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Research report

In vivo effect of chronic hypoxia on the neurochemical profile of the developing rat hippocampus

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Abstract

The cognitive deficits observed in children with cyanotic congenital heart disease suggest involvement of the developing hippocampus. Chronic postnatal hypoxia present during infancy in these children may play a role in these impairments. To understand the biochemical mechanisms of hippocampal injury in chronic hypoxia, a neurochemical profile consisting of 15 metabolite concentrations and 2 metabolite ratios in the hippocampus was evaluated in a rat model of chronic postnatal hypoxia using in vivo ^{1}H NMR spectroscopy at 9.4 T. Chronic hypoxia was induced by continuously exposing rats (n = 23) to 10% O_2 from postnatal day (P) 3 to P28. Fifteen metabolites were quantified from a volume of 9–11 μl centered on the left hippocampus on P14, P21, and P28 and were compared with normoxic controls (n = 14). The developmental trajectory of neurochemicals in chronic hypoxia was similar to that seen in normoxia. However, chronic hypoxia had an effect on the concentrations of the following neurochemicals: aspartate, creatine, phosphocreatine, GABA, glutamate, glutamine, glutathione, *myo*-inositol, *N*-acetylaspartate (NAA), phosphorylethanolamine, and phosphocreatine/creatine (PCr/Cr) and glutamate/glutamine (Glu/Gln) ratios ($P < 0.001$ each, except glutamate, $P = 0.04$). The increased PCr/Cr ratio is consistent with decreased brain energy consumption. Given the well-established link between excitatory neurotransmission and brain energy metabolism, we postulate that elevated glutamate, Glu/Gln ratio, and GABA indicate suppressed excitatory neurotransmission in an energy-limited environment. Decreased NAA and phosphorylethanolamine suggest reduced neuronal integrity and phospholipid metabolism. The altered hippocampal neurochemistry during its development may underlie some of the cognitive deficits present in human infants at risk of chronic hypoxia.
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Topic: Brain metabolism and blood flow

Keywords: Chronic hypoxia; Hippocampus; Rat; ^{1}H NMR spectroscopy; Phosphocreatine

1. Introduction

The incidence of cyanotic congenital heart disease (CHD) is approximately 2 per 1000 live births [22]. While advances in the techniques of cardiopulmonary bypass and hypothermia have improved the survival rates of these infants [31], cognitive deficits remain a significant cause of morbidity in this population [6]. Developmental delay has

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been recognized as early as 2 months old in infants with CHD [1]. In children with CHD, long-term studies using standardized tests of academic achievement have demonstrated lower scores compared with the controls [54]. Multiple risk factors such as chronic hypoxia, anesthesia, and potentially low cerebral blood flow during cardiopulmonary bypass may be responsible for these learning disabilities [35–37].

Children with CHD are in a state of chronic hypoxia prior to the repair of their cardiac lesion. Most surgical repairs for CHD are performed during the first 2 to 3 years of life [47], and therefore, the time period of chronic hypoxia encompasses the period of rapid hippocampal growth and development [4]. The hippocampus is central to recognition memory and plays an important role in cognitive development. Early hippocampal damage due to chronic hypoxia may lead to memory deficits, which could present as learning disabilities in children with CHD.

In rats, the rapid phase of hippocampal development occurs postnatally in the first 4 weeks and corresponds to the first two to three postnatal years of rapid hippocampal development in humans [4]. Rodents that have been hypoxic during the early postnatal period demonstrate evidence of disrupted synaptic development [13], alterations in NMDA receptor expression and function [42], and alterations in key enzymes of glucose oxidative metabolism in the brain [26]. The activity of neuronal cytochrome *c* oxidase, a key enzyme in oxidative phosphorylation, is decreased in chronic hypoxia [11,27].

Given the effects of chronic hypoxia on brain energy metabolism and neurotransmitter function, a longitudinal evaluation of neurochemical changes during hippocampal development may aid in the understanding of the potential mechanisms by which chronic hypoxia alters hippocampal development and function. In vivo high-field ^{1}H NMR spectroscopy is a sensitive and non-invasive method that can be used for the simultaneous quantification of multiple metabolites from distinct regions of the developing brain under physiological and adverse perinatal conditions [46,50]. At high magnetic field strengths of 9.4 T, a neurochemical profile consisting of metabolite markers of energy metabolism, neuronal integrity, myelination, and neurotransmitters can be reliably and longitudinally measured from specific brain regions, such as the developing hippocampus [46,50].

The objective of the present study was to evaluate in vivo the hippocampal neurochemical profile in a rat model of chronic postnatal hypoxia. We hypothesized that chronic hypoxia would adversely affect the metabolite markers of energy metabolism, neuronal integrity, myelination, and neurotransmitters. We used a well-described rodent model of chronic postnatal hypoxia in our study [11,26,32]. Rats between the ages of postnatal day (P) 14 and P28 were studied because of the similarity between their hippocampal development and that of, respectively, a full-term human newborn infant and a 2- to 3-year-old child [4].

2. Materials and methods

2.1. Animal preparation

The study was approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee at the University of Minnesota. Pregnant Sprague–Dawley rats were purchased (Harlan Sprague–Dawley; Indianapolis, IN) and housed with free access to food and water in the local animal care facility. The dams and the pups were maintained on a regular diet (Teklad 4% Mouse/Rat Diet 7001, Harlan-Teklad; Madison, WI). On P3, the pups were randomly culled to eight per litter to ensure adequate nutrition. Twenty-three pups from three litters were studied by ^{1}H NMR spectroscopy. Thirteen pups were studied longitudinally at all three time points, and the rest were studied in a cross-sectional manner, resulting in 16–17 spectra at each of the three postnatal ages. The neurochemical profile during development was compared with our previously published data of normoxic controls [50]. Historical controls were used because of the expense of performing the studies and the precision of the metabolite concentrations generated in that study. Briefly, the data of the normoxic controls had been obtained from fourteen age-matched rats from three litters that were raised under conditions identical to the chronic hypoxia group, except for the O_2 concentration in the environment (21% instead of 10%). Seven of these rats were studied by ^{1}H NMR spectroscopy at each of the three postnatal ages, and the remaining rats were studied in a cross-sectional manner, resulting in 8–11 spectra at each of the three postnatal ages. A separate set of pups in the chronic hypoxia (n = 12 from two litters) and normoxia groups (n = 16 from two litters) were used for measurement of body and brain weights, brain water content and hematocrit on P14 and P28, and brain iron concentration on P14.

2.2. Induction of postnatal chronic hypoxia

The dam and the pups in the chronic hypoxia group were subjected to continuous postnatal hypoxia from P3 to P28. They were placed in an airtight Plexiglas chamber (Scientific Apparatus; University of Minnesota; Minneapolis, MN). The concentration of O_2 inside the chamber was maintained at 10% by mixing air and nitrogen and was monitored using an oxygen analyzer (Miniox II; Catalyst Research Corporation; Owings Mills, MD). The degree of hypoxia was based on rodent models of CHD [8,34]. Excess humidity from the chamber was eliminated using calcium bisulphate (Drierite; W.A. Hammond Drierite Company; Xenia, OH). The chamber was opened for maintenance twice a week for approximately 10 min each time. The rats

were kept in a temperature-controlled room with a 12-h dark and light cycle.

2.3. ^{1}H NMR spectroscopy

Spontaneously breathing rat pups were studied using ^{1}H NMR spectroscopy on P14, P21, and P28 under inhalation anesthesia using previously published methods from our laboratory [46,50]. The mean ± SD duration of anesthesia was 56 ± 8 min on P14, 49 ± 5 min on P21, and 48 ± 5 min on P28. The mean total duration that the animals were removed from the hypoxia chamber for ^{1}H NMR spectroscopy was 125 ± 6 min on P14 and 110 ± 6 min on P21. P28 animals were maintained in normoxia after the conclusion of the study. A uniform temperature was maintained inside the magnet using circulating warm water.

In order to assess whether acutely placing the rats in 50% O_2 during NMR studies altered markers of energy metabolism, P28 pups from the chronic hypoxia group (n = 6) underwent ^{1}H NMR spectroscopy under both 50% O_2 and 10% O_2. Additionally, to evaluate whether repeated exposure to normoxia and anesthesia had affected the neurochemical profile, four animals from a litter in the chronic hypoxia group were studied longitudinally at all three postnatal ages and their four littermates were studied only on P28.

All experiments were performed on a 9.4 T/31 cm magnet (Magnex Scientific; Abingdon, U.K.) equipped with 11 cm gradient coil (300 mT/m, 500 μs) and custom-designed shim coil (Magnex Scientific; Abingdon, U.K.) with second-order shim strength up to 0.04 mT/cm^2 [51]. The magnet was interfaced with a Varian INOVA console (Varian, Inc.; Palo Alto, CA). A quadrature transmit/receive surface RF coil with two geometrically decoupled single-turn coils was used. Homogeneity of the magnetic field was automatically adjusted by FASTMAP shimming [15,17]. The water signal was suppressed by VAPOR method [49] and localization was done by ultra-short echo-time STEAM (TE = 2 ms, TM = 20 ms, TR = 5 s) combined with outer volume suppression. The positions of volume of interest (VOI) 9–11 μl were centered on the left hippocampus based on multislice RARE images.

3. Analysis

3.1. Quantitation of metabolites

^{1}H NMR spectra were analyzed using LC Model [43], including a macromolecule spectrum in the database, according to previously described procedures [41,50]. The unsuppressed water signal from the same VOI and age-dependent brain water content were used for metabolite quantification. The brain water content was measured on P14 and P28 using the weight difference between freshly removed brain and its residue after lyophilization. The following 18 metabolites were quantified from each spectrum: alanine, aspartate, creatine (Cr), γ-aminobutyric acid (GABA), glucose, glutamate, glutamine, glutathione, glycerophosphorylcholine (GPC), lactate, *myo*-inositol, *N*-acetylaspartate (NAA), *N*-acetylaspartylglutamate, phosphocreatine (PCr), phosphorylcholine (PCho), phosphorylethanolamine, *scyllo*-inostol, and taurine. The phosphocreatine/creatine (PCr/Cr) and glutamate/glutamine (Glu/Gln) ratios were calculated.

3.2. Brain iron assay

Animals were deeply anesthetized with sodium pentobarbital (120 mg/kg IP) and blood samples for hematocrit were taken from the left ventricle. Following in situ transcardial perfusion with normal saline, the brain was dissected out, flash-frozen and stored at −80 °C. Brain iron concentration was assayed by atomic absorption spectroscopy [45] and was expressed as μg of elemental iron/g wet-tissue weight.

3.3. Statistical analysis

Group means of body weight, brain weight, brain water content, and hematocrit on P14 and P28, and brain iron concentration on P14 in the two groups were compared using the two-tailed unpaired t test. The effects of age, chronic hypoxia and age-dependent effect of chronic hypoxia (age * chronic hypoxia) on the metabolites were determined using multivariate analysis of variance (MANOVA). For metabolites that demonstrated a difference, the age-specific effect of chronic hypoxia was determined by performing post hoc analyses using the Bonferroni-adjusted t test at each postnatal age. A software package (SPSS; Chicago, IL) was used for the statistical analysis. The data are presented as mean ± SD. Statistical significance was set at $P < 0.05$, except for the post hoc analyses, where significance was set at $P < 0.02$.

Table 1
Effect of postnatal chronic hypoxia on body weight, brain weight, hematocrit, brain iron concentration, and brain water content of the developing rats

	Postnatal age (days)	Chronic hypoxia group	Normoxia group	P
Body weight (g)	14	23.5 ± 2.6	32.5 ± 2.1	<0.001
	28	61.1 ± 5.0	97.5 ± 11.0	<0.001
Brain weight (g)	14	0.70 ± 0.06	0.86 ± 0.06	<0.001
	28	0.73 ± 0.03*	1.14 ± 0.03	<0.001
Hematocrit (%)	14	45.0 ± 2.8	40.0 ± 1.7	0.001
	28	65.1 ± 3.1	39.3 ± 1.4	<0.001
Brain iron concentration (μg/g)	14	6.3 ± 1.0	8.2 ± 1.1	0.004
Brain water content (%)	14	82	85	0.014
	28	80*	80	NS

Values: mean ± SD (n = 8/group at each age, except *n = 4).
Significance by two-tailed unpaired t test.

4. Results

The pups in the chronic hypoxia group had lower body and brain weights and higher hematocrits when compared with the normoxia group on P14 and P28. The mean brain water content and brain iron concentration on P14 were lower in the chronic hypoxia group (Table 1).

Representative in vivo ^{1}H NMR spectra from the hippocampus of chronic hypoxia and normoxia rats, and the corresponding MR images with selected VOI are displayed in Fig. 1. In addition to signals typical for clinical ^{1}H NMR spectra (NAA, Cr, choline), signals of other metabolites, such as aspartate, glutamate, *myo*-inositol, *N*-acetylaspartylglutamate, and taurine, could be readily identified. In addition, signals of the methylene groups of Cr and PCr were resolved (insets in Fig. 1). Fifteen of eighteen metabolites included in the LC Model basis set were reliably quantified (Cramer-Rao lower bounds [CRLB], <25%) from each spectrum and only these were used for further analysis. Alanine and *scyllo*-inositol were excluded due to CRLB > 25%. Furthermore, because of strong cross-correlation between GPC and PCho, due to their close spectral similarity, the sum of GPC and PCho was analyzed.

To verify whether multiple exposures to anesthesia and 50% O_2 concentration during NMR experiments compounded the effects of chronic hypoxia on metabolites, metabolite concentrations on P28 from four pups studied

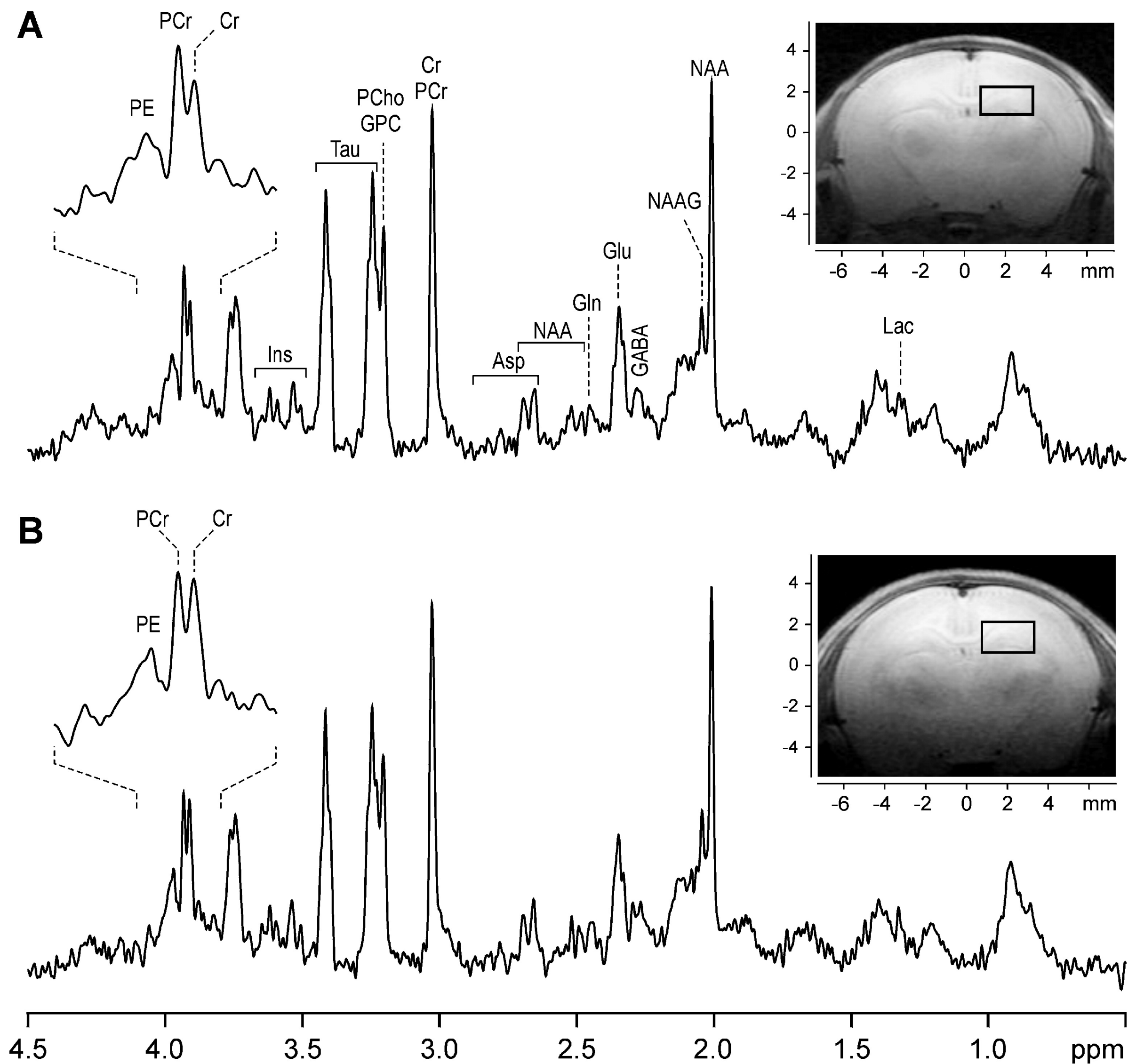

Fig. 1. MRI and ^{1}H NMR spectra from the hippocampus of chronic hypoxia (Panel A) and normoxia (Panel B) rat on postnatal day 14. The volume of interest (A: 2.5 × 1.3 × 3.0 mm^3; B: 2.5 × 1.5 × 3.0 mm^3) is placed on the left hippocampus from where ^{1}H NMR spectra were obtained. Insets show the expanded spectral regions (3.8 to 4.1 ppm) demonstrating signals of creatine and phosphocreatine. Localization sequence: STEAM, TE = 2 ms, TR = 5 s, NT = 240. Abbreviations. Asp: aspartate, Cr: creatine, GABA: γ-aminobutyric acid, Glu: glutamate, Gln: glutamine, GPC: glycerophosphorylcholine, Lac: lactate, Ins: *myo*-inositol, NAA: *N*-acetylaspartate, NAAG: *N*-acetylaspartylglutamate, PCr: phosphocreatine, PCho: phosphorylcholine, PE: phosphorylethanolamine and Tau: taurine.

longitudinally on P14, P21, and P28 were compared with those obtained from the four littermates that were studied only on P28. No significant differences were present for any metabolite (Fig. 2). Thus, the data from animals studied longitudinally and cross-sectionally were pooled together to establish the mean values at each postnatal age.

In both groups, there were developmental changes between P14 and P28 in the concentrations of all metabolites, except glucose and lactate ($P < 0.003$ each). The developmental trajectories of metabolites in the chronic hypoxia group were similar, but not identical, to those in the normoxia group. Chronic hypoxia had an effect on Cr, GABA, glutamine, *myo*-inositol, NAA, and phosphorylethanolamine concentrations, as well as on PCr/Cr and Glu/Gln ratios ($P < 0.001$ each, Fig. 3). Aspartate, PCr, glutamate, and glutathione concentrations were also affected by chronic hypoxia ($P < 0.001$ each, except glutamate $P = 0.04$; data not shown). Chronic hypoxia had no effect on taurine (Fig. 3), glucose, GPC + PCho, lactate and *N*-acetylaspartylglutamate concentrations (data not shown). Chronic hypoxia had an age-dependent effect (age * chronic hypoxia) on aspartate, PCr, glutamate, *myo*-inositol, NAA, phosphorylethanolamine concentrations, and PCr/Cr ratio ($P < 0.001$ each, except PCr/Cr ratio $P = 0.03$).

To determine whether the elevated PCr/Cr ratio resulted from acute exposure to higher O_2 concentration during the NMR studies, ^1H NMR spectra were obtained in six P28 pups under both 50% O_2 and 10% O_2. Exposure to 10% O_2 resulted in increased spectral line-width and decreased accuracy of Cr and PCr quantification (CRLB increased from 6% to 15%). In spite of this, PCr/Cr ratio was elevated at 10% O_2 concentration and was similar to that measured using 50% O_2 concentration ($P = 0.14$).

5. Discussion

Chronic hypoxia significantly altered the neurochemical profile of the developing rat hippocampus. Ten of the fifteen neurochemicals were affected as a result of exposure to chronic hypoxia, while five were spared. Such differential effects on metabolites in the hippocampus suggest that certain biochemical pathways are more sensitive than others to chronic hypoxia. Alterations in energy metabolism by chronic hypoxia may underlie many of the neurochemical changes.

The most intriguing effect of chronic hypoxia was on energy metabolism, as indexed by the elevated PCr/Cr ratio. We expected the PCr/Cr ratio to be reduced because cytochrome *c* oxidase activity is decreased in chronic hypoxia [11,27] and because PCr acts as a temporary energy buffer when ATP production is inadequate [52]. Furthermore, the effect was present during both hypoxic conditions and hyperoxic testing states, suggesting that the increase was due to chronic hypoxia rather than to the sudden hyperoxic conditions during NMR studies.

The increased PCr/Cr ratio in the presence of decreased oxidative phosphorylation suggests that energy metabolism

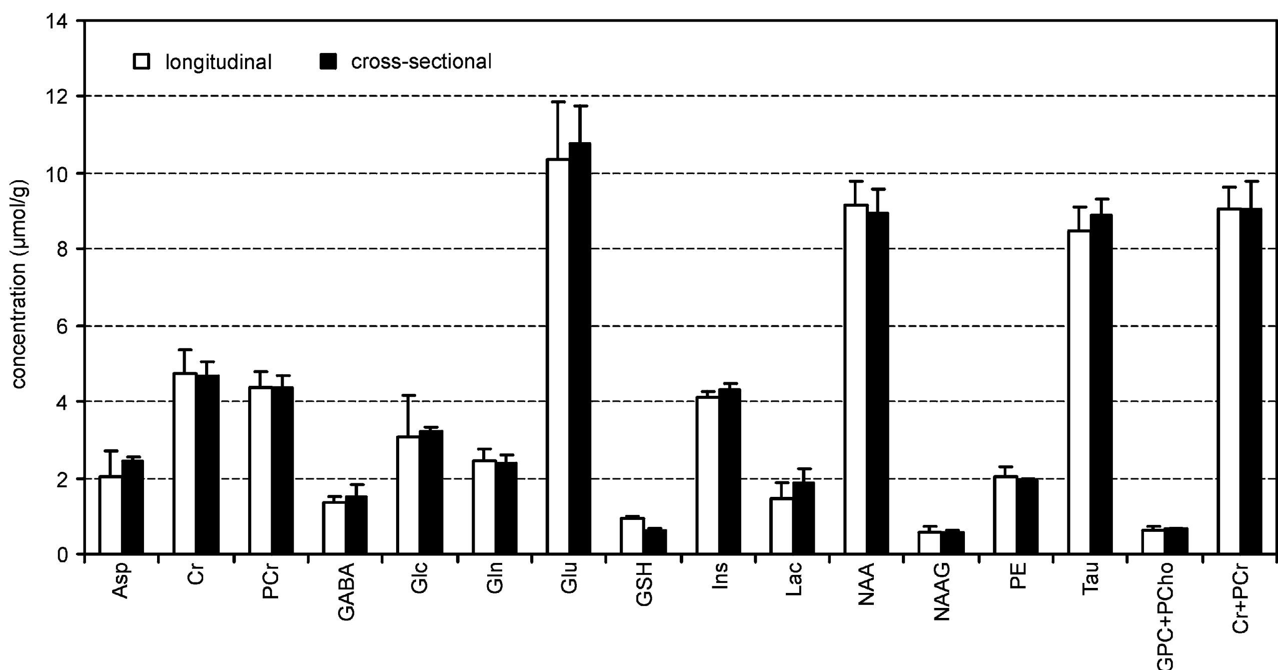

Fig. 2. Neurochemical concentrations in the hippocampus of postnatal day (P) 28 rats in the chronic hypoxia group. *Longitudinal* represents data from rats that were studied on all three postnatal days (P14, P21, and P28); *Cross-sectional* represents data from littermates that were studied only once on P28. Values are mean ± SD; $n = 4$ in each group (P not significant; two-tailed unpaired t test). Abbreviations. Asp: aspartate, Cr: creatine, GABA: γ-aminobutyric acid, Glc: glucose, Glu: glutamate, Gln: glutamine, GSH: glutathione, GPC: glycerophosphorylcholine, Lac: lactate, Ins: *myo*-inositol, NAA: *N*-acetylaspartate, NAAG: *N*-acetylaspartylglutamate, PCr: phosphocreatine, PCho: phosphorylcholine, PE: phosphorylethanolamine and Tau: taurine.

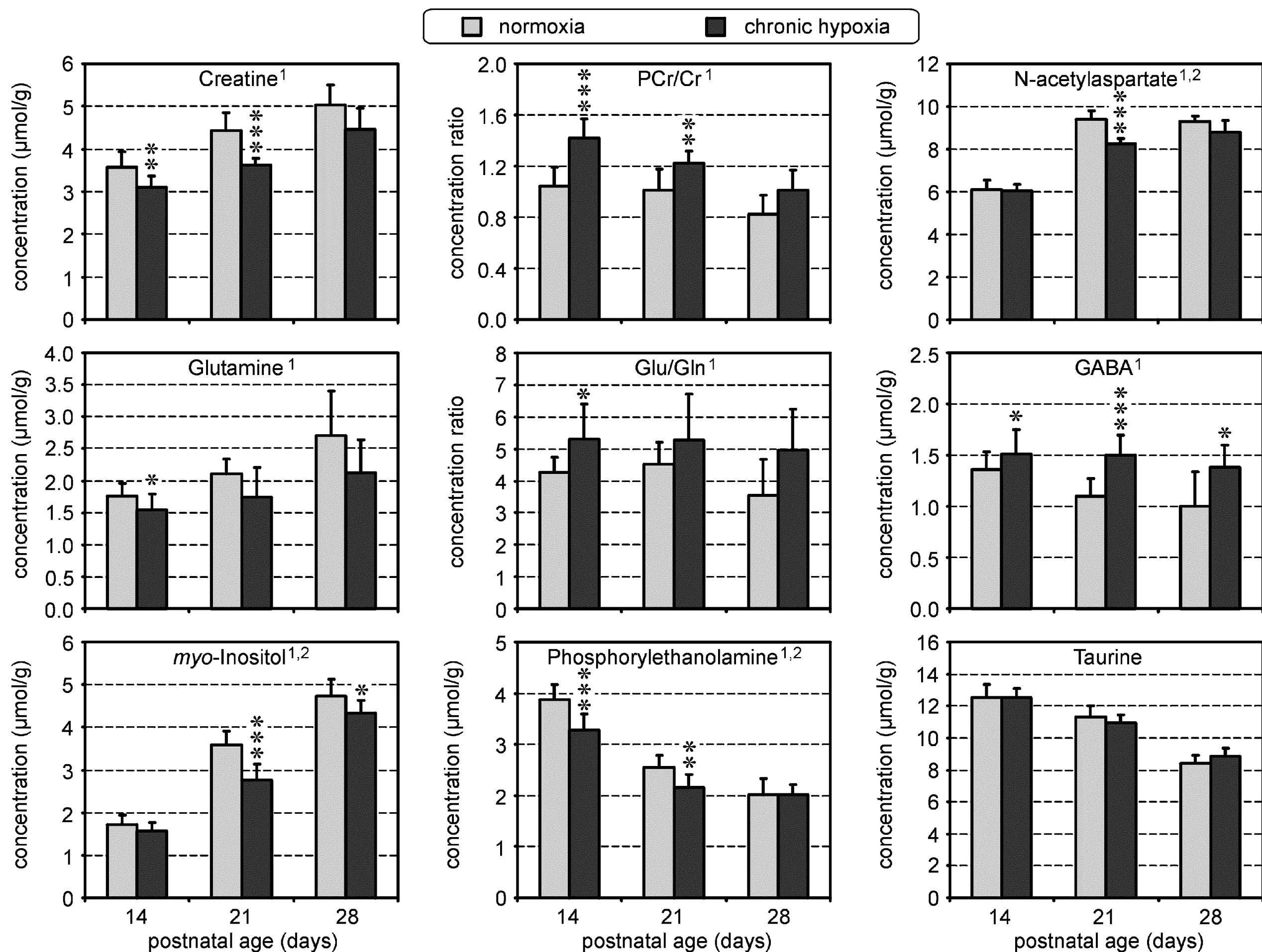

Fig. 3. Effect of chronic hypoxia on creatine, *N*-acetylaspartate (NAA), glutamine, γ-aminobutyric acid (GABA), *myo*-inositol, phosphorylethanolamine, and taurine concentrations, and phosphocreatine/creatine (PCr/Cr) and glutamate/glutamine (Glu/Gln) ratios during hippocampal development in the chronic hypoxia and normoxia groups. Values are mean ± SD; n = 23 in the chronic hypoxia group and n = 14 in the normoxia group. The normoxia data are from a previously published study [50]. There were developmental changes between postnatal days 14 and 28 in all metabolites ($P < 0.03$ each). [1]Metabolites affected by chronic hypoxia and [2]metabolites affected by age * chronic hypoxia, respectively ($P < 0.001$ each; MANOVA). Asterisks denote significant differences between groups at each postnatal age (*$P < 0.02$, **$P < 0.001$, and ***$P < 0.0001$; Bonferroni-adjusted unpaired t test).

is altered in chronic hypoxia, probably as an adaptive response. We propose that the developing hippocampus adapts to chronic hypoxia by decreasing its energy utilization. Reduced energy utilization has been documented in neuronal cell cultures in response to acute hypoxia [33] and in certain highly anoxia-tolerant species, such as turtles and fish [21]. Furthermore, it has been demonstrated that PCr concentration increases in the rat brain when cerebral energy consumption is suppressed by the ingestion of ethyl alcohol [14]. It is likely that chronic hypoxia in our model may have induced a similar effect on PCr.

Understanding altered hippocampal energy metabolism during chronic hypoxia is important because it may account for the increased glutamate and GABA concentrations, and Glu/Gln ratio in the chronic hypoxia group. These neurotransmitter findings imply that excitatory neurotransmission may be suppressed in chronic hypoxia. Glutamate plays an important role in synaptogenesis and plasticity through the stimulation of NMDA receptors [2,20,25]. Glutamate-mediated excitatory neurotransmission is a metabolically expensive process [3] and is predominantly accomplished through glutamate–glutamine cycling between neurons and astrocytes [16,18,30]. As ^{1}H NMR spectroscopy measures glutamate predominantly in the neuronal compartment [38], increased glutamate in our study implies that glutamate release from the neurons may be suppressed in chronic hypoxia, presumably in an attempt to conserve the meager energy stores [19]. The decreased expression of NMDA receptors and disrupted hippocampal synaptogenesis demonstrated in this model support our postulation [13,42].

The energy findings also were potentially responsible for the elevation of GABA concentration, a finding previously described in this model [53]. Depletion of ATP due to hypoxia results in increased synthesis and decreased catabolism of GABA in hippocampal neurons [29]. Elevated GABA concentration in our study is consistent with the premise that the overall excitatory activity of the neurons is suppressed in chronic hypoxia. For example, in patients

with intractable seizures, elevating GABA concentration in the brain suppresses brain activity and results in improved seizure control [39]. We speculate that increased GABA levels may provide such an inhibitory drive against excitatory neurotransmission in chronic hypoxia.

Changes in phosphorylethanolamine and NAA concentrations also may be due to altered energy metabolism in chronic hypoxia. Phosphorylethanolamine, a precursor of the major brain phospholipid phosphatidylethanolamine [44], was lower in the chronic hypoxia group. In humans and rats, the levels of phosphorylethanolamine are initially high and decrease with the onset of myelination [9,40]. There also is evidence to suggest that phosphorylethanolamine levels are increased in tumors of the brain that have high cell turnover rates [23]. We postulate that the decreased phosphorylethanolamine levels demonstrated in our study are due to altered myelination or decreased cell turnover in a hypometabolic state.

NAA was reduced in the chronic hypoxia group. NAA is synthesized and stored primarily in the neuron and has long been considered a marker of neuronal integrity in the adult brain [5]. However, the role of NAA in the developing brain is not well described. Even though NAA is produced in neurons, its catabolic enzyme, aspartoacylase, is found primarily in oligodendrocytes [5,7,10]. Deacetylation of NAA contributes carbons to fatty acid synthesis during myelin generation [24], a highly energy-dependent process in the developing brain. Studies have demonstrated that the rate of deposition, quantity and maturation of myelin are delayed in chronic hypoxia [28,32]. Decreased NAA in the present study may therefore represent loss of neuronal integrity, altered myelination, or both in chronic hypoxia.

The animal model used in this study is well described for studies of chronic hypoxia and results in a similar degree of hypoxia present in human infants with CHD [8,26,32]. The body weight of the chronic hypoxia rats was decreased, as has been demonstrated in all studies that have used a similar model [8,12,26]. While the associated malnutrition may have played a role in the neurochemical alterations, the effects are likely to be minor. In a rat model of malnutrition with similar loss of body weight, there were no alterations in PCr, and glutamate levels were decreased in the brain [48]. Similarly, as seen in children with CHD, iron deficiency was present in the chronic hypoxia group. While the 20% reduction in brain iron concentration may have played a role in the neurochemical alterations, it is unlikely to be responsible for all the changes. While changes in certain neurochemicals (for example, PCr/Cr, glutamate, and GABA) were similar in the two conditions, those in others (such as NAA and phosphorylethanolamine) were distinctly different from those demonstrated in perinatal iron deficiency [46].

In conclusion, the neurochemical profile of the developing hippocampus was altered in chronic hypoxia. Changes in multiple metabolites suggest involvement of several biochemical pathways consistent with adaptive changes to altered energy metabolism in chronic hypoxia. High-field ^{1}H NMR spectroscopy allowed us to longitudinally and comprehensively study these neurochemical changes. Because it is non-invasive, this method can be used in human infants at risk for chronic hypoxia. Altered hippocampal neurochemistry during its phase of rapid development may underlie some of the cognitive deficits described in human infants with chronic hypoxia.

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