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Neuroanatomic Relationships between the GABAergic and Serotonergic Systems in the Developing Human Medulla

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Abstract

γ -Amino butyric (GABA) critically influences serotonergic (5-HT) neurons in the raphé and extra-raphé of the medulla oblongata. In this study we hypothesize there are marked changes in the developmental profile of markers of the human medullary GABAergic system relative to the 5-HT system in early life. We used single- and double-label immunocytochemistry and tissue receptor autoradiography in 15 human medullae from fetal and infant cases ranging from 15 gestational weeks to 10 postnatal months, and compared our findings with an extensive 5-HT-related database in our laboratory. In the raphé obscurus, we identified two subsets of GABAergic neurons using glutamic acid decarboxylase (GAD65/67) immunostaining: one comprised of small, round neurons; the other, medium, spindle-shaped neurons. In three term medullae cases, positive immunofluorescent neurons for both tryptophan hydroxylase and GAD65/67 were counted within the raphé obscurus. This revealed approximately 6% of the total neurons counted in this nucleus expressed both GAD65/67 and TPOH suggesting co-production of GABA by a subset of 5-HT neurons. The distribution of GABA_A binding was ubiquitous across medullary nuclei, with highest binding in the raphé obscurus. GABA_A receptor subtypes $\alpha 1$ and $\alpha 3$ were expressed by 5-HT neurons, indicating the site of interaction of GABA with 5-HT neurons. These receptor subtypes and KCC2, a major chloride transporter, were differentially expressed across early development, from mid-gestation (20wks) and thereafter. The developmental profile of GABAergic markers changed dramatically relative to the 5-HT markers. These data provide baseline information for medullary studies of human pediatric disorders, such as sudden infant death syndrome.

Keywords

Autoradiography; glutamic acid decarboxylase; KCC2 co-transporter; paragigantocellularis lateralis; preBötzing complex; raphé obscurus; sudden infant death syndrome

INTRODUCTION

γ -Amino butyric (GABA), the major neurotransmitter of inhibitory synaptic neurotransmission, influences virtually every homeostatic function mediated by the medulla

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oblongata. These functions include respiration (Pierrefiche et al., 1998; Shao et al., 1997), chemosensitivity to carbon dioxide (Curran et al., 2000; Curran et al., 2001; Curran et al., 2002; Kanazawa et al., 1998; Kuribayashi et al., 2008), blood pressure regulation (Dampney, 1994; Heesch et al., 2006; Menezes et al., 2007) and the laryngeal chemoreflex (Bohm et al., 2007; Van der Velde et al., 2003). While many biogenic amines and neuropeptides modulate the effects of GABA and vice-versa, GABA's interactions with serotonin (5-HT) are particularly important and are the focus of this report. Like the medullary GABAergic system, the 5-HT medullary system is critically involved in homeostatic control. The 5-HT medullary system is comprised of the raphé (raphé obscurus, raphé pallidus, raphé magnus), extra-raphé (paragigantocellularis lateralis, gigantocellularis, intermediate reticular zone), and ventral surface arcuate nucleus in the human (Kinney et al., 2007), all sites where 5HT source neurons are located. The 5-HT medullary regions are involved in the modulation of breathing, blood pressure, chemosensitivity and/or heart rate according to the level of arousal (Kinney, 2009). The 5-HT source neurons within this region of the medulla project to nuclei that mediate cardiorespiratory control and other homeostatic functions, e.g., hypoglossal nucleus (upper airway control) and the nucleus of the solitary tract (visceral sensory input), they comprise the medullary 5-HT system. These nuclei are interconnected and have been shown to play critical roles in homeostatic processes. The majority of Sudden Infant Death Syndrome (SIDS) cases have been associated with multiple serotonergic (5-HT) abnormalities in regions of the medulla oblongata involved in cardiorespiratory control during sleep (Kinney et al., 2003; Machaalani et al., 2009; Ozawa et al., 2002; Panigrahy et al., 2000; Paterson et al., 2006). Animals studies indicate that GABA neurons and receptors are enmeshed in this 5-HT network (Holmes et al., 1994a; Holmes et al., 1994b; Kachidian et al., 1991; Stamp et al., 1995); moreover, sub-populations of 5-HT neurons in the medullary raphé co-express GABA; and GABA receptors, all suggestive of an important relationship between the two neurotransmitters (Belin et al., 1983; Cao et al., 2003; Jones et al., 1991; Lovick, 1988a; Lovick, 1988b; Nosaka et al., 2000). Yet, the anatomic relationship of these two neurotransmitter systems relative to one another is essentially unknown in the developing human medulla. This lack of knowledge is of particular concern relative to efforts to elucidate complex brainstem disorders in early human life, including the sudden infant death syndrome (Kinney et al., 2003; Machaalani et al., 2009; Ozawa et al., 2002; Panigrahy et al., 2000; Paterson et al., 2006).

In this study, we tested the hypothesis that there are marked changes in the developmental profile of GABAergic markers of the human medullary system relative to the 5-HT system in early human life. We used a combination of techniques to delineate the chemical anatomy of the GABAergic and 5-HT systems in the developing human medulla. We determined the co-localization of GABA in 5-HT neurons with antibodies to tryptophan hydroxylase and GAD65/67, the biosynthetic enzymes for 5-HT and GABA, respectively. We also applied tissue receptor autoradiography to determine the regional distribution and the density of GABA_A receptor binding sites in the infant medulla, and single and double-labeled immunocytochemistry to determine the distribution of neurons expressing KCC2, and GABA_{Aα3} and GABA_{Aα1} receptors. In these studies, we focused upon GABA_A receptors because they are strongly implicated in medullary homeostatic functions (Cao et al., 2003; Liu et al., 2006; Mulkey et al., 2007). They demonstrate extensive structural heterogeneity based upon at least 15 subunits (α 1-6, β 1-3, γ 1-3, δ , and ρ 1-2) that are encoded by distinct genes (Hornung et al., 1996; Macdonald et al., 1994); the subunit composition determines the functional and pharmacological properties of the receptor and is developmentally controlled. We specifically chose to delineate the distribution of the α 1 and α 3 subunits because of rat data indicating a developmental change in their expression at postnatal day 12 (P12) in the preBötzinger complex and nucleus of the solitary tract, i.e., nuclei critical for respiratory control (Hornung et al., 1996; Liu et al., 2006; Wong-Riley et al., 2005). We also analyzed the developmental profile of KCC2, a neuron-specific member of the K-CL co-transporter family which is known to co-localize with the GABA_A receptor and mediate the trans-membrane

extrusion of chloride ions (Miles, 1999; Rivera et al., 1999; Williams et al., 1999). Finally, we integrated the GABA findings with those related to 5-HT chemical anatomy that has been observed in our laboratory over the last decade involving the topography of 5-HT neurons, receptors, and transporter (Kinney et al., 2007; Kinney et al., 2001a; Paterson et al., 2004; Zec et al., 1996). We believe the use of this 5-HT archival database in relationship to new data about GABAergic chemical anatomy is justified because of the difficulty in accruing precious brain tissues of human fetuses and infants over time, and the need to use all tissues that are available in the most productive way. In the following study, we build upon the 5-HT database and provide new information about the spatial distribution of GABA neurons, receptors, and transporters relative to medullary 5-HT chemical anatomy across early human development.

MATERIALS AND METHODS

Clinical Database

Normative medullae brainstems were accrued from fetal and infant cases autopsied in the Department of Pathology, Children's Hospital Boston, MA, and the Office of the Chief Medical Examiner, San Diego, CA. This study was approved by the Committee on Clinical Investigation at Children's Hospital of Boston.

Tissue Preparation for GABA_A Receptor Tissue Section Autoradiography

Our procedures for tissue preparation have been described in detail previously (Kinney et al., 2001b; Panigrahy et al., 2000). Unfixed brainstem sections were stored frozen at -70°C , and subsequently sectioned at $20\mu\text{m}$ on a Leitz motorized cryostat. Alternate sections from each infant case were used for binding analysis with the radioligand for the selected GABA receptor.

Assessment of GABA_A Binding

Total GABA_A receptor binding was determined by incubation of unfixed slide-mounted tissue sections in 50nM ^3H -GABA (PerkinElmer Inc, Wellesley, MA) for 20 minutes at room temperature. The tissue sections were pre-incubated in 50mM Tris-HCL (pH 7.4) containing $100\mu\text{M}$ Baclofen (a GABA_B agonist) for 45-60 minutes at room temperature. Non-specific binding was determined by addition of $100\mu\text{M}$ isoguvacine to the solution. Following the incubation, the sections were washed in 50mM Tris-HCL (pH 7.4) for 10 seconds. The sections were then rinsed in ice cold water for 10 seconds before drying in a warm stream of air. Tissue sections were placed in cassettes and exposed to ^3H -Hyperfilm, (Amersham) for 8 weeks at 4°C with a set of tissue equivalent ($\mu\text{Ci/g}$) ^3H standards (Amersham, Buckinghamshire, England) for conversion of optical density of silver grains to femtomoles/milligram of tissue (fmol/mg tissue) to determine GABA_A binding. Film autoradiograms were generated according to standard laboratory procedure for development of light sensitive film. For each specimen, receptor binding density was analyzed in 10 medullary nuclei at a defined level (see below) of the brainstem (2 autoradiograms for each nucleus) according to previously published methods (Kinney et al., 2001a; Panigrahy et al., 2000; Paterson et al., 2004). Quantitative densitometry of autoradiograms was performed using a MCID 5+ imaging system (Imaging Research, Ontario, Canada).

The medulla was analyzed at standardized levels based upon the human brainstem atlas of (Olszewski et al., 1982; Paxinos et al., 1995; Törk et al., 1990) according to published protocols from our laboratory (Kinney et al., 2007; Kinney et al., 2001a). The brainstem atlas of Paxinos and Huang (1995) was utilized for the modern nomenclature of the brainstem raphé nuclei. The mid-medulla at the level of the nucleus of Roller was analyzed, as depicted in Plate XII in the brainstem atlas of (Olszewski et al., 1982); the rostral medulla at the level of the nucleus paragigantocellularis (PGCL) was also analyzed, as depicted in Plate XIV. For the anatomic analysis of 5-HT chemical anatomy the medullary 5-HT system was defined by us as the regions

of the medulla that contain 5-HT cell bodies and those that receive the major projections from these 5-HT cell bodies. The 5-HT 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) (Kinney et al., 2007). The major projection sites of the medullary 5-HT neurons include the hypoglossal nucleus (HG), nucleus of the solitary tract (NTS), and dorsal motor nucleus of the vagus (DMX).

GABA-Related, Single-label Immunocytochemistry

Formalin-fixed, slide mounted paraffin-embedded tissue sections were deparaffinized using a series of incubations in xylene, and graded alcohol concentrations, and immediately washed in running water for 2-3 minutes. An Antigen retrieval method was employed; sections were placed in 1× citrate buffer (pH 6.0) and heated to 195°C in a microwave (with a temperature probe to maintain constant temperature) for 10 minutes. Sections were removed from the microwave and allowed to cool at room temperature in the 1× citrate buffer for 5 minutes, after which they were rinsed under running water for 2-3 minutes. Sections were incubated with a commercial peroxidase blocking reagent (DakoCytomation) for 6 minutes at room temperature to block endogenous peroxidase activity. To block non-specific staining, the slides were placed in a solution containing of BSA: 4% normal goat serum in 1× phosphate buffered saline (PBS) for 1 hour (modified for the GABA receptor immunocytochemistry by adding 3% BSA and 0.1% triton x-100). Specific sections were incubated with individual antibodies at various dilutions (Table 2) overnight at 4°C. Sections were next incubated in the secondary antibodies: Biotin-SP-conjugated AffiniPure Anti-Rabbit IgG (Jackson ImmunoResearch) at 1:500 dilution in the modified blocking solution for 30 minutes at room temperature, followed by incubation with peroxidase-conjugated streptavidin at 1:500 dilution for another 30 minutes. Immunoreactivity was detected using a DAB peroxidase substrate kit (Vector laboratories); the reaction was stopped with cold PBS (pH 7.4). Sections were then washed in running water, dehydrated, and coverslipped. The finished stained slides were visualized with an Olympus BX51 microscope (Olympus America Inc., Melville, NY) with image capture using an Optronics Microfire S99808 camera and Microfire 1.0 and Neurolucida 5.0 software (Microbrightfield, Colchester, VT).

We devised a semi-quantitative grading system to compare immunostaining intensity in cases at different ages, the grading system has been described in detail previously (Rognum et al., 2009). We first identified upon survey of all cases a site as an internal standard which was immunostained with the same intensity irrespective of age; this site was the ependymal cells, known to express GABA_A receptors (Casellas et al., 2002), lining the fourth ventricle. These standards were assigned after a complete review of all slides in a blinded fashion, demonstrating the uniformity of staining intensity of these regions across all cases and ages. We designated the intensity of this “standard” as grade 2 and visually scored neurons in the selected nuclei relative to this standard as: Grade 0 = no neuronal immunostaining; Grade 1 = less intense immunostaining than standard, Grade 2 = equal immunostaining to standard; Grade 3 = more intense immunostaining than standard, as illustrated for the GABA_{Aα3} antibody (Fig. 1). The staining was assessed in a blinded fashion, without knowledge of age of the subject. Intensity scoring was performed in the following regions: raphé obscurus (midline site of 5-HT neurons); extra-raphé (nucleus gigantocellularis and paragigantocellularis, lateral site of 5-HT neurons); arcuate nucleus (ventral medulla, embedded subset of 5-HT neurons); hypoglossal nucleus (upper airway patency, receives 5-HT terminals), dorsal motor nucleus of vagus (preganglionic outflow of parasympathetic system, receives 5-HT terminals) and nucleus of the solitary tract (visceral sensory input, receives 5-HT terminals). Three (3) sections at the correct anatomical levels for each case were investigated blindly by two observers (KGB and KDR). The grades of the intensity of immunostaining are presented as the mean ± standard error of the mean.

Linear and quadratic regression analysis was used to determine the effects of postconceptional age on the expression of the different receptors subtypes shown by immunohistochemistry. P-values <0.05 were considered significant.

Immunocytochemical Tissue Controls

In all assays, closely adjacent tissue sections to that with the primary antibodies GAD65/67, GABA_{Aα1}, GABA_{Aα3}, and KCC2, as well as the other selected markers (Table 1), were incubated in 4% NGS overnight in the absence of the primary antibody in order to determine that the immunohistochemical staining observed was specific for the target protein. A pre-absorption assay for GAD65/67 antibody, because of the well-recognized particular difficulty in working with this antigen was performed in the tissue sections which were incubated with primary antibody pre-incubated with a saturating concentration of the target protein (Invitrogen, Carlsbad, CA); we observed absent immunostaining after pre-absorption (Fig. 1). Western blot analysis revealed the specific protein band size (65 kDa) for the GAD65/67 antibody, as also shown by the company (ABCAM, Cambridge, MA) (Fig. 3).

GABA-Related and 5-HT Markers with Double-Label Immunocytochemistry

The same protocol used for single label immunocytochemistry was employed up to the addition of using multiple primary antibodies. The sections were used to determine co-localization and distribution of GABAergic markers, 5-HT neurons, and other markers (Table 1). Double-label immunofluorescence was performed for: (1) tryptophan hydroxylase (TPOH) and GAD65/67; 2) TPOH and GABA_{Aα3} receptors; 3) TPOH and GABA_{Aα1} receptors; 4) KCC2 and myelin basic protein (MBP); 5) KCC2 and neurofilament; and 6) GAD65/67 and choline acetyl transferase (ChAT). The same methods were employed as mentioned in the description of the single label immunocytochemical experiments previously with the exception of a cocktail mixture of primary antibodies, at the developed concentrations (Table 1) applied to the tissue and incubated at 4°C for 24hrs. The following day, the sections were rinsed for 30 minutes in 1× PBS and incubated with the appropriate Alexa-Fluor secondary antibodies (Invitrogen Molecular Probes, Eugene, Oregon, USA) for 1 hour at room temperature (Table 1). The antibody PH8 (Chemicon International, Temecula, CA) against TPOH was used to identify 5-HT neurons. The PH8 antibody (Chemicon, Temecula, CA) was used for localization of 5-HT cell bodies (Kinney et al., 2007); this antibody is a murine monoclonal antibody that binds a common epitope of TPOH, tyrosine hydroxylase, and phenylalanine hydroxylase (Cotton et al., 1988). In human tissue fixed in formalin or formaldehyde, the PH8 antibody only binds to TPOH due to an apparent formaldehyde-induced change in the antigenic determinant of tyrosine hydroxylase (Baker et al., 1991; Haan et al., 1987; Halliday et al., 1990; Törk et al., 1990). The caveats of the PH8 antibody and the laboratory controls for the use of this antibody in human tissue sections has been previously discussed by us in detail (Kinney et al., 2007). Immunofluorescence was visualized with an Olympus BX51 microscope (Olympus America Inc., Melville, NY) using FITC and TRITC filters with image capture using a Coolsnap fx camera (Photometrics, Tuscon, AZ) and MCIF Elite 6.0 software (Imaging Analysis Inc., Ontario, Canada). From each term birth case used in the study, 3 sections were analyzed. The number of 5-HT positive neurons and GAD67/65 positive neurons in the raphé obscurus were counted in each case. The raphé obscurus was photographed using the FITC and TRITC filters, red GAD67/65 positive cells and green PH8 positive 5-HT neurons were counted followed by neurons co-labeled under a separate filter. Immunofluorescent images were captured, and imported into Photoshop 6.0 (Adobe Systems, San Jose, CA) where they were analyzed and appropriate labels and scale bars were added. We determined the percent of 5-HT neurons that co-expressed GABA by first photographing the entire raphé obscurus for 5-HT neurons only, GABAergic neurons only, and merged 5-HT and GABAergic neurons, and the number of each cell type and total cell numbers were assessed. We then calculated the percent of each cell type as a proportion of the total number of cells counted.

RESULTS

Clinicopathologic Dataset

A total of 15 medullae from infants dying from causes other than SIDS (controls) were obtained from the San Diego Medical Examiner's office and Department of Pathology at Children's Hospital Boston. Ten (10) formalin-fixed, paraffin-embedded medullae were obtained from the archives of the Department of Pathology, Children's Hospital Boston, MA, for immunocytochemical analysis. The 10 cases ranged in postconceptional age from 15 weeks to 92 weeks (i.e., 2 years), with a median of 36 weeks. There were no significant pathologic changes in the brainstem or elsewhere in the brain. The causes of death were: spontaneous abortion (n=1); placental abruption (n=1), prematurity (n=3); congenital heart disease (n=3), congenital neuroblastoma (n=1), and pulmonary aspiration of a foreign body (n=1). Five cases were available for autoradiographic analysis. The cases ranged in age from 38 to 47 postconceptional weeks, with a median of 44 weeks. There was no significant brainstem pathology. The causes of death were: Arthrogryposis multiplex congenita (n=1), disseminated (systemic) viral infection (n=1), meconium aspiration (n=1), and congenital heart disease (n=2).

The Distribution of GAD65/67 neurons in the Fetal and Infant Human Medulla

GAD65/67 neurons were identified at all ages studied; from 15 gestational weeks to 10 postnatal months. The GABAergic neuronal population identified was morphologically diverse, a sub-population of small-round neurons was identified and a sub-population of spindle-shaped cells also. GAD65/67 immunopositive neurons were distributed throughout the medullary nuclei, including in the components of the medullary 5-HT system that contain 5-HT neurons. In the raphé obscurus, a small subset of small, round neurons (presumed interneurons) were positively stained with GAD67/65. There was also a subset of medium, spindle-shaped neurons immunostained with GAD65/67; these neurons were morphologically similar to 5-HT neurons immunostained for TPOH. In three representative cases at term gestation, we determined that a subset of the spindle shaped cells in the raphé obscurus was immunopositive for both TPOH and GAD65/67, 6% (20/308) of the total neurons counted co-expressed both GAD65/67 and TPOH (Fig. 4). Of the neurons co-expressing both neurotransmitters, the population differed morphologically such that 61% (11/18) were small round neurons and approximately 39% (7/18) were spindle-shaped. In the arcuate nucleus at the ventral surface, a subset of an estimated 25% of the round neurons was positive for GAD67/67 immunostaining. Alpha motor neurons in the hypoglossal nucleus contained only surface GAD65/67 punctate immunostaining around the edge of the soma (Fig. 5), presumably indicative of GABAergic terminals.

GABA_A Receptor Analysis in the Medullary 5-HT System

Utilizing tissue autoradiography, we found that GABA_A receptor binding was ubiquitous across nuclei in the infant medulla, with the highest level of binding in nucleus of the solitary tract (80.8 ± 6.1 fmol/mg) and raphé obscurus (80.7 ± 10.8 fmol/mg) (Fig. 6). The lowest binding levels were observed in the paragigantocellularis lateralis (62.4 ± 7.3 fmol/mg) and arcuate nucleus (50.4 ± 10 fmol/mg). Statistical analysis revealed that postmortem interval had no effect on ³H-GABA_A binding. At all ages examined in both the fetal and infant medulla with immunocytochemistry, we found that GABA_{Aα1} and GABA_{Aα3} receptor subtypes localized to nuclei containing 5-HT neurons, as well as those that received 5-HT projections (Fig. 7). In the infant medulla, the regional distribution of GABA_{Aα1} and GABA_{Aα3} was consistent with that found for the GABA_A binding in the autoradiographic study. However, the developmental profile of the GABA_{Aα1} and GABA_{Aα3} subtypes differed significantly from each other in different nuclei within the medulla (Fig. 8). In the seven 5-HT nuclei studied the GABA_{Aα3} receptor changed quadratically with age, its expression increased until 40-50 weeks and then

leveled out, up to the oldest case studied which was 10 months (raphé obscurus ($p < 0.001$), paragigantocellularis lateralis ($p = 0.003$), arcuate nucleus ($p = 0.004$), HG ($p = 0.005$), DMX ($p = 0.025$), NTS ($p = 0.009$), and GC ($p = 0.005$); in contrast, there was a significant decrease in the expression of the GABA_{A α 1} receptor in the raphé obscurus with age ($p = 0.002$). Other medullary nuclei studied also exhibited trends to decrease with age. The GABA_{A α 1} receptor expression significantly increased quadratically with age ($p = 0.01$) in the arcuate nucleus. Thus, there were changes occurring only in the raphé obscurus for each receptor subtype, with an increase in GABA_{A α 3} and decrease in GABA_{A α 1}. Using double-label immunocytochemistry in the representative medulla of a term infant, we observed that both the GABA_{A α 1} and GABA_{A α 3} were expressed by 5-HT neurons in the raphé obscurus and extra-raphé (paragigantocellularis lateralis) (Fig. 9). The staining was punctate on soma of neurons, suggestive of receptor staining.

KCC2 Analysis in the 5-HT Medullary System

At 15 weeks gestational age, KCC2 was expressed by differentiating 5-HT and non-5-HT neurons throughout the medullae. KCC2 expression was also present in all neuronal types and in all nuclei within the medulla from 15 gestational weeks to the end of the first postnatal year, the time-frame studied by us (Fig. 10). KCC2 expression changed quadratically with age in all nuclei except the DMX, increasing until around 40 weeks (term-birth) and then leveling out. There was also a non-significant trend towards the same in the DMX ($p = 0.18$). The cellular localization of KCC2 expression, however, changed with age from cell bodies at 38–41 weeks to also involve tegmental fibers in the reticular formation at 83 and 92 postconceptional weeks, i.e. approximately 11 and 13 postnatal months, respectively, the oldest ages studied (Fig. 10). Double label-immunofluorescence with antibodies against axonal neurofilament (Covance [SMI312] and myelin basic protein (SMI99) showed no colocalization with KCC2 (Fig. 11) suggesting that the immunopositive tegmental fibers were not axons or the myelin sheaths encasing the axons. In contrast, KCC2 co-localized with MAP-2, indicating its localization on dendrites (Fig. 12).

Comparative Analysis of the GABAergic and 5-HT Systems in the Developing Human Medulla

We found at 15 gestational weeks, the earliest time-point analyzed, that both the GABA and 5-HT neurons are present in their “final” topographic positions in the human medulla. The main difference was that GABA neurons were present throughout every nucleus, in contrast to 5-HT neurons that were restricted to discrete locations in the raphé, extra-raphé, and along the ventral medullary rim (Kinney et al., 2007). The main changes in both systems were in the expression of receptors with differential patterns pre-and/or postnatally in different nuclei (Fig. 14). Across the last part of human gestation, there was a sharp decline in 5-HT receptor binding as demonstrated with the broad radioligand ³H-lysergic acid diethylamide (LSD); after birth, the only receptor binding change was an increase in the binding in the 5-HT_{1A} receptors in the hypoglossal nucleus (Paterson et al., 2004) and decreased binding in the 5-HT_{2A} receptors in the hypoglossal nucleus, principal inferior olive, and dorsal accessory olivary nucleus (Paterson et al., 2009). In contrast, there was a gradual increase in GABA_{A α 3} expression, as demonstrated by immunocytochemistry, in the raphé obscurus, paragigantocellularis lateralis, and arcuate nucleus from midgestation to the end of the first year, and a decrease in GABA_{A α 1} only in the raphé obscurus over the same time period (Fig. 8, 13). There were changes in the expression of KCC2 seen with immunohistochemistry, paralleling the GABA_{A α 3} unlike the 5-HT transporter that did not change across infancy (Fig. 13); data were not available in the fetal period for the 5-HT transporter.

DISCUSSION

In this study, we examined the chemical anatomy of the GABAergic neurons in the developing human medulla relative to 5-HT markers, as it is known that GABA modulates 5-HT signaling in important ways in homeostatic processes (Curran et al., 2000; Curran et al., 2001; Heesch et al., 2006; Menezes et al., 2007; Pierrefiche et al., 1998; Shao et al., 1997). Indeed, GABA is critical for respiration itself, as mouse fetuses lacking GAD67 have an abnormal respiratory neural network and die from respiratory failure (Fujii et al., 2007; Kuwana et al., 2004; Kuwana et al., 2003). In the developing human medulla, we found that GABAergic neurons are intermingled among the neurons of the medullary 5-HT system which are in turn spatially restricted to the raphe, extra-raphé and ventral surface. In addition, a small subset (approximately 6%) of 5-HT neurons in the raphe obscurus co-expresses GABA. Moreover, the developmental profiles of 5-HT receptors and transporter and GABA_A receptors and transporter change dramatically relative to one another in precise regional sequences. In the following discussion, we highlight the potential significance of our findings relative to human medullary development in early life.

Potential Limitations of the Study

The main limitation of this study is the small sample size of the fetal and infant medullae. This limitation results from the major difficulty in the accrual of normative autopsy samples from human fetuses and infants for research. Despite the small sample size, we found consistent developmental changes that underscore, we believe, the validity of the findings. A second potential limitation of the study is the semi-quantitative analysis of immunostaining with the GABA_A receptor subtypes and KCC2. There is no widely accepted approach to the quantitation of immunostaining, with methods depending upon generally visual assessment of intensity and/or the percent of neurons in a region with immunopositive staining. We addressed the issue of quantitation by the visual comparison of intensity of an internal standard that we found on pilot examination to be the same in all cases, i.e., the ependymal lining. This region is of particular value, we believe, because ependymal cells are not known to express GAD67/65, GABA_A receptors, or KCC2. While perhaps not ideal, we believe this method provides a reasonable means to compare the intensity of immunostaining among regions and different developmental ages, as indicated by our findings of significantly different profiles.

Distribution of GABAergic Neurons in the Human Infant Medulla

We found that GABAergic neurons, receptor subunits, and KCC2 are present in nuclei critical for respiratory and autonomic control as early as midgestation, including the components of the medullary 5-HT system, e.g., raphe obscurus, and its major projection sites, e.g., nucleus of the solitary tract. We found GABAergic neurons, receptors, and KCC2 in the ventral medullary surface within the arcuate nucleus, of interest as this site is postulated to be the human homologue of the respiratory chemosensitive fields in the ventral medulla of experimental animals (Filiano et al., 1990; Paterson et al., 2004). We also found that GABA neurons identified by GAD67/65 immunostaining are located within the 5-HT medullary system, i.e., those nuclei that contain 5-HT neurons and those that receive 5-HT projections, from 15 gestational weeks, the youngest case analyzed by us, to 1 postnatal year, the oldest age studied. We also found that 5-HT neurons in the raphe (raphe obscurus) and extra-raphé (paragigantocellularis lateralis) express GABA_A receptors, underscoring the potential for GABAergic receptor-mediated inhibition of 5-HT neurons. Moreover, 5-HT receptor subtypes are expressed by GABAergic neurons in these same regions. In addition, GABA_A receptor subunits are expressed by multiple non-5-HT neuronal types throughout the medulla, and thus, GABA exerts its effects upon non-5-HT as well as 5-HT neurons. While the specific mechanisms are not always clear, animal studies indicate different interactions between GABA and 5-HT neurons: GABAergic disinhibition of 5-HT neurons in the raphe pallidus in rats

activate, for example, sympathetic outflow to brown adipose tissue involved in thermoregulation (Cao et al., 2003); microinjection of the GABA_A antagonist bicuculline into the 5-HT neurons in the feline paragigantocellularis lateralis increases the frequency of neuronal firing, producing a rise in blood pressure and analgesia (Lovick, 1988a; Lovick, 1988b). Moreover, 5-HT and GABA neurons act independently of one another to influence the same function: in rats, for example, caudal raphe-induced inhibitory effects upon respiration are transmitted to the phrenic motor nucleus by a direct GABAergic descending pathway, whereas its facilitatory effects are transmitted via a 5-HT descending pathway (Cao et al., 2006). GABA and 5-HT also interact with one another via co-expression in single raphe neurons (Belin et al., 1983). In our study, we observed that about 6% of 5-HT neurons in the human raphe obscurus express GABA as early as term birth. In adult mice and rats, approximately 2-4% of 5-HT neurons in the caudal raphe co-express GABA (Belin et al., 1983; Nanopoulos et al., 1982; Nanopoulos et al., 1981; Stamp et al., 1995). The physiologic significance of the co-existence of GABA and 5-HT in human raphe and extra-raphe neurons is unknown.

Expression of KCC2 in the Developing Human Medulla

The neuron-specific KCC2, a K-Cl coupled active Cl⁻ extrusion mechanism, is responsible for maintaining the Cl⁻ reversal potential less than the membrane potential, and is thus required for the proper functioning of chloride-ion channel GABA_A receptors (Williams et al., 1999). In neurons in the adult nervous system, KCC2 function is closely linked to maintenance of GABAergic inhibitory synaptic potentials during repetitive stimulation (Ueno et al., 2002). In neurons in the developing nervous system, on the other hand, GABA has an excitatory action because of a high intracellular chloride concentration. With maturation, the effect of GABA “switches” from excitatory to inhibitory due to a decrease of intracellular chloride ion concentration and an increase in the activity of KCC2 (Kakazu et al., 1999; Kakazu et al., 2000; Plotkin et al., 1997; Rivera et al., 1999; Ueno et al., 2002; Zhu et al., 2005), as well as a decrease in the activity of Na⁺-dependent Cl⁻ transport (Kakazu et al., 1999; Kakazu et al., 2000). In rats, the transition from excitatory to inhibitory effects upon respiratory rhythmogenesis occurs around embryonic day 19; by birth, GABA induces hyperpolarization of the membrane potential in respiratory neurons and suppresses respiratory frequency (Ren et al., 2006); this age-dependent change in chloride-mediated conductance is regulated by the development of chloride transporters, notably KCC2 (Ren et al., 2006). Given that KCC2 expression is considered a marker of the developmental switch from excitation to inhibition by GABAergic neurons (Plotkin et al., 1997; Rivera et al., 1999), it is noteworthy that KCC2 immunostaining in the medulla of the human fetus is in place by midgestation and its intensity in immunostaining does not change significantly from midgestation through infancy. Given that KCC2 is responsible for inhibition at the GABA synapse; its presence early throughout the medulla in the human fetus suggests that inhibitory physiological effects are well in place in the brainstem at least 20 weeks prior to birth (Lee et al., 2005; Li et al., 2008; Rivera et al., 2005). In this study, we found that KCC2 immunostaining was present in neuronal cell bodies at all ages studied. Yet, at 38 gestational weeks, there was a striking shift to include dendrites and neuropil in the cranial nerve nuclei and reticular formation subnuclei. Studies in adult animal models suggest that KCC2 is located in axons (Malek et al., 2003), dendrites (Williams et al., 1999), and/or cell somata (Williams et al., 1999).

Inter-relationships in the Development of the GABAergic and 5-HT Systems in the Human Medulla

In this study, we examined the sequences of different tissue markers in the GABAergic and 5-HT systems relative to one another from fetal life through infancy, i.e., the critical period in homeostatic development when the fetus makes the transition to extrauterine life. While the development of the different markers in these two systems is not dependent upon one another,

it is instructive to examine their developmental sequences to understand inter-related, age-dependent changes. At 15 gestational weeks, the earliest time-point analyzed, we found that both the GABA and 5-HT neurons are present in their “final” topographic positions in the human medulla (Kinney et al., 2007). The spatial organization of the GABA and 5-HT cell bodies underscore that GABAergic neurons are in a position to influence virtually all adjacent neurons via the short connections as interneurons, whereas the 5-HT neurons influence other medullary neurons via diffuse and intermediate-to-long projections. The most striking findings in the development of the two systems relative to one another are in the raphe obscurus in which there is an increase in GABA_{Aα3} expression and decrease in GABA_{Aα1} expression over the same time-period, suggesting a dynamic period in the GABAergic development in a site heavily concentrated with 5-HT neurons. The raphe obscurus is implicated in respiratory control (Feldman et al., 2006; Pierrefiche et al., 1998; Shao et al., 1997), carbon dioxide chemosensitivity (Curran et al., 2000; Curran et al., 2001; Nattie, 1999; Nattie, 1991), heart rate and blood pressure regulation (Dampney, 1994; Heesch et al., 2006; Menezes et al., 2007), baroreflexes (Callera et al., 2000; Callera et al., 2005), temperature control (Darnall et al., 2001; Darnall et al., 2006), and pain (Stamford, 1995). Our studies suggest that the development of 5-HT markers is “static” in the raphe obscurus from birth into infancy, yet changes continue to occur in GABA_A receptor subunits; thus, potential developmental changes in 5-HT-mediated function in the raphe obscurus after birth may reflect, at least in part, changes in GABA modulation. In summary, the combined data in this study provides baseline information for future neuropathologic studies of complex human pediatric medullary disorders, as suggested in the sudden infant death syndrome (Paterson et al., 2006).

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Figure 1.

Semi-quantitative grading system to compare immunostaining intensity within and across different cases and at different ages. (A). Schematic showing 5-HT nuclei. We first identified upon survey of all cases a site as an internal standard which was immunostained with the same intensity irrespective of age; this site was the (B) endymal cells in the lining of the fourth ventricle. We designated the intensity of this “standard” as grade 2 and visually scored neurons in the selected nuclei relative to this standard as: C. Grade 0 = no neuronal immunostaining; D. Grade 1 = less intense immunostaining than standard, E. Grade 2 = equal immunostaining to standard; F. Grade 3 = more intense immunostaining than standard, as illustrated for the GABA_{Aα3} antibody (Fig. 1). Scale bars = 100µm.

Figure 2.

GAD65/67 immunoreactivity in neurons in the hypoglossal and in the raphé obscurus and pre-absorption assay with GAD65/67 peptide which blocks antibody staining. (A) Immunostaining for GAD65/67 localized to the raphé obscurus (ROb); at 41 postconceptional weeks (term). (B) Immunostaining for GAD65/67 in raphé obscurus blocked when incubated with GAD65/67 peptide. (C) Immunostaining for GAD65/67 localized to the neurons in the hypoglossal nucleus at 41 postconceptional weeks (term). (D) Immunostaining for GAD65/67 in hypoglossal nucleus blocked when incubated with GAD65/67 peptide. Scale bars = 100µm.

Figure 3.

Western blot analysis using infant medulla lysate of GAD65/67, with band seen at 65kDa. We incubated with 2 concentrations of immunizing peptide 7×, and 20×; protein was completely blocked as seen in the immunohistochemistry when incubated with peptide also.

Figure 4.

The co-expression of GAD65/67 and TPOH in neurons in the raphe obscurus (ROb), with the site indicated in blue in the diagram at 41 weeks postconceptional weeks (term) $\times 40$. (A) TPOH positive neuron; (B) GAD65/67 positive; (C) Merged images. Scale bars = 100 μm .

Figure 5. GAD65/67 immunoreactivity in the hypoglossal nucleus at 41 postconceptional weeks (term) with the site demonstrated schematically ($\times 40$). (A) Punctate GAD65/67 immunostaining localizes around the edge of the somata consistent with GABAergic terminals projecting upon the somata. (B) Positively-stained alpha motor neurons for choline acetyl transferase (ChAT) indicative of the cholinergic phenotype of these motoneurons; (C) alpha motor neurons with only surface punctate immunostaining around the edge of the somata. Scale bars = 100 μm .

Figure 6.

^3H -GABA binding (fmol/mg tissue) to GABA_A receptors with tissue autoradiography, at the (A) mid (level of hypoglossal nucleus) level section of the medulla (B), rostral (level of prepositus nucleus) level section of the medulla; (C) Non-specific section (incubated with high concentration of ligand displacer) of medulla. The distribution of GABA_A binding is ubiquitous across nuclei (see text).

Figure 7.

GABA_A receptor immunoreactivity in 5-HR Medullary Nuclei at 41 postconceptional weeks. The regional distribution of the GABA_A receptor subtypes studied is consistent with that found for the GABA_A receptor binding. Immunostaining is punctate and localized to neuronal cell bodies and processes, consistent with postsynaptic localization of the receptors. Scale bars = 100 μ m.

Figure 8.

Developmental profile of GABA_A receptor subtypes in 5-HT medullary nuclei in the medulla of the human fetus and infant. From 15 gestational weeks to the end of the first postnatal year, there is a significant increase in GABA_Aα₃ expression in the raphe obscurus ($p < 0.001$), gigantocellularis ($p = 0.005$) and the paragigantocellularis lateralis ($p = 0.003$). The GABA_Aα₁ receptor expression changes with age with a significant decrease in the raphe obscurus ($p = 0.002$) and non-significant trends to decrease in the gigantocellularis and paragigantocellularis.

Figure 9. Double-label immunofluorescent images showing the co-localization of the GABA_Aα₃ with 5-HT neurons in the raphe obscurus and paragigantocellularis lateralis. (A) 5-HT positive neurons in raphe obscurus, B. GABA_Aα₃ positive neurons in raphe obscurus; C. Merged images, D. 5-HT positive neurons in paragigantocellularis lateralis; E. GABA_Aα₃ positive neuron in paragigantocellularis; F. Merged images. Scale bars = 100 μm.

Figure 10.

Developmental profile of KCC2 in 5-HT medullary nuclei in the medulla of the human fetus and infant from cases of different ages 22 and 34 gestational weeks, 41 postconceptional weeks (term) and 80 postconceptional weeks. KCC2 immunostaining is punctate and localized to neuronal cell bodies and processes in early fetal development, but through development KCC2, immunoreactivity increases to the neuropil. Scale bars = 100 μ m.

Figure 11.

Double-label immunofluorescent images showing KCC2-positive fibers are not axons, as KCC2 immunoreactivity did not colocalize with fibers positive for axonal neurofilament (NFT) and myelin basic protein (MBP) in the raphé obscurus and paragigantocellularis lateralis, ($\times 40$) A. KCC2 positive fibers in the PGCL; B. NFT positive fibers in PGCL; C. Merged images. D. KCC2 positive fibers in the PGCL; E. MBP positive fibers in PGCL; F. Merged images. Scale bars = 100 μm .

Figure 12.

Double-label Immunofluorescent images showing the co-expression of KCC2 and MAP2 in neurons in 5-HT nuclei raphé obscurus ($\times 10$) and paragigantocellularis lateralis, ($\times 40$) **A.**

KCC2 positive neurons in the ROb; **B.** MAP2 positive neurons in ROb; **C.** Merged images. Scale bars= 500 μ m; **D.** KCC2 positive neuron in PGCL; **E.** MAP2 positive neuron in PGCL; **F.** Merged images. Scale bars = 100 μ m.

Figure 13.

Summary diagram of the inter-relationships of the GABAergic and 5-HT systems during early development of the human medulla. Profiles for the GABAergic system (GABA_A receptor subtypes studied, transporters) and 5HT system in the (A) raphe; (B) paragigantocellularis lateralis; (C) gigantocellularis; (D) arcuate showing the dynamic changes that occur in different nuclei across development for the two systems. The diagram highlights the complexity of the developmental changes in the GABAergic and 5-HT systems in the human medulla relative to one another.

Table 1
Antibodies used to assess GABAergic profiles in the medulla oblongata of the human fetus and infant

Antibody	Host	Source / cat #	Immunogen	Dilution	Peptide Sequence	Labels	References
GABA A ($\alpha 1$)	Rabbit Polyclonal IgG	Sigma / G4416	Purified peptide to AA residues 1-16 of mouse or rat GABA _A receptor $\alpha 1$	1:200	QPSQDELKDNT-TVFTTR with an additional C-terminal cysteine	recognizes GABA(A) receptor $\alpha 1$ and shares little homology with $\alpha 5$ and $\alpha 2$ and no homology with any known protein	Kerr, D.I. and Ong, J. et al., 1995
GABA A ($\alpha 3$)	Rabbit Polyclonal IgG	Sigma / G4291	Purified peptide to AA residues 1-15 of mouse or rat GABA _A receptor $\alpha 3$	1:200	QGESR RQEPGDEVKQ with an additional C-terminal cysteine	Specifically recognizes the GABA(A) receptor $\alpha 3$ and shares no homology with any other known protein	Kostowski, W., Pol, J., et al., 1995
GAD65/67	Rabbit Polyclonal IgG	Abcam / ab49832	Synthetic peptide to C-terminal AA 579-594 of Human GAD67 & AA 57-585 of Human GAD65.	1:500	K- DIDFLIEIERLGQDL conjugated to KLH by a Glutaraldehyde linker.	Reacts specifically with GAD 65 and Gad 67 isoforms from brain.	N/A
Anti-K⁺/Cl⁻ Cotransporter (KCC2)	Rabbit Polyclonal IgG	Upstate / 07-432 /	N-terminal His-tag fusion protein corresponding to residues 932-1043 of rat KCC2	1:200	Residues 932-1043 of rat KCC2	Recognizes rat KCC2	Verdi, N, et al., 2000).
PH8 for tryptophan hydroxylase, key biosynthetic enzyme	Murine Monoclonal IgG	Chemicon, MAB5278	Immunoglobulin fraction from murine ascites fluid	1:1000	Common epitope of tryptophan hydroxylase (TPOH), tyrosine hydroxylase and phenylamine hydroxylase	In fixed human tissue PH8 binds specifically to TPOH due to antigen changes induced by fixation	Haan et al., 1987; Baker et al., 1991
Myelin basic protein (MBP)	Murine Monoclonal IgG2b	Covance/ SMI199	Purified IgG from mouse ascites.	1:1000	SMI 99 reacts with the sequence Ala-Ser-Asp-Tyr-Lys-Ser in position 131-136 of human myelin basic protein.	Detects myelin basic protein from most mammalian species.	Mendez G, et al., 1984, Carnegie PR, et al., 1983.
Neurofilamen	Murine Monoclonal IgG1 Cocktail	Covance/ SMI1312	Immunoglobulin fraction from murine ascites fluid	1:1000	No sequence available	Marker for axons in human tissue	Bussière T., et al., 2004, Chung RS., et al., 2003.