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Original Article

Altered 5-HT$_{2A/C}$ receptor binding in the medulla oblongata in the sudden infant death syndrome (SIDS): ~~part~~ I. Tissue-based evidence for serotonin receptor signaling abnormalities in cardiorespiratory- and arousal-related circuits

Robin L. Haynes, PhD[1,2,*], Felicia Trachtenberg, PhD[3], Ryan Darnall, MD[1], Elisabeth A. Haas, MPH[4], Richard D. Goldstein, MD[2,5], Othon J. Mena, MD[6,†], Henry F. Krous, MD[7,8], Hannah C. Kinney, MD[1,2]

[1]CJ Murphy Laboratory for SIDS Research, Department of Pathology, Boston Children's Hospital and Harvard Medical School, Boston, Massachusetts, USA
[2]Robert's Program on Sudden Unexpected Death in Pediatrics, Division of General Pediatrics, Department of Pediatrics, Boston Children's Hospital, Boston, Massachusetts, USA
[3]Carelon Research, Newton, Massachusetts, USA
[4]Department of Research, Rady Children's Hospital, San Diego, California, USA
[5]Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA
[6]San Diego County Medical Examiner Office, San Diego, California, USA
[7]University of California, San Diego, San Diego, California, USA
[8]Rady Children's Hospital, San Diego, California, USA
[†]Present address: Ventura County Medical Examiner Office, Ventura, California, USA.

*Send correspondence to: Robin L. Haynes, PhD, CJ Murphy Laboratory for SIDS Research, Boston Children's Hospital, Boston, MA 02115, USA; E-mail: robin.haynes@childrens.harvard.edu

ABSTRACT

The sudden infant death syndrome (SIDS), the leading cause of postneonatal infant mortality in the United States, is typically associated with a sleep period. Previously, we showed evidence of serotonergic abnormalities in the medulla (e.g. altered serotonin (5-HT)$_{1A}$ receptor binding), in SIDS cases. In rodents, 5-HT$_{2A/C}$ receptor signaling contributes to arousal and autoresuscitation, protecting brain oxygen status during sleep. Nonetheless, the role of 5-HT$_{2A/C}$ receptors in the pathophysiology of SIDS is unclear. We hypothesize that in SIDS, 5-HT$_{2A/C}$ receptor binding is altered in medullary nuclei that are key for arousal and autoresuscitation. Here, we report altered 5-HT$_{2A/C}$ binding in several key medullary nuclei in SIDS cases (n = 58) compared to controls (n = 12). In some nuclei the reduced 5-HT$_{2A/C}$ and 5-HT$_{1A}$ binding overlapped, suggesting abnormal 5-HT receptor interactions. The data presented here (Part 1) suggest that a subset of SIDS is due in part to abnormal 5-HT$_{2A/C}$ and 5-HT$_{1A}$ signaling across multiple medullary nuclei vital for arousal and autoresuscitation. In Part II to follow, we highlight 8 medullary subnetworks with altered 5-HT receptor binding in SIDS. We propose the existence of an integrative brainstem network that fails to facilitate arousal and/or autoresuscitation in SIDS cases.

KEYWORDS: Autoresuscitation, Cardiac and respiratory coupling, Gasping, Hypotension, Olivocerebellar pathways, Ventral medulla

INTRODUCTION

The sudden infant death syndrome (SIDS) is the leading cause of postneonatal infant death in the United States today, with an overall rate of 0.39/1000 live births (1, 2). It is defined as the sudden unexpected death of an apparently healthy infant under 1 year of age that remains unexplained after a thorough case investigation, including a complete autopsy with ancillary testing, examination of the death scene, and review of the clinical history (3). In rare, witnessed cases, death is silent, without evidence of struggle (4, 5). Death is associated with a sleep period (6), occurring either during a period of sleep itself, or coincident with one of the many arousals from sleep that normally occur throughout the sleep period. Our laboratory research is guided by the Triple Risk Factor model for the multifactorial basis of SIDS, which we first proposed in 1994

(7) and subsequently refined (8). According to this model, SIDS results when 3 factors impinge upon the infant simultaneously: (1) a critical period in the development of cardiorespiratory homeostasis, i.e. the first postnatal year of life, (2) an extrinsic stressor that produces life-threatening hypoxia (e.g. prone or face-down sleep position) or hypotension; and (3) an intrinsic vulnerability, i.e. biological abnormality in protective responses to hypoxia and/or hypotension in the infant. This subclinical vulnerability is now further postulated to be specifically triggered, or unmasked, by the extrinsic stressor (hypoxia/hypotension) during a sleep period. The triple risk model accounts for diverse epidemiologic, physiologic, forensic, autopsy, and laboratory data in SIDS infants and synthesizes them into one overlying framework for conceptualizing possible causes and mechanisms, with multiple risks acting synergistically to contribute to death. Despite the initial success of national public health campaigns promoting safe sleep environments and supine sleep position in infants in the 1990s and beyond in the United States, the SIDS rate has plateaued over the last 3 decades (9). Research into the cause(s) and mechanisms of the putative pathobiological vulnerability is urgently needed in SIDS today (9).

In this 2-part study (Parts I–II) of the serotonin $(5\text{-HT})_{2A/C}$ receptor in the medulla oblongata in SIDS, we focus on the overriding hypothesis that a subset of SIDS is due to an intrinsic failure of brainstem neural circuits critical for arousal from sleep or autoresuscitation in response to asphyxia. Failure results from abnormal $5\text{-HT}_{2A/C}$ and 5-HT_{1A} receptor signaling and results in sleep-related sudden death during a critical developmental period. Our laboratory has previously provided robust evidence of abnormalities in several medullary tissue markers of 5-HT function (e.g. 5-HT_{1A} receptor binding, 5-HT levels, tryptophan hydroxylase 2 levels) in SIDS cases compared to age-adjusted controls (8, 10). Serotonergic abnormalities have been corroborated by other groups, albeit with different methodology (11–14). Over the last decade there has been accumulating evidence that 5-HT, acting via medullary $5\text{-HT}_{2A/C}$ receptors, plays key roles in cardiorespiratory homeostasis in sleep including arousal (15–18), hypercapnic ventilatory responses (19, 20), and airway patency (21–23). Studies in animals have also shown that 5-HT is necessary for the appropriate cardiovascular and respiratory components of autoresuscitation (24–27), a response that preserves life in the face of severe brain tissue hypoxia subsequent to hypoventilation or hypotension in an infant during sleep. Yet, importantly, the role of this particular 5-HT receptor subtype in the pathology of SIDS is poorly understood. Here in Part I, we focus on quantifying $5\text{-HT}_{2A/C}$ receptor binding in key medullary nuclei in which we and others have previously identified serotonergic abnormalities (8, 10), with the goal of building upon previous findings to provide a deeper and more complete understanding of serotonergic dysfunction in SIDS pathology. We specifically postulate that $5\text{-HT}_{2A/C}$ receptor binding is altered in medullary cardiorespiratory- and/or arousal-related nuclei in SIDS cases versus controls using tissue autoradiography with the agonist ^{125}I-DOI for $5\text{-HT}_{2A/C}$ receptors. As a corollary, we further propose that 5-HT_{1A} and $5\text{-HT}_{2A/C}$ binding are both altered in the same cardiorespiratory-related nuclei in SIDS cases compared to controls, suggesting abnormal signaling interactions between the 2 receptor subtypes in SIDS. The major role for central 5-HT, working via several of at least 15 subtypes of the 5-HT receptor family (including 5-HT_{1A} and 5-HT_{2A} receptors), has long been recognized in state-dependent, cardiorespiratory control for decades, as summarized in several excellent reviews (28–30). The key brainstem regions involved in the circuitry of cardiorespiratory control are concentrated in, but not exclusive to, the medulla oblongata. These regions are synaptically interconnected to each other and to the 5-HT-synthesizing neurons of the caudal 5-HTergic domain in the brainstem, the so-called medullary 5-HT network (8). Based upon our previous research directly in SIDS brainstems (31, 32), and in context of the triple risk model described above, we have hypothesized that the intrinsic vulnerability, in at least a subset of SIDS, includes abnormalities in the medullary 5-HT network that in some, as yet unspecified mechanism, leads to sleep-related sudden death (8). In the following companion studies (Parts I–II), we move closer to deciphering the unspecified mechanism, looking directly for anatomic and/or neurochemical clues in SIDS autopsy tissues.

MATERIALS AND METHODS

Clinicopathologic database

Medullary tissue was collected from the San Diego Medical Examiner's Office (SDME) and was available for research under the auspice of the California Code, Section 27491.41. Cases were collected over 2 distinct periods of time (2004–2008 and 2009–2011), and comprise 2 independent and separately published datasets. In the early phase of these studies (8), we applied the Krous definition for SIDS which includes a relationship of SIDS to sleep, i.e. SIDS is defined as the sudden unexpected death of an apparently healthy infant under 1 year of age that is related to a sleep period that remains unexplained after a thorough case investigation, including performance of a complete autopsy with ancillary testing, examination of the death scene, and review of the clinical history (6). In the present study, we have changed to using the Radcliffe definition (3), based upon its recent consensus by a multidisciplinary international panel of SIDS experts, and current epidemiological and autopsy data in SIDS. This definition states that SIDS is the sudden unexpected death of an apparently healthy infant under 1 year of age that remains unexplained after a thorough case investigation, including performance of a complete autopsy with ancillary testing, examination of the death scene, and review of the clinical history. The brains in this study were examined according to the standard neuropathology protocol of the San Diego forensic office, and no consistent histopathologic lesions were reported. Of note, there were no SIDS cases captured in our dataset for the studies of $5\text{-HT}_{2A/C}$ receptor binding with sudden, unexplained death in infants occurring while awake. An *autopsy* control is defined as an infant dying suddenly of acute illness of short duration, in which an autopsy and scene investigation established a known cause of death. Cases were considered undetermined when the investigation, death scene

examination, or autopsy was substantially limited, incomplete, or insufficient, for example, due to legal/religious restrictions, delayed report of death that limits scene investigation; or when inconsistent accounts or other findings raise competing conclusions about the cause of death (3). Undetermined cases were excluded from the study analysis. The cause of death was adjudicated by review of the clinical and autopsy records by independent investigators blinded to the autoradiography data. Information from select clinical and autopsy variables were extracted for analysis.

Tissue preparation

The medulla oblongata with the pons and midbrain was transected fresh at the time of autopsy from the forebrain at the level of the mammillary bodies, and frozen at −80°C. The frozen medulla was divided into blocks approximately 1.5 cm long and embedded in OCT on a cryostat chuck without thawing. Sectioning was done from rostral to caudal. The 2 blocks were subsequently sectioned at 20 µm on a motorized cryostat, and every 5th section was thaw-mounted onto Superfrost Plus glass microscope slides (Thermo Fisher Scientific, Waltham, MA). Alternating sections at standardized intervals were labeled with different radioligands of interest to different receptor subtypes, e.g. 5-HT$_{1A}$ and 5-HT$_{2A/C}$ in the same SIDS and control cases.

Tissue receptor autoradiography

Our procedures for tissue receptor autoradiography with ^{3}H-DPAT binding to 5-HT$_{1A}$ receptors (31, 32) and ^{125}I-DOI binding to 5-HT$_{2A/C}$ receptors (33) in alternating sections of the same SIDS and controls have been published previously. The new data reported here concern 5-HT$_{2A/C}$ receptor binding with the radioligand ^{125}I-DOI, a known agonist to the 5-HT$_{2A/C}$ receptor (33–35). The autoradiography procedure for determination of ^{125}I-DOI binding to 5-HT$_{2A/C}$ receptors was performed on 20-µm-thick sections of frozen medulla according to a previously described protocol (33). Sections were preincubated in 50 mM Tris-HCl (pH 7.4), 0.1% ascorbic acid, and 4 mM $CaCl_2$ for 30 minutes at room temperature followed by incubation in the same buffer containing 86.3pM ^{125}I-DOI (PerkinElmer, Inc., Waltham, MA) for 60 minutes at room temperature to determine total 5-HT$_{2A/C}$ receptor binding density. Nonspecific binding was determined by addition of 10 µM ritanserin (which binds to the 5-HT$_{2A/C}$ receptor) to the incubation solution. Sections were then washed 2 × 10 minutes in ice cold buffer and dried in warm air before being placed in cassettes and exposed to ^{125}I-sensitive film (GE Life Science, Boston, MA) for 42 hours along with a set of ^{125}I standards (GE Life Science) for conversion of optical density of silver grains to femtomoles/milligram of tissue (fmol/mg) tissue. Film autoradiograms were generated according to standard laboratory procedure for development of light-sensitive film. Quantitative densitometry of autoradiograms was performed using an MCID 5+ imaging system (Imaging Research, Ontario, Canada). For each specimen, receptor binding density (expressed as the specific activity of tissue-bound ligand in fmol/mg protein) was analyzed in a total of 10 medullary nuclei (see below). Total receptor binding was determined in 2 sections and nonspecific receptor binding in one section for each nucleus analyzed. Specific receptor binding density was determined by subtracting nonspecific binding from total binding. When obtaining binding density of a region via MCID quantitation, optical density values from all the pixels within the area of interest are divided by the number of pixels within that area thus providing an average optical density measure for the size of the area of interest.

^{125}I-DOI binding to 5-HT$_{2A/C}$ receptors compared to [^{3}H]8-OH-DPAT binding to 5-HT$_{1A}$ receptors in the medulla in normative (control) development at a representative age

We compared the binding densities in the same medullary nuclei between 5-HT$_{1A}$ receptors (radioligand [^{3}H]8-OH-DPAT) previously analyzed by us (32), with binding densities of 5-HT$_{2A/C}$ receptors (radioligand ^{125}I-DOI) in the present cohort (see "Clinicopathologic database"). We then selected the representative age of 53 postconceptional weeks in infancy for a static age comparison of the anatomic distributions between the 2 radioligands. In addition, we normalized the binding levels in fmol/mg tissue to a scale of 0–3 because of the different absolute, noncomparable binding values with the 2 radioligands analyzed. Using the scale of 0–3, we compared the relative densities of 5-HT$_{1A}$ to 5-HT$_{2A/C}$ receptor binding to each other in the control (normative) infants.

Use of human brainstem atlases for reference

The whole brainstem was divided into 15 levels for ease in identification of anatomy, as defined by distinct anatomic nuclei and fiber tracts (fiducial landmarks) at each level (8, 36). The precise level of a medullary section in the case was oriented to the sections as presented in the primary reference of human brainstem atlas used for this study, that of the relatively recently revised edition of Olszewski and Baxter (37). We chose this atlas as primary in this study to ensure continuity with early brainstem studies published by us, as the earlier edition of this atlas was used in our initial brainstem autoradiography studies (8). Complementary observations were made in the human brainstem atlas of Paxinos et al (38). When there were discrepancies between the 2 atlases, that of Olszewski and Baxter (37) was used for interlaboratory consistency, due to our familiarity, experience, depiction of cytoarchitecture, and personal verification with this atlas over 2 decades of its use (H.C. Kinney, personal communication) (8). The third edition of Olszewski and Baxter (37) was notable for an update of the raphe topology and nomenclature, added definition of the anatomic boundaries of the intermediate reticular zone (IRZ) and the putative preBötzinger complex, and added identification of regions of overlap of the reticular formation with the catecholaminergic C1 and A1 groups.

The 2014 edition of the human brainstem atlas of Olszewski and Baxter (37) gives only a written description of the anatomic position of the IRZ, and not a graphic map of it in cross-section. Thus, we added its approximate position with a dotted red line at the appropriate level of the rostral reticular formation of the medulla in Plate 14 from the atlas of

Olszewski and Baxter (37) (Fig. 1), based on its written description (37), combined with its analogous position delineated graphically in the atlas of Paxinos (38). The IRZ is important in our designation of it as a key 5-HT-source nucleus of the medullary serotonergic homeostatic subnetwork. According to Olszewski and Baxter (37), the IRZ cuts across the borders of the central nucleus of the medulla oblongata, parvocellular reticular nucleus, and nucleus paragigantocellularis lateralis (PGCL) (Fig. 2). It is the junctional region of the alar and basal plates, and it contains the motoneurons of the nucleus ambiguous, retrofacial and facial nuclei, and the retroambiguus nucleus (37). The IRZ around this motor column is said to include the Bötzinger complex, the preBötzinger complex, and ventral respiratory group, but the evidence for this designation is not provided (37). Paxinos et al (38) also designate the preBötzinger complex as embedded in the human IRZ, based upon principles of comparative anatomy.

Nuclei of the medulla oblongata sampled by tissue autoradiography

Specific activity in 10 nuclei was measured at a total of 2 (one mid and the other rostral) levels of the medulla (Plates 12 [mid] and 14 [rostral]), respectively, according to the atlas of Olszewski and Baxter (Fig. 1) (37). The medullary nuclei analyzed were chosen for comparison to the same nuclei sampled across all autoradiography studies in SIDS brainstems by us (8). The 10 nuclei sampled have all been implicated independently in the control of breathing and gasping, upper airway patency, cardiovascular responses (heart rate, blood pressure), cardiopulmonary coupling, recovery from hypotension, chemosensitivity to hypoxia and carbon dioxide, arousal, and/or

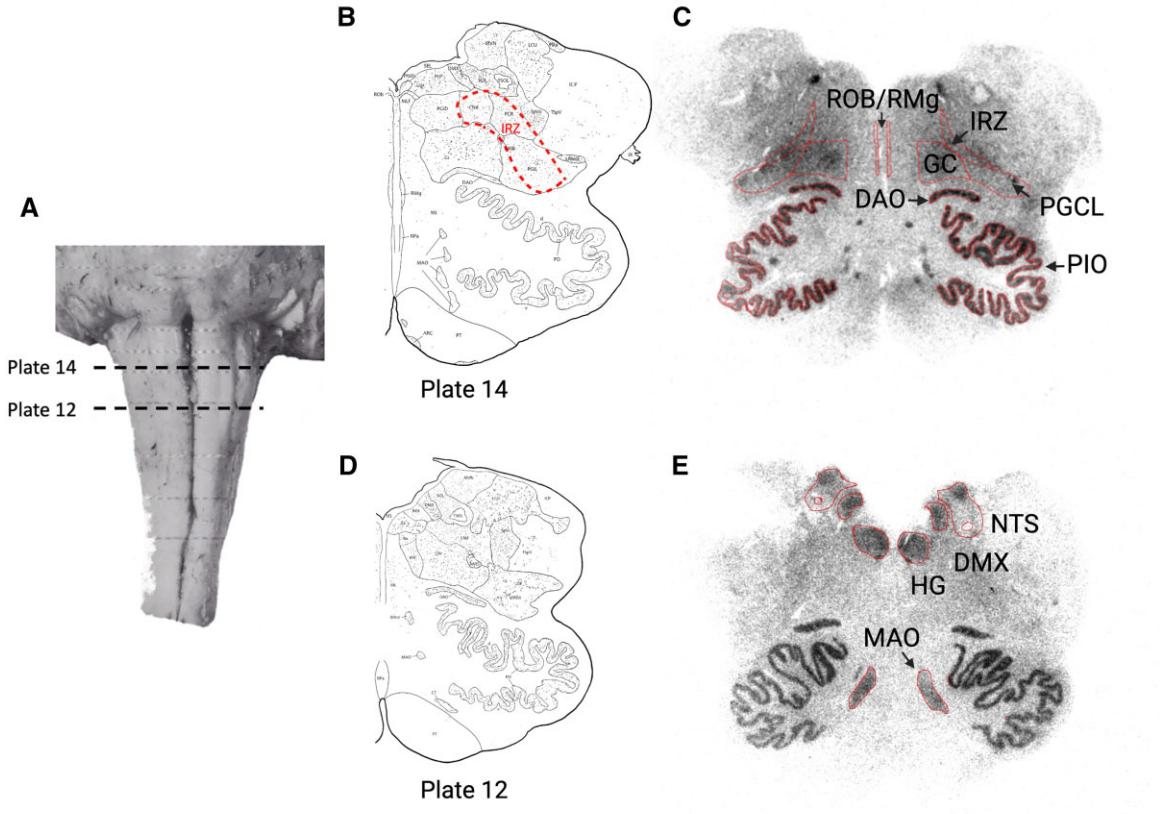

Figure 1. **(A)** The 2 cross-section levels of the human medulla oblongata, illustrating the site of the anatomic nuclei measured in this tissue autoradiographic study of 5-$HT_{2A/C}$ receptor binding in SIDS versus controls. The cross-sections are reproduced from the 2014 edition of the human brainstem atlas of Olszewski and Baxter (37), with permission from S. Karger AG, Basel. **(B)** Rostral section level 1801, level of the GC: Plate 14. The following nuclei were measured at this level: PGCL, GC, ROB/RMg, IRZ, PIO, and DAO. According to the text (37), the IRZ is located in the reticular formation at this level, forming a ventral to dorsal arc with indistinct cytoarchitecture, with boundaries across the PGCL, parvocellular nucleus, and central reticular nucleus, dorsal part, in the lateral to medial direction. We measured the IRZ at this level (denoted in red), also in reference to fiducial landmarks at this same level in the human brainstem atlas of Paxinos (38) in which the IRZ is specifically illustrated. **(C)** A representative autoradiogram with boundary contours (red) utilized in the analysis of binding in the rostral medulla. Measured nuclei are labeled. **(D)** Midsection level 1901, level of the nucleus of Roller, Plate 12. The following nuclei were measured at this level: NTS, DMX, HG, MAO. **(E)** A representative autoradiogram with boundary contours (red) utilized in the analysis of binding in the mid medulla. Measured nuclei are labeled. Composite figure was created with BioRender.com. HG, hypoglossal nucleus; NTS, nucleus of the solitary tract; DMX, dorsal motor nucleus of vagus; PIO, principal inferior olive; MAO, medial accessory olive; DAO, dorsal accessory olive; ROB, raphe obscurus; RMg, raphe magnus; GC, nucleus gigantocellularis; IRZ, intermediate reticular zone; PGCL, nucleus paragigantocellularis lateralis.

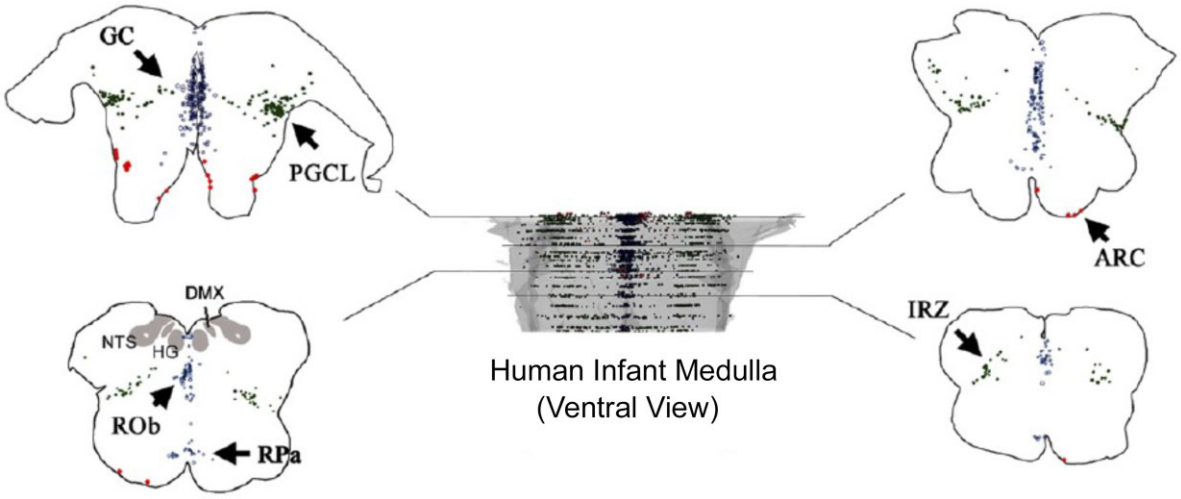

Figure 2. The topographic distribution of 5-HT-producing neurons in the human infant medulla from rostral caudal plane from the upper far right to the lower far left, reproduced from (151), with permission from Elsevier. The blue dots are 5-HT neurons embedded in the raphe (midline), the green dots are 5-HT neurons embedded in the GC (median region) and IRZ and PGCL (lateral region) of the extra-raphe, and the red dots are 5-HT neurons embedded in the arcuate nucleus at the ventral surface of the medulla (ARC) (ventral region) on the medullary surface. The IRZ forms crescent of 5-HT neurons at all levels sampled. The gray-shaded nuclei are major cardiorespiratory-related nuclei that receive projections from the medullary 5-HT source neurons. Together the 5-HT neurons and their monosynaptic terminations in the medulla are postulated to comprise the medullary homeostatic 5-HT subnetwork. See text for abbreviations. HG, hypoglossal nucleus; NTS, nucleus of the solitary tract; DMX, dorsal motor nucleus of vagus; ROB, raphe obscurus; GC, nucleus gigantocellularis; IRZ, intermediate reticular zone; PGCL, nucleus paragigantocellularis lateralis; RPa, raphe pallidus; ARC, arcuate nucleus.

sleep regulation in animal physiological and human pharmacological studies (Table 1) (19, 30, 39–73) including functions mediated by the 5-HT$_{2A/C}$ receptor.

5-HT medullary subnetwork in the rostral medulla

We focused upon nuclei in the previously defined medullary 5-HT network (8), comprised of the 5-HT source neurons and their medullary (including olivary) projection sites. The brainstem 5-HT system has historically been divided into rostral and caudal domains based upon classical ascending and descending projections, and the anatomic location of the 5-HT-synthesizing neuronal soma (29, 65, 89–97). The rostral 5-HT domain is in the midbrain (dorsal raphe) and upper pons (dorsal raphe, median raphe) and is critical for arousal, cognition, affect, learning, memory, and sensorimotor integration. The rostral 5-HT domain is also interconnected with forebrain (limbic and paralimbic) pathways of central homeostatic control. Parenthetically, 5-HT receptor binding in the rostral 5-HT domain was not studied in Parts I–II, as early 5-HT receptor binding studies by us did not show reproducible alterations within the dorsal or median raphe in SIDS cases compared to controls (98) (see below). The caudal domain, on the other hand, is comprised of the 5-HT source neurons in the caudal pons (raphe pontis) and medulla (raphe obscurus, raphe magnus, and raphe pallidus), as well as the extra-raphe 5-HT source nuclei in the ventromedial medulla (GC) and ventrolateral medulla (PGCL and IRZ), i.e. reticular formation of the rostral medulla (Figs. 1 and 2). In our work, we made boundary contours around the overlapping nuclei of the raphe obscurus (ROB) and nucleus raphe magnus, labeling the combined nuclei ROB/raphe magnus (ROB/RMg), because cytoarchitectural distinctions could not be made in the autoradiographic sections. The rostral and caudal 5-HT domains share connections with each other (72), and with cardiorespiratory- and arousal-related nuclei, including catecholaminergic neurons (99), elsewhere in the brainstem and forebrain, as documented in animal and human studies.

Statistical analysis

t-Tests were used to compare age and postmortem interval (PMI) between SIDS cases and controls. Analysis of covariance was used to test for differences in 5-HT$_{2A/C}$ binding between SIDS cases and controls while controlling for the potential effects of postconceptional age (PCA), the interaction between age and diagnosis, and dataset. Postmortem interval (PMI), sex, and prematurity were also included in the models when significant. Finally, analysis of covariance was used to examine whether the presence of a risk factor for SIDS was associated with a statistically significant difference in 5-HT$_{2A/C}$ binding in SIDS cases, controlling for the potential effects of PCA and dataset. SIDS risk factors included prenatal exposures to alcohol and/or smoking, recent history of illness, found in the prone and side sleep position, sleeping with face covered, sleeping in an adult bed or sofa, and bed-sharing. A $p < 0.05$ was considered statistically significant.

RESULTS

Clinicopathologic database

For tissue receptor autoradiography, we examined the medullae of 70 infants (58 SIDS infants and 12 autopsy controls) (Tables 2 and 3). The cases ranged in age from 36 to 76 postconceptional weeks. There was no significant difference in

Table 1. Definition of the 10 medullary nuclei sampled for autoradiography studies

Site	Level of medulla sampled	Cranial nerve root	Function	References
HG	Mid	12	Upper airway control of tongue including in gasping and sleep	(63, 74)
NTS	Mid	9,10	Preganglionic sympathetic outflow in ANS, chemosensitivity to hypoxia and hypercarbia, cardiorespiratory coupling, blood pressure	(62, 64, 65, 71, 75–79)
DMX	Mid	9,10	Preganglionic parasympathetic outflow, cardiomotor vagal neurons, blood pressure	(66, 80, 81)
PIO	Mid	None	Olivocerebellar relay, skeletal muscle coordination and timing in breathing, chemosensitivity to hypercarbia, blood pressure recovery, inputs from cerebral cortex and other brainstem nuclei (e.g. red nucleus), projections to intermediate cerebellum and neocerebellum	(82, 83)
MAO	Mid	None	Vermal cortex and fastigial nucleus connectivity involved in chemosensitivity to hypercarbia and recovery from hypotension	(82, 83)
DAO	Mid	None	Olivocerebellar relay, input from cerebral cortex, and dorsal columns, projections to vermis	(82, 83)
ROB/RMg (overlap)	Rostral	None	RVLM, 5-HT medullary subnetwork, multiple homeostatic functions, respiration, blood pressure, chemosensitivity, motor control, arousal	(19, 40, 41, 44, 61, 68, 84, 85)
GC	Rostral	None	RVLM, putative region of human preBötzinger complex, 5-HT medullary subnetwork for homeostasis, medullary arousal	(86, 87)
IRZ	Rostral	None	RVLM, putative region of human preBötzinger complex, medullary 5-HT subnetwork	(37, 75)
PGCL	Rostral	None	RVLM, 5-HT medullary subnetwork for homeostasis, putative region of human preBötzinger complex, overlaps with C1, sleep, and arousal	(70, 72, 75, 88)

HG, hypoglossal nucleus; NTS, nucleus of the solitary tract; DMX, dorsal motor nucleus of vagus; PIO, principal inferior olive; MAO, medial accessory olive; DAO, dorsal accessory olive; ROb, raphe obscurus; RMg, raphe magnus; GC, nucleus gigantocellularis; IRZ, intermediate reticular zone; PGCL, nucleus paragigantocellularis laterali; RVLM, rostral ventrolateral medulla.

Table 2. Age and cause of death of acute controls

Case number	Gestational age (week)	Postnatal age (week)	Postconceptional age (week)	Sex	Cause of death
1	36.0	0.3	36.3	F	Fatty acid oxidation disorder
2	35.0	2.0	37.0	M	Complications of prematurity
3	39.0	0.4	39.4	M	Unsuspected congenital heart disease
4	40.0	0.1	40.1	M	Car accident
5	38.0	2.3	40.3	F	Congenital heart disease
6	37.0	4.0	41.0	F	Acute bronchiolitis and focal bronchopneumonia
7	40.0	3.6	43.6	F	Heart and renal disease, not otherwise specified
8	38.0	15.3	53.3	F	Aspiration pneumonia with chronic bronchiolitis
9	40.0	17.0	57.0	M	Congenital heart disease
10	37.0	21.7	58.7	F	Pneumonitis with focal acute bronchopneumonia
11	41.0	23.0	64.0	M	Accidental wedging
12	40.0	28.6	68.6	M	Sepsis

F, female; M, male.

PCA between SIDS cases (53.4±7.3 weeks) and controls (48.3±11.4 weeks) ($p = 0.16$) (Table 3). The SIDS cases had a mean PMI of 19.4 hours with a range of 5.5–30 hours, and the controls had a mean PMI of 16.75 hours, with a range of 8–26.2 hours (nonsignificant). The causes of death in the controls were: congenital heart disease ($n = 3$), acute pneumonia ($n = 3$), car accident ($n = 1$), fatty oxidation disorder ($n = 1$), complications of prematurity ($n = 1$), cardiac and renal disease ($n = 1$), sepsis ($n = 1$), and accidental wedging ($n = 1$) (Table 2). The brainstems in all cases and controls demonstrated no pathologic changes as assessed by hematoxylin and eosin staining of the frozen tissue sections.

Normative ^{125}I-DOI binding to 5-HT$_{2A/C}$ receptors in the human infant (control) medulla

There were 12 controls in this study, ranging in age from 36 to 68 postconceptional weeks, and dying of a variety of acute causes (see "Clinicopathologic database" above). The relative level of ^{125}I-DOI binding and the anatomic localization replicated a preliminary (smaller) dataset of controls ($n = 7$)

previously published by us (33). The highest density of ^{125}I-DOI binding to the 5-HT$_{2A/C}$ receptor in the controls (n = 12) in this study was observed in the dorsal accessory olive (DAO) of the olivocerebellar system followed by the dorsal motor nucleus of vagus (DMX) (Fig. 3). Intermediate levels of binding were observed in the nucleus of the solitary tract (NTS), hypoglossal nucleus (HG), the extra-raphé regions containing 5-HT neuron cell bodies (including the GC, PGCL, and IRZ), and the medial accessory olive (MAO) and principal inferior olive (PIO) of the olivocerebellar system (Fig. 3). Across nuclei of the rostral reticular formation (IRZ, GC, and PGCL) distinctive anatomic parcellation among the 3 nuclei by visual inspection was not evident (Fig. 3). Negligible binding was observed in the ROB/RMg. Different ^{125}I-DOI developmental trajectories to 5-HT$_{2A/C}$ receptor binding were observed in the medullary nuclei sampled in the control group. Binding levels remained relatively unchanged over the first 12 months of life in the ROB/RMg, PGCL, GC, and IRZ, in the controls, compared to a steady increase in the HG, NTS, DMX, PIO, and MAO in binding over the same period (Fig. 4).

Table 3. Clinicopathologic information concerning the SIDS cases versus acute controls

	Controls Mean (SD)	SIDS Mean (SD)	
N	**12**	**58**	**p value**
Gestational age (wks)	38.4 (1.9)	38.2 (3.4)	0.71
Postnatal age (wks)	9.9 (10.5)	15.2 (7.8)	0.05
Postconceptional age (wks)	48.3 (11.4)	53.4 (7.3)	0.16
Postmortem interval (hrs): median and range	16.75 (5.6)	19.4 (5.7)	0.11

N, Number; wks, weeks; hrs, hours; SD, standard deviation.

Comparison of 5-HT$_{1A}$ and 5-HT$_{2A/C}$ receptors in the medulla in normative (control) development at a representative age

The binding of ligands for 5-HT$_{1A}$ and 5-HT$_{2A/C}$ receptors overlapped in most of the medullary nuclei sampled, but at different relative binding levels (Scale 1–3) (Table 4; Fig. 5). The most notable differences between 5-HT$_{1A}$ and 5-HT$_{2A/C}$ binding were within the: (1) ROB/RMg, which displayed the highest binding for 5-HT$_{1A,}$ (degree 3) but the lowest binding for 5-HT$_{2A/C}$ (degree 1), and (2) the PIO which displayed high binding (degree 3) for 5-HT$_{2A/C}$ receptors, compared to negligible binding for 5-HT$_{1A}$ receptors (degree 0).

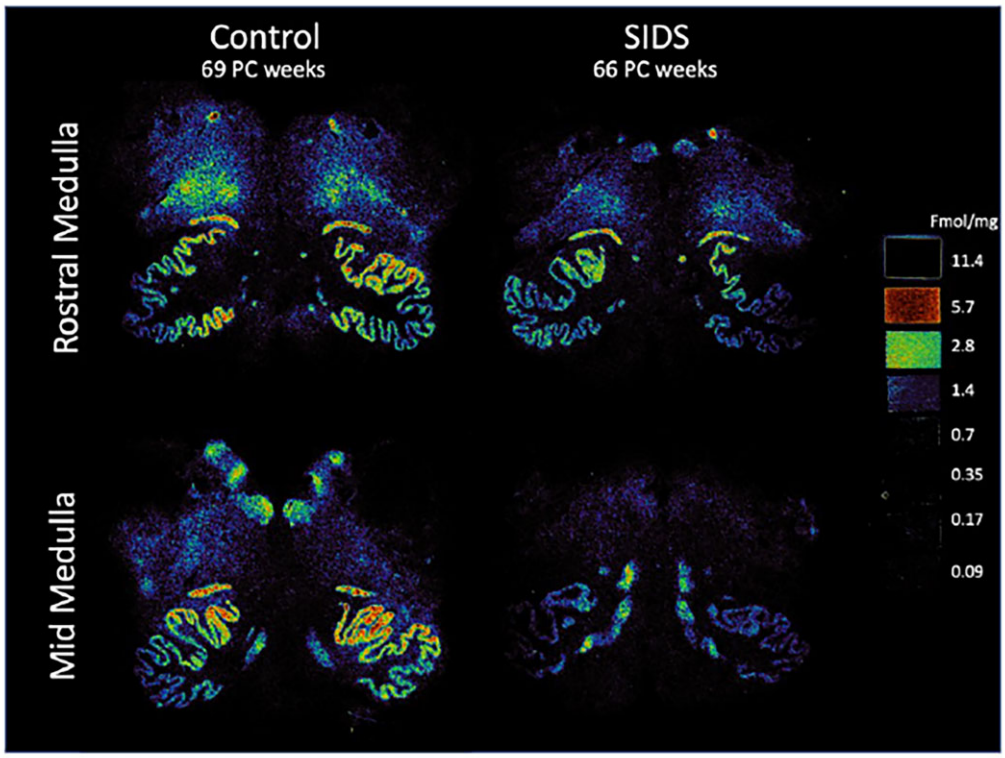

Figure 3. Comparison of pseudocolored, computer-generated mosaics of the specific activity of ^{125}I-DOI binding to the 5-HT$_{2A/C}$ receptor in an "older" SIDS case (greater than 3 months at death) and age-related control (see text). The receptor binding in each subject was measured at the standardized rostral and mid medulla levels (see Fig. 1). The same color scale of specific activity in fmol/mg tissue, and the same magnification of the cross level was used to compare binding levels between the SIDS case and control. There is a visually obvious reduction in binding in the SIDS case compared to the control in the IRZ, GC, PGCL, NTS, HG, DMX, and lamellae of PIO. HG, hypoglossal nucleus; NTS, nucleus of the solitary tract; DMX, dorsal motor nucleus of vagus; GC, nucleus gigantocellularis; IRZ, intermediate reticular zone; PGCL, nucleus paragigantocellularis lateralis; PIO, principal inferior olive.

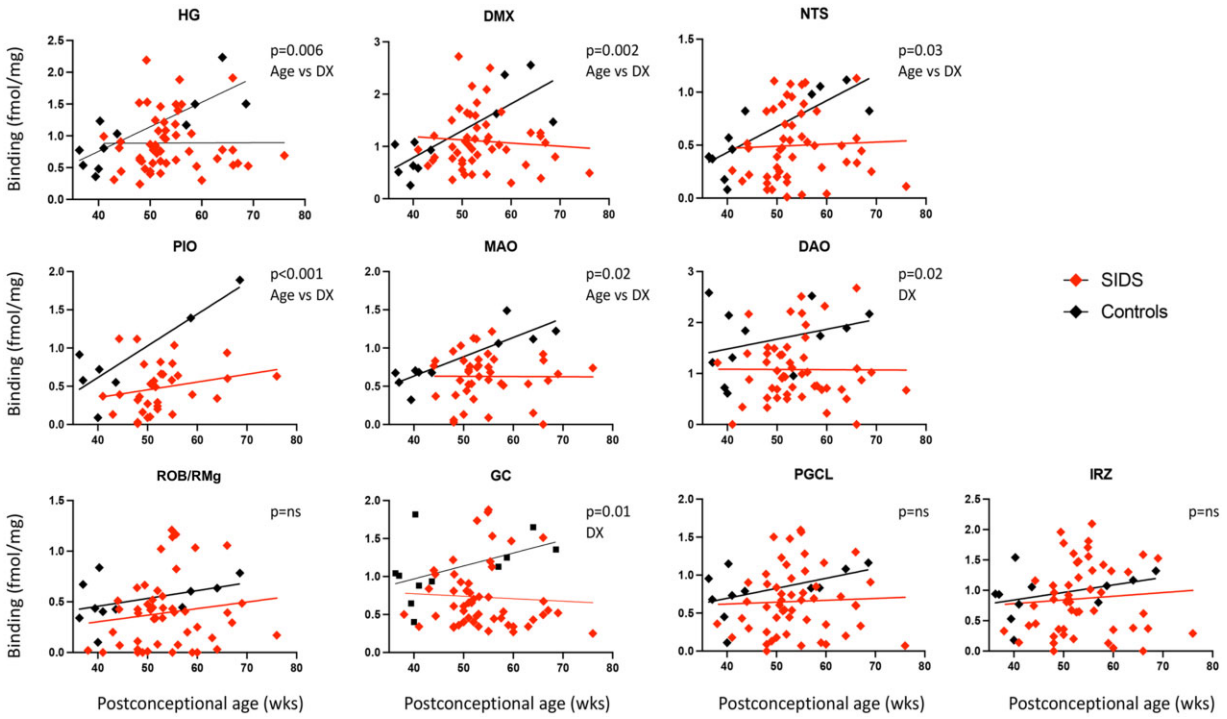

Figure 4. SIDS cases (red triangles) compared to autopsy controls (black triangles). Regression lines are added for comparison between SIDS cases (red lines) and controls (black lines). See abbreviation list and text for description. HG, hypoglossal nucleus; NTS, nucleus of the solitary tract; DMX, dorsal motor nucleus of vagus; PIO, principal inferior olive; MAO, medial accessory olive; DAO, dorsal accessory olive; ROB, raphe obscurus; RMg, raphe magnus; GC, nucleus gigantocellularis; IRZ, intermediate reticular zone; PGCL, nucleus paragigantocellularis lateralis.

Table 4. Relative binding differences in 5-HT_{1A} receptors compared to 5-$HT_{2A/C}$ receptors in controls at a representative postconceptional age of 53 weeks

Nuclei	5-HT_{1A} receptors	5-$HT_{2A/C}$ receptors
HG	0	3
NTS	1	3
DMX	1	3
ROB/RMG	3	1
GC	2	3
IRZ	2	3
PGCL	2	3
PIO	0	3
MAO	1	3
DAO	3	3

HG, hypoglossal nucleus; NTS, nucleus of the solitary tract; DMX, dorsal motor nucleus of vagus; ROb, raphe obscurus; RMg, raphe magnus; GC, nucleus gigantocellularis; IRZ, intermediate reticular zone; PGCL, nucleus paragigantocellularis lateralis; PIO, principal inferior olive; MAO, medial accessory olive; DAO, dorsal accessory olive.

5-$HT_{2A/C}$ receptor binding to ^{125}I-DOI in SIDS versus controls

We measured 5-$HT_{2A/C}$ binding in 10 medullary nuclei from 58 SIDS cases and 12 controls (Tables 1, 2, and 5; Fig. 4). There were significant alterations in binding in 7 of the 10 nuclei sampled in SIDS cases compared with controls, with differences occurring in 2 patterns (Table 5; Fig. 4). In one pattern, there was a significant reduction in binding in SIDS cases compared to controls in 2 nuclei: the GC (28% reduction, $p = 0.01$); and DAO (23% reduction, $p \leq 0.04$). In 5 nuclei, on the other hand, there was a significant age-versus-diagnosis interaction with increased receptor binding with increasing age in the HG, NTS, DMX, PIO, and MAO in the controls, compared to unchanged levels with increasing age in the SIDS cases (Table 5; Fig. 4). These significant age-versus-diagnosis interactions all occurred in medullary nuclei that receive 5-HT innervation (5-$HT_{2A/C}$ heteroreceptors), but do not contain 5-HT source neurons. In 3 of the 4 nuclei sampled that contain 5-HT source neurons, there was no significant difference in ^{125}I-DOI binding, i.e. in the ROB/RMg, PGCL, and IRZ between SIDS and controls (Table 5; Fig. 4). Of note, the GC was the only nucleus containing 5-HT source neurons with a significant binding difference in 5-$HT_{2A/C}$ (mean reduction) in the SIDS versus controls; the marginally significant ($p = 0.06$) age-versus-diagnosis interaction for GC suggests the possibility of the second pattern of binding reduction in this nucleus as well. PMI had a significant effect in the DOA only (decrease in binding with increasing PMI; $p = 0.02$), for which it was appropriately adjusted in analysis.

5-$HT_{2A/C}$ receptor binding to ^{125}I-DOI compared to 5-HT_{1A} receptor binding to [^{3}H]8-OH-DPAT in SIDS versus control

We next compared the 5-HT_{1A} and 5-$HT_{2A/C}$ receptor binding between the SIDS cases and controls in the 10 medullary nuclei sampled. Table 6 shows data from the independent datasets with cases that overlapped between 5-HT_{1A} (32) and 5-$HT_{2A/C}$ studies. The nuclei with binding alterations in both

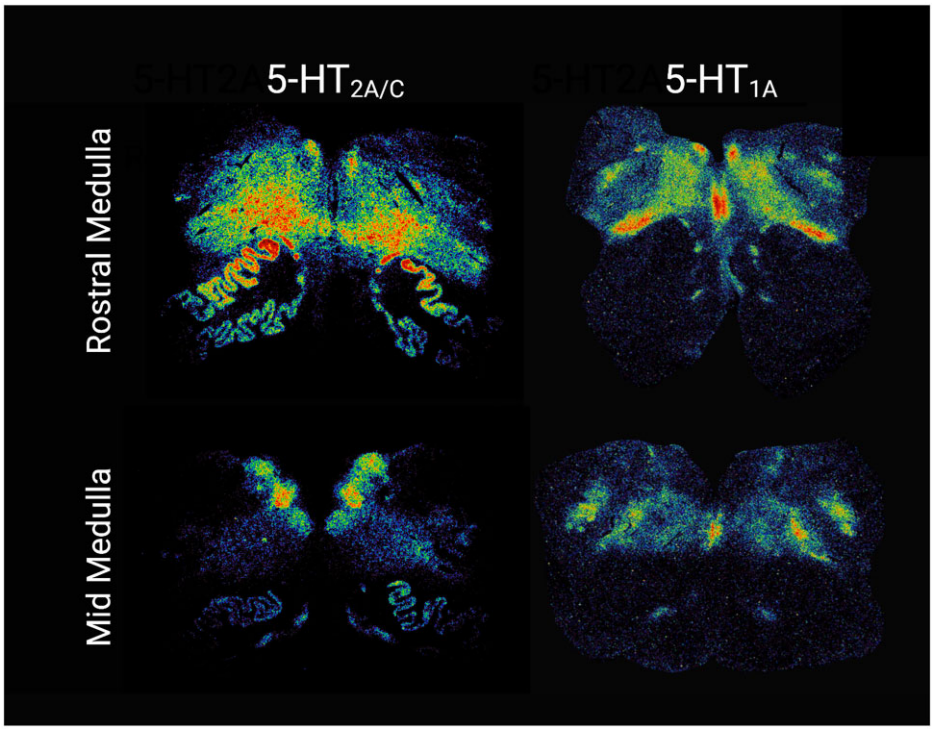

Figure 5. Representative autoradiograms of ^{125}I-DOI binding to 5-HT$_{2A/C}$ receptors compared to ^{3}H-DPAT binding to 5-HT$_{1A}$ receptors in a 53 postconceptional week SIDS infant. Mid and rostral levels of the medulla are shown. Created with BioRender.com.

Table 5. ^{125}I-DOI binding levels to 5-HT$_{2A/C}$ receptors in selected medullary nuclei, SIDS, and controls

Nucleus	SIDS N	Acute controls N	SIDS mean (SE)	Acute controls mean (SE)	p diagnosis	p age×DX interaction
HG	51	11	0.93 (0.06)	1.11 (0.13)	–	**0.006**
DMX	49	11	1.14 (0.09)	1.25 (0.18)	–	**0.002**
NTS	49	11	0.53 (0.05)	0.66 (0.10)	–	**0.03**
PIO	34	7	0.46 (0.04)	0.80 (0.10)	–	**<0.001**
MAO	41	10	0.63 (0.05)	0.89 (0.10)	–	**0.02**
DAO	51	12	1.20 (0.07)	1.56 (0.15)	**0.04**	0.12
RAPHE	50	11	0.41 (0.04)	0.54 (0.09)	0.22	0.87
GC	52	11	0.79 (0.05)	1.10 (0.11)	**0.01**	0.06
PGCL	52	11	0.69 (0.05)	0.81 (0.12)	0.37	0.38
IRZ	51	11	0.90 (0.08)	0.96 (0.16)	0.71	0.71

Bold values indicate statistical significance ($p<0.05$). HG, hypoglossal nucleus; NTS, nucleus of the solitary tract; DMX, dorsal motor nucleus of vagus; PIO, principal inferior olive; MAO, medial accessory olive; DAO, dorsal accessory olive; ROb, raphe obscurus; RMg, raphe magnus; GC, nucleus gigantocellularis; IRZ, intermediate reticular zone; PGCL, nucleus paragigantocellularis lateralis.

5-HT$_{1A}$ and 5-HT$_{2A/C}$ receptors in the SIDS cases were the HG, DMX, and NTS, and the DAO (olivocerebellar subnetwork). The patterns were different for the 2 radioligands in 3 of these nuclei (HG, DMX, and NTS) with a significant difference in the mean 5-HT$_{1A}$ binding and a significant age-versus-diagnosis interaction in the 5-HT$_{2A/C}$ binding (increase in binding with increasing age in controls but essentially steady binding with age in the SIDS cases [Table 6]). The DAO showed a significant age-versus-diagnosis effect in the 5-HT$_{1A}$ binding (a steady binding with age in the controls but a significant decrease in age in SIDS) compared to a mean reduction of 5-HT$_{2A}$ in the SIDS infants. In the caudal 5-HT domain, there was a significant age-versus-diagnosis interaction in 5-HT$_{1A}$ receptor binding in the IRZ and PGCL and a marginal significant age-versus-diagnosis interaction in the ROB/RMg and GC. In comparison, there was a mean decrease in 5-HT$_{2A/C}$ binding in the GC in SIDS compared to controls, but no significant binding differences in 5-HT$_{2A/C}$ receptors in the ROB, IRZ, and PGCL (Table 6).

5-HT$_{2A/C}$ receptor binding and risk factors for SIDS

When we examined whether the presence of a risk factor was associated with a difference in 5-HT$_{2A/C}$ binding in SIDS cases, we found no significant associations. There was also no significant effect of sex or prematurity on binding in any nucleus (data not shown).

Table 6. Comparison of patterns of ^{125}I-DOI and [^{3}H]8-OH-DPAT binding—differences between SIDS and controls

	[^{3}H]8-OH-DPAT Duncan et al (32) 5-HT$_{1A}$	^{125}I-DOI This study 5-HT$_{2A/C}$
Nucleus		
HG	S_{mean}	$S_{int}\uparrow^{c}$
DMX	S_{mean}	$S_{int}\uparrow^{c}$
NTS	S_{mean}	$S_{int}\uparrow^{c}$
RO/RMg	$MS_{int}\downarrow_{s}$	NS
GC	$MS_{int}\downarrow_{s}$	S_{mean}
IRZ	$S_{int}\downarrow_{s}$	NS
PGCL	$S_{int}\downarrow_{s}$	NS
ARC	NS	ND
PIO	ND	$S_{int}\uparrow^{c}$
MAO	NS	$S_{int}\uparrow^{c}$
DAO	$S_{int}\downarrow_{s}$	S_{mean}

S, significant; NS, nonsignificant; MS, marginally significant; ND, not determined; int, interaction age versus diagnosis; $\uparrow^{c}$, controls increase with age; $\downarrow_{s}$, SIDS decrease with age; mean, age-adjusted mean difference, serotonin, 5-HT; dataset, DS; tritiated, ^{3}H; iodinated, ^{125}I; lysergic acid diethylamide, LSD; 8-hydroxy-2-(di-*n*-propylamino)tetralin, 8-OH DPAT; 2,5-dimethoxy-4-iodoamphetamine, DOI; HG, hypoglossal nucleus; NTS, nucleus of the solitary tract; DMX, dorsal motor nucleus of vagus; PIO, principal inferior olive; MAO, medial accessory olive; DAO, dorsal accessory olive; ROb, raphe obscurus; RMg, raphe magnus; GC, nucleus gigantocellularis; IRZ, intermediate reticular zone; PGCL, nucleus paragigantocellularis lateralis.

DISCUSSION

The major finding of this study is that ^{125}I-DOI binding to the 5-HT$_{2A/C}$ receptor is altered in SIDS cases compared to (PCA) age-adjusted autopsy controls in several (n = 7) nuclei that share state-dependent cardiorespiratory regulation, including notably in survival responses to environmental hypoxia and hypotension. Thus, we confirm the a priori hypothesis of this study that 5-HT$_{2A/C}$ receptor binding is altered in medullary nuclei that are critical for the proper functioning of arousal and/or cardiorespiratory reflexes in the SIDS cases compared to controls. Importantly, these 5-HT$_{2A/C}$ receptor data further support the 5-HT brainstem hypothesis in SIDS that was previously based upon 5-HT$_{1A}$ receptor binding analysis, combined with that of other 5-HT autopsy tissue markers in our laboratory (8), as well as independent laboratories (11–13, 100, 101). Our human data in toto, coupled with findings using animal models of serotonergic dysfunction (15–27), suggest a defect in the known critical role of 5-HT$_{2A/C}$ and 5-HT$_{1A}$ receptors in arousal from sleep, chemosensitivity, cardiorespiratory coupling and, in more extreme threats to brain oxygen status, gasping and the cardiovascular components of autoresuscitation. In sum, these new data point to specific mechanisms of cardiorespiratory failure leading to sleep-related sudden death in at least an important subset of SIDS.

Serotonin, 5-HT receptors, SIDS, and tissue autoradiography

The neurotransmitter 5-HT is involved in a diverse array of brain functions, including cognition, mood, aggression, anger, anxiety, locomotion, learning and memory, impulse control, cardiorespiration, temperature, sleep/waking regulation, pain, circadian rhythm, appetite, eating, energy balance, sexual behavior, immunity, and neuroendocrine function (26, 29, 30, 58–60, 62, 65, 89, 91, 94–96, 102–117). The unifying theme of these 5-HT-mediated functions is postulated to be their critical contribution to homeostasis and neuroplastic responses to stress (29, 89, 94, 96, 113). The effects of the natural ligand 5-HT are mediated via heterogenous 5-HT receptor subtypes, of which there are at least 15 based upon physiological and neurochemical properties and amino acid sequences (118, 119). The method utilized in this study, autoradiography, allows for a "functional" analysis of key aspects of neurotransmitter receptor signaling, i.e. combined number and affinity of receptors, in a tiny circumscribed area of the brainstem (8). Thus, this method allows for the assessment of receptor binding in a nucleus, although immunocytochemistry or other methods are needed to distinguish whether the receptors are expressed pre- or postsynaptically. Nevertheless, tissue autoradiography provides a bridge between neuroanatomy and neurochemistry by pinpointing if and where there is a neurotransmitter defect.

Summary of findings of part I

In this study using ^{125}I-DOI for 5-HT$_{2A/C}$ binding, we found either lower mean binding or altered binding with age (age-versus-diagnosis interaction) in 2 and 5, respectively, out of 10 medullary nuclei examined (see below). In normative development, the 5-HT$_{2A/C}$ receptor appears to be upregulated with increasing age after ~55 postconceptional weeks in the HG, DMX, NTS, PIO, and MAO in the controls, but remains unchanged in the SIDS cases. The altered nuclei in the SIDS cases include 7 of the total of 10 nuclei sampled: HG ($p = 0.006$) (upper airway patency), DMX ($p = 0.002$) (parasympathetic outflow, regulating heart rate and underlying respiratory sinus arrhythmia), GC (suggested by us to be part of the infant preBötzinger complex involved in gasping [$p = 0.01$]), MAO ($p = 0.02$), DAO ($p = 0.02$), PIO ($p \leq 0.001$) (these latter 3 nuclei are involved in coordination of respiratory musculature, upper airway-thoracic synchronization, chemosensitivity, and blood pressure recovery), and NTS ($p = 0.03$) (chemosensitivity of respiratory and sympathetic nervous system activity and their coupling). These 7 nuclei are all components of the medullary homeostatic network previously defined by us (8), with the GC as a 5-HT source nucleus, and the HG, DMX, NTS, MAO, DAO, and PIO, as nuclei that receive monosynaptic projections from the medullary 5-HT neurons (118, 119).

Dysregulation of 5-HT receptor binding in medullary cardiorespiratory circuits in SIDS

The reported age-versus-diagnosis interaction in the HG, DMX, NTS, PIO, and MAO indicates that the effect of diagnosis on the 5-HT$_{2A/C}$ binding values varies with age in these nuclei, as is the case for 5-HT$_{1A}$ binding, albeit with different trajectories (see below). The 5-HT$_{2A/C}$ binding values overlapped in the younger SIDS and control cases, i.e. in the neonatal and early postnatal period, but became increasingly divergent between the 2 groups with increasing age. This pattern suggests 2 subsets of SIDS—a "younger" subset without a 5-HT$_{2A/C}$ binding difference from controls, and an "older" subset with 5-HT$_{2A/C}$ binding demonstrating a widening difference over the second half of the first postnatal year. The age

distribution of SIDS is unique in pediatrics, with relatively small numbers in the first month of life, a peak at 2–3 months, and approximately 90% of deaths occurring before 6 months of age—an age distribution referred to as the critical developmental period for SIDS in the triple risk model (7, 120). Age-related changes have been demonstrated in human REM sleep architecture (121), arousal responses to hypoxia (122), responses of the ventral medullary surface to changes in blood pressure in kittens (123), magnetic resonance imaging in kittens during hypotension in medullary sites, including in the ventrolateral medulla and raphe (124), and autoresuscitation defects in mouse models with 5-HT deficiencies (26).

Several SIDS studies have reported different distributions of risk factor profiles according to age at death (120, 125–128). These studies suggest that there are different underlying mechanisms for SIDS, depending on age. Such risk factors include higher incidence of bed-sharing in SIDS infants dying less than 3 months of age (129), lower birthweight in SIDS infants dying at older compared to younger ages (126), higher median age at death for SIDS infants dying in summer compared to winter months (130), and higher incidence of smoking in pregnancy for SIDS infants dying in the first few months of life compared to SIDS infants dying later in the first postnatal year (125). A recent study was conducted examining age-related risk factor profiles for sudden unexpected infant death (SUID) utilizing the Centers for Disease Control and Prevention Cohort Linked Birth/Infant Death data set (approximately 12 million live births). Based on pregnancy, birth, and demographic-related factors the following findings were reported from 9668 SUID cases: (1) younger chronological age at death was associated with reported maternal smoking during pregnancy and factors generally associated with lower socio-economic status (i.e. maternal education); and (2) older chronological age at death was associated with low birthweight, prematurity, and admission to the neonatal intensive care unit (120). When age was corrected for length of gestation, however, these latter factors (i.e. low birthweight, prematurity) were associated with a younger postnatal age at death. Nevertheless, these latter data support the idea that a premature infant will pass through the vulnerable period at a later postnatal age, because he/she was born at an earlier gestational age (120). In this large study, postnatal risk factors were not determined because data collection ended at hospital discharge after birth. Of note, it was striking that none of the individual risk factors in the multivariate analysis showed the characteristic SIDS curve for risk with age, indicating the iconic shape is likely a product of the incidence of some risk factors increasing, and some decreasing, with age (120). Sudden unexpected infant death (SUID) during the first week of life may be a different entity from SUID in the remaining weeks of the first month as well, due in part by different risk factor profiles in the spectrum of SUID (128). Of note, in this study, we found no profile of risk factors other than age that distinguished the 2 possible subsets of SIDS cases (with or without low 5-HT$_{2A/C}$ binding) from each other. However, the sample size of the 2 groups was insufficient for further meaningful analysis. The interpretation of the age-versus-diagnosis interaction as reflecting a temporal profile in receptor binding should be approached with caution, given that binding measurements were not made in the same individual over time. The binding levels were a marker of the level in each infant at the time that he/she died. The older cohort of SIDS infants may have had reduced 5-HT$_{2A/C}$ receptor binding throughout pre- and postnatal development, but nonetheless survived by avoiding extrinsic SIDS risk factors for a longer period than the younger cohort.

Given this caveat about an abnormal developmental trajectory in SIDS cases, we view the normal increase in 5-HT$_{2A/C}$ binding in the controls as representing a developmental change in receptor function reported in experimental animals (50, 123) to be critical in various homeostatic processes. It appears in our SIDS cases that this developmental change, as measured here by 5-HT$_{2A/C}$ binding, is absent or deficient, at least in some medullary nuclei (i.e. HG, NTS, DMX, PIO, and MAO). Of note, these nuclei are not comprised of medullary 5-HT-synthesizing neurons, but all receive projections from them. Given that the 5-HT$_{2A/C}$ receptor is generally a heteroreceptor on postsynaptic neurons, the relatively reduced receptor binding in older SIDS cases compared to controls likely reflects abnormalities in postsynaptic receptors. Whether these abnormalities are attributed to abnormal synaptic development and/or pruning at these sites, or to altered expression and/or affinity of the receptor protein itself is unknown. Regarding the latter, genetic alterations in 5-HT receptor genes need to be considered. Such alterations may change aspects of receptor regulation, signaling and/or trafficking. Recently, an X-linked mutation in the 5-HT$_{2C}$ receptor was reported in a cohort of SIDS infants (131). The finding of this mutation in SIDS further supports the selection of the radioligand DOI in our autoradiography studies, as it binds both 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors. Mutations in the 5-HT$_{1A}$ or 5-HT$_{2A}$ receptor genes have been sought but have not been reported in SIDS to date; they are an area for future research. Also unknown in this cohort of cases is whether 5-HT levels are abnormal in nuclei with altered 5-HT receptor binding. In a previous study, we have shown decreased 5-HT in medullary 5-HT source nuclei, using high performance liquid chromatography (32). In this study, we cannot rule out changes in 5-HT$_{2A/C}$ receptor binding compensatory to other abnormalities within the 5-HT system.

Interactions between 5-HT$_{1A}$ and 5-HT$_{2A/C}$ receptor binding in the medulla in SIDS

In most medullary regions sampled, ligand binding to 5-HT$_{1A}$ and 5-HT$_{2A/C}$ receptors colocalize to the same nuclei in the normative controls, albeit with different relative densities (Table 4). In most of the nuclei, either 5-HT$_{1A}$ and/or 5-HT$_{2A/C}$ were altered in SIDS compared to controls (Table 6) suggesting, at minimum, an imbalance of 5-HT signaling within that nucleus. Novel relationships have been characterized among membrane receptors (132–135), and it may be that these relationships are altered in SIDS. For example, isoreceptor complexes of G protein coupled receptors (GPCR) colocalize in proximity (nanometer range) within the same microdomain at the plasma membrane (132–135). Between these multi-GPCR complexes, allosteric interactions can

produce cellular signals in response to a ligand that differ from cellular signals triggered by the same ligand to a single GPCR. In regard to 5-HT, dynamic 5-HT homo- and heteroreceptor complexes within the plasma membrane contribute to the cellular response to 5-HT in different cells and under different conditions. Within the CNS, 5-HT_{1A} has been shown to exist in heteroreceptor complexes with non-5-HT receptors (i.e. FGFR1) (136) as well as isoreceptor complexes with 5-HT receptors including 5-HT_7, 5-HT_{1B}, and 5-HT_{1D} (137, 138). Of interest are studies showing the existence of 5-HT_{1A}-5-HT_{2A} isoreceptor complexes within the hippocampus (134). The dynamics of various 5-HT isoreceptor complexes in the human developing medulla are not known nor are the changes in these complexes associated with pathology such as SIDS. To assess these changes, proximity assays with specific antibodies are necessary. Our colocalization of receptor binding in a specific area of the medulla does not adequately address the presence of or changes in receptor complex dynamics. It does, however, highlight a potential new area of research in addressing questions related to 5-HT receptors and dysregulation in the pathology of SIDS.

Limitations of the study

This study is focused mainly on the role of 5-HTergic abnormalities in 5-$HT_{2A/C}$ receptor binding in postulated medullary circuits that mediate (failed) autoresuscitation in SIDS. We emphasize, however, that the regulation of cardiorespiration and sleep-waking is exceedingly complex, and involves multiple neurotransmitters, their isoreceptor complexes, transporters, and signaling pathways that may be involved in the pathogenesis of sudden death during a sleep period in a vulnerable timeframe. Indeed, the work of our laboratory and that of others have revealed abnormalities in the tissue parameters of other (non-5-HTergic) brainstem neurotransmitters, including GABA, muscarinic, nicotinic, kainate, and neuropeptides, e.g. neurokinin-1 receptor binding to substance P, in SIDS cases compared to controls (11, 12, 14, 110, 139–146). Also, regions rostral to the medulla, i.e. pons, and limbic forebrain (hypothalamus, hippocampus, and amygdala) are likewise involved in sleep, arousal, protective cardiac, and respiratory reflexes not necessarily involved in autoresuscitation (140, 147–150). Further research is needed to determine putative pontine and limbic networks of interest. At this point, however, the most robust and reproducible findings in SIDS have been in components of the 5-HT system within the medulla (12–14, 100, 145), and justify the continued focus on this region. Emphasis on the use of specific 5-HT_{2A} ligands, rather than DOI binding to the 5-$HT_{2A/C}$ receptors, is needed in the future. These specific radioligands have become readily available and increasingly reliable in the period after this study with the DOI radioligand was initiated.

An additional limitation of the study is the necessarily suboptimal selection of the control group for comparison to the SIDS group. Control tissue from deceased infants cannot be considered "normal" but, by definition, control infants have known (and varied) causes of death to compare with infants with no known cause of death (SIDS). Although we excluded cases from the control group that had major brain malformations or other neuropathological findings, the known causes of death in the control group may otherwise affect 5-HT receptor binding in currently unknown ways. We excluded cases with known hypoxic-ischemic histopathology. We also excluded cases with so-called delayed "SIDS" and those cases on life support and terminal ventilator dependence, i.e. "respirator brain." Of note, controls represent a diverse array of pathologies and, as such, the cause of death is unlikely to affect 5-HT receptor binding in any consistent way. Of interest would be 5-HT-related gene expression in mRNA and protein receptor immunological markers, for example, and neuronal counts in affected nuclei, but tissue is not consistently available in this cohort, and thus is beyond the scope of this study.

Finally, sample size, especially for the control group, was a necessary limitation of the study, given the rarity of death and autopsies in infants; a larger sample size may have allowed for detection of more subtle differences. Nevertheless, we have demonstrated an alteration of 5-$HT_{2A/C}$ receptor binding in SIDS, consistent with prior findings of altered 5-HT_{1A} in this population.

CONCLUSION

In this study, we demonstrate that there is an abnormal trajectory of 5-$HT_{2A/C}$ receptor binding in multiple nuclei of cardiorespiratory- and arousal-related circuits in the medulla in SIDS. These nuclei involve the NTS, HG, DMX, GC, PIO, MAO, and DAO. We hypothesize that abnormalities in 5-$HT_{2A/C,}$ potentially combined with abnormalities in 5-HT_{1A} receptor binding (see above) and deficiency of 5-HT (32) result in faulty 5-HT receptor signaling in critical homeostatic circuits in the medulla, with desynchronization and sudden death, presumably upon a hypoxic challenge in a sleep period, during a vulnerable developmental window. We further speculate that these pathologic and intrinsic vulnerabilities in at least a subset of SIDS leads to a failure across multiple vital respiratory, cardiovascular, and autonomic brainstem systems that are necessary for successful reoxygenation and perfusion of the developing brain in response to hypoxic threats, a concept developed and pursued in depth in Part II of this 2-part series. Finally, this present study suggests that new avenues of research involving receptor complex dynamics and serotonergic GPCRs should be sought in future research at the molecular level in SIDS tissues, to discover basic mechanisms of signaling failure and biomarkers in SIDS.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

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