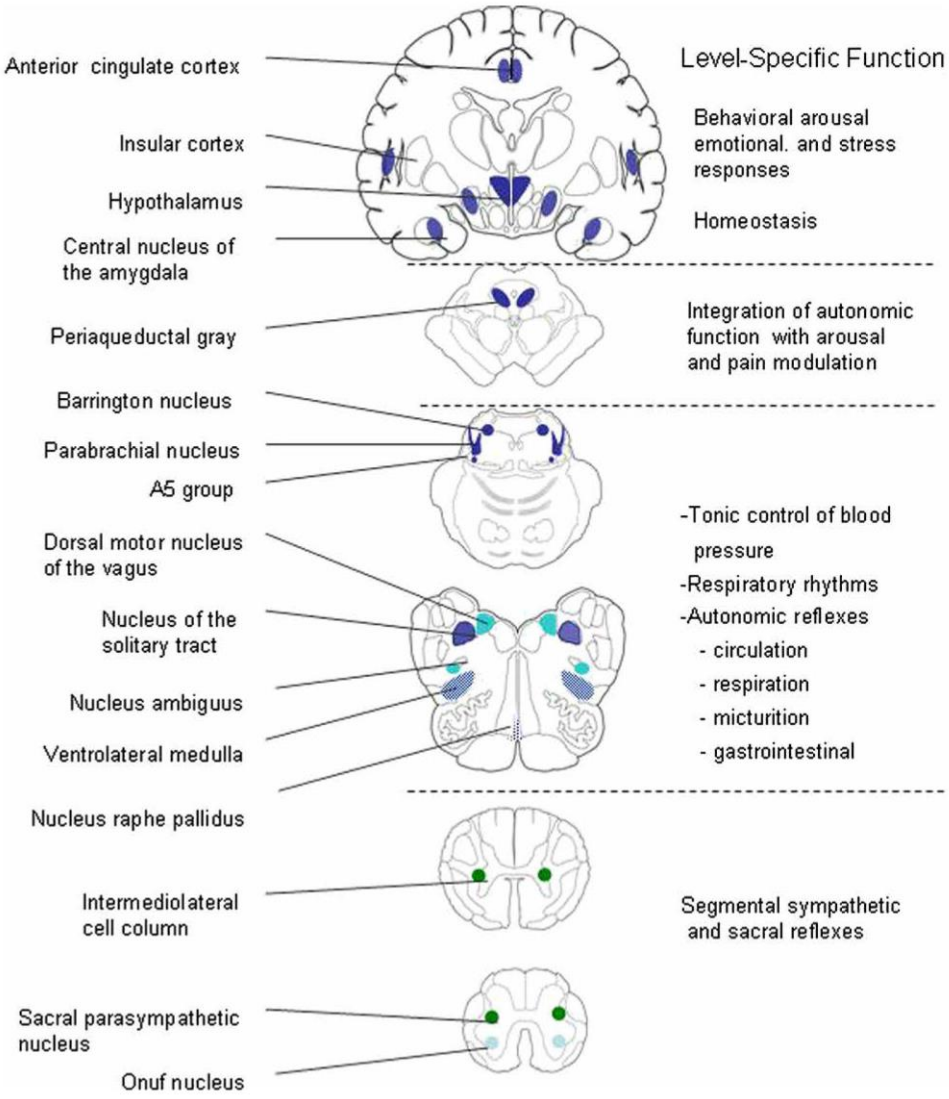


Figure 5 *Central autonomic control areas and levels of interaction of autonomic control [2]*

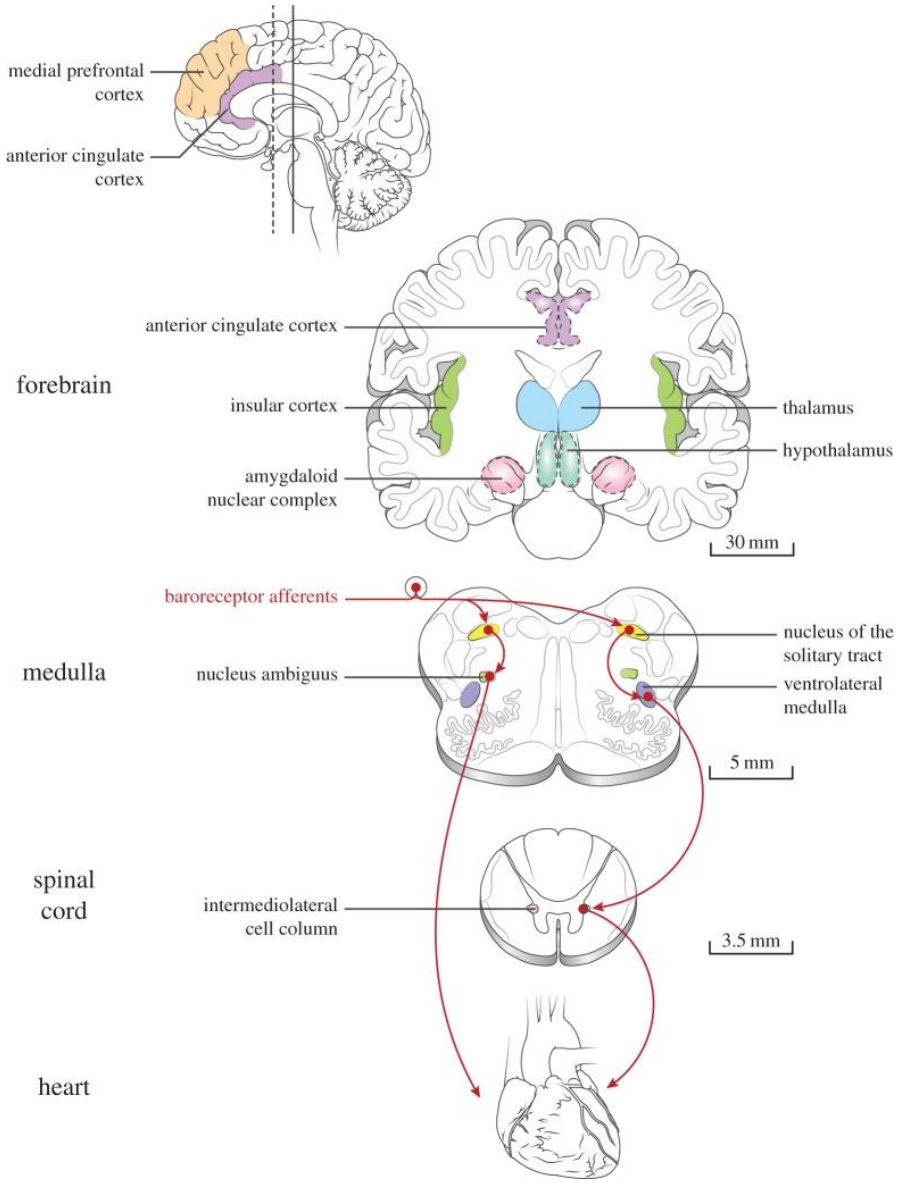


Figure 6 *Several crucial areas involved in brain–heart interactions. Selected areas of the forebrain, brainstem and spinal cord involved in human autonomic function are depicted. Arrows illustrate major pathways of the baroreceptor reflex, mediating the homeostatic control of blood pressure (left and right branches stemming from the baroreceptor afferents indicate parasympathetic and sympathetic outflow, respectively). Scale bars provide approximate dimensions in the human brain [3]*

1.3 Neural Control of the Heart

Anterior insula, anterior cingulate cortex (ACC), amygdala, hypothalamus, periaqueductal gray matter, parabrachial nucleus, and some regions of the medulla, exert a neural control on cardiac function. These areas are involved in emotional behavior, stress responses, and homeostatic reflexes too. They exert their influence on heart rate (HR) and cardiac contractility by the sympathetic and parasympathetic nervous systems [4], however a possible lateralization of this central control, remain unresolved issue.

Important examples of beat -to - beat on cardiac function are severe arrhythmias and myocardial injury in the setting of neurologic catastrophes and sudden unexplained death in epilepsy [5, 6].

The heart has intrinsic electrophysiologic properties by a pacemaker activity. This originates in specialized cardiomyocytes from the cardiac intrinsic conduction system and comprises the sinoatrial (SA) node, the atrioventricular (AV) node, the bundle of His, and the Purkinje fiber network. The HR, excitability, and contractile function of the cardiomyocytes depend on the interaction between their intrinsic characteristics and their regulation by the vagus and sympathetic nerves via the intrinsic cardiac ganglionated plexuses.

The HR (chronotropism) is produced by the spontaneous depolarization (automatism) of the SA node. This is under the control of a "voltage regulator," which is determinated through cyclic activation and deactivation of different membrane ion channels, and a "calcium regulator", triggered by rhythmic Ca^{2+} release from the sarcoplasmic reticulum by way of the ryanodine receptor 2 (RYR2); rhythmic increase of cytosolic Ca^{2+} activates the Ca^{2+}-Na^{+} exchanger current leading to depolarization.

The cardiac cycle begins with the spreading of depolarization via connexion channels from neighboring cardiomyocytes, which is followed by opening of voltage-gated Na^{+} ($Na_v1.5$) channels. They have rapidly inactivated by the depolarization, which activates both L-type calcium channels responsible for the plateau of the action potential the conduction, and voltage-gated K^{+} channels responsible for repolarization. The synchronized activity of these channels produces the excitability of the His-Purkinje system (bathmotropism), the velocity

of AV conduction, or dromotropism (PR interval) and the duration of the cardiac action potential (QT interval). The systolic contraction (inotropism) occurs by excitation-contraction coupling, whenever calcium released from the sarcoplasmic reticulum through the RYR2 binds to the troponin complex and activates the contractile apparatus. Cellular relaxation during diastole (lusitropism) follows upon removal of cytosolic Ca^{2+} by the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) uptake pump, which is negatively regulated by the protein phospholamban (inhibitor of Ca^{2+}-ATPase).

The cardiac nervous system involves areas distributed throughout the neuraxis (Fig. 6, 7) and includes intrinsic and extrinsic components. The intrinsic cardiac nervous system (ICN) is an intricate neural network composed of ganglionated plexuses embedded in the epicardial fat pads and the heart wall. Its function is controlled by extrinsic influences mediated by the vagus and sympathetic nerves. The ICN ganglia are characterised by heterogeneous population of neurons that include afferent, efferent, and local circuit neurons; their intrinsic activity is modulated by sympathetic or vagal inputs. Most ganglion cells utilize acetylcholine as their primary transmitter; others contain somatostatin, vasoactive intestinal peptide, or nitric oxide synthase. The sympathetic innervation of the heart originates in a subgroup of neurons of the intermediolateral (IML) cell column of the spinal cord (Fig. 6, 7); these neurons collect tonic excitatory glutamatergic inputs from neurons in the rostral ventrolateral medulla (RVLM). The cardiac preganglionic sympathetic neurons are cholinergic and send small myelinated axons that synapse on noradrenergic neurons of the superior, middle cervical and cervicothoracic (stellate) ganglia, which give origin to axons that contribute to the cardiac plexuses innervating the heart via the superior, middle, and inferior cardiac nerves. The left-to-right distribution of sympathetic nerves is asymmetrical [7] and shows interindividual variability; this may clarify their heterogeneous effect on cardiac electrophysiologic properties. The primary neurotransmitter of cardiac sympathetic neurons is norepinephrine (NE), however some neuromodulators co-exist, for example adenosine triphosphate, calcitonin gene-related peptide, and neuropeptide Y.

The cardiovagal innervation represents the parasympathetic output. The preganglionic cardiovagal neurons are primarily located in the NAmb, and to a lesser amount in the dorsal motor nucleus of the vagus. These neurons are cholinergic and their axons extent to the cardiac ganglia through superior cervical, inferior cervical thoracic rami, which anastomose with cardiac sympathetic nerves to form the cardiac plexus. Most of the vagal nerve fibers innervate the atrium and SA and AV nodes, however some branches innervate the wall of the ventricles too [7].

Effects of the autonomic output on cardiac function regard Sympathetic and Parasympathetic activities. Sympathetic activation elicits an increase of inotropism and dromotropism, a faster conduction through the AV node, with an intensification of excitability of the His-Purkinje system, that determines a grow of contraction force during systole, and speeder relaxation of the cardiac muscle cells during diastole.

Conversely, the main effects of the vagus, by the cholinergic neurons of the cardiac ganglia, are inhibition of the pacemaker activity of the SA node (decrease in HR), reduced AV conduction, and decreased excitability of the His-Purkinje system.

Tonic vagal influence on the automatism of the SA node prevails over that of the sympathetic system during the rest condition. In fact, the HR has a circadian pattern; it increases in early morning because the sympathetic activity surges and decreases during sleep, particularly during non-REM sleep, for the vagal predominance. However, a phasic transient vagal interruption and sympathetic activation result in HR surges during REM sleep. The vagal activity, together with increased sympathetic influence, is rapidly decresed in response to orthostatic stress, hypovolemia, or exercise. In conditions with very low basal HR (e.g., athletes, during non-REM sleep, or patients with sinus bradycardia), vagal stimulation could unexpectedly increase HR by shortening the time between atrial depolarizations. The vagal activation, particularly in the ventricles, is higher in the setting of prominent concurrent sympathetic stimulation; this so-called “accentuated antagonism” depends on presynaptic inhibition of sympathetic transmission.

HR variability (HRV) is instantaneous HR that results from interactions between the vagal and sympathetic influences on the SA node, it depends by the variation in the beat-to-beat time interval (RR interval). HRV can be evaluated during the deep breathing and during the Valsalva maneuver.

The cardiac preganglionic sympathetic IML neurons are tonically activated by premotor glutamatergic sympathoexcitatory neurons of RVLM, which acts as a common effector of descending and reflex pathways controlling blood pressure (BP) and cardiac function; some of these neurons also synthesize epinephrine (C1 group). Psychological stress, pain, hypoxia, hypovolemia, and hypoglycemia activate RVLM neurons both directly and via descending inputs from the forebrain. On the contrary, instead, RVLM is inhibited by the baroreflex via disynaptic inhibition from the NTS, mediated by γ-aminobutyric acid (GABA)ergic neurons of the caudal ventrolateral medulla (Fig. 7). The NAmb contains the majority of cardioinhibitory vagal motoneurons that control SA automatism and AV node conduction. These neurons are activated by glutamatergic inputs from barosensitive neurons of the NTS and inhibited by local GABAergic neurons and by GABAergic neurons of the medullary ventral respiratory group that are active during inspiration. In this way, the vagal control of the HR is modulated on a beat-to-beat basis by respiration: cardiovagal activity is reduced during inspiration and increased during expiration. This physiologic event is named respiratory sinus arrhythmia (RSA). RSA is an important measure of cardiovagal output and health, and declines linearly with age. In addition, the Hering-Breuer reflex generated by pulmonary mechanoreceptors via the NTS may contribute to the RSA. The caudal portion of the NTS receives afferents from baroreceptors, cardiac receptors, chemoreceptors, and pulmonary receptors, mainly via vagal and glossopharyngeal afferents. Therefore, it is the first central relay for all medullary reflexes, including the baroreflex and cardiac reflexes controlling BP and HR.

The baroreceptor reflex (baroreflex) is a fundamental BP buffering tool and is activated by mechanical deformation of vessel walls in the carotid sinus and aortic arch during systole. Increase in BP stimulates baroreceptor afferents of the glossopharyngeal and vagus nerves, from which run monosynaptic excitatory

input to the NTS. Barosensitive NTS neurons initiate sympathoinhibitory and cardioinhibitory response by means two different pathways. By the first, the sympathoinhibitory pathway controls total peripheral resistance via disynaptic inhibition of RVLM neurons mediated by GABAergic neurons of the caudal ventrolateral medulla. Through the second pathway, the cardioinhibitory signal elicits a decrease in HR via direct excitatory inputs from the NTS to cardiovagal neurons of the NAmb. Afferents from the heart and coronary and pulmonary arteries trigger a variety of cardiovascular reflexes. Among these, cardiac unmyelinated afferents with cell bodies in thoracic dorsal root ganglia follow the trajectory of the sympathetic nerve trunks and provide inputs to the dorsal horn (particularly lamina I) and intermediate gray matter of the spinal cord, and myelinated and unmyelinated vagal afferents with cell bodies in the nodose ganglion that provide inputs to the NTS. On the other side, myelinated vagal afferents are activated by atrial distension due to an increase of blood volume, which trigger reflex activation of sympathetic input to the SA node and thus increase in HR, as well as inhibition of renal sympathetic activity and arginine vasopressin release, promoting sodium and water excretion. Furthermore, unmyelinated spinal and vagal afferents innervating the ventricles are stimulated by strong mechanical or chemical stimuli, in particular products of ischemia or inflammations such as adenosine triphosphate, serotonin, and prostanoids. Spinothalamic projections from lamina I neurons elicit the sensation of cardiac pain; these afferents can also trigger excitatory cardiac reflexes via local interneurons projecting to the IML (the so called “cardio-cardiac reflex”). In response to chemical stimulation of myocardial injury, unmyelinated vagal afferents in the ventricles may trigger a decrease in BP and HR (Bezold-Jarisch reflex). Stimulation of pulmonary arterial baroreceptors at physiologic pressures produces reflex vasoconstriction and respiratory stimulation. This could be implicated in cardiovascular control during exercise or in hypoxic conditions.

The insular cortex (IC), ACC, central nucleus of the amygdala (CeA), and several hypothalamic nuclei (Fig. 7) are forebrain regions projecting to medullary and spinal nuclei and controlling cardiac function. These projections could be either direct or via a relay in the periaqueductal gray. Afferent cardiovascular

information carried by dorsal horn (layer I) or NTS neurons reaches cortical areas through the thalamus. Moreover, visceral afferents are also conveyed to the parabrachial nucleus of the pons, which relays the information to the thalamus, hypothalamus, and amygdala, and by catecholaminergic neurons of the A1/C1 group of the ventrolateral medulla. The role of these areas in control of cardiac function is yet poorly understood, as the same is regarding the hemispheric lateralization of cardiovascular control too. The IC has been involved in a large number of functions and in the pathophysiology of a variety of neurologic disorders. It is subdivided into dorsocaudal and rostroventral zones. The dorsocaudal zone comprises several areas that receive gustatory, viscerosensory, somatosensory, pain, and vestibular afferences relayed by thalamus. The rostroventral zone is interconnected with the ACC and the amygdala and is primarily involved in emotional processing. Electrical stimulation of the insula in patients undergoing surgical treatment for intractable epilepsy elicits a variety of visceromotor phenomena, including changes in BP and HR. Over simplifying, stimulation of the left IC more frequently elicited a small decrease in HR and BP whereas stimulation of the right IC elicited the opposite effects. This, supported by fMRI, suggests that the left IC primarily regulates the parasympathetic and the right IC the sympathetic influence on the heart.

The ACC integrates autonomic responses with behavioral arousal via its wide connections with the IC, prefrontal cortex, amygdala, hypothalamus, and brainstem autonomic nuclei. Functional MRI studies show that the ventral ACC is involved in “default mode network”, activated in the resting state in conditions of self-monitoring. Whereas, the dorsal ACC, together with the anterior IC, is a core component of the so-called “salience network”; it is primarily recruited during tasks that demand cognitive control, including conflict resolution and is associated with an increase in sympathetic drive, resulting in HR increase. On the contrary, the subgenual ACC and adjacent ventromedial prefrontal cortex become at the same time inactivated. Pharmacologic and neuroimaging studies show that subgenual ACC activity is associated with vagally mediated HRV, particularly in the right hemisphere.

The amygdala provides emotional meaning to sensory stimuli and is involved in mechanisms of fear conditioning. The amygdala includes several subnuclei, among these, the basolateral nuclear complex and the CeA. The medial part of the CeA projects to the hypothalamus and brainstem and triggers the autonomic, endocrine, and motor manifestations of the fear responses; sympathoexcitatory responses involve excitatory connections to the RVLM, and inhibition of barosensitive neurons of the NTS. Functional neuroimaging studies have demonstrated a coactivation of the lateral and medial amygdala in relationship to changes in HRV both at rest and during emotional tasks. The orbitofrontal and ventromedial prefrontal cortices exert an inhibitory effect on the amygdala via GABAergic neurons in the lateral CeA and in the intercalate nucleus between the basolateral amygdala and the CeA; these prefrontal influences is involved in mechanisms of emotional regulation, including fear extinction. In fact, in addition to promoting vagal output, these prefrontal areas tonically inhibit sympathoexcitatory responses initiated in the amygdala.

Sympathoexcitatory responses during stress are mediated by projections from the hypothalamus. It controls autonomic output to the heart via inputs that originate primarily from the paraventricular nucleus, dorsomedial nucleus of hypothalamus (DMH), and lateral hypothalamic area; these hypothalamic projections reach the periaqueductal gray parabrachial nucleus, RVLM, NAmb, dorsal motor nucleus of the vagus, NTS, and IML. Hypothalamus modulates the baroreflex via NTS or NAmb connections. Experimental studies indicate that the inputs from the IC to the hypothalamus are mostly ipsilateral.

Reduced HRV and reduced baroreflex sensitivity (BRS) are relevant markers of cardiovascular risk, including predisposition toward ventricular arrhythmias in patients with primary cardiac disease because they reflect abnormalities in forebrain vagal or sympathetic drive, brainstem reflexes, or vagal or sympathetic output (as occurs in diabetic or amyloid neuropathy). Reduced HRV, mainly at the expense of decreased high-frequency HF (vagal) component and sometimes associated with indices of increased sympathetic activity, has been described in patients with ischemic stroke, epilepsy, multiple sclerosis, and Parkinson disease (PD).

A wide spectrum of cardiac arrhythmias, some of them life-threatening, can be determinated by central autonomic disorders. For example, vagal hyperactivity leads to bradyarrhythmias, including AV block, while sympathetic hyperactivity triggers both supraventricular and ventricular tachycardia and sympathetic or vagal hyperactivity may lead to atrial fibrillation (AF). In the ventricles, sympathetic activity is proarrhythmic and vagal activation is antiarrhythmic.

Seizures and cardiac dysfunction have numerous common traits; these include cardiac arrhythmias as a localizing ictal event, seizures and cardiac arrhythmias as coincident manifestations of channelopathies, nevertheless cardiovascular dysregulation and ictal arrhythmias as a mechanism of sudden unexpected death in epilepsy. Sinus tachycardia occurs in 80% to 100% of patients before, during, or after a temporal lobe seizure; paroxysmal AF, supraventricular or ventricular tachycardia, and ventricular fibrillation may also occur. Furthermore, temporal lobe seizures may lead to ictal bradycardia and asystole. Seizures, more common in patients with left-sided seizures, can also produce alterations of cardiac repolarization as manifested by acute changes in corrected QT (QTc) duration, which may be in part due to ictal hypoxemia. Mutations in genes encoding Na^+ or K^+ channels are associated with coexistence of long QT syndrome (LQTS) or Brugada syndrome and epilepsy. Autonomic cardiovascular manifestations of seizures may have a role in the pathophysiology of sudden unexpected death in epilepsy, but this causal relationship is yet to be established.

The dysfunction of the autonomic nervous system is a common phenomenon that links the major cardiac pathologies to neurologic disorders. These profound effects on the heart may contribute to the mortality rates of many neurologic conditions such as ischemic stroke, sudden cardiac death and epilepsy.

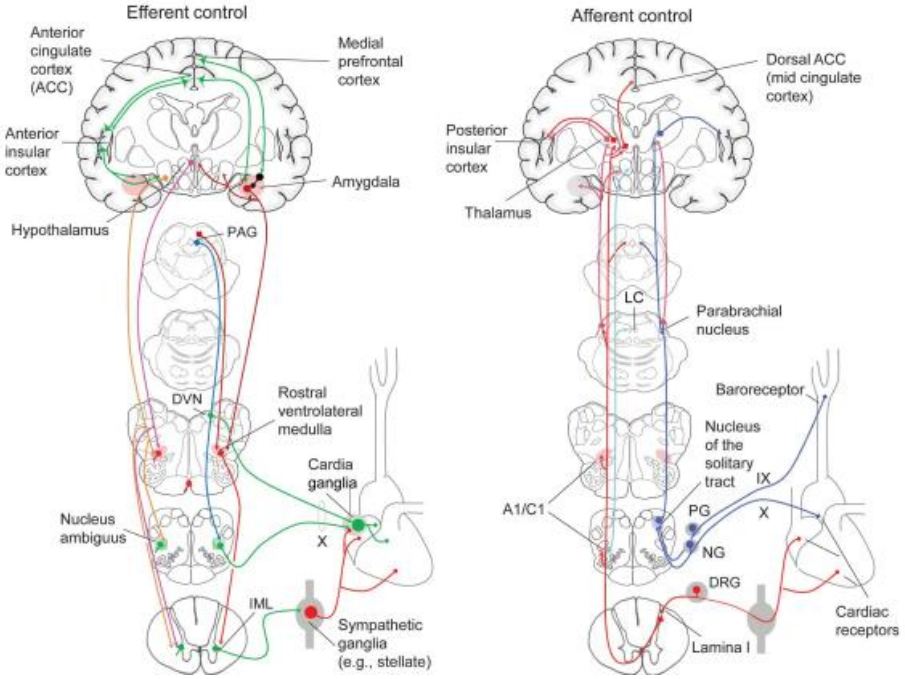


Figure 7 *Neural control of the heart is integrated at all levels of the neuraxis [4]*

1.4 Overview of Respiratory Control

Respiration is a rhythmic motor behavior analogous to locomotion that is generated by semiautonomous neural networks, located in the medulla and pons. This complex network of neurons is named the Central Pattern Generator [8]. These neurons depolarize during the three phases of respiration: inspiration, postinspiration, and expiration, coincident with activity motoneurons innervating the muscles of respiration. Inhibitory interneurons that discharge during specific phases of the respiratory cycle control the timing of the respiratory cycle. The autonomic control of breathing generate a respiratory rhythm by means a core group of synaptically coupled excitatory neurons in the brainstem located in the pre-Bötzinger complex (PBC) (Fig. 8, 9) [9]. PBC has pacemaker properties and is the putative site of genesis of inspiratory activity. Its neurons express the homeobox gene DBX1 and are immunopositive for neurokinin-1(NK1) receptor and somatostatin.

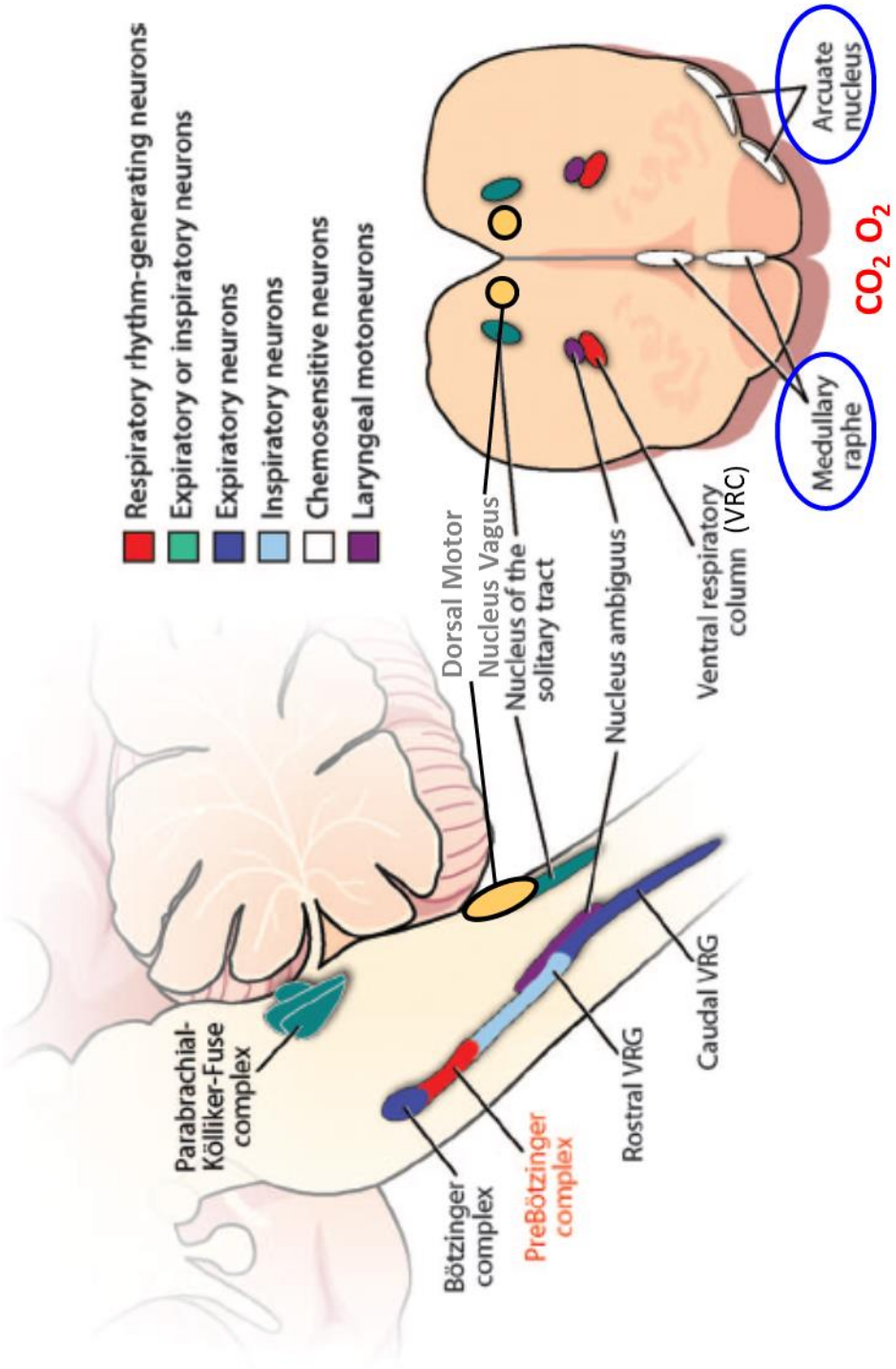


Figure 8 *The anatomic relationship between the regions in the human brainstem that constitute the respiratory network [9, modified]*

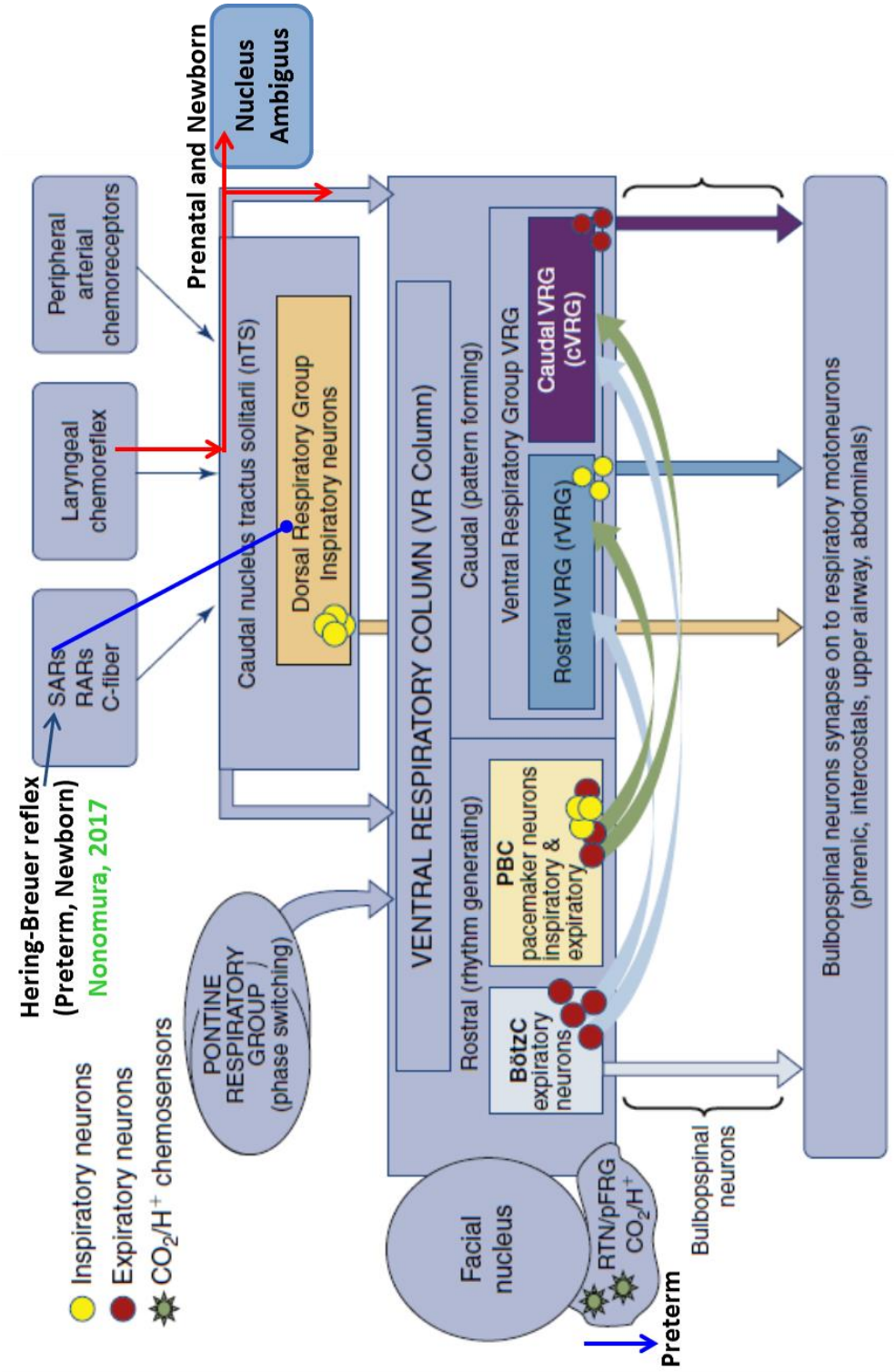


Figure 9 *Simplified schematic illustrating the major pontine-medullary brainstem network controlling respiration and afferent and efferent projections [9, modified]*

The respiratory-related neurons are grouped in three main areas in the brainstem: 1) the dorsal respiratory group within the NTS; 2) the ventral respiratory column (VRC), which extends from the facial nucleus to the ventrolateral medulla at the spinal–medullary junction, that should not be confused with the ventral respiratory group (VRG); 3) the pontine respiratory group within the dorsolateral pons (Fig. 8).

The VRC consists into a rostral part, involved in rhythmogenesis, and a caudal part, involved in pattern development. Bulbospinal neurons are neurons that originate in the medulla (bulbo) and synapse with motoneurons in the spinal column such as the phrenic motoneurons. The rostral VRC contains both the rostral VRG, comprising a large proportion of bulbospinal inspiratory neurons that project directly to the phrenic and external intercostal motoneurons, and the caudal VRG, holding bulbospinal expiratory neurons that project to abdominal and internal intercostal motoneurons. Propriobulbar neurons are intrabulbar neurons that send short projections to other neurons in the brainstem. The rostral VRC consists of two areas that are essential to the formation of respiratory rhythm: the PBC and the Bötzinger complex. The PBC contains coupled excitatory neurons with pacemaker property. These pacemaker cells are rostral to the NAamb, have both intrinsic inspiratory and expiratory bursting properties, and are essential to sustaining respiratory rhythm rhythmogenesis.

In individuals who died of multiple system atrophy, a neurodegenerative disease with central respiratory deficits, reduced numbers of neurons were identified in the area of the putative PBC as compared with the brains of individuals with spinocerebellar ataxia 3, a neurodegenerative disease without central respiratory deficits [10]. Another area with autonomous rhythm-generating properties is the postinspiratory complex (PiCo). It controls postinspiratory activity and is localized medially to the NAmb and caudallly to the nucleus of cranial nerve VII. The Bötzinger complex contains propriobulbar expiratory neurons that provide strong inhibitory inputs to inspiratory and expiratory bulbospinal neurons in the VRC.

The retrotrapezoid nucleus (RTN)/ parafacialrespiratory group (pFRG) located along the ventral medullary surface beneath the facial nucleus (Fig. 9) holds

neurons with chemosensitive properties, which depolarize in response to increasing carbon dioxide (CO_2) concentration and decreasing pH and synapse with rhythm- and pattern-generating neurons in the VRC. In 10 weeks' gestation human fetuses have been registered episodic spontaneous fetal breathing movements. Respiratory rhythmogenesis is a well-known phenomenon in embryonic animal models; the occurrence of this respiratory-related activity in rats is coincident with the characteristic expression of NK1 receptors of the PBC. In the human fetus, according to new concepts and animal model based theories, episodic respiratory activity aimed at promoting lung development is generated by the intermediolateral nucleus (ILN) in the upper spinal cord [11, 12]. At the same time, during intrauterine life, the Kölliker–Fuse nucleus (KFN), located in the rostral pons, shows an essential 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. The RTN/ pFRG, in the caudal pons, starts working at birth, under the stimulation of the KFN, which radically changes its function, giving rise to the first inspiratory act. The activity of the RTN/ pFRG is called "pre-inspiratory" because it is exclusively assigned to activating, in its turn, the proper inspiratory nucleus in the medulla oblongata: the PBC, responsible for starting postnatal breathing (Fig. 10).

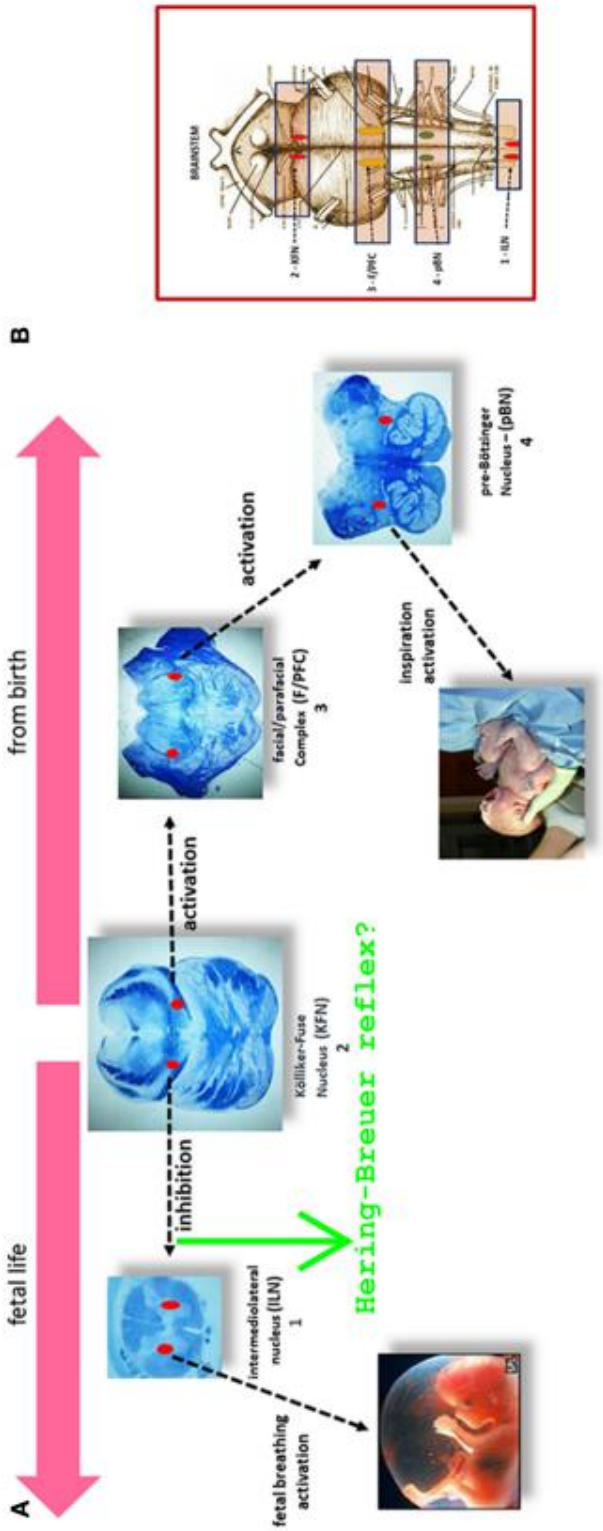


Figure 10 *The steps of the respiratory control in human perinatal [11, modified]*

In conclusion, respiratory rhythm and inspiratory–expiratory patterns occur from dynamic interactions between (1) excitatory neuron populations in the PBC and rostral VRG, which are active during inspiration and form the inspiratory motor output, (2) excitatory neurons in the PiCo, which are active during postinspiration, (3) inhibitory neurons in the PBC that provide inspiratory inhibition within the network, (4) inhibitory neurons in the Bötzinger complex, which are active during expiration and provide inhibitory inputs to inspiratory and expiratory neurons within the network and to phrenic motor neurons (Fig. 11) and (5) the RTN/ pFRG region in the pons-medulla junction, which contains key elements of the central pattern generator for breathing that are important in central CO_2–chemoreception and for gating active expiration. Contiguous brain regions associated with a given behaviour are increasingly being divided into subregions associated with distinct aspects of that behavior. Recently two functionally separate parafacial nuclei have been recognized in mammalians: ventral (pF_V) and lateral (pF_L). pF_V provides a generic excitatory drive to breathe, even at rest, whereas the pF_L is a conditional oscillator quiet at rest that, when activated, e.g., during exercise, drives active expiration (Fig. 12, 13, Table 1) [13, 14].

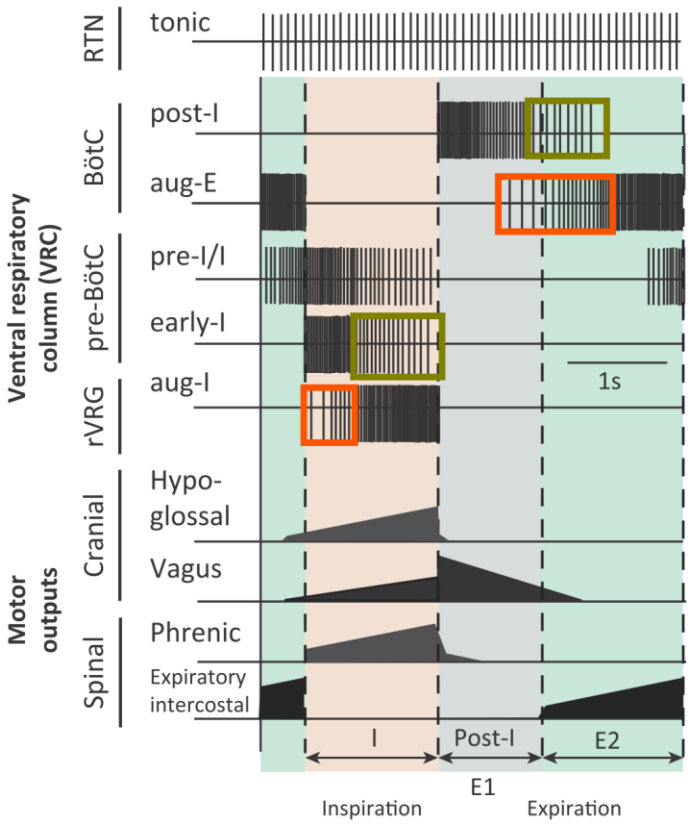


Figure 11 *Three-phase organization of neuronal spiking and motor output patterns during the respiratory cycle. Neuronal spiking patterns in different brainstem nuclei (top) and motor outputs (bottom), illustrated schematically, are typical of those recorded in anesthetized mammalian animal models. The motor output patterns illustrated represent the activity of cranial hypoglossal, central vagus, and spinal phrenic and expiratory intercostal nerves. Respiratory neurons active during the three phases are characteristically categorized based on profiles of their firing patterns (e.g., decrementing, in green, or augmenting, in red, spiking frequency) and their predominant activity phase. As shown, pre-I/I neurons in the PBC start firing before and continue into inspiration; early-I neurons have peak spiking in early inspiration followed by a decrementing pattern; rVRG aug-I neurons have an augmenting or ramping firing pattern; post-I neurons have a decrementing firing pattern; and aug-E neurons have a ramp firing pattern during E2. The scheme shows activity phases, that are not strictly sequential, but exhibit considerable temporal overlap [9, modified]*

Failure to coordinate postinspiration with inspiration can result in aspiration pneumonia, a prominent cause of death in Alzheimer's disease, Parkinson's disease, dementia, and other neurodegenerative diseases. Infact, breathing must be tightly coordinated with other behaviors such as vocalization, swallowing, and coughing, that happen during the postinspiration phase by PiCo network [13]. Glutamatergic-cholinergic neurons form the basis of this network, while GABAergic inhibition establishes the timing and coordination with inspiration. PiCo has autonomous rhythm generating properties and is necessary and sufficient for postinspiratory activity in vivo. PiCo also has distinct responses to neuromodulators when compared with other excitatory brainstem networks.

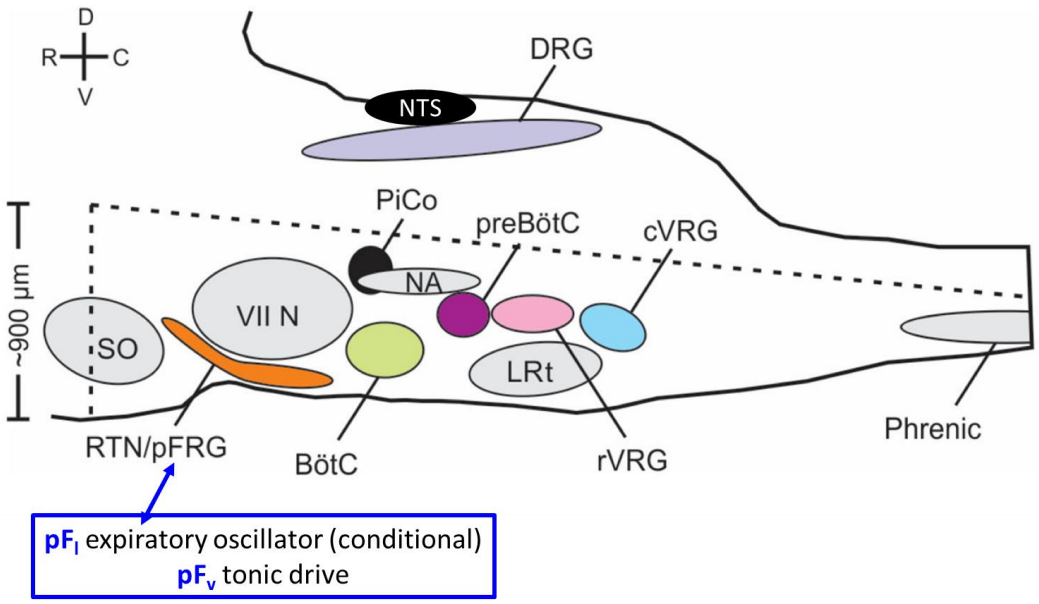


Figure 12 *Schematic sagittal view of the medullary ventral respiratory column in the brainstem. Slice retains part of the superior olive (SO), and the entire retrotrapezoidal nucleus/para-facial respiratory group (RTN/pFRG), divided in two functionally separate parafacial nuclei: ventral (pFV) and lateral (pFL). pFV provides a generic excitatory drive to breathe, even at rest, whereas the pFL is a*

conditional oscillator quiet at rest that, when activated, e.g., during exercise, drives active expiration; facial nucleus (VII N); Bötzinger Complex (BötC); Post-inspiratory Complex (PiCo); Nucleus ambiguus (NA); preBötzinger Complex (preBötC); lateral reticular nucleus (LRT), and the rostral and caudal ventral respiratory groups (rVRG and cVRG, respectively). The slice also retains a portion of the spinal cord and includes part of the phrenic motor nucleus and the dorsal portion of the medulla including the dorsal respiratory group (DRG). Moreover, the nucleus tractus solitarius (NTS), located in the dorsal medulla, helps to co-ordinate respiratory and sympathetic responses to hypoxia. Legend: dorsal (D), ventral (V), rostral (R), caudal (C) [13 modified]

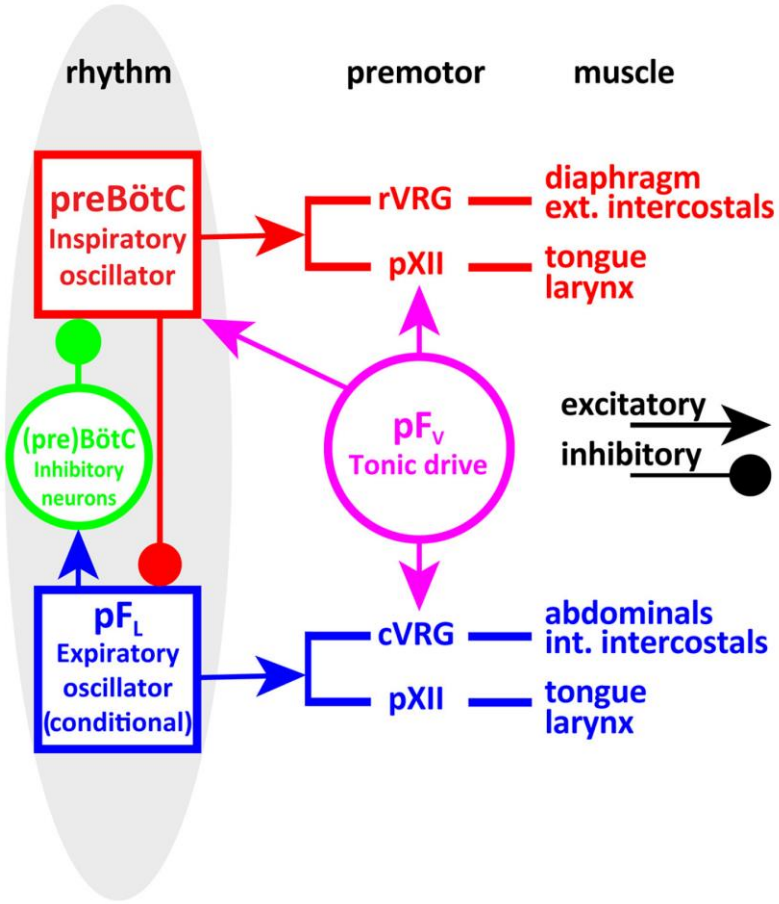


Figure 13 *Schematic of minimal respiratory central pattern generator, which at its core consists of three essential components: (1) an inspiratory oscillator in PBC that drives inspiration by exciting inspiratory premotor neuronal populations, e.g., rVRG and parahypoglossal region (pXII), and inhibits* pF_L*; (2) a (conditional) expiratory oscillator in* pF_L *that gates and drives expiration by exciting expiratory premotor neuronal populations, i.e., cVRG and pXII, and assures alteration of phases by exciting neurons that inhibit PBC, e.g., inhibitory neurons in either the PBC or BC; and (3) a source of tonic drive in* pF_V *that is responsive to* CO_2*/pH and integrates other sensory afferents affecting respiratory drive, via excitatory connections to PBC, BC, and respiratory premotorneurons, e.g., rVRG, cVRG, and pXII [14]*

Neuroanatomy: Brainstem Respiratory Network	Characteristics	Comment
Dorsal respiratory group	1. Located in the medulla 2. Receives sensory input from mechanoreceptors and peripheral chemoreceptors 3. Contains primarily inspiratory neurons 4. Synapses with nerves innervating the diaphragm and intercostal muscles	
Ventral respiratory group	1. Located in the medulla 2. Contains inspiratory neurons in the rostral ventral respiratory group and expiratory neurons in the caudal ventral respiratory group 3. Sends inspiratory impulses to laryngeal and pharyngeal muscles, diaphragm, and external intercostal muscles 4. Sends expiratory impulses to the abdominal and internal intercostal muscles	Do not confuse the ventral respiratory group with the ventral respiratory column.
Pontine respiratory group	1. Includes the Kölliker–Fuse and other parabrachial nuclei 2. Important in timing of inspiration: sends inhibitory signals to the dorsal respiratory group 3. Innervates laryngeal premotor neurons controlling upper airway resistance 4. Innervates spinal projecting neurons controlling phrenic motoneurons	Overstimulation causes apneustic breathing.
Retrotrapezoid nucleus (humans)/parafacial respiratory group (rodents)	1. Located in the ventral medulla in the parafacial region 2. Parafacial respiratory group separated into ventral and lateral areas with different effects on respiration 3. Contains glutamatergic neurons expressing the transcription factor paired-like homeobox 2b 4. Express neurokinin 1 receptor 5. Activity regulated by CO_2 or pH 6. Synaptic inputs from peripheral chemoreceptors modify output of this area. 7. Enhances expiratory activity of abdominal muscles during hypercapnia 8. Thought to be insensitive to opioids	Congenital central hypoventilation syndrome Individuals have insensitivity to hypercapnia—retrotrapezoid nucleus is affected. Genetic mutation: polyalanine expansion mutation in the *PHOX2B* gene
Bötzinger complex	1. Located in the medulla 2. Contains expiratory neurons 3. Inhibits inspiratory neurons 4. Involved in controlling the alteration between inspiration and expiration 5. Augments expiratory activity (second phase of expiration)	
Pre-Bötzinger complex	1. Located in the medulla 2. Intrinsic rhythmic inspiratory excitatory drive 3. Glutamatergic neurons have widespread projections 4. Also inhibits expiratory neurons during inspiration	
Nucleus tractus solitarii	1. Located in the dorsomedial medulla 2. Receives inputs form pulmonary mechanoreceptor, peripheral chemoreceptor, and other visceral sensory afferent inputs 3. The dorsal respiratory group (contains inspiratory neurons) is part of the nucleus tractus solitarii	
Brainstem raphe nuclei	1. Located near the midline of the brainstem with extensive rostrocaudal extension from the caudal medulla to the pons 2. Extensive projections throughout the brain and spinal cord 3. Rich source of serotonergic neurons with extensive projections 4. Synapse with neurons that are involved in motor, somatosensory, and limbic systems	Abnormalities in serotonergic neurons in the raphe have been found in infants who died of sudden infant death syndrome.
Bulbospinal neurons	Neurons originating in the brainstem that synapse with motoneurons in the spinal column	For example, bulbospinal neurons synapse with phrenic motoneurons and with motoneurons for the intercostal muscles
Propriobulbar neurons	Neurons originating in the brainstem that send projections to other neurons in the brainstem	For example, the Bötzinger complex contains propriobulbar expiratory neurons that provide strong inhibitory inputs to inspiratory and expiratory bulbospinal neurons in the ventral respiratory column

Modified from Smith JC, Abdala AP, Borgmann A, Rybak IA, Paton JF. Brainstem respiratory networks: building blocks and microcircuits. *Trends Neurosci.* 2013;36:152–162.

Table 1 *Major Anatomic Regions in the Brainstem Involved in the Pattern and Timing of Respiration [9]*

Neurochemical control of respiration regards glutamate, as the major neurotransmitter mediating excitatory synaptic input to brainstem respiratory neurons and respiratory premotor and motor neurons through binding to α-amino-3- hydroxy-5-methylisoxazole-4-propionic acid kainite receptors and metabotropic glutamate receptors. On the other hand, GABA and glycine are the two major inhibitory neurotransmitters mediating inhibitory synaptic input in the respiratory network; they have a crucial role in pattern generation and termination of inspiratory activity. GABA (via GABA A receptors) and glycine (via glycine receptors) mediate fast synaptic inhibition via activation of chloride channels. Throughout development, glutamate always functions as an excitatory neurotransmitter; however, it is not the case that GABA and glycine are always inhibitory neurotransmitters. In early development, GABA and glycine mediate excitatory neurotransmission in many neuronal networks, including the respiratory network (Fig. 14) [15]. An early excitatory effect of GABA has been discovered for hippocampal neurons, neocortical neurons, and phrenic motor neurons [16]. GABA and glycine signaling changes the level of chloride in the cell. Activation of the sodium (Na^+)–potassium (K^+)–chloride (Cl) cotransporter (NKCC1) and the potassium–chloride transporter (KCC2) on the cell modulates intracellular ion concentrations. Specifically, NKCC1 carries Na^+, K^+, and $2Cl^-$ into the cell, while activation of KCC2 moves K^+ and Cl^- outside the cell. During early development, the high NKCC1/KCC2 ratio causes high intracellular chloride concentrations in immature neurons. When GABA then binds to GABA A receptors, there is net outward movement of Cl^- ions, coming out membrane depolarization. With maturation, the KCC1/KCC2 ratio reverses, and there is less chloride in the cell. Now when GABA binds to GABA A receptors, more chloride comes into the cells, leading to hyperpolarization. With brain injury, NKCC1 expression increases, producing the GABAergic system less inhibitory. Moreover, myoclonic jerks are associated with midazolam exposure (GABA A receptor

agonist) in premature infants. GABA B receptors, which are metabotropic G protein–coupled receptors, also have an important role in inhibiting respiratory rhythm in adult animals as compared with new-born animals.

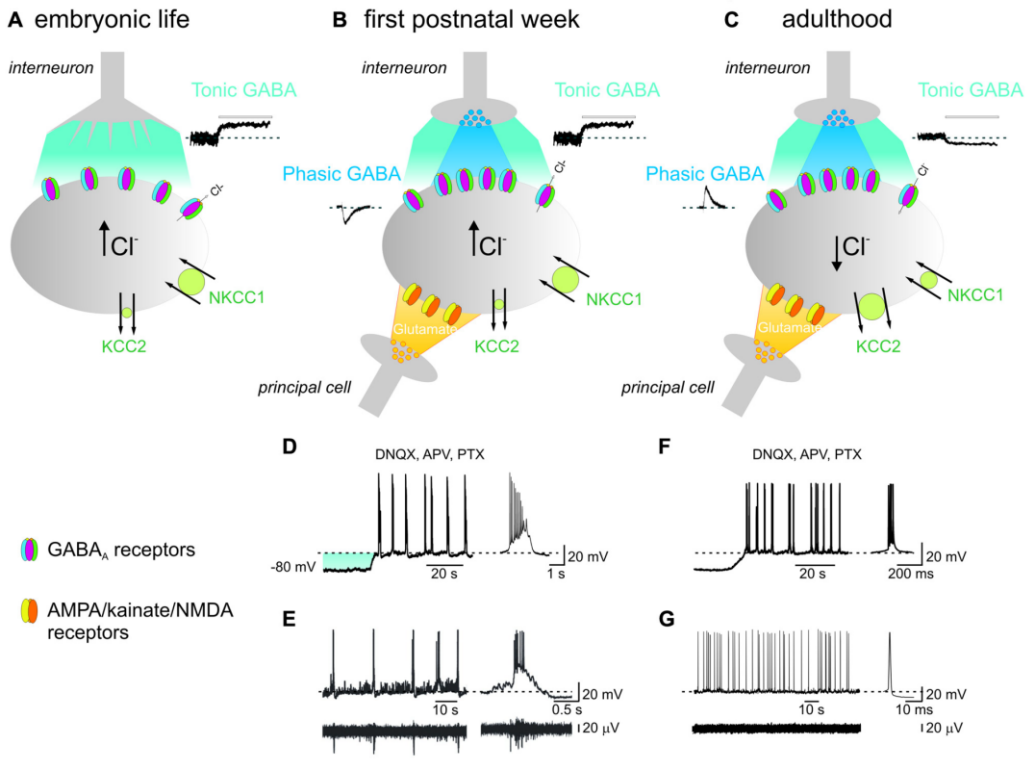


Figure 14 *In the immature hippocampus, ambient GABA depolarizes targeted cells and contributes to generate network-driven GDPs (giant depolarizing potentials) [15]*

Many endogenously released neuromodulators influence the excitatory and inhibitory output mediated by glutamate and GABA–glycine respectively on major neuronal respiratory networks (Fig. 15, Table 2) [17]. Acetylcholine, substance P, cholecystokinin (CCK), and thyrotropin-releasing hormone all exert an excitatory drive, whereas opioids, somatostatin, and prostaglandin E 2 exert an inhibitory drive on respiratory-related neurons. Norepinephrine, dopamine, serotonin, and adenosine can have excitatory and inhibitory influences depending

on the specific neuromodulator receptors. Rhythmogenic neurons within the PBC are distinctly identified by being immunopositive for glutamate transporter, NK1, μ-opioid, and GABA B receptors. These PBC neurons produce and are excited by brain-derived neurotrophic factor (BDNF). Apnea—a common syntom of illness in infants—is mediated, in part, by an increase in prostaglandin E 2 levels in the central respiratory network.

Neuromodulator	Receptor Subtype	Source of the Endogenous Ligand	Excitatory or Inhibitory on Respiratory Rhythm	Comment
Glutamate	NMDA, AMPA, GluR		Excitatory	Major excitatory neurotransmitter
Ach	M3	PAG, LC, X	Excitatory	
NE	α1-Adrenergic	LC	Excitatory	
Serotonin	5-HT2A2B, 5-HT3, 5-HT4	Raphe	Excitatory	
Dopamine	Likely D_1	PVN, hypothalamus	Excitatory	
ATP	P2X2	Ventral medulla; CO_2/H^+-sensitive cells in the RTN	Excitatory	
Adenosine	P2Y1	Ventral medulla	Excitatory	
Substance P	NK1	nTS, NA	Excitatory	
CCK	CCK1	nTS, raphe	Excitatory	
TRH	TRH-R (1 and 2)	Raphe	Excitatory	
GABA	$GABA_A$, $GABA_B$		Inhibitory	Major inhibitory neurotransmitter (can be excitatory during fetal life)
Glycine	GlyR		Inhibitory	Can be excitatory during fetal life
NE	α2-Adrenergic	Pons	Inhibitory	
Dopamine	D4	PVN, hypothalamus		
Adenosine	A_1, A_2	Ubiquitous from metabolism of ATP that increases during hypoxia	Inhibitory	Contributes to respiratory depression at the baseline (A_1), and mediates HVD
Opioid	μ, δ, κ	nTS, PBN, PVN, raphe	Inhibitory	Prominent inhibitory effect during early development
PDGF	PDGF-β	nTS	Inhibitory	Contributes to HVD

Data from Doi A, Ramirez JM. Neuromodulation and the orchestration of the respiratory rhythm. *Respir Physiol Neurobiol.* 2008;164:96; and Simakajornboon N, Kuptanon T. Maturational changes in neuromodulation of central pathways underlying hypoxic ventilatory response. *Respir Physiol Neurobiol.* 2005;149:273.
Ach, Acetylcholine; *AMPA*, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; *ATP*, adenosine triphosphate; *CCK*, cholecystokinin; *GABA*, γ-aminobutyric acid; *GluR*, glutamate receptor; *GlyR*, glycine receptor; *HVD*, hypoxic ventilatory depression; *LC*, locus ceruleus; *NA*, nucleus ambiguus; *NE*, norepinephrine; *NMDA*, *N*-methyl-D-aspartate; *nTS*, nucleus tractus solitarii; *PAG*, periaqueductal gray; *PBN*, parabrachial nucleus; *PDGF*, platelet-derived growth factor; *PVN*, paraventricular nucleus; *RTN*, retrotrapezoid nucleus; *TRH*, thyrotropin-releasing hormone; *TRH-R*, thyrotropin-releasing hormone receptor; *X*, vagal nucleus.

Table 2 *Neurotransmitters and neuromodulators that mediate respiratory rhythm [9]*

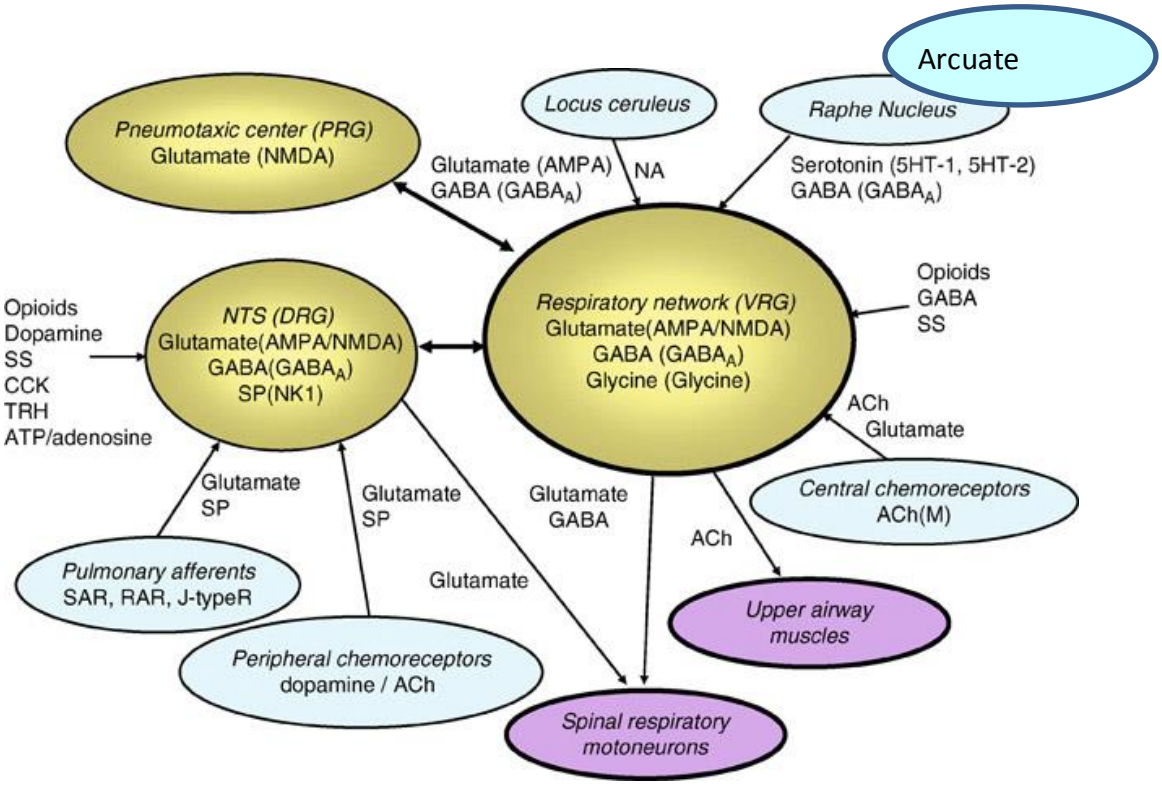


Figure 15 *Respiratory Neurotransmitters and Neuromodulators [17 modified]*

There are many peripheral inputs that can modulate the Central Cardio-Respiratory Network. The NTS in the brainstem, is an important relay point (Fig. 9, Table 3) in which sensory information from vagally mediated reflexes and chemical signals from the blood (arterial chemoreceptors) and cerebrospinal fluid (central chemoreceptors) and information from higher brain regions are integrated. Neurons from the NTS synapse onto respiratory related neurons, so augmenting or attenuating minute ventilation.

Bronchopulmonary reflexes modify the depth and duration of inspiration and expiration; they are mediated through the myelinated and unmyelinated fibers of vagus nerve. Myelinated vagal afferent fibers are activated via slowly adapting stretch receptors (SARs), which are activated by volume and stretch of the lung (mediating the Breuer–Hering reflex), or rapidly adapting receptors (RARs), which are activated in response to inhaled irritants (e.g., ammonia, cigarette smoke) and large inflations or deflations of the lung. Activation of SARs modifies the duration of inspiration and expiration, whereas activation of RARs causes

sighs (i.e., augmented breaths) and cough. Unmyelinated vagal afferents of airway are C-fibers that are activated by a multitude of chemical stimuli, including CO_2 and capsaicin, but also lung edema and elevated temperature. Activation of C-fibers in the lung produces rapid shallow breathing and apnea.

Receptor	Characteristics	Stimulant	Responses	Comment
Slowly adapting stretch receptors	1. Mechanoreceptors 2. Mediated by fast-conducting, myelinated vagal fibers 3. Located in lung parenchyma	Lung volume and transmural pressure	1. Breuer–Hering reflex 2. Termination of inspiration and prolongation of expiration 3. Bronchodilation 4. Tachycardia	Breuer–Hering reflex more active in infants than in adults
Rapidly adapting receptors	1. Mechanoreceptors 2. Irritant receptors 3. Located throughout the airways 4. Mediated by fast-conducting, myelinated vagal fibers	1. Inhaled irritants 2. Low lung volumes	1. Cough 2. Mucus production 3. Augmented breaths (sighs)	Responsible for inducing sighs in premature infants—restoring functional residual capacity
Bronchial and pulmonary C fibers	1. Located throughout the airway from the nose to alveoli 2. Stimulated by substances in the pulmonary circulation and inhaled 3. Slowly conducting, nonmyelinated vagal fibers	1. Capsaicin 2. Respiratory irritants 3. Lung edema 4. Inflammatory mediators	1. Rapid, shallow breathing 2. Apnea 3. Bronchoconstriction 4. Laryngoconstriction 5. Mucus secretion 6. Vasodilatation (pulmonary C fibers) 7. Bradycardia	J-receptors located in alveoli activated by lung edema
Laryngeal chemoreflex	1. Potent airway-protective reflex from aspiration 2. Receptors in laryngeal mucosa 3. Mediated by sensory fibers in the superior laryngeal nerve	1. Hyposmolarity 2. Low chloride content	Response in newborns: 1. Hypoventilation/apnea 2. Laryngoconstriction 3. Swallowing 4. Bradycardia 5. Shunting of blood flow to brain, heart, adrenals Response in adults: 1. Cough 2. Arousal 3. Swallowing	May contribute to apnea and bradycardic events associated with oral feedings in premature infants. Immature responses are exacerbated during hypoxia

Modified from Kubin L, Alheid GF, Zuperku EJ, McCrimmon DR. Central pathways of pulmonary and lower airway vagal afferents. *J Appl Physiol (1985).* 2006;101:618–627; Thach BT. Maturation and transformation of reflexes that protect the laryngeal airway from liquid aspiration from fetal to adult life. *Am J Med.* 2001;111 Suppl 8A:69S-77S; and Widdicombe J. Reflexes from the lungs and airways: historical perspective. *J Appl Physiol (1985).* 2006;101:628–634.

Table 3 *Airway Receptors and Reflex Responses [9]*

Breuer–Hering reflex contributes to tidal breathing; it is produced by occlusion of the airway at the end of expiration. The following occluded inspiratory effort is prolonged, and expiratory effort is shortened. Alternatively, the occlusion can be performed at end of an inspiratory effort; then the following occluded expiratory effort is prolonged and inspiratory effort is shortened. With this performance, the Breuer–Hering reflex significantly contributes to tidal breathing in infants. The reflex is strongest at birth and then decreases during the first year of life. It is

reasoned that the strength of the Breuer–Hering reflex is inversely related to gestational and postnatal age because of the excessively compliant chest wall in new-borns, which collapses at lung volumes lower than functional residual capacity. With decreasing lung volumes during expiration, the Breuer–Hering deflation reflex is triggered; the expiratory time is then shorter, and the inspiratory time is extended. Several factors increase the strength of the Breuer–Hering reflex, including premature birth, prone sleeping position, active sleep, and respiratory distress syndrome.

Otherwise, the stimulation of pulmonary C-fibers by chemical stimulants causes bronchoconstriction and apnea in new-borns. Bronchopulmonary C-fibers are stimulated by capsaicin, acidosis, adenosine, reactive oxygen species, hyperosmotic solutions, lung edema and primarily inflammatory mediators. For this reason, the pulmonary C-fiber–mediated respiratory inhibition may generate persistent apnea beyond term gestation in infants born at the limit of viability who have lung inflammation from chronic lung disease, viral infections and/ or prenatal tobacco smoke exposure.

Likewise, SIDS could be ascribed to sensitization of bronchopulmonary C-fibers from tobacco smoke exposure, mystified by acute infection leading to prolonged apnea from which the infant does not recover.

The laryngeal mucosa holds receptors that respond to changes in upper airway pressure and chemicals, but also water receptors, that are stimulated by hypoosmolarity and low chloride content, exist. These receptors can be slowly adapting, rapidly adapting irritant receptors, or C-fibers.

The laryngeal chemoreflex (LCR) is the upper airway reflex that mediates significant cardiorespiratory effects and that occurs particularly in new-borns. In fact, LCR is one of the most potent defensive reflexes protecting the respiratory tract from inadvertent aspiration. Laryngeal chemoreceptors are stimulated by liquid in the airway, which usually induces coughing, swallowing, and arousal. However, the response in immature infants is apnea followed by hypoventilation, laryngeal constriction, and swallowing, nevertheless, bradycardia, peripheral vasoconstriction, and redistribution of blood flow also occur. The associated apnea and bradycardia, enhanced by baseline hypoxemia, can be life threatening

in new-borns. Afferent fibers for this reflex run in the superior laryngeal nerve, a branch of the vagus nerve. These afferents synapse with neurons in the NTS, which then send excitatory projections to motoneurons of the recurrent laryngeal nerve in the NAmb, causing constriction of the laryngeal constrictor muscle, resulting in laryngospasm; inhibitory projections to phrenic motoneurons in the cervical spinal cord, inhibiting diaphragmatic contraction, resulting in apnea; and an excitatory pathway to cardiac vagal neurons in the NAmb causing bradycardia.

Foetus LCR likely functions to prevent aspiration of amniotic fluid, which is chloride enriched. In premature infants, the reflex may be involved in the apnea and bradycardic responses related to feeds and gastroesophageal reflux (GER) that reaches the larynx or nasopharynx. LCR, induced in new-born animal models, shows that the apnea associated with the LCR is prolonged during hyperthermia and by nicotine exposure. Whether these environmental events can produce an elongation of the reflex in human premature infants and if the immature response is still present in term infants or how the maturation of the reflex is affected by premature birth has not been determined. However, profound inhibitory cardiorespiratory effects that are also accentuated by prenatal nicotine exposure, may be important reflexes stimulated by LCR in some SIDS cases and infants with acute life-threatening events.

Respiratory rhythmogenesis is primarily driven by the level of $PaCO_2$ in the blood and cerebrospinal fluid and, to a lesser extent, by oxygen tension. Conversely, peripheral arterial chemoreceptors in the carotid body are principally responsible for modifying breathing in response to changes in oxygen tension. Several groups of neurons in the brainstem, are chemosensitive because are responsive to CO_2/H^+; specifically they are localized in the medullary raphe, RTN, NTS, arcuate nucleus, locus ceruleus, and fastigial nucleus. Elevation of $CO2/H^+$ concentration, in these chemosensitive brain regions, increase ventilation acts.

The serotonergic neurons in the caudal raphe project to phrenic motoneurons, where they modulate neuronal plasticity in response to hypoxia, while the glutamatergic RTN receives polysynaptic excitatory inputs from peripheral arterial chemoreceptors and sends projections to neurons in the VRC, including the PBC. The arcuate nucleus contains a large population of glutamatergic

neurons and a smaller population of serotonergic neurons. It depolarizes in response to hypercapnia and its absence or hypoplasia in human infants has been associated with SIDS cases [18, 19].

Purinergic signalling involving the purinergic P2 receptors and adenosine triphosphate (ATP) mediating CO_2/H^+ responsiveness exclusively to chemosensitive areas in the RTN. Both neurons and astrocytes in the RTN function as respiratory chemoreceptors. The role of astrocytes in this process appears to involve CO_2/H^+-dependent release of ATP to enhance activity of chemosensitive RTN neurons. The extracellular brain ATP is rapidly converted to adenosine by ectonucleotidase activity. Adenosine is a potent neuromodulator that into the RTN reduces the hypercapnia ventilatory response and serves to limit RTN chemoreceptor function by selective A1-receptor activity. These results, currently in animal models, may identify adenosine as a novel purinergic regulator of RTN chemoreceptor function during hypercapnia [20].

Infants with apnea of prematurity have reduced central respiratory drive to breathe when compared with infants who do not have apnea of prematurity at the same postconceptional age. Probably it depends on the maturation of synaptic inputs from chemosensitive neurons to respiratory-related neurons in the brainstem and/or maturation of intrinsic properties of chemosensitive nuclei.

The peripheral arterial chemoreceptors in the carotid body, placed at the bifurcation of the carotid artery, are involved in the reflex control of ventilation in response to changes in arterial oxygen tension. Nevertheless, specialized cells within the carotid body also are sensible to changes in blood CO_2/H^+ concentration, reflexively increasing ventilation in response to acidosis and hypercapnia and decreasing ventilation in response to hypocapnia. The major excitatory neurotransmitter in peripheral arterial chemoreceptors is ATP. Adenosine, its breakdown product, and adenosine A2a and A1 receptors, which are excitatory and inhibitory G protein–coupled receptors, respectfully form the response of the carotid sinus nerve. Xanthines block both excitatory and inhibitory adenosine receptors.

The NTS is the primary target for afferent processes from peripheral arterial chemoreceptors. Glutamate is responsible for chemical transmission of excitatory

inputs from the peripheral arterial chemoreceptors on second-order neurons in the NTS. These second-order neurons then lead tonic excitatory projections to CO2/H+-sensitive neurons in the RTN and bulbospinal neurons in the dorsal respiratory group and the VRG that synapse with respiratory motoneurons, leading to changes in the integrated output of the muscles of respiration according to the ventilatory response to hypoxia.

Because of the complexity of the ventilatory response to hypoxia and the frequent occurrence of arousals induced by hypoxic exposure, assessment of the effect of sleep state on the ventilatory response to hypoxia is difficult.

However, adequate arousal mechanisms are key in preventing respiratory failure and death, and impaired arousal mechanisms are hypothesized to contribute to SIDS and SUDEP. Prone sleeping position increases the percentage of quiet sleep, and is associated with increased time to hypoxic arousal in human infants. A hypoxic microenvironment from rebreathing with defective arousal and autoresuscitative mechanisms is theorized to have happened in infants who have died of SIDS in prone sleeping position.

1.5 Neuro-cardiac-respiratory genes

Several neuromodulator systems seem more critical in supporting respiratory rhythmogenesis than others and are associated with genetic mutations that produce marked abnormalities in respiratory control. Serotonergic neurons in the caudal medullary raphe nuclei have wide projections to phrenic and hypoglossal motoneurons, the NTS, the RTN, and the PBC. The serotonergic system influences diverse homeostatic functions, including cardiorespiratory responses and thermogenesis. Individuals with Prader–Willi syndrome, who may exhibit breathing abnormalities at birth, have mutations in the necdin gene (NDN) on chromosome 15 leading to abnormalities in the brainstem serotonergic system. Medullary serotonergic neurons are also CO_2 sensitive, in fact, genetically modified mice that do not develop medullary serotonergic neurons, have a CO_2 sensitivity reduced by 50%. The brainstem serotonergic system appears disrupted in some brains of SIDS cases. While specific single-gene mutations that regulate serotonin production and function have not been identified in infants who have died of SIDS, some studies have shown a higher proportion of specific polymorphisms in the 5′ regulatory region of the SLC6A4 gene, which encodes serotonin transporter, which regulates the reuptake of serotonin from the extracellular space. Infants who have died of SIDS have an increased frequency of the long allele variant and the variable number 12-tandem repeat in intron 2 polymorphisms in the promoter. The long allele occurs more frequently in African Americans; African Americans also have a 2.7-fold greater incidence of SIDS than whites. In fact, the polymorphisms produce an increased activity of serotonin transporter, thereby decreasing the time that serotonin stays in the synapse, leading to a relative serotonin deficiency causing dysregulation of the cardiorespiratory system. Kinney [19] described unifying clinical 5-HT-related disorders of early life under the title of "developmental serotonopathies' that have not previously been considered together. (Fig. 16, Table 4). These disorders are produced by dominant (nevertheless not necessarily exclusive) defects in 5-HT metabolism which originate during gestation or soon after birth, and which are associated with a variety of genetic and/or environmental factors. Developmental serotonopathies act predominately on the rostral 5-HT domain, caudal 5-HT

domain, or both domains (Table 4). Several developmental serotonopathies involving the caudal 5-HT domain and are associated with sudden death in early life, including SIDS, Rett Syndrome and Prader–Willi syndrome. Diagnosis of the developmental serotonopathies is ever increasing by means of identification of gene or enzymatic defects, and/or by measurements of blood and/or CSF levels of 5-HT metabolites. Rett syndrome is an X-linked disorder with mutations in several genes, but the most common genetic defect (90%) is in the methyl CpG binding protein 2 gene (MECP2). Affected individuals are normal at birth and then experience progressive worsening leading to severe motor, cognitive, and autistic behavioral systems. They also present characteristic severe respiratory disturbances with prolonged apnea and hyperventilation that can be lethal. Genetically modified mice with deficiency of the MECP2 gene have reduced levels of norepinephrine and serotonin in the medulla and have breathing patterns similar to those of humans with Rett syndrome. Pharmacologic treatment to increase brain norepinephrine and serotonin levels stabilizes breathing and prolongs the life of these mice. Fluoxetine and buspirone have been reported to be able to reduce breathing dysregulation in a patient with Rett syndrome [21]. Altered GABA neurotransmission might also be contributory as suggested by experiments using stem cells from a patient with Rett syndrome that demonstrated that the functional switch of GABA neurotransmission from excitation to inhibition was impaired [22]. Congenital central hypoventilation syndrome (CCHS), also notorious as Ondine's curse, is another rare autosomal dominant genetic disorder, occurring in 1 in 200,000 live births. Affected individuals typically have satisfactory ventilation during wakefulness but significant hypoventilation during sleep as well as impaired ventilatory responses to CO_2 and hypoxia during sleep and wakefulness. Although the disorder most commonly presents during infancy, milder forms may present later in childhood or even during adulthood. More than 90% of individuals with CCHS have mutations in the PHOX2B gene. PHOX2B is a homeobox gene located on chromosome 4 that is specifically expressed in limited types of neurons involved in autonomic processes. It is expressed in chemosensitive glutamatergic neurons in the RTN that collect polysynaptic inputs from peripheral arterial chemoreceptors; its

expression is also required for the development of the carotid body, NTS, and catecholaminergic neurons. The RTN contains putative central chemoreceptors that have intrinsic pH sensitivity and release the excitatory neurotransmitter glutamate, thereby stimulating breathing during hypercapnia. Consequently, mutations in the PHOX2B gene alter the development of key structures that regulate chemical control of breathing. This mutation is classified as polyalanine repeat mutations (PARMs). Fewer affected individuals with CCHS have nonpolyalanine repeat mutations (NPARMs) resulting in deletions in exon 3 that cause frameshift mutations. Depending on the mutation, some patients require tracheostomy and long-term ventilation and/or diaphragmatic pacing. Although respiratory stimulants are uneffective in increasing respiratory drive, drugs that cause respiratory depression may induce a profound respiratory depression. However, abnormalities throughout the autonomic nervous system are often observed in patients with CCHS. Specifically, patients can present with Hirschsprung disease and neuroblastomas (neuroblastoma–Hirschsprung disease–CCHS syndrome) (Fig. 17) [23]. A subset of CCHS cases with the least number of repeats might die in infancy and could be misidentified as SIDS.

Genetic studies in SIDS have been moved by clinical, epidemiological, and/ or neuropathological observations. Five categories of candidate genes in SIDS victims have been considered [24], most of them are involved in Channelopathies. Channelopathies are a heterogeneous group of disorders with more than one entity and with more than one possible genetic aetiology, resulting from the dysfunction of ion channels situated in the membranes of all cells and many cellular organelles. These comprise diseases of the nervous system (e.g., generalized epilepsy, familial hemiplegic migraine, episodic ataxia, and periodic paralysis), the cardiovascular system (e.g., LQTS, short QT syndrome, Brugada syndrome, and catecholaminergic polymorphic ventricular tachycardia, CPVT), the respiratory system (e.g., cystic fibrosis), the endocrine system (e.g., neonatal diabetes mellitus, familial hyperinsulinemic hypoglycemia, thyrotoxic hypokalemic periodic paralysis, and familial hyperaldosteronism), the urinary system (e.g., Bartter syndrome, nephrogenic diabetes insipidus, autosomal-dominant polycystic kidney disease, and hypomagnesemia with

secondary hypocalcemia), and the immune system (e.g., myasthenia gravis, neuromyelitis optica, Isaac syndrome, and anti-NMDA receptor encephalitis).

Summarizing, the five groups of putative genes involved in SIDS can be divided in: (1) genes for ion channel proteins based on electrocardiographic evidence of prolonged QT intervals in SIDS victims, (2) gene for serotonin transporter based on decreased serotonergic receptor binding in brainstems of SIDS victims, (3) genes pertinent to the early embryology of the ANS (and with a link to the 5-HT system) based on reports of ANS dysregulation in SIDS victims, (4) genes for nicotine metabolizing enzymes based on evidence of cigarette smoking as a modifiable risk factor for SIDS, and (5) genes regulating inflammation, energy production, hypoglycemia, and thermal regulation based on anamnestic information of postnatal infection, low birth weight, and/or overheating in SIDS victims.

However, any associations observed with genes in these categories have not been consistently identified through multiple studies and consequently will require further studies. In fact, neither the variable number of tandem repeat area in the promoter of the MAOA gene, nor rs25531 in the gene encoding 5-HTT, is involved in SIDS. Nevertheless, as medullary serotonergic abnormalities most likely contribute to the death in at least some SIDS cases, it is imperative to investigate these genes, as well as other genes involved in the serotonergic network, in more detail [25, 26].

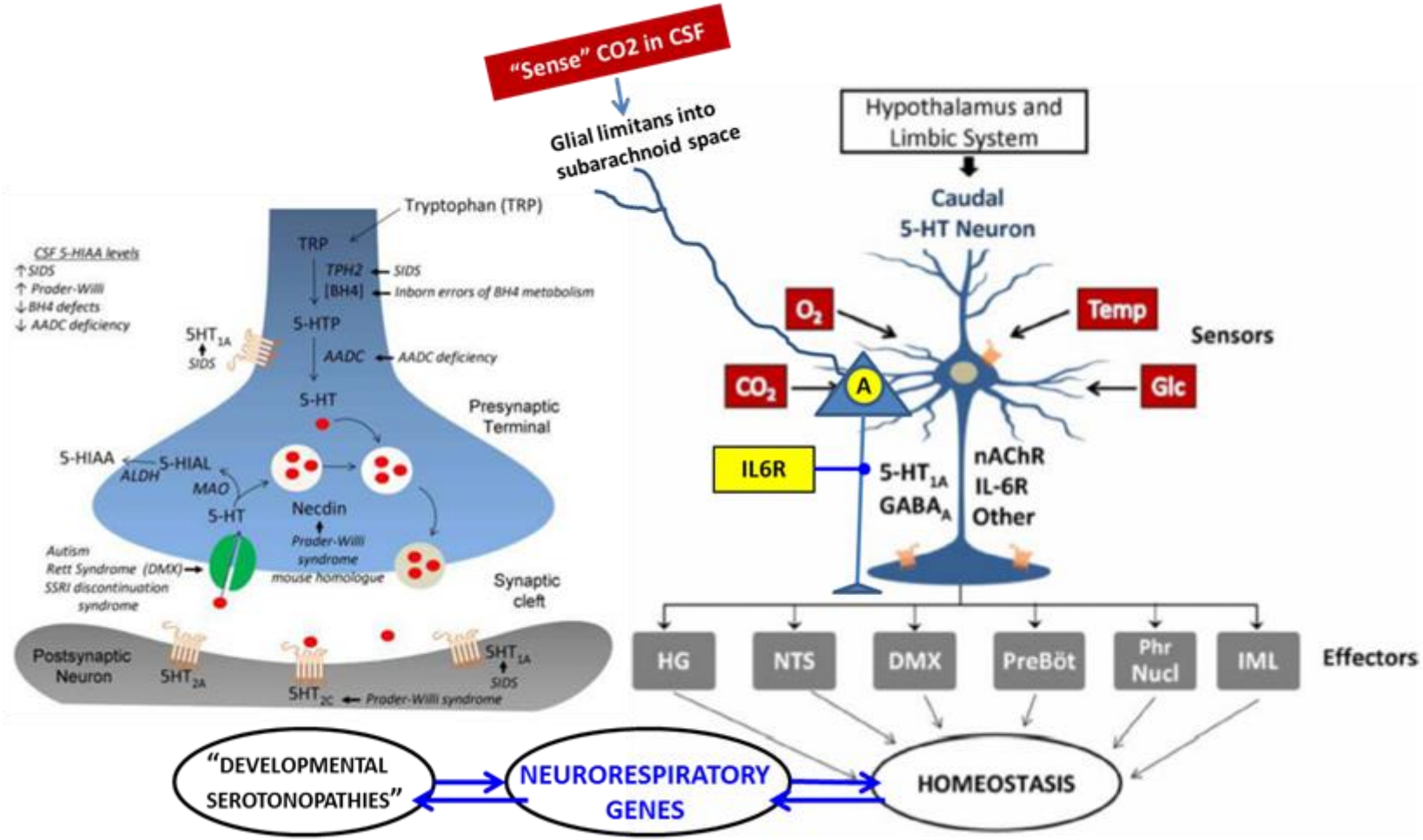

"Sense" CO2 in CSF
Glial limitans into subarachnoid space
Hypothalamus and Limbic System
Caudal 5-HT Neuron
Tryptophan (TRP)
TRP
TPH2
SIDS
[BH4]
Inborn errors of BH4 metabolism
5-HTP
AADC
AADC deficiency
5-HT
CSF 5-HIAA levels
↑ SIDS
↑ Prader-Willi
↓ BH4 defects
↓ AADC deficiency
5HT1A
SIDS
Presynaptic Terminal
5-HIAA
ALDH
5-HIAL
MAO
5-HT
Necdin
Prader-Willi syndrome mouse homologue
Autism
Rett Syndrome (DMX)
SSRI discontinuation syndrome
Synaptic cleft
Postsynaptic Neuron
5HT2A
5HT2C
Prader-Willi syndrome
5HT1A
SIDS
O2
Temp
Sensors
CO2
A
Glc
IL6R
5-HT1A
GABAA
nAChR
IL-6R
Other
HG
NTS
DMX
PreBöt
Phr Nucl
IML
Effectors
"DEVELOPMENTAL SEROTONOPATHIES"
NEURORESPIRATORY GENES
HOMEOSTASIS

Figure 16 *Neurochemistry mediating respiratory control. Schematic diagram of the caudal 5-HT system and its relationship to homeostatic regulation. This system: 1) receives sensory input about the internal milieu via the nucleus of the solitary tract (visceral sensory) in the autonomic nervous system, as well as its own chemo-and glucose receptors in close relationship to arteries and/or cerebrospinal fluid; 2) modulates adjustments to homeostatic stresses via its projections to the major medullary effector nuclei (HG, hypoglossal nucleus; NTS, nucleus of the solitary tract; DMX, dorsal motor nucleus of the vagus; preBöt, preBötzinger complex, Phr Nucl, phrenic nucleus in the cervical spinal cord; and IML, intermediolateral column in the thoracic spinal cord); 3) receives modulatory input itself from the hypothalamus other limbic forebrain sites relevant to sleep/wake cycle regulation and cardiorespiratory effects via receptor-mediated interactions with diverse neurotransmitters and neuromodulators; 4) interfaces with the cytokine system which is critical to homeostasis in its mediation of "protective" sickness behaviors and cellular defenses against tissue damage, and 5) the arcuate nucleus contains a large population of glutamatergic neurons and a smaller population of serotonergic neurons. It depolarizes in response to hypercapnia and its absence or hypoplasia in human infants has been associated with SIDS cases. Interleukin 6, an inflammatory mediator, inhibits the arcuate nucleus. Abbreviations: O2, oxygen, CO2, carbon dioxide; temp, temperature; glc, glucose; A, arcuate nucleus; IL6R, interleukin 6 receptor. Existence of neuro-cardiac-respiratory genes has been hypothesized [19 modified]*

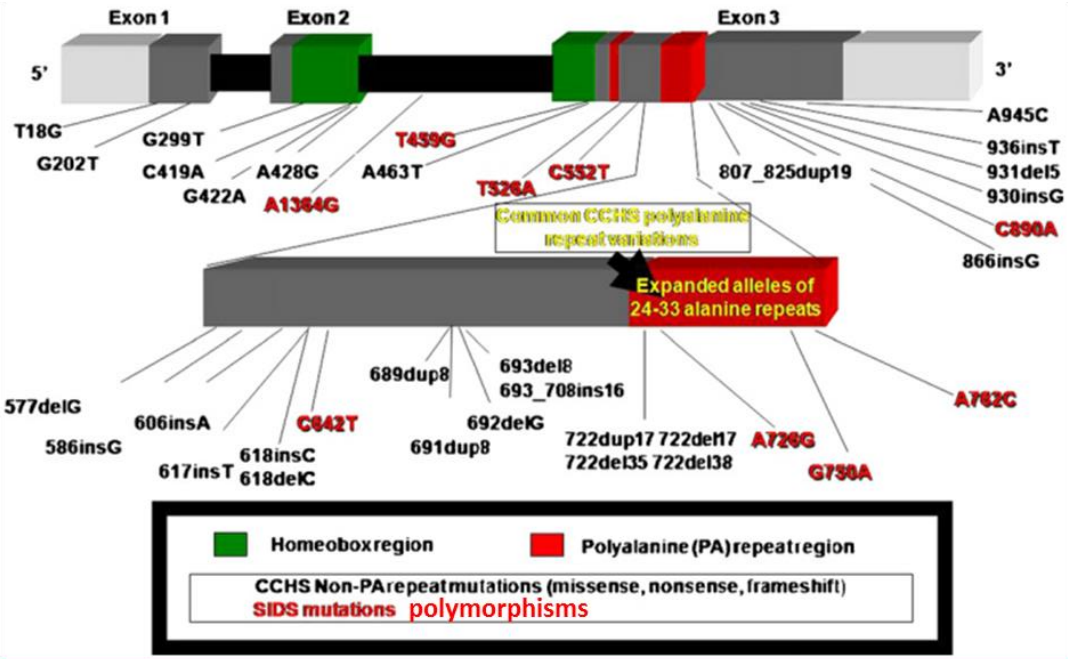
Disorders of the Caudal and Rostral 5-HT Domains
• Inborn errors of 5-HT metabolism
• Autism spectrum disorder (ASD)
• Rett Syndrome
• Fetal Alcohol Syndrome (FAS)
• Neonatal SSRI Discontinuation Syndrome
Disorders Mainly of the Caudal 5-HT Domain
• Prader-Willi Syndrome
• Sudden Infant death syndrome (SIDS)
Disorders Mainly of the Rostral 5-HT Domain
• Unknown

Table 4 *Human developmental serotonopathies with predominant involvement of the caudal, rostral, or both 5-HT domains [19]*

Genomic dysregulation

PHOX2B mutations in Congenital Central Hypoventilation Syndrome (CCHS) and SIDS

PHOX2B binds directly to the regulatory regions of the dopamine βhydroxylase



SIDS: Developmental Serotonopathies, Autonomic Nervous System (ANS) dysregulation, Necdin, MECP2, Nicotine metabolizing, Ion Channel Protein genes, Sarcomere genes

Figure 17 *PHOX2B mutations in CCHS and SIDS. Patients with CCHS have heterozygous mutations in the PHOX2B gene. CCHS-related mutations comprise polyalanine repeat expansion mutations in exon 3 (more than 90% of the cases) as well as non-polyalanine expansion mutations (missense, nonsense, and frameshift mutations) (less than 10% of the cases). SIDS mutations include an intron 2 polymorphism and 8 discrete mutations in exon 3. The SIDS mutations have not been identified in CCHS subjects. Nevertheless, it is estimated that a subset of CCHS cases with the least number of repeats might die in infancy and will be misidentified as SIDS [23 modified]*

Genetic testing is not currently routine at autopsy because the cost would be prohibitive. From 2008, the New York Office of the Chief Medical Examiner has screened autopsy-negative SUD (sudden unexplained death) cases for the six genes most frequently associated with a cardiac channelopathy (KCNQ1, KCNH2, SCN5A, KCNE1, KCNE2, and RyR2). The results from testing of 274 SUD cases revealed that 13.5% of infants and 19.5% of non-infants were positive for a total of 22 previously classified channelopathy-associated variants, along with 24 novel channelopathy variants. The SCN5A gene accounted for 68% of infant and 50% of non-infant positive results [27]. Whereas, researchers in Australia evaluated screening of autopsy-negative sudden arrhythmic death syndrome (SADS) and unexplained cardiac arrest (UCA). The targeted genetic testing of more than 100 SADS families had a diagnostic yield of 18%, while the yield of UCA families was 62%. The majority findings in both groups were LQTS and Brugada syndrome [28].

It is very interesting to note that a commonly proposed mode of death in SUDEP is cardiac arrhythmia. The inherited cardiac arrhythmias, such as LQTS and CPVT, are known to cause syncope, arrhythmias, and sudden unexplained death. Transgenic mice with a point mutation in the most common LQTS gene, KCNQ1, express cardiac arrhythmias and epileptic seizures, with sudden death recorded during electrocardiogram and electroencephalogram (EEG) monitoring. Respiratory dysfunction is another proposed factor that may contribute to SUDEP,

in fact, video EEG monitoring of inpatient patients with SUDEP showed that terminal seizures are followed by respiratory and cardiac anomalies with terminal apnea and cardiac arrest [29]. Therefore, serotonergic and respiratory systems are implicated in SUDEP and SIDS. However, no study, currently, has uncovered variants in genes or promoters involved with serotonergic signaling or respiratory function associated with SUDEP or SIDS [30]. It is likely that additional candidate genes for respiratory dysfunction exist, for example NOS1AP encoding the nitric oxide synthase 1 (neuronal) adaptor protein [29]. Nevertheless, a postulated mechanism for SUDEP is that seizures initiate pathogenic signalling between the brain and heart, inducing lethal cardiac arrhythmias.

Assumed that defects in membrane excitability can result in both epilepsy and cardiac arrhythmias, ion channels co-expressed in the brain and heart, but eventually in respiratory apparatus too, are leading candidates for SUDEP and SIDS.

Selected genes for SUDEP, where a link to SIDS has been supposed, include SCN1A, SCN1B, and DEPDC5 [31, 32, 29]. SCN1A and SCN1B are voltage-gated sodium channels; they are heteromeric proteins that function in the generation and propagation of action potentials in muscle and neuronal cells. Mutations in SCN1A can cause generalized epilepsy with febrile seizures plus (GEFS+) and Dravet syndrome. Mutations in SCN1B are associated with generalized epilepsy with febrile seizures plus, Brugada syndrome, and defects in cardiac conduction. DEPDC5 is linked to familial epilepsies such as familial temporal lobe epilepsy, epileptic spasms, familial focal epilepsy with variable foci, autosomal dominant nocturnal frontal lobe epilepsy, and cortical dysplasia. Pathogenic variants in DEPDC5 have been reported in SUDEP. DEPDC5, encodes the GATOR1 (GTPase-activating protein (GAP) activity toward RAGs) subcomplex 1, which is a critical regulator of the pathway that signals amino acid sufficiency to mTORC1 (protein synthesis regulator).

Recently, rare SCN4A variants that directly alter NaV1.4 function, have been detected in infants who had died from SIDS [33]. These variants are predicted to significantly alter muscle membrane excitability and compromise respiratory and laryngeal function. Variants in NaV1.4 that directly modify skeletal muscle

excitability can produce myotonia, periodic paralysis, congenital myopathy, and myasthenic syndrome. SCN4A variants have also been found in infants with life-threatening apnoea and laryngospasm. The presence of an SCN4A gene variant can impair sodium channel function and may exacerbate an infant's susceptibility in a period in which the developmental regulation of respiratory muscle fibre types and muscle sodium channel expression may define a critical period of vulnerability to respiratory stressors.

The putative pro-arrhythmic effect of SCN4A mutations is substantiated by the demonstration of Nav1.4 expression in human cardiomyocytes. Although that its tangible electrophysiological relevance remains to be defined, this result suggests a potential role of modifier genes in the determination of Brugada phenotype linked to SCN4A mutations. Nevertheless, rare variants with complete segregation in the SCN4A, were identified in SUDEP [34, 35].

In conclusion, several genes have been linked to SUDEP, SIDS and SCD, making them candidate genomic biomarkers. Since SUDEP involves some combination of abnormal brain, cardiac, and respiratory physiology, any gene with expression or phenotypes in these tissues could be a potential biomarker. Brain-heart dysfunction leading to SUDEP could arise from genetic variation in two main classes of neuro-cardiac genes based on their differing expression patterns: 1) genes with primarily neural expression that influence the heart extrinsically via the autonomic nervous system, and 2) genes with high levels of expression in both brain and heart that can impact cardiac function either intrinsically or extrinsically. 3) Neuro-respiratory genes associated with SIDS, could present some rare variations in SUDEP too.

For this reason, we intend named the emergent class of rare genetic variation markers involved in sudden death-channelopathies, as neuro-cardiac-respiratory genes (Fig. 16, 17, Tables 5, 6) [36, 37, 38, 39, 40].

Brain–heart expression patterns and associated phenotypes of neurocardiac genes linked with SUDEP. Richerson and Buchanan, 2011 ; Glasscock, 2013 (modified)

	Gene	Expression		Phenotypes		
		Brain	Heart	Epilepsy	Arrhythmias	SUDEP
"Brain"genes	*KCNA1*	h m	h m	h, m	m	m
Brain stem?	*SCN1A*★			h, m	m	h, m
	SCN8A			h, m	m	h
"Respiratory" genes	*HCN2*			h, m	m	h
	PRRT2			h		h
"Heart"genes	*KCNQ1*			m	h, m	m
Brain stem?	*KCNH2*★			h	h	h
	SCN5A	SIDS		h	h, m	h
"Respiratory" genes	*RYR2*			h, m	h, m	h

Table 5 *The circles represent gene expression levels in humans (h) and mice (m) based on expressed sequence tags (ESTs) from brain and heart cDNA pools as reported by the UniGene database maintained by the National Center for Biotechnology Information (NCBI). The darkness of the fill is proportional to the expression level. The association of each gene with epilepsy, cardiac arrhythmias, or SUDEP in human patients or mouse models is showed by an h or m, respectively. Existence of neuro-cardiac-respiratory genes has been hypothesized [36 modified]*

	Clinical disorders	Gene product or function	Expression sites (RNAseq)	Potential SUDEP mechanisms
Primary epilepsy or brain gene				
SCN1A[8]	Dravet syndrome or epileptic encephalopathy, generalised epilepsy with febrile seizures plus	Sodium channel $Na_v1.1$	Brain, heart, lung	Postictal parasympathetic hyperactivity; increased epilepsy severity
SCN2A	Epileptic encephalopathy	Sodium channel $Na_v1.2$	Brain, heart, lung	Increased epilepsy severity[36]
SCN8A[41]	Epileptic encephalopathy	Sodium channel $Na_v1.6$	Brain, heart, lung	Increased epilepsy severity[42]
PRRT2[43]	Benign familial infantile seizures	Proline-rich transmembrane protein 2	Brain	Potentially via interaction with SNAP-25, involved in presynaptic neurotransmitter release
DEPDC5[44,45]	Focal epilepsy (broad spectrum of phenotypes)	G-protein signalling pathway; component of the GATOR1 complex, which inhibits the mTORC1 pathway	Brain, heart, lung	Uncertain; potentially increased epilepsy severity
CSTB	Unverricht-Lundborg disease	A stefin that inhibits intracellular thiol protease; might prevent protease leakage from lysosomes	Brain, heart, lung	Increased epilepsy severity neurological impairment due to progressive myoclonic epilepsy
TSC2, *TSC1*	Tuberous sclerosis complex	Hamartin (TSC1), tuberin (TSC2); downregulate the mTORC1 pathway	Brain, heart, lung	Potentially increased epilepsy severity
HCN2[43]	Generalised epilepsy	Hyperpolarisation activated cyclic nucleotide gated potassium channel 2; contributes to spontaneous rhythmic activity in heart (sinoatrial node) and brain	Brain, heart, lung	Unknown; potential impairment of brainstem or cardiac pacemaker cells
Primary cardiac genes				
KCNQ1[42]	Long QT syndrome	Potassium channel KvLQT1/$K_v7.1$; ventricular repolarisation	Brain, heart, lung	No definite SUDEP cases; potential arrhythmogenic effect
KCNH2[42,43]	Long QT syndrome	Potassium channel hERG1/$K_v11.1$; repolarisation of cardiac action potential	Brain, heart, lung	Unknown[42]
SCN5A[42,43]	Long QT syndrome	Sodium channel, $Na_v1.5$; rapid depolarising sodium current underlying cardiac action potential upstroke	Brain, heart, lung	Identified in SUDEP cases; potentially combined epilepsy and arrhythmia
NOS1AP[42]	Long QT syndrome	Cytosolic protein that binds to neuronal nitric oxide synthase	Brain, heart, lung	Identified in SUDEP case; potentially combined epilepsy and arrhythmia[42]
RYR2[42,43]	Sudden cardiac death	Cardiac ryanodine receptor 2, acts as intracellular calcium release channel, coupling excitation–contraction	Brain, heart, lung	Identified in SUDEP case; potentially combined epilepsy and arrhythmia[43]
HCN4[42,43]	Bradycardia; sick sinus syndrome	Hyperpolarisation activated cyclic nucleotide gated potassium channel 4; slow kinetics of activation and inactivation, cardiac pacemaker role	Brain, heart, lung	Variant identified in SUDEP case
Genetic disorders*				
Dup15q11[8]	Epileptic encephalopathy; variable epilepsy phenotype	Supernumerary isodicentric chromosome 15; extra copies of *UBE3A* (Angelman syndrome) and *GABRB3* (GABA receptor)	*UBE3A* and *GABRB3*: brain, heart, and lung	Increased epilepsy severity; neurological impairment
5q14.3 deletion[46]	Variable severity of epilepsy and neurodevelopmental disability	Haploinsufficiency of *MEF2C* (role in myogenesis) and *EFNA5*, receptor protein-tyrosine kinases involved in neurodevelopment	*MEF2C* and *EFNA5*: brain, heart, and lung	Increased epilepsy severity; neurological impairment

SUDEP=sudden unexpected death in epilepsy. *No animal models exist for these genetic disorders; with most genes, it is uncertain whether the risk of SUDEP is solely accounted for by the severity of epilepsy and developmental delays.

1.6 Blocks and microcircuits of the Brainstem cardio-respiratory network

An emerging concept is that each of the cardio-respiratory brainstem regions has specialized roles in controlling breathing and heartbeat. Smith et al., 2013 refers to each of these regions as a "microcircuit" that is burst by cellular properties, synaptic and intrinsic membrane properties that generate a specific function of cardiorespiratory control (Fig. 18) [8, 41, 42].

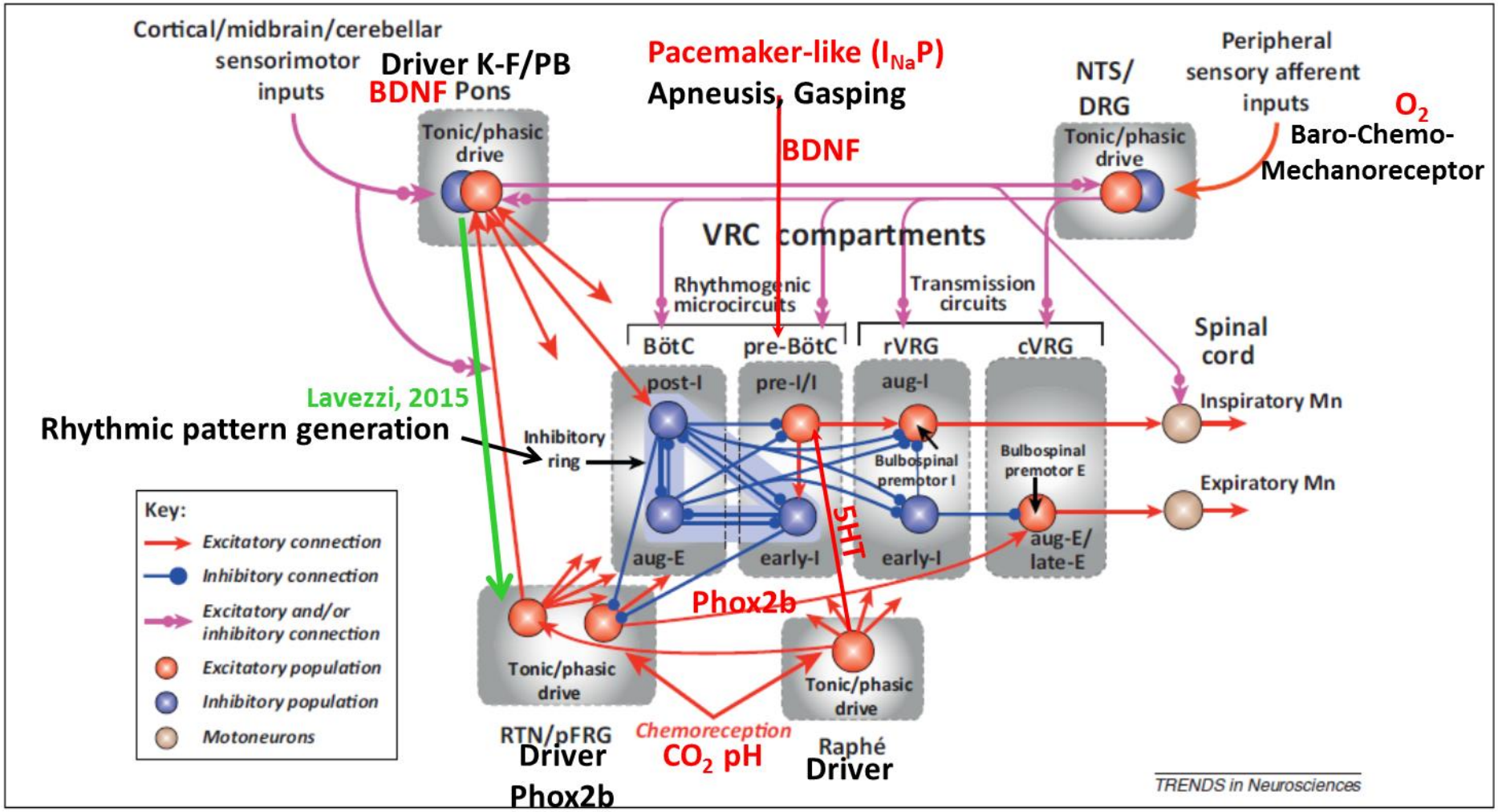

Cortical/midbrain/cerebellar
sensorimotor inputs
Driver K-F/PB
BDNF Pons
Pacemaker-like ($I_{Na}P$)
Apneusis, Gasping
BDNF
NTS/
DRG
Peripheral
sensory afferent
inputs
O_2
Baro-Chemo-
Mechanoreceptor
Tonic/phasic drive
Tonic/phasic drive
VRC compartments
Rhythmogenic microcircuits
Transmission circuits
BötC
pre-BötC
rVRG
cVRG
Spinal cord
post-I
pre-I/I
aug-I
Inspiratory Mn
Lavezzi, 2015
Rhythmic pattern generation
Inhibitory ring
Bulbospinal premotor I
Bulbospinal premotor E
Expiratory Mn
5HT
aug-E
early-I
early-I
aug-E/
late-E
Phox2b
Tonic/phasic drive
Tonic/phasic drive
Key:
Excitatory connection
Inhibitory connection
Excitatory and/or inhibitory connection
Excitatory population
Inhibitory population
Motoneurons
RTN/pFRG
Driver
Phox2b
Chemoreception
CO_2 pH
Raphé
Driver
TRENDS in Neurosciences

Figure 18 *Schematic representation of compartmentalized microcircuits in the brainstem assumed to be involved in respiratory rhythm and pattern generation. Individually populations (shown as spheres) consisted of 20–50 single-compartment neurons described in the Hodgkin–Huxley style. Each side of the brainstem contains these multiplexes with extensive bilateral circuit interconnections (not represented). Pre-Bötzinger complex (pre-BötC) and Bötzinger complex (BötC) are the major components of the ventral respiratory column (VRC) with rhythmogenic microcircuits generating multiple respiratory patterns. These circuits include the excitatory glutamatergic (red) pre-inspiratory-inspiratory (pre-I/I) population in pre-BötC and three inhibitory GABAergic or glycinergic (blue) populations within the pre-BötC–BötC that are hypothesized to mutually inhibit each other [inhibitory ring of early-I, post-I, and augmenting expiratory (aug-E) neurons]. BötC expiratory neurons are a major source of synaptic inhibition of inspiratory neurons during expiration. The pre-I/I excitatory neurons of the pre-BötC, which is the excitatory core of the respiratory network, project via premotor circuits to cranial motoneurons (e.g., hypoglossal and vagus, respectively for the tongue and pharynx muscles) and to rostral ventral respiratory group (rVRG) excitatory [augmenting inspiratory (aug-I)] bulbospinal premotor neurons with projections to spinal cord inspiratory (phrenic) motoneurons (Mn, brown). The excitatory bulbospinal expiratory neurons of the caudal VRG (cVRG) project to thoracic and abdominal spinal expiratory motoneurons. According to the circuit model, neurons within these compartments receive tonic, phasic, or rhythmic excitatory drive from the pontine, dorsal respiratory group (DRG), retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG), and raphe compartments. Drives from the second two compartments are regulated in part by blood or brain CO_2 levels (chemoreception), which may involve modulatory connections from the raphe to RTN/pFRG as represented. Phox2b gene mutation deletes neurons expressing this transcription factor in the RTN/pFRG. This decreases phrenic nerve activity, modifies respiratory frequency, and reduces system responses to CO^2. Recently preBötC rhythmogenic neurons have been found to produce, and be excited by, brain-derived neurotrophic factor [42]. It is believed that trophic factors from*

peripheral arterial chemoreceptors acting on central mechanisms that control breathing during early postnatal development are the key to stable rhythmogenesis throughout life [8, 11, 41, 42 modified].

A computational model of the spatially distributed brainstem respiratory network has been developed to reproduce experimental findings and explanations regarding to the rhythm-generating mechanism with sequential reproduction of the network (Fig. 18) [41]. The model includes the pons and the major medullary compartments. For simplicity, some respiratory neuron types, (e.g., post-I, aug-E) have not been localized in particular compartments (for example PiCo), but rather distributed throughout the VRC.

Among the microcircuits that have been identified, the primary networks are the many time mentioned before: the PBC which controls inspiration, the postinspiratory complex (PiCo) which controls postinspiratory activity, and a subset of the parafacial respiratory group (lateral parafacial, pFL) controlling active expiration (Fig. 12, 13) [13, 14, 43].

Other brainstem microcircuits are specialized to control heartbeat; they include the NAmb, a nucleus that contains cardiac vagal neurons and exerts parasympathetic control of the heart, and the RTN, containing Phox-2B neurons, which have a robust influence on sympathetic control of the heart (e.g. indirectly on KFN). The RTN neurons are critical for sensing CO_2 too, however, a second CO_2 chemo-sensible area is the raphe nucleus, which contains GABAergic and serotonergic neurons. The NTS, in the dorsal medulla, collects important peripheral sensory information from the carotid bodies and aortic bodies, which are very sensitive to changes in blood oxygen levels and CO_2, via glossopharyngeal nerve and the vagus nerve, respectively. Mechanoreceptors are located in the airways and lung parenchyma, and are responsible for a variety of reflex responses (Fig. 18). These include the Hering-Breuer reflex that terminates inspiration to prevent over inflation of the lungs. Latest discoveries suggest that the neurons of the NTS are crucial for the processing and coordination of respiratory and sympathetic responses to hypoxia.

Moreover, various noradrenergic nuclei, such as the C1 region, are critical for the control of arousal and the sleep-wake cycle so, a functional cardiorespiratory control requires the tight and operative co-ordination between these important lower brainstem microcircuits and the upper diencephalic-telencephalic stations. In fact, there are many additional important microcircuits that also present fundamental activities in the homeostatic regulation of breathing and the heart. These can be found not only in the medulla and the pons, but also in the cerebellum, neocortex, hippocampus, amygdala, the hypothalamus, and the PAG. Each of these areas has specific roles in the control of breathing and heart rate and in particular have been implicated in SIDS as well as in other sudden unexplained death events.

The best-understood microcircuit controlling breathing is the PBC, a precise brainstem region known to be critical for the generation of inspiration (Fig. 12, 13, 18). Lesioning of this microcircuit leads to the cessation of breathing. Indeed, since 1976, pathological abnormalities in the form of astrogliosis have been described in SIDS victims in PBC [44]. However, the PBC also contains inhibitory neurons, which are essential not only for the generation of inspiration, but also for the afferent control of the PBC, that reconfigures itself into different states. In fact, under normal baseline conditions, the PBC contributes to the generation of normal breathing (also mentioned as eupnea). Nevertheless, the PBC also spontaneously generates sighs both in babies and adults, but not as frequently as infants. The majority of neurons in this microcircuit are activated during both eupneic and sigh activity, however what seems to drive these differences are cellular mechanisms that differentially control sighs versus eupneic activity. It has, for example, been demonstrated that sighs are finely sensitive to a specific calcium channel subtype (P/Q-type channel) that is critical for glutamatergic, i.e. excitatory, synaptic transmission [45]. Mutation of this particular channel subtype in an animal model does not affect normal breathing, but abolishes the ability to sigh and besides, these animals ultimately die. This last event is interesting in the context of SIDS. Eupnoeic and sigh activities are differentially modulated by neuromodulators during sleep. Acetylcholine acting

on muscarinic receptors activates sighs but inhibits eupneic activity; Serotonin and substance P, which have both been implicated in SIDS, are sigh activating.

The PBC also remodels itself in response to hypoxia; this microcircuit responds stereotypically to reduced oxygen levels with an early increase and the generation of sighs, followed by a secondary depression and the generation of gasps. Much has been learned about the neurons involved in the generation of the gasps in the PBC and its underlying cellular mechanisms (fig. 19, 20, 21) [46, 47].

Arousal and autoresuscitation follow stereotypical patterns that defend infants sleeping in the prone position from SIDS. In the course of prone sleeping, re-breathing breathe out air can increase CO_2 and decrease O_2 levels. The anomalous blood gas composition initiates the arousal response that begins with sigh generation during sustained eupneic breathing. Efficacious arousal results in head lifting and repositioning which restores the O_2 supply. If arousal fails, a more severe hypoxic state is obtained and eupneic breathing will shift to gasping. This transition is mediated by network reconfiguration of the PBC. Gasping is an autoresuscitation response that can lead to arousal and a return to normal blood gas levels. Should an infant fail to both arouse and autoresuscitate, the irreversible hypoxic insult leads to asphyxiation and the occurrence of SIDS. Brainstem anomalies, however, may alter PBC network and impair sigh and gasp generation which may increase SIDS vulnerability. Noteworthy it is that also the entity SUDEP usually occurs during sleep as an unwitnessed event and young individuals are mostly found in the prone position.

Gender, genetic polymorphisms, prenatal nicotine exposure, inflammation and temperature can increase this susceptibility because of direct altered function of the PBC on the ability of this microcircuit to generate sigh and gasping rhythms, as well as coordinate cardiorespiratory coupling between the PBC and NAmb.

High IL-6 levels in CSF, produced in a simple upper airway infection, in vulnerable infants may initiate an immune reaction in the laryngeal and tracheal mucosa, resulting in a fatal IL-6 liberation within the CNS. IL-6 receptors on serotonergic neurons in the arcuate nucleus in the brainstem of SIDS cases, could be involved in the modulation of breathing, blood pressure and heart rate according to the level of arousal. One reason for such vulnerability may be certain

patterns of polymorphisms in genes encoding components in the immune system. The expression of IL-6 receptors (IL-6R) and gp130 (involved in IL-6R signaling) in the brainstem have been analysed in SIDS. The mean IL-6R intensity grade in the arcuate nucleus was significantly higher in the SIDS group than in the controls and a positive correlation between IL-6 CSF levels and immunostaining of gp130 have been detected in the arcuate nucleus in SIDS. This, together with the studies finding elevated levels of ILs in the CSF, indicates a connection between the mucosal immune system and dysfunction of the serotonergic network in the brainstem.

Also high neuronal IL-1β immunoreactivity has been found in the brainstem in SIDS, with a region-specific pattern of cytokine expression in SIDS brains in comparison to non-SIDS brains. An overexpression of IL-1β in the brainstem can determinate a disturbed homeostatic control of cardiorespiratory and arousal pathway, possibly leading to SIDS. An overexpression of IL-2 in cardiorespiratory brainstem nuclei have been detected in SIDS, further suggesting that there is a neuro-cytokine connection.

During infection, peripherally cytokines may cross the blood-brain barrier, bind to cytokine receptors on neurons that determine stress responses in the hypothalamus and/or brainstem, and thereby determine sickness behaviors, including blunted arousal and depressed respiration. In an infant with an underlying vulnerability in the brainstem, as reported in SIDS, a mild infection that initiates a cytokine cascade might trigger sudden death [48].

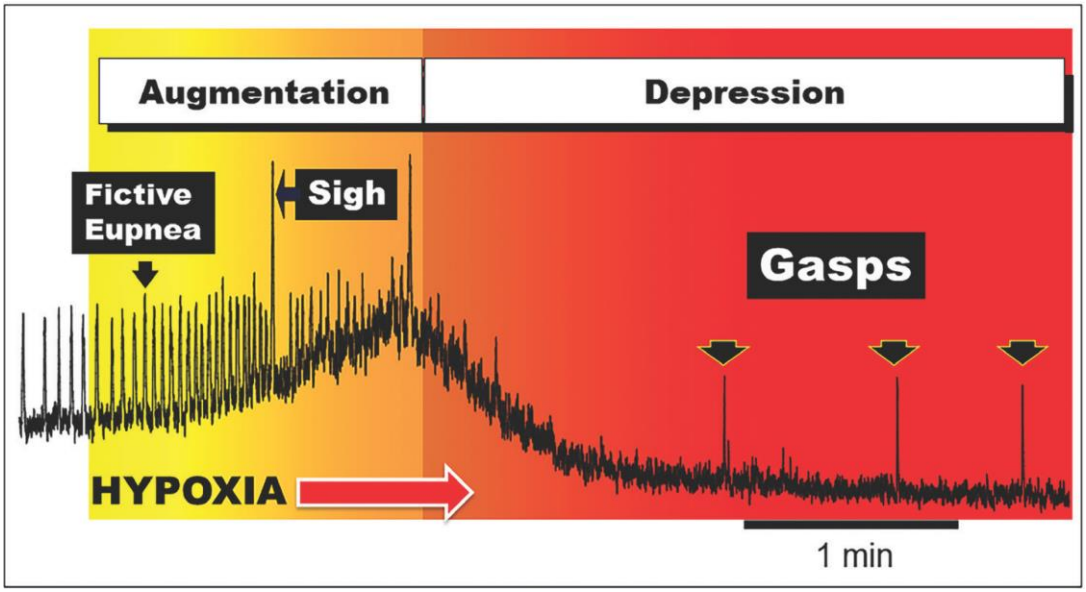


Figure 19 *The isolated PBC network reconfigures in response to hypoxia. During normal oxygenation, the PBC autonomously generates a rhythmic, fictive eupneic pattern of activity. When exposed to hypoxia, the network responds by initiating an augmentation period typified by an increase in eupneic burst frequency and the generation of sighs. This period is followed by a secondary depressive phase in which gasps are generated. This response pattern of the PBC is thought to be the neuronal correlate to the stereotypical hypoxic response observed in humans [46]*

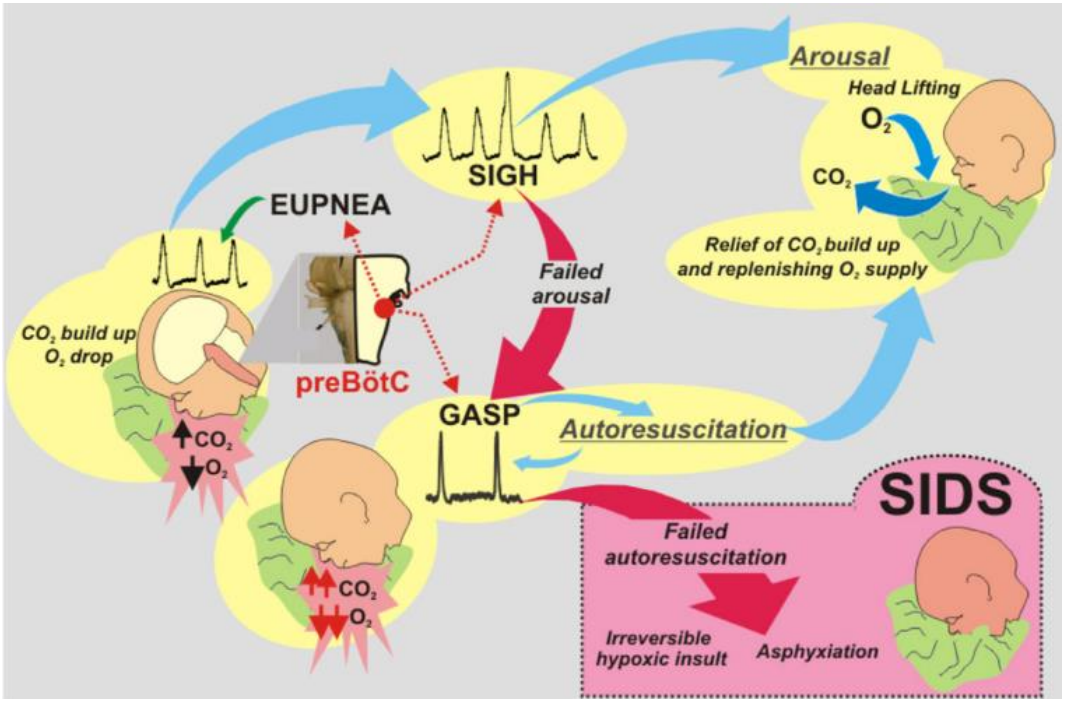


Figure 20 *Arousal and autoresuscitation follow stereotypical patterns that defend infants sleeping in the prone position from SIDS. In the course of prone sleeping, re-breathing breathe out air can increase CO_2 and decrease O_2 levels. The anomalous blood gas composition initiates the arousal response that begins with sigh generation during sustained eupneic breathing. Efficacious arousal results in head lifting and repositioning which restores the O_2 supply. If arousal fails, a more severe hypoxic state is obtained and eupneic breathing will shift to gasping. This transition is mediated by network reconfiguration of the PBC. Gasping is an autoresuscitation response that can lead to arousal and a return to normal blood gas levels. Should an infant fail to both arouse and autoresuscitate, the irreversible hypoxic insult leads to asphyxiation and the occurrence of SIDS. Brainstem anomalies, however, may alter PBC network and impair sigh and gasp generation which may increase SIDS vulnerability [47]*

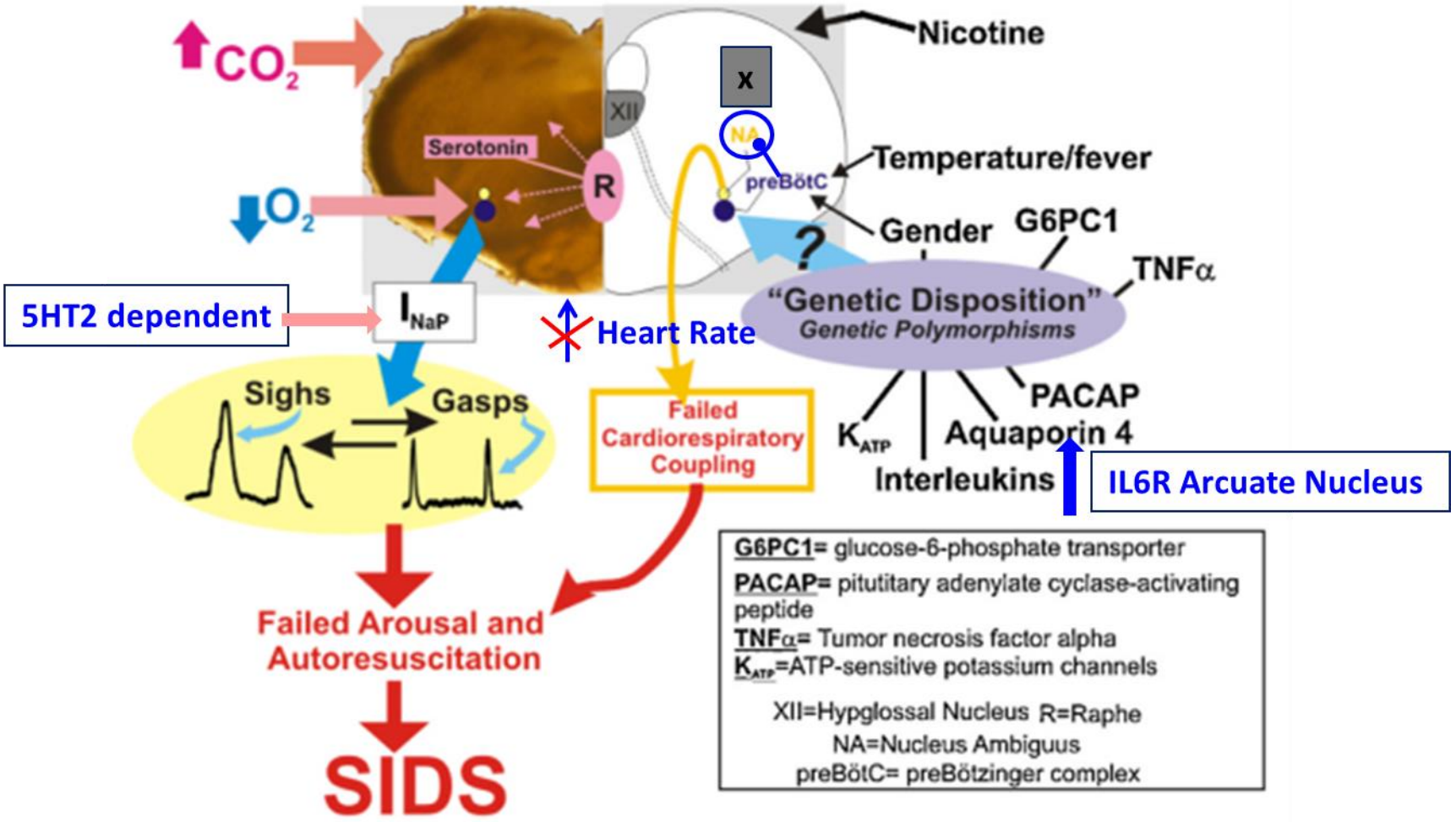

CO_2
O_2
Serotonin
XII
R
X
NA
preBötC
Nicotine
Temperature/fever
Gender
G6PC1
TNFα
?
"Genetic Disposition"
Genetic Polymorphisms
PACAP
Aquaporin 4
K_{ATP}
Interleukins
IL6R Arcuate Nucleus
5HT2 dependent
I_{NaP}
Heart Rate
Sighs
Gasps
Failed Cardiorespiratory Coupling
Failed Arousal and Autoresuscitation
SIDS
G6PC1= glucose-6-phosphate transporter
PACAP= pitutitary adenylate cyclase-activating peptide
TNFα= Tumor necrosis factor alpha
K_{ATP}=ATP-sensitive potassium channels
XII=Hypglossal Nucleus R=Raphe
NA=Nucleus Ambiguus
preBötC= preBötzinger complex

Figure 21 *The final common pathway for SIDS consists of a failure to arouse and autoresuscitate in response to environmental challenge. Gender, genetic polymorphisms, prenatal nicotine exposure, inflammation and temperature can increase the SIDS susceptibility. These risk factors can directly alter the function of the PBC and impair the ability of this network to generate sigh and gasping rhythms, as well as coordinate cardiorespiratory coupling between the PBC and NAmb. Thus, the function of the PBC appears to be an important physiological determinant for SIDS [25, 47 modified]*

In the "biphasic response" to hypoxia, there is close coordination between neuronal circuits controlling the heart and breathing. In fact, during hypoxia, there is an initial increase in both the heart rate and respiratory rate. During this initial "augmentation phase" there is also the generation of sighs, which cause further transient increases in heart rate. The augmentation phase is followed by a depression phase during which respiration and heart rate decrease. The general heart rate decrease (bradycardia) is interrupted by transient periods of tachycardia that coincides with the generation of gasps. Mechanistically, it is hypothesized that this cardiorespiratory coupling is mediated through an interaction between the PBC, the microcircuit controlling inspiration, and the anatomically proximate NAmb, a nucleus that contains the cardiac vagal neurons that generate the parasympathetic control of the heart rate. Indeed, these cardiovagal neurons are located at the same level of the NAmb as the PBC. It is hypothesized that during each inspiration, inhibitory inspiratory neurons within the PBC inhibit cardiac vagal neurons in the NAmb (Fig. 21), which results in the disinhibition at the level of the heart, thus leading to an inspiratory-related heart rate increase. Any disturbance in this core interaction between the respiratory and the cardiac system will result in dysautonomia. Arousal is directly linked to the change in heart rate occurring during a sigh and gasp, therefore, a vulnerable infant is likely characterized by a disturbance of this core circuitry. One possible consequence is that in these infants cardiovagal neurons are not as excitable, which would lead to

an increased heart rate and decreased cardiorespiratory coupling, all typical signs of dysautonomia.

The cellular building blocks of breathing presents a distinct phase named “the postinspiratory complex” or PiCo, that occurs just after an inspiration. It constitutes a brake on the passive release of expiratory airflow and protects the larynx and upper airways from aspirating particulate matter and fluid. During the postinspiratory phase, laryngeal adductor muscles in the neck are activated and are involved in multiple non-ventilatory behaviors including swallowing, vocalization, and coughing. These behaviors must be tightly coordinated with breathing to prevent aspiration. Stimulating sensory laryngeal receptors activates a laryngeal adductor reflex comprising of a prolonged postinspiratory apnea, a postinspiratory vagal motor output, which innervates the larynx and a dramatic decrease in heart rate. While this is normally cardioprotective, in vulnerable individuals exaggeration of the laryngeal adductor reflex can induce a fatal apnea due to prolonged glottal closure. This has been proposed as a possible cause of death for SIDS victims. PiCo, a medullary population rostral to the PBC has been considered as an autonomous oscillator thought to control postinspiration. It is also in close proximity to the NAmb and has similar rhythm-generating characteristics to the PBC.

In conclusion, pathophysiological changes in the cardiorespiratory response to exogenous stressors such as hypoxia or hypercapnia are associated with alterations in brainstem anatomy and microcircuit functionality.

1.7 Cardiorespiratory-Cardioventilatory coupling

Brainstem is the core of the cardiovascular and respiratory control. From the early 1900 to the late 1970, it has been considered that cardiac and respiratory centres (i.e. pneumotaxic and apneustic, cardio accelerator and cardio inhibitor centres) were autonomous and independent, although related, entities. By Cohen in 1979, a unified theory of cardiorespiratory centres of the brainstem has been postulated [49]. Cardiorespiratory centres belong to a single integrated network of nuclei, named "Connectome", that have been recognized by means of tractography with Diffusion Tensor Magnetic Resonance Imaging (Fig. 22, 23, 24) [50, 51, 52].

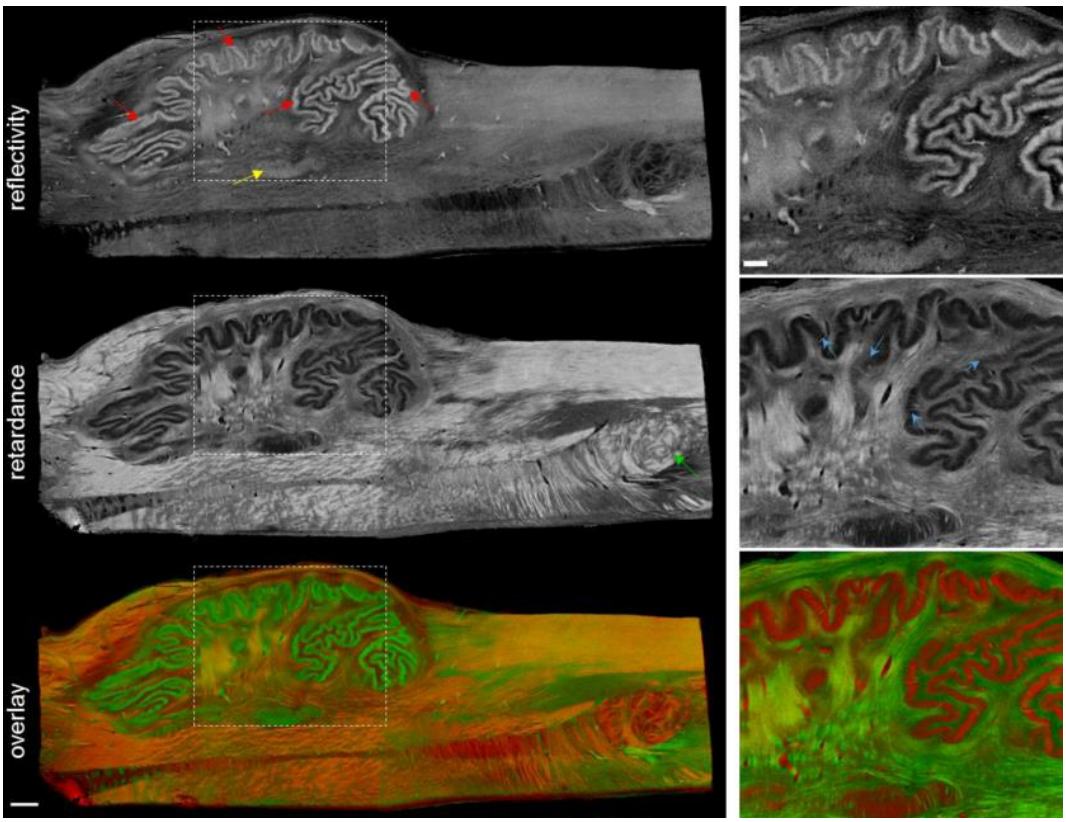


Figure 22 *Fiber tracts and nuclei of human medulla oblongata by diffusion tensor imaging (DTI) [50]*

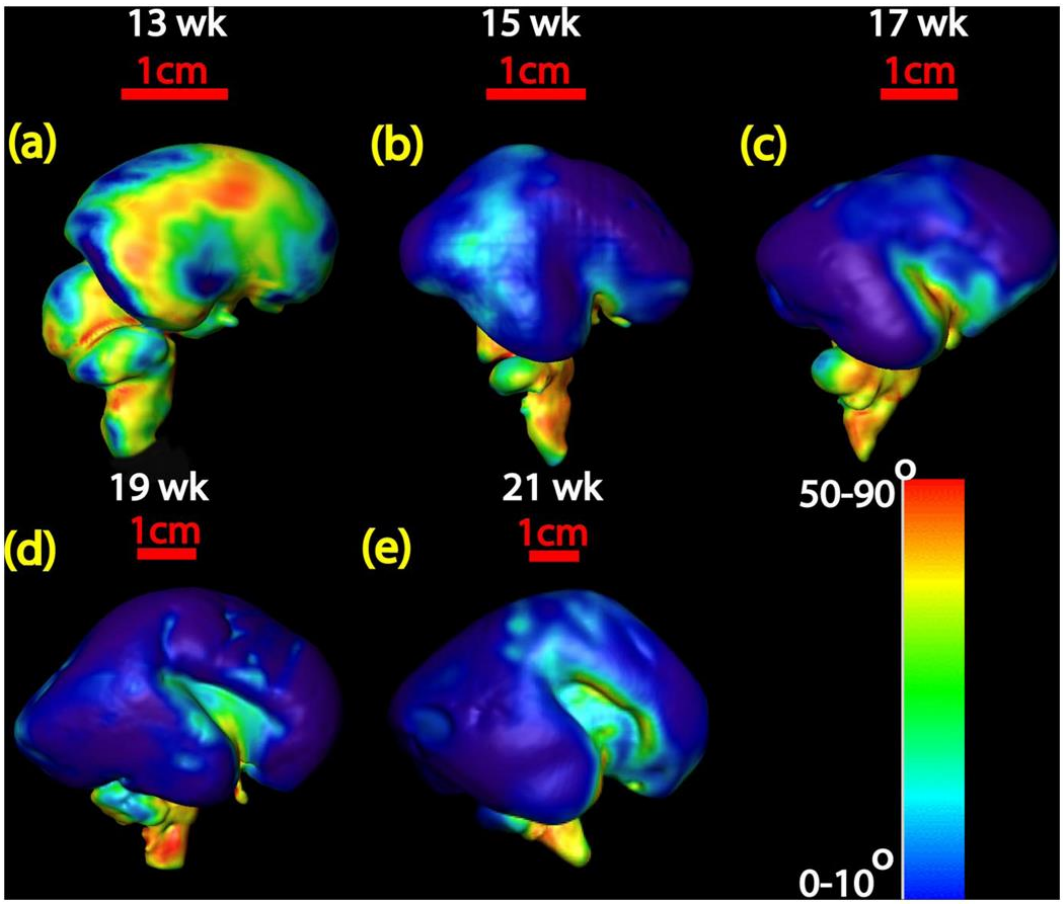


Figure 23 *Anatomical characterization of human fetal brain development with diffusion tensor magnetic resonance imaging [51]*

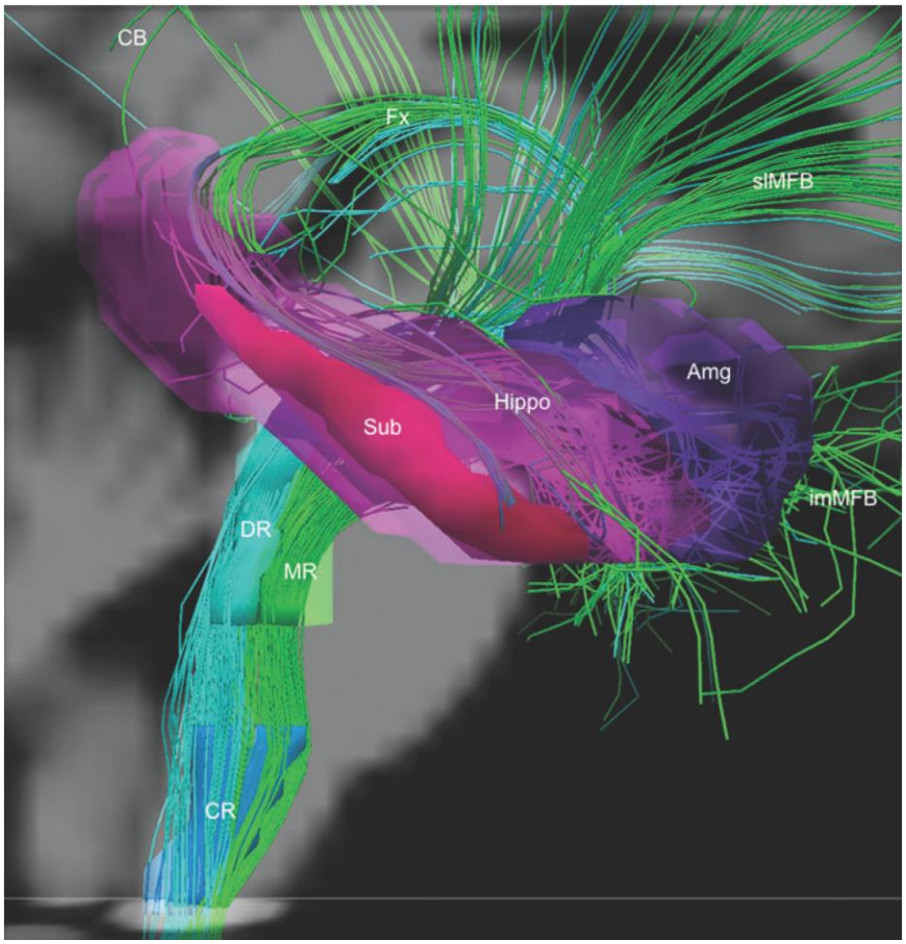


Figure 24 *The Structural Connectome of the Human Central Homeostatic network [52]*

Cardiac, sympathetic, and respiratory motor activities can be viewed as a unified rhythm controlled by brainstem neural circuits for effective and efficient gas exchange. The final aim of this neuro- cardiovascular- respiratory system consists in oxygen releasing and carbon dioxide capturing to/ from tissues. Specifically, cardiorespiratory and cardioventilatory coupling can be understood as a unified vital rhythm organized by brainstem neural circuits to maintain and rationalize this equilibrium, even if both couplings have only a small effect on the efficiency of gas exchange. Rather, the cardiorespiratory and cardioventilatory control system may act as weakly coupled oscillators to maintain rhythms within a bounded variability and to preserve the Energy Conservation [53].

By cardiorespiratory coupling, we mean the Respiratory Sinus Arrhythmia (RSA) that is characterized by a heart rate (HR) increasing during inspiration and a HR decreasins during expiration. Conversely, CardioVentilatory Coupling (CVC) is considered the influence of heartbeats and arterial pulse pressure on respiration with tendency for the next inspiration to start at a preferred latency after the last heart beat in expiration. Nevertheless, CVC is feeble and becomes apparent during quiet sleep and anesthesia.

The cardiorespiratory coupling functionally acts by reflex arc with cardiac vagal neurons in the NAmb and the NTS, located in the dorsal medulla; it helps to coordinate respiratory and sympathetic responses to hypoxia (Fig. 9, 25) [53].

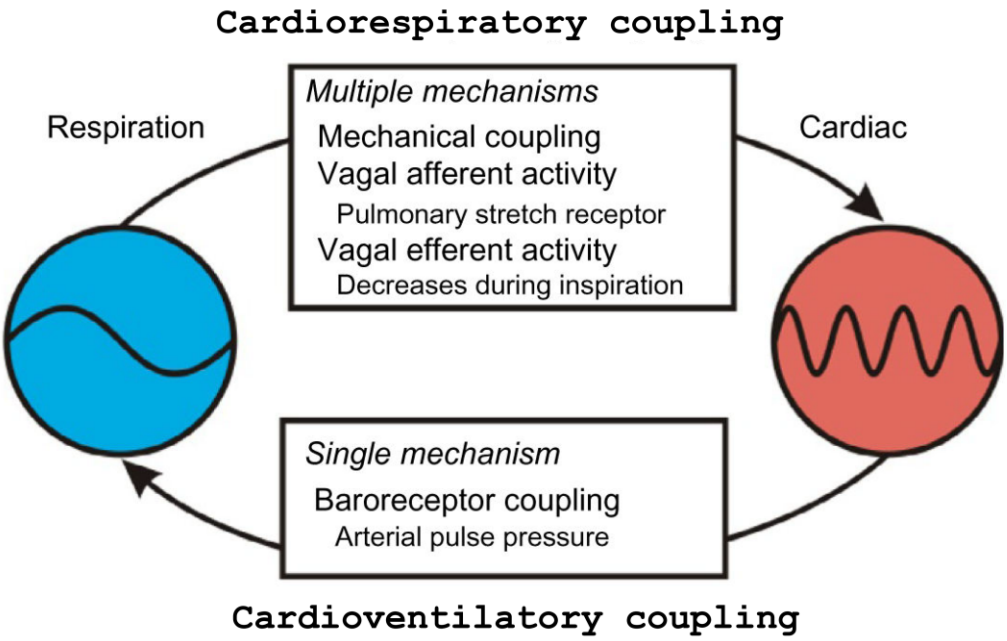


Figure 25 *Schematic of cardiorespiratory and cardioventilatory coupling. Each limit cycle (blue (left), respiratory and red (right), cardiac) represents the reciprocal coupling scheme between respiration and autonomic, cardiac rhythms. Here the cardiac: respiratory rhythm is 4:1 entrainment, symbolized as harmonics. There are multiple mechanisms mediating the respiratory influence on the cardiac cycle, while a single mechanism, through baroreceptors, mediates the influence of the cardiac/sympathetic activity onthe respiratory cycle [53 modified]*

In 1733 Rev. Stephen Hales reported that respiration modulates HR and BP. This observation was confirmed by Carl Ludwig (1847) who measured the increases in HR and BP during inspiration [54]. The increase in HR during inspiration is mentioned as respiratory sinus arrhythmia (RSA) and the increase in BP as Traube–Hering waves. Heart rate and BP are regulated neurally, and both parasympathetic and sympathetic nerves have respiratory-modulated activity patterns. Multiple factors, including mechanical coupling, underlie the increases in HR and BP. Mechanical coupling in the cardiorespiratory system is due to the location of the lungs and heart in the thoracic cavity. Inspiration relies on a decrease in thoracic pleural pressure which draws blood to the heart, increases venous return and, increases HR and cardiac output by mechanoreceptor stimulation. Respiratory sinus arrhythmia is due to a combination of three factors (Fig. 26) [53, 54, 55, 56]: (1) the direct influence of central respiratory neurones on cardiac neurones, (2) an indirect effect on heart rate of changes in blood pressure and (3) a reflex cardiac response to mechanical inflation of the lung chronic intermittent hypoxia (CIH). Since stimulation of pulmonary afferents also activates post-I neurons via P cells of NTS [55], these cells may be involved in both Hering–Breuer and barosympathetic reflexes.

In animal models, acute intermittent hypoxia evoks a progressive increase in SNA and phrenic nerve activity (PNA) that persisted for at least 60 min following the last hypoxic exposure. The recruitment of activity after acute intermittent hypoxia and in the presence of maintained normoxic and eucapnic blood gas levels is referred to as long-term facilitation and occurs during CRC. Methysergide, a serotonergic (5HT2) receptor antagonist, blocks the development of sSNA (and PNA) long-term facilitation [53], so it has been hypothesized that serotonergic patway influences the CRC (Fig. 26).

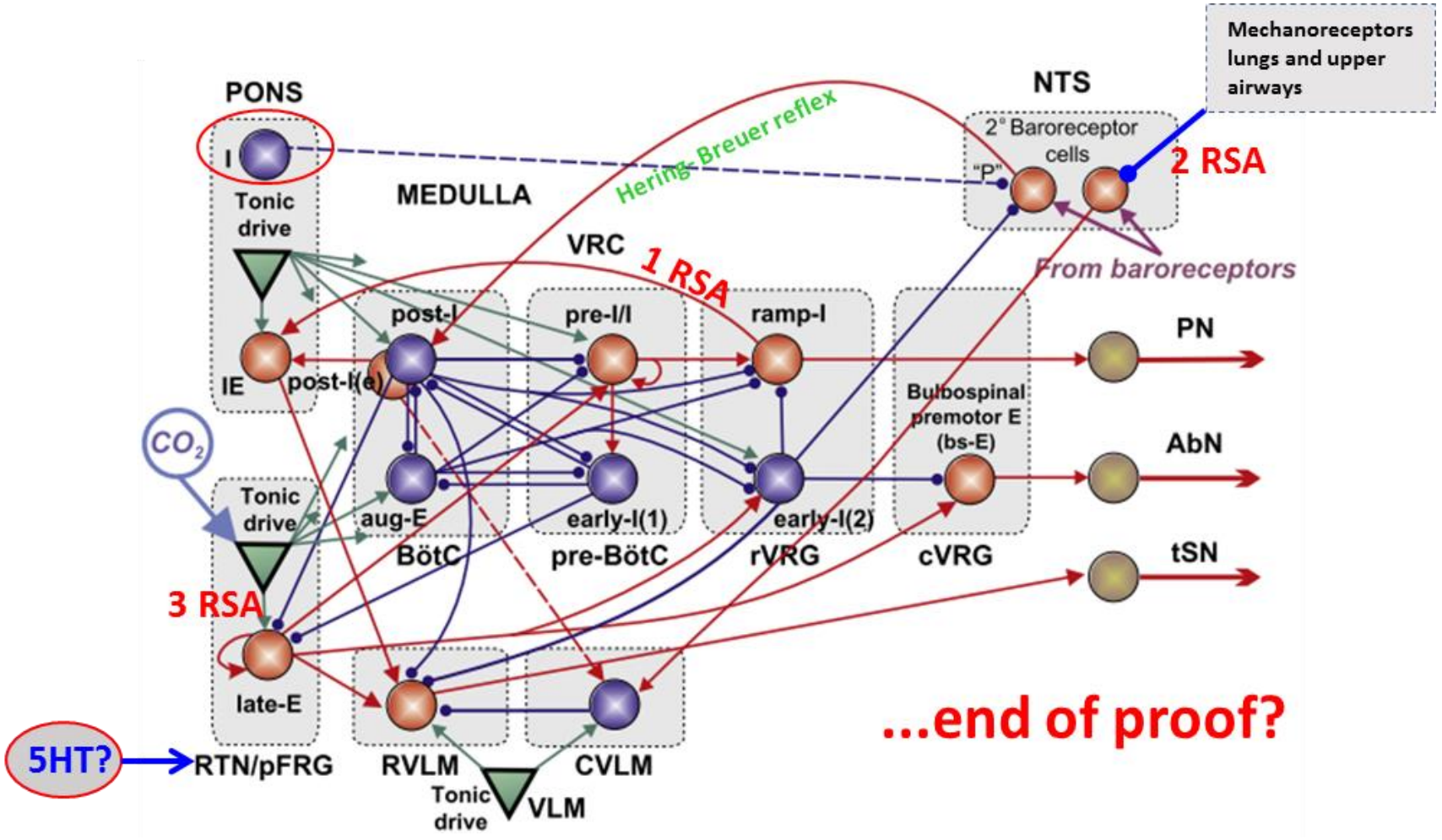
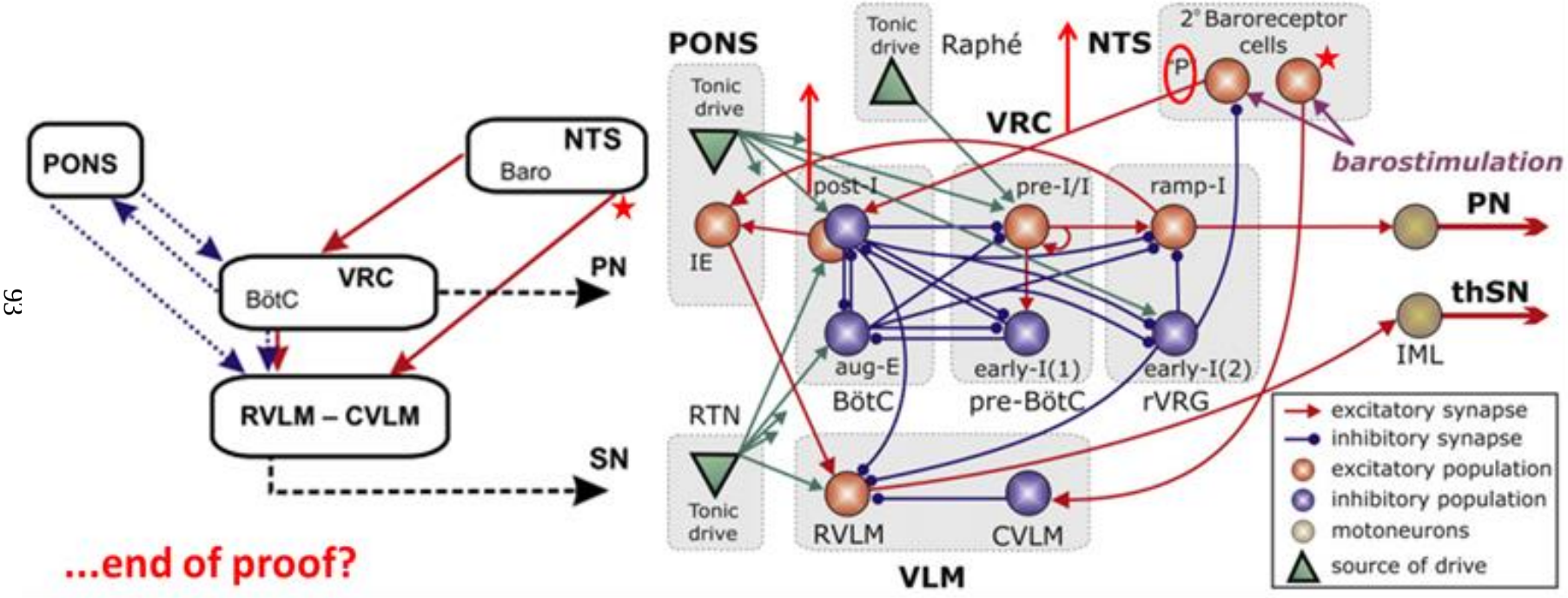

PONS
I
Tonic drive
MEDULLA
Hering-Breuer reflex
NTS
2° Baroreceptor cells
"P"
Mechanoreceptors lungs and upper airways
2 RSA
From baroreceptors
VRC
1 RSA
post-I
pre-I/I
ramp-I
IE
post-I(e)
PN
Bulbospinal premotor E (bs-E)
AbN
tSN
CO_2
Tonic drive
aug-E
early-I(1)
early-I(2)
BötC
pre-BötC
rVRG
cVRG
3 RSA
late-E
...end of proof?
5HT?
RTN/pFRG
RVLM
CVLM
Tonic drive
VLM

Figure 26 *The model by Molkov et al. (2014) [56] based on the earlier model of Smith et al. (2007) [8, 41] combines the circuitry responsible for the respiratory modulation of sympathetic activity at the level of brainstem. It simulated neural interactions between different populations of respiratory neurons within major brainstem compartments involved in the control of breathing: interaction between respiratory-related activity of Pre-Bötzinger complex (pre-BötC) and Bötzinger complex (BötC), the ventral respiratory column (VRC), rostral and caudal ventral respiratory group (rVRG, cVRG), pontine circuits (PONS), sensory network in the nucleus tractus solitary (NTS), and rostral and caudal ventrolateral medulla (RVLM/CVLM). These compartments included the populations of postinspiratory (post-I) and augmenting inspiratory (aug-E) neurons of BötC, preinspiratory/inspiratory (pre-I/I) and early-inspiratory (early-I(1)) neurons of pre-BötC, and ramping inspiratory (ramp-I) and early-I(2) neurons in rVRG. Baekey et al. (2010) [57] extended this model by incorporating NTS containing populations of 2nd-order baroreceptor cells, VLM containing the excitatory RVLM and inhibitory CVLM populations, and the phase-spanning inspiratory–expiratory (IE) population in the pons [56, 57 modified]*

In addition to respiratory timing influencing heartbeats, Elder, Baekey, and Larsen, separately in 2010, studied the cardiac timing effect on respiratory timing, i.e. the CVC in human adult and infants (Fig. 27) [54, 57, 58].

They hypothesized that barostimulation prolongs expiration by exciting expiratory neurons in BC. Since this prolongation seems to depend on pons, they proposed that the second barosensitive population (marked "P" in Fig. 26, 27) excites pontine-dependent post-I populations. In line for the evidence that barostimulation had virtually no effect on respiratory patterns when applied during inspiration, they introduced in their hypothesis an inhibitory connection from the inspiratory early-I(2) population in rVRG to the "P" barosensitive population in NTS. Summarizing, barostimulation prolongs expiration by exciting expiratory neurons in BC and determining the triggering of inspiratory onset by a preceding heartbeat. These data imply an augmentation of cardiac influence on ventilatory

rhythm in infants in quiet sleep. In preterm infants, CVC may have a role in supporting oxygenation. In conclusion, CRC and CVC could be remarkable physiologic controls involved in the pathogenesis of infant and adult sudden deaths [59].


PONS
NTS
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BötC
PN
RVLM – CVLM
SN
...end of proof?
PONS
Tonic drive
Raphé
NTS
2° Baroreceptor cells
P
VRC
barostimulation
Tonic drive
post-I
pre-I/I
ramp-I
PN
IE
thSN
IML
aug-E
early-I(1)
early-I(2)
BötC
pre-BötC
rVRG
RTN
Tonic drive
RVLM
CVLM
VLM
excitatory synapse
inhibitory synapse
excitatory population
inhibitory population
motoneurons
source of drive

Figure 27 *Cardiac activity of baroreceptor afferents can effect respiratory activity (CVC). Barostimulation prolongs expiration by exciting expiratory neurons in BC and determining the triggering of inspiratory onset by a preceding heartbeat ("P" populations of barosensitive cells in the NTS) [57 modified]*

2. Brainstem and Sudden Death

2.1 Brainstem and Sudden Cardiac Death (SCD)

Sudden death (SUD) is a major health problem all over the world. Estimations are that SUD is responsible for one fifth of all deaths. The most common causes of SUD are cardiac, i.e. sudden cardiac death (SCD) [60], but there are also other causes such as neurological conditions (stroke, epileptic attacks, and brain trauma), drugs, catecholamine toxicity, etc. A common feature of all these diverse pathologies underlying sudden death is the disequilibrium of the autonomic nervous system control on the cardiorespiratory system. The sympathetic and vagal-parasympathetic, work together to modulate electrical and mechanical properties of the heart. Their balance is regulated by the brainstem, which processes the inputs from the periphery (baro-reflexes, chemo-reflexes, etc.) and higher brain structures (cortex, limbic system, hypothalamus, etc.) [61]. Autonomic cardiac innervation plays a significant role in SCD, modulating the circuit of cardiac excitability and propagation. Significant neural remodeling in the setting of dysautonomic heart disease predisposes to malignant ventricular arrhythmias by causing alterations at the level of the myocardium, the intrinsic cardiac ganglia, extracardiac intrathoracic sympathetic ganglia, extrathoracic ganglia, spinal cord, and the brainstem, as well as the higher centres and the cortex. [62].

Conversely, acute stroke can disturb central autonomic control, resulting in myocardial injury, electrocardiographic abnormalities, cardiac arrhythmias, and eventually sudden death. In fact, autonomic imbalances are recurrent after infarcts involving the insular cortex, a limbic area inter-connected with the brainstem regions involved in the sympathetic and parasympathetic autonomic control. Cardiovascular comorbidities increase the risk of cardiac morbidity and mortality after stroke. Thus, many sudden deaths and serious non-fatal cardiac events after stroke are most likely due to an interaction between cardiovascular and neurological events. The exact mechanisms producing sudden death remain incompletely understood and further researches are needed to examine the

autonomic consequences of acute stroke and to identify patients at high risk of sudden death [63].

This important neurovisceral/autonomic nervous system also influences in the pathophysiology and progression of heart disease, including heart failure and arrhythmias leading to SCD. Leukemia inhibitory factor (LIF) and other members of the interleukin 6 family can induce fetal gene expression (so-called rejuvenation) in adult cardiomyocytes and determine transdifferentiation of neurons during cronic heart failure (CHF). For example, they induce functional denervation, cardiac and extracardiac neural remodeling, the cholinergic transdifferentiation of cardiac adrenergic neurons into cholinergic neurons, via a gp130 signaling pathway in CHF (Fig. 28) [64]. As we previously mentioned (Fig. 16), IL-6 inhibits the arcuate nucleus of medulla oblongata; it is an important inflammatory mediator of the autonomic dysregulation of brainstem during SIDS.

On of the most devastating event of sudden cardiac death (SCD) is dependent on the cardiac channelopathies, heritable cardiac arrhythmia syndromes caused by abnormal ion channel function clinically leading to syncope, seizures, and SCD, often in the setting of a structurally normal heart, but with molecular abnormalities [65]. These inherited and potentially lethal arrhythmia disorders include a variety of diseases, of which the most common ones—long QT syndrome (LQTS), Brugada syndrome (BrS), and catecholaminergic polymorphic ventricular tachycardia (CPVT) [66].

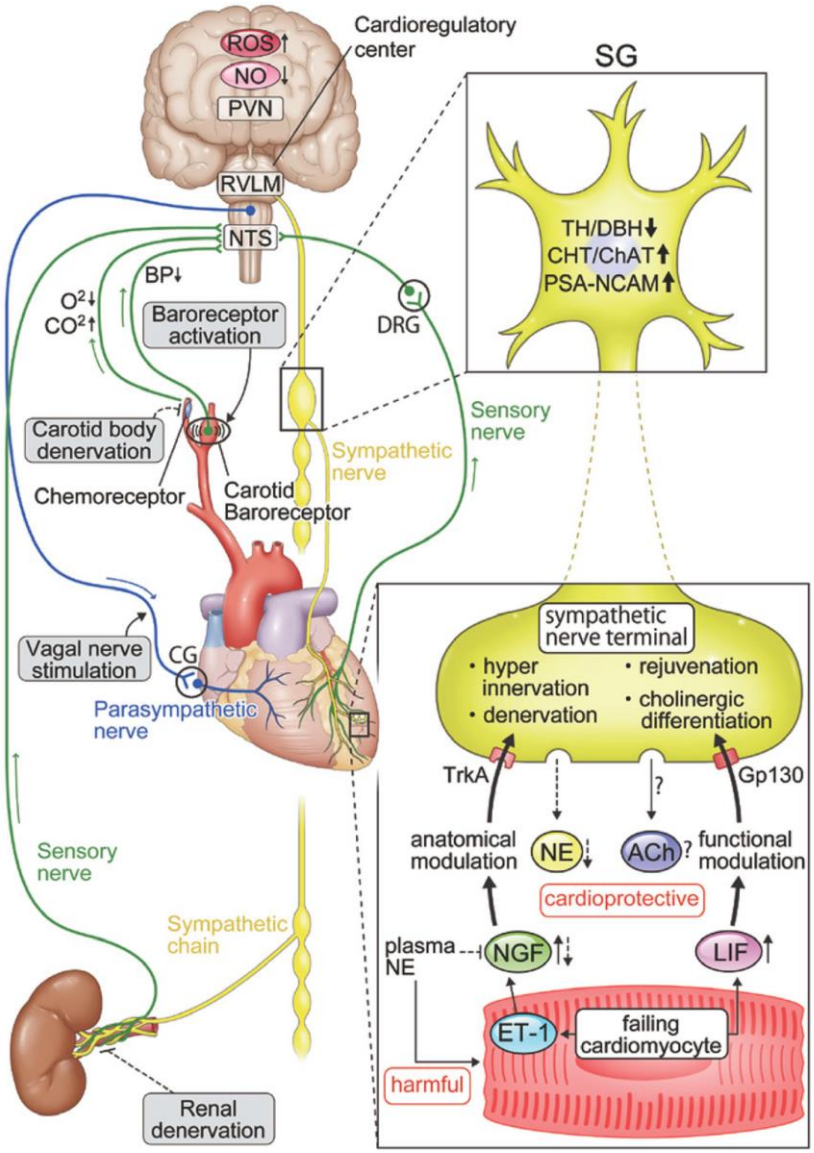

Figure 28 *Systemic autonomic interactions and crosstalk between cardiomyocyte and sympathetic nerve terminal via humoral factors in diseased heart. This figure shows that central and peripheral mechanism of the heart and brain interaction including the cardiac autonomic efferent (sympathetic and parasympathetic) and afferent (sensory) nerves. Representative promising interventional therapies are also described in the figure. In addition, alteration of cardiac sympathetic nerves occurs in postganglionic fiber. Failing cardiomyocytes induces nerve growth factor (NGF) via endothelin-1 (ET-1)–mediated pathway and leukemia inhibitory factor (LIF). NGF and LIF lead to hyperinnervation (anatomic modulation) and rejuvenation/cholinergic differentiation (functional modulation), respectively.*

This phenomenon shows the expression of catecholaminergic markers such as tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) reduced and of cholinergic (choline transporter [CHT], choline acetyltransferase [ChAT]) and juvenile (polysialylated neural cell adhesion molecule [PSA-NCAM]) increased. Ach indicates acetylcholine; BP, blood pressure, CG, intrinsic cardiac ganglia; DRG, dorsal root ganglia; NE, norepinephrine; NTS, solitary tract; PVN, paraventricular nucleus; ROS, reactive oxygen species; RVLM, rostral ventrolateral medulla; SG, stellate ganglia; and TrkA, tropomyosin-related kinase A [64].

2.2 Brainstem and Sudden Infant Death Syndrome (SIDS)

The Sudden infant death syndrome (SIDS), has been originally proposed to describe those sudden and unexpected infant deaths under 1 year of age, for which no sufficient explanation could be found, however this concept has been lately criticised on the basis that there is little evidence that all such deaths arise from the same or similar mechanisms [67]. In fact, the term SIDS represents a diagnosis by exclusion and thus is only valid if all correct evaluations have been carried out to look for all known etiologies and circumstances that may lead to sudden and unexpected death. The deaths are exceptional in the first week or two after birth, rise to a peak incidence between the second and fourth months and then decrease to revert rare after 6 months of age. There is a typical excess of males who constitute around 60% of the deaths and a marked winter peak of passing, although fortunately have both decreased in the 20 years simultaneously to the 'Back to Sleep' campaign introduction. The dramatic fall in infant mortality rates in the 20th century in England and Wales from 95 per thousand live births in 1912 to 10.8 in 1982 and to 4 per thousand live births in 2012 and shows an apparent association with the (relatively recently introduced) practice to dissuade parents from using the prone sleeping position for babies (Fig. 29) [68]. In the Australian context the number of SIDS deaths reduced from over 500 per year in 1988 to 134 per year in 1999, which corresponded to a decrease in the average number of SIDS deaths per 100,000 live births from 196 in the 1980s to 52 deaths between 1997 and 2002. In California in the United States, the number of SIDS deaths per year fell from 110.5 deaths per 100,000 live births in 1990 to 47.2 deaths per 100,000 live births in 1998 (Fig.30) [69, 70].

In more recent years, SIDS death rates have globally levelled. Although SIDS is fortunately a rarity, it is responsible for a large number of infant deaths and in USA, the 2014 estimates suggest that SIDS is the cause of death for about 3.9 of every 10,000 infant born each year.

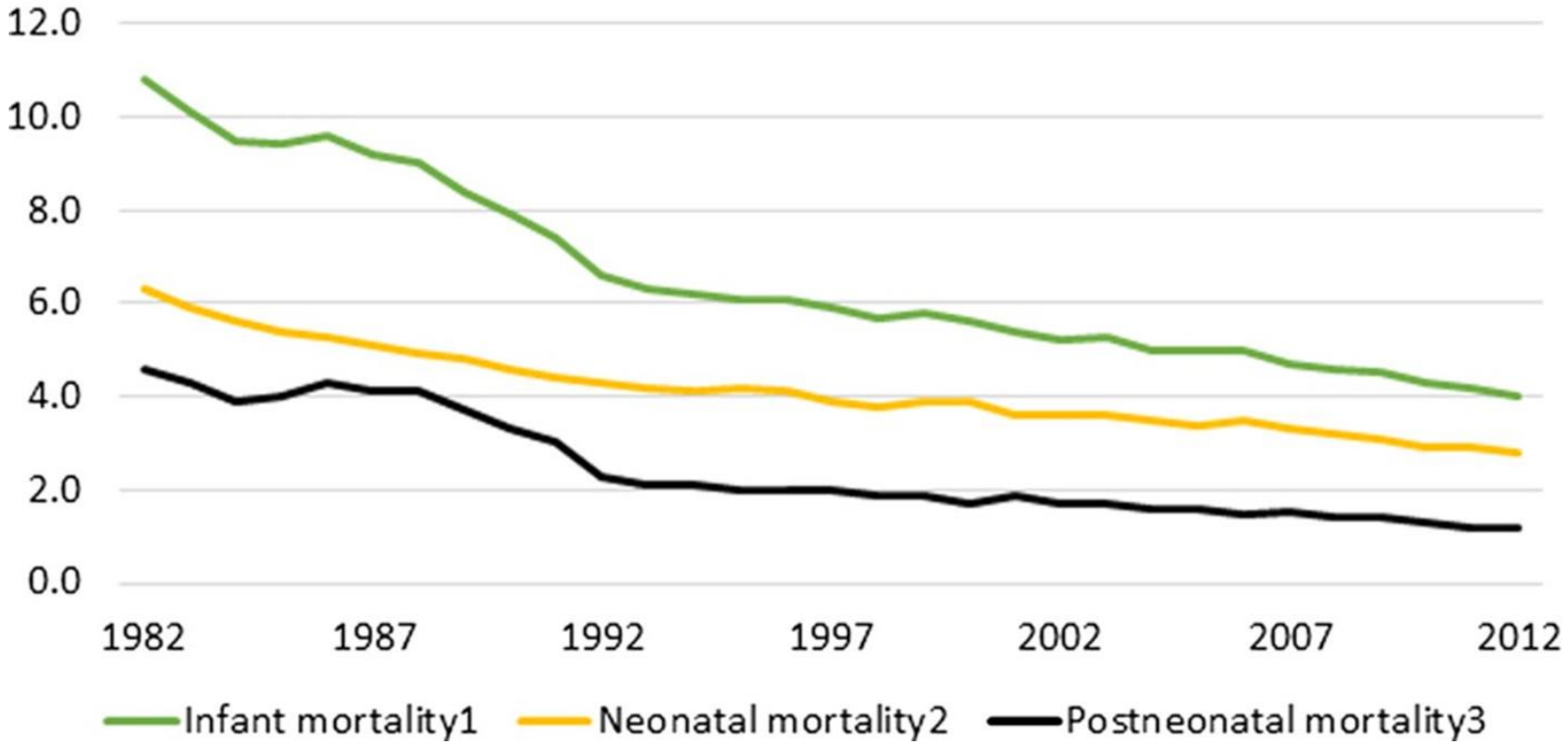
12.0
10.0
8.0
6.0
4.0
2.0
0.0
1982
1987
1992
1997
2002
2007
2012
Infant mortality1
Neonatal mortality2
Postneonatal mortality3

Figure 29 *Neonatal, postneonatal and infant mortality per thousand live births, England and Wales 1982–2012 (Office for National Statistics) [68]*

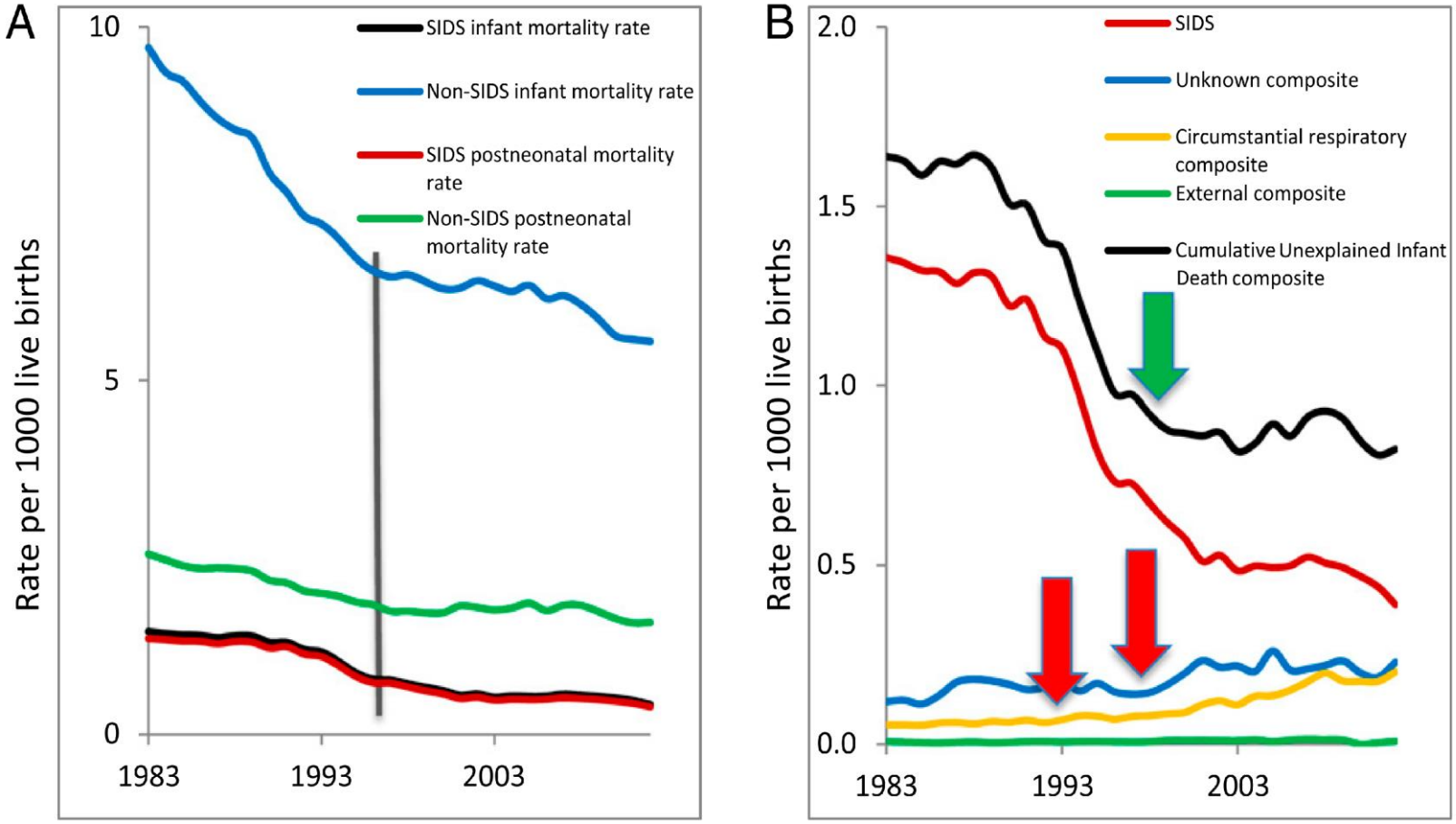
A
10
5
0
Rate per 1000 live births
1983
1993
2003
SIDS infant mortality rate
Non-SIDS infant mortality rate
SIDS postneonatal mortality rate
Non-SIDS postneonatal mortality rate
B
2.0
1.5
1.0
0.5
0.0
Rate per 1000 live births
1983
1993
2003
SIDS
Unknown composite
Circumstantial respiratory composite
External composite
Cumulative Unexplained Infant Death composite

Figure 30 *Mortality rates from SIDS, non-SIDS, and composites of diagnoses hypothetically reallocated from SIDS, over the 30-year period in USA. Composites are Unknown Combined events, Circumstantial Respiratory Composite, External Causes Composite, and Cumulative Unexplained Infant Death (CUID) Composite. A, Comparative trends of SIDS and non-SIDS mortality. The vertical gray line defines the beginning of a plateau in postneonatal mortality. B, Comparative trends in diagnostic composites. The red arrows represent inflection points of increase in mortality in postneonatal composites. The green arrow denotes the beginning of the plateau in mortality for the CUID Composite [70]*

In 1994, Kinney and Filiano suggested that unexpected infant deaths could arise because of the combination of three concurrent factors: 1. a vulnerable infant 2. a vulnerable phase of development 3. a final insult occurring in this window of vulnerability [71].

The widely accepted 'triple risk' hypothesis, includes infants with underlying vulnerability that born preterm or of low birth weight, foetuses with alcohol exposure-related vulnerability, those exposed to maternal smoking in utero and infants with certain metabolic abnormalities or common polymorphisms that may make them more vulnerable to certain environmental factors or infectious agents [72, 73, 74]. The manifest male preponderance in SIDS victims may also represent an intrinsic vulnerability, as well as the normal progression of development of thermal, respiratory and cardiovascular control may lead to periods of increased vulnerability to external stressors such as infection, thermal stressor, the prone sleeping position, overbundling, bedding surfaces, and bed sharing.

Studies, based on physiological recordings, about the relationship between respiratory control, normal developmental physiology and brainstem neurophysiology, (Fig. 19, 20) [46, 47] have demonstrated that a number of SIDS show periods of worsening bradycardia with gasping before complete cessation of respiratory efforts. This event suggests a failure of arousal and autoresuscitation

as a final event in these infants, furthermore such infants have shown a lower frequency of sighs during sleep than age-matched control infants, suggestive of some underlying abnormality of respiratory control.

Sighs are commonly triggered by a slight fall in blood oxygen saturation. An increase in the frequency and extent of sighs is associated with cerebral cortical arousal, which is an important protective mechanism under conditions of mild to moderate hypoxaemia. However, during conditions of progressive and heavy hypoxaemia, eupnoea gives way to gasping respiratory efforts with resuscitation, compensatory tachycardia, and awakening, otherwise with progressive bradycardia and finally to apnoea or cessation of all respiratory efforts. This complex neural network is particularly dependent on the activity of serotonergic neurons in the brainstem. The PBC of medulla is responsible for both maintenance of eupnoea, but also the generation of sighs and gasping. It also has important effects on cardiorespiratory coupling, a crucial component of the autoresuscitation response to hypoxia because it carries out to an inspiratory-related heart rate increase.

The critical importance of serotonergic neurons on brainstem homeostatic mechanisms has been emphasized by the consistent findings of abnormalities of different nuclei of brainstem involved in serotonergic pathways observed in autoptic brains of SIDS cases. ~40% of SIDS deaths are associated with abnormalities in serotonin in regions of the brainstem critical in homeostatic regulation. Furthermore, a significant increased serum 5-HT levels in 31% of SIDS cases has been reported, compared to controls too. This high serum 5-HT could be utilized as a peripheral marker of 5-HT metabolism, in autopsied infants to differentiate SIDS deaths due to serotonin related defects from other causes of sudden death (Fig. 31) [75].

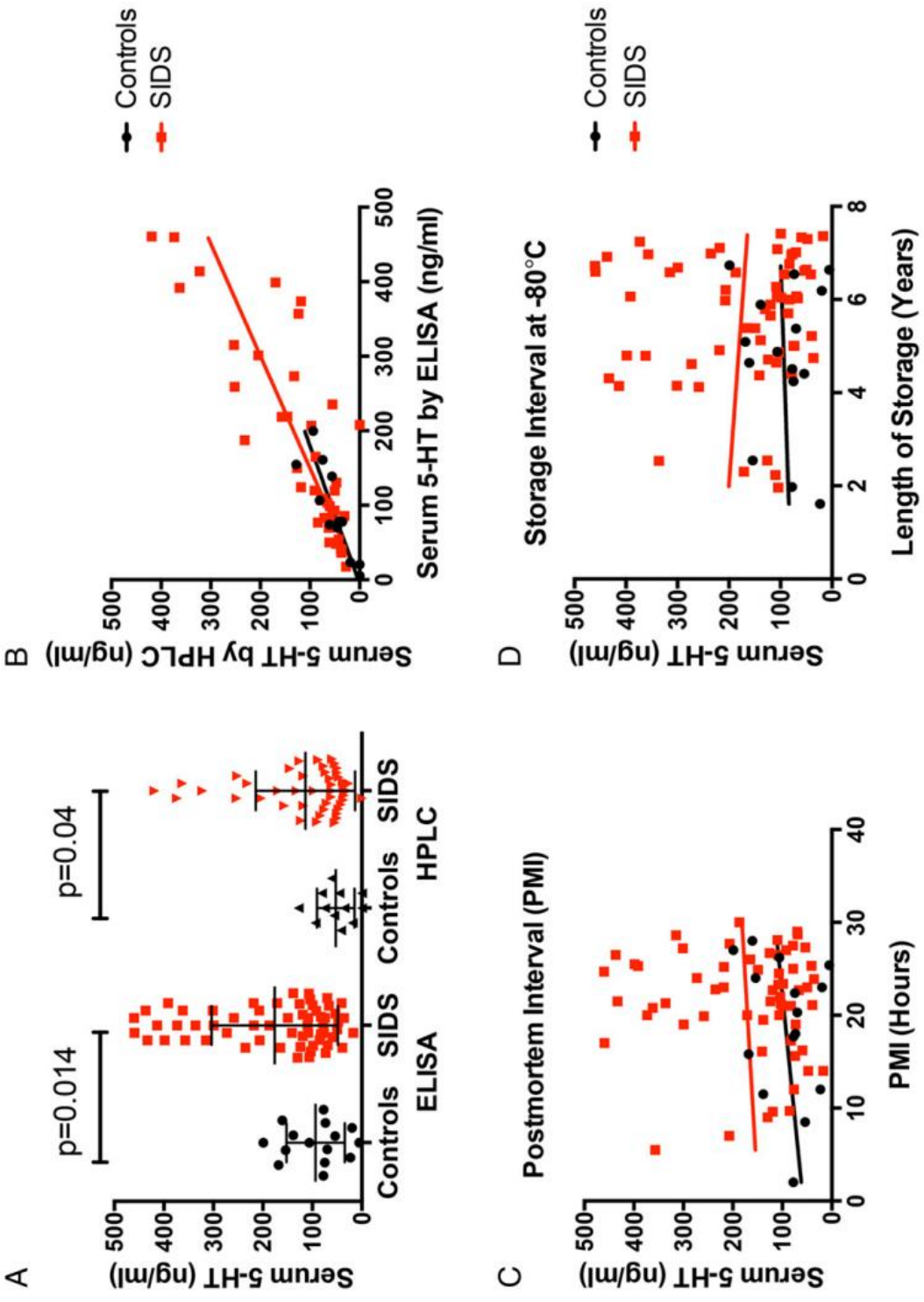

Figure 31 *Serum 5-HT values determined by ELISA and HPLC. (A) There was a significant elevation (average of 95%) in SIDS infants [177.2 ± 15.1 (SE) ng/mL] compared with controls [91.1 ± 30.6 (SE) ng/mL], P = 0.014, by ELISA. HPLC*

confirmed this finding in a subset of cases. The bars in the graph represent SD. (B) Serum 5-HT levels obtained with two different methods, ELISA and HPLC, showed significant correlation in SIDS cases [Spearman correlation of 0.84 (P <0.001)] and controls [Spearman correlation of 0.87 (P < 0.001)]. There is no significant effect of (C) postmortem interval or (D) storage length at −80 °C on the levels of serum 5-HT in control or SIDS infants [75]

It has been formulated the hypothesis that SIDS is due to a developmental disorder of the medullary serotonergic network occured prenatally but that then exerts its effects in the postnatal period [76]. Abnormalities that have been identified include alterations in 5-HT receptor binding patterns (5-HT 1A and 5-HT 2A receptors), reduced brainstem levels of 5-HT and tryptophan hydroxylase (TPH2, the rate limiting enzyme regulating 5-HT synthesis), decreased binding to the 5-HT transporter relative to 5-HT cell density, increased 5-HT cell number and density of 5-HT neurons, morphological immaturity of 5-HT neurons, and reductions in the level of the signal transduction family of proteins in regions of the medulla oblongata involved in the regulation of homeostatic function. Given the complex role of 5-HT within the medulla, associated abnormalities are likely responsible for impaired reflexes and responses of critical autonomic respiratory defence mechanisms to exogenous stressors such as hypoxia.

However, other abnormalities in various brainstem neurochemicals including catecholamines, neuropeptides, acetylcholinergic, aminoacids (predominantly glutamate), growth factors including brain-derived neurotropic growth factor, adenosine and some cytokine systems have all been reported in infants who died of SIDS. In fact, the multitransmitter hypothesis for SIDS proposes that neurochemical anomalies are not necessarily limited to one neurochemical system such as that of 5-HT. Recently, by Proteomic MALDI-TOF/TOF-IMS examination of peptide expression in brainstem of autoptic SIDS cases, has been reported abnormal expression patterns in 41 peptides corresponding to 9 proteins within key brainstem nuclei in SIDS cases compared to controls [77]. A significant abnormality has also been identified in the peptide neurotransmitter

substance P (SP) binding to its neurokinin-1 receptor in multiple nuclei intimately related to cardiorespiratory and autonomic control in SIDS cases. In detail, reduction of SP in SIDS infants in areas of the olivary nuclei that control head and neck movement by olivo-cerebellar system, may explain why SIDS infants fail to lift their heads away from challenging environments when prone. Defective interaction of certain medullary mechanisms with cerebellar sites might result in aninability of SIDS infants to illicit appropriate respiratory and motor responses to life threatening challenges during sleep [78].

During hypoxia there is an accelerated consumption of the purine adenosine monophosphate (AMP) because cells try to maintain energy state. As AMP breaks down during hypoxia, hypoxanthine accumulates. Increased hypoxanthine concentrations in plasma, urine, cerebrospinal fluid, amniotic fluid, and vitreous humor have been detected significantly higher in hypoxic individuals, including hypoxic infants, compared to non-hypoxic individuals [79]. Activation of presynaptic adenosine A2a receptors seems to enhance GABA release in NTS. Thus, this is a speculation, when the LCR is elicited, in addition to activation of glutamatergic second order neurons that elicit the apnea, there are parallel inhibitory GABAergic processes acting on second order neurons within the NTS that normally drive or support eupnea. These second order neurons, such as pump neurons, receive information from the carotid body, or CO_2-sensitive neurons within the NTS (Fig. 26). During LRC, eupnea-promoting processes must be inhibited to allow apnea to occur. GABA receptors and A2a receptors in the NTS, tend to shorten the duration of LCR with early re-emergence of eupnea [80].

Obviously, more than one transmitter has to be studied in the same medullary region and in the same case, because not all of the proposed abnormalities have been confirmed by other investigators. Multiple neurochemical abnormalities, are present in specific brainstem nuclei, particularly within the ventrolateral medulla. These areas include the arcuate nucleus, midline raphe, hypoglossal nucleus, olivary nuclei, nucleus tractus of solitary tract, and dorsal motor nucleus of vagal nerve.

Some authors thought astrogliosis in the brainstem of SIDS cases, as produced by hypoxia [81]. Significant gliosis and decreased density of neurons specifically

within the inferior olivary nuclei of the brainstem have also been reported. As well as scarring of the brainstem, researchers have demonstrated an abnormal, immature developmental pattern of neurons with significantly increased dendritic spine density in SIDS brainstems. Therefore, the brainstem immaturity may produce an abnormal central respiratory and arousal control [82]. According to this theory, recent proteomic investigations have shown abnormalities in SIDS infants within the brainstem raphe and hypoglossal nuclei and in the medullary pyramids, relating to neuronal development, neuronal/glial/axonal growth, metabolism, and apoptosis [77] (Fig. 32, 33, 34).

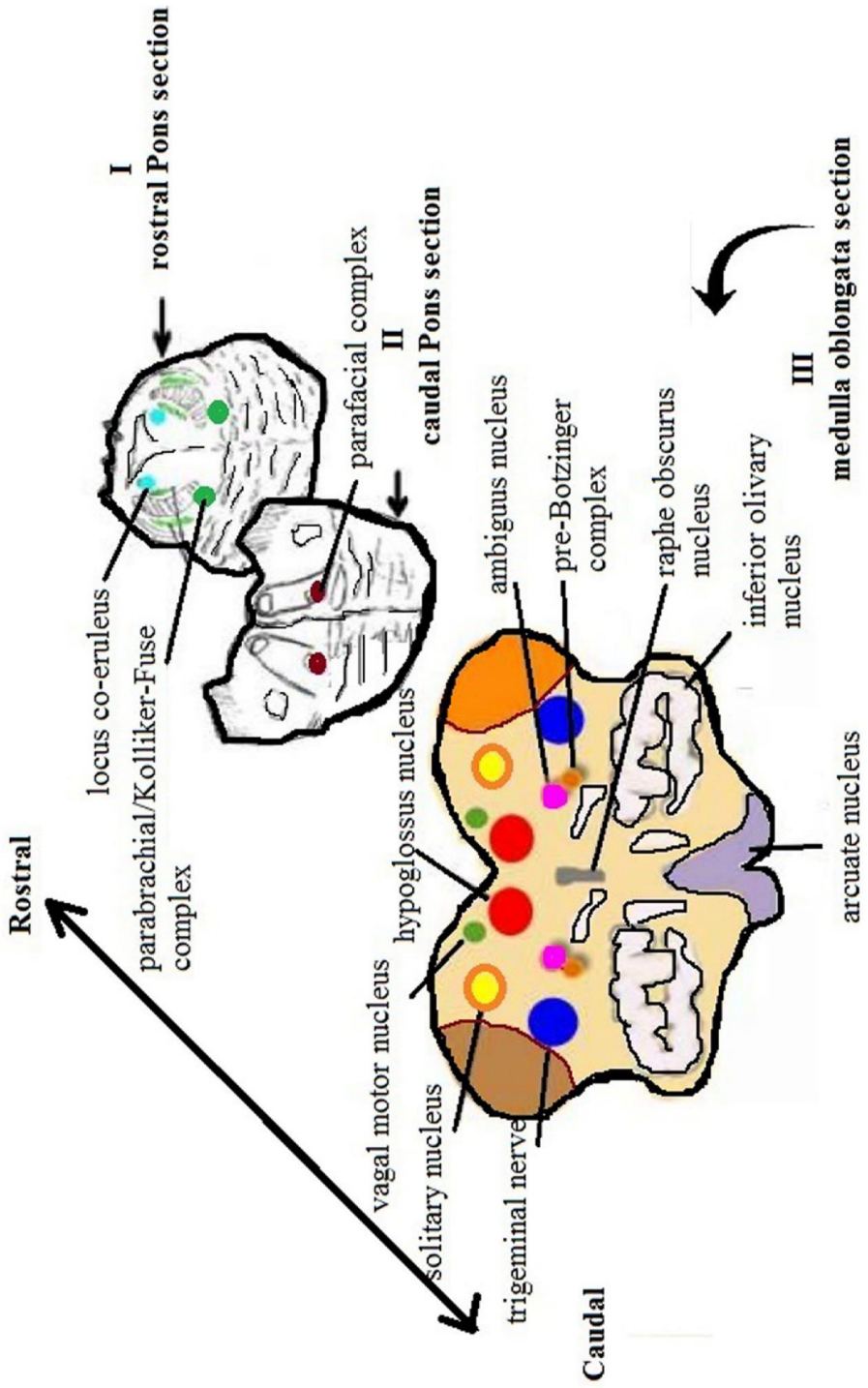

Figure 32 *Schematic representation of the main histological sections obtained from the brainstem for the anatomopathological examination [82]*

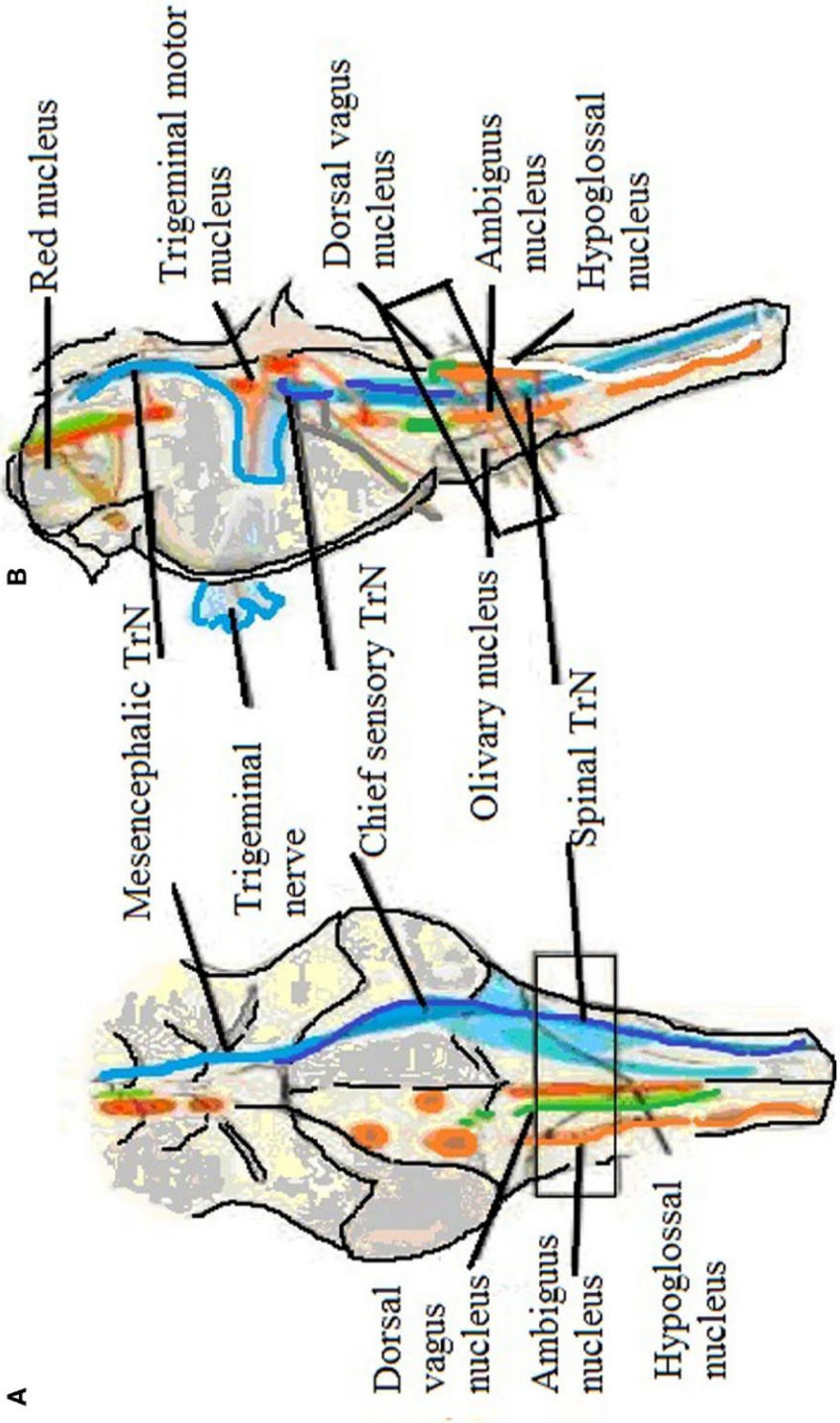

Figure 33 *Brainstem showing trigeminal nucleus and also shows the level of sampling. (A) Ventral view and (B) side view showing trigeminal nerve, mesencephalic, chief sensory, and spinal trigeminal nucleus [82]*

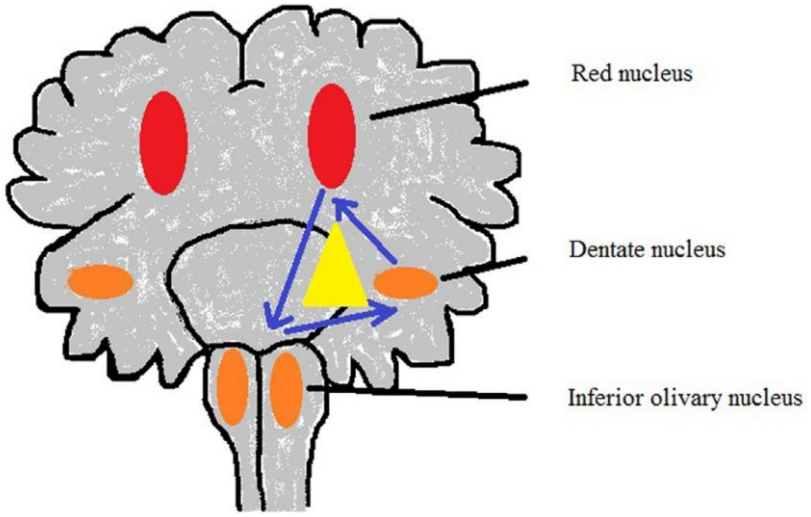

Figure 34 *Sketch of Guillain–Mollaret triangle [83]*.

The role of pontine KFN in breathing control is well known: it is interconnected with the prevalent serotonin and noradrenaline neurons in the brainstem. KFN is a key element of the orexin system that is involved in arousal; KFN contributes to providing a breathing rhythm and coordination of sleep-to-wake transition. Any defect in orexin expression in KFN could be responsible for prevention of arousal and can be a crucial factor in causing SIDS. Orexin has a robust effect on the brainstem raphe nuclei and locus coeruleus, in arousal from sleep. BDNF pathway dysfunctions may interfere with the normal KFN development in SIDS victims, in fact 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.

Locus coeruleus is a pons complex mainly responsible for the physiological responses to conditions of stress and panic. It produces noradrenaline, tyrosine hydroxylase, and neuromelanin (NM). It is involved in vital activities related to the brain interconnections and behavioral regulations, including coordination of the sleep–wake cycle and control of the cardiorespiratory functions. Strong correlations concerning defects in noradrenaline system, low levels of NM,

hypoplasia of locus coeruleus, along with a high neuronal death rate, are associated with SIDS.

The RTN is part of caudal pons and comprises cluster of glutamatergic and non-aminergic neurons that are responsible for the expression of the homeodomain transcription factor Phox2b, transcriptional factor involved in CCHS (Fig. 17) [23]. It is involved in the chemoreception control, therefore, its dysgenesia may perform a vital part in the pathogenesis of SIDS.

The Spinal Trigeminal Nucleus (STrN) is part of medulla, and it transmits information related to pain and temperature in the orofacial region. The cranial nerves spread pain stimuli from peripheral regions to the STrN. There is a relevant association between SIDS victims and reduced SP expression levels in the fibers of STrN [78].

The sympathetic preganglionic neurons reside in the IML. This is group of columnar cells, present between the first thoracic spinal region and the third lumbar region, organized longitudinally, in the gray matter of the lateral horn of spinal cord. Experimental studies have demonstrated the role of IML in the breathing motor activities, development of a spinal cord–brainstem network and "cardio-cardiac reflex" (Fig. 6, 7, 16). In SIDS, IML fails to mature progressively. In fact, SIDS victim neurons do not morphologically 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 IML such as neuronal immaturity in a normal structure, hypoplasia, and agenesis was demonstrated [83].

Guillain–Mollaret Triangle (G-Mt) or Dentato-Rubro-Olivary Network, is organized in three parts: the ipsilateral RN, the inferior olive, and the contralateral dentate nucleus, respectively in the midbrain, medulla, and cerebellum to form dentato-rubro-olivary pathway. G-Mt may be involved in the pathogenetic mechanisms of the palatal myoclonus and a significant increase of lesions of these three nuclei were found in SIDS victims [83].

The hypoglossal nerve is a motor nerve that controls extrinsic and intrinsic muscles of the tongue. It arises from the medullary Hypoglossal Nucleus (HGN) controls swallowing, chewing, vocalization, and inspiration. HGN anomalies such

as hypo/hyperplasia, absence of interneurons and/ or somatostatin positivity have been demonstrated in SIDS cases. Even if HGN is not a main respiratory regulatory centre, however it contains motoneurons with respiratory-related rhythmical discharges. HGN stimulates the genioglossus, an extrinsic muscle of the tongue, which contributes to maintain a patent airway during inspiration.

Raphe Nuclei and PBC are the main brainstem respiratory nuclei, of which we have amply described their physiopathological significance in cardiorespiratory control. In response to stress such as hypoxia, these neuron clusters are able to reconfigure, to generate multiple breathing patterns, and to facilitate autoresuscitation. The raphe nuclei 5-HT system regulates cardiovascular and cardiorespiratory function, chemosensitivity, thermoregulation, arousal, and pain. Thus, respiratory anomalies identified in infants who subsequently died from SIDS, particularly decreased chemoreception and altered hypercapnic responses, may really reflect altered expression of 5-HT and its receptor in the raphe nuclei. The NTS is among nuclei of the brainstem where 5-HT receptor binding deficiencies have been detected in infants who died of SIDS. The NTS receives projections from serotonergic neurons in the raphe pallidus and raphe obscurus, and neurons in the NTS express 5-HT 1A, 1B, 2A, 3, 4, and 7 receptors. SIDS victims have been found to have reduced levels of brainstem 5-HT and tryptophan hydroxylase 2 metabolizing enzyme from tryptophan, but retain producing 5-HT neurons.

PBC is a cluster of interneurons present in the ventrolateral medulla of the brainstem. It has a pacemaker property and has both intrinsic inspiratory and expiratory bursting properties that are essential to sustaining respiratory rhythm rhythmogenesis. Neuropathology of the PBC, as hypoplasia with a low neuronal number with dendritic hypodevelopment, defective neuronal morphology, immunonegativity of neurotransmitters, and agenesis, so as altered neurokinin-1 receptors, and somatostatin expression were observed in a subset of SIDS victims as compared to the controls. These abnormalities are directly interconnected with the neonatal deaths and still births.

Asymmetry and microdysgenesis of the hippocampus, decreased orexin levels in the hypothalamus have also been demonstrated in SIDS cases compared to control infants [78, 84].

2.3 Brainstem and Sudden Unexpected Death in Epilepsy (SUDEP)

Sudden unexpected death in epilepsy (SUDEP) is most common in younger adults (aged 20–45 years), however can indiscriminately hit all ages [37]. The greatest risk factor for SUDEP are generalised tonic-clonic seizures (GTCS); frequently, SUDEP occurs after this type of seizure in bed during sleep hours and the person is found out in a prone position. Death after single febrile, unprovoked seizures or status epilepticus are not-SUDEP forms of seizure-related sudden deaths.

The SUDEP risk per 1000 patients with epilepsy per year sorts from 6.3 to 9.3 in epilepsy surgery candidates or in patients after epilepsy surgery, 1.1 to 5.9 in epilepsy clinic populations—most with large percentage of patients with refractory seizures—and 0.35 to 2.3 in community-based populations. A meta-analysis study of SUDEP produced a pooled estimate of 1.2 cases of SUDEP per 1000 people with epilepsy per year. Once epilepsy begins in early childhood and runs without a complete remission, the average cumulative risk of SUDEP is about 8% by age 70 years.

SUDEP might be underdiagnosed in older adults in whom sudden death might be ascribed to cardiac events without suspect of any alternative causes. Conversely, among infants and children, some cases of SIDS and sudden unexplained death in childhood (SUDC) could be SUDEP. In early childhood, unobservable non-motor seizures that induce apnoea can be lethal, muddling through the distinction of SIDS and SUDC from SUDEP.

SUDEP may be compared to other sudden death syndromes providing valuable insights into epidemiology, risk factors, and mechanisms. SCD differs from SUDEP, in fact SCD occurs most often in the morning while the individual is awake, in contrast with cardiac patients with obstructive sleep apnoea, who most often die during sleep [85]. Otherwise, SIDS and SUDC share with SUDEP unwitnessed death in bed during sleep, are more common in males, and are associated with lying in a prone position (Table 7).

	SIDS	SUDC	SUDEP
Occurrence during a sleep period	++	++	++
Prone position	+ to ++	++	++
Premature birth or low birth weight	+	+ or –	Unknown
Male sex	+ (61%)	+ (63%)	+ (59%)
Illness or fever shortly before death	+	+	–
Exposure to smoking	+	Unknown	Unknown
Microscopic hippocampal abnormalities	+	+	+
Routine autopsy	Normal*	Normal*	Normal*†
Method of diagnosis	Exclusion	Exclusion	Exclusion
Theoretical mechanisms	Cardiac, respiratory, and arousal	Unknown	Cardiac, respiratory, and arousal
Potential link to serotonin system	Yes‡	Unknown	Yes§ ss
History of epilepsy	–	–	++
History of febrile seizure	Unknown	+	+ or –
Age at death	<1 year	1–5 years	Any age

Effects are shown as: negative (–), positive (+), or strongly positive (++). SIDS=sudden infant death syndrome. SUDC=sudden unexplained death in childhood. SUDEP=sudden unexpected death in epilepsy. NA=not applicable. *Minor findings are common but do not explain the cause of death. †Pulmonary edema common; brain often shows localised pathology and eosinophilic neuronal changes—but no change explains the immediate cause of death, some are likely effects of the terminal process. ‡Based on some neuropathological studies, but genetic studies are negative. §Based on animal models and very limited human data. ss Patodia et. Al., 2018

Table 7 *Shared and different features in SIDS, SUDC, and SUDEP [37 modified]*

In addition, hippocampal pathology contribute to fuzz boundaries between SIDS, SUDC, and SUDEP. Granule cell dispersion and bilamination occur in adult patients with epilepsy and hippocampal sclerosis; however, SUDC cases have

more focal granule cell bilamination in the dentate gyrus than controls too. In animal models, seizures and even interictal epileptiform activity might cause granule cells to disperse. Therefore, in some SIDS and SUDC cases, the individual might have had seizures and the mechanisms causing death might overlap with those that cause SUDEP.

Scanty control of primary or secondary GTCS is the strongest SUDEP risk factors. Most witnessed SUDEPs have occurred following seizures, especially GTCS and interventions to reduce seizure frequency and severity are the most effective preventive strategy. In pooled data collected from four case-control studies with living epilepsy controls, male sex, epilepsy onset before age 16 years, disease duration of more than 15 years, and number of GTCS per year were each significantly associated with SUDEP. Antiepileptic drug polytherapy was not a SUDEP risk factor when the analysis controlled for GTCS frequency. In children, drug-resistance leading to continued seizures over 5 years increased the risk of SUDEP 5.2 times, while intellectual disability appears a risk factor for SUDEP in England and the USA but not in all studies. Anxiolytics, but primarily alcohol misuse were associated with an increased SUDEP risk in adults but not in children. SUDEP occurs during sleeping hours in 58.5% of all cases, and a 2.6 times increased risk for SUDEP is associated with nocturnal seizures after rectification for other SUDEP risk factors as total number of antiepileptic drugs ever taken, current use of carbamazepine, and concurrent asthma. Obviously, nocturnal supervision (checks at night or with a listening device) is associated with reduced risk of SUDEP.

Early age epileptic encephalopathies contribute to developmental delays and carry a high risk of SUDEP; however, the most common risk of SUDEP involves patients with Dravet syndrome, in which the risk of SUDEP is estimated to be 15 times higher than in other paediatric epilepsies.

In many unwitnessed cases of SUDEP, there is circumstantial evidence of a terminal seizure, eg tongue bite. However, in a minority of witnessed SUDEP cases there is no clinical seizure prior to death as recently reported regarding 3 sudden deaths in patients with epilepsy who were undergoing electroencephalography (EEG) monitoring [86]. In all 3 cases, autopsies revealed

no structural or toxicological cause of death, and they were classified as definite SUDEP according to criteria of Nashef [87]. These SUDEP cases also had no electrographic evidence of terminal seizure recorded by the brain-responsive neurostimulation with RNS System (NeuroPace, Mountain View, CA, USA). Even if patients may have had a seizure arise in a cerebral location not sampled by the device, it was likely that this SUDEP was seizure-independent. Sudden cardiac death due to arrhythmia may be responsible for a minority of SUDEPs and furthermore, whole-exome sequencing in SUDEP cases demonstrated that 7% of decedents had mutations in genes associated with long-QT syndrome, a cause of lethal cardiac arrhythmias [29, 30]. Contemporary, in the same study cohort were also identified six previously unreported variants, unique to SUDEP cases, in glutamateric and GABAergic neurotransmission. These variants could affect on excitatory-inhibitory equilibrium by increasing risk of epilepsy and/ or producing centrally mediated autonomic dysfunction (Fig. 35).

Pathway	Genes/Patients	ACMG/AMP	SUDEP.05	SUDEP.13	SUDEP.09	SUDEP.01	SUDEP.07	SUDEP.15	SUDEP.03	SUDEP.11
GABA/Glutamate Signalling	ITPR1:p.A1760T	VUS	■							
	GABRR2:p.A118S	VUS		■						
	SSTR5:p.A332S	VUS			■					
	GRIK1:p.M330V	VUS			■					
	CNTNAP2:p.E683K	VUS				■				
	GNAI2:p.S22F	VUS					■			
	GRM8:p.I83V	VUS					■			
	GRIK5:p.F758Y	VUS						■		
Cardiac Arrythmia	KCNMB1:p.M177T	VUS							■	
	DPP6:p.R54G	VUS							■	
	JUP:p.I165V	VUS								■
	F2:p.H479Y	Likely pathogenic								■
	TUBA3D:p.Y185C	VUS								■

Figure 36 *Variants in GABA/glutamate signaling and cardiac transduction genes in SUDEP patients. Following the ACMG/AMP guidelines, most novel variants are identified as variants of unknown significance (VUS). Variant in F2 is*

identified as likely pathogenic. Novel variants in tissue from both in SUDEP (blue) and living epilepsy (orange) patients [89]

A SUDEP gene might be best defined as a gene mutation or a pathogenic variant that causes epilepsy and increases SUDEP risk through central or peripheral nervous system or end-organ effects on respiratory, cardiac, or additional autonomic functions.

Epileptic patients, who have mutations in cardiac channelopathy genes might be prone to seizure-induced arrhythmias. However, these genes are also expressed throughout the brain, and could predispose patients to seizures or postictal apnoea or bradycardia.

Several mutations in the neurocardiogenic channelopathy genes have been identifed as a possible cause of epilepsy and increased SUDEP risk. Patient groups have revealed at least nine different neuro-cardiac genes that may contribute to a genetic susceptibility for SUDEP, making them potentially useful as genomic biomarkers (Table 5, 6) [36, 37, 40]. Most of these genes, principally coding for differention channels, have been linked to epilepsy in rare Mendelian pedigrees. However, some types of epilepsies prevalently involve multiple co-inherited or de novo genetic variants that associate to cause disease. Recognizing which gene variant combinations are beneficial or deleterious is complicated by the large number of non-synonymous variants, even of identified epilepsy genes, in both epilepsy patients and non-epilepsy control groups. New studies reveal that SUDEP involves polygenic interactions between genes related to epilepsy, cardiac arrhythmias, and respiratory dysfunction. Complex combinations of ion channel gene variants occur in both epilepsy patients and healthy controls. One method to begin dissecting the specific “channotype” and phenotypic causation among this genetic complexity is to identify potential genetic modifiers. Genetic modifiers perform a substantial role in influencing the clinical severity of SUDEP and other type of sudden deaths. Modifier loci usually segregate independently from the primary mutation and can influence penetrance, age of onset, progression, or severity of disease. Identification of genetic modifiers is important for

understanding the pathophysiology of inherited Channelopathies and may suggest genes and pathways that contribute to complex disease. Furthermore, beneficial modifiers that mask disease phenotypes, known as genetic suppressors, represent potential gene targets for therapeutic intervention (Fig. 36) [88, 89].

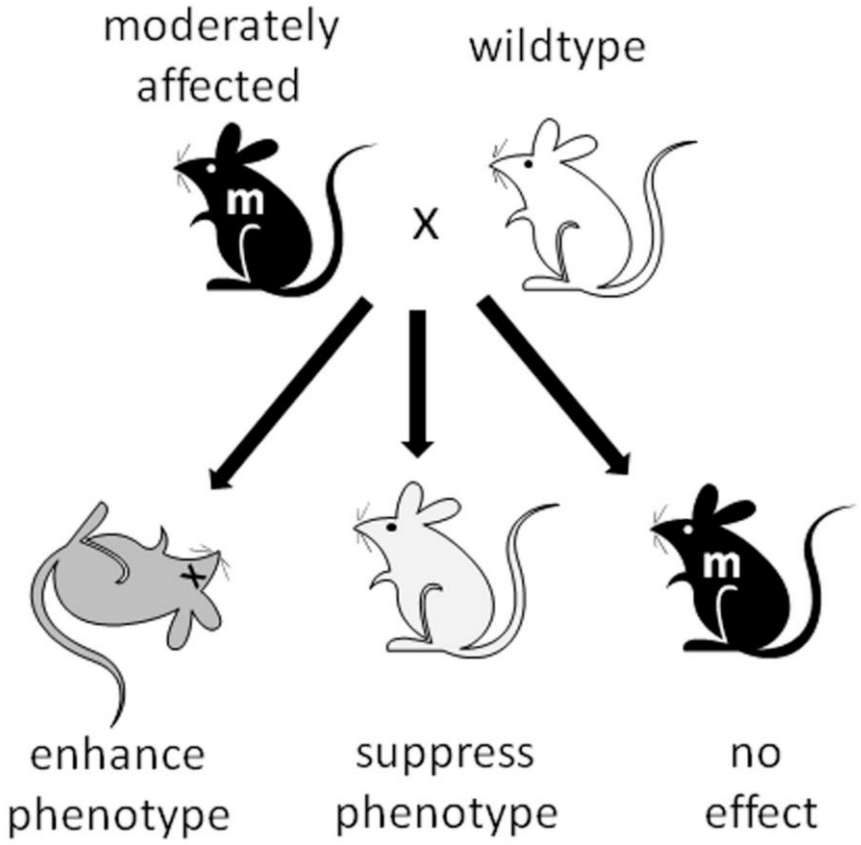

Figure 36 *Strain-dependent variation of phenotype in mouse models due to genetic modifiers. To search for modifier effects, a mouse mutant (m; black) can be crossed to a wildtype mouse from a different inbred strain background (white). Modifiers can act as enhancers that exacerbate the phenotype or suppressors that abrogate the phenotype. Alternatively, an inbred strain that does not carry modifier variants will have no effect on the phenotype [88].*

Regarding the pathophysiology of SUDEP, pathological changes are so variable and unspecific than nothing could be assumed as pathognomonic. However,

cardiac and pulmonary anomalies are frequent in cases of SUDEP at autopsy. From the Litterature have been referred mild-to-moderate myocyte hypertrophy, multifocal fibrosis in deep and subendocardial myocardium and moderate to severe pulmonary congestion and oedema in patients who had SUDEP [37]. Therefore, ictal autonomic effects might cause structural changes from which functional alterations can be determined in the cardiopulmonary system and contribute to SUDEP.

Neuropathological abnormalities associated with SUDEP comprise both causes of epilepsy and consequences of seizures. Thom and colleagues reported in 2016 (Fig. 37) [90], a study of 145 SUDEP patients, in which neuropathological alterations were typically unremarkable in generalised epilepsies, whereas in symptomatic and focal epilepsies, 52% of cohort patients presented macroscopic lesions eg, contusions, old infarcts, hippocampal sclerosis, and cortical dysplasia and 89% showed microscopic abnormalities eg, hippocampal sclerosis and cortical dysplasia. All of these abnormalities have to be considered relevant to epilepsy cause. Again, mild brain swelling occured in about 28% of SUDEP cases, while in 55% of deceased patients presented acute hypoxic neuronal changes as eosinophilic neuronal change, most often in the hippocampus, but also throughout cortical and subcortical regions. These acute changes were more numerous when a seizure happened within 24 h of death, the body was in a prone position, the external airways were occluded, or brain swelling was present, compared with cases without these features. Moreover, a precious detection of HSP-70 positive hippocampal neurons can suggest that neuronal injury was due to a seizure shortly before death. Nonetheless, nothing of these lesions or their locations associated with an increased risk of SUDEP. In SUDEP cases compared with controls, imaging studies demonstrated increased right mesial temporal volumes and decreased thalamic grey matter volumes, indicating possible seizure-induced damage, or a pre-existing feature of the epileptic network involving the autonomic and respiratory functional structures (Fig. 38, 39) [91, 92].

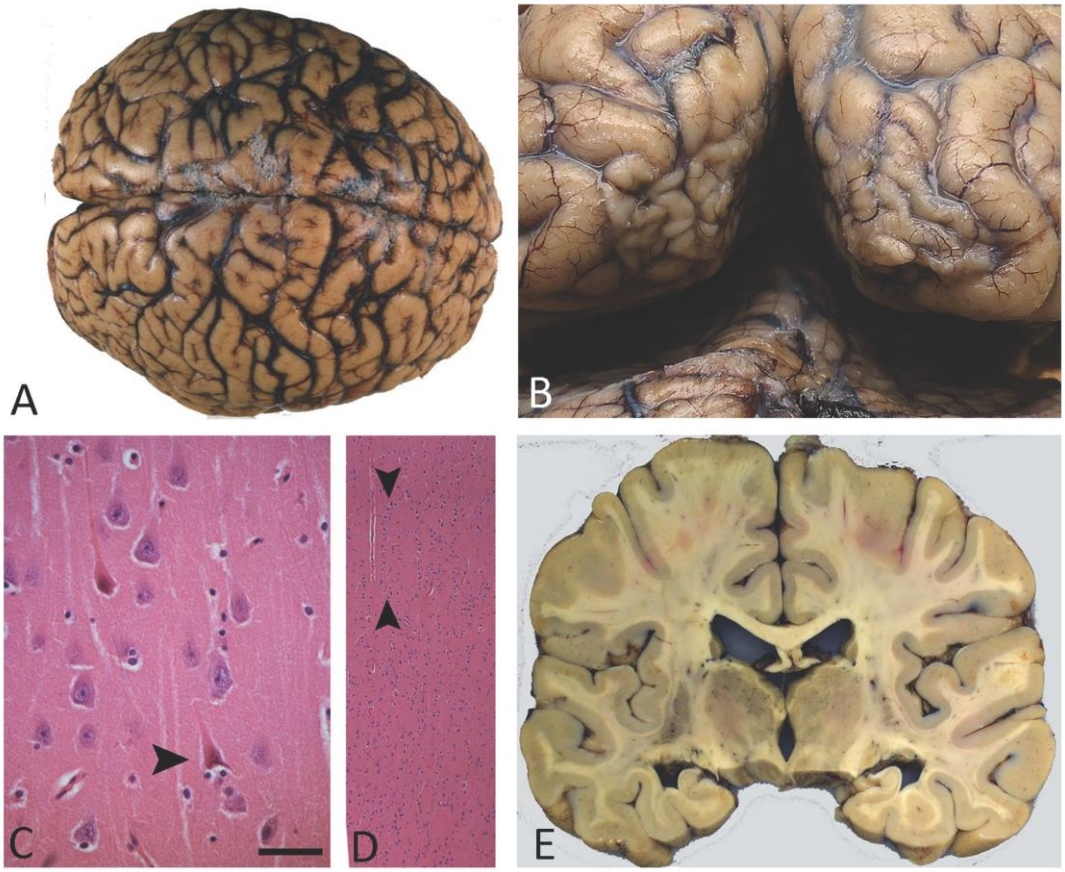

Figure 37 *Examples of some of the neuropathological abnormalities in SUDEP. (A) Evidence of brain swelling in SUDEP with gyral flattening over convexities in a 39-year-old female. (B) Bilateral occipital ulegyric malformation following a focal perinatal ischaemic event, simulating polymicrogyria, in a 52-year-old male with SUDEP. (C) Acute eosinophilic neuronal change in the CA1 sector with scattered pyramidal neurones showing this alteration in SUDEP (arrow) (D). An individual with a clinical diagnosis of DiGeorge syndrome showing an impression of exaggerated micro-columnar cytoarchitecture (arrows showing columns of > 10 neurones and loss of horizontal lamination) in the middle temporal lobe gyrus; also in this case, bilateral hippocampal atrophy was due to hippocampal sclerosis (E) with an impression of incomplete hippocampal inversion with an upward pointing hilus. Bar is equivalent to 40 microns approximately in (C) and 250 microns in (D) [90].*

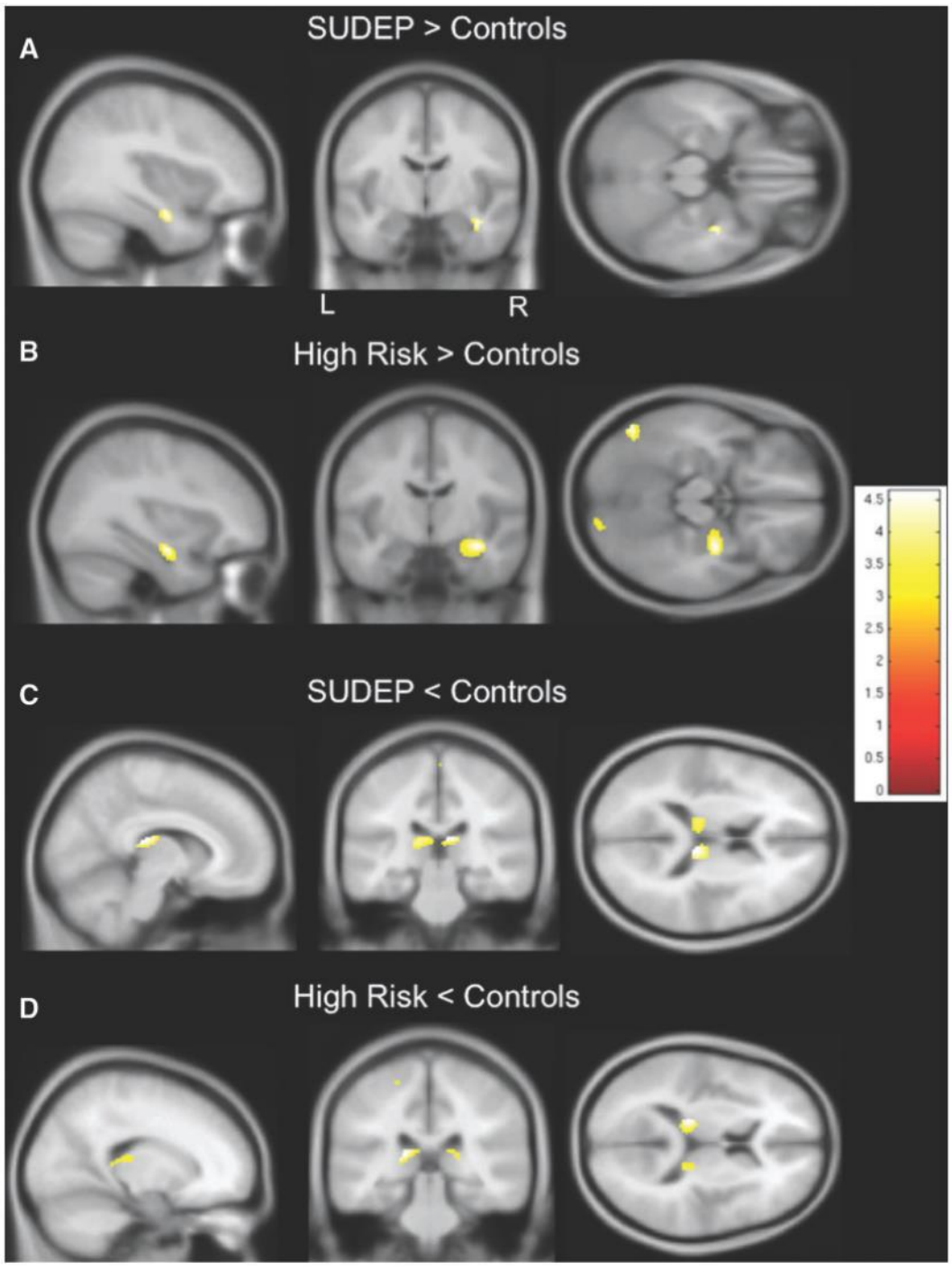

Figure 38 *Regional grey matter volume differences between SUDEP and people at high risk and controls. (A) SUDEP cases show increased grey matter volume in the right hippocampus and parahippocampal gyrus compared to healthy subjects. (B) Similarly to SUDEP cases, subjects at high risk show increased grey matter volume in the right hippocampus and parahippocampal gyrus compared to healthy controls. (C) Compared to controls, grey matter volume is decreased in*

SUDEP cases in the pulvinar bilaterally. (D) Likewise, grey matter volume is decreased in those at high risk in the left pulvinar, compared to healthy controls. T-values are represented in the coloured bars. P 5 0.001, 30 voxel threshold extent; L = left; R = right [91]

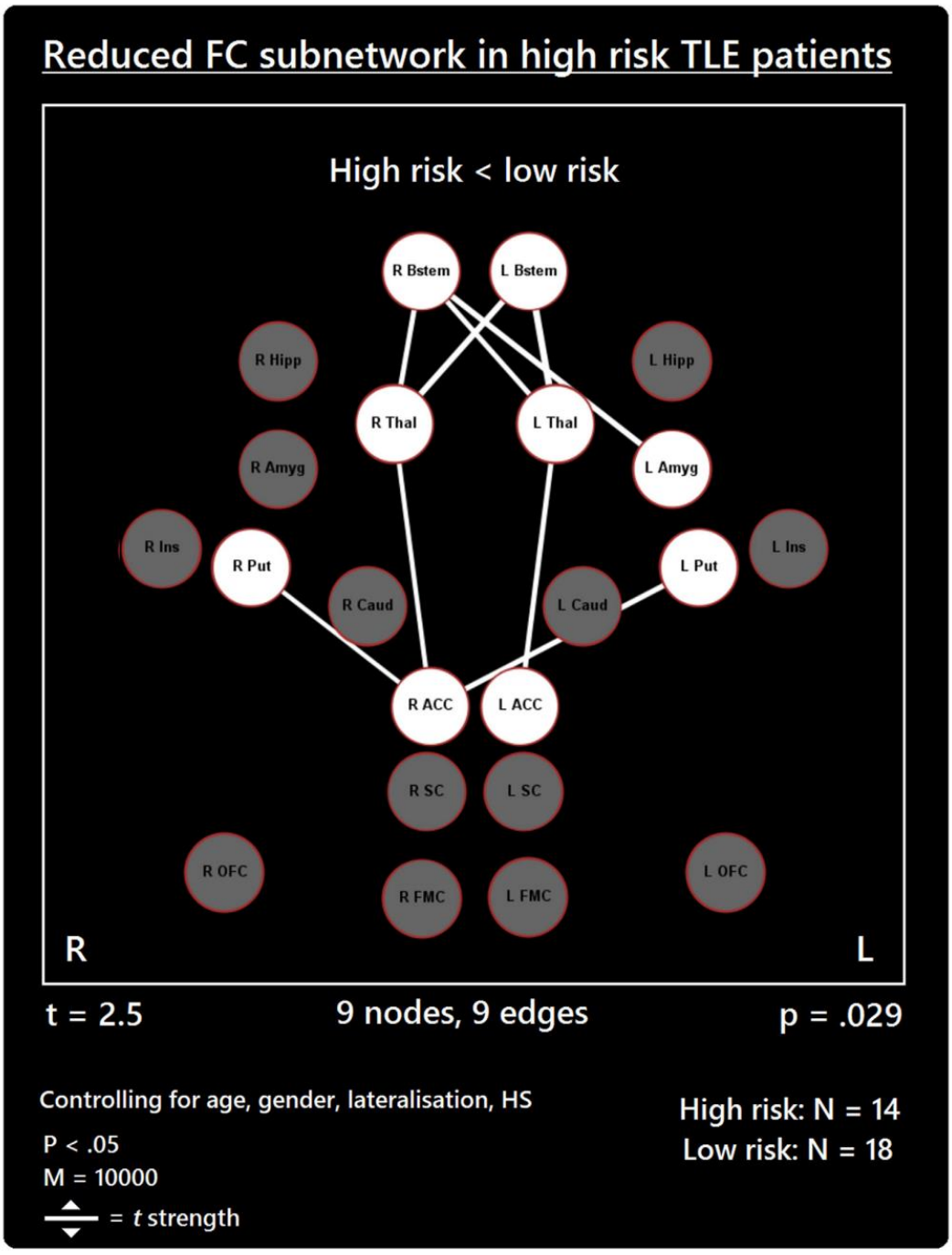

Figure 39 *Reduced functional connectivity (FC) subnetwork in high risk over lower risk of sudden unexpected death in epilepsy (SUDEP) patients. Subnetwork of reduced FC involving the bilateral brainstem (Bstem), bilateral thalamus (Thal), bilateral putamen (Put), bilateral ACC, and left amygdala (Amyg). L, left; r, right; HS, hippocampal sclerosis; t, t-statistic threshold; M, number of permutations; p value was set at <0.05, family-wise error rate (FWER) corrected. Nodes in white are those which were involved in the significant subnetwork. Red node outline represents search for reduced connectivity (high<low). Visualization using Gephi (https://gephi.org/). Typically, postictal apnoea and bradycardia progress to asystole and death. A crucial element of SUDEP is brainstem dysfunction, for which postictal generalised EEG suppression might be a biomarker. Dysfunction in serotonin and adenosine signalling systems, as well as genetic disorders affecting cardiac conduction and neuronal excitability, might also contribute. Because GTCS precede most cases of SUDEP, patients must be better educated about prevention. The value of nocturnal monitoring to detect seizures and postictal stimulation is unproven but warrants further study [92]*

Recently, Mueller and colleagues [93] have expanded the findings of the previous imaging studies [91, 92] by demonstrating a correlation between volume loss in the mesencephalon and autonomic nuclei in the medulla oblongata and reduced HRV. In particular, volume loss in the area of the mesencephalon/raphe nuclei and the upper medulla oblongata at the level of the obex fit together with the region in which alterations of neuromodulatory, neuropeptidergic, and monoaminergic systems have been shown in a histopathological study of patients who died of SUDEP [94].

These findings support the theory of a potential “ two-hit ” pathomechanism [93]. Excitotoxic effects of spreading seizure activity, are responsible for the mesencephalic abnormalities that, causing structural abnormalities beyond the focus, represent the “first hit”. Periaqueductal gray, colliculi, raphe, and cuneiform nucleus are dorso-mesencephalic structures involved in cardiorespiratory control but also in seizure control and arousal [95]. Their

structural abnormalities could, therefore, increase the severity of seizures but also may possibly prolong phases of impaired consciousness that prevent the patient from perceiving and reacting to a post-ictal respiratory depression. Impaired consciousness is observed both in witnessed SUDEP cases with preceding seizure, and also in those without [96]. Then, it suggests that loss of consciousness is not an epiphenomenon of a severe seizure but a crucial factor in the pathomechanism of SUDEP. Prolonged and more severe seizures extend the excitotoxicity and cause damage in previously unaffected brainstem regions. These last events are also more likely associated with cardiac arrhythmias and respiratory impairment that in turn could cause additional abnormalities in vulnerable regions, for example, hypoxemic damage in the watershed area that involves the nucleus of the solitary tract [97, 98]. Time by time, all of the "second hit" abnormalities will accumulate, structurally and functionally remodelling brainstem network and thereby destabilize the system [99, 100] until they are severe enough to result in the complete failure of the autonomic control in a situation of extreme demand. This two-hit scenario could at least play a role in a subset of patients with focal epilepsy without known genetic risk factors for SUDEP (Fig. 40, 41) [101, 102, 29].

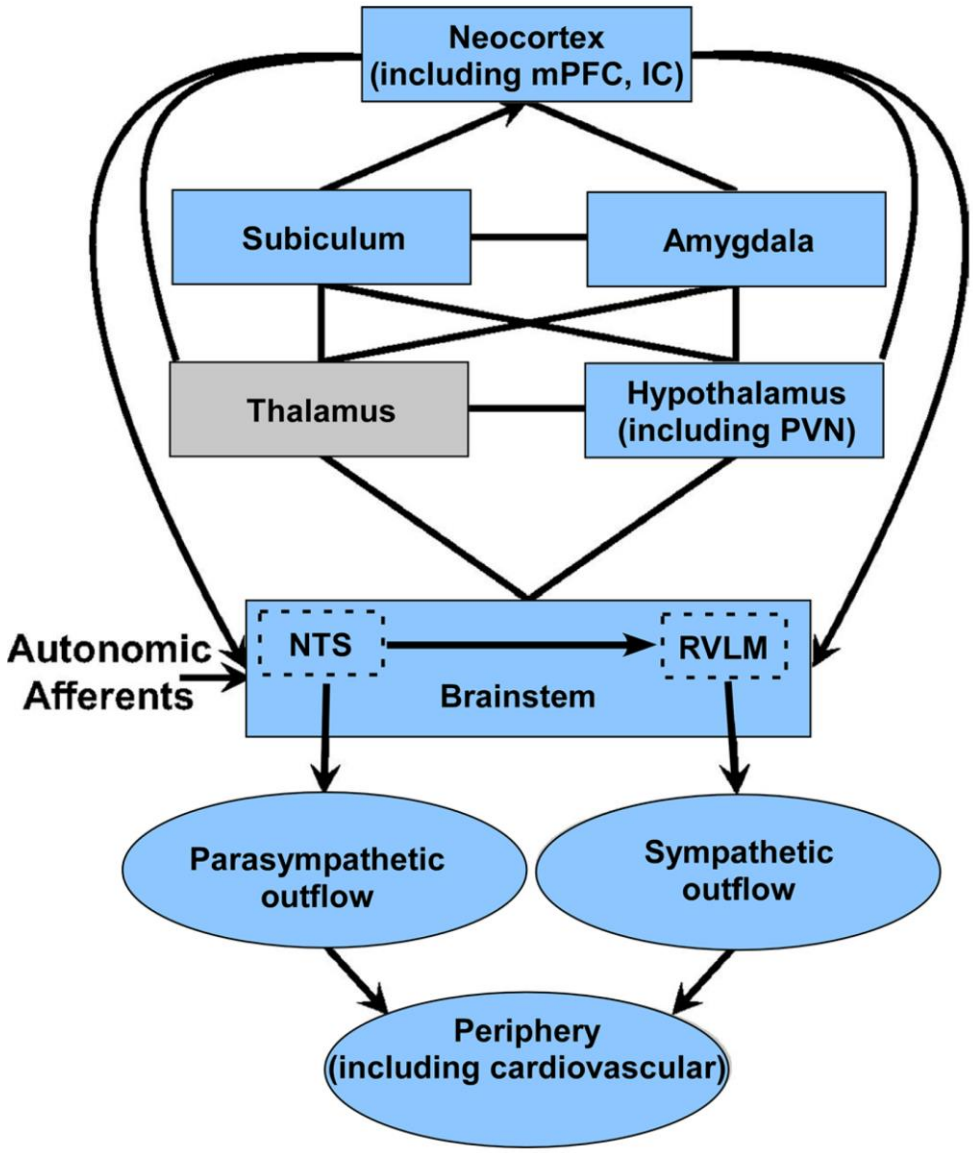

Figure 40 *Simplified schematic of pathways from cortical regions to the brainstem to influence autonomic outflow. Lines denote bidirectional connections, and arrows denote monodirectional projections. The key point is that pathways exist for seizure spread from limbic cortical areas (via subiculum) to hypothalamus (including paraventricular nucleus, PVN) and to brainstem regions serving as parasympathetic motor and sympathetic pre-motor functions. Relayed projections through the amygdala are even more prominent. Projections from neocortical regions, including insular cortex, have their own access to the hypothalamus and brainstem nuclei. The result is a multitude of pathways for seizure spread to*

impact autonomic and respiratory brainstem regions. NTS nucleus of the tractus solitarius, RVLM rostral ventrolateral medulla [102]

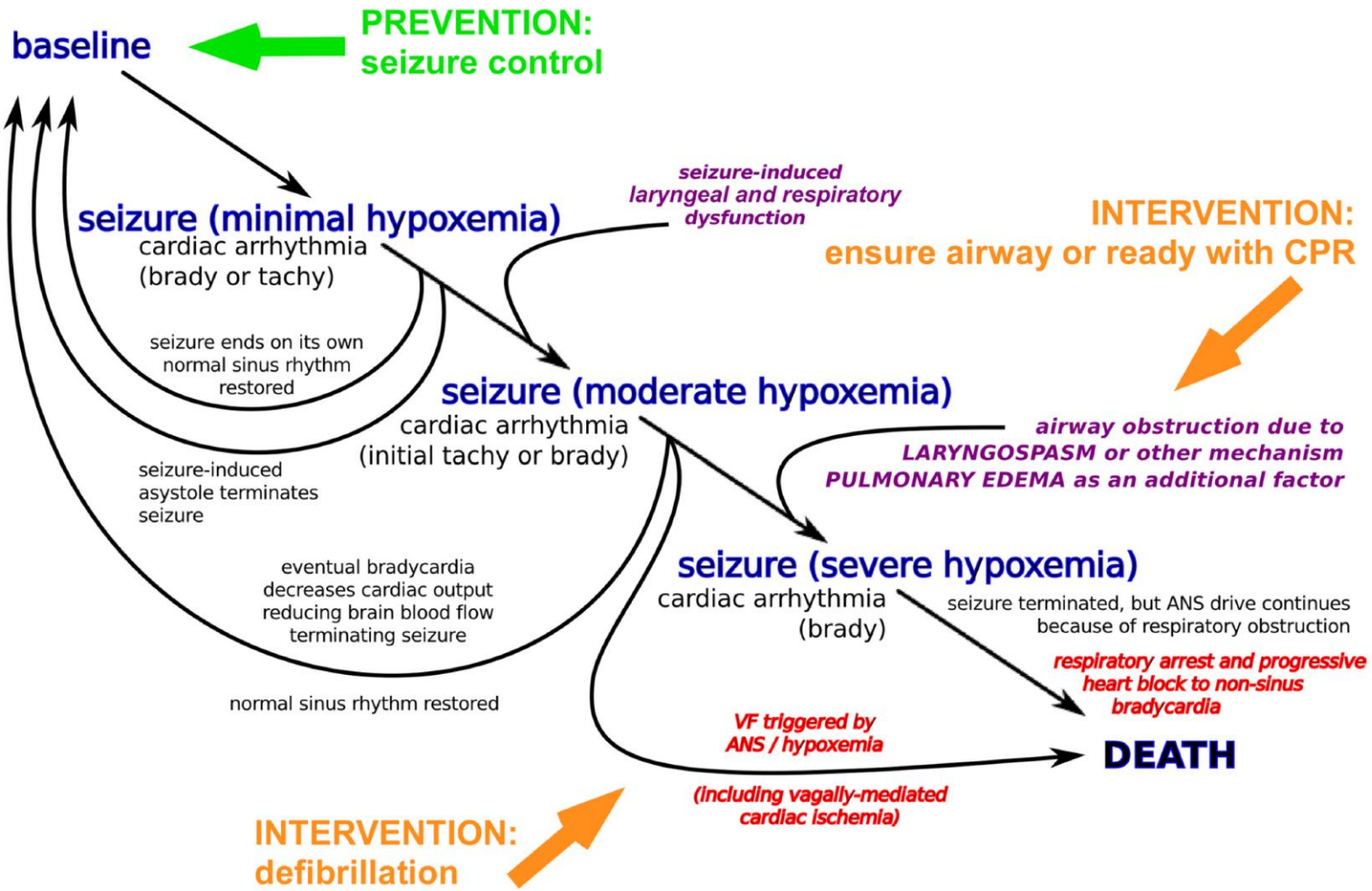
baseline
PREVENTION:
seizure control
seizure (minimal hypoxemia)
cardiac arrhythmia
(brady or tachy)
seizure-induced
laryngeal and respiratory
dysfunction
INTERVENTION:
ensure airway or ready with CPR
seizure ends on its own
normal sinus rhythm
restored
seizure (moderate hypoxemia)
cardiac arrhythmia
(initial tachy or brady)
airway obstruction due to
LARYNGOSPASM or other mechanism
PULMONARY EDEMA as an additional factor
seizure-induced
asystole terminates
seizure
eventual bradycardia
decreases cardiac output
reducing brain blood flow
terminating seizure
seizure (severe hypoxemia)
cardiac arrhythmia
(brady)
seizure terminated, but ANS drive continues
because of respiratory obstruction
respiratory arrest and progressive
heart block to non-sinus
bradycardia
normal sinus rhythm restored
VF triggered by
ANS / hypoxemia
DEATH
(including vagally-mediated
cardiac ischemia)
INTERVENTION:
defibrillation

Figure 41 *Schematic representation of possible outcomes mediated by autonomic overactivity associated with seizures. The majority of seizures will conclude on their own and permit a spontaneous recovery of autonomic derangements. Asystole will terminate the seizure and carry out to the same kind of recovery once the seizure ends. Ventricular fibrillation is one path to death, but this is a difficult condition to achieve and actually gets harder as the heart dilates with repeated seizures. The cause of death that could be the most likely, given that laryngospasm is a feature of every convulsive seizure, is seizure-induced laryngospasm sufficient to cause obstructive apnea. The apneic condition can persist beyond the end of the seizure (the severe bradycardia and poor ejection fraction will lead to decreased brain blood flow and terminate the seizure). Once the point of respiratory arrest is reached, relaxation of the laryngospasm or artificially opening the airway will not be sufficient for resuscitation. There is clearly a window of chance for cardiopulmonary resuscitation (CPR) to resuscitate patients at this point, but resuscitation depends on how quickly CPR can be applied. As a preventative measure, the best prevention remains good control of seizures. As interventions, the opportunity for resuscitation after VF or laryngospasm is short. Attention to differentiating between these two possibilities will save additional time [102]*

SUDEP- associated GTCS might lead to prolongation or intensification of symptoms as depressed level of consciousness and reflex responsiveness to environmental risks. With GTCS, the ictal sympathetic storm extends into the postictal period, increasing cerebrospinal and serum adrenaline and noradrenaline concentrations for more than 30 min in wich also hyperthermia, tachycardia, hypertension, electrodermal activity, and pulmonary oedema can be present. However in the meanwhile, also excess parasympathetic activity may be simultaneous activated, suggesting that postictal activation of both sympathetic and parasympathetic systems might contribute to SUDEP pathogenesis (Fig. 40, 42). Why some seizures and postictal states progress to SUDEP while the vast majority recover, have to be still explained.

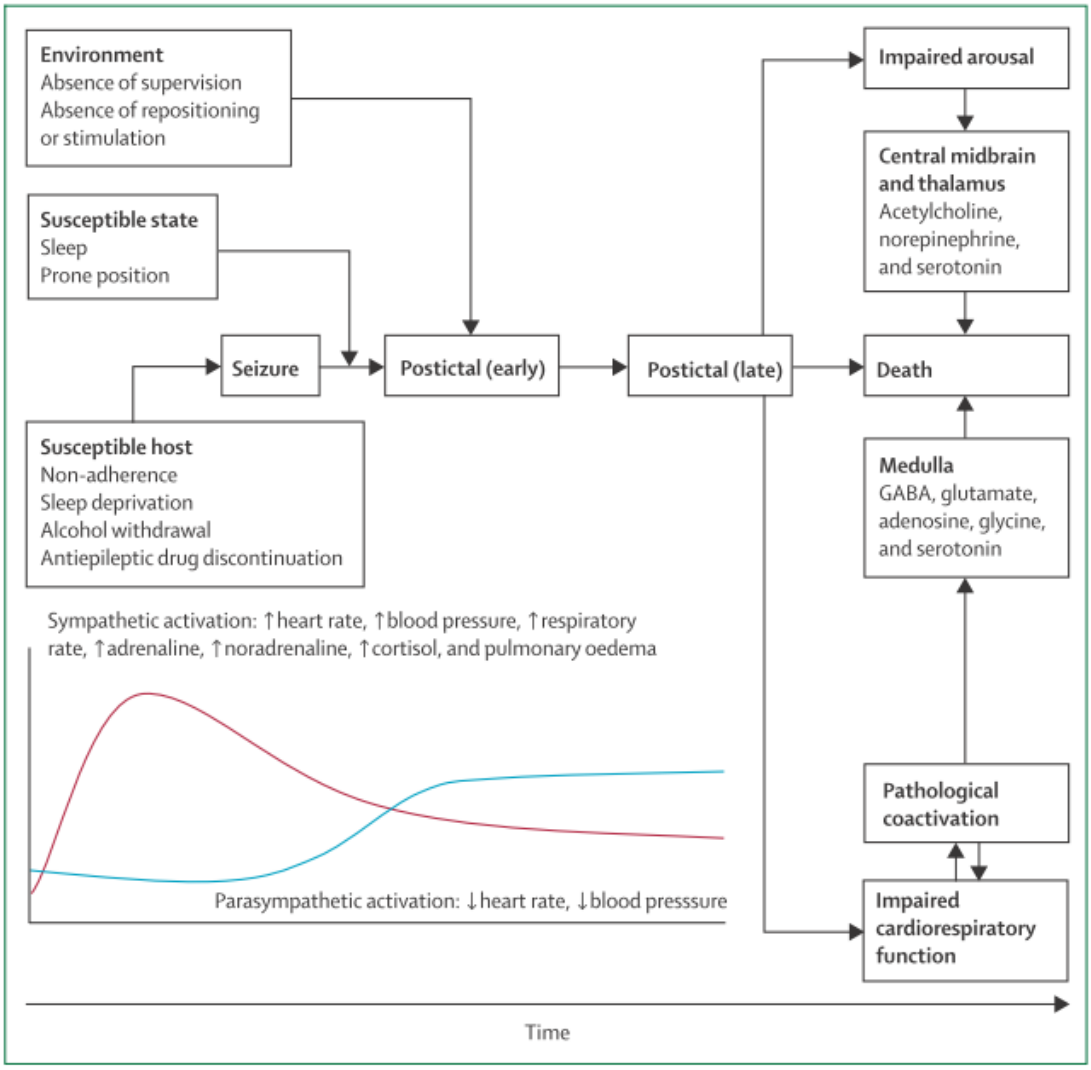

Figure 42 *Model of sudden unexpected death in epilepsy pathophysiology Suppression of brainstem function, arousal, and respiration seem to be crucial mechanisms, along with many other factors that can contribute to risk of sudden unexpected death in epilepsy [86]*

During postictal phase of a GTCS, SUDEP can take place as an heterogeneous event, with combinations of respiratory disorder, arousal failure, non-tachyarrhythmic cardiac dysfunction, and postictal generalised EEG suppression (PGES).

The MORTEMUS study, the MORTality in Epilepsy Monitoring Units Study, recorded SUDEPs in the course of epilepsy monitoring unit admissions in which

antiepileptic drugs were reduced or discontinued [103]. All of these cases began with an early, centrally mediated, severe dysfunction of both respiratory and cardiac activity after GTCS. Many of these patients had identical high-risk markers (epilepsy onset before 16 years, epilepsy duration >15 years, and frequent GTCS) and usually two or more GTCS in the day before death. However, no biomarkers could irrefutably differentiate SUDEP cases from survivors. This could significance the total phenotypic and genotypic heterogeneity of SUDEP patients.

Deaths resulted from postictal respiratory deficit, arousal impairments, and bradycardia leading to asystole; however tachyarrhythmias were never observed. Thus, most SUDEPs in patients with refractory epilepsy are not seizure-induced sudden cardiac death, are extremely rare and involves additional patho-physiological mechanisms or external stressors. Therefore, reduced heart rate variability and increased sympathetic tone-driven tachyarrhythmias are not main pathogenic factors in SUDEP. When parasympathetic hyperactivity is superimposed, possibly reflecting impaired brainstem autonomic regulation, pathological postictal coactivation of sympathetic and parasympathetic systems might make SUDEP more probable (Fig. 40). According to this theory, anxiolytics, alcohol, and withdrawal from brain depressants can cause seizures, autonomic instability, and death. Furthermore, a severe postictal hypotension can occur and might contribute to brainstem dysfunction and PGES. Peri-ictal apnoea with oxygen desaturation is frequent and can be severe, and on the other hand, postictal hypoxia might be due totally to apnoea, but pulmonary oedema might also add. Higher cortical areas, under the epileptic event, can influence the breathing, and can activate descending pathways to the lower brainstem. For example, it is well known that electrical stimulation of the human ventromedial prefrontal, insular, temporal cortex, hippocampus, and amygdala can each affect brainstem cardiorespiratory control and cause respiratory arrest. Nevertheless, seizure propagation in amygdala can produce apnoea and decrease oxygen saturation without the dyspnoea sensation. In conclusion, postictal respiratory effects of seizures seem those respiratory events most strongly related to SUDEP.

Postictal neurogenic pulmonary oedema can be sympathetically mediated, as shown by stellate gangliotomy in animal models, or can be also produced from hypoxaemia due to central apnoea. Infrequently, the negative pressure pulmonary oedema might also result from ictal laryngospasm. Although pulmonary oedema is unlikely to cause SUDEP, however, together to positional asphyxia, they might contribute to pathophysiology of the sudden death. Seizures most often cause sinus tachycardia, can determine asystole, bradycardia, atrioventricular nodal block, and peri-ictal atrial or ventricular tachyarrhythmias. Indeed, the really rare lethal peri-ictal tachyarrhythmia might more likely be caused by ischaemic heart disease. In fact, 90% of sudden cardiac arrest cases occurred in epileptic people with an underlying cardiovascular disease (Fig. 43).

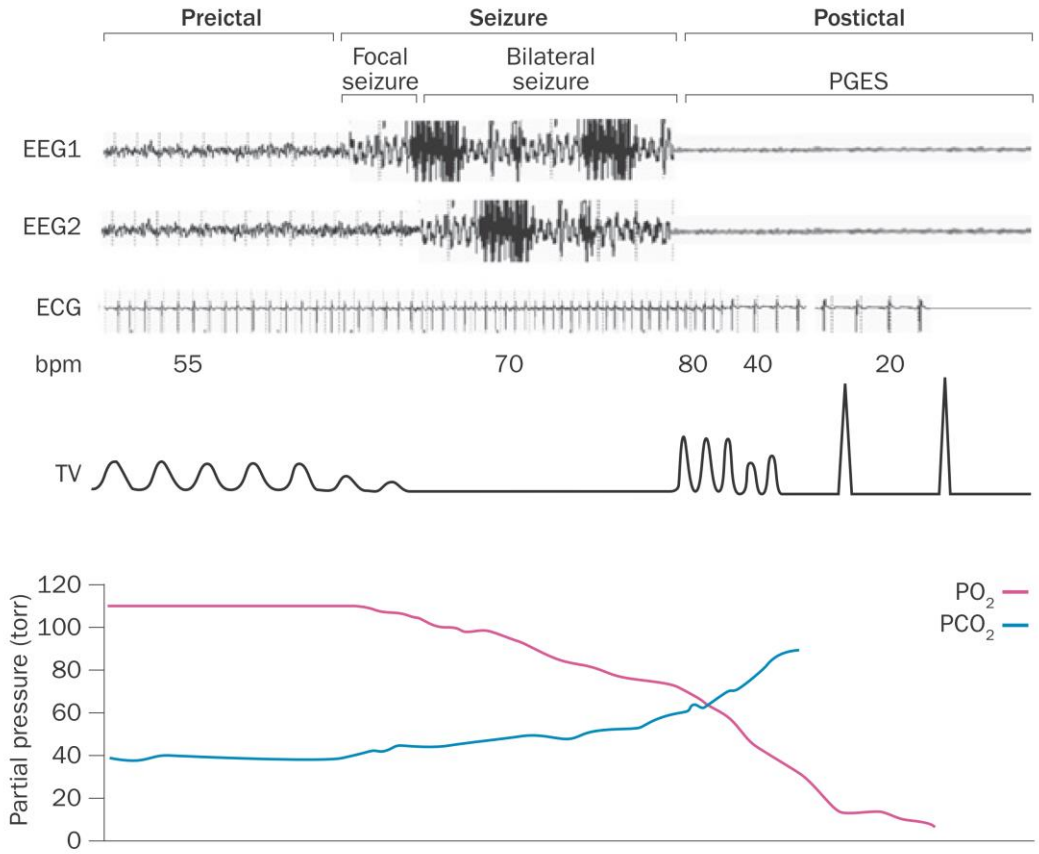

Figure 43 *Proposed sequence of events surrounding a theoretical case of 'respiratory SUDEP'. When a focal seizure begins, hypoventilation causes a fall*

in PO_2 and a rise in PCO_2. On seizure generalization, apnoea occurs, causing acceleration of the blood gas changes, and tachycardia. In the postictal state, PGES occurs due to loss of drive from the ascending arousal system, but there is transient tachypnoea due to powerful drive from decreased PO_2 and increased PCO_2. However, ventilation is inadequate due to either airway obstruction or decreased effort. PO_2 drops to dangerously low levels causing terminal gasps, but autoresuscitation fails. The patient's heart slows due to parasympathetic drive and blood gas derangements, and then stops. Abbreviations: ECG, electrocardiogram; PCO_2, partial pressure of carbon dioxide; PGES, postictal generalized EEG suppression; PO_2, partial pressure of oxygen; TV, tidal volume [40]

Through neuro-cardiac genes involved in cardiac channelopathies, patients with arrhythmia and sudden cardiac death risk, might be predisposed also to epilepsy if the affected ion channel is expressed in their brain [29, 36, 40]. So, genetic causes of LQT, especially LQT2 can also cause epilepsy, and on the other hand, deleterious cardiac gene mutations might be over-represented in SUDEP cases [101, 30]. However, because neither one LQT gene is common to all cases, nor LQT mutations are common in these patients, so as LQT arrhythmias (eg, torsades de pointes) have not occurred in hospital-observed SUDEPs, and SUDEP cases do not show QTc prolongation on ECG, the importance of LQT on SUDEP is blurred. In the brainstem are expressed genes associated with LQT (eg, KCNH2) that might support peri-ictal central cardiorespiratory dysfunction. In conclusion, the rare but significant increased frequency of cardiac channelopathy mutations and abnormal ventricular conduction pattern (QRS <110 per ms, morphology of incomplete right or left bundle branch block or intraventricular conduction delay) in SUDEP cases matched with controls, point out that cardiac alterations might contribute in some cases.

From the MORTEMUS experience, the data indicate that most of SUDEPs result from a combination of postictal dysfunction of cardiorespiratory control and depression of consciousness. The primary integrative centres and final output

pathways for breathing and autonomic output, as well as the modulatory systems associated with maintenance of arousal, are localized in the brainstem (Fig.44).

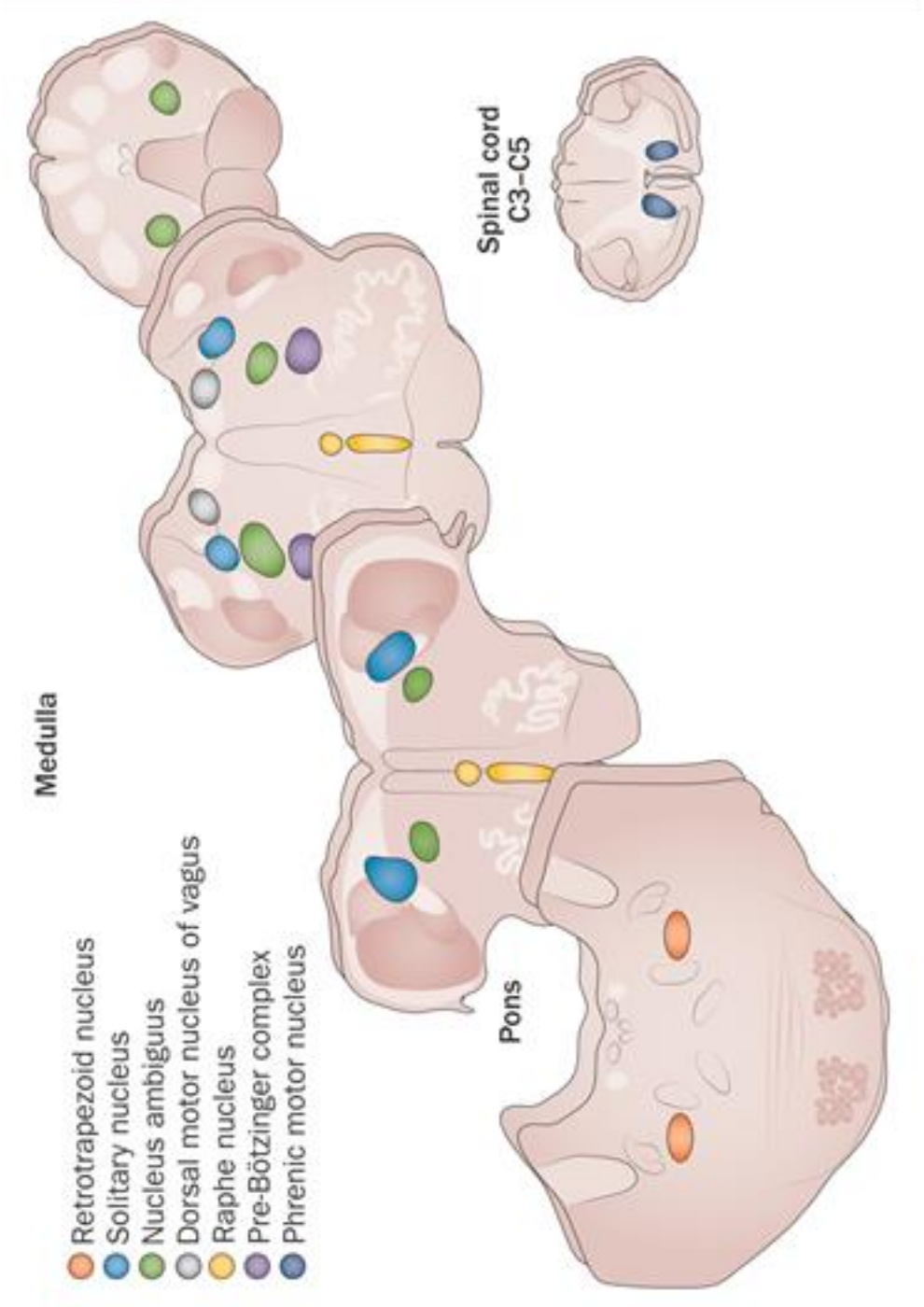

Figure 44 *Anatomical distribution of brainstem nuclei involved in cardiorespiratory control. A seizure in the forebrain activates pathways that lead*

to dysfunction of brainstem nuclei critical for cardiorespiratory control. Seizure spread into the brainstem also disrupts both ascending and descending arousal systems [40]

Forebrain and limbic seizures, that reduce the postictal modulation of brainstem cardiorespiratory networks, decrese the activity of monoaminergic, cholinergic, and glutamatergic modulatory systems that affect breathing, autonomic control, and arousal, are heavily implicated in SUDEP.

Severe hypercapnia occurs after GTCS in epileptic patients, and slowly resolves over about 20 min. This prolonged recovery persists long after breathing resumes, it represents the postictal depression of carbon dioxide chemoreception, which could involve the inhibition of serotonergic neurons as well as other monoaminergic, cholinergic, and glutamatergic neurons implicated in respiratory chemoreception.

Serotonin dysfunction might increase the risk of SUDEP; the 5-hydroxytryptamine (5-HT) type 2C receptor knockout mouse was one of the first animal models of SUDEP to be developed, with spontaneous and audiogenic seizures followed by respiratory arrest. Only a study with preliminary data on epileptic patients suggested that selective serotonin reuptake inhibitors are associated with reduced peri-ictal oxygen desaturation in focal seizures, although not in generalised tonic-clonic seizures, the seizure type most associated with SUDEP [104]. However, it has been recently hypothesized that peripheral serum serotonin levels in epileptic patients are inversely correlated with potential biomarkers of SUDEP [105]. In fact, it was observed an inversely associated interictal serum serotonin level with a shorter period of PGES. Furthermore, a proportional variation in serotonin (postictal-interictal) was inversely associated with a shorter duration of tonic phase of generalized seizures. These data suggest that peripheral serum serotonin levels may play a role in seizure and earlier post-seizure outcome.

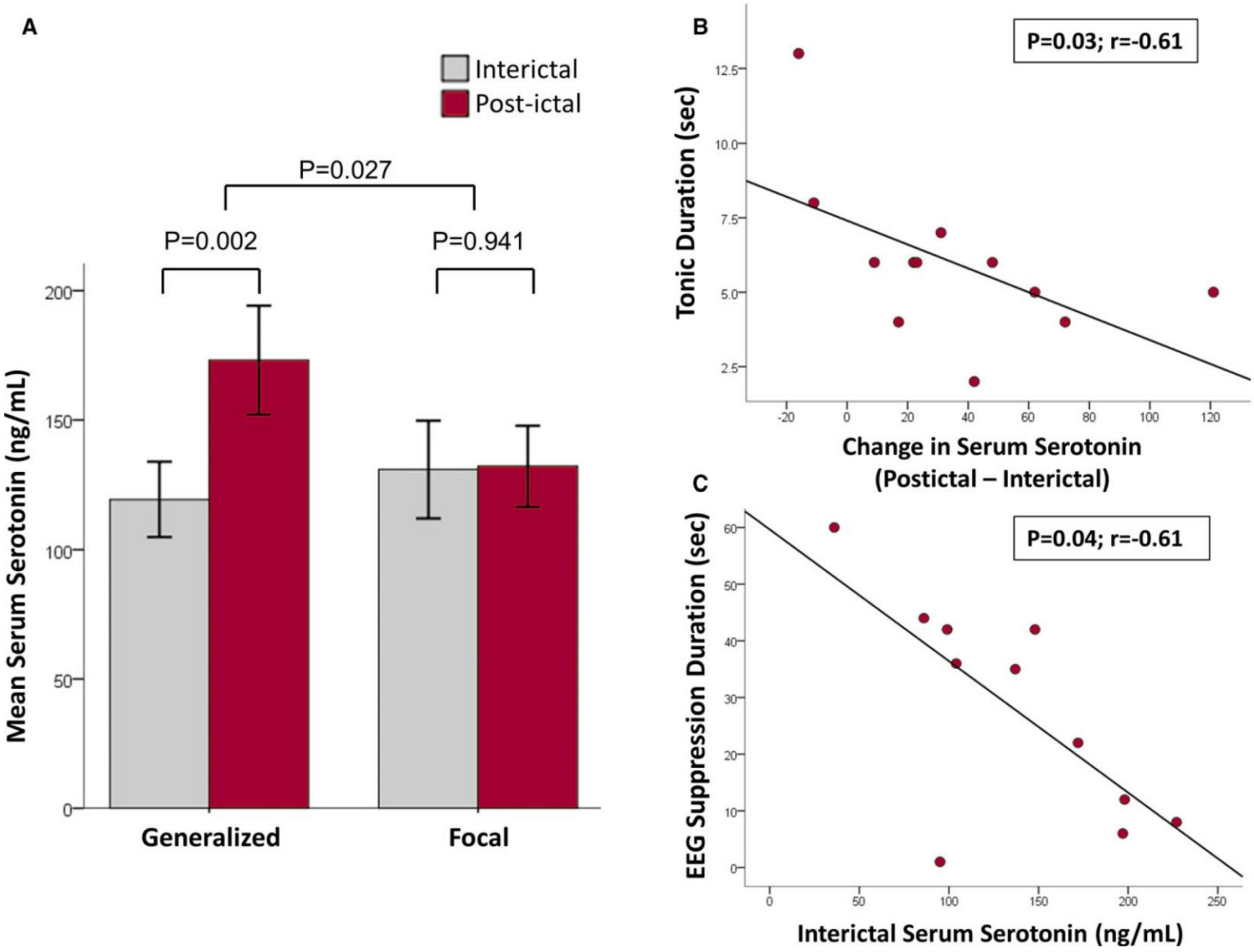
A
Interictal
Post-ictal
P=0.027
P=0.002
P=0.941
Mean Serum Serotonin (ng/mL)
200
150
100
50
0
Generalized
Focal
B
P=0.03; r=-0.61
Tonic Duration (sec)
12.5
10.0
7.5
5.0
2.5
-20
0
20
40
60
80
100
120
Change in Serum Serotonin
(Postictal – Interictal)
C
P=0.04; r=-0.61
EEG Suppression Duration (sec)
60
50
40
30
20
10
0
0
50
100
150
200
250
Interictal Serum Serotonin (ng/mL)

Figure 45 *A, Serum serotonin levels increase after seizures. The mean serum postictal serotonin levels (in ng/mL) are shown in red bars and interictal serotonin levels are shown in gray bars for the 2 seizure groups: generalized (n = 19) and focal (n = 26). Elevated levels of postictal serum serotonin after generalized seizures were statistically significant when compared to interictal levels (P = .002), but not after focal seizures (P = .941, paired sample t test). Also, the change in serum serotonin level (postictal-interictal) was statistically significant (P = .027, independent 2-sample t test) between the generalized and focal seizure groups. B, Association of tonic duration with serum serotonin. The differences between postictal and interictal serum serotonin levels were plotted against the tonic duration of the seizure. Increased levels of serotonin were significantly associated with reduced duration of tonic phase during generalized seizures (n = 12). C, Association of postictal generalized electroencephalographic (EEG) suppression (PGES) duration with serum serotonin. The interictal serum serotonin levels were plotted against the PGES duration of the seizure. Higher interictal serotonin was significantly associated with shorter period of EEG suppression during generalized seizures (n = 11) [105]*

Serotonergic neurons are central chemoreceptors that stimulate breathing and induce arousal from sleep in response to hypercapnia. Their function is important to prevent asphyxia during postictal apnoea with airway obstruction. Medullary serotonergic neurons are inhibited by seizures, and this event might contribute to ictal apnoea and prolonged postictal hypoventilation. For this reason exists a strong link among serotonin and SIDS with SUDEP (table 7). Thus, serotonergic therapies have the potential to reduce SUDEP risk.

Patodia and co-workers [94], have recently explained and clarified the main role of neuronal populations in the ventrolateral medulla, including in particular the putative human PBC, and the medullary raphe. It is the first time that are described anatomo-clinical correlation between brainstem nuclei and serotoninergic patway failure in SUDEP and or with duration of seizures. Significant findings included also a reduction in somatostatin neurons and

neurokinin 1 receptor labelling in the ventrolateral medulla in sudden death in epilepsy compared to controls. They also noted a significant reductions in medullary neuromodulatory systems of serotoninergic and galaninergic networks. These evidences could have a translational value because the epilepsy-mediated pathology in medullary respiratory neuronal groups might be associated with a vulnerability meaning for SUDEP.

Other brainstem neurotransmitters and neuromodulators might also perform an important part in SUDEP pathophysiology. There is considerable interconnection between the ascending and descending projections and functions of brainstem neurons that release serotonin, noradrenaline, acetylcholine, and glutamate. These brainstem neurons represent the ascending arousal system associated with control of wakefulness, and also characterize the modulatory respiratory output for the carbon dioxide chemoreception. Nevertheless, adrenergic fibers acting on the heart, might also contribute to SUDEP mechanisms. Various other contributors to cardiorespiratory control resulted relevant for SUDEP. For example, adenosine is important for seizure-induced inhibition of breathing, effect prevented by caffeine, an adenosine receptor antagonist. Adenosine receptors are expressed throughout the nervous system, and activation of inhibitory A 1 receptors has anticonvulsant effects. In the course of seizures, adenosine levels increase in the brain and suppress seizure activity. However, activation of adenosine receptors, in particular A 1 receptors of RTN in the brainstem, also induces severe respiratory depression. If seizures caused an increase in extracellular adenosine in the brainstem, this would lead to hypoventilation, apnoea, and arrhythmias in the postictal period. At the same time, caffeine can lower seizure threshold via effects on cortical excitability or by impairing restorative sleep. Reuptake of adenosine into the astrocyte is mediated via two types of equilibrative nucleoside transporters and driven by metabolic clearance of adenosine via phosphorylation into AMP by ADK (Fig. 46). After a damage to the brain, as following ischemia or seizures, astrocytic ADK expression undergoes a biphasic response: acute downregulation of the enzyme within hours as an acute neuroprotective response and later astrogliosis associated with overexpression of ADK within days or weeks. Consequently, ADK may be upregulated and cause adenosine deficiency in

epileptogenic temporal lobe and sclerotic hippocampus from epileptic patients (Fig. 47) [106].

Nevertheless, it was also very recently discovered in animal models, that a high morphological complexity of PBC astrocytes correspond to their functional role in providing structural/metabolic support and modulation of the crucial neuronal circuits essential for breathing [107, 108]. It has been hypothesized that astrocytes can modulate neuronal excitability, synaptic transmission, and plasticity. An increase of intracellular [Ca^{2+}] triggers releasing of signalling molecules or "gliotransmitters" (such as ATP/adenosine, D -serine, and others). By this release of gliotransmitters, astrocytes may influence activities of neural circuits controlling sleep, feeding, and chemosensing.

Whereas activation of inhibitory A 1 receptors is advantageous in epilepsy, chronic pain and cerebral ischemia, inhibition of facilitatory A 2A receptors has profound neuroprotective effects, which approved for Parkinson's disease (Fig. 49) [109]. Adenosine represents a promising candidate for restoring the functional imbalance between brain dopamine and glutamate activities in these network believed to underlie the genesis of schizophrenia symptoms, as well as restoring homeostasis of bioenergetics.

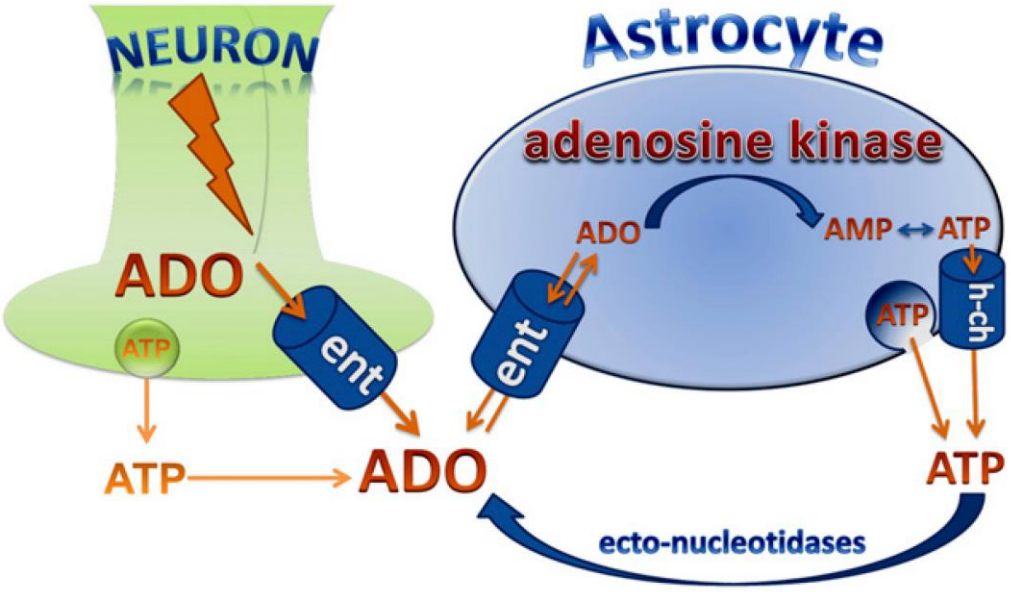

Figure 46 *Astrocytes constitute a sink for the metabolic clearance of adenosine in the brain. Whereas neurons are capable of releasing adenosine directly, astrocytes can release ATP via vesicular release and/or by direct release through hemichannels (h-ch). Extracellular ATP is rapidly degraded into adenosine (ADO) by a series of ectonucleotidases. Adenosine can also be released directly via equilibrative nucleoside transporters (nt). Intracellular adenosine levels are largely controlled by adenosine kinase, which phosphorylates adenosine into AMP. Small changes in adenosine kinase activity rapidly translate into major changes in adenosine. Intracellular astrocytic adenosine kinase is considered to be a metabolic reuptake system for adenosine [106]*

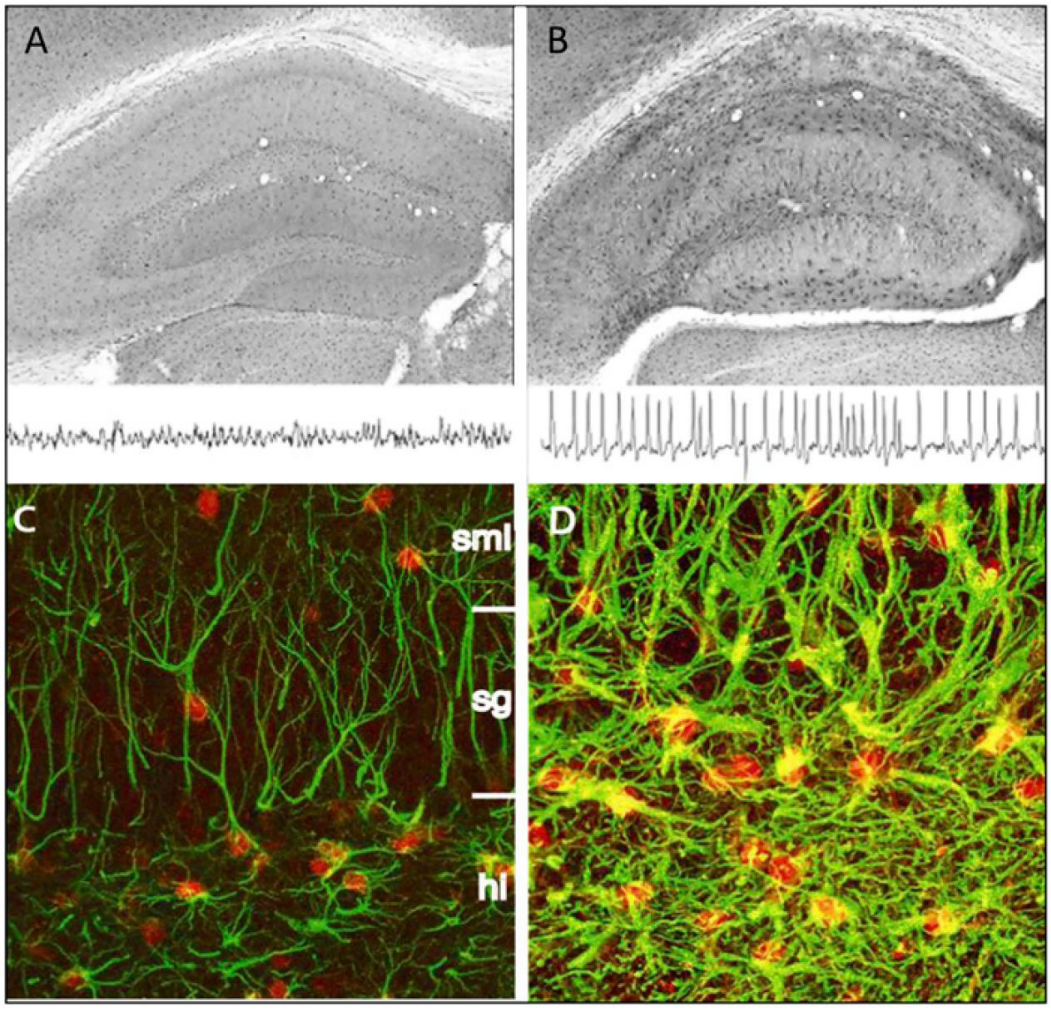

Figure 47 *Astrogliosis and overexpression of ADK in a mouse model of temporal lobe epilepsy. (A and B) Brains from kainic acid (KA)-treated mice were taken at 4 weeks after either intrahippocampal KA or saline injection. Transverse brain sections of the KA-injected brain hemisphere were stained for ADK-immunoreactivity. Note prominent overexpression of astrogliosis in association with spontaneous seizures activity in B. (C and D) Colocalization of ADK and GFAP immunofluorescence, as seen by confocal laser scanning microscopy. Transverse brain sections of a KA-injected animal taken 4 weeks after the injection and those from a naïve control animal were double stained for ADK (red) and the astrocyte marker GFAP (green). Optical sections were digitized at high magnification and superimposed for display. (C) Dentate gyrus of a control animal. Note that the cell bodies of individual astrocytes (green processes) are stained for ADK (red). (D) Dentate gyrus of a KA-injected animal. Note the*

massive gliosis characterized by the swelling of cell bodies, the enlargement of astrocytic processes, and the expansion of ADK-immunoreactivity into the processes (colocalization of ADK and GFAP, yellow). sp, Stratum pyramidale; sml, stratum moleculare; sg, stratum granulosum [106]

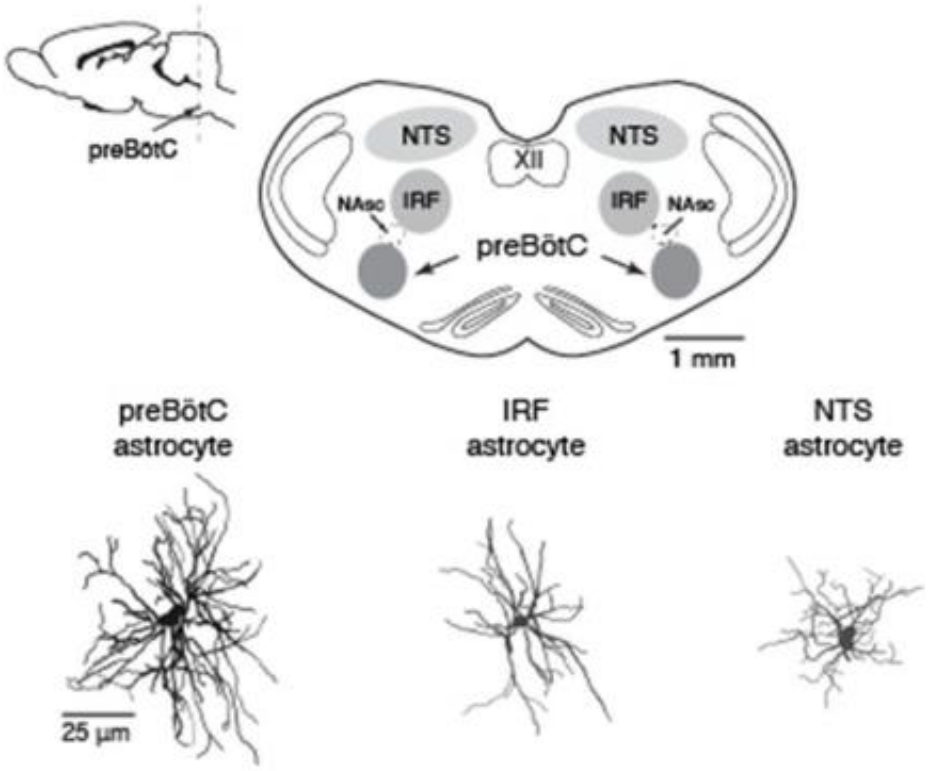

Figure 48 *In the rodent brainstem, astrocytes (the most abundant and structurally complex glial cells of the CNS) have been shown to play a role in chemosensing and modulation of respiratory circuit activity, including the rhythm-generating circuits of the preBötzinger complex (preBötC) located within the ventrolateral medullary reticular formation. However, structural properties of astrocytes residing within different brainstem regions are unknown. In this study astrocytes in the preBötC, an intermediate reticular formation (IRF) region with respiratory-related function, and a region of the nucleus tractus solitarius (NTS) in adult rats were reconstructed and their morphological features were compared. Detailed*

morphological analysis revealed that preBötC astrocytes are structurally more complex than those residing within the functionally distinct neighboring IRF region, or the NTS, located at the dorsal aspect of the medulla oblongata. Authors posit that morphological complexity of preBötC astrocytes reflects their functional role in providing structural/metabolic support and modulation of the key neuronal network essential for breathing [107]

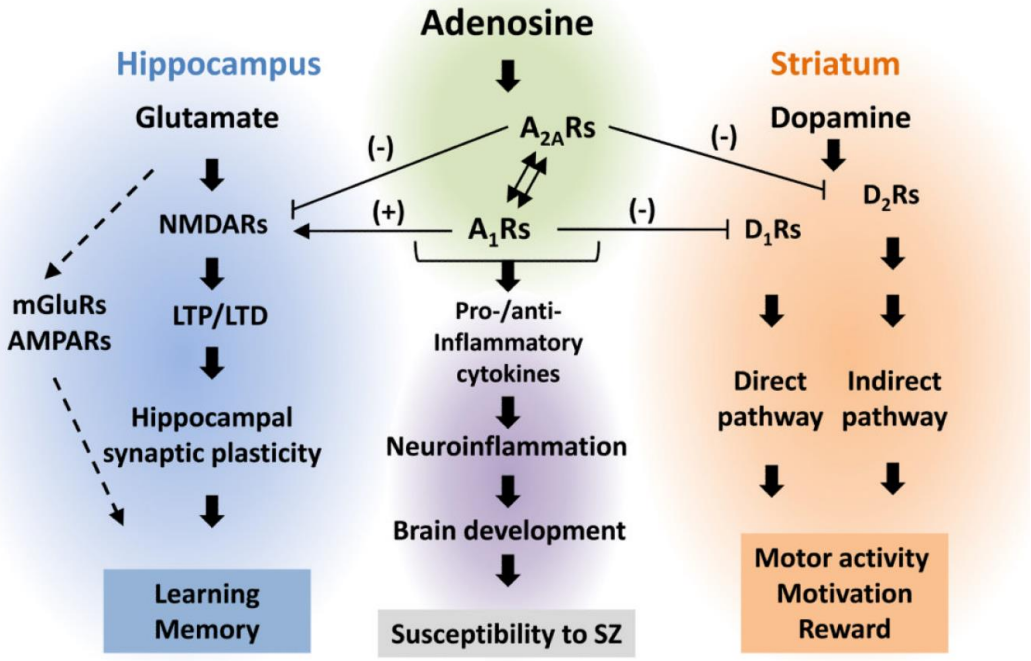

Figure 49 *Molecular basis and pathways of adenosine-based manipulation of glutamatergic and dopaminergic neurotransmission: dopamine receptors (DRs), long-term potentiation (LTP) and depression (LTD), N-methyl- D -aspartic acid receptors (NMDARs), schizophrenia (SZ) [109]*

GABA and opioid receptors could also be essential for SUDEP because GABA and opioid receptor activation can inhibit breathing. These receptors might be related to active inhibition of cardiorespiratory control centres by descending projections during seizures and, importantly, activation of these receptors by

pharmacological treatment or recreational drugs (including alcohol) could increase the risk of postictal cardiorespiratory collapse.

These and other data suggest that drugs that modulate serotonin, adenosine, or other neurochemical systems might contribute to prevent SUDEP, although the finest pharmacological objectives remain unclear.

Objectives

SIDS is characterized by the death of an infant that cannot be explained, despite a systematic case examination, including death scene investigation, autopsy, and review of the clinical history. Several differing and sometimes contradictory hypotheses of the underlying mechanisms of SIDS have been proposed. The most reliable seems to be the "triple risk hypothesis". Based on this theory, unexpected infant deaths might arise as a consequence of the combination of three factors coming together: a vulnerable infant, a vulnerable phase of development and a final insult occurring in this window of vulnerability. Recently, a unified neuropathological theory contributes to describing SIDS. According to this, serotonergic neurons play a crucial homeostatic function in the cardiorespiratory brainstem centres. In SUDEP, which has clinical parallels with SIDS, alterations to medullary serotoninergic neural populations and autonomic dysregulation have been shown too. Cardiac, sympathetic, and respiratory motor activities can be viewed as a unified rhythm controlled by brainstem neural circuits for effective and efficient gas exchange. Nevertheless, evidence suggests likely genomic complexity and a degree of overlap among SIDS, Sudden Intrauterine Death (SIUD), SCD and SUDEP.

The main objective of present research has been to describe abnormalities in brainstem nuclei, in part because robust molecular or functional examination of these nuclei has not been carefully performed. We intended to perform detailed functional mapping of these brainstem nuclei. Specifically, the cardiorespiratory and cardioventilatory coupling can be understood as a unified vital rhythm controlled by brainstem neural circuits. By cardiorespiratory coupling, we mean the Respiratory Sinus Arrhythmia (RSA) that is characterized by a heart rate (HR) increasing during inspiration and an HR decreasing during expiration. Conversely, CardioVentilatory Coupling (CVC) is considered the influence of heartbeats and arterial pulse pressure on respiration with the tendency for the next inspiration to start at a preferred latency after the last heartbeat in expiration. We hypothesized that these two reflex systems are not separate, but constitute an integrated network. We defined this last concept as "unifying theory". By studying all the

maps of the cardiorespiratory nuclei of the Literature, we integrated this concept into a reworking map of brainstem nuclei that could also explain the gasping and blocking cardiorespiratory of sudden deaths. Thus, understanding the theory of a unique, unifying cardiorespiratory network may help to identify the mechanisms underlying the sudden deaths. Moreover, brainstem nuclei anomalies and/ or malformations may represent biomarkers that identify specific subpopulations of infants, cardiopathic or epileptic patients, holding a prognostic value.

The final ambition of the present study has been to investigate possible etiopathogenetic implications and potential roles of RSA-CVC coupling disarrangement, as a prognostic clinical-pathological signature of sudden death. The theory of a unique, unifying cardiorespiratory network, it has been recently demonstrated in some cases of arrhythmia, in some cases of SUDEP with striking systolic hypotensive changes and in some cases of SIDS too.

Material and Methods

We investigated articles, reviews indexed in PubMed describing putative neuro-cardiac-respiratory genes and cardiorespiratory, and cardioventilatory coupling theories. Specifically, we evaluated cardiorespiratory brainstem nuclei and whole brains of fetal, infant, and adult autopsies respectively to detect congenital errors in the cerebral development or malformations, but also to identify the "normal" or "dysplastic" brainstem centres.

1. Meta-methods

We have decided to entitle this chapter "meta-methods" because we do not want to describe all the used experimental methods that will be illustrated in the original paper. It is our intention here only to explain, by our recently published article "From fix to fit into the autoptic human brains", the rational and/or the "behind the scenes" of the methods which have particularly influenced our research.

1.1 "From fix to fit into the autoptic human brains"

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From fix to fit into the autoptic human brains

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Abstract

Formalin-fixed, paraffin embedded (FFPE) human brain tissues are very often stored in formalin for long time. Formalin fixation reduces immunostaining, and the DNA/RNA extraction from FFPE brain tissue becomes suboptimal. At present, there are different protocols of fixation and several procedures and kits to extract DNA/RNA from paraffin embedding tissue, but a gold standard protocol remains distant. In this study, we analysed four types of fixation systems and compared histo- and immunostaining. Based on our results, we propose a modified method of combined fixation in formalin and formic acid for the autoptic adult brain to obtain easy, fast, safe and efficient immunolabelling of long-stored FFPE tissue. In particular, we have achieved an improved preservation of cellular morphology and obtained success in *post-mortem* immunostaining for NeuN. This nuclear antigen is an important marker for mapping neurons, for example, to evaluate the histopathology of temporal lobe epilepsy or to draw the topography of cardiorespiratory brainstem nuclei in sudden infant death syndrome (SIDS). However, NeuN staining is frequently faint or lost in *post-mortem* human brain tissues. In addition, we attained Fluoro Jade C staining, a marker of neurodegeneration, and immunofluorescent staining for stem cell antigens in the postnatal human brain, utilizing custom fit fixation procedures.

Introduction

It is often difficult to obtain reproducible immunohistochemical signals and to perform genomic analysis from *post-mortem* brain tissues, because the elapsed time from the death of the patient, the time of fixation, and the time of embedding may influence the preservation of antigens and nucleic acids.[1-5] In fact, researchers frequently prefer to exclude autoptic brain tissues from their experimental cases because the quality of nucleic acids varies in different *post-mortem* samples. However, it would be very interesting to obtain good quality autoptic tissue, both to have autoptic "normal" controls and to compare the genomic data from surgical and pathological specimens *versus* autoptic brain tissues.

The effect of *post-mortem* delay and fixation time are crucial preanalytical variables that can influence genomic analysis and immunohistochemistry outcome. A delay on fixation, as well as the time of formalin fixation can reduce the immunostaining efficiency.[1,6] Preservation of proteins in relation to the *post-mortem* delay is sporadically calculated, and, in the routine setting, the *post-mortem* delay might range from a few hours to several days. Several biochemical changes occur in the cadaver, including a decrease in the pH, which influences the exposure of target proteins and epitopes. However, this detection loss has received little attention from the scientific community.[7,8] The usual fixative is 10% formalin, which consists 3.7% formaldehyde in water with 1% methanol. Formaldehyde is a reactive electrophilic substance; it reacts promptly with various functional groups of biological macromolecules in a cross-linking mode.[9] Formalin degrades DNA and masks protein epitopes by forming covalent bonds between neighbouring proteins or nucleic acids. The initial cross-linking is formed by 24 to 48 h after penetration while the concluding may take about 30 days to generate the stable covalent cross linkages. The initial phase of the reaction is reversible while the later reaction becomes irreversible when there is high number of covalent bonds produced. This modifies the physicochemical state of tissue such as redox and membrane potentials of the tissue, changing surface charges and therefore alters the reactivity of cellular components. The reversible nature of the initial phase reaction is the basis of antigen retrieval in molecular techniques. To unmask epitopes for antibody binding in formalin-fixed tissue, several enzymatic and heat-induced antigen retrieval methods have been introduced. These methods are not completely successful for all antibodies and not all unmasking procedures are usable for every antibody.[9-14] Formic acid is generally used to virtually eliminate the risk of handling infectious material of autoptic tissues from patients with prionic diseases.[15] Formic acid pre-treatment may also produce an antigen retrieval effect, enhancing immunostaining.[16-17]

The present study aims at examining the effect of four different types of fixation on the morphological preservation, histostaining and immunoreactivities of several antibodies, against formalin-sensitive antigens, that do not produce optimal result in *post-mortem* human brain tissue. In other words, we intend to compare formalin fixation, *i.e.* the standard and more common method, *versus* the traditional fixative solution mixed with formic acid and/or acid acetic respectively. We chose formic acid for its anti-prionic property and putative unmasking capacity. At the same time, we selected acetic acid, an efficient dehydrating agent,

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Key words: Formalin fixation; formic acid; glacial acetic acid; ethanol; *post-mortem* brain tissue; NeuN; Fluoro Jade C; neural stem cell immunofluorescence staining.

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to reduce oedema of cerebral tissues. Clarke (1851) was the inventor of using a combination of alcohol and acetic acid for fixation, yet many authors attribute Carnoy (1887).[18,19] Currently, the formalin, alcohol and acetic acid mixed protocol is utilized for foetal brain fixation.[20,21] We decided to modify and utilize the foetal protocol for perinatal and postnatal brains, dividing the time of fixation in steps of four days for each passage (Table 1). We adapted protocols for adult brain fixation according to this postnatal protocol, to unify all of protocols in time point of multiple of four days. We chose the final time of formalin fixation in twenty days, according to the time course for both generation of stable covalent cross linkages, global volume shrinkage and formalin-induced changes of myelin.[9,22,23] We cut every fresh human brain in coronal macroslices of 1 cm of thickness to have fast penetration and fixation of the tissue.[9] Every macroslice for each brain has been treated with different protocols to be tested, so, each brain has been fixed by all of our different protocols. The standard formalin fixation has been considered our internal negative control, because from this tissue we have never obtained positive immunostaining against formalin-sensitive antigens, as it usually happens with *post-mortem* brain tissues.

We propose a protocol to standardize the NeuN staining of autoptic brains by automatic machine staining. Furthermore, we propose to use Fluoro Jade C, a histochemical technique currently used only to detect neurodegeneration in animal models, for human autoptic diagnoses.[24,25]

Materials and Methods

Human adult brain tissue

The study was approved by the "S. Anna" University Hospital Ethical Committee within the Project for Human White Matter Study of the Ferrara's University.[26] *post-mortem* cerebral tissue was obtained from five adult patients without any previous history or symptoms of neurological disease. To over cross ethical problem related to the precocious extraction of the brains, and to reduce the variability of preanalytical phase, all tissues were extracted 40 h *post-mortem*.[8] During autopsy, the fresh brain was cut in coronal macro-sections of 1 cm thickness. In this way, four serial sections included the hippocampi.[27] Following *post-mortem* macro-dissection, adjacent coronal slices were immersed in four different containers according to four different protocols of fixation (four containers for each *post-mortem* brain). One additional coronal macro-section at the level of the hippocampus was obtained from a patient who died for a non-neurological cause and was affected by monolateral temporal post-traumatic epilepsy. This macro-section was fixed in 10% neutral-buffered formalin for almost three months.

All tissue blocks used in this study were from the temporal lobe and the hippocampus. To exclude any variability of antigen expression related to the Brodmann areas considered, we preliminary prepared different cortex blocks, taken from all cortical lobes of the brain fixed in formalin, therefore we compared antigens expression and nucleic acids extraction for each area.

Human postnatal brain tissue

Five autoptic postnatal brains were obtained from patients of about one month, who died for sudden unexpected infant death (SUID). We included a postnatal brain from a hypoxic ischemic encephalopathy case as our positive control. Again, brain was extracted 40 h *post-mortem*.

Types of fixation

We used four different methods of fixation for the adult human brain, which we named A, B, C and D type. In the A protocol, i.e. the traditional protocol of *post-mortem* tissue fixation, we put macro-slices in 10% neutral-buffered formalin for 21 days. In the B protocol, we used 10% neutral-buffered formalin to fix but, at the 9th day, we put the macro-slices in a 98% formic acid solution for 1 hour and then again in formalin. At the 21st day, after the final dissection of the tissue blocks, we repeated the incubation in 98% formic acid for 1 h, just before processing and paraffin embedding. In both the A and B protocols we changed the formalin after 24 h and then every four days, for a total of five times.

In the C protocol we used a liquid fixative (LF) based on a 10x glacial acetic acid solution in 10% neutral-buffered formalin. The LF was changed after 24 h and then every four days, for a total of four times. In the last four days we moved the slices in 70% ethanol. The D protocol was identical to the C, except at the 9th and 17th day we incubated slices in formic acid for 1 h, just before the last passage in ethanol for four days (Table 1).

The macro-slice from the epileptic post-traumatic brain was fixed using the A protocol for three months. For postnatal brains, we used the LF described above for protocol C, which was changed every day for 8 days. Thereafter, brains were plunged into 70% ethanol for 4 days, then in fresh 80% ethanol for another 4 days and finally cut in macro-slices to conclude the fixation using a 95% ethanol solution for 4 days (Table 2).

Paraffin embedding

To exclude the variability due to post-fixation processing, all tissue blocks were processed and embedded in paraffin within 1 week. Paraffin embedded blocks were cut in 7 µm sections for histology and immunohistochemistry, or in 10 µm sections for genomic analysis, using a microtome (Leica, Wetzlar, Germany). Sections were mounted on adhesive microscopy slides (Superfrost plus, Thermo Scientific, Waltham, MA, USA) and assigned for staining and RNA purification and amplification to researchers whot were blind of the group to which each section belonged.

Table 1. Adult fixation protocols.

A	1 day + 5 x 4 days in formaline (21 days)
B	1 day + 2 x 4 days (9 days) in formaline + formic acid 1 h + 3 x 4 days (12 days) in formaline + formic acid 1 h (before processing)
C	1 day + 4 x 4 days (17 days) in LF (Formaline + acetic acid) + EtOH 70X 4 days
D	1 day + 2 x 4 days (9 days) in LF + formic acid 1 h + 2 x 4 days in LF + formic acid 1 h + EtOH 70X 4 days

Table 2. Perinatal fixation protocol.

Liquid fixative*	EtOH 70X	EtOH 80X	Cutting in	EtOH 95X macroslices
8 days in liquid fixative 4 days (changed each day)	4 days	4 days		

*Glacial acetic acid (10x solution) in 10% neutral-buffered formalin

Histology and immunohistochemistry

Automatized staining and immunostaining

The haematoxylin and eosin (H&E) stain was automatically performed using Leica Biosystems for two sections of each block. For immunostaining, we used an automatic and clinically validated instrument (Ventana Benchmark Ultra Systems from Roche Tissue Diagnostics). For all antibodies, we used the automatic CC1 Ventana pretreatment (Cell Conditioning Solution, Ventana Catalog Number 950-124) for 52 min. We used two Ventana prediluted custom-fit antibodies, the anti-Neurofilament antibody (clone 2F11, mouse monoclonal, Roche Number 05267714001, by Cell Marque CMC26610021) and the anti-Glial Fibrillary Acidic Protein antibody (GFAP, clone EP672Y, rabbit monoclonal, Roche Number 05269784001, by Cell Marque CMC43450021). For NeuN, neuronal nuclear antigen, we chose the anti NeuN/Fox3 antibody (clone N/A60, mouse monoclonal, by Immunological Sciences MAB-90228; 1:100). All three antibodies were incubated for 1 h. The UltraView DAB procedure (Benchmark Ultra System) was used for detection.

Manual NeuN and Nestin immunostaining

Manual immunohistochemistry was performed as previously described on the long-term fixation slides from the epileptic post-traumatic brain.[28,29] Sections were dewaxed (2 washes in xylol for 10 min, 5 min in ethanol 100%, 5 min in ethanol 95%) and then rehydrated in distilled water for 5 minutes and in PBS 1x for 10 min. The neuronal nuclear antigen was unmasked using a commercially available kit (Unmasker, Diapath), according to the manufacturer's instructions. We then employed the Dako Cytomation EnVision® + Dual Link System-HRP (DAB+) kit. After washing in PBS 1x, sections were incubated at room temperature for 10 min with Endogenous Enzyme Block, to quench endogenous peroxydase activity, and then incubated with the primary antibodies. For NeuN, we used the anti NeuN/Fox3 antibody (clone N/A60, mouse monoclonal, by Immunological Sciences MAB-90228; from 1:25 to 1:200). We used anti-nestin (mouse monoclonal, Immunological Sciences MAB-10430; 1:20), without unmasking, in the postnatal brain. For NeuN, we performed an overnight incubation at 4°C in humid atmosphere; for nestin, 100 minutes at room temperature in humid atmosphere. Slices were then rinsed twice in PBS 1x and incubated for another 30 min with Labeled Polymer-HRP [Dako Cytomation EnVision® + Dual

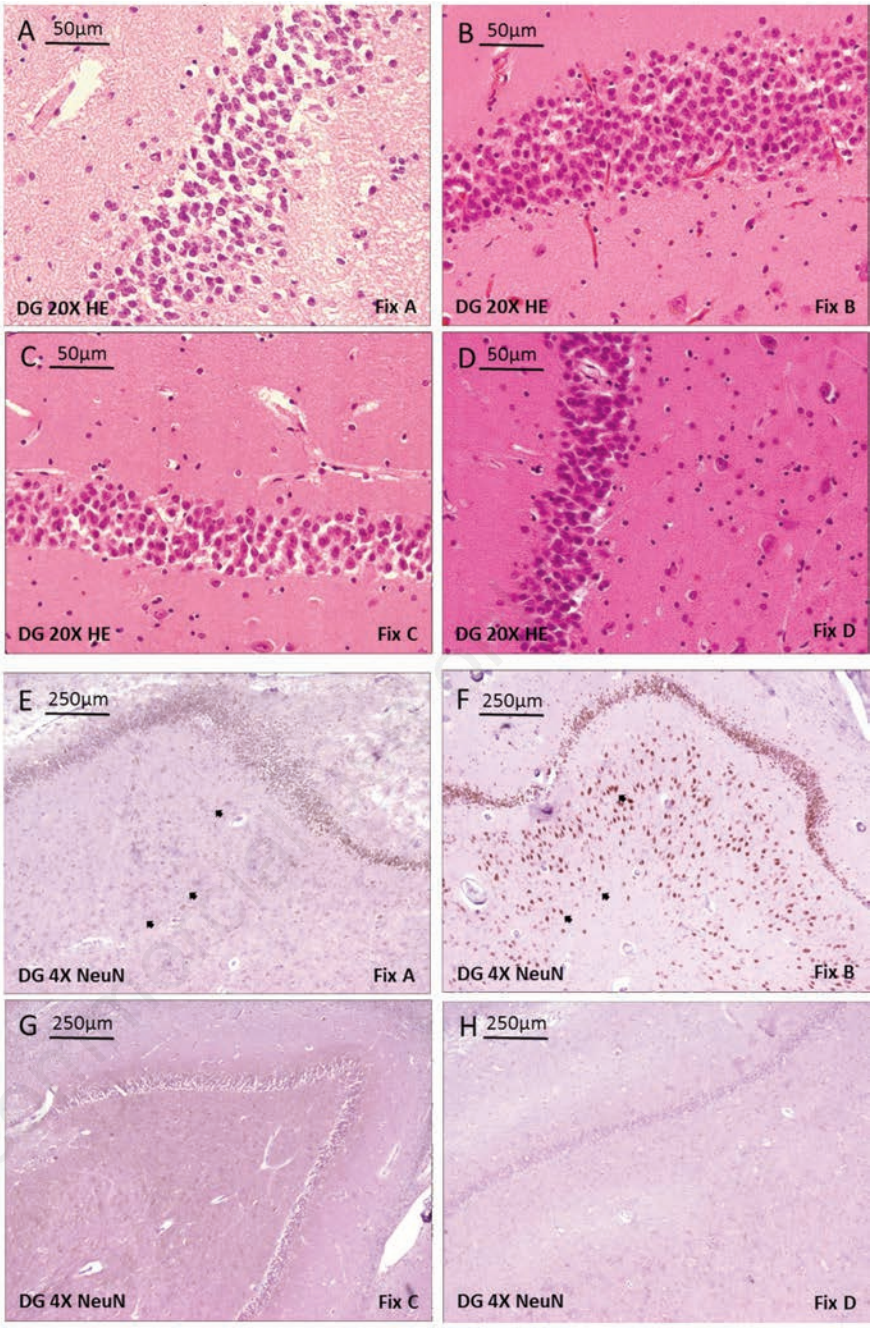

Figure 1. Human dentate gyrus in *post-mortem* cases. Hematoxylin and eosin staining (HE), magnification 20x and automatized NeuN immunohistochemistry performed in four different fixation protocols obtained from the same autoptic case, magnification 4x. A) HE in formalin fixation (classic protocol); B) HE in formalin fixation and formic acid treatment; C) HE in liquid fixative (LF) based on glacial acetic acid and formalin solution; D) HE in LF and formic acid treatment; E) NeuN staining in A protocol; F) NeuN staining in B protocol; G) NeuN staining in C protocol; H) NeuN staining in D protocol. HE staining shows that by B protocol the tissue matrix is more compact, without empty and irregular artefactual spaces, nuclei and nucleoli are more visible and better preserved and cell shape appears well represented (B). Automatized staining for NeuN results surely positive with little background exclusively in B protocol (F). Automatized NeuN immunostaining in fixation A protocol shows high level of background and paler staining for CA4 neurons (black arrow heads) and more loose parenchyma (E), compared to automatized NeuN staining in B protocol (F).

Link System-HRP (DAB+)]. The reaction product was detected as a brown substrate using 3,3-diamino-benzidinetetrahydrochloride (DAB). Finally, sections were mounted using a water-based mounting medium (Shur Mount™, TBS). The specificity of immunolabeling was verified with a control in which the primary antibody was omitted.

Fluoro-Jade C (FJC) staining

Sections were dewaxed as described above and incubated in a solution containing 1% NaOH in 80% ethanol (5 min), then in 70% ethanol (2 min), then in distilled water (2 min). They were then incubated for 10 minutes in 0.06% potassium permanganate, washed for 2 minutes in distilled water, and transferred to a 0.001% FJC staining solution (IS-0012, FJC solution 1000x, Immunological Sciences). After staining, sections were rinsed in distilled water, counterstained with 0.0001% 4',6-diamidino-2-phenylindole (DAPI) for 15 min, washed again, and air dried.

Manual immunofluorescence

Sections were dewaxed, rehydrated, and unmasked as described above. After washing in PBS, sections were incubated with Triton X-100 (0.3% in PBS 1x, room temperature, 10 min), washed twice in PBS 1x and incubated with 5% BSA and 5% serum of the species in which the secondary antibody was produced, for 30 min. They were then incubated with the primary antibodies overnight at 4°C, as follows: nestin (mouse monoclonal, Chemicon) and SOX2 (rabbit polyclonal, Immunological Sciences) 1:10 and 1:25 respectively. After 5-min rinses in PBS, sections were incubated with Triton X-100 (as above, 30 min), washed in PBS and incubated with the goat anti-mouse, Cy2 (Cyanine)-conjugated, secondary antibody (1:50 dilution; Jackson ImmunoResearch) for mouse primary antibodies, or with a goat anti-rabbit, Texas red-conjugated, secondary antibody (1:100; Jackson ImmunoResearch) for rabbit primary antibodies, at room temperature for 3.5 h. After staining, sections were washed in PBS, counterstained with 0.0001% DAPI for 15 min, and washed again. Coverslips were mounted using water-based anti-fading Gel/Mount (Biomeda).

DNA/RNA extraction

Total DNA/RNA were extracted using a total nucleic acid purification kit (RecoverAll Total Nucleic Acid Isolation Kit, Ambion Life Technologies, Foster City, CA, USA), from each paraffin embedded block by 10 µm thickness slices. The concentration and the quality of nucleic acid were investigated using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and an Agilent 2100 Bioanalyzer. All of the procedures were produced according to our previously published protocols.[30,31]

Analysis

Sections were analysed using a Leica microscope (DMRA2), by three expert pathologists under double blind conditions.

Cell shape, consistency of parenchymal

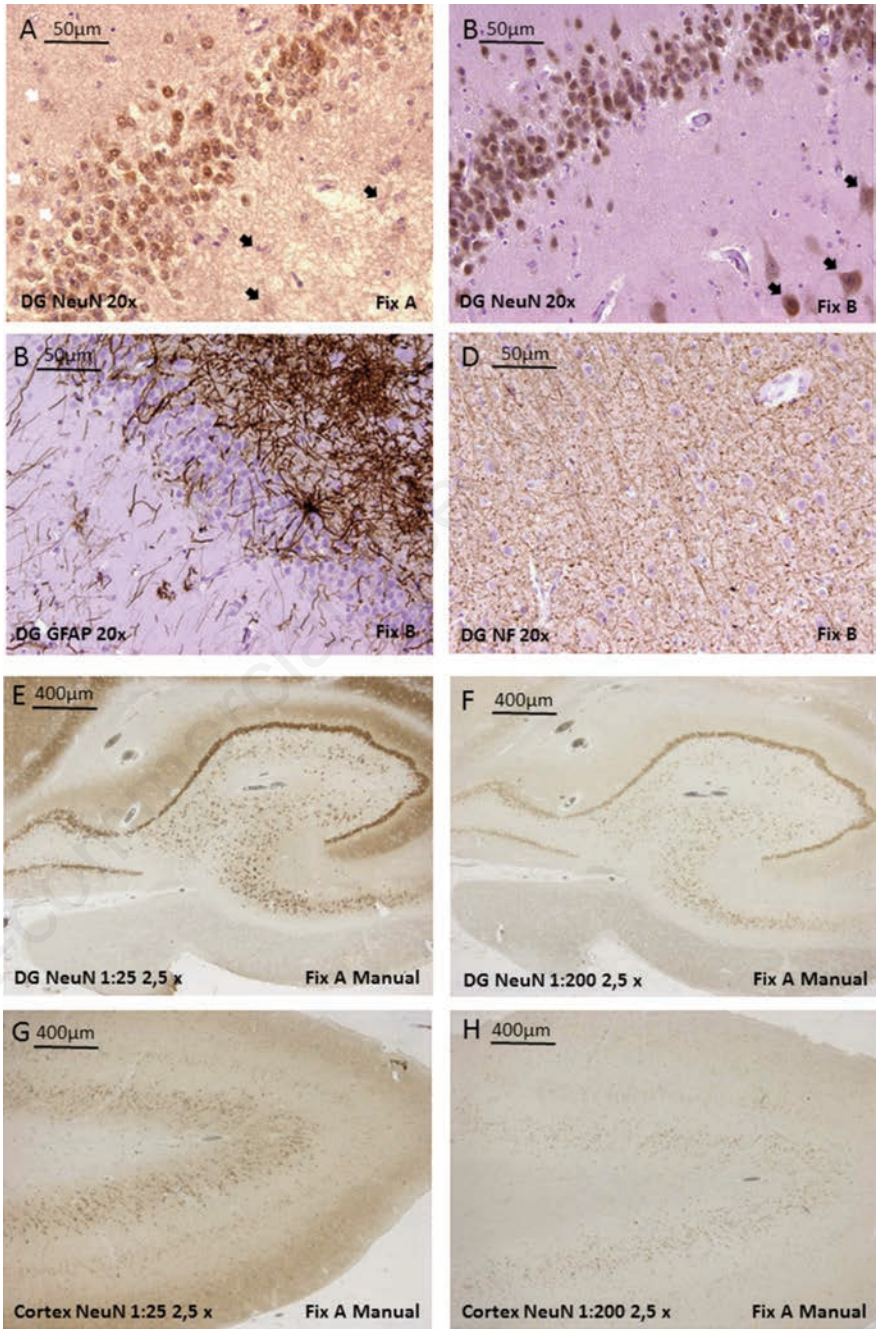

Figure 2. Human dentate gyrus in *post-mortem* cases. Automatized immunohistochemistry performed against NeuN, GFAP and NF brain antigen markers, magnification 20x (A-D) and manual staining against NeuN antigen, magnification 2.5x (E-H). A) Automatized NeuN immunostaining in A-type fixation protocol appears paler with more background, false positivity for glia and astrocytes (white arrow heads) and negativity of CA4 area neurons (black arrow heads), compared with B. B) Automatized NeuN immunostaining in B-type fixation protocol. C) Automatized GFAP immunostaining in B-type fixation protocol. D) Automatized NF immunostaining in B-type fixation protocol. E-H) Human Hippocampus in long-term fixation of the same *post-mortem* case. Manual immunostaining in A-type fixation protocol, with different antibody concentration respectively in E,G) and F,H).

matrix, nucleolar and nuclear preservation as well as immunohistochemistry positivity, have been evaluated using objectives from 5x to 20x by means a semiquantitative method based on score among 0 and +++. The degree of neurodegeneration was analysed on Fluoro-Jade C sections, based on a modification of a previously described "neurodegeneration score" for animal models.[32] The score range spanned from 0 (absence of Fluoro-Jade C-positive neurons) to +++ (maximal neurodegeneration pattern in the hippocampus). +++ was given with many positive cells with confluent fluorescence.

Results

We compared four different fixation protocols. Comparison was made between: A protocol (10% neutral-buffered formalin for 21 days); B protocols (10% neutral-buffered formalin for 21 days with two intervals of 1 hour each, at day 9 and 21, in which tissue was dipped in a 98% formic acid); C protocol (LF every four days and 70% ethanol for the last four days); D protocol (as C except at the day 9 and 17, when tissue was dipped in a 98% formic acid solution). We chose A protocol, traditionally utilized for most of the histochemical techniques, as our negative internal control to confirm and evaluate morphological observations obtained by others.

The automatically performed H&E staining was produced on the four differently fixed brain tissues, obtained from the same patient (Figure 1 A-D). We observed a better preservation of the cell shape, more compact parenchymal matrix and nucleolar and nuclear preservation with the formalin-formic fixation (B protocol, Figure 1B). For the perinatal and postnatal brain, characterized by a high content of water,[33] we utilized only one approach of fixation, slightly modifying the fixation procedure of C protocol (C modified protocol). In fact, we used acetic acid addition to formalin with different ethanol steps, dehydrating agents. For immunostaining, we used an automatic and clinically validated procedure based on Ventana Benchmark Ultra systems from Roche Tissue Diagnostics. Both for NeuN and for easier to detect antibodies like GFAP, the quality of immunohistochemistry was superior by using the B protocol. Also the neurofilament and GFAP staining were better in B-type-tissues compared with the other fixations (Figures 1 E-H and 2 A,B).

Manual immunohistochemistry for NeuN (Figure 2 C-F) and nestin (Figure 4A) was performed on adult and postnatal cerebral tissues, respectively, by means a stronger and drastic manual unmasking protocol. We were able to perform an excellent staining on macroslices fixed in classic formalin protocol (A protocol), for adult brain tissue, whereas we utilized the previously described foetal/ perinatal fixation protocol (C modified protocol), for the nestin immunostaining of postnatal brain tissue.

Regarding identification of the best antigen retrieval protocol, we verified different treatments: 1. CC1 Ventana pre-treatment; 2. microwave irradiation by means of a commercially available kit (Unmasker, Diapath); 3. formic acid pre-treatment; 4. Triton X-100 permeabilization; 5. a combination of 1 and 3, or 2 and 4.

We tested neurofilament (Figure 2C), GFAP (Figure 2D) and NeuN antigen expression in adult brains (Figures 1 E-H and 2 A,B,E-H) and Nestin and SOX2 in postnatal brain (Figures 4A and 5 A-E). For *post-mortem* adult brains, we applied automatized and manual immunostaining. The first gave us the best results with formic acid and CC1 Ventana combination (1 and 3), suggesting that the B protocol is the preferable approach for automatized antigen detection. Microwave irradiation and Unmasker Diapath solution produced an excellent result for manual NeuN detection in adult *post-mortem* tissue; however, the best fixation for this approach was the A protocol, exclusively formalin.

Concerning the postnatal brain, the unmasking procedure was necessary only for immunofluorescent staining, in which we utilized microwave irradiation plus Diapath Unmasker and Triton X-100 tissue permeabilization (2 and 4).

Regarding Fluoro-Jade C staining, the A protocol for adults (Figure 3 A-F) and the C modified protocol for newborn tissues (Figure 4 B,C) has been utilized without

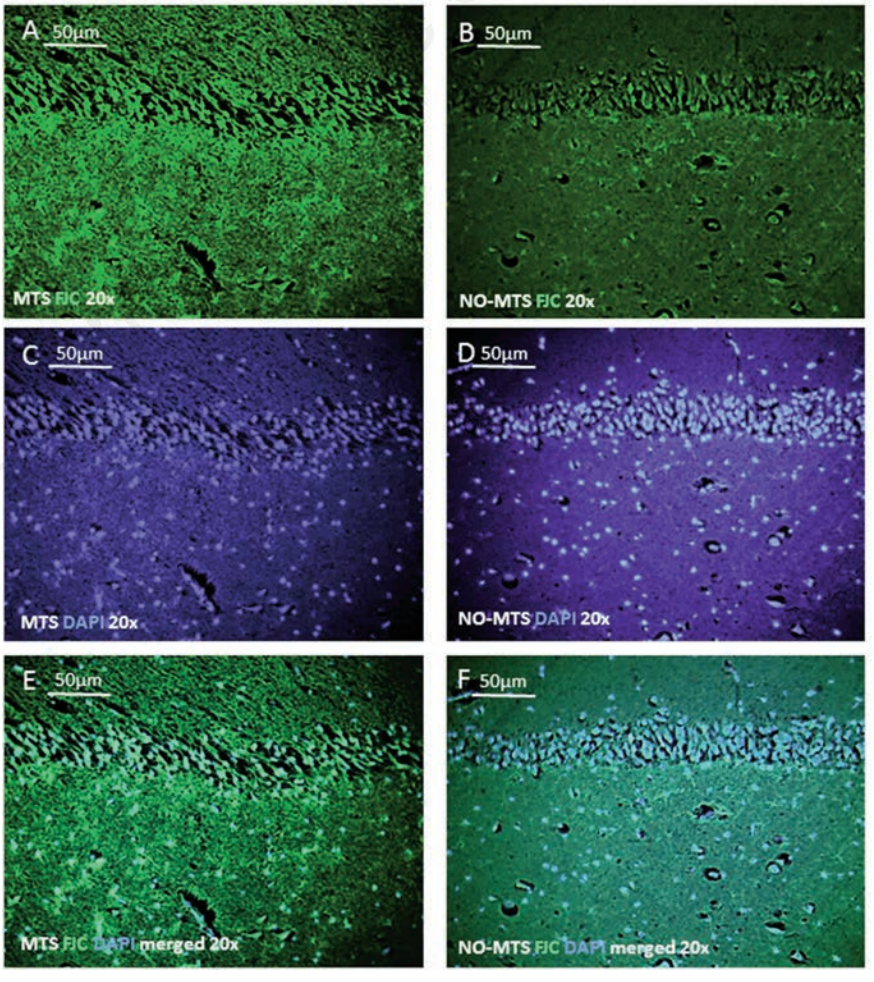

Figure 3. Human adult Fluoro Jade C (FJC) staining of hippocampal neurodegeneration, magnification 20x. A) Monolateral temporal post-traumatic epilepsy with left mesial temporal sclerosis (MTS), FJC in green; B) Controlateral temporal lobe in absence of neurodegeneration, FJC in green; C) DAPI in blue of left MTS; D) DAPI in blue of contralateral side. E, F) merged view of A-C and B-D.

any problems. Score for FJC positivity was totally in advantage for temporal lesion in post-traumatic epilepsy and for postnatal brain with hypoxic ischemic encephalopathy on the not-lesioned contralateral hippocampus (+++/+) and respectively on SUID cases (+++/+). By means of this fixation method, concomitant with the immunofluorescence protocol, we detected neural stem cells positive for nestin and SOX2 (Figure 5 A-E).

Initial data of RNAs extraction and DNA amplification display apparently homogeneous and similar results with the four types of fixation (Table 3). Randomly we repeated the NeuN staining by automatized Ventana procedure, on different *post-mortem* brain samples arrived to our attention for diagnostic purpose and obtained the same result.

Table 3. RNA-DNA extraction.

	RNA, DNA				
Fixations	74/13	15/13	D5/13	80/13	81/13
A	***,***	***	*/**		
B	**** ***				***
C				*/**	
D	***,*			**	**,*

RNA ratio: *1.55-1.64; **1.65-1.74; ***1.75-1.84; **** 1.85-1.99; DNA ratio: *1.55-1.64; **1.65-1.74; ***1.75-1.80.

Discussion

The study of brain histology and protein expression represents a fundamental step in the understanding of brain development and physiopathology of the Central Nervous System diseases. Protein expression in FFPE tissue has been analysed, however, despite advances in molecular technologies, the quality of RNA and protein from fixed paraffin-embedded tissue remains variable. In particular, in autoptic cases with long-term fixation and high variability deriving from the coroner *post-mortem* practices, both the immunohistochemistry and gene and RNA detection, request more accurate and detailed validation of custom fit procedures.[7,8,34,35] Regarding to the preanalytical phase of *post-mortem* delay of autopsy, we choose the interval between death and autoptic procedures around 40 hours. We standardized this *interim* period on 40 h, because it corresponds to the time of brain extraction that is considered adequate for the ethical problems (usually 24 h) and in which we do not observe any alteration in the intensity of staining of the more common neuronal and glial antigens (between 33 and 48 h).[8] Recently, Thom and co-workers have published a review, in which they provide a framework for best practices, integration of clinical, pathological and molecular genetic investigations in Sudden Unexpected Death in Epilepsy (SUDEP).[36] Authors underlined the lack of sufficient numbers of suitable control cases and tissues and lack of standardized guidance documents for conducting autopsies. Our intent has been to follow these suggestions by evaluating different techniques of fixation, antigen retrieval, immunohistochemistry and/or fluo-staining and RNA extraction in formalin-fixed paraffin-embedded tissue

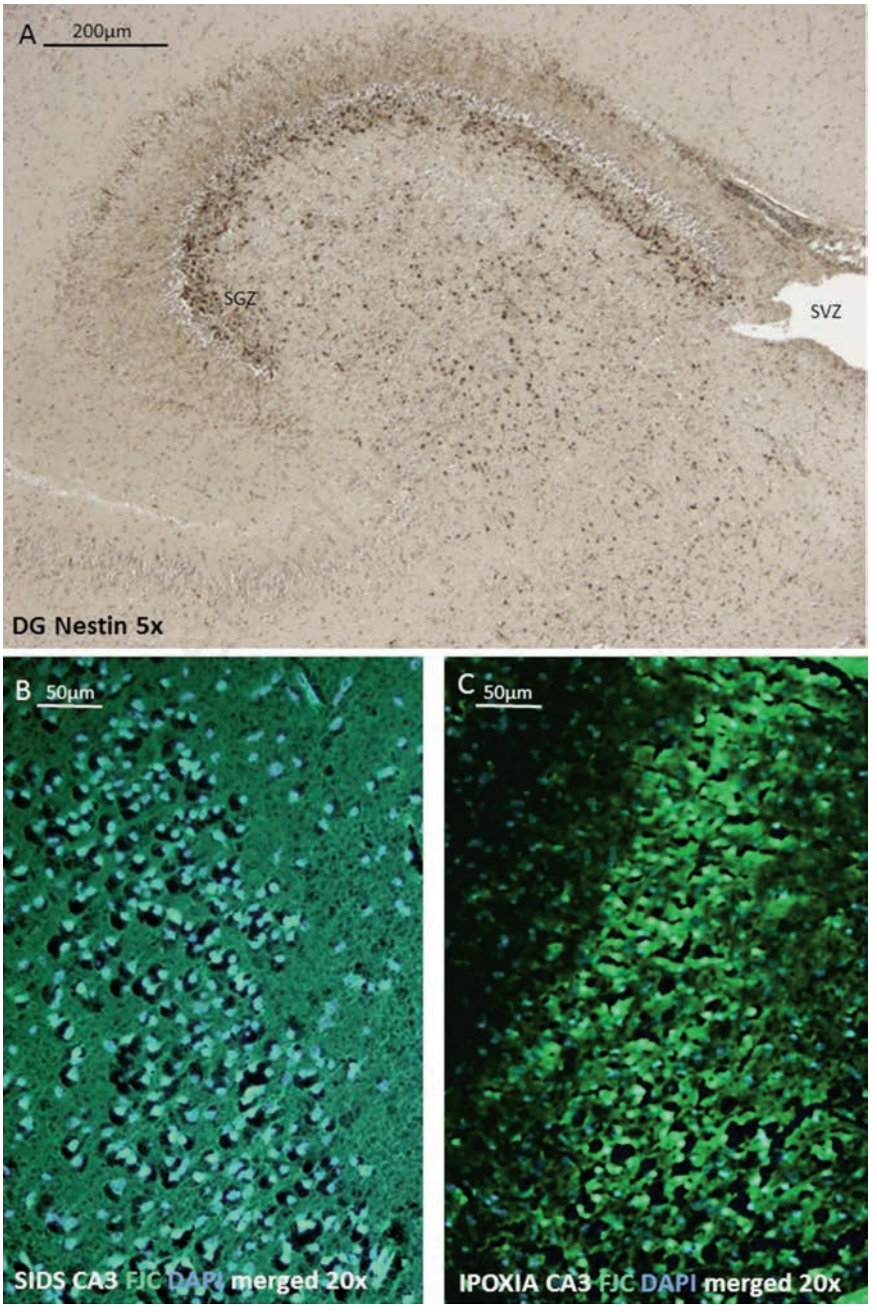

Figure 4. Human postnatal dentate gyrus in SIDS case. Nestin immunostaining, magnification 5x and Fluoro Jade C (FJC) neurodegeneration detection, magnification 20x. A) Nestin immunostaining in LF protocol: nestin- positive neural precursors are migrating from the subgranular (SGZ) and subventricular zone (SVZ); B) Human postnatal FJC staining (in green) of CA3 area of hippocampus in SIDS case, without any aspect of neurodegeneration, compared with CA3 area of perinatal FJC positive hypoxic ischemic encephalopathy in the control case (C), nuclei stained in blue by DAPI.

from *post-mortem* brains in long-term fixation.

It is a consolidated and firm data that long-term formalin fixation for *post-mortem* human brain produces an impairment of immunolabeling with formalin-sensitive antibodies.[1] Many expensive procedures have been yet tested without definitive good results. NeuN automatized immunostaining persists, for example, a prohibitive immunostaining for *post-mortem* brain tissues, as you can see by traditional protocol of formalin fixed brain macroslices (A protocol), in which NeuN staining carries on negative (Figure 1E). In other words, we chose A protocol as our negative internal control. Therefore, we decided to compare different substances to try to improve the staining results for formalin-sensitive antibodies. We considered formic acid and glacial acetic acid because respectively are normally utilized as antiprionic and dehydrating agents. Therefore, to determine the best strategy to maintain the integrity of *post-mortem* brain tissue, cell morphology and nuclear structure, we compared four different fixation protocols.

Comparison was made between: A protocol, exclusively formalin; B protocols, formalin and formic acid solution; C protocol, procedure with foetal autopsy dedicated LF, appropriate to dehydrate brain tissues and a mix method between B and C protocol (D protocol), to eventually test their synergism.

Preliminary data showed that the *post-mortem* brain tissue in formalin fixed and formic acid treated (B-type protocol) lone preserved good morphology in H&E. We scored histochemical results by microscope analysis of pathologists, however results were totally in favour of the B-type protocol. Tissue matrix is more compact, without empty and irregular artefactual spaces, nuclei and nucleoli are more visible and better preserved and cell shape appears well represented (Figure 1 A-D).

Regarding identification of the best antigen retrieval protocol, it is well known that formalin and paraffin embedding both abolish or drastically reduce immunoreactivity with the majority of the antibodies used routinely in neuropathological studies, especially in autoptic *post-mortem* tissues. We verified different treatments: 1. CC1 Ventana pre-treatment; 2. microwave irradiation by means of commercially available kit (Unmasker, Diapath); 3. Formic acid pre-treatment; 4. Triton X-100 permeabilization; 5. a combination of 1 and 3, or 2 and 4.

Our study evidenced that, for adult *post-mortem* brain tissue, the B protocol is the preferable approach for the automatized antigen detection, while A protocol, *i.e.* exclusively formalin, resulted the best fixation for manual immunostaining only when it is associated with a particular strong procedure of unmasking by microwave irradiation and Unmasker Diapath solution. Excellent automized NeuN staining (Figures 1 E-H and 2 A,B) appears true positive with B protocol, totally negative by C and D fixation and false positive with classic A fixation. Indeed, granule cells of the dentate gyrus showed the same level of pale staining as astrocytes and glial cells (white arrow heads in Figure 2A), furthermore, neurons of CA4 area are NeuN positive

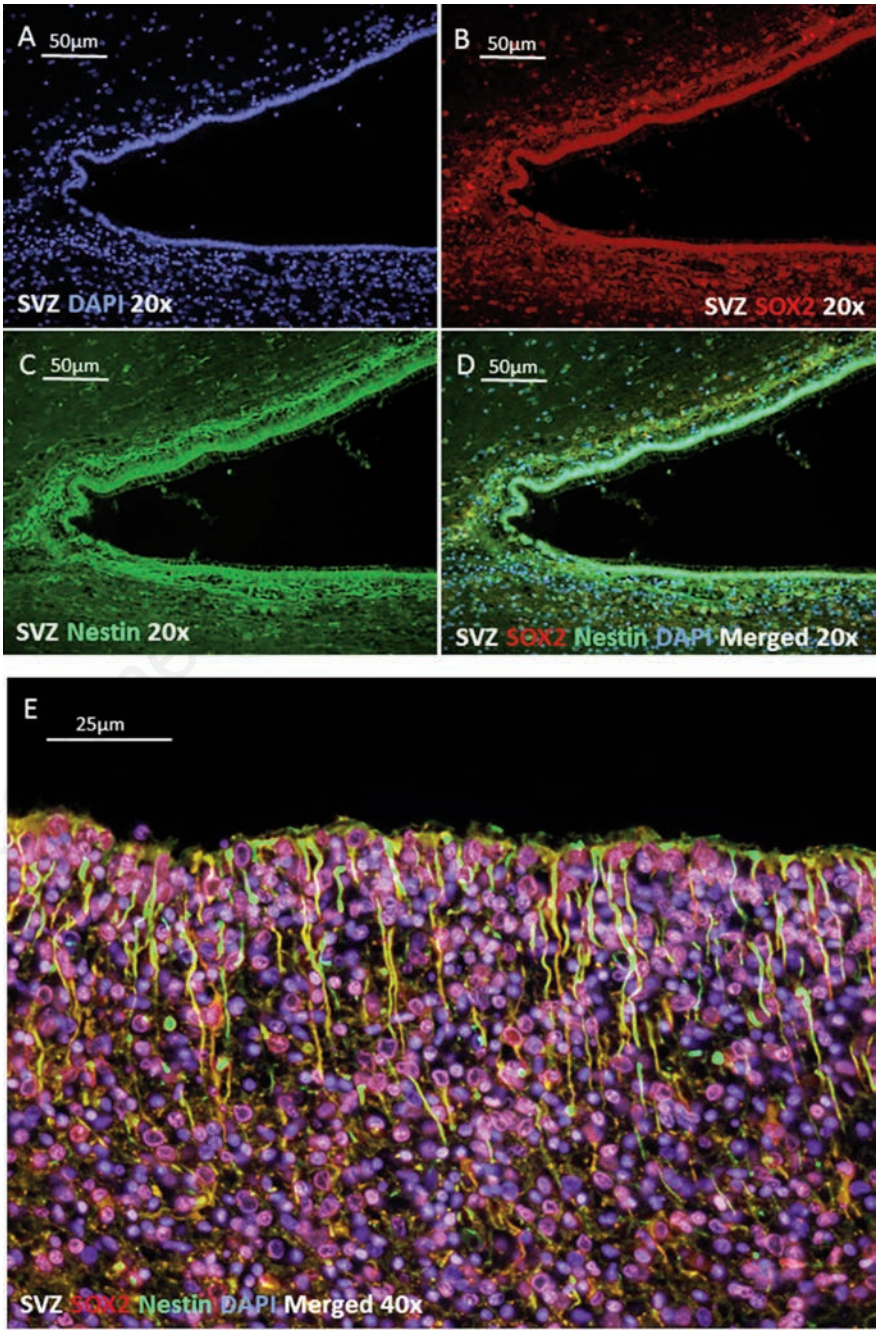

Figure 5. Human postnatal neurogenesis in subventricular zone (SVZ) by antigen of stemness in SIDS case. Immunofluorescence staining in LF fixation protocol, magnification 20x. A) Nuclei in blue, DAPI; B) SOX2 in red; C) Nestin in green; D) merged image of A, B, C; E) Human postnatal neurogenesis in subventricular zone (SVZ) in SIDS case. Merged image of immunofluorescence staining: nuclei in blue, DAPI; nestin in green; SOX2 in red, in LF fixation protocol, magnification 40x.

exclusively by B fixation (black arrow heads in Figures 1 E,F and 2 A,B). In addition, GFAP and the neurofilament staining were better in B-type-tissues compared with the other fixations too (Figure 2 C,D). We consider our automatized B protocol an interesting method to quantify clinical and research data from *post-mortem* tissue and a proficient approach to standardize NeuN detection according to shared validation system in different Institutions.[36] Obviously, this approach has to be tested on long-fixed brains from brain bank collections. On the other hand, the manual protocol of immunostaining allows to control the development of the staining during the DAB exposure (checking under microscope) and reduce the problem of background (Figures 2 E-H). For the same reason the use of manual colorimetric staining could be suggested for paediatrics cases. Nevertheless, manual NeuN staining could be the first choice for dysplasia or brain cortical malformation and dysmorphisms in perinatal deaths, an optimal approach to study the hippocampal sclerosis and granule cell pathology in *post-mortem* epileptic cases and SUDEP. The manual technique, more accurate than the automatic one, could be necessary to distinguish hypoplasia or dysgenesia of cardiorespiratory nuclei implicated in SIDS. For the first time in the Literature, we demonstrated an optimal fixation with LF (C protocol) of postnatal brain. This type of protocol, in general, is utilized for embryonic and foetal brains because it has a great power of dehydration.[37] In fact, according to Widdowson and Dickerson, the postnatal brain maintains a similar water content as foetus of 20-22 gestational weeks.[38]

The striking decrease in brain water in adult aging resembles the decrease in brain water observed in early life through adulthood: 20-22 week foetus 92%, new-born 90% and adult 77%. By combined approach of double antigen retrieval in immunofluorescent staining, we distinguished nestin and SOX2 antigens of stemness in neurogenetic area of postnatal brain, confirmed by colorimetric approach (Figure 4A).

There are very few data in the Literature regarding the development and application of novel histochemical tracer of neurodegeneration in human brain, for this reason we tested Fluoro-Jade C, usually utilized in animal models of neurodegeneration. According our result, we propose Fluoro-Jade C staining in adult and babies autopsies (Figure 3 A-F) as an easy and fast histofluostaining to suppose the presence of neurodegeneration cells in such amount as to exclude the sudden death.[24,32]

Moreover, nucleic acid information does not seem lost through these four fixation techniques. Our current data of RNAs extraction show apparently homogeneous and similar results for the four types of fixation protocol and these outcomes are very interesting especially because it is possible utilize LF and ethanol for perinatal brain tissue without lose the genomic information of cerebral samples. In fact, has to be considered mandatory for SIDS cases to realize the genomic analysis of possible chanellopathies or potential eredopathies in infant sudden death. Furthermore, by our LF fixation protocol for postnatal brain tissue we were able to detect infective diseases utilizing Altona Diagnostics assay in efficient way (*data not shown*).

The processing parameters that determine the quality of RNAs and proteins in fixed paraffin-embedded tissues have been traditionally recognized. There are four important factors that are involved in RNA and protein preservation: time before fixation, methods of fixations, length of fixation and exclusively for protein detection, antigen retrieval techniques. The results of the different methods of fixation confirm that *post-mortem* brain tissue exhibited different affinity for antibodies or histo and/or fluostaining and it is possible to adjust different protocols according to molecular requirement or to the patient ages. It is advisable in the future to test the different protocols for very long-lasting fixations, such as those of tissue banks, and drafting of the procedural guidelines.

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Results

1. "Tullio Terni (1888-1946): the life of a neurocardioanatomist with a tragic epilogue" 2018, International Journal of Cardiology submission

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Abstract

Tullio Terni was a pioneer of neuroanatomy at the University of Padua. He gave milestone contributions in the knowledge of cardiac innervation with the discovery of the "Terni column", a preganglic autonomous nervous center.

Due to "racial laws" introduced in Italy in 1938 by the Nazi-Fascist government of Mussolini, he, being Jewish, was expelled from the University of Padua like more than hundred from other Italian universities. At the end of the 2nd World War, he was reinstated to his chair of Anatomy. However, having belonged to the Fascist party, paradoxically he was dismissed from the Lincei Academy, a paradox that deteriorated his depression up to the suicide.

Biography

Tullio Terni was born in Livorno on January 21, 1888 (fig. 1). In 1910, he graduated in medicine in Florence at the School of Giulio Chiarugi (1859-1954), becoming pupil of Giuseppe Levi (1872-1965), whom he followed as assistant at the University of Sassari (1911-1915), Palermo (1915-1919) and Turin (1919-1924) [1]. He married Mary Sforni and had two children: Maurizio and Rachele (tenderly nicknamed "Lullina"). He took part of the 1st World War as Medical Captain and was rewarded with the Merit Crux. In 1919, when in Turin, he

obtained the Private Professorship. At the early age of 36, he became Full Professor and was called to the University of Padua to hold the novel chair of Histology and Embryology, first instituted for him due to his outstanding scientific achievements. He created in Padua an internationally acknowledged center for biomedical research. He was voted Academician of the prestigious Lincei Academy in 1932. On 1933, he moved to the chair of Anatomy in Padua and became chief of the Institute, when the former Director Dante Bertelli (1858-1936) retired.

With the advent of the "racial laws" on October 13, 1938, he was expelled from the University being Jewish. However, with the support of the Rector Carlo Anti (1889-1961), he was allowed to continue his ongoing research until 1940. Thereafter with the family he moved to Florence and lived hidden in the Tuscany village of Tutignano. He fell into a state of depression and needed to be cured in a Clinic in Merano (Bozen) for six months.

Soon after the end of the war, with the defeat of the Nazi-Fascist Regime, on May 13, 1945 Terni was reinstated to his chair at the University of Padua, but the was hesitant to be back for his nervous breakdown. Meanwhile, the Committee of the newly established Lincei Academy, which was charged to select those to be readmitted, decided that Tullio Terni was not worth to be confirmed, with other 35 previous members, having been compromised with the Fascism. Terni received the sad news on January 1946. On February of the same year, he signed the form of acceptance to accept the previous position at the University of Padua. On April 25, at 7 o'clock in the morning, he was found dead, following ingestion of a cyanuric phial, that he always had with him to use with the family in case of Nazism arrest [2], [3].

Terni, the scientist

Tullio Terni research ranged in several fields of neuroanatomy, descriptive or experimental cytology, embryology and teratology, human and comparative cytogenesis, cell culture regenerative biology and transplantation techniques.

The most famous paper of Tullio Terni, relevant to cardiovascular medicine, was published in 1923 on the *Archivio Italiano di Anatomia e di Embriologia* (Italian

Archive of Anatomy and Embriology), when he was still assistant, entitled *Ricerche anatomiche sul sistema nervoso autonomo degli Uccelli* (Anatomical researches on autonomous nervous system in birds) [4] (fig. 2). It concerns the discovery of a lateral column at spinal cord T1-L1-2, representing pre-ganglic neurons to the sympathetic chain and stellate ganglion. Studying chick embryos, reptiles, birds and mammals by applying Cayal method, he discovered the existence in the thoracic lumbar region of the spinal cord, a preganglionic nervous center, located in the retro paracentral region in mammals and in intermediolateral site in humans (fig. 3). He demonstrated the existence of a spinal source of sympathetic innervation in the cardiac system, represented by preganglionic neurons located in the first five to six segments of the thoracic spinal cord at the lateral gray column, which synapse with neurons of the superior cervical, medium cervical and stellate ganglia and from the second to the fourth thoracic ganglia of the sympathetic trunk (fig. 4). This center, then called "Terni column", constitutes a longitudinal column of nervous cells from the first thoracic to the second lumbar segment [5]. In the Gray's Anatomy the reference to Terni in the autonomous nervous system is quoted in the last 5^{th} edition [6].

From the clinical viewpoint, the neurons of Terni's column may play a role in several cardiac disease, including long QT and Catecholaminergic Polymorphic Ventricular Tachycardia, a still neglected possibility. In the human fetus, according to new concepts and animal model based theories, episodic respiratory activity aimed at promoting lung development is generated by the intermediolateral nucleus (ILN) in the upper spinal cord [7]. During fetal life, Terni's column generates motor stimuli for the intercostal respiratory muscles to favor the compliance of the thoracic cavity. This autonomic nucleus seems also to be involved in cardiovascular response to hypoxia, but its real function is probably not yet well understood [8].

Terni was a renewed international scientist, publishing both in Italian and German journals. He had several scientific relationships, including the Carnegie Institution of Washington in Baltimore and the Rockefeller Institute in New York, from which he received a significant grant that allowed him to purchase advanced equipment and to set up tanks of breeding of Axolot, a salamander known as

“small monster”, to investigate the regeneration of dorsal pin (fig. 5) [9]. He anticipated the tanks of zebrafish for modern molecular genetic investigation.

As pointed out in a previous paper [5], the research activity of Terni and his subsequent discoveries deserve to be remembered as they were carried out in a relatively short period of time and were made in a dramatic social and political context.

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Figures

Figure 1 *Portrait of Tullio Terni. At his right, the Terni column is depicted*

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DA

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Vol. XX.

(Con 10 tavole e 166 figure nel testo).

DITTA EDITRICE LUIGI NICCOLAI
FIRENZE

Istituto Anatomico della R. Università di Torino

Ricerche anatomiche sul sistema nervoso autonomo degli Uccelli.

1. Il Sistema pregangliare spinale.

(Con 14 figure nel testo)

Dott. Tullio Terni
Aiuto e Libero Docente

SOMMARIO.

CAPITOLO I.

Introduzione. Materiale e tecnica.

Alcuni anni or sono, scorrendo delle serie di giovani embrioni di pollo, trattati col metodo Cajal, mi ero accorto che, a determinati livelli del midollo spinale, molte fibre radicolari anteriori, anzichè originare dal ben noto nucleo motore situato superficialmente nella testa del corno anteriore, nascevano invece assai più profondamente da un cospicuo e denso raggruppamento cellulare, situato in prossimità del canale centrale. Però, io non mi ero al-

Figure 2 *Frontispiece of the Journal Archivio Italiano di Anatomia e di Embriologia (Italian Archive of Anatomy and Embriology), where Terni published in 1923 his famous paper Ricerche anatomiche sul sistema nervoso autonomo degli Uccelli (Anatomical researches on autonomous nervous system in birds)*

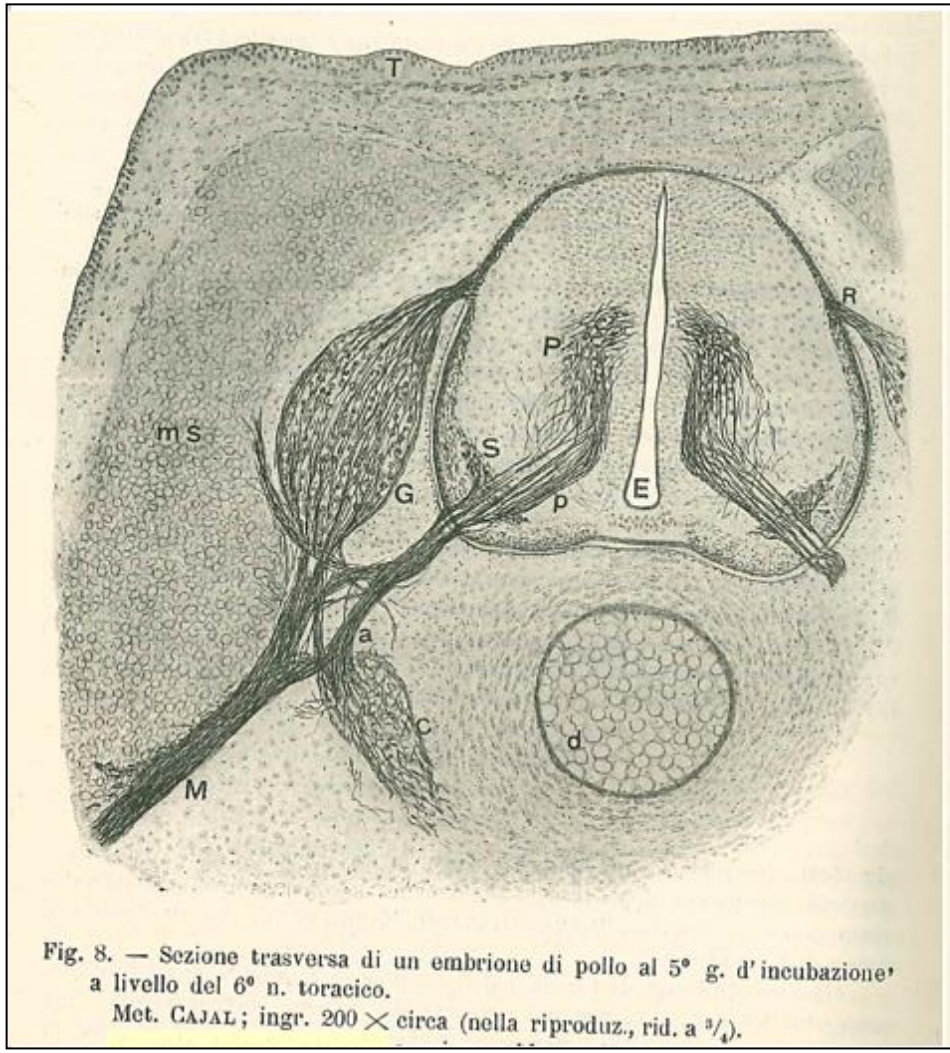

Fig. 8. — Sezione trasversa di un embrione di pollo al 5° g. d'incubazione, a livello del 6° n. toracico.
Met. CAJAL; ingr. 200 × circa (nella riproduz., rid. a $^3/_4$).

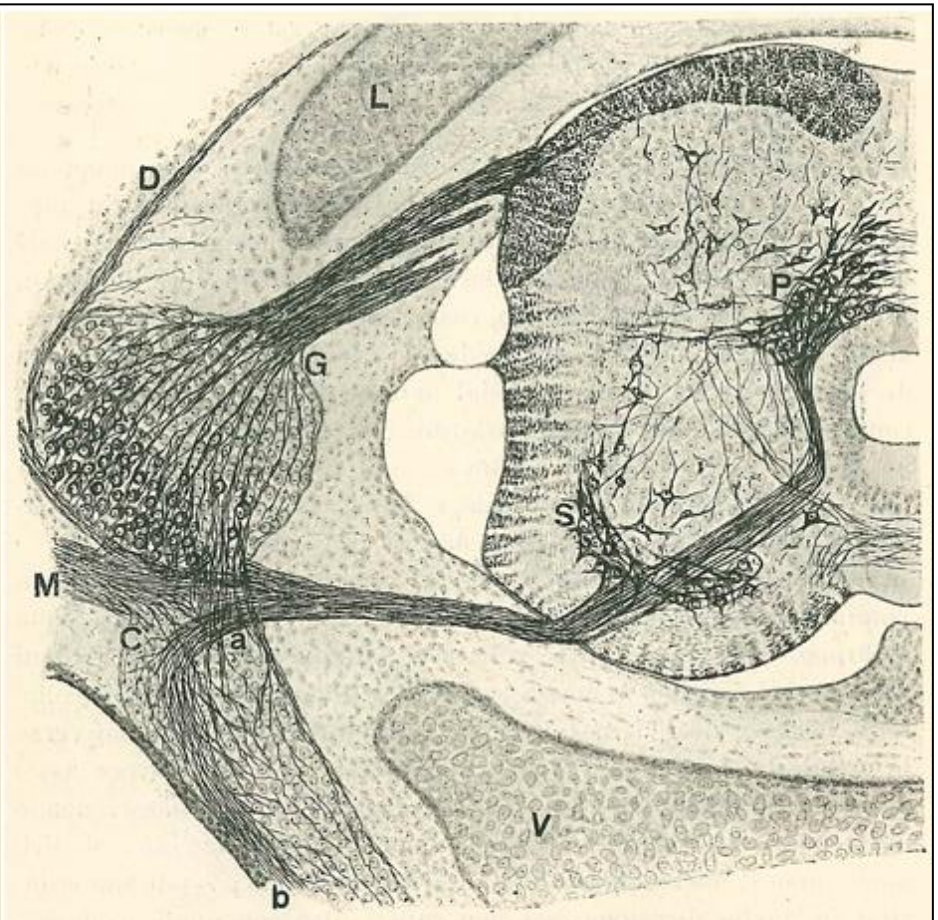

Fig. 3. — Embr. di pollo di 9-10 g., sezionato trasversalmente a livello del 5° n. toracico.
Met. CAJAL; ingr. 250 × circa (nella riproduz., rid. a $^2/_3$).

Figure 3 *Illustrations from Terni's Ricerche anatomiche sul sistema nervoso autonomo degli Uccelli (Anatomical researches on autonomous nervous system in birds) (1933)*

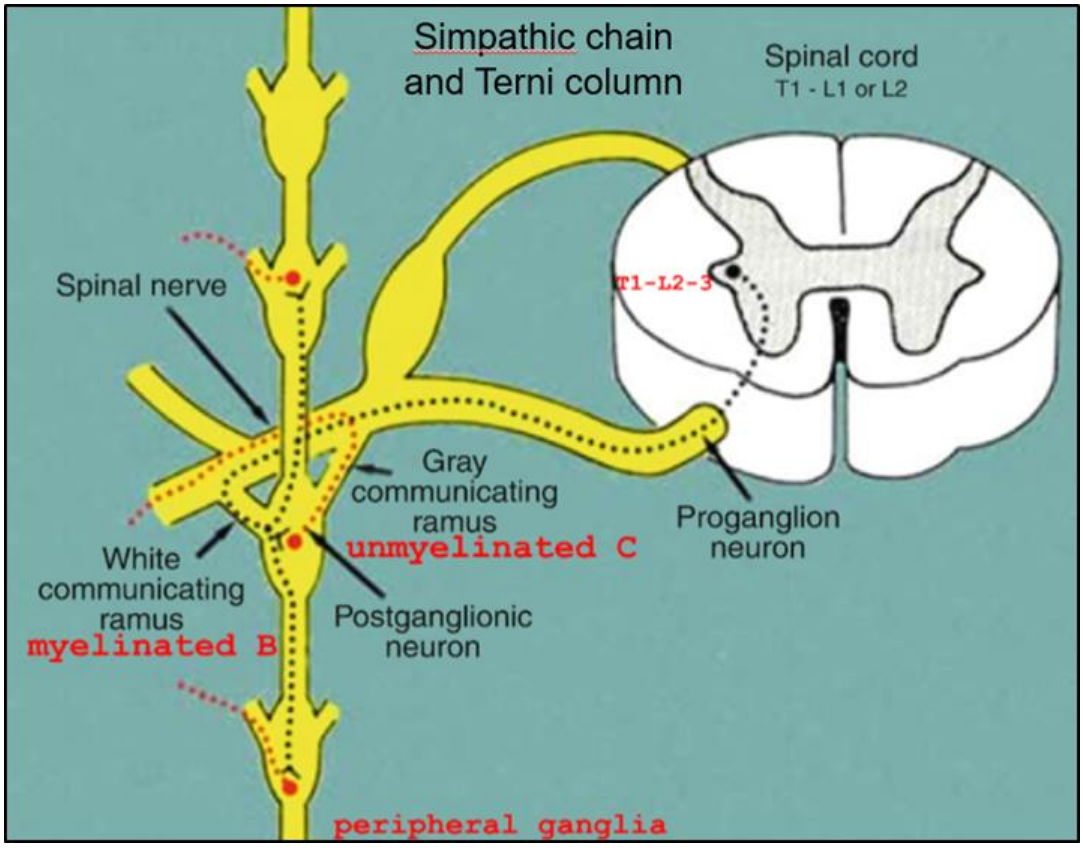

Figure 4 *Preganglionic nervous center in the thoracic lumbar region of the spinal cord, located in intermediolateral site in humans and in the retro paracentral region in the other mammals*

Figure 5 *The Axolot, salamander known as "small monster"*

2. "Neuropathology of early Sudden Infant Death Syndrome – Hypoplasia of the pontine Kölliker-Fuse Nucleus: a marker of unexpected collapse during skin care"

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Neuropathology of Early Sudden Infant Death Syndrome—Hypoplasia of the Pontine Kolliker-Fuse Nucleus: A Possible Marker of Unexpected Collapse during Skin-to-Skin Care

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Am J Perinatol

Abstract

Objective To find a possible pathogenetic mechanism of the early sudden infant death occurring in newborns during the skin-to-skin care (SSC), through the examination of neuronal centers regulating the vital activities.

Study Design This is an in-depth examination of the brain stem in 22 healthy term newborns, suddenly died in the first hour of life without the identification of a cause at autopsy (early sudden infant death syndrome [eSIDS]), 12 of them concomitantly with SSC, and 10 with age-matched controls died of known pathology.

Results Developmental alterations of neuronal structures of the brain stem were highlighted in 19 of the 22 eSIDS, but not in control. The hypoplasia of the pontine Kölliker-Fuse nucleus (KFN), an important respiratory center, was diagnosed at the histological examination, validated by morphometric quantifications, in 11 of the 12 eSIDS while they were placed on the mother's chest and in 2 of the 10 SSC unrelated neonatal deaths.

Conclusion The delayed development of the KFN could represent a specific finding of eSIDS occurring during SSC. Therefore, it is necessary to point out that the SSC represents a further risk factor that must be added to others already known for sudden infant death syndrome. Then this practice needs appropriate monitoring strategies of the infant's conditions.

Keywords
- skin-to-skin care
- sudden unexpected postnatal collapse
- sudden infant death syndrome
- early sudden infant death syndrome
- risk factors
- neuropathology
- brain stem
- Kölliker-Fuse nucleus

Skin-to-skin care, or skin-to-skin contact (SSC), that is, the "early, continuous, and prolonged SSC between the newborn and mother," is currently encouraged not only for premature and low birth weight infants but even for healthy term infants with an appropriate weight for gestational age.[1,2] This practice, previously called "Kangaroo care," in 1979 has been introduced in Colombia as an alternative to incubators for high-risk infants,[3–5] has had since then many

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supporters for a widespread implementation, given many benefits offered to newborns. This low-cost intervention, in fact, seems to protect against a wide variety of neonatal complications without contraindications.[6–8] However, SSC incurs considerable risks for newborns. Reports of sudden unexpected postnatal collapse (SUPC) in newborns subjected to SSC treatment, with varying severity, from neonatal apnea and bradycardia episodes to sudden infant death, most often occurring in the first hour of life (the so-called early sudden infant death syndrome [eSIDS]), are becoming increasingly frequent.[9–11] Several explanations have been proposed for this disastrous outcome, such as an unpredictable cardiorespiratory distress, or the prone positioning of the infant strengthened by SSC.[12,13] But what could be the real cause? In this study, we propose at the base of the pathogenetic mechanism of SUPC occurring in newborns during SSC the presence of a specific developmental alteration of a nervous center of the brain stem essential in the modulation of the respiratory rhythm.

Materials and Methods

Twenty-two cases, 13 males and 9 females, who died suddenly from apparently unknown cause within the first 7 hours of life, were selected from a large series of victims collected over a 10-year period, in accordance with the Italian Law 31/2006 "*Regulations for Diagnostic Post Mortem Investigation in Victims of Sudden Infant Death Syndrome (SIDS) and Unexpected Fetal Death*."[14] This law decrees, in particular, that all infants suspected of SIDS who died suddenly in Italian regions within the first year of age, as well as all fetuses who died without any apparent cause, must be submitted to in-depth postmortem investigation. For each case of the study, all available information about pregnancy, fetal development, and delivery and about the circumstances in which the death occurred were collected. Particular attention was paid to the potential risk factors for SUPC, namely, prematurity ($<$ 35 gestational weeks), perinatal asphyxia, congenital malformations, primiparous mother, first breast feeding, regional or general anesthesia administered during delivery,[10,15] and to the more universal factors involved in SIDS pathogenesis as prone position and maternal smoking.[16–18] Regarding a possible exposure to tobacco smoke in utero, mothers were asked to complete a questionnaire inquiring about their smoking habit. Furthermore, according to the guidelines of the "Lino Rossi" Research Center, the removal of a lock of victims' hair during the autopsy was required for all cases of the study to search for the cotinine, the main metabolite of nicotine, which has a long half-life. This test was aimed especially at verifying the negative assertions of the mothers, being aware that the retrospective assessment of maternal smoking habit, if performed after the fatal event, is sometimes unreliable, because of feelings of guilt.[19] Eight of the 22 eSIDS mothers (36.4%) resulted active smokers by their own admission, all claiming to have the smoking habit from before pregnancy, or after the nicotine test. The remaining 14 mothers (63.6%) had no history of cigarette smoking, verified through the same analysis.

All the newborns had standard basic values as regard to birth weight (range values: 2,990–4,250 g), Apgar score at 5 minutes (range: 7–10), and gestational age (range: 36–41weeks). The maternal age range was 22 to 37 years, and only three mothers were at their first pregnancy. The course of pregnancy and labor was normal and without any maternal or fetal complication in all cases. Only in two cases, the epidural anesthesia was used for pain relief during labor. The newborns at birth appeared well developed with no physical anomalies or evidence of trauma. However, a sudden collapse with full respiratory and cardiac arrest unexpectedly happened a few hours from birth. Twelve of these newborns had died while they were placed prone or on the side on their mothers' bare chests during SSC, three of these during the first breast feeding. ►**Table 1** summarizes the main characteristics of the 22 eSIDS cases. At the postmortem examination, performed approximately within 24 to 30 hours after death, the internal organs were unremarkable on gross and microscopic examination. Therefore, all eSIDS were regarded as being totally unexplained, after having ruled out any possible underlying causes. As controls, we used 10 age-matched newborns suddenly died in the first hour of life, initially classified as probable eSIDS, but in which a plausible cause of death was found at autopsy (precisely congenital heart disease, observed in 5 cases, pulmonary dysplasia in 2 cases, severe bronchopneumonia in 2 cases, and myocarditis in 1 case) (►**Table 2**). Only 2 of the 10 mothers in the control group (20%) had a proven smoking habit.

Neuropathologic Analysis

The brains appeared normal on external examination and weighed between 323 and 405 g. According to the guidelines specifically developed by the "Lino Rossi" Research Center, an in-depth anatomopathological examination of the autonomic nervous system was performed, paying particular attention to the brain stem, where the main structures controlling the vital functions are located. After fixation in 10% phosphate-buffered formalin, the brain stems were processed and embedded in paraffin. Three specimens were then taken (►**Fig. 1**): the first specimen (I) included the upper third of the pons and the adjacent caudal portion of midbrain; the second specimen (II) was taken from the caudal portion of the pons; and the third specimen (III) was taken from the medulla oblongata around the obex. These samples, on the basis of our previous observations performed on serial histological sections of the brain stem, contain entirely the main component of the respiratory network which must be examined (►**Fig. 2**). Transverse serial 4 µm sections from the samples were then made. Two of these sections, every 30 µm, were routinely stained with hematoxylin-eosin and Klüver–Barrera for histological examination. Other sections were saved and stained as deemed necessary for the morphometric evaluations, as indicated later, and also for further investigations, such as the immunohistochemical characterization of specific receptors that are differently expressed in sudden perinatal death victims and controls.[20–22]

Histology

The histological assessment of the brain stem was mainly addressed to the locus coeruleus, the linear, median raphe

Table 1 Features of the 22 eSIDS

Case	Sex	Gestational age (wk)	Birth weight (g)	Apgar (5 min)	Time of death (hours after birth)	Infant position at discovery	Mother age (y)	Primiparous mother	Maternal smoking
1[a]	M	38 + 4	3,050	8	2	Prone	35	−	−
2[a]	M	39 + 3	3,020	9	6	On the side	32	−	−
3	F	40 + 3	4,000	[b]	6	Supine	25	−	+
4[a]	F	41	3,750	8	5	Prone[c]	37	−	+
5	M	39 + 1	4,250	9	1	Prone	22	−	+
6[a]	F	39	3,590	8	3	Prone[c]	34	−	+
7	M	37 + 2	[b]	7	7	Prone	[b]	+	+
8	M	37	3,950	8	2	Supine	26	+	−
9[a]	M	39 + 3	[b]	9	4	On the side	24	−	−
10	F	38	3,750	[b]	2	On the side	31	−	−
11[a]	M	38 + 2	3,480	10	4	Prone[c]	25	−	+
12[a]	M	41	3,980	8	2	Prone	30	−	−
13	F	38	3,800	10	4	[b]	26	−	−
14	M	38 + 2	3,570	[b]	5	Prone	36	−	−
15[a]	M	37 + 2[d]	3,800	10	2	On the side	28	−	−
16[a]	F	40	3,450	10	4	On the side	29	−	−
17[a]	F	40 + 1	3,020	[b]	6	Prone	27	−	−
18[a]	F	40 + 2	3,320	9	2	On the side	31	−	−
19	F	40	[b]	10	7	Prone	32	+	−
20	M	40 + 3	2,990	7	3	Prone	[b]	−	+
21[a]	M	38[d]	4,010	10	6	Prone	31	−	−
22	M	36	3,920	9	5	Prone	29	−	+

Abbreviation: eSIDS, early sudden infant death syndrome.
[a]Died during skin-to-skin contact.
[b]Data not recovered.
[c]Died during the first breast feeding.
[d]Labor with epidural anesthesia.

nuclei, and the Kölliker-Fuse nucleus (KFN) in the caudal mesencephalon/rostral pons; to the retrotrapezoid nucleus, superior olivary complex, magnus raphe nucleus, and facial/parafacial complex in the caudal pons; to the hypoglossus, dorsal motor vagus, tractus solitarius, ambiguus, pre-Bötzinger, inferior olivary, arcuate, obscurus, and pallidus raphe nuclei in the medulla oblongata.

A diagnosis of "hypoplasia" of a given nucleus among the above-listed structures was formulated at microscopic observation when it showed a significantly decreased number of neurons and/or decreased area in serial transverse histological sections, compared with the mean values previously obtained in a wide range of age-matched controls died for a well-defined cause and specifically collected and examined to define the normal cytoarchitecture of the different nuclei. The histological examinations were performed by groups of two independent and blinded observers and comparison of the results performed employing K statistics (Kappa index [KI]) to evaluate the interobserver reproducibility. The Landis and Koch system[23] for the K interpretation was used, where 0 to 0.2 is slight agreement, 0.21 to 0.40 indicates fair agreement, 0.41 to 0.60 moderate agreement, 0.61 to 0.80 strong or substantial agreement, and 0.81 to 1.00 indicates very strong or almost perfect agreement (a value of 1.0 being perfect agreement). The application of this method in this study revealed a very satisfactory KI (0.85).

Quantitative morphometric investigations were performed, whenever necessary, to verify the reliability of the microscopic diagnoses.

Morphometric Analysis

A quantitative method was applied to adjacent histological sections stained with Klüver–Barrera. The basic investigation was performed with an Image-Pro Plus Image Analyzer (Media Cybernetics, Silver Spring, MD). The following parameters were evaluated for each nucleus of interest: (1) transverse area (expressed in mm^2), (2) neuronal density

Table 2 Features of the 10 controls

Case	Sex	Gestational age (wk)	Birth weight (g)	Apgar (5 min)	Time of death (hours after birth)	Infant position at discovery	Mother age (y)	Primiparous mother	Maternal smoking	Death cause
1	F	38 + 3	3,040	9	2	Prone	36	–	+	Congenital heart disease
2	F	[a]	2,950	7	6	Supine	[a]	–	–	Pulmonary dysplasia
3	M	39	[a]	10	7	On the side	24	+	+	Severe bronchopneumonia
4	F	38 + 1	3,400	10	5	On the side	26	+	–	Myocarditis
5	M	32[b]	3,500	8	5	Prone	35	[a]	–	Severe bronchopneumonia
6	M	38	[a]	[a]	3	Prone	29	–	–	Congenital heart disease
7	M	36	3,830	9	2	On the side	29	–	–	Congenital heart disease
8	F	39	3,600	10	1	Supine	[a]	+	–	Pulmonary dysplasia
9	M	39	[a]	10	7	Prone	34	–	–	Congenital heart disease
10	M	37 + 3	3,880	9	4	On the side	34	–	–	Congenital heart disease

[a]Data not recovered.
[b]Prematurity (< 35 gestational weeks).

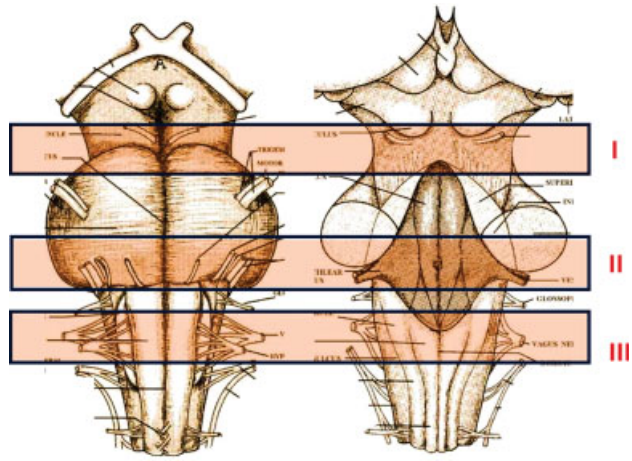

Fig. 1 Schematic representation of the brain stem sampling (on the left: ventral surface; on the right: dorsal surface): (I), pontomesencephalic specimen, including the upper third of the pons and the adjacent portion of mesencephalon; (II), caudal pontine specimen; and (III), medulla oblongata specimen, including the obex.

(number of neurons with clearly defined edges, nucleus, and nucleolus per mm^2), and (3) volume (in mm^3). The transverse area was calculated for every case as mean value of the measurements obtained, after having drawn the nucleus boundaries, on three slides: a histological section at rostral pole of the nucleus, a section at the median level, and a section at the caudal pole. The evaluation of the neuronal density was performed on the same selected histological sections with the same criterion, that is, mean value of the three measurements. A computer program developed by Voxblast (VayTek, Fairfield, IA) was used on all the available sections to obtain the three-dimensional reconstruction of the whole structure, from the caudal to the rostral pole. For every section, the outer boundaries of the nucleus were

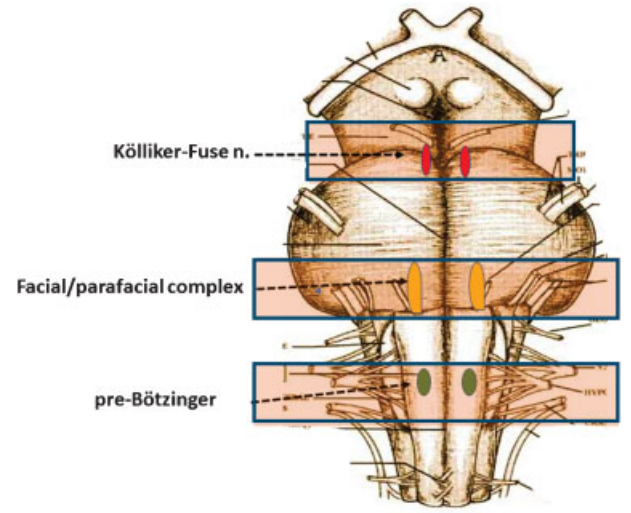

Fig. 2 Schematic representation of the localization of the main structures to be examined, component of the respiratory network, fully included in the three brain stem specimens of ►**Fig. 1**.

traced. The tracings were digitized by computer and then registered to re-establish their original positions relative to one another. The fourth ventricle was used in general as landmark for registration.

Statistical Analyses

Statistical calculations were performed with the Statistical Package for the Social Science (SPSS) statistical software (version 11.0; SPSS Inc., Chicago, IL). All the morphometric results were overall expressed as mean values and standard deviation (SD). The statistical significance of direct comparisons between groups with normal and defective nucleus was determined using the analysis of variance (F-test). The Fisher's exact test was calculated to find associations between categorical variables of interest, and *t*-test was used to find mean differences in continuous variables. The selected threshold level for statistical significance was $p < 0.05$.

Results

The in-depth microscopic examination of the brain stems allowed us to detect, for 19 of the 22 eSIDS of the study, developmental alterations of major neuronal structures that preside over vital functions. More precisely, hypoplasia of nuclei of the raphe system and of the arcuate nucleus in the medulla oblongata, and of the facial/parafacial complex and the KFN in the pons were frequently highlighted in these cases. Individual victims not rarely displayed combination of these alterations (►**Table 3**). Hypoplasia of the KFN, in terms of decreased neuronal number and extension, was the most frequent finding at microscopic observation (13/22 cases) (►**Fig. 3C**), The normal cytoarchitecture of this nucleus, which extends longitudinally throughout the first entire brain stem sample (see ►**Fig. 2**), is shown in the upper part of ►**Fig. 3**. It is well analyzable in the more cranial transverse sections of the pons (►**Fig. 3A**), precisely in the tegmentum, between the decussation of the superior cerebellar peduncles and the medial lemniscus. At high magnification, a small population of large neurons frequently showing a distinct, eccentric nucleus with evident nucleolus and abundant cytoplasm and Nissl substance at the cell periphery, is well discernible (►**Fig. 3B**). ►**Figs. 4** to **6** refer to the alterations most frequently observed in eSIDS beyond the hypoplasia of the KFN, that is, the hypoplasia of the raphe nuclei (in particular, the raphe obscurus nucleus, observed in six cases) and of the arcuate nucleus (present in six cases). In

Table 3 Neuropathologic findings in the 22 eSIDS

Case	Developmental alterations of brainstem centers
1[a]	Hypoplasia of the Kölliker-Fuse nucleus; hypoplasia of the raphe obscurus nucleus
2[a]	Hypoplasia of the Kölliker-Fuse nucleus; hypoplasia of the facial/parafacial complex
3	Agenesis of the area postrema; desquamation of the fourth ventricle ependymal cells
4[a]	None
5	Hypoplasia of the facial/parafacial complex
6[a]	Hypoplasia of the Kölliker-Fuse nucleus; hypoplasia of the raphe magnus nucleus
7	Severe hypoplasia of the arcuate nucleus; hypoplasia of the raphe obscurus nucleus
8	Agenesis of the pre-Bötzinger nucleus; agenesis of the arcuate nucleus
9[a]	Hypoplasia of the Kölliker-Fuse nucleus; hypoplasia of the arcuate nucleus
10	Hypoplasia of the Kölliker-Fuse nucleus
11[a]	Hypoplasia of the Kölliker-Fuse nucleus; hypoplasia of the raphe magnus nucleus; agenesis of the arcuate nucleus
12[a]	Hypoplasia of the Kölliker-Fuse nucleus; hypoplasia of the raphe median nucleus
13	Hypoplasia of the Kölliker-Fuse nucleus; hypoplasia of the raphe obscurus and pallidus nuclei
14	None
15[a]	Hypoplasia of the Kölliker-Fuse nucleus; hypoplasia of the raphe obscurus nucleus; severe hypoplasia of the arcuate nucleus
16[a]	Hypoplasia of the Kölliker-Fuse nucleus; hypoplasia of the raphe obscurus nucleus
17[a]	Hypoplasia of the Kölliker-Fuse nucleus; hypoplasia of the facial/parafacial complex; hypoplasia of the arcuate nucleus
18[a]	Hypoplasia of the Kölliker-Fuse nucleus; hypoplasia of the pre-Bötzinger nucleus
19	None
20	Agenesis of the retrotrapezoid nucleus; hypoplasia of the superior olivary complex
21[a]	Hypoplasia of the Kölliker-Fuse nucleus
22	Hypoplasia of the raphe obscurus nucleus

Abbreviation: eSIDS, early sudden infant death syndrome.
[a]Died during skin-to-skin contact.

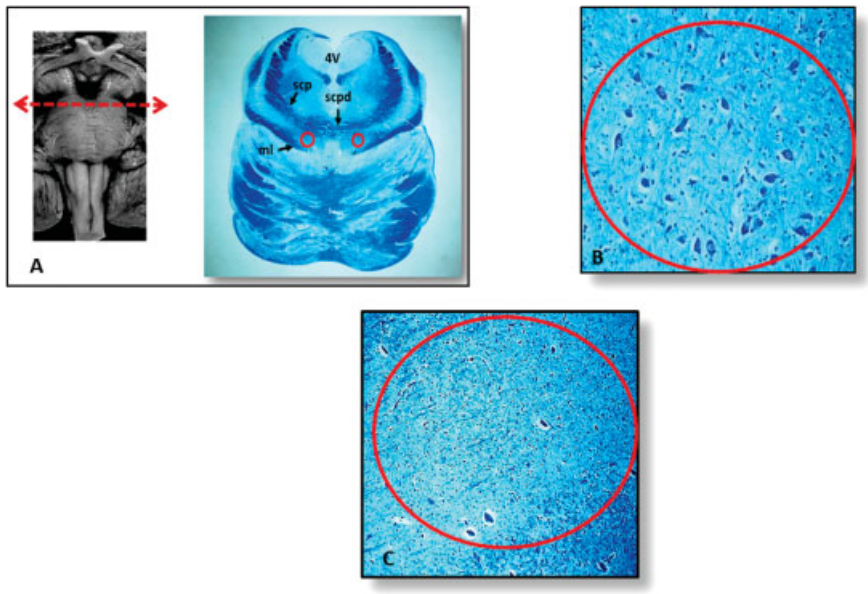

Fig. 3 An image series related to the Kölliker-Fuse nucleus (KFN). (**A**) On the left: ventral brain stem image with the indication of the level for the optimal sampling for the examination of the KFN; on the right: histological section at the above-mentioned level. The circles show the bilateral localization of the KFN, represented at higher magnification in (**B**). At this magnification, large KFN neurons can be seen, with distinct nucleus and evident nucleolus, intermixed with smaller cells (interneurons and astrocytes). (**C**) Hypoplasia of the KFN observed in a newborn died 2 hours after birth (case no. 1 of ►**Table 1**). (**A**) On the right, Klüver–Barrera's staining, magnification: ×0.5—(ml, medial lemniscus; scp, superior cerebellar peduncle; scpd, decussation of the superior cerebellar peduncles; 4V, fourth ventricle); (**B**, **C**) Klüver–Barrera's staining, magnification: ×100.

the control group, no significant developmental alteration of the brain stem nuclei was found.

The morphometric analysis, applied specifically to the KFN, given its frequent involvement in developmental defects in eSIDS, allowed to obtain valid and objective quantitative results, in support to the histological findings. Notably, the hypoplasia of the KFN was diagnosed in 11 of the 12 eSIDS while they were placed on the mothers' chest (91.7%) and in 2 of the 10 SSC unrelated neonatal deaths (20%). The quantitative values related to transverse area, neuronal density, and volume of the KFN, evaluated in all eSIDS and controls, are reported in ►**Table 4** as mean ± SD. The cases were subdivided in three groups: (group I) eSIDS associate to SSC; (group II) eSIDS unrelated to SSC; and (group III) controls. While between groups II and III, there were no significant differences, group I showed mean values

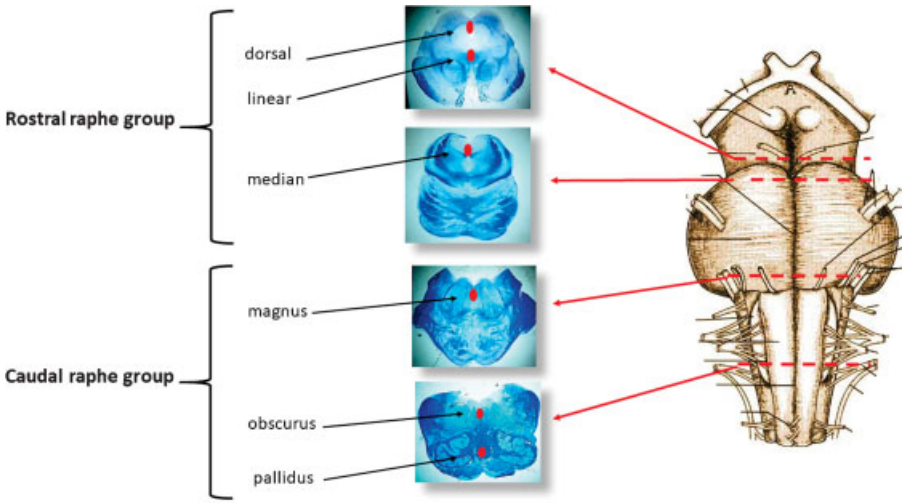

Fig. 4 Schematic representation of the localization of the raphe nuclei in the brain stem. The nuclei are subdivided in two main clusters located in the midbrain/rostral pons (the rostral raphe group, including three nuclei: the linear raphe nucleus, the dorsal raphe nucleus, and the median raphe nucleus) and in the caudal pons/medulla oblongata (the caudal raphe group, including other three nuclei: the magnus raphe nucleus, the obscurus raphe nucleus, and the pallidus raphe nucleus). Histological sections: Klüver–Barrera's staining, magnification: ×0.5.

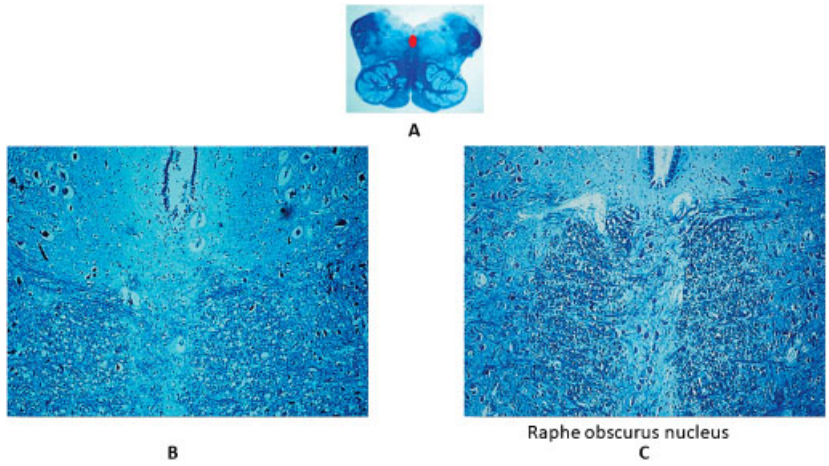

Fig. 5 (**A**) Histological section of medulla oblongata with the indication in the circled area of the localization of the raphe obscurus nucleus. (**B**) Hypoplasia of the raphe obscurus nucleus (case no. 15 of ►**Table 1**). (**C**) Normal structure of the raphe obscurus nucleus in a control case (case no. 3 of ►**Table 2**). (**A**) Klüver-Barrera's staining, magnification: ×0.5; (**B, C**) Klüver-Barrera's staining, magnification: ×20.

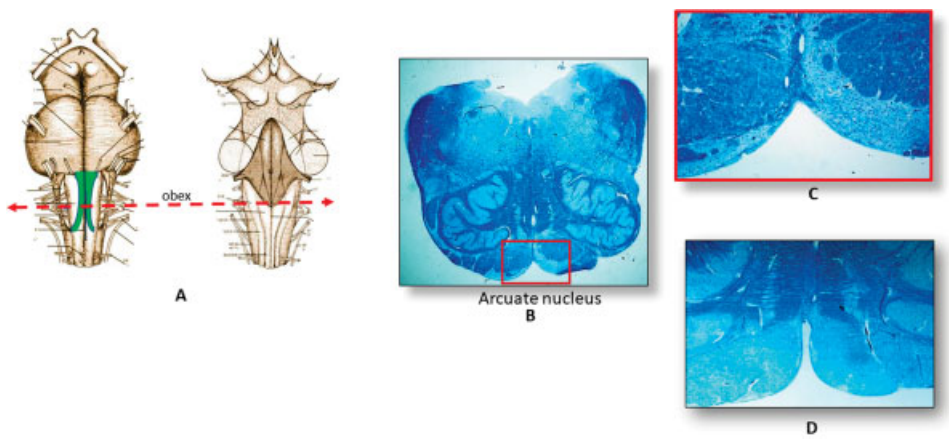

Fig. 6 An image series related to the arcuate nucleus. (**A**) On the left: ventral brain stem schematic image with the indication of the localization of the nucleus on the ventral surface of the medulla oblongata (in green), extending from the caudal border of the pons to the caudal pole of the olive and of the optimal sampling at the obex level; on the right: dorsal brain stem surface. (**B**) Histological section at this level. The framed area is represented at higher magnification in (**C**). (**D**) Severe hypoplasia of the arcuate nucleus (case no. 15 of ►**Table 1**). (B) Klüver–Barrera's staining, magnification: ×0.5; (**C, D**) Klüver–Barrera's staining, magnification: ×10.

of the three parameters (area, neuronal density, and volumetric reconstruction) up to three to four times lower than those obtained in groups II and III ($p < 0.01$). These results allowed to recognize objectively the KFN hypoplasia and to establish that this alteration is a very common finding in sudden neonatal deaths occurred in association with SSC.

Correlation of the Results with the Main Risk Factors for SUPC

A significant association was found between SSC, prone/on the side position of newborns and hypoplasia of the KFN. In fact, 11 of the 12 SUPC cases associated to SSC showed the concomitance of these three situations (cases 1, 2, 6, 9, 11, 12,

Table 4 Morphometric analysis of the KFN in 22 eSIDS and 10 controls

Groups	No. of cases	Transverse area (mm^2)	Neuronal density (no. of cells/mm^2)	Volume (mm^3)
I (eSIDS died during SSC)	12	0.81 (0.43)[a,b]	4.66 (1.23)[a,b]	0.98 (0.74)[a,b]
II (eSIDS unrelated to SSC)	10	2.54 (0.47)	23.1 (3.57)	2.33 (0.56)
III (Controls)	10	2.57 (0.31)	23.7 (3.8)	2.36 (0.21)

Abbreviations: eSIDS, early sudden infant death syndrome; KFN, Kölliker-Fuse nucleus; SSC, skin-to-skin care.
Note: Values are expressed as mean (standard deviation).
[a]Significance related to groups II and III: $p < 0.01$.
[b]Diagnostic values for KFN hypoplasia.

15–18, and 21 of ►**Tables 1** and **3**) (Fisher's exact test, $p = 0.002$). Newborns died face down during SSC ($n = 11/12$, 91.7%) were then more likely to get a hypoplasia of the KFN than those died not concomitantly with SSC ($n = 2/10$, 20%—cases 10 and 13).

No statistically significant association was found between SSC in SUPC infants with hypoplasia of the KFN and (1) maternal smoking ($n = 2/12$, 16.7%; Fisher's exact test, $p = 1.00$—cases 6 and 11); (2) anesthesia ($n = 2/12$, 16.7%; Fisher's exact test, $p = 1.00$—cases 15 and 21); and (3) breast feeding ($n = 2/12$, 16.7%; Fisher's exact test, $p = 1.00$—cases 6 and 11).

►**Table 5** shows the statistical evaluation of the main characteristics of the case–control status. The subjects of the study have been divided in two groups: newborns died from apparently unknown cause within the first 7 hours of life (22 cases) and age-matched newborns died of known cause (controls: 10 cases). The average gestational age (weeks) was statistically different in the two groups (cases: mean [SD] = 40.1 [1.9], controls: mean [SD] = 38.1 [2.7]; *t*-test, *p*-value = 0.03). Statistically significant univariable association was found between case–control status and (1) SSC (Fisher's exact test, $p = 0.004$) and (2) hypoplasia of the KFN (Fisher's exact test, $p = 0.002$). All other variables did not provide statistically significant results.

Discussion

The main value of this study is to have highlighted a developmental alteration of a specific brain stem center associated to the unexpected sudden death of newborns occurring during SSC in the first hour of life. Precisely, through the in-depth examination of the nervous systems, we have observed in the brain stem of the great part of these victims the hypoplasia of the KFN, a pontine center essential in the breathing control.[24,25]

Recognition of the importance of a close and direct contact between mothers and infants after delivery and the related significant benefits, such as improved cardiorespiratory stability, especially in preterm infants, and improved breast feeding and thermoregulation,[26–28] has led to the widespread adoption of SSC. In addition, the Cochrane review pointed out that this practice has "no short- or long-term negative effects."[6] However, over time, SUPC was reported as being a rare event associated with SSC, mainly occurring in infants of primiparous mothers left unobserved by medical and nursing personnel, often during the first breastfeed and/or associated to maternal regional or general anesthesia during labor.[9–11] The most convincing cause of SUPC has been identified in the prone position (face down) of the newborn, with its face often pressed against the mother's chest,[13] contrary to the 1994 universal recommendation to place infants in the supine position.[29,30] The prone position is one of the components of the triple risk model for SIDS, that is, when three events occur simultaneously: underlying vulnerability of infants, critical developmental period, and exogenous stressors.[31] All of these factors are present in newborns who die during SSC. In fact, according to

Table 5 Main characteristics of the sample by the case–control status

Continuous covariates	Cases[a]		*p*-Value[b]
	Yes ($n = 22$)	No ($n = 10$)	
	Mean (SD)		
Gestational age, wk	40.0 (1.9)	38.1 (2.7)[c]	**0.030**
Birth weight, g	3,615.8 (387.8)[d]	3,457.1 (359.3)[d]	0.355
Apgar score at 5 min	8.8 (1.0)[e]	9.1 (1.1)[c]	0.522
Maternal age, y	29.5 (4.2)	30.9 (4.5)	0.446
Time of death, hours after birth	4 (1.9)	4.2 (2.1)	0.789
Categorical covariates			
	n (%)		
SSC[f]			
Yes	12 (54.5)	0	**0.004**
No	10 (45.5)	10 (100)	
Position[g]			
Prone	19 (90.5)[c]	8 (80.0)	0.577
Supine	2 (9.5)[c]	2 (20.0)	
Hypoplasia of the KFN			
Yes	13 (59.1)	0	**0.002**
No	9 (40.9)	10 (100)	
Primiparous mother			
Yes	3 (13.6)	3 (33.3)[c]	0.320
No	19 (86.4)	6 (66.7)[c]	
Maternal smoking			
Yes	8 (36.4)	2 (20.0)	0.440
No	14 (63.6)	8 (80.0)	
Gender of infants			
Female	9 (40.9)	4 (40.0)	1.000
Male	13 (59.1)	6 (60.0)	
Labor with epidural anesthesia			
Yes	2 (9.1)	0	1.000
No	20 (90.9)	100 (0)	
Died during the first breast feeding			
Yes	3 (13.6)	0	0.534
No	19 (86.4)	10 (100)	

Abbreviations: KFN, Kölliker-Fuse nucleus; SD, standard deviation; SSC, skin-to-skin care.

[a]Cases—Yes: newborns who died suddenly from apparently unknown cause within the first 7 hours of life. Controls—No: age-matched newborns suddenly died of known pathology in the first hour of life.

[b]Fisher's exact test was calculated to find associations between categorical variables and *t*-test was used to find mean differences in continuous variables. The statistically significant *p*-values are in bold.

[c]One missing value.

[d]Three missing values.

[e]Four missing values.

[f]SSC between the newborn and mother.

[g]Position of newborns during the SSC.

"Ending Newborn Deaths," a report by Save the Children,[32] the initial hours of postnatal life are the most dangerous as this is the time of transition from intrauterine to extrauterine life, when the newborns are less responsive and more

vulnerable to the outside world and stressors, including the prone position which reduces the amount of oxygen supplied to the brain. Gnigler et al[33] first coined the appropriate acronym "ESUDI" (early sudden unexpected death in infancy) for SIDS which occurs in the first 4 hours of life when the newborns are placed in prone position on mother's chest due to SSC. We preferred to classify the victims of sudden death included in this study as "eSIDS," as reported by Herlenius and Kuhn in an exhaustive literature review of SUPC cases.[9] Like many other researches, we have asked ourselves what could be the cause of these deaths suddenly happening during SSC. We believe that the reduced levels of oxygen due to the face down, and also on the side position, frequently imposed by the SSC, put newborns at inauspicious risk, above all, in the presence of developmental alterations of a specific brain stem center involved in breathing control. In fact, hypoplasia of the KFN was here observed in 11 of the 12 cases died within the first 6 hours of life during SSC.

The role of the human KFN in the motor pattern of breathing and respiratory rhythm control is, at this moment, not established. The current knowledge on the physiology and function of this nucleus arises from animal studies; therefore, we can only hypothesize a similar behavior in humans, even though aware of the existence of differences between species.[34]

Breathing in rats is controlled by a complex of neurons arranged in a lateral column extending from the rostral pons to the caudal medulla oblongata (the so-called lateral respiratory column [LRC]).[35] The KFN, located at the rostral end of the LRC, is a fundamental component of this circuitry. Despite that at birth the respiratory network must be fully functional to ensure the vital function of breathing, the KFN shows postnatal immaturity and requires the brain-derived neurotrophic factor (BDNF), a member of the neurotrophin class of growth factors, for regulating the maturation of its neurons and for stimulating their connections.[36] The mature KFN, through excitatory synaptic inputs to medullary respiratory regions,[37,38] modulates the phase transition from inspiration into expiration and the dynamic control of upper airway patency, particularly during the expiratory airflow.[39–41] Furthermore, the KFN orchestrates the postapnea airway reflexes frequently elicited by noxious stimulation of the nasal or laryngeal mucosa.[42–45] Most protective effects are in fact expiratory and require initially postinspiratory glottal closure to protect the lower airways from aspiration.[46–48] At the same time, the consequent abdominal muscle contraction generated as compulsory reflex to oppose the closed upper airways, causes high abdominal and thoracic pressure to return the organism to homeostasis.[49] It has been demonstrated that ablation of the KFN inputs prolongs the inspiratory duration producing apneusis.[50,51] Assuming the same functions of the KFN in humans, we could explain how the KFN defects in newborns can easily lead to sudden collapse when their nose and mouth are crushed on the mother's chest. The hypothesis of a similar behavior of the KFN in rats and humans is supported by our recent study showing the indispensability of BDNF for its maturation.[52] A difference would seem to exist instead with regard to the complete maturity of KFN which is reached at the end of fetal life in humans[24,52] but in early stages of life in rats.[36] In fact, we have observed that the KF neurons normally display intense BDNF immunoreactivity before birth and only in several newborns suddenly died in the first hour of life with KFN hypoplasia.[52]

We therefore have good reason to propose the hypoplasia of this nucleus, when associated to prone or on the side position of the infant imposed by the SSC, as a possible marker of these deaths. Our assumption nevertheless requires further validation on more extensive case studies. Correlations with other risk factors for SUPC, as primiparity, use of epidural during labor, first breast feeding, or maternal smoking in pregnancy were not statistically significant. Noteworthy, however, was the association found, even if only in two cases of SSC-related SUPC, between administration of epidural during delivery and hypoplasia of the KFN, especially in view of the experimental studies of Levitt et al.[53] These authors demonstrated in anesthetized rats that opioids are able to depress respiratory rate by activating the μ opioid receptors of a population of neurons in the KFN, thus inhibiting the critical role of this nucleus in maintenance of eupneic respiratory activity. Therefore, our purpose is even to carefully investigate in future studies the role of maternal opiate analgesia or regional/general anesthesia on the newborn respiratory disturbances.

A doubt arises from our results is that if the reduced size and the decreased neuron number of the KFN are the result of delayed intrauterine development or if the KFN hypoplasia is caused by apoptotic or atrophic mechanisms occurred in a normal developed nucleus. It is not possible at the moment to give an answer. We are however for the first hypothesis that the KFN hypoplasia is a congenital intrinsic defect, probably due to the interference of particular factors that predispose the neonates to death, particularly when combined with the prone position.

It is also difficult to establish whether the hypoplasia of other nuclei found sometimes in our case study may have contributed to the death. We have in fact observed the hypoplasia of one or more nuclei of the raphe system in six cases related to SSC (group I of ►**Table 4**) and in three unrelated to SCC (group II), agenesis/hypoplasia of the arcuate nucleus in four eSIDS of group I and in two of group II, agenesis of the retrotrapezoid nucleus (one case of group II) and hypoplasia of the superior olivary complex (one case of group II). In a further case of group II, the agenesis of the area postrema associated to desquamation of the ependymal cells of the fourth ventricle has been found. Information about the location, morphology, function, and developmental defects related to these structures can be found in our previous articles.[54–59] Here, we report brief considerations about the most frequent alterations observed in the brain stem of eSIDS, beyond the KFN hypoplasia, that is, the hypoplasia of nuclei of the raphe system and of the arcuate nucleus.

All these nuclei are chemosensitive structures involved in ventilatory drive. In fact, respiratory activity must respond also to chemosensory stimuli to maintain O_2 and CO_2 homeostasis in blood and tissues. The raphe system is a complex of

nuclei distributed along the median line of the brain stem. Our anatomopathological examination allowed for identification in the human brain stem of the normal topography and cytoarchitecture of these nuclei (the caudal linear raphe nucleus, the dorsal raphe nucleus, the median raphe nucleus, the raphe magnus nucleus, the raphe obscurus nucleus, and the raphe pallidus nucleus).[54] These nuclei contain serotonergic neurons acting as CO_2 sensors that are capable of maintaining pH homeostasis, so playing an important role in breathing control. Different studies have reported a reduced expression of serotonin receptors in neurons of the raphe system among SIDS cases versus controls.[60,61]

The arcuate nucleus is a group of neurons located in the brain stem on both sides of the midline at the ventral surface of the medulla oblongata, which extends between the caudal border of the pons and the caudal pole of the olive.[55,62] The underdevelopment of this nucleus, in all its forms (delayed neuronal maturation, hypoplasia, and agenesis), has been observed in more than 50% of the victims of SIDS, as reported in previous studies performed at our center[55,63] and in similar studies conducted by other groups of researches,[64,65] thus supporting the hypothesis that the arcuate nucleus is necessary to live. However, our recent observation (yet unpublished data) of the total absence of the arcuate nucleus in healthy adult humans (aged from 34 to 89 years) who died due to major trauma, led us to question its vital importance. Therefore, also in this study, we are cautious in affirming the contribution of the hypodevelopment of the arcuate nucleus to the pathogenetic mechanism of the sudden death of newborns during SSC.

In conclusion, although the current trend is to encourage the SSC practice, clinicians should be advised of the possible presence in the brain stem of newborns of developmental alterations of neuronal centers that play an important role in breathing control, particularly the hypoplasia of the KFN. This defect could compromise the vital functions in situations of oxygen deficiency conceivably caused by the infant prone positioning during SSC. Therefore, above all in the first few hours of life, it is essential to carefully monitor the infant's conditions during SSC, by placing him in a safe position and making sure that its nose and mouth are not occluded. However, when the KFN is severely defective and therefore not working, there is a very poor chance of survival because this nucleus is essential in monitoring the ventilatory activity.

Our opinion is that the SSC can be considered as a new risk factor for sudden neonatal death, to be added to others already well known for SIDS, as infant prone position and maternal cigarette smoking, and even others only recently proposed as potential triggers for sudden perinatal death as pesticides and in particular the endocrine disrupting compounds.[66,67] Finally, this report emphasizes the importance of a wide application of the Italian law 31/2006, above all, in cases of early sudden death which happens in concomitance with SSC. The in-depth study of the nervous system, according to the guidelines here presented, could in fact demonstrate that these deaths are due to the defective development of the Kölliker-Fuse, an indispensable nucleus for life, and therefore, to protect clinicians from any legal consequence.

Note

Parents of all the infants included in the study provided written informed consent to autopsy and related research.

Ethical Approval

Permission from the Ethics Committee was not required for this study as our research center ("Lino Rossi" Research Center of the Milan University) is the national referral center for sudden unexplained fetal and infant death, in accordance with the aforementioned Italian Law no. 31. Study approval was anyway granted by the Institutional Review Board of Milan University.

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None.

Conflict of Interest

None.

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3. "Variability of the medullary Arcuate Nucleus in humans"

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Abstract

The arcuate nucleus is a component of the ventral medullary surface involved in chemoreception and breathing control. The hypoplasia of this nucleus is a very frequent finding in victims of sudden unexplained fetal and infant death (from the last weeks of pregnancy to the first year of life). On the contrary, this developmental alteration is rarely present in age-matched controls who died of defined causes. These observations lead to hypothesize that a well-developed and functional arcuate nucleus is generally required to sustain life. The aim of this study was to investigate whether the arcuate nucleus maintains the same supposed function throughout life. We carried out neuropathological examinations of brainstems obtained from 25 adult subjects, aged between 34 and 89 years, who died from various causes. For almost half of the cases (44%) microscopic examinations of serial histological sections of medulla oblongata showed a normal cytoarchitecture of the arcuate nucleus, extending along the pyramids. For the remaining 56% of cases, various degrees of hypodevelopment of this nucleus were observed, validated through the application of quantitative morphometric investigations, from decreased area, neuron number, and volume, to full aplasia. These unexpected findings indicate that the involvement of the arcuate nucleus in chemoreception in adulthood is questionable, given the possibility of living until late age without this nucleus. This opens new perspectives for researchers on the role and function of the arcuate nucleus in humans from birth to old age.

Key words: human brainstem, medulla oblongata, arcuate nucleus, hypoplasia, aplasia, chemoreception, adulthood.

Introduction

In humans, the arcuate nucleus (AN) is a group of neurons located in the brainstem on both sides of the midline at the ventral surface of the medulla oblongata, which extends between the caudal border of the pons and the caudal pole of the olive (Mikhail and Ahmed, 1975). This nucleus has been proposed as the homologue of the chemoreceptive areas in the ventral medullary surface

(VMS) reported in experimental researches, responsible for mediating the ventilator responsiveness to carbon dioxide (CO2) (Bruce and Cherniack, 1987; Filiano et al., 1990; Paterson et al., 2006a; Millhorn, et al. 1986; Zec, et al.,1997). In addition, functional magnetic resonance imaging performed on 11 human healthy volunteers (Gozal et al., 1994), revealed patterns of activation in the AN area during hypercapnic ventilatory test, further suggesting that the AN could be involved in respiratory regulation in humans.

The AN has been the focus of numerous studies performed on subjects who died in late fetal and infant age (from the last weeks of pregnancy to the first year of life). In particular, different groups of researchers have indicated that, in the first months of life, some infants are at risk of Sudden Infant Death Syndrome (SIDS) when their response to increased blood CO2 levels, particularly in arousal phase, is reduced (Biondo et al., 2003; Filiano and Kinney, 1992; Kinney et al. 2011; Kinney, 2009; Machaalani and Waters, 2014; Matturri et al., 2000; 2002; 2004; Paterson et al., 2006; Rubens and Sarnat, 2013). In a high percentage of cases, this abnormal reaction of SIDS infants has been correlated to the hypodevelopment of the AN, as well as other brainstem nuclei abnormalities.

These data are consistent with the systematic review on the neuropathological features of SIDS reported by Paine et al. (2014). The authors analyzed 153 studies, of which only 70 (46%) met the three essential diagnostic criteria for SIDS which are: 1) the death is sudden; 2) death occurs in infants between the ages of 1 week and 1 year; 3) no alternative cause of death is found after a full post mortem and clinical assessment, including investigation of the death scene. Nine studies that have been discussed in this review concern the AN, 5 of which performed at the "Lino Rossi" Research Center of the Milan University (Biondo et al., 2003; Lavezzi et al., 2004; Matturri et al., 2000; 2002;2004). The authors conclude that it is not possible to establish, on the basis of the reports on the literature, whether or not the arcuate nucleus has an aetiological role in SIDS.

Overall, our many previous researches performed on a large SIDS cohort (over 150 cases), reported the presence of AN hypoplasia, sometimes even in mild form (delayed neuronal maturation, monolateral hypoplasia or hypoplasia confined to one third of its extension), in more than half of the SIDS cases (approximately

60%). On the contrary, the AN hypodevelopment was a rare conclusion in age-matched control cases died of known causes. These observations led us to hypothesize that a proper development and functionality of the AN could be necessary to sustain life. Nevertheless, the casual observation of the total absence of this nucleus in the medulla oblongata of a healthy adult subject who died for a major trauma, led us to revise our hypothesis. Therefore, with the aim to determine whether this was merely an isolated, inexplicable finding or, on the contrary, that a well-developed and functional AN could be essential throughout the entire lifespan, we decided to analyze the AN in a cohort of adults who died of various causes.

Material and Methods

Study Subjects

The study was conducted on 25 subjects (including the person who prompted this study), 18 males and 7 females aged between 34 and 89 years, who died from various causes (Table 1). In-depth analyses of their brainstems were carried out at our Research Center in accordance with the guidelines established for the anatomopathological examination of perinatal deaths, under Italian law n.31/2006 "Regulations for Diagnostic Post Mortem Investigation in Victims of SIDS and Unexpected Fetal Death" (available at: http://users.unimi.it/centrolinorossi/files/gazz_ufficiale.pdf) and according to guideline for autopsy investigation of adult sudden cardiac death (Basso et al., 2017).

After fixation in 10% phosphate-buffered formalin, the brainstems were processed and embedded in paraffin from the lower third of the midbrain to the caudal pole of the olive. Two main specimens were obtained. The first specimen included the medulla oblongata, isolated through an upper cut at the caudal border of the pons and a lower cut at the beginning of the spinal cord; the second specimen included the pons and the lower third portion of the midbrain.

Transverse serial sections of the two samples were made at intervals of 50-60 μm. For each level, serial 5 μm sections were obtained, two of which were routinely

stained for histological examination using hematoxylin-eosin and Klüver-Barrera. The remaining sections were stained as deemed necessary and saved for further investigations.

The histological evaluation of the brainstem nuclei, according to the guidelines established by the Italian law, are focused on identifying the hypoglossus, the dorsal motor vagal, the tractus solitarius, the ambiguous, the pre-Bötzinger, the inferior olivary, the raphé nuclei (obscurus and pallidus), the trigeminal and above all the arcuate nucleus, the focus of this study, in the medulla oblongata; on the locus coeruleus, the median raphé and the Kölliker-Fuse nucleus in the rostral pons/caudal mesencephalon; on the retrotrapezoid nucleus, the superior olivary complex, the magnus raphé nucleus and the facial/parafacial complex in the caudal pons.

Protocol for the brainstem examination

Specific protocol for the examination of the Arcuate Nucleus (AN)

A first examination was performed under a light microscope at different magnifications (from 0. x to 100x) on serial histological sections obtained from the medulla oblongata, with the plates from VIII to XVIII of the classic Atlas of Olszewski and Baxter (1982) and the sections from E to J of the online Atlas of the Brainstem of Swenson (available at: http://www.dartmouth.edu/~rswenson/Atlas/BrainStem/index.html) used as references for the AN analysis.

Every time that the morphological examination of the AN largely corresponded to that represented in the Atlases, the nucleus was classified as "normal". A diagnosis of "hypoplasia" was formulated when the AN showed decreased area in serial histological sections compared to the corresponding reference images at the same levels.

The examination of slides was performed double blinded, with no prior knowledge of the cause of death. Comparison of diagnoses achieved by every pathologist was obtained employing the K Index (KI) to evaluate the inter-observer reproducibility. A specific system for the K interpretation was used, where 0 to 0.2 is slight agreement, 0.21 to 0.40 indicates fair agreement, 0.41 to 0.60 moderate agreement, 0.61 to 0.80 strong or substantial agreement, and 0.81

to 1.00 indicates very strong or almost perfect agreement (a value of 1.0 being perfect agreement) (Landis and Koch, 1977). The application of this method in the present study produced a satisfactory KI (0.85).

In order to verify the reliability of the microscopic examinations, all the cases were submitted to quantitative morphometric investigations.

Morphometric analysis of the AN

A quantitative method was applied on the serial sections obtained from the medulla oblongata, stained with Klüver-Barrera. The basic investigation was carried out with an Image-Pro Plus Image Analyzer (Media Cybernetics, Silver Spring, Maryland, USA) on both sides of the AN. The following parameters were evaluated: 1) AN transverse area (expressed in mm2), 2) AN neuronal density (number of neurons with clearly defined edges and with a distinct nucleolus per mm2), and 3) AN volume (in mm3). The transverse area was calculated for every case as mean value of the measurements of the AN area, after having drawn its boundaries, simply on three slides: a histological section at high level of the medulla oblongata (corresponding to plate XIV and section I, respectively, of the aforementioned Atlases), a section at the median level (plate XII and section H) and a caudal section (plate X and section G). The evaluation of the neuronal density was performed in the same selected histological sections with the same criterion, ie as mean value of the three measurements. A computer program developed by Voxblast (VayTek, Fairfield, Iowa) was employing to obtain the three-dimensional reconstruction (3-D) of the whole structure, from the caudal pole of the olives to the pontomedullary junction, by using all the serial sections and in which the outer boundaries have been traced. The tracings were digitized by computer and then registered to reestablish their original positions relative to one another. The fourth ventricle was used as landmark for registration. Statistical calculations were carried out with the SPSS statistical software (version 11.0; SPSS Inc., Chicago, IL, USA). All the morphometric results were expressed as mean values and standard deviation (m ± SD). The statistical significance of direct comparisons between groups with normal and defective AN was determined using the analysis of variance (F-test). The selected threshold level for statistical significance was $p < 0.05$.

Ethics statement

Permission from the Ethics Committee was not required for this study as our Research Center (The Lino Rossi Research Center at Milan University) is the national referral center for neuropathological studies under the Italian Law n. 31/2006. The study was approved by the institutional review board of Milan University.

Results

Examination of the Arcuate Nucleus (AN)

Histological examinations of medullary serial sections highlighted in 11 cases (44%) (see Table 1) a clear, large and well delineated AN, subdivided in two units, extending along the ventral median fissure, superficial to the pyramidal tract, between the caudal border of the pons to the caudal pole of the olives. The extreme caudal part of the lateral extension of the AN was either triangle-shaped or formed a narrow band. It was largest in the rostral medulla oblongata where it frequently appeared histologically continuous with the caudal pontine nuclei. The cytoarchitecture of the AN was clearly visible at the obex level (Figure 1A). At higher magnification, its neurons appeared loosely arranged, medium sized, oval, polygonal or elongated with eccentric large nuclei and long dendrites frequently well discernable (Figure 1B). Occasionally, small groups of neurons, isolated or connected with the main structure, appear to be embedded among the fascicles of the pyramids. The death diagnoses of these cases were: carcinoma (5 cases), accidental polytrauma (2 cases), myocardial infarction (2 cases), aspergillus pneumonia following lung transplantation (1 case) and arrhythmogenic right ventricular cardiomyopathy (1 case).

For the remaining 14 cases (56%), whose deaths were not caused by respiratory diseases but due to cardiac pathologies (8 cases), carcinoma (3 cases) and polytrauma (3 cases), various degrees of AN hypodevelopment were highlighted at the histological examination. Precisely a “bilateral hypoplasia”, characterized by small size of the medial and lateroventral sides of the nucleus with neuronal depletion compared with the AN features observed in the previous group, was

diagnosed in 3 cases; a “partial hypoplasia”, including both hypoplasia of the caudal two thirds of its extension and monolateral (right) hypoplasia in 3 cases, and an “aplasia”, i.e. total absence of the nucleus, in 8 cases (Figure 2). No difference was found between AN alterations and sex and age of the subjects. In fact AN hypodevelopment was found in 10/18 males (56%) with a mean age of 62,3 years, and in 4/7 females (57%), mean age 63,2 years.

The morphometric analysis allowed to obtain valid and objective quantitative results in support of the microscopic diagnoses. The measurements related to the AN transverse area, neuronal density and volume, allowed to subdivide the cases in three distinct categories: (I) normal (11 cases); (II) hypoplasia (with significant reduction of the AN values compared with those of the first group) (6 cases) and (III) aplasia, without any trace of the AN (8 cases).

Table 2 shows the mean values related to the three parameters (area, neuronal density and volumetric reconstruction) obtained in Groups I and II, the only groups in which it was possible to make measurements. The mean values related to the transverse area in the first and second groups were 2.76 ± 0.5 and 1.06 ± 0.3 and the mean neuronal density 91.2 ± 4.6 and 45.3 ± 6.2, respectively. The decreased area and number of neurons observed in group II were associated to a three-dimensional reduction of the nucleus, compared with the corresponding values obtained in group I. The mean 3-D results were in fact 26.7 ± 6.9 in group II and 46.7 ± 1.1 in group I ($p<0.05$). Table 3 shows the groups II and III (hypoplasia + aplasia) joined in the same category, considering into the whole as altered expression of the AN. This enlarged group was compared to the normal one, obtaining even more significant statistical differences regarding the transverse area, neuronal density and volume ($p<0.01$).

Examination of the other brainstem nuclei

It is important to note that it is sometimes difficult to identify other nuclei in the brainstems of adult men, unlike those in fetuses and newborns for whom the neuropil is still underdeveloped, due to the presence of numerous fibers (neuronal axons, dendrites and glial cell processes) that are synaptically intertwined and consequently conceal neurons. However, in this study it was possible to identify the locus coeruleus, the medial nucleus of the superior olivary complex and the

facial/parafacial complex in the pons; the hypoglossus, the dorsal motor vagal, the tractus solitarius, the ambiguous, the inferior olivary and the obscurus raphé nuclei in the medulla oblongata of all the 25 cases. In two cases, one with AN hypoplasia and one with AN aplasia, a very high number of neurons in the raphé obscurus nucleus (hyperplasia) was evident as compared with the other 23 cases in which the nucleus was present with a normal cythoarchitecture, i.e. a moderate group of neurons arranged along the midline of the medulla oblongata (Figure 3). Moreover the Kölliker-Fuse nucleus and the retrotrapezoid nucleus in the pons, and the pre-Bötzinger and Pallidus Raphé nucleus in the medulla oblongata were detectable in 5 cases, despite the presence of numerous nervous processes. Notably was the observation in two cases with AN hypoplasia and one with AN aplasia, of an overdeveloped pre-Bötzinger nucleus (hyperplasia), characterized by a marked increased area and number of neurons compared with the same nucleus highlighted in the other two cases, in which the cytoarchitecture looked like normal, according to our previous study (Lavezzi and Matturri, 2008) (Figure 4). These results are reported in Table 1.

Discussion

This paper concerns the variable and surprising behavior in adult men of the medullary AN, which has been hypothesized to have a central chemoreceptive function, and discusses the possible significance of its hypoplasia in advanced age.

Respiratory activity must respond to chemosensory stimuli in order to maintain O2 and CO2 homeostasis in blood and tissues. In particular, the CNS mechanism for CO2 detection and ventilator regulation, mainly deduced from experimental studies, involves the neurons located in chemosensitive areas of the VMS. Since neurons of the human AN have similar characteristics to those of VMS, it has been hypothesized that the AN contributes to the regulation of respiration in humans. The implication of the AN in various lung dysfunctions reinforces this hypothesis (Ono et al., 2001; Folgering et al., 1979; Matturri et al. 2003). However, at this moment, no proven functional information was provided about

the role of the AN in chemoreception and breathing control. Also in the study of Zec et al. (1997) performed on human fetal brainstems, the involvement of the AN in ventilation is only supposed but not demonstrated. These authors, by using a lypofilic dye (DiI) in order to examine anatomic relationships of the human AN with cardiorespiratory-related brainstem regions, observed the presence of labeled fibers from the AN to the caudal raphé, a region implicated in respiratory control. However, because the staining density obscured the underlying cytoarchitecture, this study could not confirm that the DiI fibers originate from AN neurons. Furthermore, Filiano et al. (1990) comparing the human and feline superficial ventral medulla, only supported the idea that the neurons of the human AN are homologous to the neurons in chemosensitive areas of the cat.

The underdevelopment of the AN, in all its forms (delayed neuronal maturation, hypoplasia, aplasia), has been observed in over 50% of the victims of sudden perinatal death, as reported in previous studies carried out at our center (Biondo et al., 2003 Matturri et al., 2000; 2002; 2004) and in similar studies conducted by other groups of researches (Kinney, 2009; Kinney et al., 2011; Machaalani et al., 2014; Paterson, et al., 2006a; Rubens and Sarnat, 2013), thus supporting the hypothesis that this nucleus could be vitally important for the chemoreception, though however without trying a causal relationship with SIDS. We must also consider that other developmental irregularities could contribute to the pathogenetic mechanism of death.

The results obtained on the AN in adult human led us to hypothesize that the function of any brainstem nucleus may not be constant and equally important throughout life, but it can markedly change with age. We wish to suggest that, while some nuclei could give an essential contribution through the entire lifespan, others, likes the AN, only during the earliest developmental stages. This is perhaps just our likely hypothesis, based on our previous research on the intermediolateral nucleus (ILN) in the spinal cord (Lavezzi et al. 2010). In fact, the activity of the ILN is essential in the first phases of fetal life, as it is able to promote spontaneous bursts of rhythmic respiratory-related activity, that are essential for the lung development, even in the absence of excitatory synaptic drive, through the release of neurotrophic signals and modifications of the

intracellular milieu (Ren and Greer, 2003; Ren et al., 2006; Spanswick and Logan, 1990). Moreover, when completely differentiated, in postnatal life, the ILN no longer plays a leading role in the respiratory pattern, but it acquires a new function as effector of orthosympathetic reflexes (Powley, 2013).

Besides, we believe that the role and structure of a given nucleus may change from one subject to another in adulthood, and this applies above all to the AN, since its morphology is extremely variable in age-matched subjects, as shown in this study. Baizer (2014), in an interesting review on the cytoarchitectonic organization of the human brainstem compared to that of several other species, specified that the AN is a unique structure in humans, not present on other species (as cat, squirrel monkey, macaque monkey) with the exception of the chimpanzee in which it is sometimes observable. She reported, though in a limited number of cases (4 females and 4 males aged from 45 to 71 years) provided by the Witelson Normal Brain Collection (Witelson and mcCulloch, 1991), individual variability of the AN in the size and shape and frequent asymmetry with left–right differences, without any mention of its hypodevelopment or aplasia.

Our observations raise an important question, i.e., are the AN anomalies reported in this study developmental in origin or acquired? If congenital, the hypothesis of an important role of the AN in chemoreception and gas homeostasis control would be unsustainable, given the possibility of survival for a long time in absence of this nucleus. If acquired, they could be secondary to an injury, as already suggested for SIDS infants with AN hypoplasia (Lavezzi et al., 2004). In these cases, death was attributed to a failure in homeostatic control in presence of a stressor, such as hypoxia or increased CO2, which are frequently associated to tobacco smoke exposure. Or more simply, hypoplasia of the AN could be the result of a normal physiological aging process leading to neuron loss. For many years it has been reported in the literature that extensive neuron death is an inevitable result of normal aging (Brody, 1992; Dayan, 1970a, 1970b). Morrison and Hof (1997) highlighted however in a wide review on the aging brain, how the application of stereological techniques allowed to demonstrate extensive loss of neurons in cerebral cortex of subjects affected by Alzheimer's disease (AD). On the contrary, in neurologically normal elderly individuals there was no evidence

of neuron number decline in the same region. More recently, Bishop et al. (2010), in another review on the same topic, refer that, although neuronal loss is minimal in most cortical regions, age-dependent changes, as reduced synaptic connectivity and loss of integrated functions, can occur in different brain regions.

Anyway, in support of the hypothesis of a diminished important role of NA in adulthood is the observation, here triggered by the morphometric investigation, of a significant decrease on neuronal density, even in a normal AN structure, compared with that of infants died in the first year of life. In fact, the mean neuronal number observed in infants with a well-structured AN in a previous study of our Research Center (Matturri et al. 2000) was 236 ± 14, more than twice greater than that here obtained in adults (mean value: 91.2 ±4.6).

Noteworthy is the association here showed, although in a few cases, between AN hypoplasia/aplasia and hyperplasia of two chemosensitive centers of the medulla oblongata involved in ventilator drive, the pre-Bötzinger nucleus and the raphé obscurus nucleus. The pre-Bötzinger is a nucleus that contains a limited number of small neurons very essential for rhythmogenesis (Lavezzi and Matturri, 2008; Ramirez, 2011). Experimental investigations have in fact shown that this neuronal structure generates constantly the inspiratory phase of respiratory rhythm (Lü et al., 2017; Reklinget al., 1998; Smith et al., 1991). Schwarzacher et al. (2010), by using comparative cytoarchitectonic criteria, confirmed the relevance of the pre-Bötzinger in the neuronal control of eupneic breathing in humans. The raphé obscurus nucleus contains instead serotonergic neurons acting as CO2 sensors that are capable of maintaining pH homeostasis, so playing an important role in breathing control (Paterson et al., 2006b; Ozawa and Okado, 2002). An increase in the number of neurons in these structures, even if observed in a very low percentage of cases, may be interpreted as an attempt to compensate the alleged reduced chemoreceptive functionality of the AN.

A limitation of this study is the low number and heterogeneity of the investigated subjects. Therefore, we intend to continue this research on a large series of cases, with the aim to perform a more detailed analysis of the AN producing a more omogeneous subgroups to highlight, in particular possible sex, age, and death cause differences. There are in fact important biological differences between men

and women regarding epidemiology and pathophysiology of many widespread diseases (Regitz-Zagrosek, 2012). Furthermore we wish to evaluate the neuroanatomic connections of the AN, via interneuronal synapses, with other nerve centers with the aim to provide with valuable insight on the variable behaviour of the AN in human adults. Already Edlow et al. (2016), using ultra-high resolution diffusion spectrum imaging tractography, have recently demonstrated in healthy adults the presence of integrated central homeostatic networks (CHN) between autonomic and cardiorespiratory nuclei in the human brainstem and forebrain sites critical to homeostatic control. A "CHN connectome" has been defined in particular by these authors between the raphé nuclei in the brainstem and the medial temporal lobe. Our aim is to carry out a similar study not only to verify the links of the AN with the raphé obscurus and the pre-Bötzinger nuclei, but also to highlight new possible connectivity patterns with higher forebrain centers relevant for the autonomic control, with the possibility to modulate one another. A reduced presence of the AN in adulthood could be counterbalanced by an increased expression and functionality of the other components of the network, to maintain a stable homeostasis. This interpretation could be an initial step towards elucidating the neuroanatomic significance of the intersubjective variability of the AN nucleus in adult humans.

In conclusion, this study does not want to give an explanation about the different morphology and behavior of the AN we have observed in adults, but only to expose our observations hoping to stimulate researches on the role and function of this nucleus from birth to old age.

Author Contributions:

AML, BP, GT and SF planned and carried out the research and contributed to the analysis and interpretation of the results. All the authors drafted and approved the final version of the manuscript.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1: (A) Klüver-Barrera histological transverse section of medulla oblongata at obex level showing a normal arcuate nucleus (case n.7 of Table 1; male, 44-year-old). Arrows indicate a large, well delineated arcuate nucleus, subdivided into two medial and lateroventral parts, is well visible. In the framed area in (A), represented at higher magnification in (B), scattered medium sized, polygonal or elongated neurons are visible. At the top, schematic representation of the brainstem indicating the level of the histological section. AN arcuate nucleus; fV fourth ventricle; ION inferior olivary nucleus; Pyr pyramid; V ventral; VMS ventral medullary surface; D dorsal. Magnification (A) 0.5x; (B) 20x.

Figure 2: (A) Klüver-Barrera histological transverse section of medulla oblongata showing bilateral aplasia of the arcuate nucleus (case n.11 of Table 1; male,78-

year-old). The framed area in (A), visible at higher magnification in (B), shows the pyramids, covered only by the meningeal pial membrane, bordering directly on the ventral medullary surface. The arcuate nucleus is completely missing. The white holes, well visible in (B) are the spaces that separate the pyramidal fascicles. fV fourth ventricle; ION inferior olivary nucleus; mm meningeal membrane; Pyr pyramid; V ventral; VMS ventral medullary surface; D dorsal. Scale bar: (A) 2mm; (B) 200µ. Magnification (A) 0.5x; (B) 20x.

Figure 3: (A) Klüver-Barrera histological transverse section of medulla oblongata showing hyperplasia of the raphé obscurus nucleus (case n.21 of Table 1; male, 67-year-old, with arcuate nucleus aplasia). The framed area, represented at higher magnification in (B), shows an increase in number of neurons compared with that of the raphé obscurus nucleus represented in (C) of an age-matched case of the study (case n.22 of Table 1 with normal arcuate nucleus). At the top, on the left there is a panoramic view of a complete histological medullary section with indication in the circled area of the localization of the nucleus; on the right a schematic representation of the brainstem indicating the level of the histological section. D dorsal; fV fourth ventricle; ION inferior olivary nucleus; ROb raphé obscurus nucleus; V ventral. Magnification: (A) 10x; (B) and (C) 20x.

Figure 4: (A) Klüver-Barrera histological transverse section of medulla oblongata showing hyperplasia of the pre-Bötzinger nucleus, (case n.9 of Table 1; female, 49 year-old with arcuate nucleus aplasia). In the framed area, at higher magnification in (B), a greater number of neurons can be seen compared with that of the pre-Bötzinger nucleus represented in (C) of an age-matched case of the study (case n.14 of Table 1 with normal arcuate nucleus). At the top, on the left there is a panoramic view of a complete histological medullary section with indication in the circled areas of the localization of the nucleus; on the right a schematic representation of the brainstem indicating the level of the histological section. D dorsal; DA dorsal accesory of the inferior olivary nucleus; pre-Böt pre-Bötzinger nucleus; ROb raphé obscurus nucleus; V ventral. Magnification: (A) 10x; (B) and (C) 20x.

Table 1. Profiles of the 25 cases of the study (18 males, 7 females; age range: 34-89 years) with death diagnosis and neuropathological results

Case no.	Sex/Age (years)	Death diagnosis	Arcuate Nucleus histology	Other brainstem nuclei alterations
1	M/60	aspergillary ulcer-necrotic pneumonitis in pulmonary transplant	normal	/
2	M/34	arythmic sudden cardiac death	aplasia	/
3	F/60	*myocardial infarction*	aplasia	/
4	M/48	polytrauma by car accident	bilateral hypoplasia (severe)	/
5	M/45	cardiac transplant rejection	bilateral hypoplasia	/
6	M/60	polytrauma by fall	aplasia	/
7	M/44	polytrauma by fall	normal	/
8	M/71	prostatic carcinoma	normal	/
9	F/49	gastric carcinona	aplasia	Pre-Bötzinger hyperplasia
10	M/57	gastric carcinona	normal	/
11	M/78	pancreatic carcinoma	aplasia	/
12	F/55	colon carcinoma	bilateral hypoplasia (moderate)	Pre-Bötzinger hyperplasia
13	M/61	colon carcinoma	normal	/
14	F/78	thyroid carcinoma	normal	/
15	M/67	*myocardial infarction*	normal	/
16	F/89	arrhythmogenic right ventricular cardiomyopathy	aplasia	/

17	F/58	arrhythmogenic right ventricular cardiomyopathy	normal	/
18	M/80	hypertrophic cardiomyopathy	aplasia	/
19	M/79	dilated cardiomyopathy	partial hypoplasia (2/3 of its extension)	raphè obscurus hyperplasia
20	M/44	hypertensive cardiomyopathy	partial hypoplasia (monolateral,right)	/
21	M/67	polytrauma by car accident	aplasia	raphè obscurus hyperplasia
22	M/41	polytrauma by car accident	normal	/
23	F/60	osteorsarcoma	normal	/
24	M/88	*myocardial infarction*	partial hypoplasia (monolateral,right)	Pre-Bötzinger hyperplasia
25	M/64	*myocardial infarction*	normal	/

Table 2- Morphometric analysis of the AN of cases with presence of AN

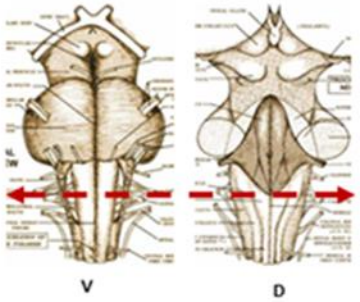
Groups	**N. cases**	**Transverse area** (mm^2)	**Neuronal density** (no.cells/mm^2)	**Volume** (mm^3)
I *(normal AN)*	11	2.76 ± 0.55	91.2 ±4.6	46.7 ± 1.15
II *(AN hypoplasia)*	6	1.06 ± 0.3*	45.3 ± 6.2*	26.7 ±6.9*

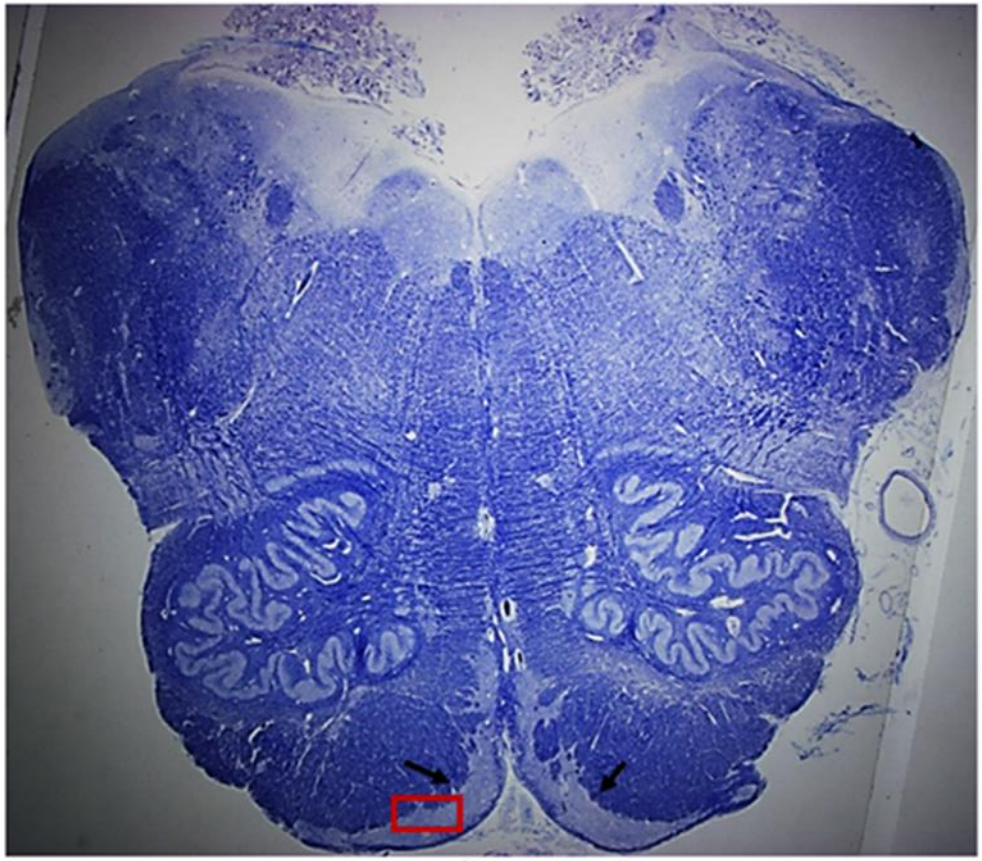
*Significance related to group I: $p < 0.05$

Table 3- Morphometric analysis of the AN in all the 25 cases

Groups	**N. cases**	**Transverse area** (mm^2)	**Neuronal density** (no.cells/mm^2)	**Volume** (mm^3)
I *(normal AN)*	11	2.76 ± 0.55	91.2 ±4.6	46.7 ± 1.15
II enlarged *(AN hypoplasia + AN aplasia)*	14	0.45 ± 0.3*	19.42 ± 14.7*	11.45 ± 9.1*

*Significance related to group I: $p < 0.01$





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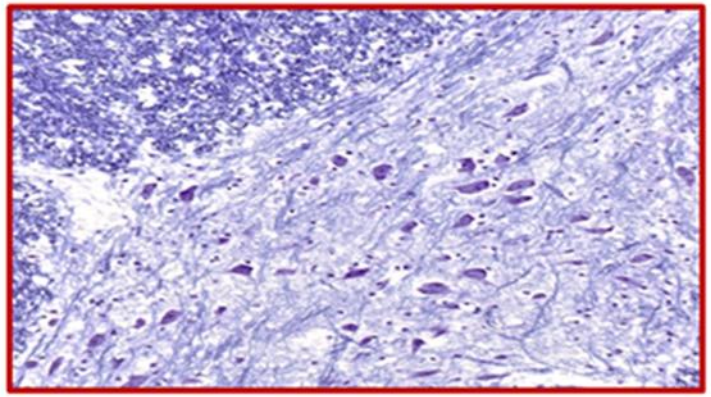
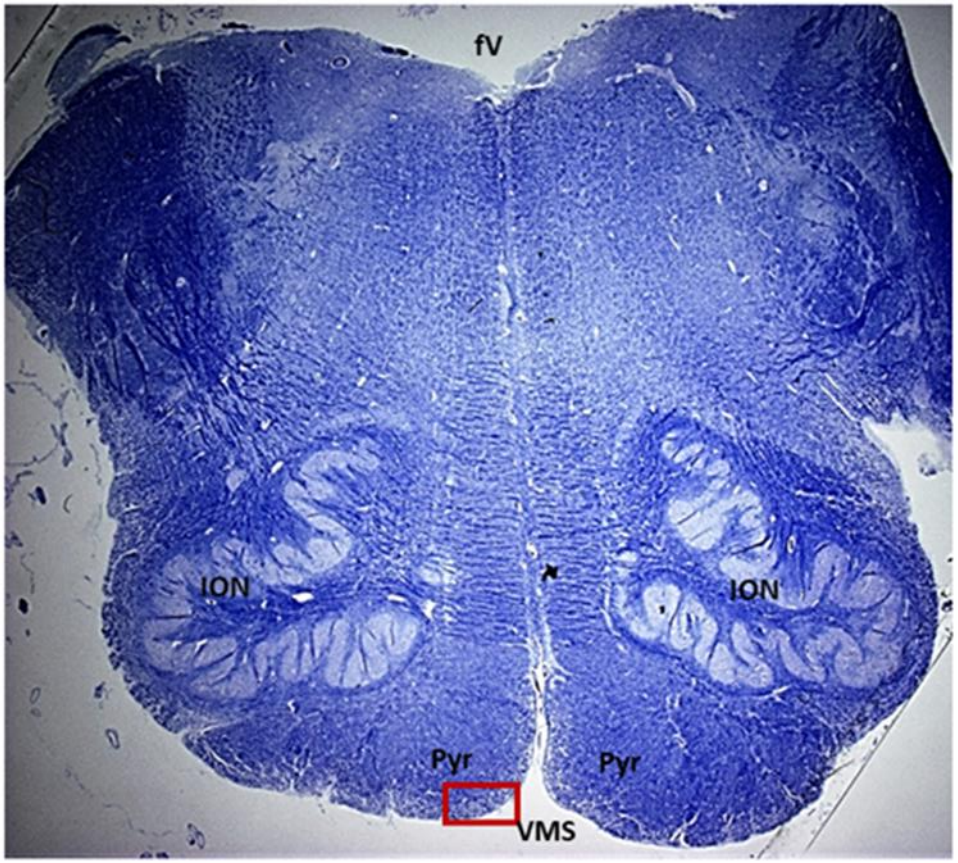


B

Fig.1



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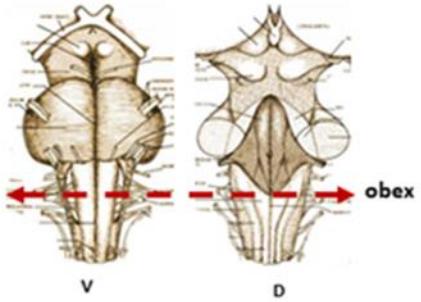
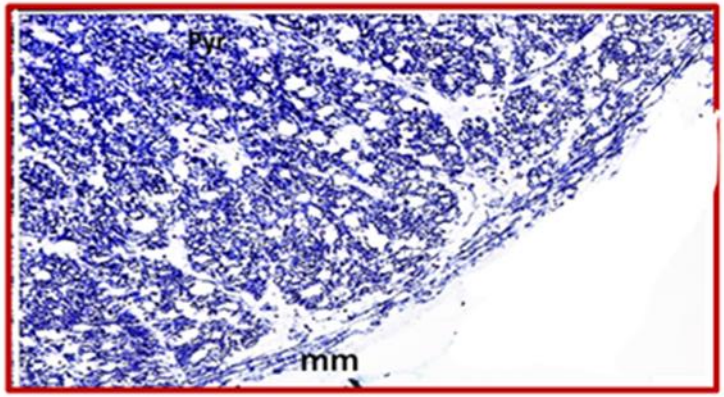
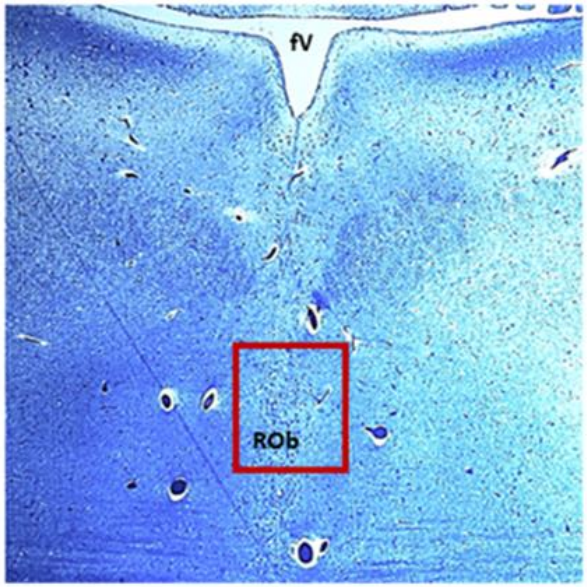
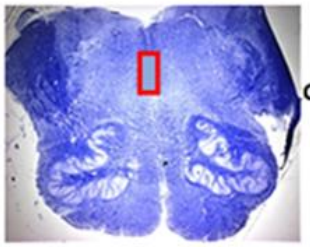


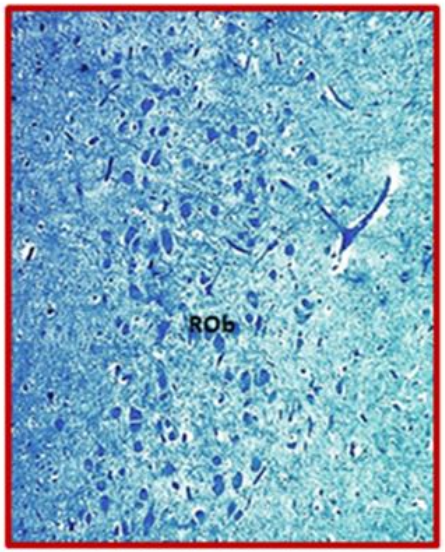


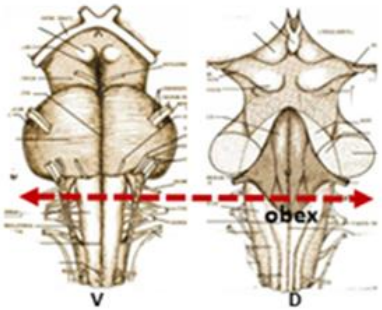
B

Fig. 2









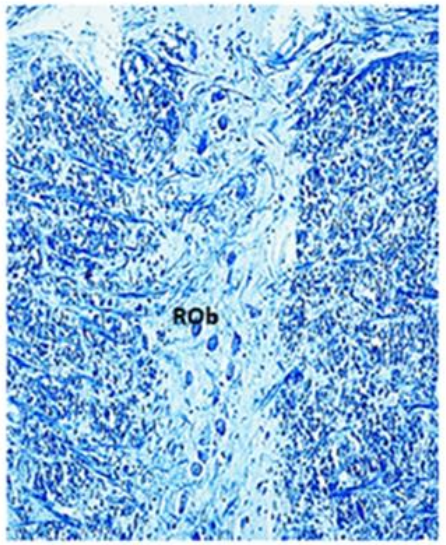
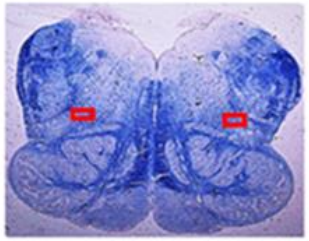


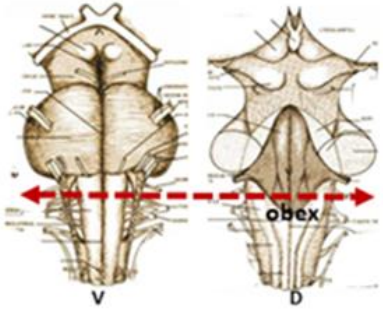
Fig. 3

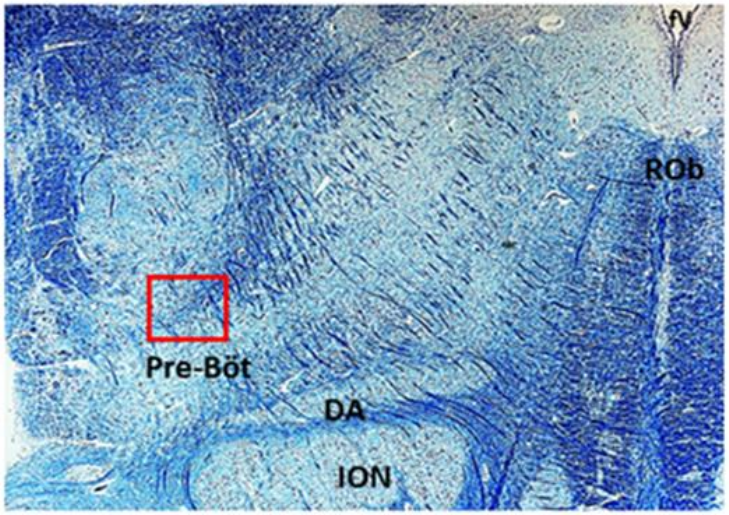
A

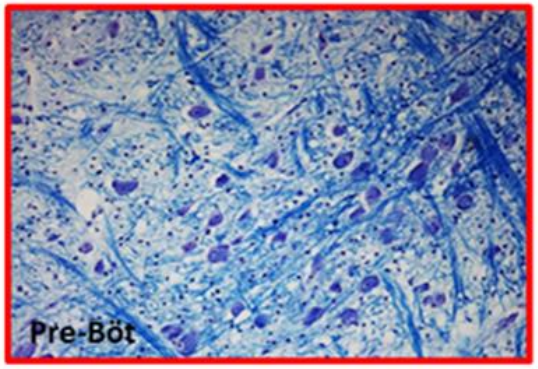
B

C









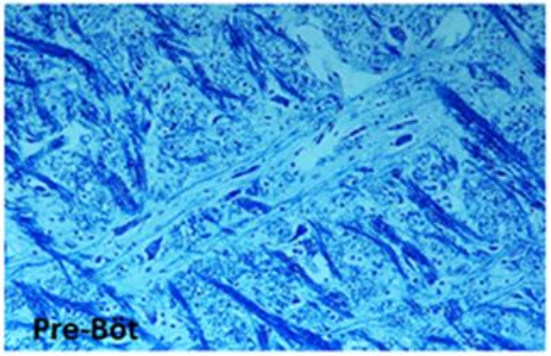


Fig. 4 A B C

4. "Unexpected in utero exposure to psychotropic medications"

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Unexpected in utero exposure to psychotropic medications

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Introduction: Pregnancies in the world each year involve women who have or who will develop psychiatric illness during the pregnancy. Psychotropics in gestation could produce adverse perinatal and postnatal outcomes, however counseling these women to discontinue medication presents new risks associated with untreated or inadequately treated mental illness, such as poor prenatal care, inadequate nutrition, and increased alcohol and tobacco use. The single administration at a higher dosage over multiple medications, active pharmaceutical compounds with fewer metabolites and higher protein binding are preferred. Nevertheless all psychotropic medications cross the placenta, are present in amniotic fluid, and can enter breast milk.
Case description: The FDA, the Australian Drug Evaluation Committee and Micromedex have categorized medications according to risk during pregnancy. Based on these classifications Benzodiazepines, have been demonstrated possibly teratogenic however they are still used for treating anxiety, panic, seizures and insomnia. International Pharmacopoeia reported an increased risk of intrauterine growth retardation, hypotonia, bradycardia, respiratory depression, low Apgar and preterm delivery for fetal plasma drug concentrations equivalent to the therapeutic range of maternal prescription. Very few data are published on sudden intrauterine death (SIUD) for intrauterine exposure to psychotropic medication. We describe a SIUD correlated with unexpected toxic plasmatic fetal concentration of Lorazepam.
Results and conclusions: The pregnant manifested anxiety, panic attack and deficiency of emotional transport for the fetus. She was treated with Lorazepam evening dose of 2mg from 24 to 40 gestational weeks, according to therapeutic range prescription, when suddenly and unexpectedly fetus died in utero just before the delivery. We evaluated the maternal and fetal pharmacological plasmatic concentration and observed a fetal plasmatic level of Lorazepam of 330mcg/l greater the toxic cutoff of 45mcg/l, suggested by Micromedex to cause "floppy infant" syndrome. By autoptic diagnosis we hypothesized neuropathological signs of sudden cardiac arrest in health fetus.
Take-home message: We propose that the monitoring of the maternal plasma levels of benzodiazepines during pregnancy and assessment of the concentration umbilical cord at birth, have to be correlated with the fetal vital signs. Nevertheless, it is difficult to define the metabolic fate of drugs in utero. Each of the major metabolic pathways can be promoted by placental and/or fetal enzymes and the metabolite concentration in the fetus does not ineludibly reflect the ability of the fetus to metabolize drugs. For this reason it has to be strictly evaluated the toxicological etiopathogenesis of some cases of SIUD and still birth.

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Marcus Gunn Syndrome and implications for Oral and Maxillofacial surgery (OMFS)

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Introduction: Marcus Gunn Syndrome, also known as Jaw Wink Syndrome or trigemino-oculomotor synkinesis, was first reported in 1883. It typically presents at birth with unilateral ptosis and eyelid elevation on jaw opening. Pathophysiology is explained by an oculofacial synkinesis. There is an aberrant connection of the oculomotor nerve and the mandibular branch of the trigeminal nerve resulting in eyelid elevation on mouth opening. The typically congenital syndrome is exceptionally rare. It is often diagnosed in infancy with complete ophthalmic examination and ptosis evaluation. This syndrome does not often require surgical intervention but it may still have an impact in clinical management.
Case description: A 32-year-old male presented in the OMFS outpatient clinic in Countess of Chester Hospital for extraction of his lower third molars. His past medical history included a known diagnosis of Marcus Gunn Syndrome but he was otherwise fit and well. He had resting ptosis of the left and elevation of the left eyelid on jaw protrusion.
Results and conclusions: Third molars were removed uneventfully under local anesthesia and no further treatment was required. Literature suggests that patients with Marcus Gunn Syndrome may have an atypical oculocardiac reflex during their surgical procedure and patients are at increased risk of malignant hypothermia. In this case, the procedure was performed under local anesthesia but this condition may impact on surgical planning if general anesthesia was to be considered.

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Rash or infection? An uncommon case of fever with skin lesions

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Introduction: Acute generalised exanthematous pustulosis (AGEP) is rare form of late hypersensitivity syndrome that can be sometimes mistaken as a skin infection. The differential diagnosis of infectious pustular lesion is large but it can also appear in the setting of a complete non-infectious estate.
Case description: We present a 40-year-old woman from a French-Canadian background who developed pustular lesions all over her body in the setting of fever, weakness and headache. She was previously affected by an Henoch–Schönlein purpura and developed secondary chronic infectious leg skin lesions.
Result and conclusion: Two months before the apparition of the pustules, she was treated by many different antibiotics (cephalexin,

5. "The brain beating and the heart breathing. Cardiorespiratory nuclei analysis and brainstem mapping in sudden infant death"

Clinical Neuropathology, Vol. 36 – No. 3/2017 (p. 135) Joint meeting 53rd Congress of the Italian Association of Neuropathology and Clinical Neurobiology (AINPeNC) 43rd Congress of the Italian Association for Research on Brain Aging (AIRIC), Abstract of Oral Presentation

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The brain beating and the heart breathing. Cardiorespiratory nuclei analysis and brainstem mapping in sudden infant death

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Objective: Sudden infant death syndrome (SIDS) is characterized by the death of an infant that cannot be explained, despite a systematic case examination, including death scene investigation, autopsy, and review of the clinical history. Sudden unexpected infant death (SUID) is a wide-ranging concept used to describe any sudden and unexpected death, whether explained or unexplained, including SIDS, which occurs during the first year of life. Differing hypotheses of the underlying mechanisms of SIDS have been proposed. The most reliable seems to be the "triple risk hypothesis". Based on this theory, SIDS might arise by the combination of three factors: a vulnerable infant, a vulnerable phase of development and a final insult occurring in this window of vulnerability. In the past, disjoined causes were recognized as main pathogenic conditions: primary cardiac alterations or primary respiratory anomalies or primary neurological predispositions. Recently, a unified neuropathological theory contributes to describe SIDS. Thanks to that, serotonergic neurons have a crucial homeostatic function in the cardiorespiratory brainstem centers. Materials: We investigated articles and reviews indexed in PubMed, queried databases of digital brain atlases to understand brainstem microcircuits involved in respiratory rhythm and in pattern generation for cardiorespiratory coupling. Contextually, we started to analyze brains and principally brainstems of SUID cases from 2014 until today. Methods: Cardiac, sympathetic, and respiratory motor activities can be viewed as a unified rhythm controlled by brainstem neural circuits for effective and efficient gas exchange. We improved the usual postnatal brain fixation, utilizing liquid solutions of glacial acetic acid in neutral-buffered formalin and ethanol. Results: By cardiorespiratory coupling theory and data from the literature, we obtained a scheme of cardiorespiratory brainstem nuclei network, and finally we mapped the principal cardiorespiratory nuclei of the human postnatal brainstem in 26 SUID cases. About 30% of our SUID cases presented anomalies of brainstem nuclei. Discussion: Many intrinsic and extrinsic factors increase SIDS susceptibility including prone sleeping position, inflammations, gender, prenatal nicotine exposure, and temperature. The final common pathway for SIDS involves a failure to arouse and autoresuscitate in response to environmental challenge. These risk factors can directly alter the function of cardiorespiratory nuclei and impair the ability of this network to coordinate cardiorespiratory coupling. Conclusions: Neuropathological analysis of the infant brainstem represents a good tool to infer on the final events of SUID and SIDS. An integrated study of postmortem functional imaging could help to understand the network of this beating-breathing-thinking unit.

Myo-miRNA and inflammatory microRNAs in muscle of sporadic and genetic ALS

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Objective: MicroRNAs are small non-coding RNAs that are associated with stress granules, mitochondria, and other subcellular organelles in muscle. Few studies have explored the role of microRNAs in muscle atrophy in amyotrophic lateral sclerosis (ALS). MiR-206, miR-133a, miR-133b, miR-1, and miR-27a are called "myo-miRNA" and are considered as markers of muscle regeneration, myogenesis, and fiber type differentiation. MiR-155, miR-146a, miR-221, miR-149* are involved in the inflammatory/angiogenic process. We previously observed that there is different serum microRNA profile in spinal versus bulbar ALS. We have investigated muscle biopsies in a series of ALS cases both sporadic and genetic and imply muscular regeneration at the level of neuromuscular junctions. Material and methods: We studied, in EI Escorial-proven ALS cases, muscle biopsies obtained for diagnostic purposes and requested to TMTB Biobank, myomicroRNAs (MiR-1;MiR-206; MiR-133a; MiR-133b; MiR-27a), and inflammatory microRNAs (MiR-155; MiR-146a; MiR-221; MiR-149*) by qRT-PCR. ALS cases were divided according to gender and age of onset. A series of cases had mutation of SOD and C9orf. The biopsies' morphologies were scored as + (slight atrophy), ++ (small group atrophy), +++ (group of atrophic fibers and connective tissue increase). Morphometric analysis of muscle fiber size was done to correlate muscle atrophy with molecular parameters. Results and discussion: All microRNAs studied were strongly upregulated in muscle biopsies of ALS patients versus controls with the exception of miR-149*. Significant overexpression of miRNAs was present in genetic versus sporadic ALS and in male versus female gender. The morphologic score utilized confirmed a muscle fiber atrophy in ALS patients compared to controls. Genetic ALS (SOD, C9orf) were atrophic with high fiber variability. Conclusions: These results provide evidence of the molecular role of microRNAs in correlation to muscle atrophy. In addition, we observed an increased expression of microRNAs in genetic ALS and dysregulation of inflammatory microRNAs. The upregulation of myomiRNAs we found correlates with the degree of atrophy and possible regeneration process of neuromuscular junction.

TSC-associated central nervous system lesion: different lesions with a common pathogenesis

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Objective: Tuberous sclerosis complex (TSC) is a dominantly inherited disease caused by mutations in either TSC1 or TSC2 genes leading to hyperactivation of mTORC complex with development of hamartomas in different organs and se-

6. **Descriptive epidemiology on Sudden Unexpected infant death (SUID) and Sudden Infant Death Syndrome (SIDS) of Veneto Regional Center: Neuropathological evidences.**

PROCEEDINGS 7° Congresso Triennale di Anatomia Patologica SIAPEC-IAP 2016. Pathologica (2016) Vol 108 (4) p. 276-277, Abstract of Oral Presentation

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removed as usual with the help of a hammer and chisel.
The same method is used for the removal of the ethmoid bone and the opening of the floor of the anterior cranial fossa, required to perform targeted samples of the ophtalmic nerve, the upper rectus muscle and retina (see image).
Results. In the numerous cases in which we have applied the method, starting from routine cases on which the tests have been carried out, it was sufficient to use a small amount of water directly in the cutting zone in order to obtain a total dust suppression with a minimum contamination of easily washable equipment (saw + plastic cap) or materials destined for incineration (absorbent cross). The procedure also allows us to perform the task without the help of a second operator.
Conclusion. To our best knowledge this is the safest method to avoid environmental pollution and occupational exposure, although it requires some experience by the operator.

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DESCRIPTIVE EPIDEMIOLOGY ON SUDDEN UNEXPECTED INFANT DEATH (SUID) AND SUDDEN INFANT DEATH SYNDROME (SIDS) OF VENETO REGIONAL CENTER: NEUROPATHOLOGICAL EVIDENCES

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Introduction. Sudden infant death syndrome (SIDS) is characterized by the death of an infant that cannot be explained, despite a systematic case examination, including death scene investigation, autopsy and review of the clinical history (1). SIDS has been known since the beginning of recorded history, in fact it was the topic of the Old Testament's description about the wisdom of Solomon (2). Solomon had to judge by the dispute between two mothers. One of their babies was found dead during the night. Solomon offered to cut the surviving infant in half with his sword but only the true mother, of course, supplicated him not to. Sudden infant death syndrome was originally defined in 1969 focusing interest on sudden death in infants without an recognized cause (3). Nowadays, sudden unexpected infant death (SUID) is a wide- ranging concept used to describe any sudden and unexpected death, whether explained or unexplained, including SIDS, which occurs during the first year of life (4, 5). Sudden unexpected death that remains unexplained after complete postmortem evaluation is considered to be equivalent to deaths classified as SIDS; in other words SIDS represents an unexplained SUID. A statement of reaffirmation for this definition and its rules was published by the Task Force on Sudden Infant Death Syndrome in October 2014 (6). Several differing and sometimes contradictory hypotheses of the underlying mechanisms of SIDS have been proposed. The most reliable seems to be the "triple risk hypothesis". Based on this theory, proposed in 1994 by Kinney and Filiano, unexpected infant deaths might arise as a consequence of the combination of three factors coming together: a vulnerable infant, a vulnerable phase of development and a final insult occurring in this window of vulnerability. In the past, different causes were recognized as main pathogenetic conditions, in particular they were proposed or primary cardiac alterations, or primary respiratory anomalies or primary neurological predispositions. Recently, an unified neuropathological theory contributes to describe SIDS. According to this, serotonergic neurons play a crucial homeostatic function for the cardiorespiratory brainstem centers. 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 with control infants dying of other causes (7). This brain region includes the main nuclei and structures that coordinate the vital activities, such as cardiovascular function and breathing, before and after birth (8).
Methods. By the logical inference of Charles Sanders Peirce (1839-1914) we intend to apply the abductive reasoning on SUID of Padua Reference Centre of Veneto Region during the last two years. To abduce means to "guess" a hypothetical explanation from an observed circumstance. In abductive reasoning, unlike in the deductive one, premises do not guarantee the conclusion. One can comprehend abductive reasoning as "inference to the best explanation". Diagnostic expert systems frequently employ abduction and that is why, in the scientific method, the causes of events are still unknown, although several even conflicting pathogenetic hypotheses of the underlying mechanisms have been proposed. It is thus possible to perform abductive analysis in the presence of missing or incomplete input evidence, which normally results in degrees of uncertainty in the output conclusions. Obviously, this method would will fit on SIDS pathogenesis because it is in the "nature and nurture" of unpredictable biological events, asking help to the "guessing". According to our assumption, we intend to use the tool of neuropathological investigation to analyze all of SUID cases of Padua Centre from 2014 to 2016. By means of this approach, the neuropathological abduction becomes an inferential method to obtain some additional information for "bona fide SIDS". Therefore, based on our abductive and descriptive epidemiology approach, we finally propose the "fourfold risk hypothesis" because we suggest to add into whole risk the "sine materia" condition too (9, 10, 11). Neurocardiac genes of susceptibility could explain brain-heart expression patterns and molecular phenotypes of neurocardiac genes linked with some of the "true unexplained SIDS" (12). In order to obtain this, we utilized the inferior olives of medulla oblongata as neuropathological biomarker of sudden cardiorespiratory arrest, to assess the full extent of time course of hypoxic-ischemic brain injury during unexpected sudden death in infancy. In fact, as reported in the literature (13, 14), neurons of inferior olive nucleus are particularly vulnerable during subacute hypoxic-ischemic insult, perhaps by retrograde trans-synaptic neurodegeneration from cerebellum connections. On the contrary, sparing of the inferior olivary nuclei, may be associated with cases of cardiac arrest encephalopathy (15, 16).
Results. We analyzed 14 cases of SUID, 5 of which were finally classified as SIDS. By double blind we counted the percentage of morphologically "normal" cells on apoptotic ones of medulla oblongata inferior olive nuclei. We obtained a high significant difference of alive cell percentage between 9 SUID cases (high level of apoptotic cells) and 4 SIDS cases (high level of alive cells), respectively. One "bona fide SIDS" case has been segregated from the other four SIDS, because it shown high level of apoptotic cells in inferior olive nuclei,

according to clinical history of hypoxic-ischemic insult due to prone sleeping position.

Conclusions. Neuropathological analysis of bulb inferior olives represents a good tool to infer on the final events of SUID and in particular of "bona fide SIDS". Among SIDS cases, only those with high rate of live cells in inferior olives nuclei could be considered the consequence of cardiorespiratory arrest and, therefore, part of "true SIDS" sine materia.

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A HISTOPATHOLOGICAL AND CLINICAL ANALYZE OF THE GLIOSARCOMA CASES OF A SINGLE INSTITUTIONS

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Introduction. Gliosarcoma is a biphasic tumor that has both glial and mesenchymal component or the glial component gained subsequently a mesenchymal phenotype. Over the time these tumors were considered as two distinct entities, the first one has been considered as the real "gliosarcoma" and the latter as glioma with desmoplastic metaplasia(1, 2). The pathogenesis and the diagnosis of these tumors has been a topic of controversy.

Methods. We analyzed the pathological report of 632 consecutive glioblastoma cases of our files from, 2002 to 2014, and retrieved from the archive 25 (3.9%) cases that has been described as "de novo" gliosarcoma. All cases has been reviewed and clinically and morphologically analyzed retrospectively. GFAP, Actin, Desmin, p53, S100 has been revaluated.

Results. The mean age of the cohort in the current study was 57 (range 5-80) and there were 20 men and 5 women. In all cases that has been analyzed both glial component (GFAP+, S100+) and the so called " mesenchymal" part has been identified. 16 cases had a predominant histology of smooth muscle like cells with mostly intersecting fascicles with spindled shape, eosinophilic cytoplasm. Some of this cases show somehow bland cytomorphological features with brisk mitotic activity. In 9 cases there was a prominent pleomorphism of the cells with lipoblast like or rhabdoid like cells and hyperchromatic nuclei. In all cases mitotic activity and KI67 (range from 10%-60%) was extremely heterogeneous with no effect on the prognostic outcome on the patients. Actin and desmin shows some focal stain in most of the cases with no specificity while myogenin stain was negative. In 19 of this patients follow up was available showing DOD much shorter in comparison of the classical GBM that is available in the literature (Mean 9.94 ranging from 1-35 months after the first diagnosis).

Conclusions. In our study we analyzed 25 gliosarcoma that although histologically is a separate entity due to the limited experience and the rarity of this subgroup patients continue to be managed in the same manner as GBM patients. To our experience no IHC stain except GFAP that help to distinguish the glial component from the non-glial component. We could not find any morphological features that correlate with a more favorable prognosis reinforcing the belief that as in GBM in this tumors as well we need more molecular datas to improve the management of this patients.(3)

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PATOLOGIA CARDIOVASCOLARE

EOSINOPHILIC ALVEOLITIS IN A NEARLY-DROWNED YOUNG MAN AFFECTED BY PAPILLARY FIBROELASTOMA: A CASE REPORT

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Introduction. A pulmonary inflammatory infiltration rich in eosinophils can be present in many different conditions, therefore a differential diagnosis is needed. Among recog-

8. Profiles of brainstem nuclei from 28 infant sudden death cases of Veneto Regional Center (unpublished data)

28 infant sudden deaths (15 M, mean age 72 days), collected from 2014 to 2017 by the Veneto Regional Center of Cardiovascular Pathology Unit of Padua University (Italy).

According to the general definition of SIDS of San Diego panel in 2004, we considered "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" [110]. Indeed, we did not intend to utilize SIDS sub-categories as Sudden Unexpected Early Neonatal Death (SUEND) and Sudden Unexplained Death in Children older than a year (SUDC) [69], rather we planned to sub-categorize our 28 sudden infant death cases respectively in "SUIDs", "SIDSs", "CONTROLS" and "Ex SIDS" categories. This last class represented a "workable" category in which included autoptic cases initially considered in SIDS group, and then, by the molecular autopsy and/ or genetic analysis, classified as syndromic diseases and "ex"-cluded from SIDS patients.

Summing up, we observed 11 SUIDs, 7 SIDSs, 4 EX SIDSs and 6 CONTROLS.

In the 11 infants with a certain cause of death, predominantly males, the main findings were interstitial pneumonia and bronchiolitis with airway obstruction in 9 (82%), lymphocytic myocarditis in 1 (9%) and Pulmonary Interstitial Glycogenosis (PIG) in the last one (9%). Molecular analysis was positive for viruses in 6/11 (54.5%) of cases.

4 male EX SIDSs counted respectively in 1 Arnold Chiari I syndrome, 1 Cornelia de Lange syndrome, 1 Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency and 1 Costello syndrome by kras mutation. Amongst 6 CONTROLS, of which 5 were females, 3 died for congenital cardiopathies (50%), 2 for intramural rhabdomyomas in tuberous sclerosis complex (33%) and 1 subsequently maternal uterine rupture (17%).

Histological examination performed on medullary and pontine serial sections highlighted, in 43% (25% SUIDs, 7%SIDSs, 11% EX SIDS) of the whole cohort

of 28 infants, different degrees of monolateral/ bilateral Arcuate Nucleus (AN) hypodevelopment and/ or aplasia and similar anomalies of the other nuclei of infant brainstem, in particular the locus coeruleus, the medial nucleus of the superior olivary complex and the facial/parafacial complex in the pons; the hypoglossus, the dorsal motor vagal, the tractus solitarius, the ambiguous and the obscurus raphé nucleus in the medulla oblongata. We were also able to consider the presence or not, and possible alterations of the Kölliker-Fuse nucleus, the retrotrapezoid nucleus, the other nuclei of the superior olivary complex in the pons and the pre-Bötzinger and pallidus raphé nucleus in the medulla oblongata (Fig. 50, 51, 52, 53). Alterations of brainstem nuclei were found so distributed in the three different sub-categories: 7 on 11 (64%) SUIDs, 2 on 7 (29%) SIDSs, 3 on 4 (75%) EX SIDSs. On the contrary, CONTROLS showed the total absence of brainstem nuclei anomalies. Complessively, and according to the Literature data [19, 111, 112, 113, 114, 115], 55% of sudden unexpected infant deaths (12 on 22 SUIDs, SIDSs, EX SIDSs) were affected, compared to 6 negative controls (0 on 6).

All of the results are reported in Table 8 and Graphics 8a, 8b.

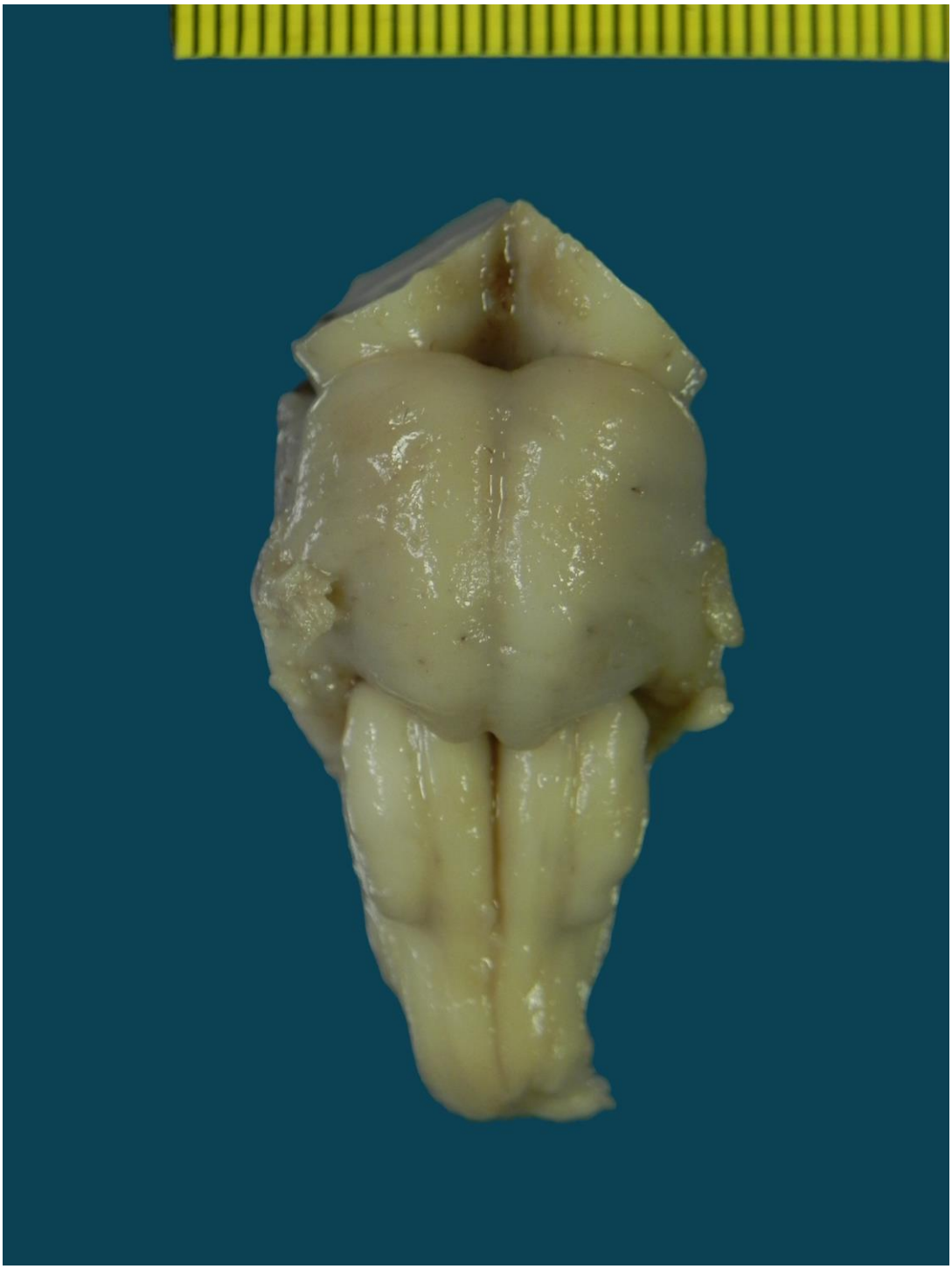


Figure 50 *Ventral surface of brainstem of 9 days old, female died for aspiration pneumonia. Sudden unexpected early neonatal death (SUEND)*

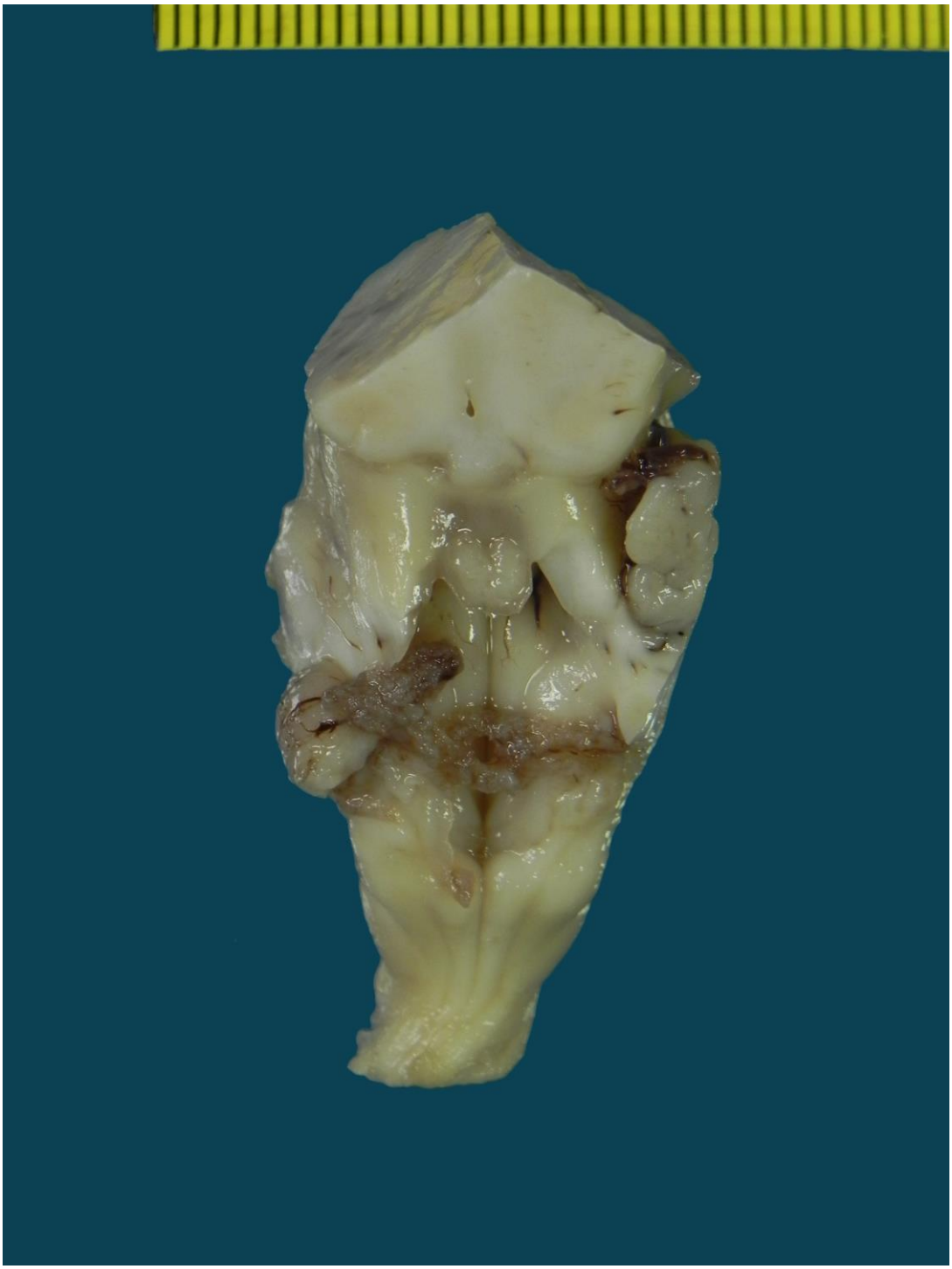


Figure 51 *Dorsal surface of brainstem of 9 days old, female died from aspiration pneumonia. Sudden unexpected early neonatal death (SUEND)*

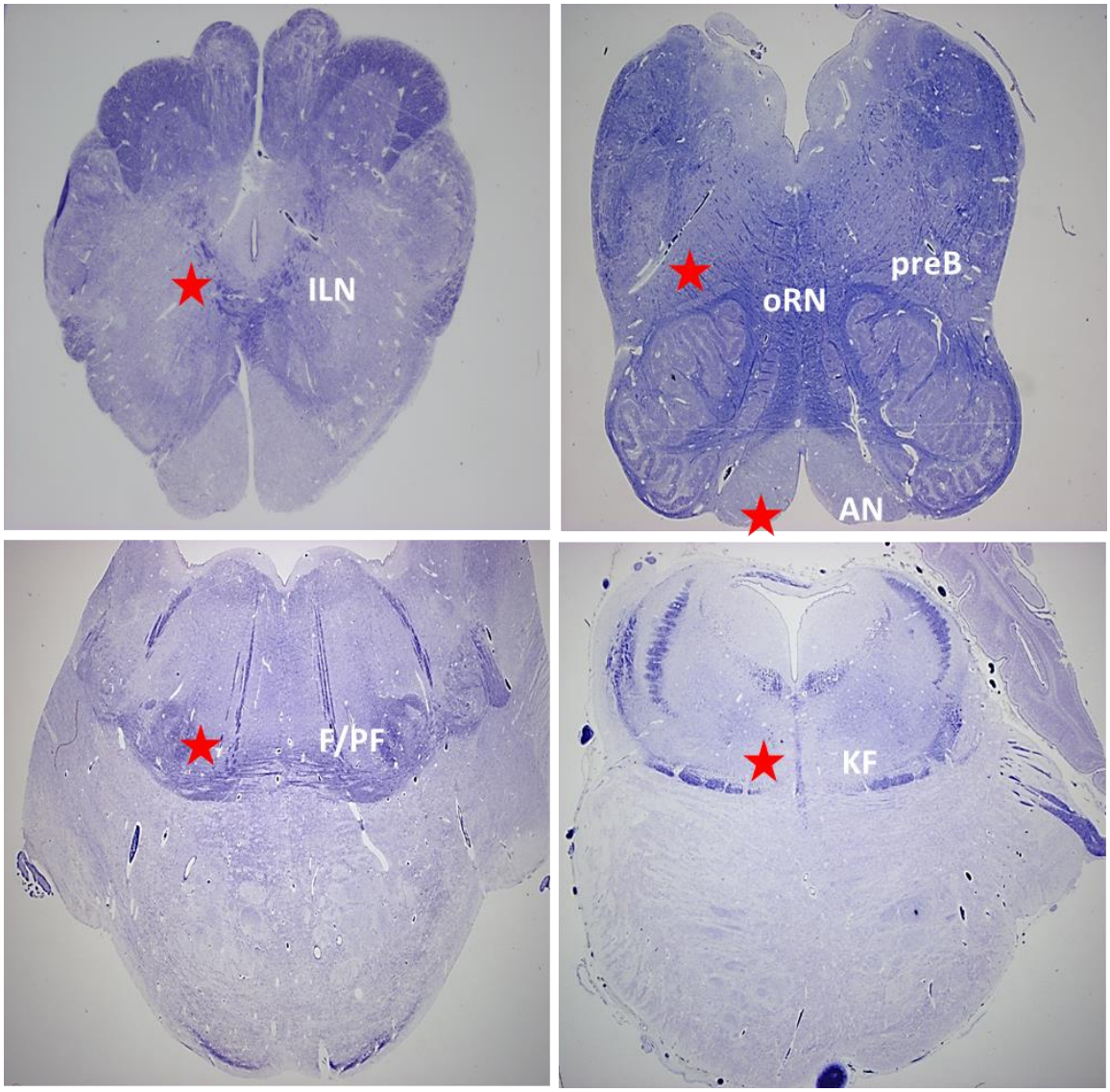


Figure 52 *Histological spinal cord, medullary and pontine serial sections highlighted the principal brainstem cardiorespiratory nuclei of a 3 months old infant. ILN, Intermediolateral Nucleus in the spinal cord; AN, Arcuate Nucleus; preB, pre-Bötzinger Nucleus, oRN, obscurus Raphé Nucleus in the medulla oblongata; F/PF, Facial/Parafacial complex and KF, Kölliker-Fuse Nucleus in the pons*

Table 8. Profiles of brainstem nuclei from 28 SUID/ SIDS/ EX SIDS CONTROL cases of Veneto Regional Center

Case no.	Sex/ Gestational weeks/ Age	Death diagnosis	Arcuate Nucleus histology	Other brainstem nuclei alterations
1	F/ 39 gw/ 9 days	Aspiration pneumonia. Sudden unexpected early neonatal death (SUEND)	partial hypoplasia (monolateral,right)	Pre-Bötzinger hyperplasia (right)
2	F/ 41 gw/ 48 days	SIDS	normal	normal
3	F/ 35 gw/ 3 months 21 days	SIDS	bilateral aplasia	Pre-Bötzinger monolater aplasia (right)
4	F/ ?/ 96 days	Bed sharing, lymphocytic myocarditis and diffuse inflammation, (SUID)	partial hypoplasia (monolateral, left)	Pre-Bötzinger monolater aplasia (left); raphè obscurus aplasia
5	M/ 37 gw/ 20 days	Pulmonary Interstitial Glycogenosis (PIG) (SUEND)	bilateral hypoplasia	Pre-Bötzinger monolater aplasia (right)/
6	F/ preterm/ 25 days	Intramural rhabdomyomas, cortical tubers in tuberous sclerosis complex	normal	normal
7	M/ 30 gw/ 8 months	Bronchopulmonary dysplasia of premature baby, bronchopneumonia and mitral valve calcifications in previous endocarditis (SUID)	bilateral hypoplasia	normal
8	M/ 39 gw/ 19 days	Cytomegalovirus-positive lymphocytic pneumonia, gastroenteritis, meningoencephalitis (SUEND)	bilateral hypoplasia	normal
9	F/ 39 gw/ 33 days	Bed sharing and lymphocytic bronchiolitis (SUID)	normal	Raphè obscurus hypoplasia
10	M/ 35 gw/ 48 days	Costello syndrome by kras mutation (ex SIDS)	bilateral hypoplasia with intra-pyramidal distribution	right hypoplasia of Pre-Bötzinger; Facial/ Peri-facial nuclei; Raphè obscurus hypoplasia
11	M/ 36 gw/ 1 day	Intramural rhabdomyomas, cortical tubers in tuberous sclerosis complex (Control)	normal	normal
12	F/ term/ 3 days	Oedema and pulmonary haemorrhage; (SUEND), metabolic disease?	normal	normal
13	M/ term/ 2 months	Prone sleeping (SIDS)	normal	normal
14	M / term/ 4 months	Prone sleeping with pacifier (SIDS)	normal	Inferior olivary nucleus ectopia; Raphè obscurus hypoplasia
15	F/ 37 gw/ 2 die	Maternal uterine rupture (Control)	normal	normal
16	F/ term/ 6 months 11 days	Congenital cardiopathy (Control)	normal	normal
17	F/ 35gw/ 6 months	Congenital cardiopathy (Control)	normal	normal
18	F/ 34gw/ 2 months	Congenital cardiopathy (trigemellar	normal	normal

		pregnancy)		
19	M/ 40gw/ 7 months	Prone sleeping, recurrence of Brief Resolved Unexplained Events (BRUE)	bilateral hypoplasia	Facial/ Peri-facial nuclei hypoplasia
20	M/ 42gw/ 2 days	Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (ex SIDS)	bilateral aplasia	Raphè obscurus and Raphè pallidus hypoplasia
21	M/ 40gw/ 28 days	Respiratory Syncytial Virus (RSV) - positive bronchitis and bronchiolitis (SUID)	normal	normal
22	M/ term/ 25 days	RSV-positive lymphocytic pneumonia; subdural and subarachnoid haemorrhage undefined cause (SUEND)	normal	normal
23	F/ 38gw/ 28 days	Bed sharing, drug addict mother (SIDS)	normal	normal
24	M/ 41gw/ 7 days	Sudden Unexpected Postnatal Collapse (SUPC) 2 hours after birth	normal	normal
25	F/ 37gw/ 16 hours	Cornelia de Lange syndrome (ex SIDS)	normal	normal
26	M/ term/ 2 months 23 days	Bed sharing; human Herpesvirus 6 (HHV6)- positive intersitial pneumonia SUID)	normal	normal
27	M/ term/ 1 year 23 days	HHV6- positive interstitial pneumonia and ependymitis (SUID)	bilateral hypoplasia	Raphè obscurus and Raphè pallidus hypoplasia
28	M/ 37gw/ 8 months 18 days	Prone sleeping; Arnold Chiari I syndrome (ex SIDS)	normal	Pre-Bötzinger bilateral aplasia, Raphè obscurus hypoplasia

Table 8. *Profiles of brainstem nuclei from 28 SUID/ SIDS/ EX SIDS CONTROL cases of Veneto Regional Center*

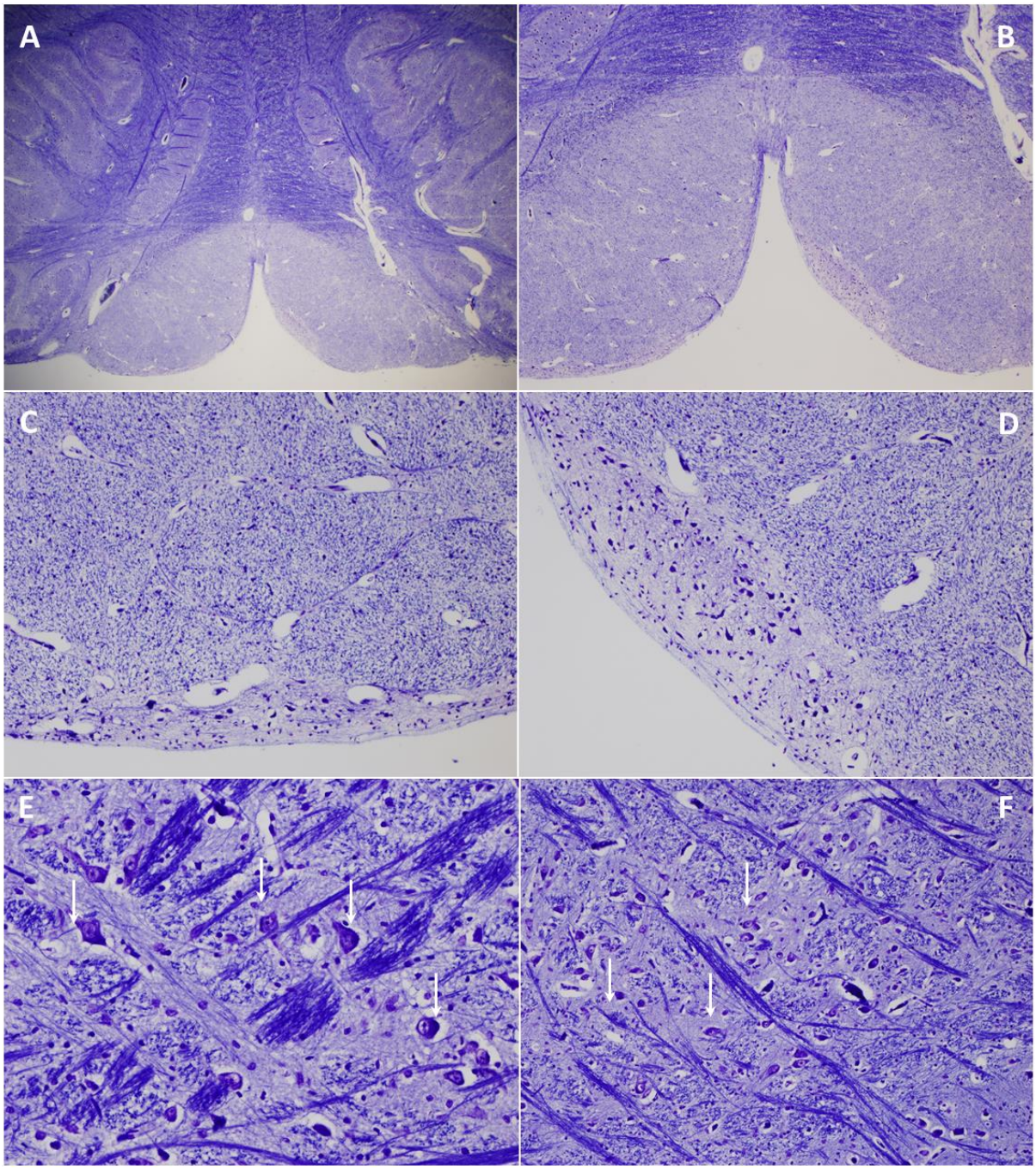
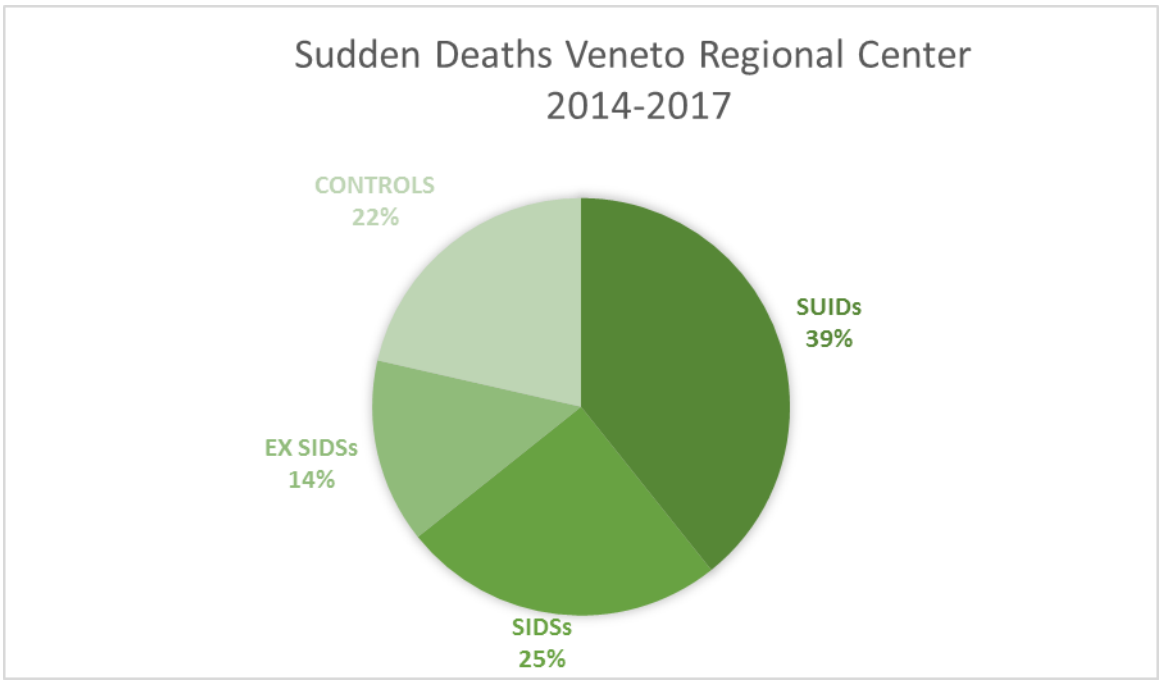
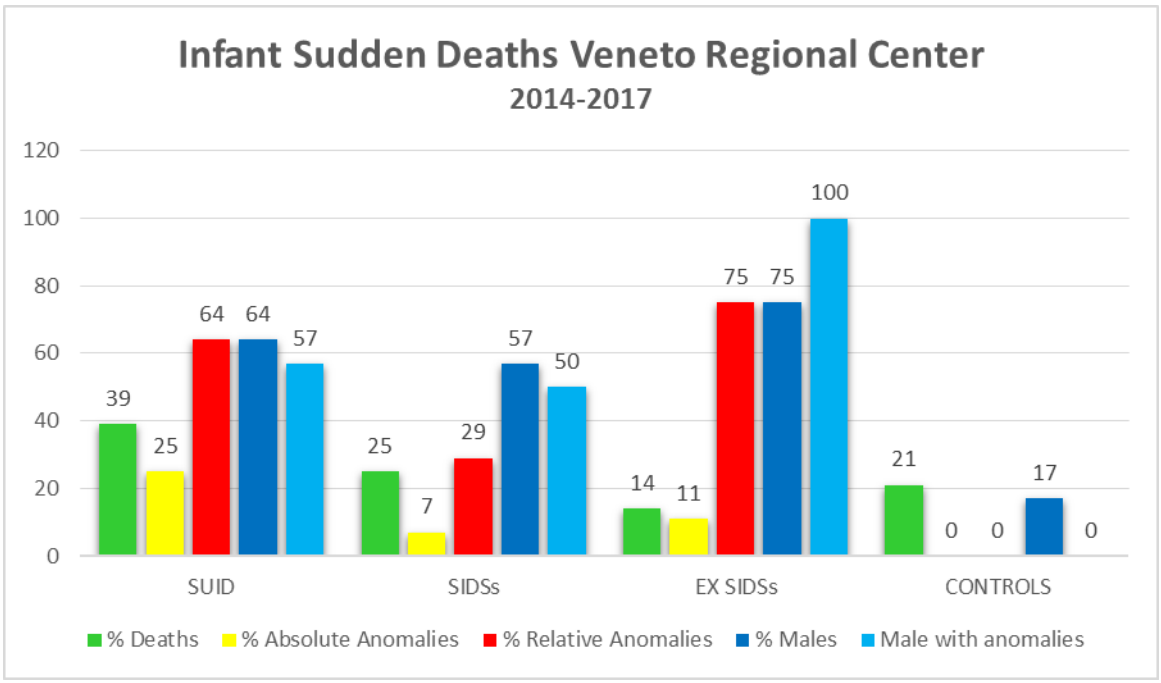


Figure 53 *Cardiorespiratory nuclei of the Brainstem. Case n. 1 Table 8: partial hypoplasia (monolateral, right) of the Arcuate nucleus (A, B, C, D) and Pre-Bötzinger hyperplasia (right) compared to the normal contralateral (left) (E, F: arrows show in E hyperplastic neurons of Pre Bötzinger nucleus). A, 12,5x; B, 50x; C, D 100x; E, F, 200x*



Graphics 8a



Graphics 8b

Graphic 8a can be submitted to differents considerations. Firstly, controls are primary females and with no anomaly of brainstem nuclei, whereas genetic syndromic events, as 4 cases of EX SIDS, are all males and brainstem anomaly-affected. This could be in line with the concept that exists a manifest male preponderance in SIDS victims. Therefore, it may depend on the gene- disease association, but also represents an intrinsic vulnerability, such as infection, thermal stressor, the prone sleeping position, overbundling, bedding surfaces, and bed sharing. All of the affected three groups presented anamenestic history of bed sharing and prone sleeping position and this emphasizes the absolute vulnerability, typical of all of the infant sudden death sub-categories.

Ex SIDSs is an archetype workable sub-class; it could be considered to derive from the original concept of SIDS because may represent those otherwise old unexplained SIDS cases, that nowadays are discrimined and diagnosed by molecular autopsy [116].

Our cohort' true SIDS group appears the weakest class of our analysis because it is too large, it has too many unexplained deaths compared to epidemiological data [24]. Moreover, it is not well correlated with the vulnerability according to gender, and presents the minor percentage of anomalies of the brainstem nuclei too. From this, it derives that, the SIDS sub-category is a heterogeneus class in which we have to improve our data analysis and molecular detection.

Finally, because 55% of SUIDs, SIDSs and EX SIDSs manifest alterations of brainstem nuclei, it significant that more of half cases have a brainstem defect-associated vulnerability, compared to the whole negativity of controls. Based on this Regional evidence, it is mandatory for the local Scientific Community to recruit new external centers to improve our knowledge on infant sudden death etiopathogenesis.

Discussion

Molecular profiling of SUDEP cases and the investigation of genetic models have directed to the identification of putative SUDEP genes of which most are ion channel active along the neurocardiac, neuroautonomic, and neurorespiratory pathway. Concurrently, anomalous time- activation, transcription or regional expression of candidate neuro-cardiac-respiratory genes implicated for SUDEP, could be similarly involved in other unexpected sudden deaths.

Limits and future milestones of these researches regard the ability to utilize the defective molecular pathways, the neuronal mapping of anomalies of brainstem nuclei and mutations of neuro-cardiac-respiratory genes, as autopsy-based biomarkers in infant and adult sudden deaths.

The growing use of powerful detection methods such as massive sequencing has given a significant impulse to the examination for minimally invasive disease indicator. In addition, the discovery of the existence of free or exosomal circulating nucleic acids in blood and CSF has also raised the research in this direction. Although this is still a relatively young field, it is rapidly evolving and promises great advances in the field of biomarker discovery, especially for nervous system pathology. The CNS is the least accessible of all tissues and would therefore greatly benefit from advances in this arena. Current limitations to this approach include all those intrinsically associated with biomarker discovery i.e., working with material from different sources, patient history, post-mortem brain tissue in paraffin-embedded, as well as those specifically associated with sequencing-based detection methods and extraction strategies. Not less important are the discrepancies observed with human tissue: samples from different sources show wide variability in profile as a result of handling, sample preparation and preservation. These are especially pronounced when a highly sensitive technique like sequencing is used. Moreover, the source of human tissue, in SUDEP cases, is primarily from patients who may be on medication. Therapy, in fact, may be a potentially confounding cofactor because it pursues restoration of the biological balance and may target molecules of interest [117]. On this background, research groups worked and are still working to find theranostic biomarkers of sudden deaths. Respectively, the study of Temporal Lobe Epilepsy (TLE) based on

human surgical specimens, and SUDEP, based on human post-mortem tissues, is affected by two main limitation: first, the lack of appropriate controls; second, the fact that most of the specimens are taken from pharmaco-resistant patients, making it not ever possible to discriminate between the phenomenon directly related to epilepsy and sudden death or those related to drug therapy.

Genes differentially expressed in SUDEP and/ or SIDS, brainstem neuronal pathways SUDEP/ SIDS- dependent, may represent prognostic biomarkers and help to identify the mechanisms underlying the disease.

Many intrinsic and extrinsic factors increase fetal, perinatal, infant, and adult sudden death susceptibility. The final common pathway for SIDS and SUDEP involves a failure to arouse and autoresuscitate in response to environmental challenge. The different risk factors, among these a prone position, associated to the alterations of the medullary serotoninergic neural populations and autonomic dysregulation, can influence the function of cardiorespiratory nuclei and impair the ability of this network to coordinate cardiorespiratory–cardioventilatory coupling (CRC-CVC).

Contrary to classical cardiorespiratory models, where respiratory modulation of the heart is well known to be under the guise of respiratory sinus arrhythmia (RSA), the CVC, instead, is a temporal alignment between the heartbeat and inspiratory activity. It is a major determinant of breath-to-breath variation in observed respiratory rate. The cardiac-trigger theory attributes this to tunings of respiratory timing by baroreceptor afferent impulses to the central respiratory pattern generator [118].

We intended demonstrated that this double coupling is involved in unexpected sudden deaths by the presentation of Literature data and through the anatomo-clinical correlation between brainstem nuclei mapping and clinical evidence published.

Pathogenesis of SUDEP is a complex mechanism that appears challenging to define because most cases occur unwitnessed, and cardio-encephalic-respiratory clinical data recorded in only a handful of cases [119]. Theories of SUDEP supposed seizures beginning in the cortex can sometimes induce changes in brainstem cardiorespiratory control mechanisms. In fact, postictal hypoventilation

determinates a major contributor to the cause of death in SUDEP and brainstem serotonin and adenosine pathways could be primary involved. Strong parallelisms exist between SIDS and SUDEP, and undoubtedly similarities pointing out the possibility of shared pathophysiology concerning the combined failure of respiratory and cardiovascular control mechanisms.

Clinical reports and experimental studies have demonstrated changes in cardiac, respiratory, gastrointestinal, and genitourinary function before, during, and after a seizure, by producing a panautonomic activation during limbic cortical seizure activity [102]. Seizures that cause death must do so by spreading to autonomic brain regions into medullary areas for both sympathetic premotor and parasympathetic preganglionic activation. This synergic, parasympathetic-orthosympathetic co-activating dysautonomia alters the cardiorespiratory-cardioventilatory coupling unified circuit.

These observations motivated the aim of our thesis and confirmed our working hypothesis regarding the identification of physiopathology of SIDS and SUDEP.

HRV is CRC-dependent of the heartbeats and follows from RSA. A diminished HRV is, for example, typical of asphyctic and distressed newborns. Such pathophysiological CRC alterations become most evident with the sigh reflex, during gasping and the autoresuscitation. Children that later died of sudden infant death syndrome had a diminished heart rate change during the sigh, so as, in a similar way, sigh-related heart rate decreasing were reported in Familial Dysautonomia [120, 121].

RSA generation is mainly related to the direct brainstem modulation of the cardiac vagal preganglionic neurons and by inhibition of cardiac vagal efferent activity by lung inflation. RSA is also considered to improve pulmonary gas exchange. However, the physiological mechanisms involved in CRC-CVC coupling and their synchronization are still not fully understood. These physiological circuits might coordinate cardiovascular and respiratory rhythms in the brainstem through the control of phase synchronization between cardiorespiratory centres and nerve discharges, thus improving energy efficiency.

RSA is observed in full-term infants, demonstrating the presence of cardiorespiratory coupling even at this early life stage. This coupling increases in

parallel to gestational age at birth, reflecting the shift from sympathetic to parasympathetic dominance during the postnatal period. Therefore, it produces an increasing influence of respiration on modulation of HR. On the contrary, immaturity in linear cardiorespiratory coupling observed in preterm infants, mostly in the form of lower HRV with high-frequency range, impairs responses to cardiorespiratory stress and increases the sudden death risk.

Mrowka et al [122] hypothesized that the directionality of CRC-CVC coupling depends on respiratory frequency. In particular, respiration rate would work as a low pass filter, i.e. below a set respiratory frequency directionality is mainly from respiration to HR, whereas above a certain respiratory frequency threshold the interaction becomes bidirectional. The underlying mechanisms for this low pass filter effect could be linked to lower information transmission to the cardiac oscillator caused by reduced information from the vagal nerve to the atrial pacemaker cells when respiration frequency is above a certain threshold, such as 0.6 Hz. Given that this cutoff frequency is potentially related to vagal nerve regulation, these findings would be consistent with previous studies showing immature vagal function in premature infants. One possible elucidation is that this type of synchronization involves an energy consumption benefit, namely, the reduction in intrathoracic pressure during inspiration increases cardiac filling and consequently cardiac output. This indicates that the development of cardiorespiratory autonomic control is a postnatal age-dependent phenomenon. The increased occurrence of apparent life-threatening apneic events in premature infants during the first months of life, suggests a longer window of adaptation associated with several high-risk conditions for SIDS. In fact, it has been shown that the increased incidence of SIDS in premature infants is related to an underlying cardiorespiratory vulnerability. SIDS have fewer spontaneous arousals from sleep as compared to controls. Moreover, many SIDS victims, as also described in our Veneto Regional Center cases, evidenced abnormalities in the brainstem networks responsible for cardiorespiratory control. This phenotype can increase risk vulnerability to exogenous stressors, such as the prone sleeping position [123].

The sleeping position and time after feeding can influence the autonomic system of preterm infant. It has recently demonstrated that heart rate is higher and heart period variability is lower in the prone position, and the effects of sleeping position on cardiac functioning are more marked during the middle of the intrafeed interval. In preterm infants, autonomic responses to nutrition modify the cardiorespiratory effects of sleeping position. Prone sleeping risk may vary with time after feeding [124].

The anatomy and muscles of the upper airway, the threshold for arousals from sleep, and ventilatory control adapted by central and peripheral chemoreceptor sensitivity can influence the development of obstructive sleep apnea (OSA) during childhood and as adults. Preterm-born children are three to five times more likely to have OSA during childhood and at least twice as likely to have OSA as adults. Important risk factor could be represented by cardioventilatory control, including chemoreceptor sensitivity, the blood gas response to change in ventilation, and coupling between cardiovascular and respiratory brainstem neurons [125]. Contemporary, an existing clinical analysis of cardiorespiratory arrests in epilepsy monitoring unit, the MORTEMUS project [103], showed severe cardiovascular and respiratory failures (apnea/hypoventilation and bradycardia/asystole) to occur terminally in cases of SUDEP.

Furthermore, individuals who are at high risk of SUDEP exhibit FC compared with low-risk patients (Fig. 39) [92]. This functional dysautonomic neural network involves the thalamus, brain stem, anterior cingulate, putamen and amygdala, and a second subnetwork spread out to the medial/orbital frontal cortex, insula, hippocampus, amygdala, subcallosal cortex, brain stem, thalamus, caudate, and putamen.

Combined peri-ictal apnea and bradycardia, therefore, in the context of FC analysis is a rare epiphenomenon, however, when it happens, may characterize a potentially deleterious, high vagal tone phenotype in seizure patients in the SUDEP perspective [126]. Prolonged ictal central apnea (>60 s) is associated with severe hypoxemia (SpO_2 < 75%). This combination may be a biomarker of SUDEP that merits prospective study. Persistent apnea could also represent, so as previously demonstrated for SIDS, a phenomenon of CRC-CVC derangement due

to dysfunction or “ exhaustion ” in the major breathing control sites in the human brainstem.

Concluding remarks

Neuropathological analysis of the infant brainstem and neuro-cardiac-respiratory gene mapping represents a good tool to infer on the final events of SIDS and SUDEP, although nothing it is clear regarding the role of adult cardiorespiratory centres. An integrated study of postmortem neuropathology and molecular autopsies could help to understand the network of this beating-breathing-thinking unit (Fig. 54).

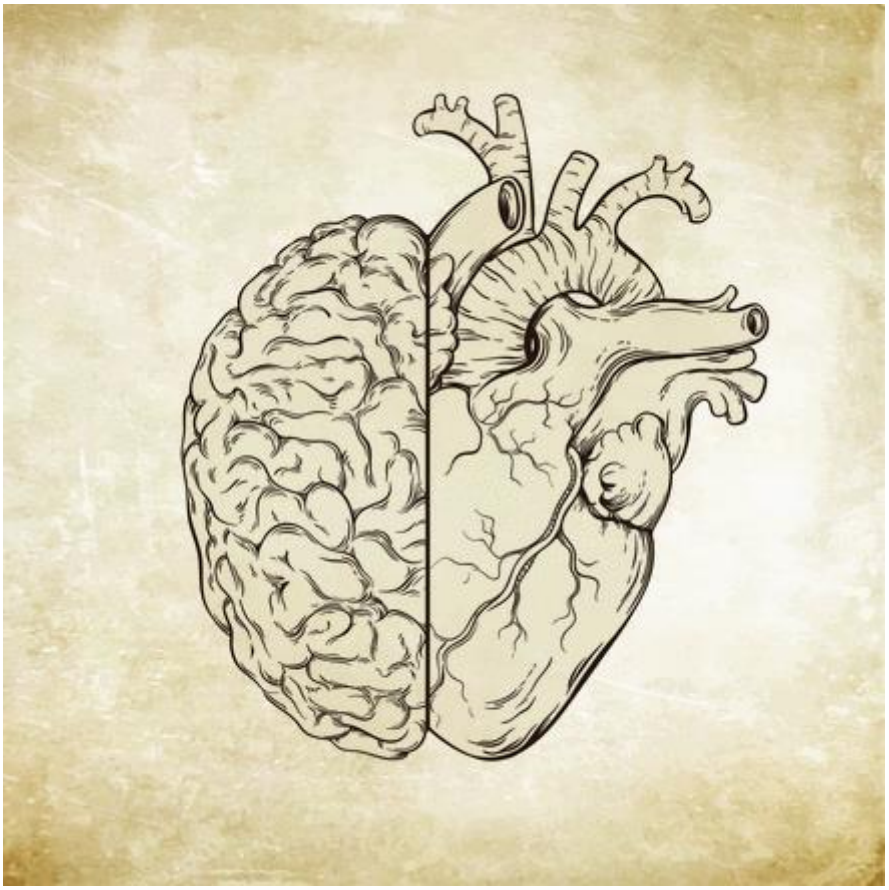


Figure 54 *The "beating-breathing-thinking unit"*

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Appendix

1. Certificates

UCL HUMAN RESOURCES DIVISION
Employment Contract Administration

20 December 2017

PRIVATE & CONFIDENTIAL

Dr Beatrice Paradiso
c/o Juliet Solomon
Institute of Neurology
6th Floor
Queen Square Hospital
London
WC1N 3BG

Please quote on all correspondence your employee number: N2067357

For enquiries contact Amanda Crawford on 0203 108 (5) 9592 or a.crawford@ucl.ac.uk

Dear Dr Paradiso

UCL Institute of Neurology - Clinical and Experimental Epilepsy

I have pleasure in informing you that the title of Honorary Research Associate in the above department has been conferred on you with effect from 08 January 2018 to 08 July 2018.

General arrangements for your activities as Honorary Research Associate should be made in consultation with the Head of Department. Your contact within the Faculty/Department will be Libby Bertram.

The appointment is an honorary one and will be for the period of your active association with UCL until 08 July 2018 and as determined by your Head of Department. Further periods of appointment and renewal will be based on your contribution to the University.

On arrival at UCL you should contact the Departmental Administrator with regards to obtaining an IT account and ID card.

During your appointment with the University you are expected to comply with UCL's standards, behaviours, procedures and regulations and Equal Opportunity Policy statement. The University may terminate this appointment with immediate effect in the event that you are in breach of any of its provisions or if your conduct brings yourself or the University into disrepute.

I would like to welcome you to this honorary appointment and trust this association will be mutually rewarding.

Yours sincerely

Anita Gorasia
Employment Contracts Supervisor

cc: Libby Bertram

UCL HUMAN RESOURCES DIVISION
Employment Contract Administration

15 August 2018

PRIVATE & CONFIDENTIAL
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C/o Juliet Solomon
Ion 6th Floor Qsh
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WC1N 3BG

Please quote on all correspondence your employee number:
N2067357

For enquiries contact Santhi Rajoo on 020 3108 7160 or s.rajoo@ucl.ac.uk

Dear Dr Paradiso

UCL Institute of Neurology

I have pleasure in informing you that the title of Honorary Research Associate in the above department, conferred on you with effect from 08 January 2018, has been extended to 09 October 2018.

General arrangements for your activities as Honorary Research Associate should be made in consultation with the Head of Department.

The appointment is an honorary one and will be for the period of your active association with UCL, as determined by your Head of Department.

On arrival at UCL you should contact your Departmental Administrator with regards to obtaining an IT account and an ID card.

Yours sincerely

Santhi Rajoo
HR Services Administrator
Human Resources Division

cc: Katy Pestell

CERTIFICATE

This is to certify that

Beatrice Paradiso

was a participant of the seminar & workshop

8th International Summer School for Neuropathology and Epilepsy Surgery

INES 2018

July 26th– July 29th, 2018

Universitätsklinikum Erlangen

Institute of Pathology

Krankenhausstr. 8/10, 91054 Erlangen, Germany

Course Director

Ingmar Blümcke, MD

The 8th Summer School (SNR 795546) is awarded with 18 CME credits (category B) from the Bavarian board of physicians (Bayerische Landesärztekammer)