Environmental Enrichment Contributes to Neurodevelopmental Recovery After Hypoxia

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Premature, very low birth weight infants often suffer from hypoxia, a deficiency of oxygen that can result in lasting cognitive and behavioral impairments. The present study used a mouse model to explore hypoxia-induced neurodevelop mental deficits and the role of environmental enrichment in reducing these abnormalities. It was found that chronic neonatal hypoxia decreased parvalbumin-expressing cortical interneurons and impaired spatial memory. However, hypoxia-reared mice that were environmentally enriched had greater cortical volumes than those that were not, and their parvalbumin-expressing interneuron number and spatial memory task performance were not different from controls. These findings suggest that low oxygen levels significantly alter brain cellular composition, neurotransmitter levels, volume, and function. Environmental enrichment may be a critical factor in reducing hypoxia-induced effects.

Introduction

Neurodevelopment can be drastically affected in infants born preterm (before 37 weeks) with very low birth weight (VLBW) (less than 1500 g; Aylward, 2002). Major disabilities such as moderate to severe mental retardation, sensorineural hearing loss or blindness, cerebral palsy, and epilepsy occur in 12 to 14% of this population. However, high prevalence, low severity dysfunctions – such as learning disabilities and borderline mental retardation – occur in 50 to 70% of preterm VLBW infants (Aylward, 2002). During school-age years, preterm VLBW children demonstrate significantly lower cognitive scores and are more likely to suffer from neuropsychological deficits when compared to normal-birthweight children (Bhutta et al., 2002). Behavioral handicaps such as attention deficit hyperactivity disorder (ADHD), increased externalization and internalization of behaviors, and depression are observed more often in premature VLBW children than in their normal-birthweight counterparts (Bhutta et al., 2002; Räikkönen et al., 2008; Sommerfelt et al., 1993).

Abnormal development of lung tissue and bronchopulmonary dysplasia are complications in preterm VLBW infants which result in chronic hypoxemia, a condition of decreased oxygen carriage in the blood (Fagel et al., 2006; Hack et al., 2002; Ment et al., 1998). Low oxygen may be a key instigator in the mental and behavioral disabilities mentioned above (Fagel et al., 2006; Hack et al., 2002). A mouse model of chronic perinatal hypoxia has been developed to investigate hypoxemia and to manifest the neurodevelopmental patterns of preterm VLBW infants (Fagel et al., 2006; Ment et al., 1998). Even though preterm VLBW infants exhibit cognitive and psychological disabilities early in life, these handicaps often decrease with age, indicating that some mechanism(s) occurs in the developing brain to reduce hypoxia-induced deficits (Fagel et al., 2006; Ment et al., 2003). One mechanism through which this recovery may occur is cortical neurogenesis, or the development of new neurons in the brain. Even after birth, a small number of neurons are generated in the juvenile and adult brain, especially following injury (Fagel et al., 2006; Magavi et al., 2000). Previous research has shown that increased cortical neurogenesis can occur after a period of hypoxia to reverse the effects of low perinatal oxygen levels, including loss of cortical neurons, cortical volume, and brain weight (Fagel et al., 2006).

In addition to cortical neurogenesis, another mechanism that may serve to compensate for hypoxia-induced effects is environmental enrichment, which has the potential to produce significant cellular, molecular, and behavioral changes in the brain (Praag et al., 2000). An enriched environment is a setting that facilitates inanimate and social interactions, increases physical activity, and stimulates sensory and motor pathways. In animal models, such a setting is created by introducing items such as a larger cage, exercise equipment, larger groups of individuals, toys, nesting materials, and tunnels. The experimental setting may be altered by frequently replacing toys and changing food locations to ensure novelty and stimulation (Mora et

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al., 2007; Praag et al., 2000). Enrichment can induce neural plasticity, thus contributing to neurodevelopmental recovery in damaged or diseased brains (Praag et al., 2000). For example, enrichment drastically improves cognitive functions such as learning and memory, an effect that correlates with the positive effects caused by an increase in neurogenesis (Mora et al., 2007; Segovia et al., 2009). Similar to the effects of cortical neurogenesis, normal rodents placed in enrichment manifest increased brain weight and size (Fagel et al., 2006; Praag et al., 2000).

It seems possible, then, that environmental enrichment may also facilitate the recovery from neurodevelopmental deficits in the mouse brain after chronic perinatal hypoxia. The purpose of the present research was to determine the consequences of chronic sublethal perinatal hypoxia on gamma-aminobutyric acid (GABA)ergic interneuron development and spatial memory, and to explore environmental enrichment as a possible mechanism of recovery after the hypoxic insult. We found that cortical interneurons exhibit significant deficits of the calcium-binding protein parvalbumin (PV) after hypoxia. However, this strong effect on hypoxia-reared mice disappears if they are placed into environmental enrichment; in fact, levels of cortical PV-positive interneurons and cortical volume were not significantly different in hypoxic, enriched mice, and normoxic mice. Data gathered from two behavioral tests, the Delayed-Alternation task and Morris water maze, also indicate that enrichment can eliminate spatial memory impairments that result from chronic perinatal hypoxia.

Because the mouse model of hypoxia parallels the neurodevelopmental patterns of preterm VLBW infants, these findings as well as this entire line of research are relevant to clinical human therapies. By understanding how low oxygen levels affect brain chemistry and cellular composition, and by discovering enrichment conditions that reverse these effects, we may understand how environmental alterations and therapies may be used to improve the cognitive, behavioral, and psychological well-being of individuals born preterm with VLBW. Furthermore, this study elaborates upon disruptions in GABAergic interneuron development, a phenomenon that is characteristic of a spectrum of illnesses such as epilepsy, schizophrenia, autism, Tourette's, anxiety disorders, and more (Kalanithi et al., 2005; Levitt, 2005). By studying the role and importance of GABAergic interneurons in early development, we may gain more knowledge of many developmental disorders and the mechanisms by which they act.

Materials & Methods

Animals

All procedures in these experiments were approved by the Yale Animal Resources Center and Institutional Animal Care and Use Committee (IACUC). Animals in the behavioral studies were standard C57B mice; animals in the stereological studies were transgenic GAD67-GFP mice. In the latter cohort, green fluorescent protein (GFP) was transcribed under the promoter for the glutamate decarboxylase isoform 67 gene (GAD67-GFP). Upon birth, mice were randomly assigned to one of four experimental conditions: normoxic, non-enriched (NX/NE); normoxic, enriched (NX/E); hypoxic, non-enriched (HX/NE); and hypoxic, enriched (HX/E).

Rearing in Hypoxia and Enrichment

Mice in the hypoxic conditions were placed in an airtight Plexiglas chamber with a continuous flow of air with O2 displaced by N2; the overall concentration of O2 was 9.5 to 10.5%. Oxygen levels were monitored minute-to-minute by sensors coupled to a computer. For all analyses, the period of hypoxia began at postnatal Day 3 (P3) and continued for eight days to P11. Hypoxic chambers were inspected twice daily to ensure appropriate oxygen levels.

Mice in the enrichment conditions were placed into large Plexiglas cages measuring 24 cm wide × 20 cm high × 46 cm long. A running wheel, a series of clear and colored plastic "habit-trails" of different configurations, and several small plastic, hard rubber, or wooden balls and objects

of different shapes were scattered on the cage floor. Small wooden blocks or metal link chains were suspended from the cage roof. Every three days, objects were changed, cleaned, disinfected, and rearranged to ensure novelty. Prior to weaning, 5 to 13 pups were housed per cage. After weaning and gender separation, no more than eight pups were housed per cage. Enrichment cages were inspected twice daily. The enrichment period began at P20 and continued until immunohistochemical examination or behavioral testing. Mice not in the enrichment conditions were housed in standard rack mount Plexiglas cages measuring 18 cm wide × 13 cm high × 29 cm long. The bottoms of all enriched and non- enriched cages were lined with pine shavings. All mice were exposed to a 12:12-hour light-dark cycle and were provided ad-libitum access to water and food. Prior to weaning, food was located on the cage floor; after weaning, food was provided via a wire bar cage-top food hopper. Cages were changed weekly, and infant mortality, maternal cannibalism, and other health and well-being issues were addressed in a timely manner.

Immunohistochemistry

At P47, animals intended for immunohistochemical analysis were deeply anesthetized with a ketamine-xylazine injection and perfused transcardially with 10 mL of 0.01 M Phosphate Buffered Saline (PBS) and 35 mL of 4% paraformaldehyde (PFA). Brains were dissected from the skull, post-fixed in PFA for 24 hours, and cryoprotected in a 20% sucrose solution for 24 hours. The brains were then embedded in 4% agarose gel for vibratome sectioning or optimal cutting temperature compound for cryostat sectioning and stored at -80 ºC. Brains were serially sectioned either coronally or sagittally at 50 µm thickness with a Leica VT 1000S vibratome at room temperature or Leica CM1900 at -20° C. Free-floating sections were stored at +4°C in a 0.04% sodium azide (NaN_3/PBS) solution until subsequent staining.

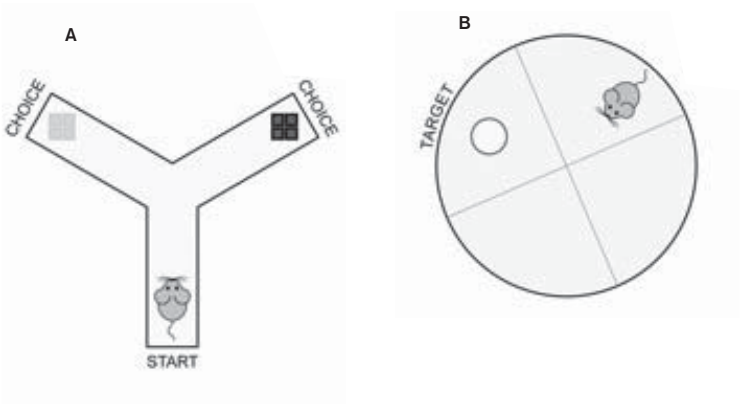
Sections were washed in PBS and blocked in a 0.1% Tween-20 / 0.2% Triton-X solution containing 10% normal goat serum (NGS/PBS++). They were incubated overnight in primary antibodies in 5% NGS / PBS++ at +4°C. Primary antibodies included mouse anti-PV (1:2500 or 1:1000, Sigma); rabbit anti-GFP (1:1000, Abcam); and chicken anti-GFP (1:2000, Abcam). After primary antibody incubation, sections were washed three times with PBS and incubated for 1 hour in secondary antibodies in 5% NGS / PBS++ at room temperature. Secondary reagents included goat anti-mouse Alexa 594 (1:1000 or 1:500, Invitrogen); goat anti-rabbit Alexa 488 (1:1000, Invitrogen); and goat anti-chicken Alexa 488 (1:500, Invitrogen). Sections were washed in PBS, mounted, and coverslipped using Vectashield mounting medium with DAPI (4', 6-diamidino-2-phenylindole) (Vector, Burlingame, CA).

Stereology and Cell Counting

Stereological analysis was completed using the StereoInvestigator software and a Zeiss Axioskope 2 Mot Plus equipped with a motorized stage, coupled to a computer. Contours of cerebral cortex and basal ganglia (counted as a whole region including the striatum, nucleus accumbens, and globus pallidus) were delineated with visual aid of DAPI and reference to The Mouse Brain in Stereotaxic Coordinates. StereoInvestigator software allowed for systematic, random sampling for cell counts using the optical fractionator method. Cells positive for PV were visualized by a 594 nm filter, GFP was visualized by a 488 nm filter, and DAPI was visualized by a 350 nm filter. Cells were counted using a three-dimensional 100 × 100 × 5 µm counting frame in a sampling grid of 1000 × 1000 µm or 700 × 700 µm for cortex and 300 × 300 µm for basal ganglia. Approximately 101 sampling sites in each coronally sectioned cortex, 200 sites in each sagittally sectioned cortical hemisphere, and 78 sites in basal ganglia were counted.

A Y-maze with three identical arms was used in the Delayed-Alternation (DA) spatial memory task, which took place starting between P45 and P60 (Figure 1A). During the training period, each animal was placed at the start arm and allowed to choose a

Figure 1

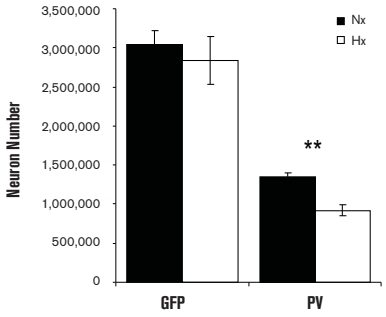


Spatial Memory Tasks

A. Delayed-Alternation spatial memory task

B. Morris water maze. Start indicates start position of each trial. Choice indicates two possible paths; dark gray square represents small chocolate reinforcements in choice arms; light gray square represents alternate baiting; Target indicates target quadrant; small circle represents hidden platform.

Figure 2



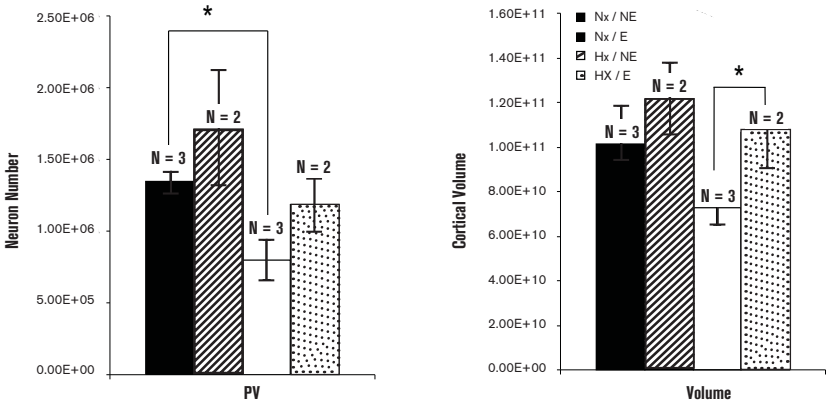
Mice Reared in Hypoxia Exhibit 32% Fewer GABAergic Interneurons Expressing PV

Hx mice were exposed to chronic sublethal hypoxia from postnatal Day 3 (P3) to P11. Nx, normoxia; Hx, hypoxia; GFP; green fluorescent protein; PV, parvalbumin.

**P < 0.01, comparing Nx versus Hx by t-test.

Nx = normoxia
Hx = hypoxia
NE = non-enriched
E = enriched
PV = parvalbumin
*P < 0.05, t test.

Figure 3



Even Though Hypoxia Induces Negative Effects in the Mouse Cortex, Compensation Can Occur by Enrichment

A. Number of PV+ cortical interneurons

B. Volume of cortex (µm3). Hx mice were exposed to chronic sublethal hypoxia during postnatal Day 3 (P3) to P11. E mice were reared in environmentally enriched cages from P20 to P47.

Table 1

Total Number of Neurons of the Indicated Phenotype as Assessed by Stereological Analysis

Hx mice were reared in chronic sublethal hypoxia from postnatal Day 3 (P3) to P11. % indicates the percent difference between Hx and Nx mice.

	Normoxia		Hypoxia		
	N =	Total Cell Number	N =	Total Cell Number	%
Cerebral cortex					
GFP	4	3,035,926	3	2,837,059	NS
PV**	3	1,347,712	3	921,998	−32**
Basal ganglia					
GFP	3	619,377	3	673,908	NS
PV	3	1,371	3	2,157	NS

Nx = normoxia; Hx = hypoxia; GFP = green fluorescent protein; PV =parvalbumin.

** P < 0.01, NS, not significant, comparing Nx versus Hx by t test.

path to one of two choice arms, where small chocolate reinforcements were placed. Then animals rested for an inter-trial interval. Successful learning of the task required selection of the choice arm that was not selected on the previous reinforced trial. After the initial placement of reinforcements in both arms, only the choice arm opposite the previous choice arm was baited. Animals participated in this training for ten trials per day until they accomplished the criterion performance of 80% correct choices over two consecutive days. Mice that successfully completed the 80% correct choices criterion were tested again using the next higher inter-trial delay period; these were 25 s, 1 min, and 5 min. In scenarios in which an animal did not reach criterion performance before 200 trials at a particular delay period, testing was terminated.

The circular Morris water maze was 1.5 m in diameter and filled with opaque water (Figure 1B). Testing took place between three to five months of age. Animals were allowed four trials per day for eight days to swim to a transparent Plexiglas platform hidden within a consistent target quadrant of the plastic tub beneath the water's surface. Path and time to reach the platform was monitored by a video tracking system (Coulbourn Instruments, Inc.). On Days 9, 16, and 23 of testing, probe trials of 60 s were completed in which the hidden platform was removed. Total path length and percent time in the target quadrant were calculated from the video tracking system data. The probe preference (PP) score, which demonstrates the time spent in the target quadrant relative to the time in the other three quadrants, was also calculated.

Statistics

In the comparison of normoxia and hypoxia, p values were calculated using Student's t-test. Comparisons of select means of the four normoxia and hypoxia, non-enriched and enriched conditions were also calculated similarly. In the DA spatial memory task, data were analyzed using a Survival Analysis test. In the eight-day training period of the Morris water maze as well as the probe trial, comparisons between the four conditions and time to reach the platform were analyzed using a repeated measures multivariate analysis of variance test (MANOVA). A post-hoc comparison using the Scheffe test was also completed for these data.

Results

Hypoxia-Reared Mice Exhibit Fewer Parvalbumin-positive Interneurons in the Cortex

To compare the potential for inhibitory neurotransmission in hypoxia- and normoxia-reared animals,

GFP+ interneurons were analyzed in the cerebral cortex and basal ganglia. Total cortical GFP+ cell numbers were similar in all mice, regardless of oxygen rearing, which suggests that GABAergic neuron number is not affected by hypoxia (Figure 2, Table 1). This finding indicates that the potential for inhibitory control is present; however, a neurodevelopmental mechanism hinders the function of GABAergic neurons. Therefore, PV+ cells (a subset of GABAergic interneurons) were examined. Despite no difference in GFP+ cells, PV+ cortical GABAergic interneurons of HX mice were significantly decreased by 32%, an absolute number of approximately 425,000 cells in total, compared to NX mice ($p < .01$) (Figure 2, Table 1). The number of GFP+ and PV+ neurons in the basal ganglia of the NX and HX conditions did not differ significantly (Table 1).

Hypoxia-Reared Mice Exhibit Decreased Performance in the Delayed-Alternation Task

To assess the effect of oxygen rearing on spatial memory, a DA task was performed. With a short 25s inter-trial delay, HX and NX mice were no different in number of trials required to achieve criterion performance or number of mice that failed the task before 200 trials (Table 2). With a 1-min inter-trial delay, no HX mice failed the task; however, they did take significantly more trials to succeed at the task than controls ($p < .05$) (Table 2). With a long 5-min inter-trial delay, Hx mice again required more trials to reach criterion performance and also failed the DA task significantly more often than NX mice ($p < .05$) (Table 2).

Enrichment After Hypoxia Increases Cortical Volume and Parvalbumin-Positive Cells

With the knowledge that hypoxia induced cortical PV+ interneuron and spatial memory deficits, we investigated the effects of environmental enrichment on interneurons. As previously observed, HX / NE mice exhibited a decrease in the number of cortical PV+ interneurons by approximately 547,000 cells in total ($p < .05$) (Table 3, Figure 3A). However, HX / E animals did not exhibit this effect; the cortical PV+ neuron number of HX / E mice did not differ significantly from controls (Table 3, Figure 3A). The cortices of HX / NE mice showed a trend to be of smaller volume than NX / NE mice, but the result was not significant (Figure 3B). The effect of enrichment in HX mice was significant, however, as cortical volume of HX / E mice was 32% greater than that of HX / NE mice ($p < .05$) (Figure 3B). This compensation of the HX-induced deficit, similar to the effect on PV+ cells, was enough to make the difference between HX / E and NX / NE cortical volumes not significant (Table 3, Figure 3B).

Environmental Enrichment After Hypoxia Improves Performance in the Morris Water Maze

To determine whether environmental enrichment induced a recovery in spatial memory similar to the recovery in PV+ cell number, a Morris water maze was conducted. No statistically significant differences in time for animals to reach the hidden platform were detected among the four conditions during the training period (Figure 4). To assess the strength of learning, further analysis was completed for the 60 s probe trial conducted on Day 9. Information from the video data tracking system demonstrated that NX mice swam in the target quadrant where the platform had been removed more than HX mice, which lacked this preference and instead swam at the edges of the tub (Figure 5). In an analysis of all four conditions, significant differences were found for the percent time in the target quadrant ($F(3, 44) = 8.2$; $p = .001$) and for the probe preference (PP) score ($F(3, 44) = 6.6$; $p = .001$) (Figure 6A, B). NX / NE mice spent significantly less time in the target quadrant (16.6% less, $p < .001$) and had a significantly lower PP score (12.3 less, $p < .001$) than mice in the other three conditions (Figure 6A, B). No significant difference was found among groups in total distance traveled (Figure 6C).

Discussion

Hypoxia induces neurodevelopmental deficits but enrichment can reduce these effects. The calcium-

Table 2

Mice Reared in Hypoxia Experience Deficits in Spatial Memory Retention During the Delayed-Alternation Task

Hypoxic mice were reared in chronic sublethal hypoxia from postnatal Day 3 (P3) to P11. DA testing began between P45 and P60. Failed indicates the number of mice not reaching criterion performance before 200 trials. Trials indicates the mean trials to reach criterion performance.

	N =	Failed	Trials	Logrank p value
25 second delay				
Normoxia	13	0	64	p = 0.8388
Hypoxia	11	0	71	
1 minute delay				
Normoxia	13	0	51	p = 0.0485*
Hypoxia	11	0	110	
5 minute delay				
Normoxia	13	4	119	p = 0.0218*
Hypoxia	11	8	180	

*P < 0.05, comparing Normoxia versus Hypoxia by t-test.

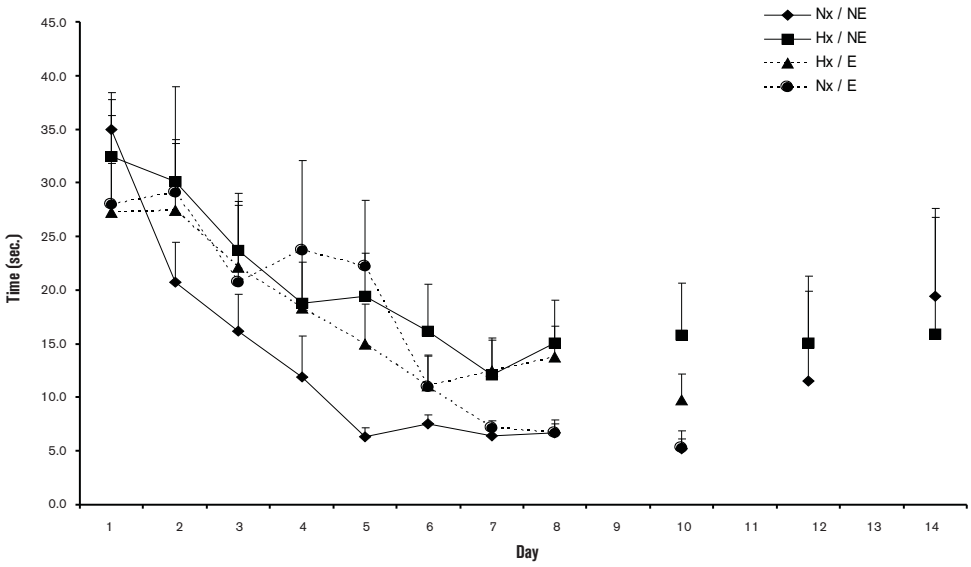
binding protein PV, expressed in approximately half of cortical GABAergic neurons, allows for fast-spiking transmissions that modulate synaptic activity, affect glutamate levels, and regulate overall neural function (Chow et al., 1999; Woo & Lu, 2006). Established studies suggest that neurological and behavioral impairments caused by abnormal GABAergic neuron development can be considerable if they occur in early embryonic or postnatal periods (Levitt, 2005; Woo & Lu, 2006). The present research emphasizes the importance of GABAergic interneuron development by demonstrating that a short-term insult by hypoxia at a critical period of development can drastically alter the circuitry of the mouse brain and can result in permanent defects observable near adulthood. HX and NX mice demonstrated similar numbers of GABAergic interneurons that were positive for GAD67-GFP, suggesting that GABAergic interneurons retained the potential for inhibitory neurotransmission. However, the fact that the number of GABAergic interneurons that expressed PV was decreased in HX mice indicates a deficit in the function of inhibitory neural control. Thus, the cortical PV+ cell data suggest that a neurodevelopmental mechanism that affects GABAergic interneuron function is dependent on perinatal oxygen levels.

Chronic perinatal hypoxia decreased the number of cortical PV+ GABAergic cells by almost one-third ($p < .01$) (Table 1, Figure 2). This abnormality could conceivably alter cortical synchronous oscillatory activity and impair behavior in individuals such as preterm VLBW infants (Lodge et al., 2009). Previous research has shown that cortical PV+ cell number is inversely correlated with hyperactivity (Smith et al., 2007). Additionally, our preliminary behavioral findings reveal hypoxia causes transient hyperactivity from age P16 to P18. Our animal model may accurately portray the development of preterm VLBW individuals, since hyperactivity disorders such as ADHD more frequently occur in this population than in children of normal birth time and weight (Bhutta et al., 2002; Sommerfelt et al., 1993). The fact that GAD67-GFP+ GABAergic cell number was not different between NX and HX mice suggests that the number of neurons with GAD67 message may be normal, even though the GAD67 protein may still be decreased. However, there is clearly a

Figure 4

Performance in the Morris Water Maze as Indicated by Time to Platform

Hx mice were reared in chronic sublethal hypoxia from postnatal Day 3 (P3) to P11. E mice were reared in environmentally enriched cages from P20 until testing between 3-5 months of age. After eight days of training, probe trials took place on Days 9, 16, and 23.



phenomenon that limits GABAergic interneuron development, since HX brains have decreased cortical PV+ GABAergic interneuron number.

In future studies, other markers of PV interneuron development may be investigated. For example, decreases in neurotrophins such as brain-derived neurotrophic factor (BDNF) or ion channels such as potassium-chloride cotransporter KCC2 or voltage-gated potassium channel Kv3.1b may be mechanisms that explain decreased PV+ cell numbers. Lower levels of these chemicals may hinder dendritic arborization, synapse formation, postsynaptic inhibitory potential, or fast-spiking ability of interneurons (Gulyás et al., 2001; Woo & Lu, 2006).

Preterm VLBW infants experience motor impairments ranging from motor difficulties or developmental coordination disorder to cerebral palsy (Schmidhauser et al., 2006). Even though mice in the HX condition experienced a PV+ neuron deficit in the cortex, no such effect was observed in the basal ganglia. Therefore, the circuitry of the cortex and basal ganglia are fundamentally different and the effects of hypoxia on each brain region may differ. For example, motor deficits due to hypoxia may not be affected at the primitive level of the basal ganglia, but may occur instead at the higher processing controls in the cerebral cortex.

Interestingly, the number of PV+ cells of HX mice that were placed into environmental enrichment were not significantly different than number of cells of NX mice (Figure 3, Table 3). This result suggests that even though chronic perinatal hypoxia induced PV+ cell deficits, a stimulating environment is sufficient to reverse the negative effects of the insult. One mechanism of PV+ cell number enhancement by enrichment may be increased neurotrophins, which increase in cortex and other brain regions with long-term environmental enrichment (Ickes et al., 2000). There was also a trend for the PV+ neuron number to be higher in HX / E than in HX /NE, and higher in NX / E than in NX / NE (Figure 3A, Table 3). With the addition of more animals into the experiment, statistical significance may be achieved.

Another trend observed in this study was decreased cortical volumes of HX mice when compared to NX controls (Figure 3, Table 3). Such a loss

Figure 5

Video Data Tracking of Morris Water Maze Probe Trial

View of Morris water maze from above. Circles indicate hidden platform in target quadrant. Lighter shades of gray indicate longer amounts of time spent in a particular location in the maze.

A. Hypoxia

B. Normoxia. Hypoxic mice were eared in chronic sublethal hypoxia from postnatal Day 3 (P3) to P11. Testing took place between 3 and 5 months of age. After an eight-day training period, this 60 s probe trial took place on Day 9.

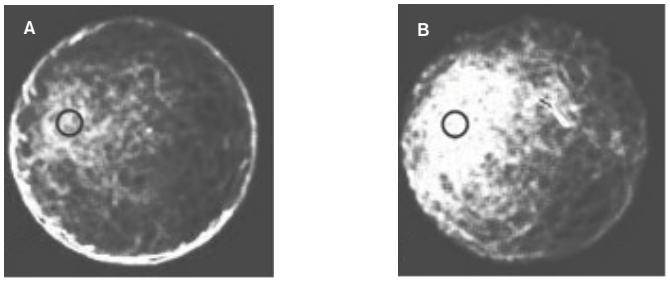


Figure 6

Hx / NE mice perform worse in the Morris water maze than Hx / E and control mice.

A. % time in target quadrant

B. Probe preference score

C. Total distance traveled. Hx mice were reared in chronic sublethal hypoxia from postnatal Day 3 (P3) to P11. E mice were reared in environmentally enriched cages from P20 until testing between 3 and 5 months of age. After the training period, this 60 s probe trial took place on Day 9.

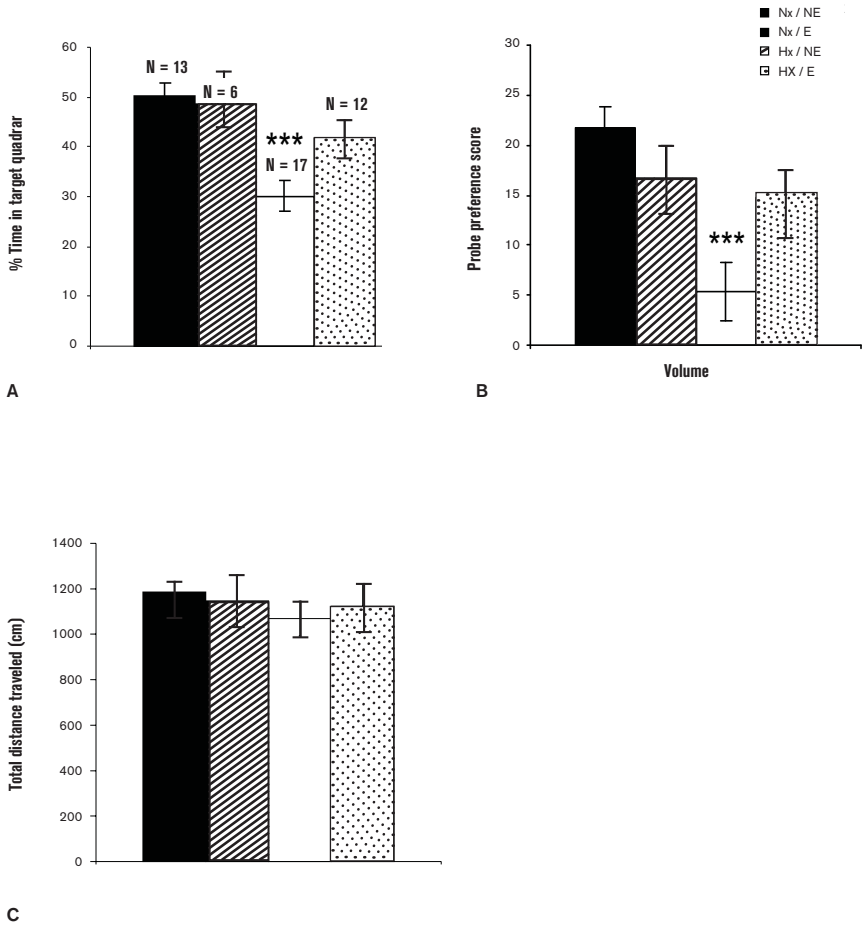
Nx = normoxia

Hx = hypoxia

NE = non-enriched

E = enriched

***p < 0.001, MANOVA



in cortical volume may be attributed to smaller cell size and may decrease efficiency in neural communication. However, the cortical volume decrease between HX mice and NX controls was not seen when HX mice were placed into enrichment. Environmental enrichment after hypoxia rearing significantly increased cortical volume ($p < .05$) (Figure 3, Table 3). The reversal of the deficits caused by hypoxic insult was sufficient to bring the cortical volume of HX / E mice to that of controls, since differences in the cortical volumes of these groups were not significant (Figure 3, Table 3).

Behavioral Data Parallel Stereological Findings

The stereological findings of hypoxia-induced deficits and enrichment-facilitated recovery correspond with behavioral testing of spatial memory. In the Y-maze DA task, mice performed similarly with a short 25 s delay, regardless of the oxygen levels in which they were reared (Table 2). This finding suggests that hypoxia does not affect initial spatial learning, and short-term spatial memory is preserved. However, with an increased 1 min delay HX mice required more trials to succeed at the task ($p < .05$), and with a long 5 min delay HX mice required more trials and failed more often ($p<0.05$) (Table 2). The declining performance in the DA task as inter-trial interval lengthened indicates that even though spatial learning and memory formation is intact, the HX animals' ability to retain these spatial memories over time is impaired.

In the Morris water maze training, differences in time to the hidden platform and distance traveled were not significant among HX and NX animals; therefore spatial learning and memory formation did occur (Figure 4). However, in later probe trials which assessed the animals' strength of learning, NX mice exhibited a preference for the target quadrant from training, as indicated by video tracking data, percent time in the target quadrant ($p<0.001$), and PP score ($p < 0.001$) (Figure 5, 6A, and 6B). The fact that HX mice have a decreased strength of learning parallels the learning deficits found in preterm VLBW school-aged children. These individuals have IQ scores that are 12 to 14 points below normal birth weight children, and more than 50% of these individuals require special assistance in education (Aylward, 2002).

The DA task and Morris water maze revealed that spatial learning strength and memory retention are limited by hypoxia, but initial learning and memory formation are intact. We found that environmental enrichment may improve retention of memory and strength of learning in HX mice (Figure 6). Compared to non-enriched mice, HX /E mice were more successful as assessed by percent time in target quadrant and PP score during the Day 9 probe trial ($p < .001$); in fact, their performance was similar to NX mice, indicating that enrichment can offset the insult of hypoxia (Figure 6). The enhancement facilitated by enrichment may be accomplished by neurogenesis, which contributes to functional recovery (Fan et al., 2007). Since the hippocampus is critical in spatial tasks, it is possible that hippocampal neurogenesis was the mechanism in the improvement of behavioral task performance of HX / E mice (Figure 6). In future studies, immunohistochemical analysis should be conducted in the hippocampus. Small amounts of neurogenesis also occur in the cortex, and it is accelerated after an injury such as hypoxia (Fagel et al., 2006; Magavi et al., 2000). The addition of cells may have been sufficient to explain the significant increase in cortical volume of HX / E mice over HX / NE mice (Figure 3, Table 3).

Environmental Enrichment Has Positive Effects on Brain and Behavior

We have shown that chronic sublethal perinatal hypoxia is detrimental to the mouse brain. We have also presented a means to recuperate the developing brain and behavior: a stimulating environment. Therefore, the biochemical implications of the external environment can be drastic. Mice reared in complex environments have longer dendrites and more synapses per neuron, and enrichment results in altered distribution of metabolic resources to synapses and neurons through changes in vascular,

Table 3

Number of Cortical PV Neurons and Cortical Volume
Hypoxic mice were exposed to chronic sublethal hypoxia from postnatal Day 3 (P3) to P11. Enriched mice were reared in environmentally enriched cages from P20 to P47. PV, parvalbumin.

	Normoxia, Non-Enriched	N =	Normoxia, Enriched	N =	Hypoxia, Non-Enriched	N =	Hypoxia, Enriched	N =
Total number of PV+ neurons	1,347,711	3	1,707,150	2	800,984	3	1,182,538	2
Cortical volume (μm3)	5.07E+10	2	6.07E+10	2	3.63E+10	3	5.34E+10	2

mitochondrial, and glial support (Sirevaag & Greenough, 1987; Wallace et al., 1992). With a decrease of PV, synaptic activity is hindered, but the increased contact of astrocytes and synapses caused by enrichment can compensate for this effect and produce a functionally normal mouse brain (Galarreta & Hestrin, 2002; Jones & Greenough, 1996). Since the animal model used in the present research parallels the neurodevelopment of preterm VLBW individuals, the positive effects of enrichment are highly encouraging in consideration of human therapies. Aspects of the environmental enrichment of the lab setting can be adapted for preterm VLBW children in the home and classroom settings.

We have tested only a small number of animals but with more mice, conclusions may be solidified and more revelations may emerge. Future research is also necessary to determine if hypoxia and enrichment affects a particular cortical region, and if volume deficits are caused by shrinkage of cells, early cell death, or the development of fewer cells. We have discovered that the cortex is vulnerable to hypoxia but could not detect differences in the basal ganglia (Figure 3A, Table 3). Future studies could examine effects of hypoxia on other brain regions, such as the hippocampus, which is a site of neurogenesis critical to spatial memory, or specific regions of the cerebral cortex, such as the parietal cortex, which is critical for spatial memory.

Today, infants weighing less than 1000 g (2.2 lb) represent almost 1% of live births in the United States (Bassan et al., 2006; Guyer et al., 1999). Animal studies such as the present research demonstrate trends of lasting negative effects initiated by perinatal low oxygen rearing, and how such effects may contribute to the cognitive and behavioral disabilities of preterm VLBW children. However, we show that the deficits are not necessarily permanent – for the animals of the study or for the parallel human population. There are encouraging treatments that may reduce hypoxia-induced deficits; one that we propose here is a stimulating environment. We suggest that this research may be extended to therapeutic objectives for humans, such as preterm VLBW infants, who have endured hypoxemia. Environmental enrichment may be applied to human therapies in the form of educational toys, exciting teaching methods, colorful classrooms, social interaction provided by recess, and novel settings provided by field trips. Using the animal model of chronic sublethal perinatal hypoxia and environmental enrichment, research can provide valuable insights into neural, cognitive, and behavioral effects of hypoxemia. With further studies, we can work towards developing therapies to reduce hypoxemia-induced disabilities. ■

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