Intermittent severe hypoxia induces plasticity within serotonergic and catecholaminergic neurons in the neonatal rat ventrolateral medulla

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Running Head: 5-HT deficiency, intermittent hypoxia and neuroplasticity

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5-HT neurons contribute to autoresuscitation and survival during intermittent severe hypoxia (IsH). In adults, catecholaminergic neurons in the ventrolateral medulla (VLM) contribute to the autonomic response to hypoxia. We hypothesized that: 1) catecholaminergic neurons in the neonatal VLM are activated following IsH; 2) this activation is compromised following an acute loss of brainstem 5-HT, and 3) IsH induces cellular and/or transcriptomic plasticity within catecholaminergic and serotonergic neurons that are within or project to the VLM, respectively. To test these hypotheses we treated rat pups with 6-fluorotryptophan (6-FL), a tryptophan hydroxylase (TPH) inhibitor, and then exposed treated and vehicle controls to IsH or air. Along with immunohistochemistry to detect tyrosine hydroxylase (TH)- or Fos-positive neurons, we used RNA-sequencing to resolve the effects of IsH and 5-HT deficiency on the expression of serotonergic and catecholaminergic system genes in the VLM. 5-HT deficiency compromised autoresuscitation and survival. IsH significantly increased the number of identifiable TH-positive VLM neurons, an effect enhanced by 5-HT deficiency (p=0.003). Contrary to our hypothesis, 5-HT-deficient pups had significantly more Fos-positive neurons following IsH (p=0.008), and more activated TH-positive neurons following IsH or air (p=0.04). In both groups the expression of the 5-HT transporter and TPH2 were increased following IsH. In 5-HT-deficient pups, the expression of the inhibitory 5-HT1A receptor was decreased following IsH, while the expression of DOPA decarboxylase was increased. These data show that the serotonergic and catecholaminergic systems in the VLM of the neonatal rat are dynamically upregulated by IsH, potentially adapting cardiorespiratory responses to severe hypoxia.

Keywords: serotonin, intermittent hypoxia, ventrolateral medulla, plasticity, catecholamines, transcriptomics
NEW AND NOTEWORTHY

Brainstem serotonin (5-HT) contributes to autoresuscitation during intermittent severe hypoxia (IsH).

We demonstrate that 5-HT deficiency is associated with a greater, IsH-induced increase in Fos- and TH-positive neurons. Irrespective of 5-HT content, IsH upregulated tryptophan hydroxylase 2 and 5-HT transporter expression. In 5-HT-deficient pups, the expression of 5-HT$_{1A}$ receptors and DOPA decarboxylase were decreased and increased, respectively, by IsH. The neonatal VLM exhibits significant serotonergic and catecholaminergic plasticity in response to IsH.
INTRODUCTION

Young mammals, especially those born pre-term, are prone to apnea, bradycardia and episodic hypoxia (36, 47), and thus are at risk for neurodevelopmental impairment, cognitive dysfunction, and even sudden death (14, 27, 38, 46). Severely hypoxic conditions (i.e. when tissue PO$_2$ drops below ~5-10 mmHg) induce apnea and bradycardia due to direct effects of hypoxia on respiratory neurons and the sinoatrial node, respectively. A sequence of cardiovascular and respiratory responses – collectively termed “autoresuscitation” – is initiated to restore normal breathing and heart rate (16). Gasping, or large inspiratory breaths, is the respiratory component of autoresuscitation that rapidly increases pulmonary gas exchange. In isolation, however, gasping is insufficient for surviving severe hypoxia; also required is an increase in sympathetic nerve activity to the heart and vessels that, in the face of hypoxia-induced vasodilatation, preserves arterial blood pressure (BP) to expedite the re-oxygenation of the heart and brain (20, 52). There are documented instances of failed autoresuscitation in cases of the Sudden Infant Death Syndrome (SIDS). An analysis of some of these data, including records of tissue PO$_2$, breathing and heart rate, suggests that hypoxia is an antecedent, primary factor leading to prolonged apnea, bradycardia and ultimately death (45, 46, 53).

In adult animals, catecholaminergic neurons within the rostral (RVLM) and caudal (CVLM) ventrolateral medulla (VLM) contribute heavily to the autonomic, respiratory, and neuroendocrine responses to hypoxia and hypotension (23). Within the RVLM are baro- and chemosensitive pre-sympathetic neurons that send projections to the intermediolateral cell column of the spinal cord to increase sympathetic nerve activity in response to hypoxia and/or hypotension. These neurons are activated by hypoxia via glutamatergic inputs from the commissural aspect of the nucleus of the solitary tract (NTS), following stimulation by afferents arriving from the carotid chemoreceptors (22). Other groups of catecholaminergic neurons within the RVLM and CVLM innervate the paraventricular
nucleus of the hypothalamus (PVN) where they synapse on spinally-projecting pre-sympathetic parvocellular neurons, as well as magnocellular neurons that contribute to the neuroendocrine response to hypoxia (28). Recent immunohistochemical and tracing analyses suggest that in adult rats, PVN-projecting catecholaminergic neurons originating in the CVLM and NTS are involved in the cardiorespiratory response to severe hypoxia (30, 31). Whether catecholaminergic neurons in the VLM are important in neonatal life has not been investigated.

Several recent studies have identified serotonin (5-HT) neurons as critical for cardiovascular and respiratory responses to intermittent severe hypoxia (IsH) (7, 9, 15). Within the first postnatal week, mice and rats with a chronic loss of 5-HT neurons and/or 5-HT content display a marked inability to generate gasping and a delay in the recovery of heart rate once gasping begins (6, 7, 9, 15). In the second postnatal week – an age that is roughly equivalent to infancy), mice deficient in tryptophan hydroxylase-2 (TPH2), the enzyme catalyzing the rate-limiting step in central 5-HT biosynthesis, remain susceptible to IsH, a phenotype associated with an inability to recover heart rate (6). In addition, we have recently shown that an acute, global loss of 5-HT (i.e. from the CNS and peripheral tissues) compromises autoresuscitation; two week-old rats treated systemically for 2 days with 6-fluorotryptophan (6-FL), a competitive antagonist of tryptophan hydroxylase (TPH), experience a pronounced, premature loss of BP and have high mortality in response to IsH (57). Again, this phenotype is associated with delayed heart rate and respiratory recovery, ostensibly due to the delay in the re-oxygenation of the heart and brain following such a large drop in BP.

The degree to which the serotonergic and catecholaminergic signaling within the VLM contribute to the cardiorespiratory response to IsH in neonatal life has not, to our knowledge, been investigated. This study addresses three related hypotheses: first, that catecholaminergic neurons in the neonatal VLM are activated in response to IsH; second, this activation is compromised following an
acute loss of 5-HT; and third, that IsH induces cellular and transcriptomic plasticity within
catecholaminergic and serotonergic neurons that reside in or project to the VLM, respectively. To
address these hypotheses we performed immunohistochemistry and RNA-sequencing on medullary
tissue taken from rat pups following an acute loss of brainstem 5-HT and exposure to IsH. Our findings
provide evidence that IsH induces cellular and transcriptomic plasticity within both the serotonergic and
catecholaminergic systems of the VLM and that some, but not all of this plasticity is influenced by 5-HT
deficiency.
MATERIALS AND METHODS

Ethical Approval

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Missouri at Columbia, MO.

Animals and treatments

Postnatal day (P)-9-10 6-FL-treated Sprague-Dawley rat pups were used for all experiments. 8 treated pups and 8 controls were used for measuring physiological responses to IsH and for RNAseq. For immunohistochemistry treated and control pups were exposed to IsH (n=6 of each) or air (n=4). Dams were fed ad libitum on standard rat chow, and kept on a 12 hr light-dark cycle. Pups were treated systemically with 6-fluorotryptophan (6-FL), a tryptophan hydroxylase inhibitor that has been previously shown to induce a rapid loss of 5-HT with minimal developmental effects (5, 57). 6-FL was dissolved in very dilute acid (0.1N HCL), at a concentration of 20 mg/ml. Three injections of 6-FL or vehicle were given over 2 days (each injection: 250-300uL; 200mg/kg i.p.). An injection was given every 8-12hrs, the last of which occurring 1-2hrs prior to hypoxic exposure. After each injection, pups were immediately returned to the litter and dam. Pups appeared to feed and otherwise behave normally following each injection. For each litter, pups were assigned to either 6-FL or vehicle, and on any given day, at least one pup from each group was exposed to IsH.

Experimental setups

Breathing was monitored using a head-out approach, as previously described (8). Briefly, the animal chamber (volume ~40 mL) was constructed from a water-jacketed glass cylinder. Ports in the chamber allowed it to be perfused with heated water from programmable bath/pump to control ambient temperature and thus hold body temperature at 36°C ± 0.5°C, monitored via a rectal thermocouple and thermometer. Inspiratory and expiratory airflows were detected by connecting both side-arms of the...
pneumotach to a differential pressure transducer (Validyne Engineering, Northridge, CA). Integration of
the flow trace provided tidal volume ($V_T$), calibrated by injecting 50 and 100uL of air into the sealed
chamber with a micropipetter, with flow running, at normal respiratory $f_R$. The pneumotach responded in
a linear fashion to these volumes. A pump (AEI Technologies, Naperville, IL) connected to the outlet
port of the mask pulled air (or hypoxic gas) through the pneumotach and across the animals’ face at a
flow of 150 ml.min$^{-1}$. The air was subsequently drawn through a small column of Drierite (W. A.
Hammond Drierite Co. Ltd., Xenia OH), and then an $O_2$ analyzer (AEI Technologies, Pittsburgh PA) to
allow for the determination of $V_o$. To induce severe hypoxia, anoxic gas (97% $N_2$/3% CO$_2$) was
delivered to the surrounds of the pneumotach through the open end of a 50cc syringe. 3% CO$_2$ was
added to the inspirate to prevent arterial hypocapnia during hypoxic hyperventilation, as has been done
in other studies (9, 17). Gas exchange within the mask occurred within a few seconds. Pressure in the
mask was negligible, imparting little to no respiratory load on the animal. Heart rate was determined via
ECG using surface electrodes embedded within a vest made from tensor bandage. All analog signals
were fed into a Powerlab data acquisition system (ADInstruments, Colorado Springs, CO, USA) and
analyzed in Labchart 7 (ADInstruments).

**Experimental Protocol**

Animals were allowed to rest in the warmed chamber (at least 10 min) until metabolic
cardiorespiratory variables were in steady state. One set of pups was used to assess the effects of 6-FL
on the cardiorespiratory responses to IsH and to determine the average number of hypoxic episodes
treated pups could tolerate for the subsequent immunohistochemical analysis. Thus, 6-FL-treated pups
used were subjected to IsH until death occurred (between episode 6 and 8). Littermate controls were
exposed to the same number of episodes and then sacrificed for tissue harvesting and RNA isolation.
A second set of pups was used for immunohistochemistry. Treated and control pups (n=6 of each group) rested in room air for 10 min, and were then exposed to 6 episodes of environmental anoxia (~40 sec in both groups), followed by 1 hr in room air. Control pups (n=4 of each group) rested in the chamber for an equivalent period of time, without exposure to hypoxia.

**Immunohistochemistry**

Our labelling technique identified neurons positive for Fos and/or TH. We used a previously described technique for processing and staining all tissues (29). Following 6 episodes of anoxia and 1 hr normoxic recovery, animals were transcardially perfused with 0.01 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS (500 ml pH 7.4; Sigma). Following 1-5 days of post-fixing, 40 um coronal sections were cut using a vibratome (VT 1000S; Leica, Germany). Free-floating coronal sections were rinsed three times for 10 min in 0.01 M phosphate-buffered saline (PBS, pH 7.4) and then incubated in 0.3% hydrogen peroxide for 30 min to quench endogenous peroxidases. Following pre-blocking in 10% normal donkey serum for 10 min, sections were incubated overnight in 1% normal donkey serum (NDS) and 0.3% Triton-0.01 M PBS containing primary antibodies against Fos (rabbit anti Fos; 1:3000; CalBiochem) and TH (mouse anti-TH, 1:1,000; Millipore). The following day, sections were rinsed in PBS and incubated for 2 hr in Cy3-conjugated donkey anti-rabbit IgG and Cy2-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch) with 1% NDS in 0.3% Triton-0.01 M PBS. Sections were rinsed, then air-dried, cover-slipped with ProlongGold (Invitrogen, P36930), and sealed with nail polish. Controls included omission of primary or secondary antibodies. To minimize variability due to processing conditions, 6-FL and control slices were processed at the same time using the same solutions.
Microscopy

We identified catecholaminergic neurons within the entire rostral-caudal extent of the VLM. We identified these neurons based on their distinct position throughout the VLM (Figure 1, (42)). Sections were examined using an Olympus epifluorescent microscope (BX51). Filter sets for Cy2 and Cy3 were used to visualize positive labeling. Image brightness and contrast were adjusted only for clarity. Images in the same focal plane were captured under each filter set using a cooled monochrome digital camera (ORCA-AG; Hamamatsu, Bridgewater, NJ). Images were analyzed with Image J (version 1.41; National Institutes of Health, Bethesda, MD). Fos-positive nuclei were identified as highly condensed stained nucleoli. TH-positive cells were recognizable by staining of the cytoplasm, visible processes, and a blank nuclear region. In all animals Fos and TH staining was localized within a distinct region of the VLM (Fig. 1). All cells within this region that displayed staining above background levels were counted. Counts were performed by an individual naïve to the hypothesis and experimental design.

RNA Preparation and Sequencing

Following exposure to IsH, the brains of treated and control pups were harvested. The cerebellum was removed, and brains were frozen in isopentane, chilled with liquid N2. Brains were stored in -80°C until processing. Bilateral, 1 mm slices containing aspects of the RVLM and CVLM were obtained from each medulla, extending rostrally from the obex. RNA was extracted using QAlzol Lysis Reagent (Qiagen, Germantown, MD), according to the manufacturer’s instructions, and quantified using assayed using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA libraries were constructed using TruSeq RNA sample preparation kit v2, following the manufacturer’s protocol (Illumina, Inc., San Diego, CA). Briefly, Total RNA (300 ng) was evaluated with the Agilent Bioanalzyer 2100 automated electrophoresis system to ensure RNA integrity prior to library construction. Poly-A containing mRNA was purified from total RNA. RNA was then fragmented and
used to generate double-stranded cDNA with the index containing adapters are ligated to cDNA ends. Library constructs were also confirmed using the Agilent BioAnalyzer and quantified with the Qubit fluorometer using the quant-iT HS dsDNA reagent kit (Invitrogen). Dilution and sequencing was performed according to Illumina’s standard protocols for the HiSeq 2000.

Tissue monoamine quantification

We used High pressure liquid chromatography (HPLC) to assess the effects of 6-FL and IsH on medullary 5-HT and noradrenaline (NA) content, respectively. Following exposure to IsH, whole brains were extracted and stored at -80°C. Medullae were isolated and homogenized using a tissue dismembrator in 100-750ul of 0.1M TCA containing $10^{-2}$ M sodium acetate, $10^{-4}$ M EDTA, 5ng/ml isoproterenol (as internal standard) and 10.5% methanol (pH 3.8). After centrifugation, the supernatant was removed for HPLC analysis. HPLC was performed utilizing an Antec Decade II (oxidation: 0.4) (3mm GC WE, HYREF) electrochemical detector operated at 33°C. 20µl samples of the supernatant were injected using an autosampler (model 2707, Waters Co., Milford, MA) onto a 100 x 4.60 mm HPLC column (Phenomenex, Torrance, CA). Biogenic amines are eluted with a mobile phase consisting of 89.5% 0.1M TCA, $10^{-2}$ M sodium acetate, $10^{-4}$ M EDTA and 10.5 % methanol (pH 3.8). Solvent is delivered at 0.6 ml/min using a Waters 515 HPLC pump. HPLC control and data acquisition are managed by Empower software (Waters Co.).

Data Analysis

Physiology

We measured the frequency of breathing ($f_B$, min$^{-1}$), $V_T$ (ml/kg), $\dot{V}_E$ ($f_B \times V_T; \text{ml.min}^{-1}.\text{kg}^{-1}$), $\dot{V_o}_2$ (ml.min$^{-1}$.kg$^{-1}$), $\dot{V}_E/\dot{V}_o_2$, and HR (beats.min$^{-1}$). Mass-specific $\dot{V}_o_2$ was determined using the formula: $\dot{V}_o_2 = (0.21 – \text{fractional O}_2 \text{exhausted from mask}) \times \text{flow (mL.min}^{-1})/\text{mass (kg)}$. For each hypoxic episode
we determined the gasp latency (duration of primary apnea) and the time required for both the recovery of 63% of the pre-hypoxic HR and $f_B$ (i.e. their respective time constants). Significant differences between groups were assessed with either Student’s two-tailed t-tests (baseline variables) or 2-factor repeated-measures ANOVA across the multiple episodes of severe hypoxia, followed by Tukey’s post hoc analysis to reveal significant interactions between variables.

**Cell counts**

Counts of positively labeled cells were made in six 400μm caudal-to-rostral levels of the VLM: from -1.2 to +1.2mm relative to the last section containing the central canal (i.e. approximate position of the obex and rostral pole of the area postrema). Most 400μm bins contained 6-10 sections across the 6 animals per treatment group. Due to variable section quality one bin contained only 5 sections. For immunohistochemical analyses, 3-factor ANOVAs were used to assess the effects of treatment (6-FL vs. Vehicle), gas (air vs. IsH) and position within the VLM (-1.2 to +1.2mm relative to the obex). When significant main effects were found, Holm-Sidak post hoc analysis was used to reveal significant interactions among these three variables.

**RNA Sequencing**

The RNA-Seq analysis was done as previously described (19). Briefly, latent Illumina adapter sequence was identified and removed from input 100-mer RNA-Seq data using Cutadapt, version 1.2.1. Subsequently, input RNA-Seq data were trimmed and filtered to remove low quality nucleotide calls and whole reads, respectively, using the Fastx-Toolkit, version 0.0.13. To generate the final set of quality-controlled RNA-Seq reads, foreign or undesirable reads were removed by sequence matching to the Phi-X genome (NC_001422.1), the relevant ribosomal RNA genes as downloaded from the National Center for Biotechnology Information or repeat elements in RepBase, version 20.02, using Bowtie, version 1.1.1. For the remaining steps, we used the RNA-Seq-Toolkit and TopHat software, version 2.0.13. The
final set of quality-controlled RNA-Seq reads, described above, was aligned to the Ensemble *Rattus norvegicus* genome sequence, Release 79, using Bowtie, version 2.2.3 and the default settings, except allowing for six total mismatches, a minimum intron length of 10 nt and a maximum intron length of 40,000 nt. To generate gene expression estimates, we worked with the set of annotated transcripts as defined in the Ensembl GTF file, Release 79, as downloaded from the Ensembl FTP site. We used Cufflinks, version 2.2.1, to estimate gene expression based on the aligned RNA-Seq data with the default settings, except for using fragment bias correction, multi-read correction, a minimum intron length of 10 nt, and a maximum intron length of 40,000 nt. To analyze differential gene expression between sample types, we used Cuffdiff, version 2.2.1. Again, we worked only with the set of transcripts defined by the Ensembl GTF file, Release 79. Additionally, we used the default settings for Cuffdiff, except for minimum alignment count of 6, fragment bias correction, multi-read correction and quartile normalization. A gene was identified as being differentially expressed in two conditions when the FDR-corrected p-value of its expression ratio was less than 0.05. Subsequent data were reformatted, sorted and filtered using a variety of Bash command-line scripts from the RNA-Seq Toolkit (19), which is available via Github (https://github.com/sgivan/RNA-Seq-Toolkit).

Three analyses were performed to determine: 1) the effect of IsH alone, revealed by significant differential gene expression between control or treated pups exposed to IsH and those exposed to air alone; 2) the effect of 5-HT deficiency alone, revealed by significant differential gene expression between control and treated pups exposed to air alone and 3) an interactive effect between IsH and 5-HT deficiency, revealed by differential gene expression between treated pups exposed to IsH and treated pups exposed to air, and either no differential expression, or differential expression in the opposite direction, between control pups exposed to IsH and controls exposed to air.
RESULTS

**Effects of 6-FL on medullary 5-HT, noradrenaline and on resting cardiorespiratory variables**

6-FL induced a ~50% loss of medullary 5-HT content (p<0.001; Fig. 2). In addition, compared to vehicle controls, pups treated with 6-FL had a ~70% increase in medullary noradrenaline content following IsH (p=0.002; Fig. 2).

Baseline cardiorespiratory variables of control and treated pups are shown in Table 1. Treated pups had a slight (15%), but significantly higher heart rate (p<0.01). While treatment had no significant effects on \( f_B, \dot{V}^e \) or \( \dot{V}^e / \dot{V}_{O_2} \), 5-HT-deficient pups had a 29% larger \( V_T \) (p=0.05) and a co-efficient of variation of the respiratory period (CV%; a measure of respiratory stability), that was more than double that of controls (p=0.002).

**5-HT depletion compromises autoresuscitation**

We non-invasively monitored the breathing and heart rate responses of treated and control pups to IsH in order to confirm the deleterious effects of 5-HT deficiency on autoresuscitation (7, 9). We measured the time to initiate gasping following the appearance of hypoxic apnea (gasp latency), and the time required for the recovery of heart rate and eupnea following the initiation of gasping. Confirming previous findings (7, 9), 5-HT-deficient pups took significantly longer to recover heart rate and eupneic breathing (Figs. 3, 4), presumably because of a larger drop in arterial blood pressure compared to controls (57). These defects occurred in spite of relatively normal gasping that, by the end of the experiment, actually occurred more quickly in 5-HT-deficient pups than controls (treatment x episode: p=0.01; Fig. 4a). On average, from the 4th hypoxic episode onwards, 5-HT-deficient pups required 2-3-fold more time to recover HR (treatment x episode: p=0.02; Fig. 4b) and eupnea (p<0.001; Fig. 4c). Associated with these major defects in autoresuscitation was reduced survival across the IsH. Treated pups survived a median of 7 episodes (range 5-8), whereas there were no deaths of control pups.
IsH and 5-HT deficiency increase the number of activated, TH-positive neurons in the VLM

TH-positive neurons were identified in both 6-FL-treated and control pups (Fig. 5a). For both groups, IsH significantly increased the number of TH-positive neurons, but compared to vehicle controls the effect was greater in 5-HT-deficient pups (6FL x IsH effect: \( p = 0.003 \); Fig. 5a). Whether they breathed room air or IsH, treated pups had greater numbers of TH-positive neurons in the most rostral aspect of the VLM (+1.2mm; treatment x position: \( p = 0.009 \)).

We identified Fos-positive neurons in the VLM of both control and treated pups following IsH (Fig. 5b). In both groups, significantly more Fos-positive neurons were found in pups exposed to IsH compared to those exposed to only air (IsH effect: \( p < 0.001 \); compare left and right panels of Fig. 5b). However, the effect of IsH on the numbers of activated neurons was enhanced by 5-HT deficiency (treatment x gas: \( p = 0.008 \)).

Based on their cardiorespiratory response to IsH, we hypothesized that compared to controls, 5-HT-deficient pups would have fewer activated catecholaminergic neurons in the VLM following IsH. In both groups we identified dual-labelled neurons in the VLM (Fig. 5c-e). Compared to controls, there were more dual-labelled neurons in 5-HT-deficient pups from positions -0.4mm to +1.2mm, irrespective of whether they breathed air or IsH (\( p < 0.02 \) for each region). In both groups, exposure to IsH significantly increased the number of dual-labelled neurons (IsH effect: \( p < 0.001 \); compared left and right panels of Fig. 5c). The magnitude of this IsH-induced increase, however, was unaffected by 5-HT deficiency (\( p = 0.35 \)).

5-HT-deficiency and IsH on gene expression within the VLM

In order to assess the effects of 5-HT deficiency and IsH on gene expression within the serotonergic and catecholaminergic systems, we used RNAseq technology to quantify mRNAs present in a 1mm tissue punch containing the majority of the CVLM and RVLM. Table 2 highlights
differentially expressed genes due to: 1) the effect of IsH alone, revealed by significant differential gene expression between control or treated pups exposed to IsH and those exposed to air alone; 2) the effect of 5-HT deficiency alone, revealed by significant differential gene expression between control and treated pups exposed to air alone and 3) an interactive effect between IsH and 5-HT deficiency, revealed by differential gene expression between treated pups exposed to IsH and treated pups exposed to air, and either no differential expression, or differential expression in the opposite direction, between control pups exposed to IsH and controls exposed to air.

In the VLM of control and treated pups IsH approximately doubled the expression of the tryptophan hydroxylase-2 gene (TPH2) and more than doubled the expression of the serotonin transporter (5-HTT) gene (Slc6a4). IsH also led to a 50% reduction in the expression of the vesicular glutamate transporter 1 (VGLUT-1) gene (Slc17a7).

There were no serotonergic or catecholaminergic system genes whose expression was altered by 5-HT deficiency alone. However, we did reveal that the expression of the choline transporter (Slc5a7) and choline acyltransferase (ChAT) were reduced by 50% and 30%, respectively in 5-HT-deficient pups compared to controls. In addition, 5-HT deficiency was associated with a 40% decrease in Fos expression.

In 5-HT-deficient pups only, IsH led to a ~4-fold increase in Fos expression. In addition, the 5-HT1A receptor gene (Htr1a) was downregulated by 70% in 5-HT-deficient pups exposed to IsH. We also identified a ~2-fold increase in the expression of the DOPA decarboxylase (Ddc) in the VLM of 5-HT-deficient pups exposed to IsH. A number of other neurotransmitter system genes were differentially expressed: Slc17a7 (up nearly 4-fold in treated pups exposed to IsH), Slc5a7 (up ~2-fold), ChAt (up 3-fold), and both ionotropic (Gria2; down 70%) and metabotropic (Grm5; down 60%) glutamate receptor genes.
DISCUSSION

The novel, most salient findings of the present study are: 1) the number of TH-positive and dual-labelled (Fos/TH-positive) neurons in the neonatal VLM was increased by IsH; 2) Contrary to our hypothesis, 5-HT deficiency was associated with more Fos- and TH-positive neurons following IsH; 3) the expression of genes encoding 5-HTT and TPH2 were up-regulated in the VLM in response to IsH, and 4) in 5-HT-deficient pups the expression of the 5-HT1A receptor was decreased following IsH, while the expression of DOPA decarboxylase was increased. These data suggest that the neonatal VLM exhibits significant cellular and transcriptomic plasticity in response to IsH, and that some of this plasticity is enhanced by a loss of brainstem 5-HT signaling.

IsH increases the number of catecholaminergic neurons in the neonatal VLM

In both treated and control pups, IsH increased the number of identifiable catecholaminergic neurons in the VLM. This plasticity was also reflected in an increase in medullary noradrenaline content following IsH. We are unaware of any previous reports that have demonstrated this form of IsH-induced plasticity. It is not possible for us to discern whether IsH actually induces the expression of TH de novo, thereby converting a previously non-catecholaminergic neuron into a catecholaminergic neuron, or simply increases the TH content of an existing catecholaminergic neuron. In other cells hypoxia elicits an increase in TH gene transcription as well as mRNA stability (10, 11). The TH gene has AP-1 response elements which bind members of the Fos and Jun proteins in response to hypoxia, and increased catecholamine synthesis in response to hypoxia is Fos dependent (41). However, our RNAseq analysis did not identify any differential expression of TH within the VLM in response to IsH. TH can be subjected to considerable post-translational modifications that alter its stability (12), and it may be that its stability increases following IsH. Catecholamines themselves may be responsible, as they have
been shown to bind TH and decrease the rate of its degradation (37). A loss of 5-HT magnified the effect of IsH on the number of identifiable TH-positive neurons. While this may be a direct effect of reduced 5-HT signaling, we cannot exclude the possibility that this resulted from more severe hypoxia and/or hypotension resulting from 5-HT deficiency.

5-HT deficiency increases the number of activated VLM neurons

6-FL-treated, 5-HT-deficient pups experience a considerable delay in heart rate and respiratory recovery during IsH, likely the result of a greater loss of BP during the hypoxic episodes as well as a delay in BP recovery during gasping (57). Our original hypotheses was, in part, based on the idea that this phenotype was resulted from reduced 5-HT-mediated excitation of pre-sympathetic catecholaminergic neurons in the VLM. Our data unequivocally do not support this hypothesis and, on the contrary, suggest that compared to controls, IsH activates more neurons in the VLM of 5-HT-deficient pups. This relative increase in neuronal activation was not confined to any one region along the rostral-caudal extent of the VLM, and our findings also indicate that a proportion of the activated neurons in 5-HT deficient pups are not catecholaminergic. They could be sympathetic premotor neurons of a different phenotype – substance P-, neuropeptide Y-, somatostatin- or enkephalin neurons for example (43, 55) – that are simply responding to a larger hypoxic/hypotensive stimulus due to reduced 5-HT signaling at some other location. Another possibility is that they are inappropriately activated GABAergic neurons that could inhibit that RVLM-mediated sympathetic response to IsH (4, 21).

If the VLM of 5-HT-deficient pups is simply responding to a greater hypoxic and/or hypotensive stimulus, then where is the primary defect? One possibility worth exploring is the PVN, an area that receives dense projections from the CVLM, projections that may play an important role in the cardiovascular and respiratory responses to more severe levels of hypoxia (29, 31). In addition, it has
been known for some time that there are serotonergic projections to the PVN from the medullary raphe nuclei \(^\text{(25, 34)}\), including projections to pre-sympathetic regions within the parvocellular PVN \(^\text{(35)}\).

Further, 5-HT has been shown to depolarize PVN neurons \(^\text{(26)}\), and others have found that the activation of parvocellular PVN neurons in response to other forms of physiological stress depends heavily on local serotonergic signaling \(^\text{(33)}\). It is worth noting that, even in room air, there are more activated neurons in the VLM of 5-HT-deficient pups compared to controls. This could be due to chronic blood gas disturbances resulting from increased apnea (their breathing is significantly more unstable; Table 1). Elevated catecholaminergic activity could in part underlie the higher resting heart rate (Table 1) and strong tendency for higher arterial blood pressure associated with 5-HT deficiency \(^\text{(57)}\).

**Intermittent severe hypoxia alters the expression of serotonergic and catecholaminergic system genes**

Our RNAseq analysis revealed that in control and treated pups alike, IsH increases the expression of critical 5-HT system components, including TPH2 and 5-HTT. Although we do not know for certain whether this finding is mirrored at the protein level, if so it would suggest increased 5-HT signaling in the VLM plays an adaptive role in the physiological response to hypoxia. Our previous and current findings regarding the responses of 6-FL-treated pups suggest that this is true, as do previous experiments using rodents deficient in 5-HT neurons that die prematurely when exposed to IsH \(^\text{(7, 9, 57)}\). However, what our transcriptomic data now suggest is that there is considerable serotonergic plasticity within the VLM in response to severe hypoxia. Our data also suggest that, similar to previous findings, the expression of the 5-HTT and TPH2 genes is dynamically upregulated in response to IsH \(^\text{(3, 13)}\). While the genomic mechanism(s) responsible for this regulation are unclear, we note that the
TPH1 gene contains HIF-1α response elements (41), and that its expression has been shown to be modulated by changing O2 levels (24, 44, 49).

In 5-HT-deficient pups the expression of the 5-HT1A receptor in the VLM is downregulated by IsH. Given that this receptor is inhibitory, this effect may be due to negative feedback in response to decreasing post-synaptic 5-HT signaling. Finally, the increased expression of the DOPA decarboxylase gene in 5-HT-deficient pups exposed to IsH is interesting in light of the increased numbers of catecholaminergic neurons in these pups. This may be in response to increased amounts of L-DOPA resulting from elevated TH levels.

**5-HT deficiency leads to plasticity within the glutamatergic system in response to IsH**

5-HT deficiency was associated with altered expression of major glutamatergic system components, including VGLUT-1 as well as metabotropic and ionotropic glutamate receptors. Compared to controls, there was ~5-times the amount of the mRNA for VGLUT-1, one of three vesicular glutamate transporters, in the VLM of 5-HT-deficient pups in response to IsH, suggesting increased glutamatergic signaling. Traditionally, it has been thought that the expression of VGLUT-1 and VGLUT-2 are confined to separate subsets of glutamatergic neurons, with little overlap. While there is evidence that VGLUT-2 is expressed in the RVLM, and that C1 neurons in this region rely on VGLUT-2 for appropriate glutamate-mediated autonomic and respiratory responses (1, 54), there is no such evidence for VGLUT-1. There is a report that VGLUT-1 is expressed within a region extending from the most caudal aspects of the CVLM into the upper cervical spinal cord (the medullo-cervical pressor area (51)), but our tissue punch did not contain this region. Interestingly, the excitability of 5-HT neurons in the dorsal raphe nuclei appears to be at least partially modulated by VGLUT-1-dependent glutamatergic neurotransmission (18). And VGLUT-3, an unconventional glutamate transporter that contributes to the respiratory response to hypoxia (39) co-localizes with 5-HT and has been found to
enhance 5-HT packaging and release (2, 50). However, we are unaware of any previous reports indicating that 5-HT has the potential to modulate the expression of major glutamatergic system components in the VLM.

**A role for peripheral 5-HT in the vascular response to intermittent severe hypoxia?**

In addition to the action of TPH2 in the brainstem and midbrain, 5-HT is synthesized by enterochromaffin cells of the gut by TPH1. Given that 6-FL could inhibit TPH1 and TPH2 alike, an intriguing possibility is that the hypotension, hypoxia and premature death of 6-FL-treated pups is due to insufficient 5-HT signaling in the vasculature during severe hypoxia. 5-HT produced in the gut is carried within the blood mostly in platelets – with a small amount dissolved in plasma – and this 5-HT binds receptors on vascular smooth muscle to elicit vasoconstriction (48, 56), an effect that has also been described in newborn animals (32). That being said, although a role for peripheral 5-HT in hypoxia-induced pulmonary vasoconstriction has been described (40), there are few if any studies that have examined the extent to which peripheral 5-HT participates in the control of systemic vasomotor tone in response to hypoxia. This issue warrants future investigation.

**Perspectives and Significance**

The results of this study suggest that the VLM of rat pups displays novel cellular and transcriptomic plasticity in response to IsH and 5-HT deficiency. 5-HT deficiency does not compromise the activation of catecholaminergic neurons within the VLM. On the contrary, the VLM is more heavily activated by IsH when it occurs in a background of 5-HT deficiency. The phenotype of some of these activated neurons remains to be identified. An additional novel finding is that IsH increases the number of identifiable catecholaminergic neurons within the neonatal VLM, an effect that is magnified by a loss of 5-HT.
Finally, we show that IsH increases the expression of TPH2 and 5-HTT, and in 5-HT-deficient animals the inhibitory 5-HT$_{1A}$ receptor is downregulated while DOPA decarboxylase expression is upregulated. Thus, at both the cellular and transcriptomic levels, the medullary serotonergic and catecholaminergic systems display considerable plasticity within the neonatal VLM in response to IsH, ostensibly as an adaptation to this repetitive severe stress.

ACKNOWLEDGMENTS

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55. **Strack AM, Sawyer WB, Platt KB, and Loewy AD.** CNS cell groups regulating the sympathetic outflow to adrenal gland as revealed by transneuronal cell body labeling with pseudorabies virus. *Brain Res* 491: 274-296, 1989.


Figure Legends

**Figure 1. Catecholaminergic neurons within the ventrolateral medulla (VLM).** Epifluorescent photomicrograph image showing the distribution of catecholaminergic neurons in the C1/A1 region of the VLM that were the focus of this study (-0.4 mm caudal to the obex, or -14.1 mm caudal to Bregma). Inset shows boxed region at higher magnification. cc= central canal. Scale bar= 0.250.

**Figure 2. Effects of 6-FL on medullary serotonin (5-HT) and noradrenaline (NA) content.** Following intermittent severe hypoxia the medullas of 6-FL-treated pups (white bars) had ~50% less 5-HT and ~70% more NA than controls (black bars).

**Figure 3. Raw records of heart rate and respiratory responses to severe hypoxia.** Shown are raw ECG, heart rate (HR) and respiratory activity (Resp Vol) in vehicle- and 6-FL-treated littermates (n=8 for both). Records begin during the severely hypoxic episode, when HR is suppressed and apnea is evident. The first gasp is indicated. Horizontal arrows in the HR and respiratory traces indicate the time required for HR and respiratory recovery. Note the delay in cardiorespiratory recovery in the 6-FL treated pup compared to its control littermate. Scale bar= 10 sec.

**Figure 4. Heart rate and respiratory recovery are delayed in 6-FL-treated, 5-HT-deficient rat pups compared to littermates.** Shown are the average gasp latencies (i.e. duration of hypoxic apnea) (a) and the time required for heart rate (HR) recovery (b) and the recovery of eupnea (i.e. normal breathing) (c) for 6-FL treated (open circles; n=6) and vehicle controls (closed circles; n=6) across the 10 episodes of severe hypoxia. Note in treated pups the significantly longer time required for recovery. *: significant difference between groups, p<0.05. Shown are mean data ± S.E.
Figure 5. Influence of intermittent severe hypoxia (IsH) and 5-HT deficiency on numbers of tyrosine hydroxylase (TH)-positive, Fos-positive and dual-labeled neurons in the ventrolateral medulla. Mean numbers of tyrosine hydroxylase (TH)-positive (a), Fos-positive (b) and dual-labelled neurons (c) across the rostral-caudal extent of the VLM (from -1.2mm to 1.2mm relative to the obex) are shown for controls (closed circles) and 6-FL-treated littermates (open circles) following intermittent severe hypoxia (IsH; left: n=6 for both groups) or air alone (right: n=4 for both groups). d, e. Epifluorescent photomicrographs of representative coronal sections (0.6mm rostral from the obex, within the intermediate region of the VLM), from a control (d) and 6-FL-treated pup (e) following IsH. Activated (Fos-positive) neurons have nuclei that are pseudostained red. TH-positive neurons have cytoplasm and projections that are pseudostained green. Scale bars=250nm. Insets show boxed regions at higher magnification (scale bar=75nm). Shown are mean data ± S.E.
Table 1. Resting variables of control (Veh) and 6-fluorotryptophan (6-FL) treated P9-10 rat pups. Mass (grams); HR: heart rate (beats.min⁻¹); $V_E^*$: ventilation (ml.min⁻¹.kg⁻¹); $V_T$: tidal volume (ml.kg⁻¹); $f_B$: respiratory frequency (breaths.min⁻¹); $\dot{V}_O_2^*$: metabolic rate (ml.min⁻¹.kg⁻¹); $V_E^* / \dot{V}_O_2^*$: respiratory equivalent. *: significant difference between vehicle and 6-FL-treated pups (p<0.05).

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<tr>
<th></th>
<th>Mass</th>
<th>HR</th>
<th>$V_E^*$</th>
<th>$V_T$</th>
<th>$f_B$</th>
<th>CV%</th>
<th>$\dot{V}_O_2^*$</th>
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<tr>
<td>Veh n=8</td>
<td>23.1 ±0.7</td>
<td>410 ±17</td>
<td>1131 ±79</td>
<td>7.3 ±0.5</td>
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<td>6-FL n=8</td>
<td>22.0 ±0.7</td>
<td>474* ±7</td>
<td>1426 ±158</td>
<td>9.4* ±0.9</td>
<td>118 ±13</td>
<td>17.3 ±2.4</td>
<td>30 ±1.0</td>
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<td>Gene Name</td>
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<td>6-FL-nx/ Veh-nx</td>
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<td>Tph2</td>
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**Table 2.** Differential expression (DE) of serotonin (5-HT), catecholamine (CA), or other neurotransmitter system genes (Other) within the ventrolateral medulla of control pups (Veh) or pups treated with 6-fluorotryptophan (6-FL), and exposed to either air (nx) or intermittent severe hypoxia (IsH). Our statistical approach assessed: 1) effects of IsH alone (denoted by <sup>a</sup>): significant DE between Veh-IsH and Veh-nx, or between 6-FL-IsH and 6-FL-nx; 2) effects of 5-HT deficiency alone (denoted by <sup>b</sup>): significant DE between 6-FL-nx and Veh-nx; and 3) interactive effect of IsH and 5-HT deficiency (denoted by <sup>c</sup>): significant DE between 6-FL-IsH and 6-FL-nx, with no DE between Veh-IsH and Veh-nx or when DE between Veh-IsH and Veh-nx was in the opposite direction as 6-F-IsH and 6-FL-nx. Values are expressed in base 2 and reflect the expression in the first group (e.g. Veh-hx) relative to the second group (e.g. Veh-nx).
Figure 2

[monoamine] (ng/mg protein)

- 5-HT
- NA

* Indicates significant difference.
Figure 3

Veh

ECG (V)

HR (BPM)

Resp Vol. (V.s)

6-FL

ECG (V)

HR (BPM)

Resp Vol. (V.s)

vast
Figure 4

(a) Gasp latency (sec)

(b) HR recovery (sec)

(c) Eupnea recovery (sec)

Episode #
Figure 5

(a) 

6FL x IsH: $p=0.003$
6FL x position: $p=0.009$

(b) 

6FL x IsH: $p=0.008$

(c) 

6FL x position: $p=0.04$

(d) Veh-IsH +0.8

(e) 6FL-IsH +0.8