Brainstem Deficiency of the 14-3-3 Regulator of Serotonin Synthesis: A Proteomics Analysis in the Sudden Infant Death Syndrome^{*}\(^{5}\)

Kevin G. Broadbelt‡**¶¶, Keith D. Rivera‡, David S. Paterson‡, Jodi R. Duncan‡‡‡, Felicia L. Trachtenberg§, Joao A. Paulo‡**, Martha D. Stapels‡‡, Natalia S. Borenstein‡, Richard A. Belliveau‡, Elisabeth A. Haas¶, Christina Stanley¶, Henry F. Krous¶, Hanno Steen‡**¶¶, and Hannah C. Kinney‡

Impaired brainstem responses to homeostatic challenges during sleep may result in the sudden infant death syndrome (SIDS). Previously we reported a deficiency of serotonin (5-HT) and its key biosynthetic enzyme, tryptophan hydroxylase (TPH2), in SIDS infants in the medullary 5-HT system that modulates homeostatic responses during sleep. Yet, the underlying basis of the TPH2 and 5-HT deficiency is unknown. In this study, we tested the hypothesis that proteomics would uncover previously unrecognized abnormal levels of proteins related to TPH2 and 5-HT regulation in SIDS cases compared with controls, which could provide novel insight into the basis of their deficiency. We first performed a discovery proteomic analysis of the gigantocellularis of the medullary 5-HT system in the same data set with deficiencies of TPH2 and 5-HT levels. Analysis in 6 SIDS cases and 4 controls revealed a 42–75% reduction in abundance in 5 of the 6 isoforms identified of the 14-3-3 signal transduction family, which is known to influence TPH2 activity \((p < 0.07)\). These findings were corroborated in an additional SIDS and control sample using an orthogonal MS\(^{2}\)-based quantitative proteomic strategy. To confirm these proteomics results in a larger data set \((38\ SIDS,\ 11\ controls)\), we applied Western blot analysis in the gigantocellularis and found that 4/7 14-3-3 isoforms identified were significantly reduced in SIDS cases \((p \leq 0.02)\), with a 43% reduction in all 14-3-3 isoforms combined \((p < 0.001)\). Abnormalities in 5-HT and TPH2 levels and 5-HT\(_{\text{IA}}\) receptor binding were associated with the 14-3-3 deficits in the same SIDS cases. These data suggest a potential molecular defect in SIDS related to TPH2 regulation, as 14-3-3 is critical in this process. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.009530, 1–17, 2012.

The sudden infant death syndrome (SIDS)\(^{1}\) is the sudden unexpected death of an infant less than 1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and clinical history (1). It is the leading cause of postneonatal infant mortality in the United States today; with an overall incidence of 0.53/1000 live birth(s) (2). Typically, a seemingly healthy infant is found dead after a sleep period, either in the early morning or after a day-time nap (1, 3, 4). Impaired brainstem responses to homeostatic challenges during sleep may result in the sleep-related sudden death characteristic of SIDS (4). We have reported various abnormalities in serotonergic (5-HT) receptors, 5-HT transporter, and 5-HT cellular maturation in the medullary 5-HT system in SIDS cases in three independent data sets over the last decade (5–7). This system within the medulla oblongata is a neural network comprised of 5-HT source neurons and their projection sites that help mediate homeostatic responses during the sleep-wake cycle (3). The medullary 5-HT system is defined by us as the regions of the medulla that contain 5-HT cell bodies and effector nuclei that receive 5-HT projections (3, 8). The 5-HT source cell bodies are present in the raphé (raphé obscurus, raphé pallidus, and raphé magnus), extra-raphé (paragigantocellularis lateralis, gigantocellularis, intermediate reticular zone, subtrigeminalis, and lateral reticular nucleus), and ventral surface (embedded within the arcuate nucleus) (8). The effector nuclei include the hypoglossal nu-

---

\(^{1}\) The abbreviations used are: ANCOVA, Analysis of covariance; 5-HT, 5-hydroxytryptamine (serotonin); HPLC, High performance liquid chromatography; FITC, Fluorescein isothiocyanate; PCA, Post-conceptual age; SIDS, Sudden infant death syndrome; TPH2, Tryptophan hydroxylase 2; FD, False discovery rate; PMI, Postmortem interval; TRITC, Tetramethyl rhodamine iso-thiocyanate; CE, Collision energy; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.
Brainstem 14-3-3 Deficiency in SIDS

cellus, nucleus of the solitary tract, and dorsal motor nucleus of the vagus (8). Recently, we reported in a fourth independent data set (Data set 4) a reduction in the levels of 5-HT and tryptophan hydroxylase (TPH2), the key biosynthetic 5-HT enzyme, in the medullary 5-HT system in SIDS cases compared with controls (9). These 5-HT and TPH2 abnormalities were associated in the same SIDS cases with 5-HT receptor abnormalities similar to those reported in SIDS cases in the first three data sets (5–7). The new finding in Data set 4 of a deficiency in TPH2 followed presumably by impaired 5-HT synthesis may be the “key” defect that leads to a cascade of changes in related 5-HT parameters.

In order to address the question of the potential cause(s) of the TPH2 and/or 5-HT deficiency in SIDS infants, we next decided to perform a discovery mass spectrometry-based proteomics screen to determine potential alterations in the abundance levels of proteins critical for TPH2 regulation and/or other proteins otherwise involved in 5-HT regulation. We thus applied two orthogonal mass spectrometry-based proteome analyses of the medullary 5-HT system in Data set 4 using the same medulla specimens in which we analyzed the 5-HT-related parameters. In the following study, we tested the hypothesis that proteomics would uncover proteins related to TPH2 and 5-HT regulation that could provide novel insight into the basis of the medullary 5-HT abnormalities in SIDS. We chose the gigantocellularis for study because it is a key component of the medullary 5-HT system (8), and SIDS cases consistently demonstrate altered 5-HT receptor binding within it (5–7, 9), including in Data set 4 (9). This analysis was performed in three sequential stages. In Stage 1, we first performed GeLC/MS-based proteomics and spectral counting in a pilot subset of SIDS cases (n = 6) and controls (n = 4) randomly selected from Data set 4. The findings of this analysis were then corroborated by analyzing one additional SIDS case and one control, likewise randomly selected from the Data set 4, with the MS/MS strategy (10). In this initial discovery stage, we found a down-regulation of 5 of the 6 isoforms identified in the 14-3-3 protein family in the SIDS cases compared with controls. In Stage 2, we verified the proteomics findings with Western blotting in precisely the same SIDS cases (n = 33) and acute controls (n = 5) in which 5-HT-related analysis was previously performed by us in Data set 4 (9). In Stage 3, we used analyzed Western blot data from an expanded Data set 4 with 5 additional SIDS cases and 3 acute controls accrued after the closure of the original Data set 4. We also analyzed 3 controls with chronic illness, so-called “chronic controls,” for a total of 38 SIDS cases and 11 (acute and chronic) controls combined. The rationale for this approach is based upon the goal of increasing the sample size of the controls in the Western blot analysis, and thereby increasing statistical power involving the rare human infant tissue samples that are extraordinarily difficult to obtain in a reasonable time-frame. Using immunocytochemical methods, we determined the regional and cellular localization in the infant medulla of 14-3-3γ, a major isoform with a significant reduction in abundance levels by Western blotting, in the SIDS cases. Given that the signal transduction 14-3-3 proteins are involved in the regulation of TPH2, we propose that the 14-3-3 deficits in the SIDS cases lead to or contribute to the reduced TPH2 and 5-HT levels, potentially bringing us closer to the molecular defect in the medullary 5-HT system in sudden infant death.

MATERIALS AND METHODS
Clinical Database for Stages 1–3—A total of 49 SIDS cases and controls were analyzed in Stages 1, 2, and 3 (Table I). SIDS cases were defined as the sudden unexpected death of an infant less than 1 year of age, with onset of the fatal episode apparently occurring during sleep that remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and clinical history (1). Acute controls were infants less than 12 months of age who died unexpectedly (in some instances with a minor or acute illness within 48 h of death) in whom an autopsy and death scene investigation established a cause of death (5–7). Chronic controls were infants less than 12 months of age who had clinical chronic illnesses but who nevertheless died suddenly and unexpectedly; a complete autopsy confirmed the chronic illness. Acute controls were comprised of infants with subclinical congenital heart disease (n = 3) and accidents (n = 5) (Table I). Chronic controls were comprised of infants with congenital myopathy (n = 1), acute intestinal obstruction secondary to duodenal stricture complicating a congenital syndrome with absent thumbs, hypoplasia, and plagiocephaly (n = 1), and acute infantile febrile illness complicating neonatal repair of gastroschisis and failure to thrive (n = 1) (Table I). All medullary tissues were obtained under the auspices of the Office of the Chief Medical Examiner system, San Diego, CA, in accordance to California law Chapter 955, Statutes of 1989 (SB1069), and the San Diego Research Project, which permit the use of autopsy tissues from infants with sudden death for research without direct parental permission. Biochemical analyses were performed blinded, i.e. the investigator was not informed about diagnosis, age, and all other clin- icopathologic variables recorded upon review of autopsy and death scene investigation reports. The classification of the cause of death in each case was performed in a standardized fashion under the auspice of the San Diego Research Project. This study was approved by the Committee on Clinical Investigation at Children’s Hospital Boston, MA.

Tissue Preparation for Mass Spectrometry (Stage 1) and Western Blot Analysis (Stages 2 and 3)—The anatomic focus of this study was upon the gigantocellularis, a nucleus located within the medial reticular formation of the rostral medulla that contains 5-HT neurons and is a key component of the medullary 5-HT system (8). At autopsy fresh brainstem tissue was collected and stored at −80 °C. Using a cold plate, two 3 mm thick blocks of medullary tissue were collected at 2 levels in reference to the human brainstem atlas of Olszewski and Baxter (11), (1) mid-medulla, level of nucleus of Roller (Plate X); and (2) rostral medulla, level of the nucleus prepositus and gigantocellularis (Plate XII). At these sites, the location of the raphé nuclei was classified according to the atlas of Paxinos and Huang (12). Tissue samples were collected from the gigantocellularis of each specimen using a 2-mm micropunch (Harris Uni-core, Electron Microscopy Sciences, Hatfield, PA) (Fig. 1). One micropunch (wet weight ∼ 20 mg) from each specimen was homogenized in standard 1X RIPA buffer with protease inhibitors. Protein concentrations of the homogenates were obtained using a Bio-Rad Protein Assay. The same homogenates were used for the mass spectrometry-based proteomics and Western blotting (Fig. 1). Tissue homogenates of the micropunched
**Table I**

Clinicopathologic data for all SIDS cases and controls analyzed in Stages 1–3. Legend: Preterm, gestational age at birth <37 weeks; M, male; F, Female; ** SIDS and acute controls added to the dataset after the closure of the original Dataset 4 to form the expanded Dataset; † SIDS cases and controls from Dataset 4 analyzed using GELC-MS/MS approach; ‡ SIDS case and control 4 analyzed using the LC-MS<sup>e</sup>-approach.

Abbreviations: N/A, not available (no information available on parameter); PMI, postmortem interval; PCA, postconceptional age

| Case | Diagnosis        | PCA (Weeks) | PMI (Hours) | Race/Ethnicity | Gender | Preterm | Known cause of death                      |
|------|------------------|-------------|-------------|----------------|--------|---------|-------------------------------------------|
| 1<sup>¥</sup> | SIDS             | 50          | 18          | White          | M      | No      | –                                         |
| 2    | SIDS             | 51          | 22          | White          | F      | Yes     | –                                         |
| 3<sup>¥</sup> | SIDS             | 51          | 20          | Hispanic       | M      | No      | –                                         |
| 4    | SIDS             | 56          | 21          | White          | M      | Yes     | –                                         |
| 5<sup>¥</sup> | SIDS             | 67          | 20          | African American | M     | Yes     | –                                         |
| 6    | SIDS             | 55          | 16          | White          | M      | No      | –                                         |
| 7    | SIDS             | 44          | 21          | Other          | M      | No      | –                                         |
| 8<sup>¥</sup> | SIDS             | 49          | 21          | White          | M      | Yes     | –                                         |
| 9    | SIDS             | 51          | 10          | Hispanic       | F      | No      | –                                         |
| 10   | SIDS             | 69          | 20          | Hispanic       | M      | Yes     | –                                         |
| 11<sup>¥</sup> | SIDS             | 58          | 19          | African American | M     | No      | –                                         |
| 12   | SIDS             | 50          | 21          | White          | M      | No      | –                                         |
| 13   | SIDS             | 60          | 20          | White          | F      | No      | –                                         |
| 14   | SIDS             | 43          | 14          | N/A            | F      | No      | –                                         |
| 15   | SIDS             | 44          | 20          | White          | F      | Yes     | –                                         |
| 16   | SIDS             | 48          | 30          | Hispanic       | M      | No      | –                                         |
| 17   | SIDS             | 52          | 12          | Hispanic       | F      | No      | –                                         |
| 18   | SIDS             | 41          | 14          | White          | M      | Yes     | –                                         |
| 19   | SIDS             | 48          | 19          | Hispanic       | M      | No      | –                                         |
| 20   | SIDS             | 64          | 23          | Other          | F      | No      | –                                         |
| 21   | SIDS             | 55          | 17          | Hispanic       | F      | No      | –                                         |
| 22<sup>†</sup> | SIDS             | 59          | 30          | Hispanic       | M      | No      | –                                         |
| 23   | SIDS             | 53          | 17          | White          | F      | No      | –                                         |
| 24   | SIDS             | 48          | 25          | Hispanic       | F      | No      | –                                         |
| 25   | SIDS             | 49          | 27          | Other          | M      | No      | –                                         |
| 26   | SIDS             | 52          | 12          | White          | F      | No      | –                                         |
| 27   | SIDS             | 51          | 20          | Other          | F      | No      | –                                         |
| 28   | SIDS             | 66          | 21          | Hispanic       | M      | No      | –                                         |
| 29   | SIDS             | 76          | 16          | N/A            | F      | No      | –                                         |
| 30<sup>¥</sup> | SIDS             | 52          | 22          | Hispanic       | M      | No      | –                                         |
| 31   | SIDS             | 38          | 8           | White          | F      | Yes     | –                                         |
| 32   | SIDS             | 51          | 21          | Hispanic       | F      | No      | –                                         |
| 33   | SIDS             | 51          | 10          | N/A            | F      | No      | –                                         |
| 34<sup>**</sup> | SIDS             | 48          | 7           | White          | M      | No      | –                                         |
| 35<sup>**</sup> | SIDS             | 45          | 17          | Asian          | F      | No      | –                                         |
| 36<sup>**</sup> | SIDS             | 56          | 9           | Asian          | M      | No      | –                                         |
| 37<sup>**</sup> | SIDS             | 43          | 14          | African American | F    | Yes     | –                                         |
| 38<sup>**</sup> | SIDS             | 52          | 18          | Hispanic       | F      | No      | –                                         |
| 39<sup>¥</sup> | Acute Control    | 39          | 15          | White          | F      | No      | Subclinical congenital heart disease       |
| 40<sup>¥</sup> | Acute Control    | 41          | 10          | Hispanic       | M      | No      | Subclinical congenital heart disease       |
| 41<sup>¥</sup> | Acute Control    | 57          | 10          | Hispanic       | F      | No      | Subclinical congenital heart disease       |
| 42<sup>¥</sup> | Acute Control    | 40          | 16          | N/A            | M      | No      | Accident                                  |
| 43<sup>¥</sup> | Acute Control    | 64          | 18          | N/A            | M      | No      | Accident                                  |
| 44<sup>¥</sup> | Acute Control    | 48          | 13          | Asian          | M      | No      | Accident                                  |
| 45<sup>**</sup> | Acute Control    | 64          | 8           | N/A            | F      | Yes     | Accident                                  |
| 46<sup>**</sup> | Acute Control    | 52          | 13          | White          | F      | No      | Accident                                  |
| 47   | Chronic Illness  | 76          | 20          | Hispanic       | M      | N/A     | Congenital myopathy                       |
| 48   | Chronic Illness  | 53          | 18          | White          | M      | No      | Acute intestinal obstruction secondary      |
|      |                  |             |             |                |        |         | to duodenal stricture complicating a        |
|      |                  |             |             |                |        |         | congenital syndrome, hypospadias           |
|      |                  |             |             |                |        |         | and plagiocephaly                          |
| 49   | Chronic Illness  | 46          | 23          | White          | F      | No      | Acute infantile febrile illness            |
|      |                  |             |             |                |        |         | complicating neonatal repair of             |
|      |                  |             |             |                |        |         | gastrochisis and failure to strive         |

*Brainstem 14-3-3 Deficiency in SIDS*

_Molecular & Cellular Proteomics_ 11.1 10.1074/mcp.M111.009530–3
raphe obscurus and paragigantocellularis lateralis at the same levels from the same SIDS cases and controls were used for the previously reported measurements of 5-HT and TPH2 (9). Alternate blocks from the medulla were used to obtain tissue sections for quantitative autoradiography to measure 5-HT_{1A} receptor binding in 10 nuclei (9). The methods of high performance liquid chromatography (HPLC) for 5-HT levels, Western blotting for TPH2 levels and tissue autoradiography for 5-HT_{1A} receptor binding applied in Data set 4 are previously described in detail (9).

**Proteomic Analysis for Discovery Stage 1—GeLC-MS/MS Method.**

In the SDS-PAGE electrophoresis-trypsin digestion-liquid chromatography and tandem mass spectrometry (GeLC-MS/MS) experiment, we loaded 5% of a micropunch (3 mm thick; 1 mm radius) lysate onto a 4–12% SDS-PAGE gel (NuPage, Invitrogen). The gel was stained with Coomassie blue (SimplyBlue Safestain, Invitrogen), and each lane was sectioned into six blocks of same size and positioning; the blocks were separately digested with trypsin using standard in gel digestion protocols (13). The peptides were analyzed on an...
LTOQ mass spectrometer connected to a nanoscale capillary HPLC system (microautosampler and Surveyor HPLC pump; all: Thermo Scientific). Peptides were loaded onto an in-house packed 100 μm i.d. × 15 cm C18 column (Magic C18, 5 μm, 200Å, Michrom Biore-source) and separated at ~400 nL/min (linear gradients from 5 to 35% acetonitrile in 0.4% formic acid). The ion trap was operated in a data-dependent acquisition mode, fragmenting up to six of the most intensive ions after each survey scan. The Thermo “.raw” files were converted into the Mascot generic format (mgf) by extracting the 200 most intense fragment ions for each MS/MS spectrum (14).

Database Searching—All MS/MS data were analyzed using Mascot (version 2.3, Matrix Science, London, UK) and X! Tandem (The GPM, thegpm.org; version 2007.01.01.1). X! Tandem was set up to search a subset of the iPlaG database (version 3.69 concatenated with the reversed protein sequences) defining trypsin as protease used. The Mascot searches were done using the same concatenated ipi.HUMAN database (version 3.69, 174260 entries) also defining trypsin as protease. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 1.5 Da. Carbamidomethylated cysteine was specified in Mascot and X! Tandem as a fixed modification. N-terminal pyroglutamic acid formation from E and S-carbamoylmethylcysteine cyclization, as well as methionine oxidation, was specified in Mascot as variable modifications. In X! Tandem, N-terminal pyroglutamic acid formation from E and S-carbamoylmethylcysteine cyclization, as well as methionine oxidation, aspiragine and glutamine deamidation, and acylcarnitide adduct of cysteine, were specified as variable modifications.

Criteria for Protein Identification and Spectral Counting-based Quantification—Scaffold (version 3.1.2, Proteome Software Inc., Portland, OR) was used to validate and group MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they had a greater than 65.0% probability, as specified by the Peptide Prophet algorithm (15). Protein identifications were accepted if established at greater than 50.0% probability and if they contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (16). These values resulted in an overall peptide false discovery rate (FDR) of 0.4% and protein FDR of 3.9%. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The subsequent spectral counting-based quantification utilized the Scaffold output.

The subsequent spectral counting-based quantification utilized the Scaffold output. The parameters were set to “Number of Unique Spectra,” “T-Test” (two-tailed), and “Use Normalization.” Scaffold normalized the MS/MS data between samples to allow for the comparison of abundances between samples. The sum of the “Unweighted Spectrum Counts” for each MS sample was used for the normalization resulting in a normalized “Quantitative Value.” This normalization accounted for any differences in loading and/or sampling. A difference between SIDS and control samples was considered to be significant at a p-value <0.10 in this phase (Stage 1) in order to obtain a wide range of proteins for consideration at discovery.

MS²-based Proteomics Experiment—A nanoACQUITY UPLC system (Waters Corp, Milford, MA) was employed to perform nanoflow separations. One acute control and one SIDS case from Data set 4 not analyzed in the previous GelC-MS/MS based experiment were used. Each sample was analyzed in triplicate and the value expressed as the mean ± standard deviation. The mobile phase A was water with 0.1% formic acid whereas mobile phase B was 0.1% formic acid in acetonitrile. A 75 μm by 150 mm analytical column packed with 1.8 μm HSS T3 C18 (Waters Corp., Milford, MA) was used to separate peptides with a 90 min gradient from 5 to 40% B at 500 nl/min. Glu-fibrinopeptide was used as the lock mass.

A Synapt G2 HDMS (Waters Corp., Milford, MA) was used to detect peptides as they eluted from the nanoLC column. The Synapt was operated in V mode with a mass resolution of 20,000 with ion mobility enabled. Alternating scans were used to detect precursor ions and then fragment ions. The masses of the precursors were detected with a 0.5 s scan with the relatively low collision energy (CE) of 4 eV. This scan was followed by a 0.5 s scan during which the CE was ramped from 27 to 50 eV (10). These data were analyzed with ProteinLynx Global Server (PLGS; version 2.5; Waters Corp). A human database containing 20,260 human protein sequences (SwissProt and TrEMBL) and an equal number of randomized sequences was used for the peptide identification searches. Error tolerances of 10 and 20 ppm of the theoretical masses were allowed for the precursor and fragment ion masses, respectively. Each protein was identified in at least two of three replicate samples. Score thresholds were established that resulted in a peptide and protein level FDR of 0.23% and 0.24%, respectively.

Western Blotting Analysis for 14-3-3 Isoforms in Stages 2 and 3—Given that the proteomic analysis demonstrated a decrease in 14-3-3 isoforms, we next tested the specific hypothesis that the Western blot analysis would demonstrate a decrease in the levels of the same 14-3-3 isoforms shown to be decreased in abundance with mass spectroscopy (Stage 1). Tissue samples of the gigantocellularis were homogenized and a final concentration of 25 μg was obtained using a modified Lowry method for protein quantification. After separation with SDS-PAGE, proteins were transferred electrophoretically to an Immobilon-P membrane (Millipore, Bedford, MA) overnight and incubated with a rabbit polyclonal anti-14-3-3 antibody (seven antibodies, specific to each Isoform at 1:3000; ABCAM, Cambridge, MA) (Table II). The different 14-3-3 isoforms were detected using a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:8000; Bio-Rad, Hercules, CA) followed by Chemiluminescence ECL (Perkin Elmer, Waltham, MA), and quantified from densitometry bands (MCID Elite 6; GE Life Sciences, Piscataway, NJ) standardized to human adult gigantocellularis tissue run simultaneously on the same gel. The blots were digitized using an MCID Elite 6 imaging system (GE Life Sciences, Piscataway, NJ); values were obtained from densitometry bands that were standardized to human adult gigantocellularis micropunch run on the each individual gel (17–21). The abundance levels were expressed as a percentage of an adult standard co-analyzed on every SDS-PAGE gel. We blotted for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading and normalization control.

Immunocytochemistry with an Antibody to the 14-3-3γ Isoform in the Stage 3 Data set—Because of the analysis of multiple tissue markers in the SIDS cases and controls in Data set 4, limited tissue remained for immunocytochemical analysis. For double label immunocytochemistry, tissue sections were incubated with an antibody to the 14-3-3γ Isoform (Table II) and an antibody to TPH2 using the PH8 antibody to identify 5-HT neurons (Chemicon International, Temecula, CA) (22). Fluorescence was detected with Alexa Fluor donkey anti-rabbit 594 and Alexa Fluor donkey anti-goat or donkey anti-mouse 488 (1:1000; Molecular Probes, Eugene, OR). Negative controls omitted the primary antibodies. Immunofluorescence was visualized with an Olympus BX51 microscope (Olympus America Inc., Melville, NY) using FITC and TRITC filters with image capture using a Coolsnaps fx camera (Photometrics, Tucson, AZ) and MCID Elite 6.0 software. Immunofluorescent images were captured, and imported into Photshop 6.0 (Adobe Systems, San Jose, CA) where they were analyzed and appropriate labels and scale bars were added. Single label immunocytochemistry was performed to determine if the distribution of 14-3-3γ was altered in SIDS cases compared with controls in the medullary 5-HT system. Frozen slide mounted sections were dried for 45–50 min at room temperature; they were then fixed.
| Antibody | Host         | Source/cat #   | Immunogen                                                                 | Dilution  | References                                                                 |
|----------|-------------|----------------|----------------------------------------------------------------------------|-----------|-----------------------------------------------------------------------------|
| 14-3-3  | Rabbit Polyclonal IgG | Abcam, Cat # ab69592 | Synthetic peptide derived from internal of human 14-3-3 gamma              | 1:3000 (WB) 1:250 (ICC) | Rajagopalan S et al. Mechanistic differences in the transcripational activation of p53 by 14-3-3 isoforms. Nucleic Acids Res 38: 893–906 (2010). |
| Sigma    | Rabbit Polyclonal IgG | Abcam, Cat # ab60311 | Synthetic peptide derived from internal of human 14-3-3 sigma              | 1:3000 (WB) 1:250 (ICC) |                                                                                     |
| Eta      | Rabbit Polyclonal IgG | Abcam, Cat # ab79163 | Synthetic peptide from the N terminal area of Sheep 14-3-3 eta            | 1:3000 (WB) 1:250 (ICC) |                                                                                     |
| Zeta     | Rabbit Polyclonal IgG | Abcam, Cat # ab63635 | Synthetic non-phosphopeptide derived from human 14-3-3 zeta + delta around the phosphorylation site of threonine 232 (S-d-T-P-Q-) | 1:3000 (WB) 1:250 (ICC) |                                                                                     |
| Tau      | Rabbit Monoclonal IgG | Abcam, Cat # ab10439 | Recombinant full length protein recognizes tau isoform. The antibody does not react with the 14-3-3 zeta isoform | 1:3000 (WB) 1:250 (ICC) | Liang S et al. Quantitative protein expression profiling of 14-3-3 isoforms in human renal carcinoma shows 14-3-3 epsilon is involved in limitedly increasing renal cell proliferation. Electrophoresis 30: 4152–62 (2009). |
| Epsilon  | Mouse Monoclonal IgG  | Abcam, Cat # ab71506 | Recombinant full length protein (Human) purified from E. coli              | 1:3000 (WB) 1:250 (ICC) |                                                                                     |
| Beta     | Rabbit Monoclonal IgG | Abcam Cat # ab97273 | Recombinant protein fragment corresponding to a region within amino acids 1–141 of 14-3-3 beta (AAH01359) | 1:3000 (WB) 1:250 (ICC) |                                                                                     |
| PH8 for TPH2 | Mouse Monoclonal IgG | Millipore Cat # MAB5278 | Common epitope of tryptophan hydroxylase (TPOH), tyrosine hydroxylase and phenylalanine hydroxylase, In fixed human tissue PH8 binds specifically to TPOH due to antigen changes induced by fixation | 1:1000 (ICC) | Haan et al., 1987; Baker et al., 1991. |
in 4% paraformaldehyde for 10 min. Sections were incubated with a 3% hydrogen peroxide solution with methanol as the solvent to block endogenous peroxidase activity. We identified 14-3-3-3 expression in tissue sections using commercially available antibodies to the different 14-3-3-3 isoforms (Table II). To block nonspecific immunostaining, the slides were placed in a solution containing 4% normal goat serum and 0.1% triton x-100, in 1 × phosphate buffered saline for 1 h. After 2 washes (5 min each) in 1 × phosphate buffered saline-0.1% Tween, sections were incubated with individual antibodies at various dilutions overnight at 4 °C. Sections were next incubated in Biotinylated antirabbit IgG affinity purified antibody (Vector Laboratories, Burlingame, CA) at 1:500 dilutions in the blocking solution for 30 min at room temperature. This was followed by incubation with Elite ABC Reagent (Vector Laboratories). Immunoreactivity was detected using a 3, 3′ Diaminobenzidine peroxidase substrate kit (Vector laboratories). Sections were then washed in water, dehydrated, and cover slipped. The stained slides were examined with an Olympus BX51 microscope (Olympus America) with image capture using an Optronics Microfire S99808 camera and Microfire 1.0 and Neurolucida 5.0 software (Microbrightfield, Colchester, VT).

Risk Factor Analysis in the SIDS Cases of the SIDS Cases in the Stage 3 Data Set—We analyzed 14-3-3-3 isoform abundance in the Stage 3 Data set relative to the known risk factors for SIDS. The objective was to determine if the abnormal isoform levels correlated with one or more risk factors, thereby suggesting that the risk factor influenced the probability of SIDS via a 14-3-3-3-related mechanism. We subdivided SIDS risk factors into “extrinsic” and “intrinsic” categories: extrinsic factors are considered to be physical stressors, e.g. prone sleep position, that place a vulnerable infant at risk for asphyxia, hypoxia, hypercarbia, or other homeostatic derangements at the time of death; intrinsic factors, on the other hand, are thought to influence the underlying pathophysiological process (vulnerability) in the infant and include such factors as prematurity and male gender (3, 7, 9). T-tests were used to test for differences by risk factors in SIDS cases in the Stage 3 data set.

Statistical Analysis for Stages 2–3—T-tests were used to compare age and postmortem interval between SIDS cases and controls, with Fisher exact tests used to compare sex and race. Analysis of covariance (ANCOVA) was used to test for differences between SIDS cases and controls while controlling for the potential effects of postconceptional age as well as postnatal age. ANCOVA was used to test the potential effects of postconceptional age on 14-3-3-3 isoform levels determined via Western blotting. To compare the 14-3-3-3 family between SIDS cases and controls, a repeated measures ANCOVA model, with multiple isoforms per case, controlling for postconceptional age and isoform, was used. Potential interactions between diagnosis (SIDS versus controls) and age were also considered. Adjustments for postmortem interval in all statistical analyses of the biochemical data were performed, but were never significant, suggesting that the differences in postmortem interval did not affect the outcome measures. In Stage 2, the abundance levels of the four 14-3-3-3 isoforms with significant reductions upon Western blotting in the SIDS cases compared with controls were correlated with measures of previously determined 5-HT markers (5-HT_{1A} receptor binding, and 5-HT and TPH2 levels) (9) the correlations were controlled for postconceptional age (partial correlation). Unless otherwise noted, p < 0.05 was considered statistically significant.

RESULTS

Stage 1: Discovery Proteomic Analysis of the Gigantocellularis—We first performed a GelC-MS/MS analysis of the gigantocellularis micropunches from six randomly selected SIDS cases and four controls from Data set 4 (Table I). There was no significant difference in postconceptional age (gestational plus postnatal age) between SIDS cases (53.5 ± 7.9 weeks) and the acute controls (50.4 ± 12.1 week) (p = 0.63) or in gestational or postnatal age between the two groups (data not shown). The SIDS cases had a significantly longer postmortem interval compared with the acute controls (SIDS, 19.0 ± 1.3 h; acute controls, 14.0 ± 3.9 h) (p = 0.04), and adjustments for the postmortem interval were made in the statistical analysis.

We assigned an average of 2364 (±1230) spectra in the 6 SIDS samples versus 2662 (±446) spectra in the 4 acute control samples at an overall spectral/peptide FDR of 0.4%. The average numbers of the unique peptides and proteins were 1054 ± 529 (SIDS) versus 1111 ± 172 (controls) and 254 ± 81 (SIDS) versus 280 ± 23 (controls), respectively. No statistically significant differences (two-tailed p-value) were found in the overall numbers of identified spectra, peptides and/or proteins between the SIDS and the acute controls. Combining the spectra results of the sample analysis from all 6 cases and 4 acute controls, we identified a total of 502 proteins in the gigantocellularis with a total protein FDR of 3.9%. Thirteen percent (63/502) of the identified proteins differed in peptide abundance levels between the SIDS cases and controls (p < 0.10), as determined by the method of spectral counting (23). The 63 proteins that were different between the SIDS cases and controls (p < 0.10) were annotated according to their main biologic function using the Gene Ontology tool in Scaffold. The proteins with differential abundance levels were classified in the order of decreasing proportion: metabolic—32% (n = 20); cytoskeletal—22% (n = 14); signal transduction—21% (n = 13); miscellaneous—14% (n = 9); synaptic—8% (n = 5); and vesicular-mediated transport—3% (n = 2).

Within the signal transduction category, 38% (5/13) of the proteins belonged to the 14-3-3-3 protein family, and six of the seven known 14-3-3-3 isoforms (γ, ζ, ε, θ, β, and η, but not α) were identified by mass spectrometry. Spectral count analysis revealed that the levels of 5 of these 6 identified 14-3-3-3 isoforms (γ, θ, η, ζ, and β) were reduced by 42–75% in the SIDS cases compared with controls (p < 0.02), with four isoforms showing the most significant changes (p < 0.002) (Table III). Upon combining the spectral counts of all 14-3-3-3 isoforms identified, there was a significant 68% reduction in the SIDS cases compared with acute controls (p = 0.001) (Table III).

To confirm this initial finding of reduced abundance of 14-3-3-3 proteins in the gigantocellularis of SIDS cases compared with acute controls by a second mass spectroscopy method, we randomly selected one SIDS case and one control from Data set 4 to be analyzed using the LC-MS®-approach (Table IV). This analysis identified a total of 413 proteins with 0.23% FDR at the peptide level and 0.24% FDR at the protein level. This MS®-experiment identified the same six 14-3-3-3 isoforms (β, ε, η, γ, θ, and ζ, and also not α) as the GeLC-MS/MS experiment (Tables III and IV). Analyzing each
Brainstem 14-3-3 Deficiency in SIDS

**Table III**

| 14-3-3 Isoforms | Mass Spectrometry | SIDS (n = 6) | % Decrease in SIDS* | p value** |
|-----------------|-------------------|-------------|---------------------|-----------|
| Gamma           | 6.3 ± 1.7         | 1.2 ± 0.5   | 75%                 | <0.001    |
| Eta             | 1.3 ± 0.5         | 0.5 ± 0.5   | 75%                 | <0.001    |
| Beta            | 2.5 ± 0.6         | 1 ± 0.4     | 75%                 | 0.002     |
| Theta           | 1.3 ± 0.3         | 0.8 ± 0.6   | 72%                 | 0.002     |
| Zeta            | 8.5 ± 2.2         | 4.0 ± 1.5   | 42%                 | 0.068     |
| Epsilon         | 2.5 ± 0.6         | 3.2 ± 1.7   | 31%                 | 0.380     |
| Sigma           | N/I               | N/I         | N/I                 | –         |
| 14-3-3 Family   | 22.3 ± 5.0        | 10.3 ± 5.0  | 68%                 | 0.001     |

**Table IV**

| 14-3-3 Isoforms | Control (n = 1) | SIDS (n = 1) | % Decrease |
|-----------------|-----------------|-------------|------------|
| Gamma           | 186.1 ± 37.0    | 97.4 ± 15.0 | 48%        |
| Eta             | 48.0 ± 3.5      | 4.3 ± 3.0   | 91%        |
| Beta            | 128.0 ± 21.0    | 56.2 ± 10.0 | 56%        |
| Theta           | 134.7 ± 13.0    | 67.5 ± 47.0 | 50%        |
| Zeta            | 259.3 ± 15.0    | 163.3 ± 15.0| 36%        |
| Epsilon         | 205.2 ± 20.0    | 160.1 ± 10.0| 22%        |
| Sigma           | N/I             | N/I         | N/I        |
| 14-3-3 Family   | 962.3 ± 40.0    | 551.8 ± 84.0| 43%        |

In all subsequent analyses in this study, we chose to focus upon the 14-3-3 family of signal transduction molecules because: (1) its isoforms were highly enriched in the 21% subset of signal transduction proteins, which showed altered protein abundance levels in the SIDS cases compared with controls (p < 0.10); (2) the finding was confirmed by an orthogonal mass spectrometry-based quantitative proteomics strategy; (3) the magnitude of the difference in selected and combined 14-3-3 protein levels was statistically robust; and (4) there is a known critical link between 14-3-3 and TPH2 levels (see below), thus confirming our a priori hypothesis that proteomics would provide fresh insight into the basis of the medullary TPH2 deficiency in SIDS. The analysis of the other proteins different between SIDS cases and controls, e.g. metabolic, cytoskeletal, and synaptic proteins, is under a separate ongoing study by us and is beyond the scope of the present study.

**Stage 2: Western Blotting Analysis of the 14-3-3 Isoforms in the Original Data Set 4** — We next sought to confirm the mass spectrometry findings related to the 14-3-3 isoforms in SIDS utilizing Western blotting for determination of protein abundance levels. We tested the hypothesis that there are reduced abundance levels of the 14-3-3 isoforms, individually or in combination, in the gigantocellularis of SIDS cases compared with controls in the original Data set 4 in which 5-HT-related abnormalities were found in the SIDS cases (9). There was no significant difference in postconceptional age (gestational plus postnatal age) between SIDS cases (52.8 ± 8.4 weeks) and the acute controls (48.3 ± 11.4 weeks) (p = 0.28) or in gestational or postnatal age between the two groups (data not shown) (Table I). There also was no significant difference in the mean postmortem interval between the SIDS cases (18.8 ± 5.4 h) and acute controls (13.8 ± 3.6 h) (p > 0.06). Using 14-3-3 isofrom specific antibodies in the Western blots (Table II), we found a significant reduction in protein levels in the gigantocellularis in four of seven isoforms of the 14-3-3 family of proteins (β, γ, η, and ζ) in SIDS cases (n = 33) compared with the acute controls (n = 5) adjusted for postconceptional age (p < 0.02) (Table V; Fig. 2). There was, for example, a 64% reduction in the 14-3-3β isofrom in the SIDS cases (38.8 ± 6.9%) compared with the acute controls (107.9 ± 18.1%) (p = 0.001), and a 51% reduction in the 14-3-3γ isofrom in the SIDS cases (51.5 ± 2.1%) compared with controls (104.3 ± 5.5%) (p < 0.001) (Table V). There was also a 43% reduction in all 14-3-3 isofroms combined (p < 0.001) in the SIDS cases (Table V). Thus, in four separate analyses using two different proteomics methods (Tables III and IV) and Western blotting (Tables V and VII), we found a substantial reduction in 14-3-3 isofroms overall in the gigantocellularis in the SIDS cases compared with controls (Table VIII). In all four analyses, there were consistently significant reductions in the same three of the known seven isoforms, i.e. γ, β, and η (Table VIII). For the other four isoforms, i.e. θ, ζ, ε, and σ, the results were varied among the proteomics and Western blotting methods (Table VIII). This variability in the results likely reflects the small sample sizes in the exploratory proteomics analyses. Nevertheless, in all three analyses in which statistical comparisons were appropriate (Tables III, V, and VII), the levels of all identified isoforms combined were significantly lower (p < 0.001)
**Brainstem 14-3-3 Deficiency in SIDS**

**Summary of 14-3-3 isoform levels and selected 5-HT parameters** in the SIDS cases and controls of the original Dataset 4 (Stage 2). Legend: 'The western blotting (SIDS; n = 33, Controls; n = 5) values in Stage 2 are protein levels expressed as percent of an adult human standard ± standard error of the age-adjusted mean; the standard was developed in the laboratory according to published procedures (18, 20, 54). † ANCOVA controlling for age for each individual isoform; repeated measures ANCOVA, with multiple isoforms per case, controlling for PCA and isoform for the 14-3-3 family. ‡ Reiteration of measurements of selected 5-HT parameters in Dataset 4 previously published by us, 3 standard error of the age-adjusted mean; the standard was developed in the laboratory according to published procedures (18, 20, 54). *The western blotting (SIDS; n = 33, Controls; n = 5) values in Stage 2 are protein levels expressed as percent of an adult human standard ± standard error of the age-adjusted mean; the standard was developed in the laboratory according to published procedures (18, 20, 54). ** There is a significant age versus diagnosis interaction and thus means are not given because the difference in the means between SIDS cases and controls varies by age (9). Abbreviations: HG, hypoglossal nucleus; DMV, nucleus of the motor nucleus of the vagus; ROB, raphé obscurus; PGGL, paragigantocellularis lateralis; GC, gigantocellularis; CI, confidence interval.

| 14-3-3 Isoforms | Acute controls SIDS % Decrease in SIDS Diagnosis |
|-----------------|-----------------------------------------------|
| Gamma           | 104.3 ± 5.5                                   | 51.5 ± 2.1 | 51% | <0.001 |
| Eta             | 109.1 ± 18.5                                  | 54.2 ± 7.1 | 50% | 0.009  |
| Beta            | 107.9 ± 18.1                                  | 38.8 ± 6.9 | 64% | 0.001  |
| Theta           | 91.5 ± 20.5                                   | 73.5 ± 7.9 | 20% | 0.420  |
| Zeta            | 137.7 ± 42.8                                  | 80.8 ± 16.5| 41% | 0.220  |
| Epsilon         | 67.2 ± 19.2                                   | 57.0 ± 7.5 | 15% | 0.620  |
| Sigma           | 116.5 ± 21.0                                  | 61.4 ± 8.1 | 47% | 0.020  |
| 14-3-3 Family   | 104.9 ± 9.0                                   | 59.6 ± 3.5 | 43% | <0.001 |

| Selected 5-HT parameters in different medullary nuclei | Acute Controls SIDS % Decrease in SIDS Diagnosis |
|--------------------------------------------------------|-----------------------------------------------|
| Parameter                                              | Age-Adjusted Means (95% CI)                    |
| 5-HT Levels in ROB<sup>1</sup>                         | 75.5 (54.2 to 96.8)                           | 55.4 (47.2 to 63.6) | 26% | 0.05 |
| 5-HT Levels in PGGL<sup>1</sup>                        | 40.0 (29.1 to 60.0)                           | 31.4 (47.2 to 63.6) | 26% | 0.04 |
| TPH2 Levels in ROB<sup>2</sup>                         | 193.9 (158.6 to 229.2)                        | 151.2 (137.5 to 165.0) | 22% | 0.019 |
| 5-HT<sub>1A</sub> binding in HG<sup>3</sup>            | 11.15 (7.77 to 14.54)                         | 6.87 (5.81 to 7.92) | 38% | 0.004 |
| 5-HT<sub>1A</sub> binding in DMV<sup>3</sup>           | 14.26 (10.22 to 18.30)                        | 7.82 (6.58 to 9.05) | 45% | 0.003** |
| 5-HT<sub>1A</sub> binding in PGGL<sup>3</sup>         | –                                             | –                                             | –   | 0.046** |

Correlative Analysis Between 14-3-3 Isoforms and 5-HT Parameters in SIDS Cases and Controls—We found significantly decreased 14-3-3 levels in the gigantocellularis in the same SIDS cases in the original Data set 4 for which we had previously reported 5-HT related abnormalities in the medullary 5-HT system (9). In the original Data set 4, abnormalities in 5-HT and TPH2 levels and 5-HT<sub>1A</sub> binding were reported in the medullary 5-HT system in SIDS cases (n = 35) compared with acute controls (n = 5) (Table V) (9). These previously published 5-HT findings are reiterated here in order to present the data concerning 14-3-3 proteins and 5-HT parameters together in one table, thereby underscoring the relationship of the 5-HT abnormalities to the 14-3-3 deficits in the same SIDS cases. The 5-HT abnormalities included a 22% reduction in TPH2 levels by Western blotting (p = 0.03), and a 26% reduction in the 5-HT levels in the raphé obscurus (p = 0.05) and paragigantocellularis lateralis (p = 0.04) by HPLC in the SIDS cases compared with controls (Table V) (9). There were also significant alterations in 5-HT<sub>1A</sub> binding in medullary nuclei that contain 5-HT source neurons, including the gigantocellularis, as well as nuclei that receive 5-HT projections (Table V).

Correlations between abundance levels of the four isoforms with significant reductions in the SIDS cases (β, γ, η, and θ) by Western blotting and 5-HT parameters were determined for the SIDS cases and acute controls in the original Data set 4 (Stage 2) (Table VI). The most statistically robust findings were positive correlations in the acute controls (but not the SIDS cases) between 14-3-3-γ and 5-HT levels in the raphé obscurus (correlation coefficient 0.996; p = 0.004) and paragigantocellularis lateralis (correlation coefficient 0.997; p = 0.003) (Table VI). That is, as the 14-3-3-γ levels increased in the acute controls in the raphé obscurus and paragigantocellularis lateralis, the 5-HT levels also increased. In addition, in the controls there were significant positive correlations between the 14-3-3-γ levels and 5-HT<sub>1A</sub> receptor binding in the paragigantocellularis lateralis (correlation coefficient 0.99; p = 0.010) and gigantocellularis (correlation coefficient 0.98; p = 0.016), and a marginally significant correlation in the raphé obscurus (correlation coefficient 0.95; p = 0.054) (Table VI). The statistically significant correlations (p < 0.05) between 14-3-3-γ levels and 5-HT parameters in
the SIDS cases were not compelling to us because of the low correlation coefficients (≤0.50), (Table VI).

**Stage 3: Western Blot Analysis of the 14-3-3 Isoforms in the Expanded Data Set 4 with the Addition of Newly Accrued SIDS Cases and Acute and Chronic Controls**—In Stage 3, we used analyzed Western blotting data involving the gigantocellularis from an expanded Data set 4 with five additional SIDS cases and three additional acute controls accrued after the closure of the original Data set 4 (Table I). We also analyzed three controls with chronic illness, so-called “chronic controls,” for a total of 38 SIDS cases, eight acute controls, and three chronic controls (Table I). There was no significant difference in postconceptional age (gestational plus postnatal age) between SIDS cases (52.6 ± 8.0 weeks), acute controls (50.7 ± 10.3 weeks), or chronic controls (58.3 ± 5.7 weeks) ($p = 0.45$), or in gestational or postnatal age among the three groups (Table I). There was no significant difference in the postmortem interval among the groups in Stage 3 (SIDS = 38; 18.2 ± 5.5 h; acute controls = 8; 12.7 ± 3.5 h; and chronic controls, 20.2 ± 2.3 h) ($p = 0.20$). There were significant 40–59% reductions in levels of 14-3-3γ ($p < 0.001$), 14-3-3β ($p = 0.001$), 14-3-3δ ($p < 0.001$), and 14-3-3ε ($p = 0.02$) in the SIDS cases compared with acute and chronic controls (Table VII). Post-hoc analysis revealed that all significant differences were between SIDS versus acute controls, as well as SIDS versus chronic controls, and that there were no significant differences in the mean measurements between the acute and chronic controls (Table VII). There was also a significant 37% reduction in the level of all 14-3-3 isoforms combined in the SIDS cases compared with acutely and chronically ill controls ($p < 0.001$).

**Stage 3: Immunocytochemical Analysis 14-3-3 γ**—Because of the multifaceted analysis in the SIDS cases and controls in the original Data set 4 (9), limited tissue remained for immunocytochemical analysis in this study, allowing only for single label analysis of a single 14-3-3 isof orm. We chose 14-3-3γ because: (1) mass spectrometry and Western blotting showed a significant reduction of 14-3-3γ in SIDS; and (2) this isof orm...
is known to have a high affinity for TPH2 (24), of relevance to elucidating the relationship of 14-3-3 to TPH2 deficiency in SIDS cases. We analyzed single-label 14-3-3 \( \varepsilon \)-immunostaining in 10 SIDS cases, two acute controls, and two chronic controls (total \( n = 14 \)) from the samples set used in Stage 3 of this analysis. Single and double-label analysis with 14-3-3 \( \varepsilon \) and TPH2 (7, 8, 25–28) was performed in three SIDS cases (49.3 ± 1.0 postconceptional weeks) and four controls (51 ± 5.0 postconceptional weeks). Immunostaining for 14-3-3 \( \varepsilon \) in the SIDS cases and controls was localized visually only to neurons (Fig. 3) and not to glia or blood vessels. Although almost all neuronal subtypes immunostained positively for 14-3-3 \( \varepsilon \), the staining intensity varied considerably (Fig. 3). The staining intensity was light in small neurons of the nucleus of the solitary tract and spinal trigeminal nucleus, moderate in fusiform neurons of the dorsal motor nucleus of the vagus, and heavy in round, medium neurons of the inferior olivary complex and pyramidal neurons of the hypoglossal nucleus (Fig. 3). The 14-3-3 \( \varepsilon \) isoform was not only expressed in these latter neurons that received 5-HT projections but also

### Table VI

| Correlation between 14-3-3 isoform and 5-HT parameter | SIDS (\( n = 27-31 \)) | Controls (\( n = 5 \)) |
|------------------------------------------------------|--------------------------|-------------------------|
|                                                      | Correlation*             | \( p \) value           | Correlation*             | \( p \) value           |
| Gamma                                                |                          |                         |                          |                         |
| 5-HT\( _\varepsilon \) Raphé Obscurus                | -0.22                    | 0.26                    | -0.38                    | 0.62                    |
| 5-HT\( _\varepsilon \) Paragigantocellularis lateralis| -0.32                    | 0.11                    | -0.37                    | 0.63                    |
| 5-HT\( _\varepsilon \) Gigantocellularis              | -0.27                    | 0.18                    | -0.35                    | 0.66                    |
| 5-HT Raphé Obscurus                                   | -0.15                    | 0.42                    | 0.996                    | 0.004                   |
| 5-HT Paragigantocellularis lateralis                  | -0.04                    | 0.85                    | 0.997                    | 0.003                   |
| TPH2 Raphé Obscurus                                   | 0.07                     | 0.72                    | 0.68                     | 0.32                    |
| Alpha                                                |                          |                         |                          |                         |
| 5-HT\( _\varepsilon \) Raphé Obscurus                | 0.22                     | 0.27                    | 0.38                     | 0.62                    |
| 5-HT\( _\varepsilon \) Paragigantocellularis lateralis| -0.05                    | 0.80                    | 0.39                     | 0.61                    |
| 5-HT\( _\varepsilon \) Gigantocellularis              | 0.14                     | 0.49                    | 0.40                     | 0.60                    |
| 5-HT Raphé Obscurus                                   | -0.32                    | 0.09                    | 0.72                     | 0.28                    |
| 5-HT Paragigantocellularis lateralis                  | -0.08                    | 0.63                    | 0.71                     | 0.29                    |
| TPH2 Raphé Obscurus                                   | -0.18                    | 0.34                    | 0.25                     | 0.75                    |
| Eta                                                  |                          |                         |                          |                         |
| 5-HT\( _\varepsilon \) Raphé Obscurus                | 0.42                     | 0.03                    | -0.68                    | 0.32                    |
| 5-HT\( _\varepsilon \) Paragigantocellularis lateralis| 0.36                     | 0.07                    | -0.88                    | 0.12                    |
| 5-HT\( _\varepsilon \) Gigantocellularis              | 0.49                     | 0.01                    | -0.92                    | 0.08                    |
| 5-HT Raphé Obscurus                                   | -0.09                    | 0.65                    | 0.06                     | 0.94                    |
| 5-HT Paragigantocellularis lateralis                  | -0.18                    | 0.36                    | 0.16                     | 0.84                    |
| TPH2 Raphé Obscurus                                   | -0.40                    | 0.03                    | -0.03                    | 0.97                    |
| Sigma                                                |                          |                         |                          |                         |
| 5-HT\( _\varepsilon \) Raphé Obscurus                | 0.07                     | 0.75                    | 0.95                     | 0.054                   |
| 5-HT\( _\varepsilon \) Paragigantocellularis lateralis| -0.10                    | 0.62                    | 0.99                     | 0.010                   |
| 5-HT\( _\varepsilon \) Gigantocellularis              | -0.02                    | 0.92                    | 0.98                     | 0.016                   |
| 5-HT Raphé Obscurus 5-HT                              | -0.05                    | 0.77                    | -0.21                    | 0.79                    |
| 5-HT Paragigantocellularis lateralis                  | -0.05                    | 0.81                    | -0.24                    | 0.76                    |
| TPH2 Raphé Obscurus                                   | -0.13                    | 0.49                    | -0.40                    | 0.60                    |

### Table VII

Summary of the western blotting analysis of 14-3-3 isoforms in the expanded Dataset 4 in Stage 3. Legend: *, The western blotting (WB) (SIDS; \( n = 38 \), Acute Controls; \( n = 8 \), Chronic Controls; \( n = 3 \)) values are protein levels expressed as a percent of an adult human standard ± standard error of the age-adjusted mean. The standard was developed in the laboratory according to standardized procedures (20, 54).

| 14-3-3 Isomers | Age adjusted means (S.E.) | Chronic controls | SIDS | Combined controls | % Decrease in SIDS |
|----------------|----------------------------|------------------|------|-------------------|-------------------|
|                | n 8                        | n 3              | n 38 | n 11              |                   |
| Gamma          | 70.7 ± 5.8                 | 76.1 ± 9.5       | 44.3 ± 2.6 | <0.001           | 73.4 ± 7.7       | 40%               |
| Eta            | 102.7 ± 13.7               | 110.0 ± 22.7     | 53.0 ± 6.3 | 0.001            | 106.4 ± 18.2     | 50%               |
| Beta           | 104.9 ± 15.0               | 89.9 ± 23.9      | 39.5 ± 6.6 | <0.001           | 97.4 ± 19.5      | 59%               |
| Theta          | 88.5 ± 14.6                | 64.1 ± 24.1      | 74.9 ± 6.7 | 0.600            | 76.3 ± 19.4      | 2%                |
| Zeta           | 117.5 ± 30.8               | 107.4 ± 50.8     | 81.4 ± 14.1 | 0.530           | 112.5 ± 40.1     | 28%               |
| Epsilon        | 68.3 ± 13.7                | 95.8 ± 22.5      | 58.1 ± 6.3 | 0.300            | 82.1 ± 18.1      | 25%               |
| Sigma          | 108.0 ± 15.6               | 103.5 ± 25.7     | 62.0 ± 7.1 | 0.020            | 105.8 ± 20.7     | 41%               |
| 14-3-3 Family  | 94.4 ± 6.6                 | 92.4 ± 10.9      | 59.0 ± 3.0 | <0.001           | 93.4 ± 8.8       | 37%               |

### Table V

Correlations between 14-3-3 protein isoforms and 5-HT markers in Stage 2. Legend: 5-HT\( _A \) refers to 5-HT\( _A \) receptor binding measured with tissue receptor autoradiography; 5-HT refers to 5-HT levels measured with high performance liquid chromatography, permission given by JAMA to use the data (9); and TPH2 refers to TPH2 levels measured by western blotting (9) (see text).
TABLE VIII
Stage 1—Summary of all mass spectrometry and western blotting results of the different 14-3-3 isoforms in the SIDS cases compared to controls in Stages 1–3.Legend: N/I, Not identified; * Significant decreases in 14-3-3 isoforms in SIDS cases compared to control; p value < 0.1, ** 22–91% Decrease in SIDS case compared to control based on normalized spectral counts, *** Significant decreases in 14-3-3 isoforms in SIDS cases compared to control; p value < 0.02 **** Significant decreases in 14-3-3 isoforms in SIDS cases compared to control; p value < 0.02

| 14-3-3 Isoforms | *GeLC-MS/MS (n = 10) | **MSF (n = 2) | ***Western blotting (Stage 3) (n = 38) | ****Western blotting (Stage 4) (n = 49) |
|-----------------|----------------------|--------------|--------------------------------------|--------------------------------------|
| Gamma           | +                    | +            | +                                    | +                                    |
| Eta             | +                    | +            | +                                    | +                                    |
| Beta            | +                    | +            | +                                    | +                                    |
| Theta           | +                    | +            | –                                    | –                                    |
| Zeta            | –                    | +            | –                                    | –                                    |
| Epsilon         | –                    | –            | –                                    | –                                    |
| Sigma           | N/I                  | N/I          | +                                    | +                                    |
| 14-3-3 Family   | +                    | +            | +                                    | +                                    |

in neurons in nuclei that contain the source 5-HT neurons, i.e. gigantocellularis (Fig. 3), paragigantocellularis lateralis, raphé obscurus (Fig. 3), and arcuate nucleus. Double-label immunocytochemistry revealed that not all 5-HT neurons in the 5-HT source nuclei expressed 14-3-3-3γ, nor were all 14-3-3-γ expressing neurons serotonergic (Fig. 3). Comparative analysis of 14-3-3-γ immunostaining between the SIDS cases (n = 10) and controls (n = 4) revealed that the regional distribution was similar between groups. We assessed staining intensity blinded to the diagnosis by ranking the tissue sections from the highest to lowest staining by visual inspection, and determined the distribution of cases in the top and bottom 25% of staining intensity. We found that 75% of the top cases (3/4) were either acute or chronic controls and 100% (4/4) of the lowest cases were SIDS (linear rank test; p = 0.048) (Fig. 4), confirming the findings of proteomics and Western blotting.

Risk Factor Analysis and 14-3-3 Isoform Levels in the SIDS Cases in Stage 3—Ninety-seven percent (37/38) of the SIDS cases had one or more risk factors in either category and 84% (32/38) had two or more risk factors in the expanded Data set 4 (Stage 3). Ninety-two percent (35/38) of the SIDS cases had at least one extrinsic risk factor (stressor), i.e. prone (51%) or side (14%) sleep position, face-down sleep position (39%), bed sharing (18%), and trivial illness within 1 week of death (45%). Sixty-eight percent (26/38) of the SIDS cases had at least one intrinsic (vulnerability) risk factor, with 24% (9/38) having two such risk factors. The intrinsic risk factors were prematurity (24%), male gender (53%), and African American race (9%). We found that SIDS infants found dead in the side sleep position (i.e. extrinsic risk factor), had significantly lower 14–3–3–γ levels than infants found dead in a supine (non-risk) position (p = 0.03) (Supplemental Files: Table 1.C). The association between SIDS risk factors and 14-3-3 levels as measured by western blotting in the gigantocellularis lateralis in the SIDS group of the expanded Dataset 4. There were no other associations between other risk factors and the 14-3-3 isoforms (Supplementary Tables 1A-D).

DISCUSSION
In this study of the medullary 5-HT system in SIDS, we applied broad-based proteomics as a discovery tool to uncover potential new proteins critical to the up- or downstream regulation of TPH2 and/or 5-HT, given our previous demonstration of abnormal levels of TPH2 and 5-HT in this system in SIDS cases (9). Our major discovery was a significant reduction in the abundance levels of individual and combined isoforms of the 14-3-3 protein family, which is known to influence the regulation of TPH2 (24, 29–31). Of note, the reduction of 14-3-3 isoforms in SIDS cases demonstrated by GeLC-MS/MS based proteomics and spectral counting analysis was corroborated by an orthogonal LC/MSF analysis. We subsequently confirmed these proteomic 14-3-3 findings with Western blotting for the γ, β, η, and σ isoforms in an expanded data set of SIDS cases and controls, the latter dying with a spectrum of known acute and chronic illnesses, and thereby pointing to the potential specificity of the 14-3-3 deficit to SIDS. Importantly, the abundance deficits in the γ, β, η, and σ isoforms deficit were associated with TPH2 and 5-HT deficiencies and 5-HT1A receptor binding abnormalities in the same SIDS cases, as the 14-3-3 Western blotting analysis was performed in the same SIDS cases and controls in which we previously reported the analysis of 5-HT, TPH2, and 5-HT1A receptor binding levels (Data set 4) (9). The nucleus analyzed in this study was the gigantocellularis, which comprises the ventromedial reticular formation of the rostral medulla and is the medial component of the medullary 5-HT system (6). It is implicated in cardiorespiratory regulation (32), muscle tone suppression during REM sleep (33), and pain modulation (32). Thus, a 14-3-3 deficiency in the gigantocellularis may lead to 5-HT dysfunction in the modulation of cardiorespiratory and visceral control in SIDS infants. Overall, these data suggest the possibility that 14-3-3 deficits lead to the TPH2 deficiency in SIDS, given that 14-3-3 is necessary for this enzyme's phosphorylation, activity, stability, and abundance levels (24, 29, 30) (see below), bringing us potentially closer to the mo-
Molecular landscape of 5-HT brainstem pathology in SIDS. Given 5-HT’s key role in the mediation of protective responses to homeostatic stressors, e.g. hypercarbia (34), a deficiency of 5-HT secondary to TPH2 impairment in the medullary 5-HT system may lead to sleep-related sudden death when the vulnerable infant faces a life-threatening challenge, e.g. hypercarbia in the prone (face-down) sleep position (3). Of note, 51% of the SIDS infants in the expanded Data set 4 (Stage 3) of this study were found prone, 39% were found face-down, and 9% were found with face-covered, all positions considered to be at risk for hypoxia, hypercarbia, or asphyxia (3).

Seven isoforms of the 14-3-3 signal transduction proteins are known in humans and are encoded by separate genes (35, 36). The commonality of the isoforms is that they bind to...
serine-/threonine-phosphorylated motifs that lead in turn to activity changes in bound ligands, altered associations of bound ligands with other cellular components, and changes in intracellular localization of 14-3-3-bound cargo (37). Of major importance to our finding of significantly reduced 14-3-3 isoforms in the medullary 5-HT system in SIDS is their key role in TPH2 modulation (35). Tryptophan hydroxylase converts brain tryptophan into 5-hydroxytryptophan in the rate-limiting step for 5-HT synthesis; phosphorylation of Ser19 and Ser104 residues (24) by calcium calmodulin kinase II and protein kinase A is necessary for TPH2 enzyme activation (29, 35). Both phosphorylation and enzyme activation, however, are also dependent upon the presence of 14-3-3, as it activates phosphorylated TPH2 through direct binding (31). Thus, 14-3-3 exerts a downstream effect on 5-HT biosynthesis by binding to phosphorylated TPH2 and increasing its stability and catalytic activity (24, 29), as well as abundance levels (29). Consequently, in SIDS cases we hypothesize that a deficiency of 14-3-3 is associated with a reduction in the activity and/or possible levels of TPH2, which in turn leads to impaired 5-HT synthesis and reduced 5-HT levels (the latter TPH2–5-HT pathway confirmed in TPH2 knockout mice) (38). The impaired 5-HT levels may then result in a compensatory increase in immature 5-HT neurons with concomitant immature (decreased) 5-HT1A receptor binding and 5-HT transporter levels, perhaps as early as the first or second trimester (4). Although we chose to focus upon the potential role of 14-3-3 proteins in the pathogenesis of 5-HT brainstem pathology in SIDS because of their key link to the regulation of TPH2 and the deficiency of TPH2 in SIDS cases (9), certain other proteins differed significantly in abundance levels between SIDS cases and controls, particularly metabolic, cytoskeletal, and synaptic proteins. The potential inter-relationships of any or all of these proteins with 5-HT and 14-3-3, in the SIDS cases are under separate ongoing analysis by us.

The question arises as to how the relatively ubiquitous 14-3-3 proteins relate mechanistically to the putative regional pathology in the medullary 5-HT system in SIDS cases. This challenging question is not without precedent in human neuropathology: huntingtin, the mutant protein that is causal in Huntington’s disease (HD), for example, is ubiquitous in the brain and systemic organs but the brunt of the pathology is localized to the striatum (39). Indeed, a major focus of HD research today is to explain this paradox by determining the molecular vulnerability of spiny neurons in the striatum to the mutant huntingtin (39). Yet, the 14-3-3 proteins perform separate, as well as overlapping, functions and are expressed in different brain regions according to different developmental time-tables (36, 40–42). The leonardo gene, for example, encodes a conserved member of the 14-3-3 family (Drosophila homolog of the ζ isoform) that is specifically involved in learning and is concentrated in presynaptic boutons of the neuromuscular junction where it helps regulate synaptic vesicle dynamics (43). Different isoforms are implicated in other
aspects of synaptic function (44–46). Importantly, the functions of the isoforms differ mainly via their specific binding to different ligands (47–49). The four isoforms with abnormal abundance levels by Western blotting in the SIDS cases in this study were γ, β, η, and σ; of note, all seven isoforms bind phosphorylated TPH2 (24, 50), including the γ isoform, which has been shown to have a high affinity for phosphorylated TPH2 (24). The immunocytochemical observation that 14-3-3 localizes to neurons within the gigantocellularis and other medullary sites and not to glia or blood vessels implicates 14-3-3 specifically in neuronal function in the human infant medulla. Future studies may reveal that specific 14-3-3 isoforms are restricted to or especially abundant in the medullary 5-HT system and play specific roles in 5-HT neurotransmission. Our finding that not all 5-HT neurons in the medullary 5-HT system express the γ isoform of 14-3-3 underscores this possibility. Yet, although we have found that the most robust findings in SIDS brains involve 5-HT markers in the medulla, abnormalities related to other brain regions and neurotransmitter systems have been reported (3, 4), and thus, the underlying molecular defect(s) in SIDS may impact wider brain function in as yet unknown ways.

In this study, we sought to determine potential relationships between the abundance levels of the different 14-3-3 isoforms and those of the 5-HT parameters available to use, i.e. 5-HT, TPH2, and 5-HT1A receptor binding levels, in the gigantocellularis in the same data set of SIDS cases and controls (original Data set 4). In this analysis, we reasoned that significant correlations, either positive or negative, would potentially provide insight into the role of specific 14-3-3 isoforms in the regulation and/or function of the specific 5-HT parameters. Of note, none of the 14-3-3 isoform levels correlated with TPH2 levels in any site analyzed of the medullary 5-HT system in SIDS cases and/or controls in this initial analysis. These data, however, do not preclude the possibility that 14-3-3 isoform levels correlate with TPH2 phosphorylation and/or activity, as demonstrated in experimental systems (24, 30). In the acute controls, we found, however, that the levels of 14-3-3-γ positively correlated with those of 5-HT in the raphe obscurus and paragigantocellularis, two key components of the medullary 5-HT system and the two nuclei in which 5-HT levels were measured by us (9). These data suggest that 14-3-3-γ is specifically involved in the regulation of 5-HT levels in the human infant medulla, which may or may not involve an interaction with TPH2 or 5-HT1A receptors. Although a role for 14-3-3-γ in TPH2 regulation is indicated by experimental studies in animal models, further investigation of its particular role in human 5-HT metabolism is needed. Interestingly, the levels of 14-3-3-γ and 5-HT did not correlate with each other in the SIDS cases as they did in the controls. This discrepancy suggests the intriguing possibility of a dysregulation of 5-HT levels related to an intrinsically abnormal 14-3-3-γ in the SIDS cases, in addition to an absolute reduction in 14-3-3-γ levels, an idea for testing in future studies. The correlation between 14-3-3-γ levels and 5-HT1A receptor binding in the controls only also suggests a role for this isoform in 5-HT1A receptor function/regulation, but we were unable to find specific supporting published data, although 14-3-3 proteins are known to interact with G-protein coupled receptors (51).

We analyzed 14-3-3 isoform abundance relative to known risk factors in the SIDS cases, and found a significant correlation of reduced 14-3-3-γ levels with side sleep position. Given that we found only one correlation among the many sought for all the isoforms identified, we are reluctant to place major emphasis upon this single finding. Rather, the finding that SIDS occurred in our data set with reduced 14-3-3-γ levels with or without each risk factor suggests that the 14-3-3 protein deficit is operative in potentially all SIDS deaths, irrespective of risk factors, and is therefore perhaps closer to the “primary” defect.

A potential limitation of the study is the effect of postmortem interval upon 14-3-3 levels; we found, however, no statistically significant effects. The remarkable stability of 14-3-3 proteins after death is likely a result of their highly compact well-folded structure (37). Moreover, a study of postmortem changes in proteins in a rat model indicate that 14-3-3 levels increase after 48 h (52), rather than decrease, i.e. the change we found in our SIDS cases compared with controls. Thus, if the postmortem interval had an effect on the observed differences in the 14-3-3 levels, it would have decreased, i.e. de-emphasized the observed differences.

In conclusion, we report the novel finding of reduced 14-3-3 isoforms in the gigantocellularis of the medullary 5-HT system in SIDS cases in which there are also deficiencies of TPH2 and 5-HT levels. Thus, a causal role for the 14-3-3 deficit in the reduced TPH2 and 5-HT levels in SIDS infants warrants further investigation. Indeed, this study suggests important hypotheses related to causal mechanisms for testing in experimental models that concern the molecular role of specific 14-3-3 isoforms in the regulation of TPH2, 5-HT, and/or 5-HT1A receptor binding levels. In addition, the results of this study suggest the possibility that 14-3-3 measurements may serve as an important future method to help identify living infants at risk for SIDS considering that 14-3-3 levels serve as diagnostic biomarkers in the blood and/or cerebrospinal fluid in neurodegenerative disorders in adults (53).
Brainstem 14-3-3 Deficiency in SIDS

PO1-HD036379 [HCK], and P30-HD18655 [Developmental Disabilities Research Center, Children’s Hospital Boston], National Institute of Alcoholism and Alcohol Abuse [5 U01 HD045991-06] [KGB]. We are grateful to the Jacob Neil Boger Foundation [KGB], Evelyn Deborah Barrett Fellowship for SIDS Research [KGB], First Candle/SIDS Alliance, and CJ Murphy Foundation for Solving the Puzzle of SIDS for their support.

This article contains supplemental Tables S1 and S2 and Figs. S1 and S2 and References.

To whom correspondence should be addressed: Department of Pathology, Enders 1111, Children’s Hospital Boston, 300 Longwood Avenue, Boston, MA 02115. Tel.: 617-912-2733; Fax: 617-730-0168; E-mail: kevin.broadbelt@childrens.harvard.edu.

Co-Senior Authors.

Current address of Dr. J. R. Duncan: Florey Neuroscience Institute, University of Melbourne, Parkville, Victoria, Australia, 3010.

REFERENCES

1. Krous, H. F., Beckwith, J. B., Byard, R. W., Rognum, T. O., Bajanowski, T., Corey, T., Cutz, E., Hanzlik, R., Keens, T. G., and Mitchell, E. A. (2004) Sudden infant death syndrome and unclassified sudden infant deaths: a definitional and diagnostic approach. Pediatr. 114, 234–238
2. Moon, R. Y., Horne, R. S., and Hauck, F. R. (2007) Sudden infant death syndrome. Lancet 370, 1578–1587
3. Kinney, H. C., Richerson, G. B., Dymecki, S. M., Darnall, R. A., and Nattie, E. E. (2009) The brainstem and serotonin in the sudden infant death syndrome. Annu. Rev. Pathol. 4, 517–550
4. Kinney, H. C. and Thach, B. T. (2009) The sudden infant death syndrome. N. Engl. J. Med. 361, 795–805
5. Kinney, H. C., Randa, L. L., Sleeper, L. A., Willinger, M., Belliveau, R. A., Zec, N., Raya, L. A., Dominici, L., Iyasu, S., Randa, B., Habbe, D., Wilson, H., Mandell, F., McClain, M., and Welty, T. K. (2003) Serotonergic brainstem abnormalities in Northern Plains Indians with the sudden infant death syndrome. J. Neuropathol. Exp. Neurol. 62, 1178–1191
6. Panigrahy, A., Filliano, J., Sleeper, L. A., Mandell, F., Valdes-Dapena, M., Krous, H. F., Raya, L. A., Foley, E., White, W. F., and Kinney, H. C. (2000) Decreased serotonergic receptor binding in rhombic lip-derived regions of the medulla oblongata in the sudden infant death syndrome. J. Neuropathol. Exp. Neurol. 59, 377–384
7. Paterson, D. S., Trachtenberg, F. L., Thompson, E. G., Belliveau, R. A., Beggs, A. H., Darnall, R., Chadwick, A. E., Krous, H. F., and Kinney, H. C. (2006) Multiple serotonergic brainstem abnormalities in sudden infant death syndrome. JAMA 296, 2124–2129
8. Kinney, H. C., Belliveau, R. A., Trachtenberg, F. L., Raya, L. A., and Paterson, D. S. (2007) The development of the medullary serotonergic system in early human life. Auton. Neurosci. 132, 81–102
9. Duncan, J. R., Paterson, D. S., Hoffman, J. M., Mokler, D. J., Borenstein, N. S., Belliveau, R. A., Krous, H. F., Haas, E. A., Stanley, C., Nattie, E. E., Trachtenberg, F. L., and Kinney, H. C. (2010) Brainstem serotonergic deficiency in sudden infant death syndrome. JAMA 303, 430–437
10. Silva, J. C., Gorenstein, M. V., Li, G. Z., Vissers, J. P., and Geromanos, S. J. (2006) Absolute quantification of proteins by LCMSE: a virtue of parallel sampling and estimation of relative protein abundance in shotgun proteomics. Anal. Chem. 78, 4193–4201
11. Winge, I., McKinney, J. A., Ying, M., D’Santos, C. S., Kleppe, R., Knappskog, P. M., and Haavik, J. (2008) Activation and stabilization of human tryptophan hydroxylase 2 by phosphorylation and 14-3-3 binding. Biochem. J. 410, 195–204
12. Baker, K. G., Halliday, G. M., Halasiz, P., Hornung, J. P., Geffen, L. B., Cotton, R. G., and Rörk, I. (1991) Cytoarchitecture of serotonin-synthesizing neurons in the pontine tegument of the human brain. Synapse 7, 301–320
13. Haan, E. A., Jennings, I. G., Cuello, A. C., Nakata, H., Fujiwasa, H., Chow, C. W., Kushinskiy, R., Brittingham, J., and Cotton, R. G. (1987) Identification of serotonergic neurons in human brain by a monoclonal antibody binding to all three aromatic amino acid hydroxylases. Brain Res. 426, 19–27
14. Halliday, G. M., Blumbergs, P. C., Cotton, R. G., Blessing, W. W., and Geffen, L. B. (1980) Loss of brainstem serotonergic and substance-P containing neurons in Parkinson’s disease. Brain Res. 150, 104–107
15. Törk, I., and Hornung, J. P. (1990) Raphe Nuclei and the Serotonergic System. In: Paxinos, G., ed. The Human Nervous System, pp. 1001–1022, Academic Press, San Diego, CA
16. Banik, U., Wang, G. A., Wagner, P. D., and Kaufman, S. (1997) Interaction of phosphorylated tryptophan hydroxylase with 14-3-3 proteins. J. Biol. Chem. 272, 26219–26225
17. Furukawa, Y., Ikuta, N., Omata, S., Yamauchi, T., Isobe, T., and Ichimura, Y. (1993) Demonstration of the phosphorylation-dependent interaction of tryptophan hydroxylase with the 14-3-3 protein. Biochem. Biophys. Res. Commun. 194, 144–149
18. Gao, K., and Mason, P. (1997) Somatodendritic and axonal anatomy of intracellularly labeled serotonergic neurons in the rat medulla. J. Comp. Neurol. 389, 309–328
19. Lai, Y. Y., Clements, J. R., Wu X. Y., Shalita, T., Wu, J. P., Kuo, J. S., and Siegel, J. M. (1999) Brainstem projections to the ventromedial medulla in cat: retrograde transport horseradish peroxidase and immunohistochemical studies. J. Comp. Neurol. 408, 419–436
20. Ray, R. S., Corcoran, A. E., Brust, R. D., Kim, J. C., Nattie, E. E., and Dymecki, S. M. (2011) Impaired respiratory and body temperature control upon acute serotonergic neuron inhibition. J. Comp. Neurol. 510, 517–550
36. Yaffe, M. B. (2002) How do 14-3-3 proteins work?-- Gatekeeper phosphorylation and the molecular anvil hypothesis. *FEBS Lett.* 513, 53–57

37. Murphy, K. L., Zhang, X., Gainetdinov, R. R., Beaulieu, J. M., and Caron, M. G. (2008) A regulatory domain in the N terminus of tryptophan hydroxylase 2 controls enzyme expression. *J. Biol. Chem.* 283, 13216–13224

38. Alenina, N., Kikic, D., Todiras, M., Mosienko, V., Qadri, F., Plehm, R., Boyé, P., Villanovitch, L., Sohr, R., Tenner, K., Hörttagl, H., and Bader, M. (2009) Growth retardation and altered autonomic control in mice lacking brain serotonin. *Proc. Natl. Acad. Sci. U.S.A.* 106, 10332–10337

39. Damiano, M., Galvan, L., Déglon, N., and Brouillet, E. (2010) Mitochondria in Huntington’s disease. *Biochim. Biophys. Acta* 1802, 52–61

40. Toyoooka, K., Muratake, T., Watanabe, H., Hayashi, S., Ichikawa, T., Usui, H., Washiyama, K., Kumanishi, T., and Takahashi, Y. (2002) Isolation and structure of the mouse 14-3-3 eta chain gene and the distribution of 14-3-3 eta mRNA in the mouse brain. *Brain Res. Mol. Brain Res.* 100, 13–20

41. Cao, L., Cao, W., Zhang, W., Lin, H., Yang, X., Zhen, H., Cheng, J., Dong, W., Huo, J., and Zhang, X. (2008) Identification of 14-3-3 protein isoforms in human astrocytoma by immunohistochemistry. *Neurosci. Lett.* 432, 94–99

42. Umahara, T., Uchihara, T., Nakamura, A., and Iwamoto, T. (2009) Isoform-dependent immunolocalization of 14-3-3 proteins in developing rat cerebellum. *Brain Res.* 1253, 15–26

43. Broadie, K., Rushton, E., Skoulakis, E. M., and Davis, R. L. (1997) Leonardo, a Drosophila 14-3-3 protein involved in learning, regulates presynaptic function. *Neuron* 19, 391–402

44. Chamberlain, L. H., Roth, D., Morgan, A., and Burgoyne, R. D. (1995) Distinct effects of alpha-SNAP, 14-3-3 proteins, and calmodulin on priming and triggering of regulated exocytosis. *J. Cell Biol.* 130, 1063–1070

45. Greengard, P., Valtorta, F., Czernik, A. J., and Benfenati, F. (1993) Synchronized vesicle phosphoproteins and regulation of synaptic function. *Science* 259, 780–785

46. Roth, D., and Burgoyne, R. D. (1995) Stimulation of catecholamine secretion from adrenal chromaffin cells by 14-3-3 proteins is due to reorganisation of the cortical actin network. *FEBS Lett.* 374, 77–81

47. Han, D. C., Rodriguez, L. G., and Guan, J. L. (2001) Identification of a novel interaction between integrin beta1 and 14-3-3beta. *Oncogene* 20, 346–357

48. Wang, Y., Jacobs, C., Hook, K. E., Duan, H., Booher, R. N., and Sun, Y. (2000) Binding of 14-3-3beta to the carboxyl terminus of Wee1 increases Wee1 stability, kinase activity, and G2-M cell population. *Cell Differ.* 11, 211–219

49. Wanzel, M., Kleine-Kohlfreger, D., Herold, S., Hock, A., Berms, K., Park, J., Hemmings, B., and Eilers, M. (2005) Akt and 14-3-3-Seta regulate Mlc2 to control cell-cycle arrest after DNA damage. *Nat. Cell Biol.* 7, 30–41

50. Isobe, T., Ichimura, T., Sunaya, T., Okuyama, T., Takahashi, N., Kawanoto, R., and Takahashi, Y. (1991) Distinct forms of the protein kinase-dependent activator of tyrosine and tryptophan hydroxylases. *J. Mol. Biol.* 217, 125–132

51. Prezeau, L., Richman, J. G., Edwards, S. W., and Limbird, L. E. (1999) The zeta isoform of 14-3-3 proteins interacts with the third intracellular loop of different alpha2-adrenergic receptor subtypes. *J. Biol. Chem.* 274, 13462–13469

52. Fountoulakis, M., Hardmeier, R., Höger, H., and Lubec, G. (2001) Postmortem changes in the level of brain proteins. *Exp. Neurol.* 167, 86–94

53. Goldknopf, I. L., Bryson, J. K., Strelets, I., Quintero, S., Sheta, E. A., Mosqueda, M., Park, H. R., Appel, S. H., Shill, H., Sabbagh, M., Chase, B., Kaldjian, E., and Markopoulou, K. (2009) Abnormal serum concentrations of proteins in Parkinson’s disease. *Biochem. Biophys. Res. Commun.* 389, 321–327

54. Broadbelt, K. G., Paterson, D. S., Rivera, K. D., Trachtenberg, F. L., and Kinney, H. C. (2010) Neuroanatomic relationships between the GABAAergic and serotonergic systems in the developing human medulla. *Auton. Neurosci.* 154, 30–41

In order to cite this article properly, please include all of the following information: Broadbelt, K. G., Rivera, K. D., Paterson, D. S., Duncan, J. R., Trachtenberg, F. L., Paulo, J. A., Stapels, M. D., Borenstein, N. S., Belliveau, R. A., Haas, E. A., Stanley, C., Krous, H. F., Steen, H., and Kinney, H. C. (2012) Brainstem Deficiency of the 14-3-3 Regulator of Serotonin Synthesis: A Proteomics Analysis in the Sudden Infant Death Syndrome. *Mol. Cell. Proteomics* 11(1):M111.009530. DOI: 10.1074/mcp.M111.009530.