Serotonin Receptors in the Medulla Oblongata of the Human Fetus and Infant: The Analytic Approach of the International Safe Passage Study

Citation
Haynes, R. L., R. D. Folkerth, D. S. Paterson, K. G. Broadbelt, S. Dan Zaharie, R. H. Hewlett, J. J. Dempers, et al. 2016. “Serotonin Receptors in the Medulla Oblongata of the Human Fetus and Infant: The Analytic Approach of the International Safe Passage Study.” Journal of Neuropathology and Experimental Neurology 75 (11): 1048-1057. doi:10.1093/jnen/nlw080. http://dx.doi.org/10.1093/jnen/nlw080.

Published Version
doi:10.1093/jnen/nlw080

Permanent link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:29408215

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA

Share Your Story
The Harvard community has made this article openly available. Please share how this access benefits you. Submit a story.

Accessibility
Serotonin Receptors in the Medulla Oblongata of the Human Fetus and Infant: The Analytic Approach of the International Safe Passage Study

Robin L. Haynes, PhD, Rebecca D. Folknerth, MD, David S. Paterson, PhD, Kevin G. Broadbelt, PhD, S. Dan Zaharie, MD, Richard H. Hewlett, MD, Johan J. Dempers, MD, Elsie Burger, MD, Shabbir Wadee, MD, Pawel Schubert, MD, Colleen Wright, MD, Mary Ann Sens, MD, Laura Nelsen, MD, Bradley B. Randall, MD, Hoa Tran, PhD, Elaine Geldenhuys, Amy J. Elliott, PhD, Heim J. Odendaal, FRCOG, MD, Hannah C. Kinney, MD, and the PASS Network

Abstract

The Safe Passage Study is an international, prospective study of approximately 12,000 pregnancies to determine the effects of prenatal alcohol exposure (PAE) upon stillbirth and the sudden infant death syndrome (SIDS). A key objective of the study is to elucidate adverse effects of PAE upon binding to serotonin (5-HT) 1A receptors in brainstem homeostatic networks postulated to be abnormal in unexplained stillbirth and/or SIDS. We undertook a feasibility assessment of 5-HT1A receptor binding using autoradiography in the medulla oblongata (6 nuclei in 27 cases). 5-HT1A binding was compared to a reference dataset from the San Diego medical examiner’s system. There was no adverse effect of postmortem interval ≤100 h. The distribution and quantitated values of 5-HT1A binding in Safe Passage Study cases were essentially identical to those in the reference dataset, and virtually identical between stillbirths and live born fetal cases in grossly non-macerated tissues. The pattern of binding was present at mid-gestation with dramatic changes in binding levels in the medullary 5-HT nuclei over the second half of gestation; there was a plateau at lower levels in the neonatal period and into infancy. This study demonstrates feasibility of 5-HT1A binding analysis in the medulla in the Safe Passage Study.

Key Words: Autoradiography; Brodmann areas; Prenatal alcohol exposure; Serotonin 1A receptor; Stillbirth; Sudden infant death syndrome (SIDS).

INTRODUCTION

Toxicity to the developing human brain due to prenatal alcohol exposure (PAE) results in a spectrum of cognitive, affective, and homeostatic abnormalities in the offspring, with or without associated facial dysmorphism, overall growth impairments, and/or somatic organ maldevelopment (1, 2). Altogether, these abnormalities fall under Fetal Alcohol Spectrum Disorders (FASD), which are estimated to occur in at least 2.4% of the general population in the United States today (1). Central and autonomic deficits (3, 4) occur in infants and children with PAE without such major brain malformations as cerebellar hypoplasia or agenesis of the corpus callosum, and potentially result from the harmful effects of PAE directly upon brainstem development (2–6). Increasingly, PAE is linked to the adverse outcomes of stillbirth (7–9) and sudden infant death syndrome (SIDS) (10, 11). If confirmed, this would place these problems of perinatal mortality under the rubric of FASD. In this study, the following definitions were used: 1) stillbirth, defined as an intraterine fetal demise ≥20

From the Department of Pathology, Boston Children’s Hospital and Harvard Medical School, Boston, Massachusetts (RLH, RDF, DSP, KGB, HT, HCK); Department of Pathology, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts (RDF); Department of Pathology, Faculty of Medicine and Health Science, Stellenbosch University, Western Cape, South Africa (SDZ, RHH, PS, EG); Division of Forensic Pathology and Medicine, Department of Pathology and Western Cape Forensic Pathology Services, Health Science Faculty, Stellenbosch University, Cape Town, South Africa (JJD, EB, SW); National Health Laboratory Services, Port Elizabeth, Eastern Cape, South Africa (CW); Department of Pathology, University of North Dakota, Grand Forks, North Dakota (MAS); Department of Pathology, University of South Dakota School of Medicine, Sioux Falls, South Dakota (LN, BBR); Community and Population Health Sciences, Sanford Research, Sioux Falls, South Dakota (AJE); Department of Obstetrics and Gynecology, Faculty of Medicine and Health Science, Stellenbosch University, Western Cape, South Africa (HJO); and The Prenatal Alcohol, SIDS, and Stillbirth (PASS) Research Network (PN).

Send correspondence to: Robin L. Haynes, PhD, Department of Pathology, Boston Children’s Hospital, Enders Building, Room 1107, 61 Binney Street, Boston, MA 02115; E-mail: robin.haynes@childrens.harvard.edu

The PASS Research Network is supported by the National Institute on Alcohol Abuse and Alcoholism, Eunice Kennedy Shriver National Institute of Child Health and Human Development, and National Institute on Deafness and Other Communication Disorders through the Cooperative Agreement Mechanism (U01 HD055154, U01 HD045935, U01 HD055155, U01 HD045991, and U01 AA016501). The opinions expressed in this paper are those of the authors and do not necessarily reflect the views of the Indian Health Service (IHS) or the National Institutes of Health, the Eunice Kennedy Shriver National Institute of Child Health and Development (NICHD), the National Institute on Alcohol Abuse and Alcoholism (NIAAA), or the National Institute on Deafness and Other Communication Disorders (NIDCD).

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

J Neuropathol Exp Neurol
Vol. 75, No. 11, November 2016, pp. 1048–1057
doi: 10.1093/jnen/nlw080

C 2016 American Association of Neuropathologists, Inc.

© 2016 American Association of Neuropathologists, Inc.
gestational weeks, and as unexplained stillbirth when a review of the clinical history, complete autopsy, and placental examination fail to determine the cause, and 2) SIDS, defined as the sudden unexpected death of an infant under 1 year of life that remains unexplained after a complete autopsy and death scene investigation (12).

The neurochemical, cellular, or molecular substrates of potential brainstem deficits related to PAE, (and thus potentially to unexplained stillbirth and/or SIDS), in the human brain are incompletely understood. The Safe Passage Study was designed in part to determine this substrate. It is an international, prospective study of 12,000 pregnancies to determine the effects of PAE upon fetal and infant morbidity and mortality (12). Mortality in the study is focused upon unexplained stillbirth and SIDS, both postulated disorders of central/autonomic homeostatic regulation (13–15).

A key objective of the Safe Passage Study is to elucidate the role of PAE in altered development of the human brainstem, with a particular focus on the development of the neurotransmitter serotonin (5-HT), because 5-HT helps mediate cognitive, affective, and homeostatic networks postulated to be abnormal in FASD, unexplained stillbirth, and/or SIDS (14–20). The rationale for the 5-HT1A receptor analysis in this study is that the receptor reflects a quantifiable parameter in postmortem tissue of the execution of 5-HT signaling in brain neurotransmission, including that related to homeostatic control (16). Moreover, 5-HT via the 5-HT1A receptor is a key trophic factor involved in neuronal proliferation and migration, programmed cell death, axonal path finding, and astroglial maturation in early brain development (21–23); all of these events are adversely affected in animal models by PAE (24).

In this report, we present the methodological and logistical approach, feasibility, challenges, and initial findings in the brainstem (medulla oblongata) analysis of 5-HT1A receptor binding with tissue receptor autoradiography in the Safe Passage Study. The study is currently in progress and has the goal of the evaluation of approximately 12,000 pregnancies and maternal/fetal dyads by January 2017 (12). It involves 2 major high-risk populations for PAE, stillbirth, SIDS, and FASD: (1) American Indian mothers of the Northern Plains, and (2) mixed ancestry mothers of the Western Cape, South Africa (12). It also includes a Caucasian population in the Northern Plains (12). The study provides for neuroanatomic and neurochemical correlates to the amount, pattern, and timing of PAE in demised fetuses and infants (up to 1 postnatal year) of women enrolled in the study since early gestation, and who have given consent for brain research in their offspring in the event of their demise. It is estimated that a total of 180 fetal (stillbirth) and post-hospital discharge infant brains (the latter for assessment in SIDS) will be obtained by the prospective study’s closure. The site of brain analysis is the Developmental Brain and Pathology Center (DBPC), Department of Pathology, Boston Children’s Hospital (12). The brains are accrued from fetuses (stillbirths and post-hospital discharge infants dying of all causes, explained and unexplained (i.e. sudden unexplained infant death [SUID] or SIDS) who succumb in impoverished and often isolated rural areas thousands of miles from the DBPC in Boston.Brains are also obtained from autopsies of premature and full-term infants who die in the hospital of perinatal complications, as part of a broader assessment of fetal and infant mortality. Technical issues center around long-distance shipping and tracking of frozen brain samples, and long (>24 hours) postmortem intervals (PMIs) due to unavoidable logistical complexities involving demised infants whose discovery occurs in homes in isolated and disenfranchised communities.

In this initial study, we tested the hypothesis that the analytic approach to 5-HT1A receptor binding in the developing human brainstem is technically feasible in the Safe Passage Study, and begin to yield novel information about human 5-HT brainstem development. Due to the stipulations of study design, we were blinded in this feasibility analysis to information regarding adverse gestational exposures (eg alcohol and cigarette smoke), as well as to the diagnosis at death (ie SIDS vs control), in order to prevent early bias in the 5-HT1A analyses of brains over the entire 7-year period of the study. Nevertheless, we selected for this “proof-of-concept” study only brainstems that were without major neuropathologic abnormalities. We also compared the 5-HT-related binding patterns in the brainstems accrued from the Northern Plains and South Africa to those archived in our laboratory from the San Diego, CA, medical examiner’s system obtained over 2 decades and considered by us to represent a “gold standard” of information about 5-HT1A binding (25, 26). This comparison was undertaken to identify potentially compromising technical issues.

MATERIALS AND METHODS

Design of the Safe Passage Study

The study’s hypotheses, specific aims, common protocol, enrollment, and compliance in specimen donation have been described in detail (12), as well as the approach to autopsy consent for research in socioeconomically disadvantaged populations (27). Autopsies are performed on site at the clinical centers in the Northern Plains and Western Cape (12), and frozen brain samples are sent to the DBPC, the centralized laboratory for research analysis. Each of these brains is linked to extensive, prospectively collected data related to maternal demographics, prenatal exposures, neurophysiological and neurobehavioral development, placental pathology, and genetics (12). For this study, we analyzed a sample of the 27 medul lae without major macro- or microscopic findings accrued in the early phase of the study (2006–2011). When designed, the Safe Passage Study anticipated and was powered on 37 SIDS and 37 non-SIDS infant deaths, which will allow detection of differences in receptor binding levels as small as 0.67 standard deviations between groups with 80% power. The institutional boards of the local hospitals at which the fetuses and infants were autopsied, as well as Boston Children’s Hospital, approved the use of brain tissues in the Safe Passage Study.

Brain Tissue Accrual, Shipping, and Tracking

The autopsies of the fetuses and infants in this study were performed at 4 local hospitals or medical examiner offices in the Northern Plains (Rapid City, SD, Sioux Falls, SD, Grand Forks, ND, and Bismarck, ND), and Tygerberg Hospital and Medico-legal Laboratory, Cape Town, South Africa
Consents for research were sought as soon after death as possible. Brain samples were packed in dry ice and shipped by a commercial courier company from the local sites to the DBPC. For the South Africa samples, the specimens were first shipped to New York City, where the packing containers were opened and dry ice was refreshed before continuing to Boston, for a total of approximately 3 days. The samples from the Northern Plains were packed in dry ice locally and shipped overnight to Boston according to overnight protocols. The Data Coordinating and Analysis Center (DCAC) located in Malden, Massachusetts tracked the samples according to a proprietary system (12).

Brain Dissection Method and Sampling Protocols

At autopsy, the brain was removed, weighed fresh, examined for gross developmental and acquired abnormalities, and divided into separate right and left cerebral and cerebellar hemispheres, and brainstem. The right cerebral and cerebellar hemispheres and entire brainstem were frozen in toto, and stored at -80 °C in airtight plastic containers until shipping in dry ice and dissection at the DBPC. The left hemisphere is formalin-fixed and sectioned for histopathologic examination. The entire frozen brainstem was blocked into 1.5-cm samples (Fig. 1), and embedded in OCT for sectioning, according to standard methods in our laboratory (25, 26). Serial tissue sections of the brainstem blocks were prepared at 20 μm with a Leica motorized cryostat from each frozen block, mounted on glass microscopic slides, and stored at -20 °C until incubation for radioligand binding.

Tissue Receptor Autoradiography of 5-HT1A Binding in the Medulla

The autoradiography procedures for determination of 3H-8-hydroxy-2-(di-N-propylamino)-tetralin (3H-8-OH-DPAT) binding to 5-HT1A receptors were performed according to...
previously described protocols from our laboratory (25, 26). We determined total 5-HT₁A receptor binding by incubation of tissue sections in 4 nM [³H]-8-OH-DPAT (PerkinElmer Inc., Wellesley, MA) for 60 minutes at room temperature. Non-specific binding was determined by addition of 10 μM 5-HT to the solution. Sections were then placed in cassettes and exposed to a BAS-TR2025 phosphoimaging plate (GE Healthcare Life Sciences, Marlborough, MA) for 4 weeks, with a set of [³H]-standards (Amersham, Buckinghamshire, UK). The standards allowed for the conversion of optical density of silver grains to femtomoles per milligram (fmol/mg) of tissue to determine binding levels. Film autoradiograms were generated according to standard laboratory procedure for development of light-sensitive film. A BAS-5000 Bioimaging Analyzer (FujiFilm) with Image Reader version 1.8 software (FujiFilm) was used to generate digital autoradiographic images from phosphoimaging plates. Quantitative densitometry of autoradiograms was performed using a MCID 5+ imaging system (Imaging Research). Specific receptor binding was determined by subtracting non-specific binding from total binding in individual tissue sections. Alternate sections of the block used for autoradiography were stained with hematoxylin and eosin for histopathologic examination.

Analysis of 5-HT₁A Receptor Binding in the Brainstem

Of relevance to SIDS and unexplained stillbirth as putative disorders of central homeostatic regulation, we focused upon 6 nuclei in the medulla oblongata of critical relevance to homeostatic regulation. These nuclei have been shown to be abnormal previously by us and others in infants dying suddenly, unexpectedly, and in relationship to sleep, i.e. SIDS (25, 26, 28, 29), including in a cohort of SIDS cases and control infants dying less than 12 months of age in which the cause of death was explained by a known entity upon complete autopsy and/or death scene investigation (25, 26).

RESULTS

Technical Considerations

Over the 6 years of tissue accrual and transfer to Boston from the clinical sites in the Northern Plains and South Africa, no sample of the forebrain, cerebellar, and/or brainstem were lost at the local sites, en route, or at the DBPC. One mishap occurred in shipping when the courier airplane was late arriving in New York City due to inclement weather, thereby delaying refurbishment of dry ice in the containers with the packaged brain samples. The late arrival was recognized by the DCAC tracking system, the courier was notified, the shipping container was identified, and ice restocked in the container with minimal thawing of the brain samples.

A second technical concern was maceration of stillbirth brains associated with delay to delivery following intrauterine death. We excluded from analysis those stillbirth brains that were sufficiently macerated to preclude identification of anatomic landmarks. In the total cohort to date, 23% (33/146) of stillbirth brains were thus excluded. In stillbirths with grossly non-macerated brains, the pattern of autoradiography was similar to that of age-related live-born (fetal) infants (Fig. 2), accounting for differences in age and biologic (and potentially pathologic) variability. To date, 12% (9/76) of infant brains have been excluded from neurochemical analysis because of poor tissue integrity and/or substantial tissue distortion upon freezing.

5-HT₁A Receptor Binding in the Developing Brainstem

A total of 27 medullae were examined. None of the medullae demonstrated major abnormalities upon gross and/or microscopic examination; the latter were performed on the frozen sections that generated the autoradiograms and were subsequently examined with hematoxylin and eosin staining. The 27 medullae were obtained from 7 stillbirths, 7 live born, preterm or term infants who died around the time of birth without discharge from the hospital, and 13 post-discharge infants. The cases ranged in age from 23 gestational weeks to 6 postnatal months, i.e. the youngest and oldest case accrued in this study for brainstem. The PMIs ranged from 5 to 99 hours; there was no obvious adverse effect of PMI on 5-HT₁A
receptor binding values, as illustrated in the RO and PGCL (Fig. 3). The pattern of binding in the cases from the clinical sites in the Safe Passage Study was virtually identical to that in cases in the San Diego reference dataset, as demonstrated at the mid-medulla level in which binding was highest in the RO from all infants (Figs. 4, 5). This point is underscored by the positive correlation between the GC and the PGCL binding to 5-HT1A receptors between the Safe Passage Study cases and San Diego reference database (Fig. 6). Of note, the correlation of the GC and the PGCL binding to 5-HT1A receptors in the San Diego reference database was previously published by Duncan et al (26). The Safe Passage Study cases are added to this correlation for comparison.

By midgestation in the human fetus (23 gestational weeks), the relative spatial distribution of 5-HT1A receptor binding was similar to that of the newborn, as demonstrated at the mid-medulla level (Fig. 2). At mid-gestation, the highest binding was in the RO, with intermediate binding in the reticular formation of the lateral tegmentum, and negligible in the inferior olive and arcuate nucleus at the ventral medullary surface. The quantitative binding patterns are comparable between stillbirths and live born fetuses.

FIGURE 2. Developmental images of changes in 5-HT1A receptor binding in the human fetal and infant medulla. The binding patterns are demonstrated at increasing gestational ages from midgestation (23 weeks) until 10 postnatal (PN) months, the youngest and oldest ages analyzed in this study, respectively. The binding levels are standardized to the same scale of fmol/mg tissue in the pseudo-colored autoradiographic images. The highest binding is present at all ages in the midline (RO), with intermediate binding in the reticular formation of the lateral tegmentum, and negligible in the inferior olive and arcuate nucleus at the ventral medullary surface. The quantitative binding patterns are comparable between stillbirths and live born fetuses.

The major findings of this study are that the quantitative analysis of 5-HT1A receptor binding in the developing medulla

1052
oblongata of the human fetus and infant is technically feasible in the Safe Passage Study and that the mapping of its temporal and spatial distribution adds previously unknown information about human medullary 5-HT receptor development. We show here that brains from fetuses and infants autopsied in the diverse sites of the Northern Plains in the United States and the Western Cape in South Africa can be analyzed in a central resource according to a common protocol and yield consistent quantitative measures in the same range, despite variable international locales, autopsy and forensic resources, and pediatric populations. At the biologic level, this study begins to define the spatial and temporal sequences in 5-HT1A receptor development in medullary nuclei directly in the human fetus and infant, providing such previously unknown information about the developmental profiles of 5-HT1A receptor binding in medullary reticular and non-reticular nuclei throughout the last half of human gestation into infancy.

Early Framework of 5-HT1A Receptor Binding in Early Human Medulla Oblongata

Serotonin release is inhibited from 5-HT-producing neurons in the brainstem raphe and extra-raphe via binding to 5-HT1A autoreceptors mainly located upon somata and dendrites (33). Thus, the intense 5-HT1A receptor binding in the medullary RO, with the second highest levels of binding in the GC and PGCL in the reticular formation of the lateral tegmentum in the fetus and infant corresponds to the distribution of 5-HT-producing neuronal cell bodies, and reflects, at least in part, autoreceptor localization on these neurons. We have previously demonstrated that the topographic pattern of the cell bodies (i.e. not binding) of 5-HT-producing neurons in the human medulla is relatively set in an “adult-like” configuration in raphe (midline) and extra-raphe (lateral) sites of the reticular formation by midgestation (34). The RO is critically

FIGURE 3. Comparison of 5-HT1A receptor binding values versus postmortem interval (PMI) in hours in the (A) raphe obscurus and the (B) paragigantocellularis lateralis. The levels are compared between the Safe Passage Study (PASS) (red circles) and laboratory reference cohorts from San Diego (blue squares). There is no obvious difference in binding with increasing PMI in either cohort, including up to approximately 100 h in cases from the Safe Passage Study. This analysis includes only medullary samples from post-discharge infants (0–10 postnatal months) with explained or unexplained causes of death combined. fmol/mg, femtomoles/milligram.

FIGURE 4. The relative quantitative levels of 5-HT1A binding are essentially identical in cases from the Safe Passage Study (PASS) cohort and San Diego reference cohort, as illustrated in representative cases. The same standards were utilized in the radioligand incubations, and the binding patterns were illustrated in pseudo-colored images from tissue receptor autoradiography with the same color scale in fmol/mg tissue. PN, postnatal.
involved in the modulation and integration of respiratory and autonomic functions during sleep and waking (15, 16, 35, 36). Its axonal collaterals diverge to project simultaneously to nuclei that are the final common pathways in control of the upper airway (HG), respiratory rhythm generation (PGCL, the putative human homologue of the pre-Botzinger complex) (37), chemosensitivity (retrotrapezoid nucleus embedded [we believe] in the arcuate nucleus at the ventral medullary surface (15, 34, 38, 39), NTS, locus coeruleus, and other chemoreceptive sites), and respiratory drive (phrenic nucleus in the cervical spinal cord) (16). Importantly, the RO also interfaces directly with the autonomic nervous system and plays a major role in its multifaceted automatic functions per collateralized axonal projections to the NTS (visceral sensory input in the autonomic nervous system that mediates baroreceptor and other reflexes), DMX (preganglionic outflow of the parasympathetic nervous system), nucleus ambiguous (site of cardio-motor neurons involved in heart rate and heart rate variability), and (monosynaptically) to the intermediolateral column (preganglionic outflow of the sympathetic innervation of cardiac muscle, sweat glands, and smooth muscle of blood vessels) (15, 16). Finally, the RO interconnects with more rostral 5-HT source neurons in the median and dorsal raphe of the upper pons and caudal midbrain that are critical components of the ascending arousal network (40–42), as well as the central homeostatic network involving interconnections between

FIGURE 5. Comparison of the binding values across postconceptional age (PCA) is similar in the Safe Passage Study (PASS) and reference cohorts, as illustrated in nuclei of the rostral medulla (raphe obscurus [RO], gigantocellularis [GC] and the paragigantocellularis lateralis [PGCL]) as well as nuclei of the caudal medulla (hypoglossal nucleus [HG], dorsal motor nucleus of the vagus [DMX] and nucleus of the solitary tract [NTS]). Individual cases from the PASS cohort are demonstrated by red circles, and from the San Diego reference cohort, in blue squares. The dotted line marks term-birth (40 gestational weeks). The range of values between the 2 cohorts is similar in the overlapping infant period. Binding in the fetal period is highest in the RO compared to the other nuclei illustrated in the PASS cohort (y-axis is different in each graph). Note that fetal samples are not available in the San Diego cohort.

FIGURE 6. The binding to 5-HT_{1A} receptors correlates positively between the gigantocellularis (GC) and paragigantocellularis lateralis (PGCL) in both the Safe Passage Study (PASS) cohort, shown with red circles for each case and San Diego reference cohort, shown with blue squares. This similar correlation reinforces the validity of binding results in the PASS cohort relative to the reference cohort in this feasibility study.
The localization of 5-HT$_{1A}$ receptors in the RO is a key marker of 5-HT neurotransmission, presumably in place at mid-gestation given the high concentration of these receptors at this site at 23 to 24 gestational weeks. In this study we saw a dramatic decline in binding over the second half of gestation in specific medullary nuclei. In an earlier study by us of mid-gestation and term time points only (but not time points in between as in this study) (43), we saw a similar decrease in binding. This pattern suggests that major functional changes in autonomic and respiratory control involving 5-HT neurotransmission via 5-HT$_{1A}$ receptors occur during this gestational period, and then stabilize relatively at birth, as suggested by the lack of significant change in binding levels from 1 to 10 postnatal months, the period in infancy that we have studied thus far. The stability of 5-HT$_{1A}$ in brainstem nuclei postnatally is consistent with postnatal piglet studies showing relatively little change in binding from postnatal day 4 to postnatal day 60 (44). Developmental studies in postnatal rodents vary depending on the technique [autoradiography (33) or immunocytochemistry (45)] and brainstem nuclei. Gestational data in the animal brainstem are lacking; thus we are unable to compare our high levels of gestational 5-HT$_{1A}$ binding with animal studies. In regard to the Safe Passage Study, of utmost interest in the final analyses are the correlations in changes of 5-HT$_{1A}$ receptor binding in different respiratory- and autonomic-related nuclei with functional autonomic measures in fetuses and infants who succumb to unexplained stillbirth and SIDS (12). This correlative study is currently in progress in a blinded fashion in the Safe Passage Study, and will be uncoded and reported at the end of the entire study in 2017.

Potential Limitations of the Study

An issue of potential concern is the capability to ship frozen samples long distances to the DBPC for central neurochemical processing. The standardized protocols and specifically trained and committed personnel in place at the clinical sites, combined with the comprehensive shipping and tracking system instituted by the DCAC, were instrumental to the success of addressing this challenge. The strength of the tracking system is illustrated in the rapid correction of the mishap in shipping through New York City for refurbishing of dry ice in the course of the long flights involved from Cape Town to Boston described above. A second major concern centered on unavoidably long PMIs for neurochemical brain assessments. At both the Northern Plains and South Africa sites, the sudden loss of a seemingly healthy infant not infrequently results in lengthy PMIs due to: (1) long delays in the transfer of bodies from discovery at homes in isolated communities, particularly in summer months in both the Northern Plains and South Africa in which ambient temperatures can be in the 93°F–103°F range, and (2) prolonged hours from last seen alive to discovery in unwitnessed sleep periods. Multiple published studies of the effects of PMI upon neurotransmitter receptor binding for tissue autoradiography in the human brain indicate that PMIs can affect binding (46, 47), but oftentimes not substantially; the latter observation is postulated to reflect the stability of the protein receptor in membranes. In this study, we analyzed the levels of 5-HT$_{1A}$ receptor binding relative to PMI (in hours), irrespective of the cause of death, and did not find an obvious effect up to approximately 100 h, the longest interval in the cases of this study. We also compared medullary binding levels to those in the San Diego reference database, and found them to be in the same range as the San Diego levels with PMIs generally no longer than 30 h (25, 26). We found that the spatial and temporal sequences of binding in the medulla followed those in previously published studies by us, as exemplified in the San Diego brainstem reference database (25, 26). In the final receptor binding studies of the Safe Passage Study, all quantitative values will be adjusted for PMI as a co-variant, as has been our practice in all receptor binding studies from our laboratory over 2 decades (25, 26, 29, 30).

An additional concern is the reliability of receptor binding studies in stillbirth brains due to maceration of tissues following intrauterine death. In the Safe Passage Study, we automatically excluded from neurochemical analysis stillbirth brains with obvious maceration and liquefaction. The important finding in this feasibility study, however, is that the pattern of 5-HT$_{1A}$ receptor binding is visually and quantitatively the same in live born fetal infants and stillborn fetuses in the same age brackets, thereby ensuring that neurochemical brain analysis is indeed feasible in stillbirth cases selected on the basis of grossly intact tissue integrity.

CONCLUSIONS

We conclude that 5-HT$_{1A}$ receptor binding analysis is feasible in stillbirth and infant brains in the Safe Passage Study, and yields important information about 5-HT chemobehavioral architecture in human brainstem development. The ultimate interpretation of the role of 5-HT$_{1A}$ receptors in human brain development and alcohol-related pathology will be forthcoming at the closure of the entire Safe Passage Study in 2017. Upon closure, this study will integrate information from a large number of brain samples at different ages with linkage to prenatal exposure, other harmful and beneficial genetic and environmental factors, and classification of death (e.g. unexplained stillbirth, SIDS). This preliminary study strongly supports the basic premise that 5-HT$_{1A}$ receptor binding, as mapped quantitatively with radioligands and tissue receptor autoradiography, is a valid and rational approach.

ACKNOWLEDGMENTS

This study was presented in part at the annual meeting of the American Association of Neuropathologists in June 2013. The authors gratefully acknowledge the courageous cooperation of the study mothers to participate in autopsy brain research of their beloved children. We thank Drs. Joseph J. Volpe for critical reading and helpful comments in manuscript preparation. We also thank the members of the NICHD advisory safety monitoring board: Elizabeth Thom, PhD (Chair); The Reverend Phillip Cato, PhD; James W Collins, Jr, MD, MPH; Terry Dwyer, MD, MPH; George Macones, Jr, MD, MPH; George Macones,
MD; Philip A May, PhD; Richard M Pauli, MD, PhD; Raymond W Redline, MD; and Michael Varner, MD. The PASS Network is solely responsible for the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript. The following researchers compose the PASS Network:

PASS Steering Committee Chair (University of Texas Medical Branch): Gary DV Hankins, MD.

Data Coordinating & Analysis Center (DM-STAT, Inc.): PI: Kimberly A Dukes, PhD; Co-PI: Lisa M Sullivan, PhD; Biostatistics: Tara Tripp, MA; Fay Robinson, MPH; Cheri Rafio, MPH; Project Management/Regulatory Affairs: Julie M Petersen, BA; Rebecca A Young, MPH; Statistical Programming/Data Management: Cindy Mai, BA; Elena Grillo, MBA BS, BBA; Data Management/Information Technology: Travis Baker, BS; Patti Folan; Gregory Toland, MS; Michael Carmen, MS.

Developmental Biology & Pathology Center (Children’s Hospital Boston): PI: Hannah C Kinney, MD; Assistant Director: Robin L Haynes, PhD; Co-investigators: Rebecca D Foltzker, MD; Ingrid A Holm, MD; Theonia Boyd, MD; David S Paterson, PhD; Hanno Steen, PhD; Kyriacos Markianos, PhD; Drucilla Roberts, MD; Kevin G Goldstein, PhD; Richard G Goldstein, PhD; Laura L Nelsen, MD; Jacob Cotton, BS; Perri Jacobs, BS.

Comprehensive Clinical Site Northern Plains (Sanford Research): PI: Amy J Elliott, PhD; Co-PI: Larry Burd, Ph.D.; Co-investigators: Jyoti Angal, MPH; Jessica Gromer, RN; H Eugene Hoyne, MD; Margaret Jackson, BA; Luke Mack, MA; Bradley B Randall, MD; Mary Ann Sens, MD; Deborah Tobacco, MA; Peter Van Eerden, MD.

Comprehensive Clinical Site South Africa (Stellenbosch University): PI: Hendrik Odendaal, MBChB, FRCOG, MD; Co-PI: Colleen Wright, MD, FRCPath, PhD; Co-Investigators: Lut Geerts, MD, MRCOG; Greteje de Jong, MBChB, MMed, MD; Pavel Schubert, FCPath (SA) MMed; Shabbir Wadee, MMed; Johan Dempers, FCPath (SA); Elseie Burger, FCPath (SA); MMed Forens Path; Janetta Harbron, PhD; Co-investigator & Project Manager: Coen Groe newald, MBChB, MMed, FCOG, M Comm.

Physiology Assessment Center (Columbia University): Co-PIs: William Fifer, PhD; Michael Myers, PhD; Co-investigators: Joseph Isler, PhD; Yvonne Sinner, PhD; Project Management: J David Nugent, MA; Carmen Condon, BA; Data Analysis: Margaret C Shair, BA; Tracy Thai, MA. NIH Project Scientists: Marian Willinger, PhD (NICHD); Dale Herold, MD, PhD (NIAAA); Howard J Hoffman, MA (NIDCD); Chuan-Ming Li, MD, PhD (NIDCD).

REFERENCES

1. May PA, Baete A, Russo J, et al. Prevalence and characteristics of fetal alcohol spectrum disorders. Pediatrics 2015;134:555-66
2. Norman AL, Crocker N, Riley EP. Neuroimaging and fetal alcohol spectrum disorders. Dev Disabil Res Rev 2009;15:209–17
3. Fifer WP, Ten Fingers S, Youngman M, et al. Effects of alcohol and smoking during pregnancy on infant autonomic control. Dev Psychobiol 2009;51:234–42
4. Kinney HC, Myers MM, Belliveau RA, et al. Subtle autonomic and respiratory dysfunction in sudden infant death syndrome associated with serotonergic brainstem abnormalities: a case report. J Neuropath Exp Neurol 2005;64:689–94
5. Clarrren SK, Alvord EC Jr, Sumi SM, et al. Brain malformations related to prenatal exposure to ethanol. J Pediatr 1978;92:64–71
6. Fifer J, Majewski F, Fischbach H, et al. Alcohol embryo- and fetopathy. Neuropathology of 3 children and 3 fetuses. J Neurol Sci 1979;41:125–37
7. Kesmodel U, Wisborg K, Olsen SF, et al. Moderate alcohol intake during pregnancy and the risk of stillbirth and death in the first year of life. Am J Epidemiol 2002;155:305–12
8. Strandberg-Larsen K, Nielsen NR, Gronhaeck M, et al. Binge drinking in pregnancy and risk of fetal death. Obstet Gynecol 2008;111:602–11
9. O’Leary C, Jacoby P, D’Antoine H, et al. Heavy prenatal alcohol exposure and increased risk for stillbirth. Bjoq 2012;119:945–52
10. Iyassu S, Randall LL, Welty TK, et al. Risk factors for sudden infant death syndrome among Northern Plains Indians. JAMA 2002;288:2717–23
11. O’Leary CM, Jacoby PJ, Bartu A, et al. Maternal alcohol use and sudden infant death syndrome and infant mortality excluding SIDS. Pediatrics 2013;132:1108–17
12. Dukes KA, Burd L, Elliott AJ. For the PASS Research Network, et al. The Safe Passage Study: Design, methods, recruitment, and follow-up approach. Pediatr Perinat Epi 2014;28:455–65
13. Fifer WP, Myers MM. Sudden fetal and infant deaths: shared characteristics and distinctive features. Semin Perinatol 2002;1:89–96
14. Kinney HC, Thach BT. The sudden infant death syndrome. N Eng J Med 2009;361:795–805
15. Kinney HC, Richerson GB, Dynecki SB, et al. The brainstem, serotonin, and sudden infant death syndrome. A Review. Ann Rev Pathol Mech Dis 2009;4:517–49
16. Kinney HC, Broadbelt KG, Haynes RL, et al. The serotonergic anatomy of the human developing medulla oblongata: Implications for pediatric disorders of homeostasis. (Invited). J Chem Neuroanat 2011;41:82–99
17. Mao J, Ma H, Xu Y, et al. Increased levels of monoamine-derived potential neurotoxins in fetal rat brain exposed to ethanol. Neurochem Res 2013;38:356–63
18. Druse MJ, Tajuddin NF, Gillespie RA, et al. The serotonin-1A agonist ipsapirone prevents ethanol-associated death of total rhombencephalic neurons and prevents the reduction of fetal serotonin neurons. Brain Res Dev Brain Res 2004;150:79–88
19. Sari Y, Prowrozek T, Zhou FC. Alcohol disrupts the outgrowth of serotonergic neurons at midgestation. J Biomed Sci 2001;8:119–25
20. Sari Y, Zhou FC. Prenatal alcohol exposure causes long-term serotonin neuron deficit in mice. Alcohol Clin Exp Res 2004;28:941–8
21. Laufer JM. Outgrowth of the serotonergic system in the rat: serotonin as a developmental signal. Ann NY Acad Sci 1996;800:297–313
22. Bonnin A, Torii M, Wang L, et al. Serotonin modulates the response of embryonic thalamocortical axons to netrin-1. Nat Neurosci 2007;10:588–97
23. Janusonis S, Glunick V, Rakic P. Early serotonergic projections to Cajal-Retzius cells: relevance for cortical development. J Neurosci 2004;24:1652–9
24. Sari Y, Hammad LA, Saleh MM, et al. Alteration of selective neurotransmitters in fetal brains of prenatally alcohol-treated C57BL/6 mice: Quantitative analysis using liquid chromatography/tandem mass spectrometry. Int J Dev Neurosci 2010;28:263–9
25. Paterson DS, Thompson EG, Belliveau RA, et al. Multiple abnormalities in the brainstem serotonergic system in sudden infant death syndrome. JAMA 2006;296:2124–32
26. Duncan JR, Paterson DS, Hoffman JM, et al. Brainstem serotonergic deficiency in the sudden infant death syndrome. JAMA 2010;303:430–7
27. Odendaal II, Elliott A, Kinney HC, et al. Consent for autopsy research for unexpected death in early life. Obstet Gynecol 2011;117:167–71
28. Machtelani R, Say M, Waters KA. Serotonergic receptor 1A in the sudden infant death syndrome brainstem medulla and associations with clinical risk factors. Acta Neuropathol 2011;125:37–49
29. Panigrahy A, Filiano J, Sleeper LA, et al. Decreased serotonergic activity in mice exposed to prenatal alcohol. J Chem Neuroanat 2011;41:82–99
30. Panigrahy A, Filiano J, Sleeper LA, et al. Decreased serotonergic activity in mice exposed to prenatal alcohol. J Chem Neuroanat 2011;41:82–99
31. Olszewski J, Baxter D. Cytoarchitecture of the Human Brain Stem. 2nd ed. Basel, Switzerland: Karger, 1981
32. Paxinos G, Huang X-F. Atlas of the Human Brainstem. New York, NY: Academic Press, 1995
33. Massey CA, Kim G, Corcoran AE, et al. Development of brainstem 5-HT1A receptor binding sites in serotonin-deficient mice. J Neurochem 2013;126:749–57
34. Kinney HC, Rava LA, Belliveau RA, et al. The development of the medullary serotonergic system in early human life. Auton Neurosci 2007;132:78–102
35. Lovick TA. The medullary raphe nuclei: a system for integration and gain control in autonomic and somatomotor responsiveness? Exp Physiol 1997;82:31–41
36. Mason P. Contributions of the medullary raphe and ventromedial reticular region to pain modulation and other homeostatic functions. Annu Rev Neurosci 2001;24:737
37. Schwarzacher SW, Rub U, Deller T. Neuroanatomical characteristics of the human pre-Botzinger complex and its involvement in neurodegenerative brainstem diseases. Brain 2011;134:24–35
38. Filiano JJ, Choi JC, Kinney HC. Candidate cell populations for respiratory chemosensitive fields in the human infant medulla. J Comp Neurol 1990;293:448–65
39. Paterson DS, Thompson EG, Kinney HC. Serotonergic and glutamatergic neurons at the ventral medullary surface of the human infant: Observations relevant to central chemosensitivity in early human life. Auto Neurosci 2006;124:112–24
40. Bang SJ, Jensen P, Dymecki SM, et al. Projections and interconnections of genetically defined serotonin neurons in mice. Eur J Neurosci 2012;35:85–96
41. McCormick DA. Neurotransmitter actions in the thalamus and cerebral cortex and their role in neuromodulation of thalamocortical activity. Prog Neurobiol 1992;39:337–88
42. Edlow BL, McNab JA, Witzel T, et al. The structural connectome of the human central homeostatic network. Brain Connect 2016;6:187–200
43. Zec N, Filiano JJ, Panigrahy A, et al. Developmental changes in [3H]lysergic acid diethylamide ([3H]LSD) binding to serotonin receptors in the human brainstem. J Neuropathol Exp Neurol 1996;55:114–26
44. Niblock MM, Kinney HC, Luce CJ, et al. The development of the medullary serotonergic system in the piglet. Auton Neurosci 2004;110:65–80
45. Liu Q, Wong-Riley MT. Postnatal changes in the expression of serotonin 1A, 1B, and 2A receptors in the ten brain stem nuclei of the rat: implication for a sensitive period. Neuroscience 2010;165:61–78
46. Kontur PJ, al-Tikriti M, Innis RB, et al. Postmortem stability of monoamines, their metabolites, and receptor binding in rat brain regions. J Neurochem 1994;62:282–90
47. González-Maeso J, Torre I, Rodriguez-Puertas R, et al. Effects of age, postmortem delay and storage time on receptor-mediated activation of G-proteins in human brain. Neuropsychopharmacology 2002;26:468–78