

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

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## 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-HT<sub>1A</sub> receptor binding using autoradiography in the medulla oblongata (6 nuclei in 27 cases). 5-HT<sub>1A</sub> binding was compared to a reference dataset from the San Diego medical examiner's system. There was no adverse effect of postmortem interval  $\leq 100$  h. The distribution and quantitated values of 5-HT<sub>1A</sub> 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-HT<sub>1A</sub> 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).

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## 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 intrauterine fetal demise  $>20$

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-HT<sub>1A</sub> 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-HT<sub>1A</sub> 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-HT<sub>1A</sub> 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-HT<sub>1A</sub> 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-HT<sub>1A</sub> 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-HT<sub>1A</sub> binding (25, 26). This comparison was undertaken to identify potentially compromising technical issues.

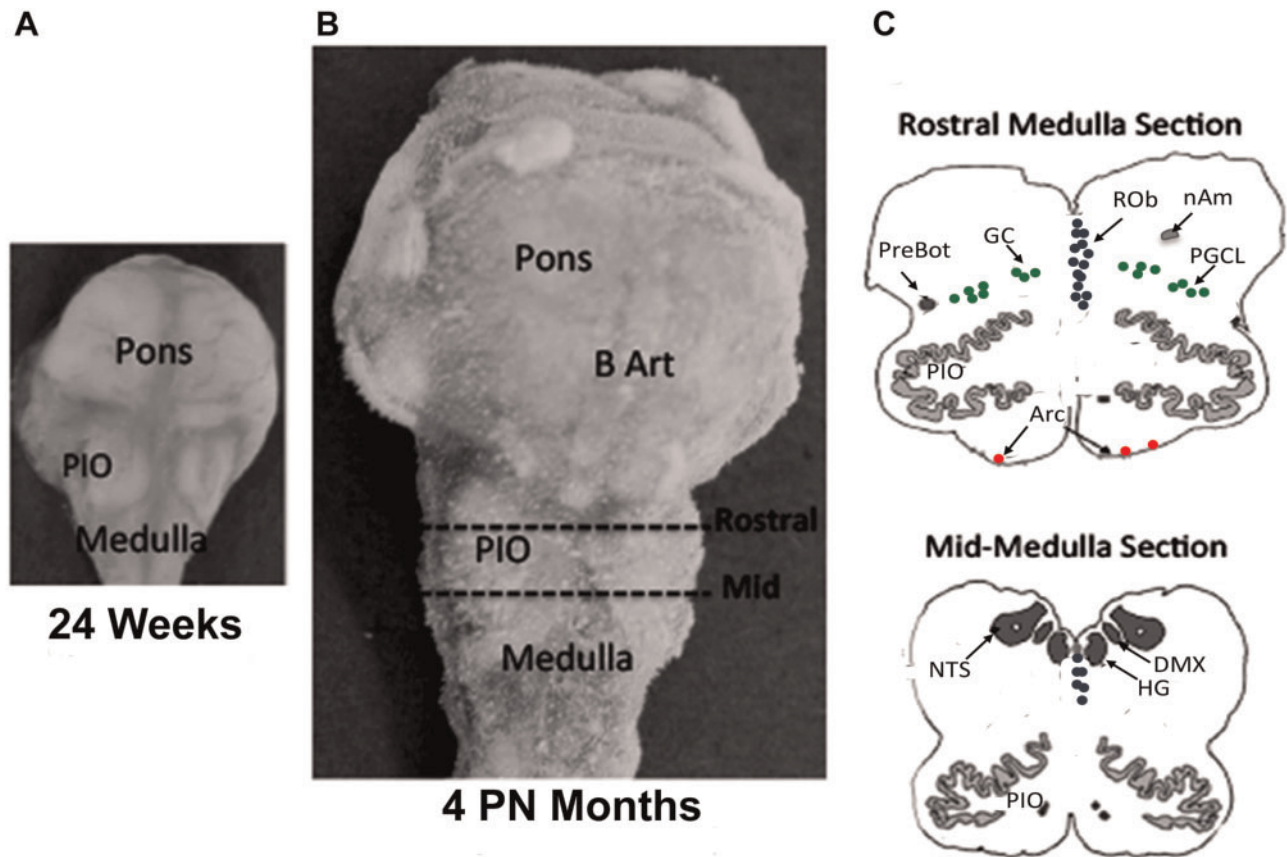
## MATERIALS AND METHODS

### 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 medullae 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



**FIGURE 1.** Dissection of the medulla oblongata for analysis in the Safe Passage Study. (**A–C**) The brainstems of stillbirths (**A**) and infant demises (**B**) are received at the Developmental Biology and Pathology Center in Boston from the local clinical sites. The medulla oblongata is analyzed at 2 levels, i.e. rostral and mid-level (dotted lines). The nuclei measured at the rostral level (**C**) are the raphe obscurus (ROb), gigantocellularis (GC), and paragigantocellularis lateralis (PGCL) in which the putative human homologue of the pre-Botzinger complex (preBot), the central rhythm generator, is embedded. The nuclei measured at the mid level (**C**) are the hypoglossal nucleus (HG), nucleus of the solitary tract (NTS), and dorsal motor nucleus of the vagus (DMX) (16). The blue circles represent 5-HT neurons in the midline (raphe), the green circles, 5-HT neurons in the lateral tegmentum (extraraphe), and the red circles, 5-HT neurons at the ventral medullary surface embedded in the arcuate nucleus (Arc) (**C**) (16). Other abbreviations: B Art, basilar artery; nAM, nucleus ambiguus; PIO, principal inferior olive; PN, postnatal.

(12). 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  $-80^{\circ}\text{C}$  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  $20\ \mu\text{m}$  with a Leica motorized cryostat from each frozen block, mounted on glass microscopic slides, and stored at  $-20^{\circ}\text{C}$  until incubation for radioligand binding.

### Tissue Receptor Autoradiography of 5-HT<sub>1A</sub> Binding in the Medulla

The autoradiography procedures for determination of  $^3\text{H}$ -8-hydroxy-2-(di-N-propylamino)-tetralin ( $^3\text{H}$ -8-OH-DPAT) binding to 5-HT<sub>1A</sub> receptors were performed according to



previously described protocols from our laboratory (25, 26). We determined total 5-HT<sub>1A</sub> receptor binding by incubation of tissue sections in 4 nM <sup>3</sup>H-8-OH-DPAT (PerkinElmer Inc., Wellesley, MA) for 60 minutes at room temperature. Nonspecific 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 <sup>3</sup>H-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 (Fuji-Film) 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 nonspecific 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-HT<sub>1A</sub> 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 controls of American Indian infants from the Northern Plains (30). The brainstem nuclei were defined according to the atlas of Olszewski and Baxter (31), with the exception of the raphe obscurus, which was defined according to the atlas of Paxinos and Huang (32). For each specimen, 5-HT<sub>1A</sub> receptor binding density was measured at 2 levels: the mid-medulla (level of nucleus of Roller [Plate XII [31]]) included the nucleus of the solitary tract ([NTS] visceral afferent processing of the autonomic nervous system, including baroreceptor reflexes and responses to hypoxia), the hypoglossal nucleus ([HG] upper airway patency, including during sleep), and the dorsal motor nucleus of the vagus ([DMX] preganglionic outflow of the parasympathetic branch of the autonomic nervous system (Fig. 1). The binding density was also measured in the rostral medulla at the level of the nucleus prepositus (Plate XIV [31]). At this level, 3 5-HT producing nuclei of the 5-HT homeostatic network of the rostral medulla oblongata were measured (16), i.e. gigantocellularis (GC), paragigantocellularis lateralis (PGCL), and raphe obscurus (RO), which are involved in cardiorespiratory integration and arousal (Fig. 1).

### Reference Archives of the San Diego Dataset

We utilized San Diego brainstem database archived in our laboratory as the reference standard for comparison of

5-HT<sub>1A</sub> receptor binding measures obtained in cases in the Safe Passage Study. This reference database was accrued from 2007 to the present. A subset of these reference cases (n = 22) were previously published by Paterson et al (25); the remaining cases (n = 40) were previously published by Duncan et al (26). The materials and methods of this dataset are identical to those in the Safe Passage Study, as previously reported in detail (25, 26). The same methodology of binding analysis was applied with the use of the same radioligand standards thereby allowing for comparison among the standard reference and Safe Passage Study datasets. In the standard reference dataset, information is available for cases dying of SIDS and autopsy controls. Control cases were 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

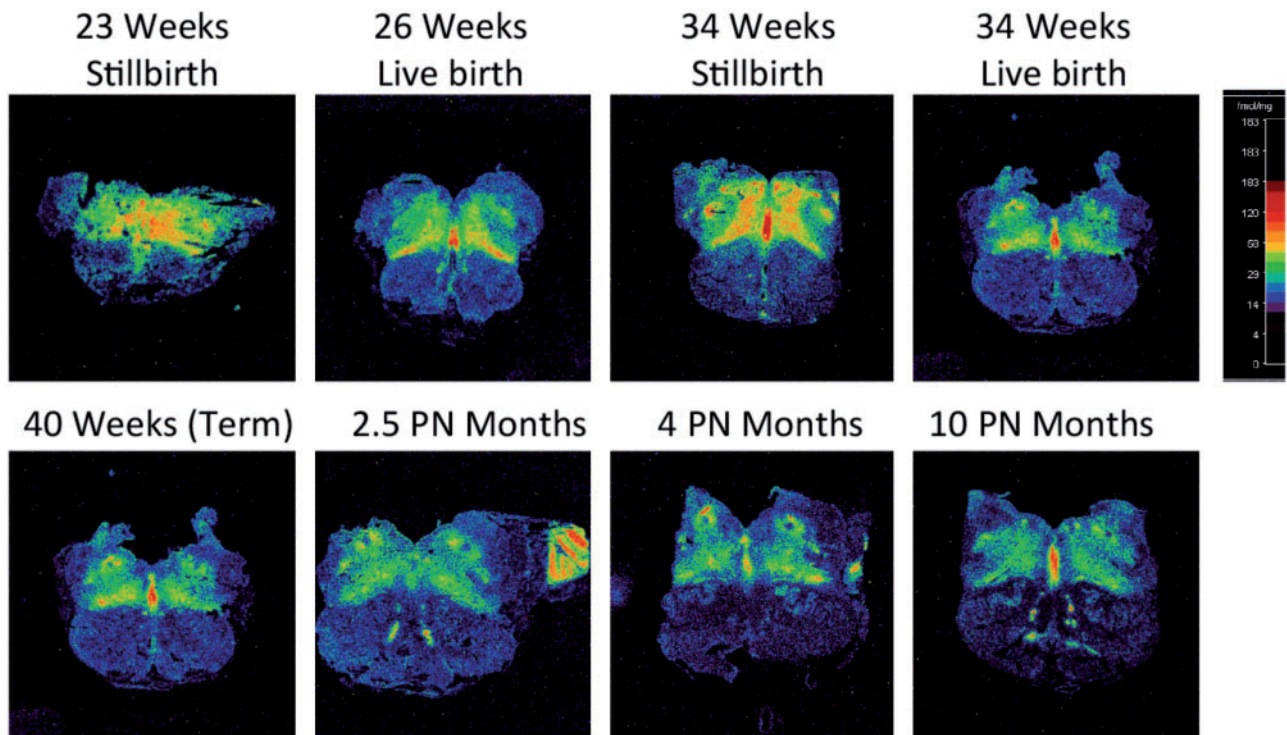
### Technical Considerations

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-HT<sub>1A</sub> 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-HT<sub>1A</sub>



**FIGURE 2.** Developmental images of changes in 5-HT<sub>1A</sub> 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.

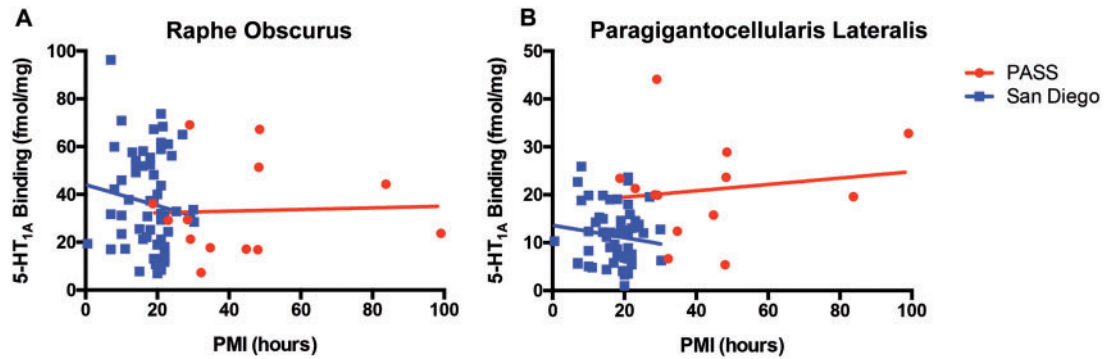
receptor binding values, as illustrated in the RO and PGCL (Fig. 3). The pattern of binding in the cases from the clinical sites in the Safe Passage Study was virtually identical to that in cases in the San Diego reference dataset, as demonstrated at the mid-medulla level in which binding was highest 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-HT<sub>1A</sub> 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-HT<sub>1A</sub> 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-HT<sub>1A</sub> receptor binding was similar to that of the newborn, as demonstrated at the mid-medullary level (Fig. 2). At mid-gestation, the highest binding was in the RO, with intermediate binding in the reticular formation of the tegmentum (including the GC and PGCL), and negligible binding in the olivary complex and arcuate nucleus at the ventral medullary surface (Fig. 2). Over the last half of gestation, however, binding tended to decrease and reach a relative plateau thereafter with binding levels between 10–20 fmol/mg tissue through 80 postconceptional weeks (10 postnatal months, oldest of the cases from the San Diego reference dataset) (Fig 5). This decrease during the last half of

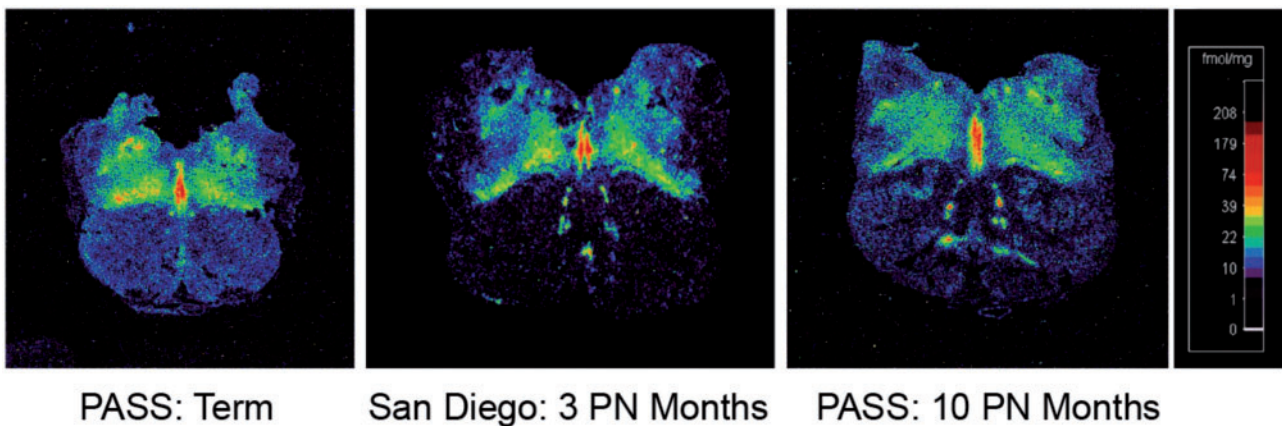
gestation is most notable in the GC, PGCL and NTS, which showed a 55–66% decrease in binding when comparing cases at mid-gestation to cases from term birth to 10 postnatal months (Fig 5). In addition, visually detectable binding appeared in the medial accessory nucleus of the olivary complex in the neonate whereas it was not apparent at midgestation (Fig. 2). Binding levels were similar between fetal, neonatal, and infant nuclei at the floor of the fourth ventricle (Figs. 2, 5), including in the DMX, HG, NTS. The spatial distribution and levels of 5-HT<sub>1A</sub> binding remained relatively constant after birth, with no dramatic changes at any site after birth (Figs. 2, 5). Across the age span studied binding levels were highest in the RO (Figs. 2, 4, 5), and were negligible in the principal inferior olive and arcuate nucleus (Fig. 2). There was variability in the postnatal 5-HT<sub>1A</sub> receptor binding levels in the cohort of the Safe Passage Study, and it remains to be determined whether this variability reflects differences in prenatal exposures and diagnosis (e.g. SIDS vs non-SIDS). Nevertheless, the binding levels fell within the range of the San Diego cohort, likewise comprised of SIDS and non-SIDS cases (Figs. 4–6).

## DISCUSSION

The major findings of this study are that the quantitative analysis of 5-HT<sub>1A</sub> receptor binding in the developing medulla



**FIGURE 3.** Comparison of 5-HT<sub>1A</sub> 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.



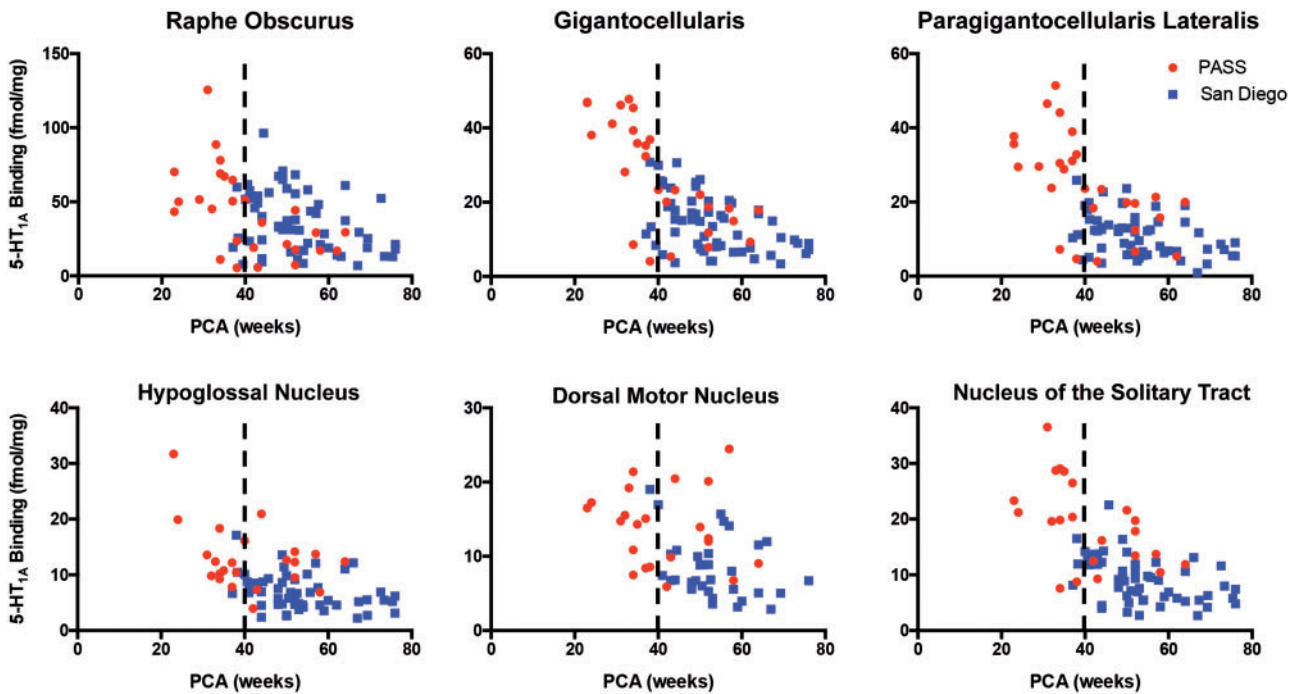
**FIGURE 4.** The relative quantitative levels of 5-HT<sub>1A</sub> binding are essentially identical in cases from the Safe Passage Study (PASS) cohort and San Diego reference cohort, as illustrated in representative cases. The same standards were utilized in the radioligand incubations, and the binding patterns were illustrated in pseudo-colored images from tissue receptor autoradiography with the same color scale in fmol/mg tissue. PN, postnatal.

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-HT<sub>1A</sub> receptor development in medullary nuclei directly in the human fetus and infant, providing such previously unknown information about the developmental profiles of 5-HT<sub>1A</sub> receptor binding in medullary reticular and non-reticular nuclei throughout the last half of human gestation into infancy.

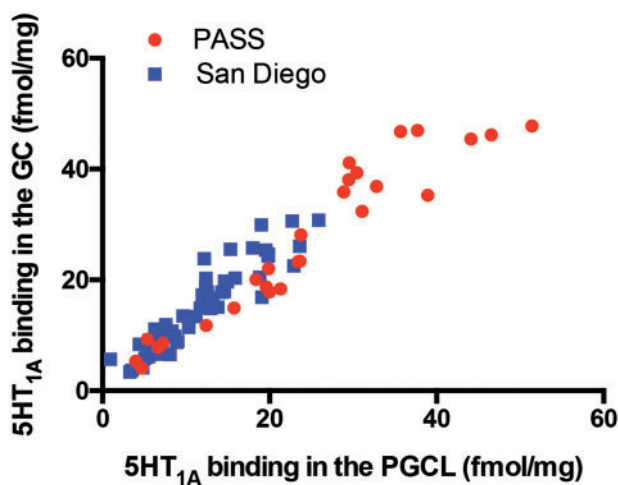
### Early Framework of 5-HT<sub>1A</sub> Receptor Binding in Early Human Medulla Oblongata

Serotonin release is inhibited from 5-HT-producing neurons in the brainstem raphe and extra-raphe via binding to 5-HT<sub>1A</sub> autoreceptors mainly located upon somata and dendrites (33). Thus, the intense 5-HT<sub>1A</sub> 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 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<sub>1A</sub> 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.

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 ambiguus (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

brainstem and forebrain sites (42). Thus, the RO is exquisitely positioned to modulate and integrate into a unified response the varied homeostatic functions thought to be impaired in unexplained stillbirth and SIDS, as set forth in the hypotheses driving the overall Safe Passage Study (12).

The localization of 5-HT<sub>1A</sub> 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<sub>1A</sub> 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<sub>1A</sub> 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<sub>1A</sub> 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<sub>1A</sub> 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<sub>1A</sub> 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<sub>1A</sub> 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<sub>1A</sub> receptor binding analysis is feasible in stillbirth and infant brains in the Safe Passage Study, and yields important information about 5-HT chemoarchitecture in human brainstem development. The ultimate interpretation of the role of 5-HT<sub>1A</sub> 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<sub>1A</sub> receptor binding, as mapped quantitatively with radioligands and tissue receptor autoradiography, is a valid and rationale approach.

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