

# Brainstem Serotonergic Deficiency in Sudden Infant Death Syndrome

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**S**UDDEN INFANT DEATH SYNDROME (SIDS) is the sudden death of an infant younger than 1 year that remains unexplained after a complete autopsy and death scene investigation.<sup>1</sup> Typically, an apparently healthy infant is found dead after a sleep period,<sup>2</sup> with death presumably occurring during sleep or one of the many transitions between sleep and waking.<sup>3,4</sup> The recognition that prone sleep position increases the risk for SIDS led to national campaigns,<sup>5</sup> but despite initial success, the overall SIDS rate has plateaued over the last decade.<sup>3,4</sup> SIDS remains the leading cause of postneonatal infant mortality in the United States, with an overall rate of 0.54 per 1000 live births.<sup>6,7</sup>

One model underlying SIDS research is the triple-risk model, which posits that SIDS results from the simultaneous occurrence in the infant of an underlying vulnerability, a critical developmental period, and an exogenous stressor.<sup>8</sup> In 3 independent data sets assessing infants with SIDS, our laboratory has consistently reported serotonin (5-hydroxytryptamine [5-HT]) receptor binding abnormalities

**Context** Sudden infant death syndrome (SIDS) is postulated to result from abnormalities in brainstem control of autonomic function and breathing during a critical developmental period. Abnormalities of serotonin (5-hydroxytryptamine [5-HT]) receptor binding in regions of the medulla oblongata involved in this control have been reported in infants dying from SIDS.

**Objective** To test the hypothesis that 5-HT receptor abnormalities in infants dying from SIDS are associated with decreased tissue levels of 5-HT, its key biosynthetic enzyme (tryptophan hydroxylase [TPH2]), or both.

**Design, Setting, and Participants** Autopsy study conducted to analyze levels of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA); levels of TPH2; and 5-HT<sub>1A</sub> receptor binding. The data set was accrued between 2004 and 2008 and consisted of 41 infants dying from SIDS (cases), 7 infants with acute death from known causes (controls), and 5 hospitalized infants with chronic hypoxia-ischemia.

**Main Outcome Measures** Serotonin and metabolite tissue levels in the raphé obscurus and paragigantocellularis lateralis (PGCL); TPH2 levels in the raphé obscurus; and 5-HT<sub>1A</sub> binding density in 5 medullary nuclei that contain 5-HT neurons and 5 medullary nuclei that receive 5-HT projections.

**Results** Serotonin levels were 26% lower in SIDS cases ( $n=35$ ) compared with age-adjusted controls ( $n=5$ ) in the raphé obscurus (55.4 [95% confidence interval {CI}, 47.2-63.6] vs 75.5 [95% CI, 54.2-96.8] pmol/mg protein,  $P=.05$ ) and the PGCL (31.4 [95% CI, 23.7-39.0] vs 40.0 [95% CI, 20.1-60.0] pmol/mg protein,  $P=.04$ ). There was no evidence of excessive 5-HT degradation assessed by 5-HIAA levels, 5-HIAA:5-HT ratio, or both. In the raphé obscurus, TPH2 levels were 22% lower in the SIDS cases ( $n=34$ ) compared with controls ( $n=5$ ) (151.2% of standard [95% CI, 137.5%-165.0%] vs 193.9% [95% CI, 158.6%-229.2%],  $P=.03$ ). 5-HT<sub>1A</sub> receptor binding was 29% to 55% lower in 3 medullary nuclei that receive 5-HT projections. In 4 nuclei, 3 of which contain 5-HT neurons, there was a decrease with age in 5-HT<sub>1A</sub> receptor binding in the SIDS cases but no change in the controls (age  $\times$  diagnosis interaction). The profile of 5-HT and TPH2 abnormalities differed significantly between the SIDS and hospitalized groups (5-HT in the raphé obscurus: 55.4 [95% CI, 47.2-63.6] vs 85.6 [95% CI, 61.8-109.4] pmol/mg protein,  $P=.02$ ; 5-HT in the PGCL: 31.4 [95% CI, 23.7-39.0] vs 71.1 [95% CI, 49.0-93.2] pmol/mg protein,  $P=.002$ ; TPH2 in the raphé obscurus: 151.2% [95% CI, 137.5%-165.0%] vs 102.6% [95% CI, 58.7%-146.4%],  $P=.04$ ).

**Conclusion** Compared with controls, SIDS was associated with lower 5-HT and TPH2 levels, consistent with a disorder of medullary 5-HT deficiency.

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ties in regions of the medulla oblongata critical to state-dependent homeostatic regulation,<sup>9-11</sup> ie, the medullary

5-HT system.<sup>3,12</sup> In the third data set, we also found increased 5-HT neuronal densities as well as decreased 5-HT

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transporter binding relative to 5-HT neuronal number.<sup>11</sup> Thus, we propose that SIDS results from an abnormality of the medullary 5-HT system that causes an inability to restore homeostasis following life-threatening challenges, eg, asphyxia, during a sleep period and leads to sudden death in the critical first year of life, when homeostatic systems are still maturing.<sup>12</sup>

The question remains as to whether underproduction or overproduction of 5-HT is associated with abnormal 5-HT receptor binding in SIDS. In this study we tested the main hypothesis that SIDS is associated with reductions in tissue levels of 5-HT, its key biosynthetic enzyme (tryptophan hydroxylase [TPH2]), or both, thereby representing a 5-HT deficiency disorder.

The 3 other study objectives were (1) to compare infants dying from SIDS with hospitalized infants who had chronic hypoxia-ischemia prior to death to evaluate the putative effects of impaired oxygenation on 5-HT tissue markers, given that some infants with SIDS experience repetitive apnea and agonal impaired gasping prior to death<sup>4,12-15</sup>; (2) to analyze 5-HT<sub>1A</sub> receptor binding to verify that this data set displays the same alterations we observed previously<sup>9-11</sup>; and (3) to examine levels of norepinephrine and dopamine and the metabolite 3,4-dihydroxyphenylacetic acid to address whether medullary abnormalities in SIDS involve the catecholamine system.

## METHODS

### Tissue Database

Tissue samples were obtained from autopsies in infants with and without SIDS between 2004 and 2008 for whom a study technician was available, obtained under the auspices of the San Diego County Medical Examiner's Office, San Diego, California,<sup>16</sup> and the San Diego Research Project. Samples from other infants who experienced chronic hypoxic-ischemic injury and died in the hospital were collected from the autopsy service of the Department of Pathology, Children's Hospital Boston, Boston, Massachusetts. None of the infants with SIDS or hospitalized infants

in this data set (referred to as the 2010 data set) were included in the 2006 data set or previous data sets published by us in 2000<sup>9</sup> or 2003.<sup>10</sup> Five of the 7 controls in the current data set were not included in the previous data sets; however, 2 of the 7 controls from the 2006 data set<sup>11</sup> were used because they had remaining available tissue. Their use was considered appropriate, owing to the difficulty in accruing control tissues from infants with acute deaths from known causes in a timely fashion (time frame <5 years). Tissue samples from the SIDS and control groups were obtained under California law that does not require parental consent for research involving sudden and unexpected infant death.<sup>16</sup> Permission for autopsy research of the hospitalized infants was given by the parents. Because of tissue limitations for the multiple parameters under examination, not all analyses could be performed in all cases. Analyses were performed blinded by the investigator to diagnosis, age, and all other recorded clinicopathological variables. The study was approved by the institutional review boards at Children's Hospital Boston, Boston, Massachusetts, and at the University of California at San Diego.

The 3 study groups were defined as (1) infants dying from SIDS<sup>1,9-11</sup> (cases) ( $n=41$ ); (2) infants who died acutely and in whom a definitive cause of death was established, as previously defined<sup>9,11,17</sup> (controls) ( $n=7$ ); and (3) hospitalized infants with chronic oxygenation disorders, as previously defined,<sup>9,17</sup> prior to death ( $n=5$ ). The SIDS cases and controls were classified without knowledge of any biochemical data generated by this study. The controls included (1) clinically unsuspected congenital heart disease and sudden death with origin of the left coronary artery from the pulmonary artery, endocardial fibrosis, and cardiomegaly at autopsy; (2) clinically unsuspected congenital heart disease and sudden death with truncus arteriosus at autopsy; (3) sudden cardiopulmonary arrest with clinical diagnosis of hypoplastic left heart, immediate surgical repair, and

immediate postoperative death (all within 48 hours); (4) acute pneumonia; (5) emergency cesarean delivery for traumatic placental abruption resulting from motor vehicle collision; (6) accidental asphyxia due to wedging of the head and airway between the wall and bed; and (7) accidental death due to drowning in a bucket. The hospitalized group included severe congenital heart disease and respiratory failure requiring chronic mechanical ventilation ( $n=2$ ), α-thalassemia, Potter syndrome with pulmonary insufficiency, and twin-twin transfusion with respiratory failure.

Clinicopathological features for the SIDS and control groups and risk factors for the SIDS group were obtained from parental interviews around the time of death, medical records, and the autopsy and death scene examination as reported by the medical examiner. Race/ethnicity was determined by the pathologist at autopsy, in conjunction with family interviews and infant medical records. Race/ethnicity was assessed because certain races/ethnicities (eg, African American, American Indian) are known risk factors for SIDS.<sup>3,4</sup>

At autopsy, fresh brainstem tissue was collected and stored at -80°C. From each brainstem, 2 blocks of medullary tissue (3 mm) were collected: 1 from the mid-medulla, at the level of the nucleus of Roller (corresponding to Plate X in the atlas of Olszewski and Baxter<sup>18</sup>) and 1 from the rostral medulla, at the level of the nucleus praepositus (corresponding to Plate XII in that atlas) (FIGURE 1). Using a 2-mm micropunch (Harris Uni-core; EMS, Hatfield, Pennsylvania), tissue was collected from 2 major components of the medullary 5-HT system, the raphé obscurus and paragigantocellularis lateralis (PGCL), according to the atlas of Paxinos and Huang,<sup>19</sup> and standardized protein samples were obtained for Western blot analysis in each SIDS case and control.<sup>20</sup> Twenty-micrometer tissue sections were collected from the remaining blocks in a standardized manner for tissue receptor autoradiography.

Because of limited available tissue, samples could not be run in duplicate.

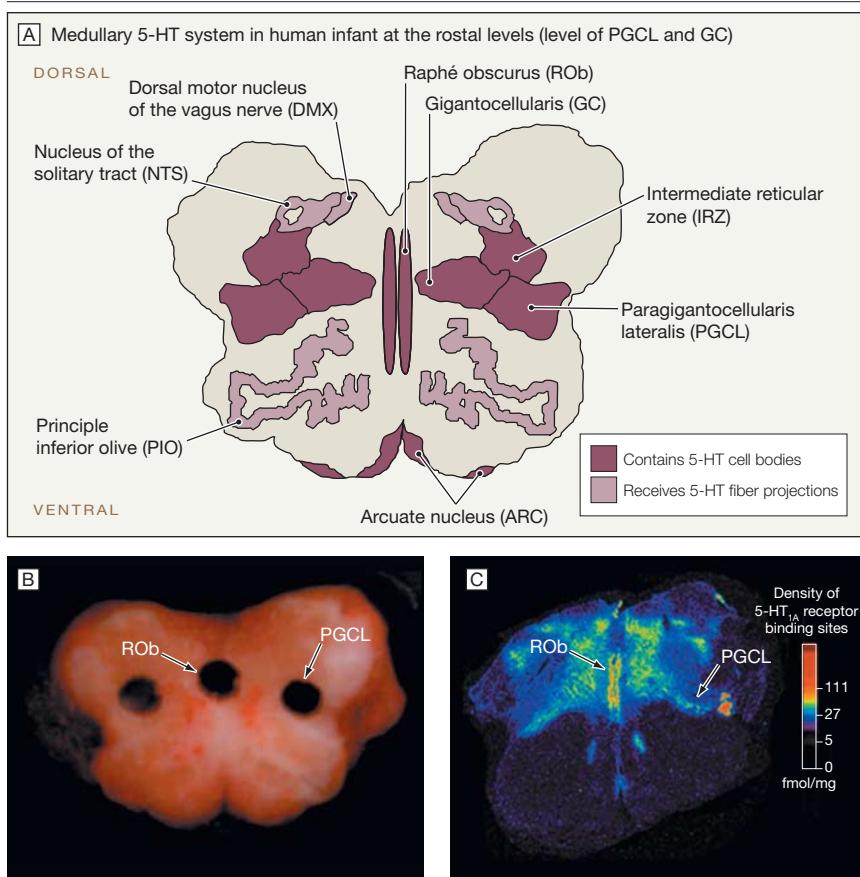
### High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) was used to measure levels of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), on micropunched samples from the raphé obscurus and PGCL. Tissue samples were homogenized on ice (Branson Sonifier 200, 1 minute) in 1mL of perchloric acid solution (0.1M HClO<sub>4</sub>, 1mM EDTA, 1mM Na<sub>2</sub>SO<sub>3</sub>) and centrifuged at 9500g and 4°C for 15

minutes (Sorvall RC-5B centrifuge; DuPont Instruments, Wilmington, Delaware). The supernatant was filtered through sterile 0.45-μm and 0.2-μm syringe filters and used immediately for analytical procedures to prevent degradation. The protein pellet was resuspended in 1M NaOH, and the protein concentration as determined by the Lowry method<sup>20</sup> was read at 660 nm. Analysis of 5-HT, 5-HIAA, norepinephrine, dopamine, and 3,4-dihydroxyphenylacetic acid levels was performed using an HPLC system with electrochemical detection (HPLC-EC; ESA Coulochem II; Bedford, Massa-

chusetts) equipped with a C-18 reverse-phase column (3 μm, 4.6 mm × 100 mm, 36°C; Microsorb MV; Varian, Walnut Creek, California). The mobile phase consisted of 90 mM NaH<sub>2</sub>PO<sub>4</sub>, 50 mM citric acid, 50 μM EDTA, 1.7 mM 1-octane sulfonic acid, and 3% acetonitrile, pH=3.0, pumped through the column at 1.0 mL/min (pressure, 270 bar). To equate for sensitivity, 25 μL run at 10 nA of sample and 5 μL run at 20 nA were used for determination of levels of 5-HT and all other compounds, respectively. This allowed for the measurement of the eluents with a sensitivity of 0.5 fmol. Serotonin, 5-HIAA, norepinephrine, dopamine, and 3,4-dihydroxyphenylacetic acid eluted from the column at 40, 8.6, 3.6, 4.6, and 11.7 minutes, respectively. Dilutions of stock standards ( $5 \times 10^{-7}$ ,  $1 \times 10^{-7}$ ,  $5 \times 10^{-8}$ , and  $1 \times 10^{-8}$  M; Sigma Chemical, St Louis, Missouri) were analyzed daily to establish a standard curve, and eluent concentrations were determined by comparing peak areas from samples with those of standards. The use of Justice Innovations ChromPerfect (Palo Alto, California) software allowed determination of regression (minimum correlation coefficient, 0.99) for the standard. Values were corrected for protein concentrations in pmol/mg protein.<sup>20</sup> The samples were run immediately following prepping without freeze-thawing.

**Figure 1.** Medullary Serotonin (5-Hydroxytryptamine [5-HT]) System in the Human Infant



A, Medullary 5-HT system in the human infant at the rostral levels (level of the PGCL and GC) for neuroanatomical reference. The medullary 5-HT system is divided into 3 components based on the location of the nuclei containing 5-HT cell bodies: (1) raphé (midline), including the ROb; (2) extrraphé (lateral), including the PGCL, GC, and IRZ; and (3) ventral surface, including 5-HT neurons embedded within the ARC.<sup>3,12</sup> B, Example of micropunched regions at the rostral medulla level for high-performance liquid chromatography and Western blot analysis in SIDS cases and controls. The regions for chromatographic analysis were the ROb and PGCL and for Western blotting was the ROb. C, Representative autoradiogram (pseudocolored computer-based image) of 5-HT<sub>1A</sub> receptor binding in a control infant at the same rostral medullary level. High binding (red) is noted in the ROb, intermediate binding (green) in the PGCL, and negligible binding (blue-black) in the PIO.

### Western Blot Analysis of TPH2 Levels

Western blot analysis was used to measure levels of TPH2 on micropunched samples from the raphé obscurus. Tissue samples were homogenized to a final concentration of 10% weight per volume, and a modified Lowry method was used for protein quantification.<sup>20</sup> After separation with SDS-PAGE, proteins were transferred electrophoretically to an Immobilon-P membrane (Millipore, Bedford, Massachusetts) overnight and incubated with a mouse monoclonal anti-TPH2 antibody (1:500; Sigma-Aldrich, Saint Louis, Missouri). TPH2 (55 KDa) was detected using a goat anti-mouse IgG horseradish peroxidase-conjugated secondary

antibody (1:10 000; Bio-RAD, Hercules, California) followed by Chemiluminescence ECL (PerkinElmer, Waltham, Massachusetts) and quantified from densitometry bands (MCID Elite 6; Imaging Research Inc, Ontario, California) standardized to human adult raphé obscurus run on the same gel. Values were expressed as a percentage of this standard.

### Tissue Autoradiography for 5-HT<sub>1A</sub> Receptor Binding

The procedure for <sup>3</sup>H-8-OH DPAT (PerkinElmer) binding to 5-HT<sub>1A</sub> receptors was based on previously described methods.<sup>11</sup> Radiolabeled sections were exposed to BAS-TR2025 phosphoimaging plates (Fujifilm Medical Systems USA, Stamford, Connecticut) for 4 weeks, along with a set of <sup>3</sup>H standards (Amersham; GE Healthcare, Piscataway, New Jersey) for conversion of optical density of silver grains in nuclei of interest to fmol/mg of tissue using an MCID Elite digital system.<sup>9-11,17</sup>

### Statistical Analysis

These studies had 80% power to detect a large effect size (1.4-SD difference between SIDS cases and controls). *t* Tests, analysis of variance, and Fisher exact tests were used to compare age, postmortem interval, sex, and race between groups. Analysis of covariance was used to test for differences between SIDS cases and controls while controlling for potential effects of postconceptional age on levels of 5-HT, catecholamine, metabolites, and TPH2, as well as 5-HT<sub>1A</sub> receptor binding. Postmortem interval and interactions between diagnosis and age were included as covariates in these models when significant. The interaction term was tested, because the effect of age on these outcomes is unknown and could potentially be different in infants with SIDS. Analysis of covariance with post hoc comparison tested differences among the 3 groups, and *t* tests were used to consider differences by risk factors. All analyses were performed on observed data only, and ad-

**Table 1.** Comparison of Clinicopathologic Features Between Sudden Infant Death Syndrome (SIDS) Cases, Controls, and Hospitalized Infants With Hypoxia-Ischemia

| Variable                          | SIDS Cases<br>(n = 41) | Controls<br>(n = 7) | Hospitalized<br>(n = 5) | P Value <sup>a</sup> |                           |
|-----------------------------------|------------------------|---------------------|-------------------------|----------------------|---------------------------|
|                                   |                        |                     |                         | 3-Way                | SIDS Cases<br>vs Controls |
| Age, mean (SD), wk                |                        |                     |                         |                      |                           |
| Postconceptional                  | 53.3 (8.0)             | 53.5 (19.5)         | 38.3 (3.4)              | .008 <sup>b</sup>    | .98                       |
| Gestational                       | 37.9 (3.4)             | 39.6 (1.3)          | 37.3 (1.9)              | .36                  | .03                       |
| Postnatal                         | 15.4 (8.5)             | 13.9 (19.0)         | 1.0 (1.7)               | .02 <sup>b</sup>     | .85                       |
| Postmortem interval, mean (SD), h | 18.6 (5.4)             | 14.9 (4.3)          | 13.6 (8.4)              | .07 <sup>c</sup>     | .09 <sup>c</sup>          |
| Male sex, No./total (%)           | 23/41 (56)             | 4/7 (56)            | 2/5 (40)                | .89                  | >.99                      |
| Race/ethnicity, No./total (%)     |                        |                     |                         |                      |                           |
| White                             | 15/39 (39)             | 3/5 (60)            | 1/2 (50)                |                      |                           |
| African American                  | 5/39 (13)              | 0/5 (0)             | 0/2 (0)                 |                      |                           |
| Hispanic                          | 15/39 (39)             | 2/5 (40)            | 0/2 (0)                 |                      |                           |
| Other                             | 4/30 (10)              | 0/5 (0)             | 1/2 (50)                | .26                  | >.99                      |

<sup>a</sup>Determined using *t* test for age and postmortem interval and Fisher exact test for sex and race.

<sup>b</sup>Hospitalized infants were significantly younger than SIDS cases and controls.

<sup>c</sup>SIDS with marginally significant higher postmortem interval.

justment for multiple testing was not performed owing to the relatively small sample size. All statistical tests were 2-sided, performed at an  $\alpha$  level of .05, and conducted using SAS version 9.2 (SAS Institute Inc, Cary, North Carolina).

## RESULTS

There was no significant difference in postconceptional age (gestational plus postnatal age) between SIDS cases (53.3 [SD, 8.0] weeks) and controls (53.5 [SD, 19.5] weeks) ( $P=.98$ ) (TABLE 1); however, postconceptional age in the hospitalized group was significantly lower (38.3 [SD, 3.4] weeks) ( $P=.008$ ), requiring adjustment for age in all analyses (Table 1). All study groups had a postmortem interval of less than 30 hours.

### 5-HT, Catecholamine, and Metabolite Levels

Samples were available from 35 SIDS cases, 5 controls, and 5 hospitalized infants. Age-adjusted mean levels of 5-HT in SIDS cases were 26% lower than in controls in both the PGCL (31.4 pmol/mg protein [95% confidence interval {CI}, 23.7 to 39.0] vs 40.0 pmol/mg protein [95% CI, 20.1 to 60.0],  $P=.04$ ) and the raphé obscurus (55.4 pmol/mg protein [95% CI, 47.2 to 63.6] vs 75.5 pmol/mg protein [95% CI, 54.2 to 96.8],  $P=.05$ ) (TABLE 2).

However, 5-HIAA levels and 5-HIAA:5-HT ratio did not indicate excessive degradation of 5-HT in SIDS cases. There were no significant differences in catecholamine levels between SIDS cases and controls. Dopamine levels, however, were 640% higher in the raphé obscurus in the hospitalized group compared with the SIDS group (81.7 pmol/mg protein [95% CI, 37.6 to 125.8] vs 11.1 pmol/mg protein [95% CI, -3.6 to 25.8],  $P=.006$ ). Moreover, 5-HT levels were 55% higher in the raphé obscurus (85.6 pmol/mg protein [95% CI, 61.8 to 109.4] vs 55.4 pmol/mg protein [95% CI, 47.2 to 63.6],  $P=.02$ ) and 126% higher in the PGCL (71.1 pmol/mg protein [95% CI, 49.0 to 93.2] vs 31.4 pmol/mg protein [95% CI, 23.7 to 39.0],  $P=.002$ ) in the hospitalized group compared with the SIDS group (Table 2).

### TPH2 Levels

Samples were available from 34 SIDS cases, 5 controls, and 4 hospitalized infants. Levels of TPH2 were 22% lower in the raphé obscurus in SIDS cases compared with controls (151.2% of standard [95% CI, 137.5% to 165.0%] vs 193.9% [95% CI, 158.6% to 229.2%],  $P=.03$ ) (Table 2). The ratio of TPH2 to 5-HT, however, did not differ. Levels of TPH2 and TPH2:5-HT ratio were lower in the hospitalized group compared with the SIDS group (102.6%

[95% CI, 58.7% to 146.4%] vs 151.2% [95% CI, 137.5% to 165.0%],  $P=.04$  and 0.9 [95% CI, -0.6 to 2.4] vs 3.1 [95% CI, 2.6 to 3.6],  $P=.01$ , respectively) (Table 2).

### 5-HT<sub>1A</sub> Receptor Binding

We measured 5-HT<sub>1A</sub> receptor binding in 10 medullary nuclei from 35 SIDS cases and 5 controls. Analysis of the hospitalized group was not possible owing to the lack of available tissue ( $n=3$ ).

There were significant alterations in SIDS cases compared with controls occurring in 2 patterns (TABLE 3). There was a significant absolute reduction in

**Table 2.** High-Performance Liquid Chromatography Measurements for Markers of the Serotonin and Catecholamine Systems and Western Blot Measurements for TPH2 Levels in Sudden Infant Death Syndrome (SIDS), Control, and Hospitalized (Hypoxic-Ischemic) Groups in the Medullary 5-HT System

| Variable          | Region            | Age-Adjusted Mean (95% CI), pmol/mg Protein |                                   |                                      | P Value <sup>a</sup>                     |  |                           |
|-------------------|-------------------|---|-----------------------------------|--------------------------------------|--|--|---------------------------|
|                   |                   | SIDS Cases<br>(n = 35)                      | Controls<br>(n = 5)               | Hospitalized <sup>b</sup><br>(n = 5) | Hospitalized <sup>b</sup><br>vs Controls | Hospitalized <sup>b</sup><br>vs SIDS Cases | Controls vs<br>SIDS Cases |
| 5-HT              | Raphé obscurus    | 55.4 (47.2 to 63.6)                         | 75.5 (54.2 to 96.8)               | 85.6 (61.8 to 109.4)                 | .52                                      | .02  | .05                       |
|                   | PGCL <sup>c</sup> | 31.4 (23.7 to 39.0)                         | 40.0 (20.1 to 60.0)               | 71.1 (49.0 to 93.2)                  | .04                                      | .002                                       | .04                       |
| 5-HIAA            | Raphé obscurus    | 294.5 (248.4 to 340.6)                      | 323.3 (204.0 to 442.7)            | 363.0 (229.2 to 496.8)               | .62                                      | .62  | .60                       |
|                   | PGCL              | 191.5 (141.6 to 241.3)                      | 222.4 (93.2 to 351.6)             | 381.3 (236.5 to 526.2)               | .06                                      | .06  | .43                       |
| 5-HIAA:5-HT ratio | Raphé obscurus    | 5.6 (5.0 to 6.3)                            | 4.4 (2.7 to 6.0)                  | 3.5 (1.7 to 5.4)                     | .08                                      | .08  | .14                       |
|                   | PGCL <sup>c</sup> | 6.4 (5.7 to 7.1)                            | 5.4 (3.7 to 7.1)                  | 6.0 (4.0 to 7.9)                     | .29                                      | .57  | .18                       |
| Norepinephrine    | Raphé obscurus    | 28.0 (22.1 to 34.0)                         | 17.1 (1.7 to 32.6)                | 19.1 (1.7 to 36.4)                   | .33                                      | .33  | .19                       |
|                   | PGCL              | 22.7 (13.3 to 32.2)                         | 12.0 (-12.5 to 36.5)              | 49.8 (22.3 to 77.3)                  | .11                                      | .11  | .05                       |
| Dopamine          | Raphé obscurus    | 11.1 (-3.6 to 25.8)                         | 14.5 (-33.2 to 62.1)              | 81.7 (37.6 to 125.8)                 | .04                                      | .006                                       | .72                       |
|                   | PGCL              | 7.7 (-14.0 to 29.5)                         | 11.3 (-46.7 to 69.3)              | 82.0 (24.0 to 140.1)                 | .08                                      | .08  | .36                       |
| DOPAC             | Raphé obscurus    | 24.7 (12.3 to 37.1)                         | 37.0 (-10.5 to 84.4)              | 24.8 (-19.2 to 68.7)                 | .88                                      | .88  | .62                       |
|                   | PGCL              | 16.4 (10.0 to 22.7)                         | 27.2 (7.7 to 46.7)                | 7.4 (-14.8 to 29.5)                  | .40                                      | .40  | .29                       |
| DOPAC/dopamine    | Raphé obscurus    | 10.4 (-1.5 to 22.3)                         | 0.3 (-54.7 to 55.3)               | 0.0 (-45.3 to 38.6)                  | .79                                      | .79  | .72                       |
|                   | PGCL              | 4.0 (1.3 to 6.7)                            | 3.1 (-4.0 to 10.1)                | 10.2 (2.2 to 18.1)                   | .32                                      | .32  | .67                       |
| TPH2 <sup>d</sup> | Raphé obscurus    | 151.2 (137.5 to 165.0)<br>(n = 34)          | 193.9 (158.6 to 229.2)<br>(n = 5) | 102.6 (58.7 to 146.4)<br>(n = 4)     | .002                                     | .04  | .03                       |
|                   | TPH2:5-HT ratio   | Raphé obscurus                              | 3.1 (2.6 to 3.6)                  | 2.9 (1.7 to 4.1)                     | 0.9 (-0.6 to 2.4)                        | .05  | .01                       |

Abbreviations: CI, confidence interval; DOPAC, 3,4-dihydroxyphenylacetic acid; PGCL, paragigantocellularis lateralis; TPH2, tryptophan hydroxylase; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine (serotonin).

<sup>a</sup>Analysis of covariance controlling for postconceptual age: model 1: SIDS vs controls (gives P value for SIDS vs controls, the primary comparison); model 2: hospitalized vs SIDS vs controls (3-way comparison with post hoc tests after overall significance to give P values for hospitalized vs control and hospitalized vs SIDS).

<sup>b</sup>Hospitalized infants with chronic hypoxia-ischemia.

<sup>c</sup>Data adjusted for significant effects of postmortem interval.

<sup>d</sup>Values for TPH2 levels are the percentage of adult human standards.

**Table 3.** 5-HT<sub>1A</sub> Receptor Binding in the Medullary Serotonin System in Sudden Infant Death Syndrome (SIDS) Cases Compared With Controls in the Current (2010) and 2006 Data Sets

|                   | 2010                                   |                     |           |   | 2006 <sup>11</sup>                     |                     |           |   |
|-------------------|--|---------------------|-----------|---|--|---------------------|-----------|---|
|                   | Age-Adjusted Mean (CI), fmol/mg Tissue |                     | P Value   |   | Age-Adjusted Mean (CI), fmol/mg Tissue |                     | P Value   |   |
|                   | SIDS Cases<br>(n = 35)                 | Controls<br>(n = 5) | Diagnosis | Age-Diagnosis<br>Interaction <sup>a</sup> | SIDS Cases<br>(n = 16)                 | Controls<br>(n = 6) | Diagnosis | Age-Diagnosis<br>Interaction <sup>a</sup> |
| HG                | 6.87 (5.81-7.92)                       | 11.15 (7.77-14.54)  | .02       | .36                                       |  |                     |           | .04                                       |
| DMX               | 7.82 (6.58-9.05)                       | 14.26 (10.22-18.30) | .004      | .73                                       | NA                                     | NA                  | NA        | NA  |
| NTS               | 8.88 (7.86-9.90)                       | 12.46 (9.13-15.79)  | .04       | .61                                       | 7.09 (5.07-9.11)                       | 14.67 (11.45-17.89) | <.001     | .58                                       |
| MAO               | 11.77 (10.05-13.49)                    | 10.14 (4.62-15.66)  | .57       | .95                                       | 9.64 (5.65-13.63)                      | 28.85 (22.70-35.01) | <.001     | .66                                       |
| Raphé obscurus    | 39.97 (33.06-46.89)                    | 29.21 (11.23-47.19) | .27       | .07                                       | 24.28 (15.68-32.88)                    | 54.68 (40.51-68.84) | .001      | .09                                       |
| Gigantocellularis |  |                     |           | .05                                       |  |                     |           | .05                                       |
| PGCL              |  |                     |           | .003                                      |  |                     |           | .002                                      |
| IRZ               |  |                     |           | .02                                       | 7.78 (6.20-9.37)                       | 13.38 (10.77-15.99) | .001      | .06                                       |
| DAO               |  |                     |           | .007                                      | NA                                     | NA                  | NA        | NA  |
| ARC               | 4.66 (3.64-5.67)                       | 3.68 (1.30-6.06)    | .45       | .82                                       | 4.13 (2.47-5.79)                       | 9.33 (6.86-11.79)   | .002      | .94                                       |
| PIO               | NA                                     | NA                  | NA        | NA  | 4.86 (3.23-6.49)                       | 4.63 (2.52-6.75)    | .86       | .63                                       |

Abbreviations: ARC, arcuate nucleus; CI, confidence interval; DAO, dorsal accessory nucleus; DMX, dorsal motor nucleus of the vagus; HG, hypoglossal nucleus; IRZ, intermediate reticular zone; MAO, medial accessory nucleus; NA, not available; NTS, nucleus of the solitary tract; PGCL, paragigantocellularis lateralis; PIO, principal inferior olive.

<sup>a</sup>With a significant age × diagnosis interaction, no means are given because the difference in means between SIDS cases and controls varies by age. When the age × diagnosis interaction was not significant, it was dropped from the final model.

binding in regions that receive projections from medullary 5-HT neurons but do not contain 5-HT neurons, ie, the hypoglossal nucleus (upper airway patency) (38% reduction), the nucleus of the solitary tract (visceral sensory input) (29% reduction), and the dorsal motor nucleus of the vagus (preganglionic parasympathetic outflow) (55% reduction). Second, in components of the medullary 5-HT system that contain 5-HT cell bodies (ie, PGCL, gigantocellularis, and intermediate reticular zone), there was a significant age  $\times$  diagnosis interaction with decreased receptor binding with increasing age in SIDS cases but no change in controls (FIGURE 2A).

We compared 5-HT<sub>1A</sub> receptor binding data between the 2006 data set<sup>11</sup> and current (2010) data set, because measurements were obtained by identical methods. Abnormal binding patterns for each nucleus were similar between the 2 data sets; the exception was reduced binding in the arcuate nucleus, raphé obscurus, and medial accessory nucleus in the 2006 but not the 2010 data set (Table 3). For further analysis with a larger sample size, we combined the 2006 and 2010 data sets ( $n=51$  SIDS cases and  $n=11$  controls) (eTable 1, available at <http://www.jama.com>) and found that the significant age  $\times$  diagnosis interactions persisted, including when identical age groups were considered (Figure 2B). Lastly, we tested the hypothesis that interrelationships exist between different medullary 5-HT-related nuclei. In SIDS cases as well as controls, altered 5-HT<sub>1A</sub> receptor binding in 1 nucleus correlated with similar alterations in other components of the 5-HT system in the same cases (FIGURE 3 and eFigure).

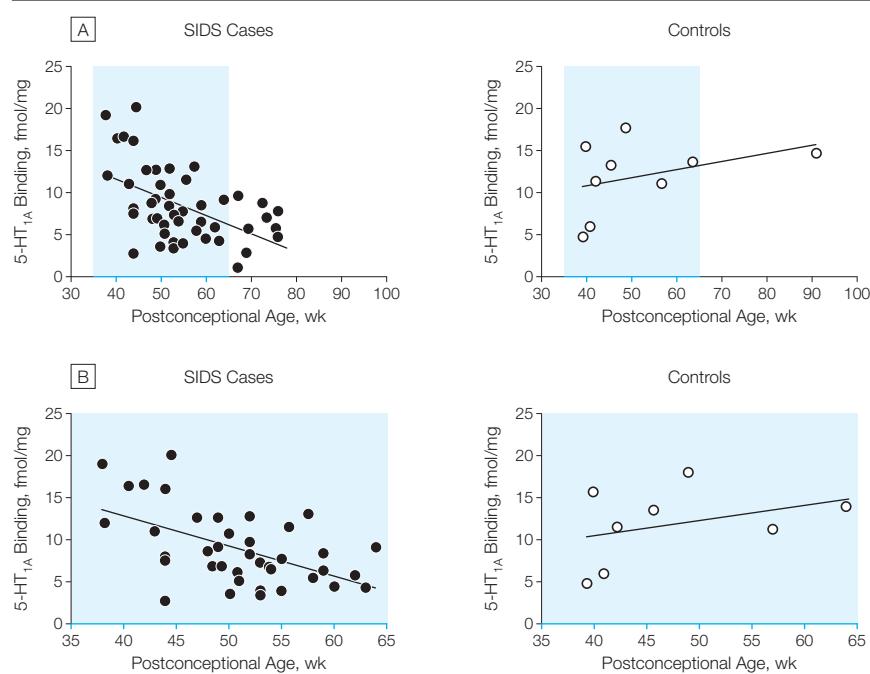
### Risk Factors in the SIDS Cases

To determine if known risk factors for SIDS were associated with abnormalities in 1 or more 5-HT parameters in the medulla, an analysis of risk factors relative to the 5-HT parameters was undertaken. Risk factors for SIDS (Table 1, eTable 2, and eTable 3) were subdivided into “extrinsic” and “intrinsic”

categories.<sup>11</sup> Extrinsic factors, eg, prone sleep position,<sup>3,4,6,7</sup> are physical stressors that place a vulnerable infant at risk for homeostatic derangements around the time of death; intrinsic factors, eg, prematurity and male sex,<sup>3,4,6,7,21,22</sup> are postulated to affect the underlying vulnerability in the infant. Ninety-five percent (39/41) of SIDS cases had 1 or

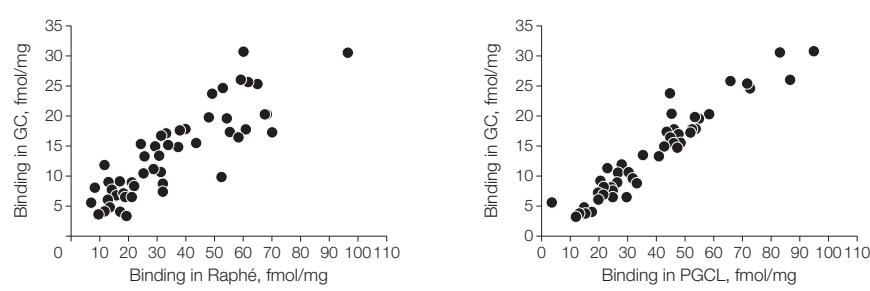
more risk factor, and 88% (36/41) had 2 or more. Ninety-three percent had at least 1 extrinsic risk factor, ie, prone (49%) and side (14%) sleep position, face down (37%), bed sharing (20%), and trivial illness prior to death (44%) (eTable 2). We found no associations between risk factors and 5-HT tissue levels (eTable 3). Significant differ-

**Figure 2.** Age  $\times$  Diagnosis Interaction in the Intermediate Reticular Zone of Sudden Infant Death Syndrome (SIDS) Cases and Controls



The intermediate reticular zone is a key extraraphé component of the medullary serotonin (5-hydroxytryptamine [5-HT]) system that contains 5-HT cell bodies. In this nucleus, 5-HT<sub>1A</sub> receptor binding decreases with age in the SIDS cases but does not change with age in the controls. A, There is a significant difference in the slopes of binding with age in the 2006<sup>11</sup> and 2010 data sets combined for all SIDS cases and controls (37–90 postconceptional weeks) ( $P=.002$ ). Sloped solid lines indicate linear regression fit. B, The significant difference between the SIDS cases and controls persists when the analysis is restricted to cases and controls that overlap in age (37–64 weeks' postconceptional age) ( $P=.004$ ). Sloped solid lines indicate linear regression fit.

**Figure 3.** Positive Correlation of 5-HT<sub>1A</sub> Receptor Binding Levels in the Gigantocellularis (GC) vs the Raphé Obscurus and Paragigantocellularis Lateralis (PGCL) in Sudden Infant Death Syndrome (SIDS) Cases (N=40)



ences were found, however, for 5-HT<sub>1A</sub> receptor binding in the raphé obscurus if the infant with SIDS was found dead in a risky sleep position (47.32 fmol/mg tissue [95% CI, 38.23 to 56.38] for prone or side sleep position vs 26.76 fmol/mg tissue [95% CI, 15.64 to 37.88] for supine position) or in an adult bed (49.06 fmol/mg tissue [95% CI, 34.20 to 63.92] vs 32.76 fmol/mg tissue [95% CI, 21.38 to 44.14] in a crib) (eTable 3). Binding levels were significantly lower if the infant with SIDS did not have the risk factor. In addition, TPH2 levels were lower in the infants with SIDS and with recent illness (165.7% [95% CI, 143.3% to 188.0%]) than those without recent illness (138.0% [95% CI, 126.6% to 149.4%]). In this data set we found no effect for male sex (eTable 3).

## COMMENT

In this article we report the presence of lower levels of medullary 5-HT and TPH2 in infants dying from SIDS, pointing to a deficiency, as opposed to an excess, of 5-HT in the pathogenesis of the disorder. The absence of changes in 5-HIAA levels or neurotransmitter turnover (5-HIAA:5-HT ratio) excludes the possibility of substantial 5-HT degradation and supports reduced 5-HT synthesis. In this data set, we also confirmed 5-HT<sub>1A</sub> receptor binding alterations, although not in the arcuate nucleus, raphé obscurus, or medial accessory olive.<sup>9</sup> There were no differences in SIDS risk factors between the 2006<sup>11</sup> and current data sets that explained this difference, nor were there any obvious differences in the controls to explain the variation in control levels between data sets. While these inconsistencies warrant further analysis, binding differences are remarkably similar in all other nuclei across our data sets and are associated with abnormalities in different parameters of 5-HT function, ie, 5-HT cell density<sup>11</sup> and 5-HT and TPH2 levels.

We also report that 5-HT<sub>1A</sub> binding alterations correlate among components of the medullary 5-HT system in the SIDS cases (and controls), substantiating our concept that the med-

ullary 5-HT system is an interrelated network that modulates respiratory and autonomic functions<sup>3</sup>—a concept likewise increasingly supported by animal data.<sup>3,23-25</sup> We now postulate that SIDS can be viewed as a disorder caused by a defect in 1 or more components of the medullary 5-HT system and that any single case need not express defects in all 5-HT markers simultaneously.

With regard to 5-HT<sub>1A</sub> receptor binding, the consistent finding over 4 data sets of several significant interactions between age and diagnosis warrants mention. Although interpretation is impossible without longitudinal study, the reduced binding in older SIDS cases may reflect a progressive decrease with age in those infants with the “SIDS abnormality.” Alternatively, it may reflect the possibility that infants with a stronger abnormality take longer to outgrow the risk period for SIDS and continue to die at older ages.

In this study, we also asked whether 5-HT abnormalities in infants with SIDS could be explained by hypoxia-ischemia. We did not observe, however, a similar pattern of abnormalities between the SIDS and hospitalized (hypoxia-ischemia) group, suggesting that the primary mechanisms underlying 5-HT abnormalities in SIDS are not mediated by chronic hypoxia-ischemia. A striking difference between the SIDS and hospitalized groups was the association of reduced TPH2 levels with reduced 5-HT levels in the SIDS group compared with unaltered 5-HT levels in the hospitalized group. These findings indicate that the SIDS cases demonstrate a different TPH2:5-HT ratio and that the SIDS profile does not mimic that of the hospitalized group; the basis of this discrepancy is currently unknown.

Catecholaminergic abnormalities in the brainstems of infants with SIDS are controversial, with reports of positive and negative findings using immunocytochemistry and tissue autoradiography.<sup>26-29</sup> Our study does not support a major abnormality in SIDS cases in medullary 5-HT nuclei that receive projec-

tions from rostral catecholaminergic cell bodies in the pons and midbrain.

The finding of at least 1 risk factor in 95% of SIDS cases underscores the importance of risk factors in the pathogenesis of SIDS, even in the era of the recommendation for supine sleep position. The finding of 2 or more risk factors in 88% of SIDS cases further underscores that SIDS results from the simultaneous occurrence of multiple events.<sup>3</sup> Infants with SIDS but without known extrinsic risk factors had significantly lower 5-HT<sub>1A</sub> receptor binding, suggesting that additional risk factors are necessary to precipitate death when the medullary 5-HT system is less compromised.

Three concerns in this study warrant consideration. The first is the possibility of compromised neurotransmitter measurements using HPLC, attributable to prolonged postmortem intervals. Animal models, however, suggest that 5-HT degradation is not significant, at least over a 27-hour postmortem delay, in cerebral cortical sites that receive 5-HT projections.<sup>30</sup> In addition, we made adjustments in this study for postmortem interval in all statistical analyses as warranted. Furthermore, we analyzed brainstem tissues only in infants with relatively short postmortem intervals (<30 hours) and avoided any freeze-thaw procedures. Second, we were unable to measure neurotransmitter levels at the synapse in postmortem tissues. Our data therefore represent combined intracellular and extracellular stores without precise cellular localization. The final concern is the small sample size of the control group, which is an unavoidable reflection of the extraordinary rareness of death as well as autopsies in infants without SIDS who die unexpectedly. Our response was to study all cases in greater depth with different modalities, to compare data from different data sets, and to combine these data when possible.<sup>9-11</sup> Independent investigators have now also reported 5-HT<sub>1A</sub> receptor deficits confirmed in SIDS cases using a different technique, ie, immunocytochemistry, thereby confirming our receptor results.<sup>31,32</sup>

These findings raise the question as to how reduced 5-HT and TPH2 levels are related to the increased 5-HT cell density,<sup>11</sup> morphologic 5-HT neuronal immaturity,<sup>11</sup> reduced 5-HT transporter binding relative to 5-HT cell number,<sup>11</sup> and altered 5-HT receptor binding<sup>9–11</sup> in the SIDS cases. We hypothesize that TPH2 levels are reduced in the medullary 5-HT system for as-yet unknown developmental, genetic, and/or environmental reasons, with a secondary reduction in 5-HT levels and impaired 5-HT neurotransmission.<sup>33</sup> We further propose that insufficient 5-HT levels early in development, potentially as early as the first or second trimester, result in a compensatory increase in immature 5-HT neurons with immature (decreased) 5-HT<sub>1A</sub> binding and 5-HT transporter levels.<sup>34</sup> That the defect is partial rather than total could explain why medullary 5-HT-mediated pathways function reasonably well at baseline or during waking but are unable to respond to homeostatic stressors during sleep when the partial deficit is potentially unmasked, thereby resulting in sudden death. Our data suggest that future animal models mimicking the 5-HT abnormalities of SIDS should focus on under-production, rather than overproduction, of 5-HT and TPH2.

**Author Contributions:** Dr Kinney had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**Drafting of the manuscript:** Duncan, Hoffman, Mokler, Nattie, Kinney.

**Critical revision of the manuscript for important intellectual content:** Duncan, Paterson, Hoffman, Mokler, Borenstein, Belliveau, Krous, Haas, Stanley, Nattie, Trachtenberg, Kinney.

**Statistical analysis:** Trachtenberg.

**Obtained funding:** Duncan, Paterson, Krous, Kinney.

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**Online-Only Material:** The eFigure and eTables 1 through 3 are available at <http://www.jama.com>.

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