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BRAIN DERIVED NEUROTROPHIC FACTOR: A FORAY INTO PREDICTING NEONATAL OUTCOMES IN PREMATURE INFANTS

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Vladimir Vladislav Glinskii

2013

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BRAIN DERIVED NEUROTROPHIC FACTOR: A FORAY INTO PREDICTING NEONATAL OUTCOMES IN PREMATURE INFANTS.

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The need to accurately predict the ability of a prematurely born infant to recover from hypoxia induced damage associated with prematurity is rooted in our need to develop better interventions, with fewer side-effects, with which to treat these complicated patients. Previous work done in murine models mimicking hypoxia induced damage and its neurological sequelae have suggested hypoxia induced factor-1α (HIF-1α), and its downstream effector molecules, brain derived neurotrophic factor (BDNF) and stromal derived factor-1 (SDF-1), may play an integral role in protecting the developing brain from the deleterious effects of low O_2. Moreover, a number of single nucleotide polymorphisms (SNPs) have been identified, which affect the expression of these proteins on an individual basis. To that end, we hypothesized that BDNF expression levels gathered from venous cord blood, and genetic analysis for the presence of the rs6265 and rs1801157 SNPs in the BDNF and SDF-1 genes could be used as biochemical and genetic biomarkers to predict short term neonatal outcomes in premature infants born at Yale New Haven Hospital.

BDNF levels, determined by quantitative ELISA in 23 patients, and the presence of SNPs, determined in duplicate by restriction fragment length polymorphism assay and Sanger sequencing in 53 patients, were correlated with the development of a variety of neonatal outcomes. Our results indicated that although not statistically

significant, the development of bronchopulmonary dysplasia, necrtotizing enterocolitis, and early onset neonatal sepsis (EONS) trended towards an association with lower cord blood BDNF levels (p-values <0.10). Likewise, the presence of the rs6265 SNP appeared to be protective against the development of culture positive EONS ($p < 0.08$), while the presence of rs1801157 SNP appeared to be protective against the development of clinical EONS. Taken together, these data suggest that while cord blood BDNF levels and the presence of the rs6265 and rs1801157 SNPs show potential as useful molecular and genetic biomarkers for predicting outcomes of premature infants, the association between these factors and neonatal outcomes is not strong enough for the number of patients examined in this study to provide conclusive results of the utility of these biomarkers in guiding clinical decision making. Consequently, further research of these, and other, biomarkers is necessary to better elucidate their usefulness.

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INTRODUCTION

Gestational prematurity (<37 weeks gestation) and low birth weight (< 2500g) in newborns is associated with an increased risk for a variety of complications in multiple organ systems, which results in a significant increase in the morbidity and mortality of these infants when compared to their full term counterparts (1). Of these complications, disorders of the developing nervous system are of particular significance, and include deficits ranging from cerebral palsy and intellectual disability to more subtle disabilities such as ADHD and social-emotional difficulties (2). As medical science progresses, the incidence of preterm birth has increased by more than 30% over the past several decades, from 9.4% in 1981 to over 12.5% in 2004 (1). This increase is partially due to our improved abilities at keeping alive preterm infants who would not have survived past the perinatal period a decade earlier. Interestingly, one prospective cohort study done in Dorset, England showed an 11% increase in the survival to discharge from the neonatal intensive care unit of premature infants born at either 24 or 25 weeks gestation from 36% in 1994 to 47% in 2000-2005 (3). However, while the decline in the premature infant mortality rate in recent years is encouraging, the rising emotional, monitory, and social costs associated with taking care of patients afflicted with these adverse outcomes, as they develop and grow into adulthood, presents a number of significant challenges.

The incidence and severity of adverse neurodevelopmental outcomes has been shown to be strongly associated with the gestational age and birth weight of premature infants (4, 5). These abnormalities are thought to be, at least in part, secondary to

cerebral hypoxemia resulting from apnea and respiratory distress syndrome which is prevalent in over two-thirds of very low birth weight (< 1500g) preterm infants (6, 7). The extent to which the damage from these insults manifests itself in later neurological development, however, varies widely (8). Further, multiple studies have demonstrated that the observed variations in responses to sublethal hypoxia associated with premature birth can be mimicked in CD-1 and C57BL6 mouse strains using specific insults (9-11).

Using such models, mice reared under hypoxic conditions have shown an increased expression of genes subserving presynaptic function, and a concomitant decrease in expression of genes involved in synaptic maturation, synaptogenesis, and glial differentiation (12). These effects, combined with the elevated rates of neuronal cell death seen in hypoxic brain injury, are thought to contribute to the deleterious effects of low O_2 (13). Consequently, recovery from such an insult is likely to be, at least in part, dependent upon replacement of lost neurons by stimulation of neuronal stem cells (NSCs) and renewed synaptogenesis. A differential response from known regions of neurogenesis such as the subventricular zone (SVZ) and the subgranular zone (SGZ) could underlie the variability of recovery to hypoxia induced damage that is observed in premature infants. Importantly, proper functionality within neurovascular compartments of SVZ and SGZ including neuronal regeneration, NSC self-renewal, differentiation and guidance is inseparable from the status and function of microvasculature. Effects of endothelial cells lining the blood vessels in the SVZ and SGZ are indispensable in the regulation of stem cell self-renewal and neurogenesis.

Endothelial cells reside in close proximity to clusters of proliferating neurogenic cells, provide attachment points for the latter, and share with NSCs multiple signaling pathways induced by VEGF, BNDF, and/or SDF-1/CXCL12 that mediate both angiogenesis and neurogenesis. Consequently, studies showing that neurogenesis and angiogenesis share several common signaling pathways, imply that neurogenic cells and endothelial cells in the SVG and SGZ could have a reciprocal effect on one another

Over the last decade our group has shown the uncoupling of the tightly controlled involution/ stabilization of the SVZ vasculature that occurs following premature delivery in animal models (14, 15). This decoupling results in the involution of the SVZ vasculature without vascular stabilization. The resulting disruption in the normal formation of basement membranes, tight junctional complexes and vessel wall envelopment by glial and pericyte cell processes in turn leads to increased rates of intraparenchymal and interventricular hemorrhage (16). More recently, SVZ and SGZ vasculature has been shown to not only participate in promoting a neurogenic environment, but also participate more directly in guiding newly generated neuronal precursor cells along the blood vessels to areas of ischemic damage (17).

The molecular mechanism of SVZ vascular stabilization and increased neurogenesis is thought to, at least in part, involve the HIF-1α signaling cascade, and its downstream effector molecules, including BDNF, VEGF, and SDF-1. Activation of HIF-1α in response to hypoxic insult results in the initiation of a complex cross-talk between neuronal cells and vascular endothelial cells within the neurovascular niche, which

contributes to neural stem cell activation, induction of angiogenesis, and microvascular stabilization (18). Hypoxia-resistant CD-1 pups reared under 9.5% O_2 until post-natal day P11 showed increased expression of BDNF, VEGF, and SDF-1 in whole brain lysates, as well as increased NSC proliferation both *in vivo* and *in vitro*, as compared to their C57BL6 counterparts. Further, C57BL6 NSCs co-cultured with brain endothelial cells and treated with rBDNF, rVEGF, and rSDF-1 showed enhanced NSC proliferation to levels equal to those of CD-1, and conversely, sequestration of these factors in CD-1 cultures blunted NSC proliferation rates to those of C57BL6, suggesting that it is the increased responsiveness of HIF-1α to hypoxic insult by endothelial cells and neural stem cells in the SVZ of the CD-1 mouse strain, and the subsequent induction of downstream signaling pathways involving BDNF, VEGF, and SDF-1, that is thought to underlie CD-1's resilience to low O_2 (9, 18).

Based on this information, it is reasonable to hypothesize that systemic (serum) levels of HIF-1α downstream effector proteins such as BDNF, VEGF, and SDF-1 could potentially serve as biochemical markers reflecting the neurovascular response of prematurely born neonates to a hypoxic insult and help predicting the risk of developing related complications associated with the premature birth in these patients.

Further, there is an evolving body of evidence in a current literature strongly suggesting that in various pathologic conditions associated with changes in BDNF, SDF-1, and VEGF levels, such changes could be linked to genetic variations defined by single nucleotide polymorphism (SNP) mutations within their respective genes. For example,

the rs6265 SNP, a G to A mutation of the *BDNF* gene at the nucleotide 196, producing a val66met amino acid substitution in the 5' pro-*BDNF* region, has been shown to affect the protein's intracellular processing and secretion via the mechanism involving alterations in pro-BDNF interaction with sortilin, a protein responsible for sorting BDNF into the regulated secretory pathway (19, 20). Similarly, the association between the rs1801157 polymorphism, a G801A mutation located in the 3' untranslated region of the *CXCL12/SDF-1* gene, and susceptibility to several different diseases correlated with changes in SDF-1 levels has been proposed (21-23). Thus, SNP mutations within the genes encoding for the HIF-1α downstream effector proteins such as BDNF, VEGF, and SDF-1 could potentially play a role in underpinning the ability of prematurely born patients to resist and/or recover from hypoxic insults and related complications. If that is the case, then such SNPs could potentially serve as genetic markers that may help to identify the patients that are at a greater risk of developing complications associated with premature birth and, perhaps, even predict their response to therapeutic interventions.

In this study, we begin the search for biochemical and genetic markers associated with neurovascular complications in premature newborns by investigating the potential relationship between systemic BDNF protein levels and the presence of rs6265 (val66met) and rs1801157 (G801A) SNP mutations in *BDNF* and *SDF-1* genes respectively with a range of neonatal complications in a cohort of 53 premature singleton neonatal patients born to mothers at Yale New Haven Hospital.

METHODS

Cord blood acquisition

Cord blood samples used for this study were generously provided by Dr. Irina Buhimschi. The protocols for acquiring fetal cord blood and associated patient information was approved by the Yale University Human Investigational Committee, and all mothers provided written and informed consent. Fetal cord blood was acquired from a cohort of 180 consecutive preterm singleton newborns born to mothers at Yale New Haven Hospital between May 2004 and September 2009, as well as 19 healthy non-laboring women undergoing elective cesarean delivery at term. Gestational age of infants was calculated by time since last menstrual period and ultrasound evaluation prior to 20 weeks gestation. Exclusion criteria for maternal enrollment included: chronic medical conditions (*i.e.* hypertension, diabetes, thyroid disease), HIV, hepatitis or other viral infections, fetal structural abnormalities or fetal heart rate abnormalities requiring immediate intervention. Mothers of all preterm infants underwent amniocentesis to rule out intra-amniotic infection and/or inflammation prior to delivery using standard clinical, biochemical, and microbiological amniotic fluid tests including glucose concentration (cut-off of ≤15 mg/dL), lactate dehydrogenase activity (LDH, cut-off ≥419 U/L), white blood cell count (WBC, cut-off ≥50 cells/mm3), Gram stain and microbiological cultures for aerobes, anaerobes, Ureaplasma, and Mycoplasma species, as described in (24). Cord blood was acquired by sterile puncture of the umbilical vein after delivery, centrifuged for 10 min (1,000g, 4°C), alquioted and stored at -80°C. Fifty-

three samples from this larger cohort were then chosen at random for further SNP analysis.

Genomic DNA extraction/purification

Genomic DNA (gDNA) was purified from fetal cord blood by use of DNeasy Blood and Tissue Kit (Quiagen, Cat# 69506) according to manufacturer's instructions. Briefly, 50µL of cord blood was treated with 20µL of proteinase K, brought to a volume of 220µL with PBS buffer solution. 200µL of Buffer AL were then added to each sample, vortexed, and incubated at 56°C for 10 min. An additional 200µL of 100% ethanol was added to each sample, and the samples were all loaded into DNeasy Mini spin columns to bind DNA. Samples were centrifuged at 8000rpm for 1 min, and the remaining contaminants were washed out using Buffers AW1 and AW2, and spun dry at 14000rpm for 3 min. Genomic DNA was eluted from the spin columns in 200µL of Buffer AE elution buffer, provided in the kit. The resultant eluent was stored at -20°C.

Genomic DNA quantification

Quantification of gDNA purified from cord blood RBCs was done by means of densitometry against a standard curve. 2µL of each gDNA sample was mixed with 10x DNA gel-loading dye and distilled water (ddH_2O) to a total volume of 10µL, and loaded onto a 1% agarose gel made with TAE buffer solution and stained with SYBR Safe DNA gel stain (Invitrogen, Cat# S33102). A standard curve was generated by serial dilutions of λ DNA to achieve concentrations of 20ng/mL, 10ng/mL, 5ng/mL, 2.5ng/mL, and 1.25ng/mL and loaded onto the gel. The gel was run at 80V for 100min and visualized in

the ChemiDoc imaging system (BioRad). Densitometry was performed using Quantity One software (ver 4.6.9), and final gDNA concentrations were quantified in relation to the standard curve.

PCR (BDNF, SDF-1)

Gene amplification was performed using the Advantage 2 PCR Kit (Clontech, Cat# 639207) according to manufacturer's instructions. PCR reactions were set up containing 25ng of gDNA from each sample, and a master mix consisting of 2.5µL 10x SA Buffer, 0.5µL of 50x dNTP, 0.5µL of 10µM Forward Primer, 0.5µL of 10µM Reverse Primer, and 0.5µL of 50x Advantage 2 Polymerase Mix. Primer sequences and expected amplicon sizes can be seen in Table A. Each sample was then brought up to a total volume of 25µL with PCR grade water and put into a thermal cycler. BDNF thermal cycler settings were to heat the samples to 95°C for 5 min, 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, followed by cooling the samples to 72°C for 10 min, and then maintain samples at 4°C indefinitely. SDF-1 thermal cycler settings were to heat the samples to 94°C for 8 min, followed by 35 cycles of 94°C for 30 sec, 62°C for 1 min, and 72°C for 1 min, followed by cooling the samples to 72°C for 5 min, and then maintain the samples at 15°C indefinitely.

Amplicon extraction/purification

All PCR products were visualized on a 2% GPG agarose gel made with TAE buffer solution and stained with SYBR Safe DNA gel stain (Figs 1a and 2a). Each 25µL PCR sample was mixed with 2µL of 10x DNA gel-loading dye and run at 100V for 45 min. A

100bp DNA ladder was also run with each gel to verify the expected size of each amplicon. PCR products were visualized using the ChemiDoc imaging system, and then extracted using the QIAquick Gel Extraction Kit (Quiagen, Cat# 28706) according to manufacturer's instructions. Briefly, each sample was carefully excised from the agarose gel with a clean scalpel under a UV light, ensuring a clear visible margin of the gel in between samples. The samples were then treated with Buffer QG and incubated for 10min at 50°C, until the gel had completely dissolved. Isopropanol was added to each sample to increase yield of DNA fragments, and each sample was loaded onto a QIAquick spin column and centrifuged for 1min at 13000 RPM. Another 500µL of Buffer QG was added to each step to ensure the removal of all traces of the agarose gel, and the centrifugation repeated. Each sample was then washed with 750µL of Buffer PE, and eluted with 50µL of Buffer EB, provided in the kit. The eluate was stored at -40°C.

Amplicon Quantification

Quantification of PCR product was done by means of densitometry against a standard curve. 2µL of each PCR product was mixed with 10x DNA gel-loading dye and ddH_2O to a total volume of 5µL, and loaded onto a 2% GPG agarose gel made with TAE buffer solution and stained with SYBR Safe DNA gel stain. A standard curve was generated by serial dilutions of a 1Kb DNA ladder (Invitrogen, Cat# 15615-024) to concentrations of 80, 40, 20, 10, and 5 ng/µL. 5µL of each dilution was loaded onto the gel, resulting signal intensities corresponding to 40, 20, 10, 5, and 2.5ng, respectively, to be seen at the 1636-bp band. The gel was run at 75V for 60min and visualized in

ChemiDoc imaging system. Densitometry was performed using Quantity One software, and final amplicon concentrations were quantified in relation to the standard curve.

BDNF RFLP Analysis

Restriction fragment length polymorphism analysis of the BDNF amplicon was carried out with the use of the AflIII (New England BioLabs, Cat# R0541S) and PmlI (New England BioLabs, Cat# R0532S) restriction endonucleases. 50ng of each sample was mixed together with 5u of AflIII and 5u of PmlI, in BSA, NEBuffer 2, and ddH_2O, to a final reaction volume of 25µL. The reaction was run overnight at 37°C. Each sample was then loaded onto a 4% GPG agarose gel made with TAE buffer solution and stained with SYBR Safe DNA gel stain, and run alongside 100bp DNA ladder (Invitrogen, Cat# 15628-019) at 75V for 90min. The gel was visualized using ChemiDoc imaging system, and the resulting banding pattern was evaluated to determine the identity of the SNP present (Fig 1b).

SDF-1 RFLP Analysis

Restriction fragment length polymorphism analysis of the SDF-1 amplicon was carried out with the use of the NciI (New England BioLabs, Cat# R0196S) restriction endonuclease. 50ng of each sample was mixed together with 40u of NciI in NEBuffer 4 and ddH_2O, to a final reaction volume of 25µL. The reaction was run overnight at 37°C. Each sample was then loaded onto a 4% GPG agarose gel made with TAE buffer solution and stained with SYBR Safe DNA gel stain, and run alongside 100bp DNA ladder (Invitrogen, Cat# 15628-019) at 75V for 90min. The gel was visualized using ChemiDoc

imaging system, and the resulting banding pattern was evaluated to determine the identity of the SNP present (Fig 2b).

Amplicon DNA Sequencing

DNA sequencing of PCR products was done by the Sanger method at the Yale Keck DNA Sequencing Facility. BDNF sequencing was done in duplicate, using the PCR forward primer and a separate BDNF reverse sequencing primer, which was used to meet Keck primer specifications. SDF-1 sequencing was performed with the PCR forward primer only. Samples were prepared by mixing 20ng of amplicon per 200 base pairs to be sequenced (resulting in 22.3ng for BDNF samples, and 54.3ng for SDF-1 samples) with 2µL of 4µM DNA primer and ddH_2O to a total volume of 18µL. Samples were then plated onto a 96-well plate and sent to the Keck DNA Sequencing Facility. The resulting sequences were analyzed using Geneious (ver 4.8, Biomatters) software (Fig 1c-d, Fig 2c-d).

Quantitative ELISA determination of BDNF Levels

All cord blood BDNF levels were acquired from platelet poor plasma samples by Dr. Yue Wu using the DuoSet ELISA Development System (R&D Systems, [http://www.rndsystems.com]). All assays were performed in triplicate using the manufacturer's recommended buffers, diluents, and substrates, according to manufacturer instructions. The ELISAs were read using an automated microplate reader (Wallac Victor). The BDNF concentrations were corroborated with total plasma protein concentration and reported as pg/mg.

Acquisition of Clinical Data

All clinical and diagnostic patient information was gathered for an alternate study (24), and was generously provided by Dr. Irina Buhimschi, in de-identified form, for correlation analysis with the SNP and protein expression data. Diagnoses of clinical short-term neonatal outcomes were done by trained physicians in accordance with clinical guidelines at Yale New Haven Hospital.

Statistical Analysis

Statistical analysis was done using SigmaPlot statistical software (ver 12.0, Systat Softare). Normality testing was performed using the Shapiro-Wilk test, with α-value of 0.05 used as threshold for normal distribution. Data that was not normally distributed was analyzed using Mann Whitney Rank Sum test. Normally distributed data was evaluated using a 1-tailed student t-test. Comparison of categorical data between two groups was analyzed using Fisher's exact test, while comparisons of multiple groups were done using Chi-square analysis. Statistical significance was assumed with p-values <0.05, while trends towards significance were assumed for p-values <0.10.

RESULTS

BDNF G196A Allele and Clinical Outcomes

The presence of the BDNF G196A SNP was determined in duplicate using both restriction fragment length polymorphism analysis and direct Sanger sequencing. Of the fifty three samples that were analyzed, ten of them were found to have the A allele in position 196 of the BDNF gene. This frequency of 18.9% was similar to the previously published frequency of 22.9%. One out of the fifty three samples tested resulted in an equivocal RFLP signal, and the sample was recorded as being negative for the G196A SNP based on Sanger sequencing alone.

Of the maternal characteristics analyzed, no statistically significant differences between mothers who had children with the G allele vs mothers who had children with the A allele in the BDNF gene were found (Table B). Maternal characteristics analyzed included maternal age, gravidity, parity, and race, whether or not the mother clinically required medical interventions such as steroids, antibiotics, tocolysis, magnesium sulfate, or progesterone throughout her pregnancy/ the peri-partum period, and the outcomes of placental histological analysis screening for evidence of chorionic plate inflammation, amnionitis, choriodeciduitis, or funisitis. The median age of mothers with a child having the A (vs G) allele was 31 (vs 27), median gravidity 3 (vs 3), and median parity 1 (vs 1). Of the patients analyzed 34% were Caucasian, 42% African American, 17% Hispanic, and 8% Other, with no statistically significant difference in the racial classification of mothers between the two different groups.

The median Apgar score at 1 and 5 min after birth was 6.5 and 8, respectively, for infants positive for the G196A SNP, compared to 6 and 8, respectively, for infants negative for it. This difference was not found to be statistically significant. The difference in the median gestational age for infants with the A-allele (30.15 weeks vs 26.2 weeks) and the median weight of infants with the A-allele (1460g vs 930g) at the time of birth, however, though not statistically significant, did trend towards significance with p-values of <0.1. Further analysis of the presence of other known neonatal complications revealed no statistically significant difference between the two groups, with incidence rates for interventricular hemorrhage of 11% (vs 24%), bronchopulmonary dysplasia 10% (vs 18%), necrotizing enterocolitis 10% (vs 12%), retinopathy of prematurity 22% (vs 36%), periventricular leukomalacia 11% (vs 10%), clinical early-onset neonatal sepsis 50% (vs 33%), and death of 0% (vs 10%), all with p-values >0.45. The overall presence of any adverse outcome for infants with the G196A polymorphism was 30% compared to the 40% present in the control group, with a p-value of 0.722 (Table C).

SDF-1 G801A Allele and Clinical Outcomes

The presence of the SDF-1 G801A SNP was also determined using restriction fragment length polymorphism analysis and confirmed with direct Sanger sequencing. Thirteen of the fifty three patients analyzed were positive for the G801A SNP, resulting in a 24.5% frequency rate of the A-allele (as compared to the published frequency of 20.7%). One out of the fifty three samples tested showed a discrepancy between the

RFLP analysis results and the sequencing results. However, due to the clearly visible presence of multiple bands on the RFLP gel, suggesting the presence of A-allele, that sample was recorded as positive for the G801A SNP.

As with the G196A SNP analysis, no statistically significant demographic differences between mothers of infants positive or negative for the G801A SNP were found. The median age, gravidity, and parity for the subset of mothers of infants with the C-allele were 29.5, 3, and 1, respectively, while the median age, gravidity, and parity for the subset of mothers of infants with the T-allele were 25, 2, and 1, respectively. Likewise, there was also no statistically significant difference in the number of mothers who received any steroids, antibiotics, tocolytics, magnesium sulfate, or progesterone between the two groups (Table D). Subgrouping of patients based on the presence of the G801A SNP did reveal a relatively lower incidence rate of stage 2-4 choriodeciduitis (61.5% vs 89.7%, $P<0.04$), as well as a relatively lower incidence rate of chorionic plate inflammation (53.8% vs 79.5%, $p <0.09$), in mothers giving birth to infants with the A-allele (Table E). No readily apparent difference was observed in the incidence rates in this sub-grouping of mothers with respect to amnionitis and funisitis.

The median Apgar score at 1 and 5 min after birth was 6 and 8 for infants positive for the G801A polymorphism, compared to 8 and 8 for infants negative for the polymorphism, with a p-value of 0.7 and 0.8, respectively. The median gestational age at the time of delivery was 26.8 weeks and 26.2 weeks, and the median birthweight 1025g and 920g, for infants with the G-allele and the A-allele, respectively. Of the immediate

neonatal complications that were looked at, infants with the G-allele had a 45% incidence rate of clinical early-onset neonatal sepsis vs 8.3% in infants with the A-allele, $p<0.05$. Other complications, including interventricular hemorrhage (22.5% vs 18.2%), bronchopulmonary dysplasia (13.5% vs 27.3%), necrotizing enterocolitis (12.5% vs 8.3%), retinopathy of prematurity (30% vs 45.5%), periventricular leukomalacia (10% vs 9.1%), and death (7.5% vs 8.3%) did not show any significant difference between the two groups (Table E). Overall incidence rate of any adverse outcome was 35% for infants with the C-allele, and 50% for infants with the T-allele, with a p-value of 0.50.

BDNF Protein Expression Data

BDNF protein expression data was gathered by Dr. Yue Wu using a quantitative ELISA assay on cord blood samples from a subgroup of 23 patients. Protein levels were then subdivided with respect to the presence or absence of a number of neonatal complications, and analyzed using 1-tailed student t-test. Of these neonatal complications, death was the only one that yielded statistically significant differences in BDNF levels between the two groups, with a mean protein level of 183.43pg/mg in patients who died prior to discharge from the NICU as compared to 105.33pg/mg in those who survived (Table F). Presumed early-onset neonatal sepsis, which was determined to be as either high (>70%) or low (<20%) level of probability of intra-amniotic infection and/or inflammation (IAI) by means of Bayesian latent-class analysis of a variety of factors, including elevated haptoglobin and haptoglobin-related protein immunoreactivity (Hp&HpRP), cord blood IL-6 levels, and a variety of other neonatal

hematological indices, trended towards significance. Of these two groups, the average BDNF level for infants classified as highly likely to be exposed to IAI was 104.29pg/mg, while BDNF levels for those classified unlikely to have been exposed was 147.33pg/mg, p-value 0.089. While segregating the patients by clinically confirmed EONS, which was defined as infants with presumed EONS on the basis of hematological indices plus either the presence of clinical symptoms and/or positive blood cultures at ≤ 72 hours after birth, resulted in further trend towards significance, with patients positive for clinical EONS resulting in mean BDNF expression levels of 95.66pg/mg, while those classified as negative for clinical EONS reached 141.33pg/mg, with a p-value of 0.051. Likewise, the presence of bronchopulmonary dysplasia or necrotizing enterocolitis also yielded differing BDNF expression levels that trended towards significance, with those positive for either disease averaging 69.2pg/mg or 74.48pg/mg, respectively, while those negative for the disease averaging 114.36pg/mg or 124.16pg/mg, respectively, with a p-value of 0.079 and 0.089. Correlations of BDNF levels with the development of interventricular hemorrhage, retinopathy of prematurity, or periventricular leukomalacia, however, did not yield any statistically significant differences in expression.

Of note, although analyzing the patients on the basis of the presence or absence of IVH alone did not list statistically significant results, separating the patients into three distinct groups of: No IVH/no EONS, no IVH/positive EONS, or positive IVH, showed statistically significant differences in BDNF expression levels between the no IVH/no EONS and the no IVH/positive EONS groups, with a mean of 157.71pg/mg and 84.65

pg/mg, respectively, p-value <0.01, and a trend towards significance between the No IVH/no EONS, and positive IVH groups, with a mean of 157.71pg/mg and 109.46pg/mg, respectively, p-value <0.10 (Fig 3).

DISCUSSION

Searching for genetic and biochemical markers, which could help to identify patients who are at a greater risk of developing complications associated with premature birth, is an important goal of modern neonatal medicine. Indeed, with the advent of medical technology and falling infant mortality rates, increasing numbers of premature infants are surviving past the perinatal period. However, as the minimum gestational age of premature infants surviving child birth decreases, the number of neonates with serious complications being cared for continues to grow. In 2009, of the 4,130,000 live births, 12.18% were born prior to 37 weeks gestation (with 2% <32 weeks gestation), 8.16% were low birth weight (<2500g), and 1.45% were very low birth weight (<1500g). Simultaneously, the neonatal mortality rate dropped from 4.29 deaths per 1000 live births in 2008 to 4.19 in 2009, resulting in a significant number of very low birth weight premature infants requiring medical attention (25). Of the possible complications in this population group, the most common is respiratory distress with an incidence rate of 93%. Other possible complications include retinopathy of prematurity (59%), bronchopulmonary dysplasia (42%), necrotizing enterocolitis (11%), intraventricular hemorrhage (IVH, 16%), and periventricular leukomalacia (3%) (26). Long term consequences of decreased oxygenation of end organs, and more specifically the neonatal brain, range from cerebral palsy and intellectual disability to more subtle disabilities such as ADHD and social-emotional difficulties, resulting in an estimated cost of 370 million dollars per year in special education costs alone (27).

Although significant advances have been made in both preventing and treating negative outcomes associated with premature birth, these interventions are not without side-effects. Infants who are not able to maintain proper oxygen saturation levels, or who become acidotic, are intubated and mechanically ventilated to help reverse hypoxemia and prevent long term neurocognitive sequelae. Though necessary, mechanical ventilation can result in permanent lung injury through a variety of means including overdistension, barotrauma, and infection, resulting in damage to the airway epithelium, destruction of the alveoli, and fibrosis of lung tissue secondary to inflammation. It is estimated that somewhere between 30-40% of very low birth weight infants suffer from chronic lung disease (28). Moreover, increased oxygen delivery to end organs in the periphery may result in oxygen toxicity stemming from generation of reactive oxygen species with subsequent cellular injury and cell death (29). Additionally, free radical species generated from both hypoxia of respiratory distress and hyperoxia of mechanical ventilation serve to perturb angiogenesis throughout more sensitive areas of the body via its effect on the hypoxia induced factor-1alpha (HIF-1α) signaling pathway and its downstream effector molecules such as vascular endothelial growth factor (VEGF) and nitric oxide (NO). Such perturbations serve a key role in pathogenesis of neonatal complications such as retinopathy of prematurity, and may also contribute to the negative neurodevelopmental outcomes seen in very low birth weight premature infants described above (9, 18, 30, 31).

The efficacy of current first-line treatments as well as their optimal regimentation and dosing are also in need of improvement. A recent study in China

revealed that despite administration of standard of care therapy, including administration of exogenous surfactant and different conventional mechanical ventilation modalities, 31% of neonates who developed respiratory distress syndrome did not survive (32). Exogenous corticosteroid administration prior to delivery of premature infants, the cornerstone for managing preterm infants, has greatly improved neonatal morbidity and mortality. However, despite their clear benefits, more than 60% of affected premature infants go on to develop respiratory distress syndrome and more than 50% experience cerebroventricular hemorrhage even with appropriate intervention. Moreover, antenatal corticosteroids have shown no statistically significant difference in the rates of development of many long term side effects of prematurity, such as decreased lung function, behavioral/learning difficulties, or intellectual impairment (33).

Taken together, this data illustrates the need for not only the development of novel interventions to prevent and treat immediate complications of low birth weight and prematurity, but also determine which individuals would derive more benefit from current interventions than harm from their associated side-effects. The efficacy of such new treatments, however, is difficult to evaluate due to the complexity of factors involved in multiple disease processes associated with prematurity, and an infant's recovery from them. Consequently, there would be great utility in being able to delineate patients who are at greatest risk for developing significant short term and long term morbidities resulting from hypoxemia and respiratory distress, from those who would naturally return to their baseline without treatment, as early as possible. One

potential approach for accomplishing such a feat is through the identification of novel genetic and molecular biomarkers, which could help predict an individual's response to hypoxia.

The underlying ability of tissues extremely sensitive to levels of oxygenation, such as the brain, to recover from hypoxic damage is thought to lie in the neurogenesis that occurs in response to chronic sublethal oxygen deprivation. Murine models mimicking chronic hypoxia have been used to gain insights into this process. Mice reared under hypoxic conditions (9.5-10.5% O_2) for 8 days starting at post natal day three (P3) showed a 24% decrease in brain weight, and a concomitant 30% loss of neurons in the cerebral cortex, as compared to their normoxic counterparts (34). This difference in brain weight, however, completely reversed within 1 week of returning to normoxic conditions, with no apparent difference in the density of neuronal cell bodies on immunostaining. By 31 days of recovery, the only discernible difference between the hypoxic and normoxic reared mice was morphological, with a thinning and decreased regularity in the array of the pyramidal cell dendritic arbors in the cerebral cortex. Intraperitoneal injection of a thymidine analog bromodeoxyuridine (BrdU) at varying time points revealed a 40% increase in BrdU-labeled neural cells in the cerebral cortex of hypoxic-reared mice one month after BrdU incorporation, as compared to their normoxic counterparts. Moreover, there was a greater than two-fold increase in BrdU-labeled cells expressing neuronal markers within the SVZ in mice recovering from hypoxia within 1 week of return to normoxic conditions, suggesting that the recovery from hypoxia induced brain damage is at least in part dependent upon activation of

increased neurogenesis to compensate for neuronal losses following prolonged decreased oxygenation (34). However, even in this mouse model, as in human disease, the extent of recovery from hypoxia-induced damage varied significantly from one individual to the next.

To investigate this further, our lab has characterized the response to chronic hypoxia between two different mouse strains, which exhibit differing levels of abilities to recover from such an insult, in hopes of elucidating key proteins that are integral to initiating this response. Two strains of mice (CD-1 and C57BL/6), were reared under both hypoxic and normoxic conditions as described in (34). It was found that while CD-1 pups were able to survive a 30-day exposure to hypoxia, C57BL/6 pups expired at day 13 under similar conditions. Pups were thus sacrificed at postnatal day P11, while both strains were still viable, for further analysis. Examination of the difference in hepatic extramedullary hematopoiesis (EMH) between the two strains, a known response to chronic hypoxia, showed a 3.4-fold induction in hepatic EMH in the CD-1 strain as compared to CD-1 mice raised under normoxic conditions, with no such appreciable increase in EMH observed in the C57BL/6 mouse strain. Comparison of hepatic EMH between strains both raised under normoxic conditions showed no appreciable difference in activation (9). Assaying the difference in SVZ NPC proliferation between the two strains using BrdU labeling of nestin-positive cells showed a baseline increase in the percent of nestin-positive cells that were also BrdU-positive in the CD-1 pups (30.2%) vs. the C57BL/6 pups (20.6%), when raised under normoxic conditions. This difference further widened when comparing mice raised under hypoxic conditions, with

CD-1 pups exhibiting 21.2% BrdU/nestin double-labeled cells vs. only 8.2% in C57BL/6 pups, thereby supporting the notion that CD-1 pups' resistance to hypoxia is at least in part due to an increased neurogenic response. Similarly, staining tissue from the SVZ areas of each mouse strain with anti-PECAM-1 antibodies revealed both a higher baseline microvascular density in CD-1 pups as compared to C57BL/6 pups, and a higher final microvascular density, when exposed to normoxic and hypoxic conditions, respectively (9).

Prior work done by our group with neural stem cells and brain endothelial cells (BECs) in cell culture has demonstrated a complex cross-talk that occurs between the two cell types involving BDNF and VEGF molecules associated with NSC proliferation and vascular tube formation. In these experiments either co-culturing NSCs with BECs or growing BECs in the presence of NSC-conditioned media both resulted in the formation of branching endothelial cell microtubular structures (illustrative of in vitro angiogenesis) in contrast to a BEC mono-layer growth that occurs in the absence of NSC-derived soluble factors. At the same time, NSCs grown either in co-culture conditioned media or in direct co-culture with BECs exhibited a significant increase in number and size of NSCs as compared to NSCs grown in NSC media alone. As expected, western blot analysis of BEC cell lysates revealed increased phosphorylation of the brain endothelial-derived VEGFR2 receptor in response to soluble factors secreted by NSCs compared to that induced by BEC-conditioned media. The examination of BDNF and VEGF protein expression levels using ELISA revealed a relatively low baseline expression of both BDNF and VEGF in BEC mono-cultures, a relatively high baseline expression of VEGF and a low

baseline expression of BDNF in NSC mono-cultures, significantly increased BDNF and VEGF levels in BECs isolated from NSC-BEC co-cultures (compared to protein levels from BEC monocultures), and no changes in VEGF and BDNF levels in NSCs isolated from NSC-BEC co-cultures (compared to protein levels from NSC monocultures). Furthermore, treatment of NSC-BEC co-cultures with sFlt-1 and sTrkB, which serves to sequester VEGF and BDNF respectively, results in dramatic blunting of vascular tube formation, which is in turn rescued with the addition of recombinant BDNF and VEGF molecules (18). As a result, it has been hypothesized that the differential response in survivability and resistance to sublethal chronic hypoxia between the CD-1 and C57BL/6 mouse strains could be due to a difference in activation HIF1α, and its effector molecules, which include BDNF and VEGF, as well as SDF-1.

HIF1α is a transcription factor regulating multiple proteins, including erythropoietin, glucose transporters, glycolytic enzymes, and VEGF among others, that are involved in cellular adaptation to hypoxia, (35, 36). Analysis of HIF1α expression levels in CD-1 pup brains revealed significantly higher levels of HIF1α expression than in their C57BL/6 counterparts under normoxic conditions, and comparatively greater drop in HIF1α levels in C57BL/6 pups in comparison to CD-1 pups, when reared under hypoxic conditions, thus resulting in higher levels of HIF1α expression in CD-1 mice in both instances. Similarly, BDNF expression levels were also higher in CD-1 brain lysates compared to C57BL/6 lysates of mice experiencing hypoxia, with no significant difference in BDNF levels between mouse strains reared under normoxic conditions. Interestingly enough, while BDNF levels showed significantly higher expression in

hypoxic vs. normoxic CD-1 pups, BDNF expression levels remained essentially unchanged in C57BL/6 pups between the two conditions. Determination of VEGF-A_{165} expression levels using ELISA revealed that, while both strains exhibited an increase in VEGF-A_{165} expression from normoxic- to hypoxic-reared mice, under both conditions CD-1 brain lysates showed a significantly higher VEGF-A_{165} expression rate than their C57BL/6 counterparts. Lastly, the same pattern held true for SDF-1 expression levels, wherein normoxic CD-1 brain lysates showed a higher level of expression than normoxic C57BL/6 brain lysates, and hypoxic CD-1 brain lysates showed a higher level of expression than hypoxic C57BL/6 brain lysates (Fig 4). Similar findings in the expression of these proteins in NPCs harvested from P1 CD-1 and C57BL/6 pups and grown under normoxic and hypoxic conditions were also found in cell culture (9).

Together, these data are consistent with the potential molecular mechanism which governs the differential ability to recover from chronic hypoxia between the CD-1 and C57BL/6 mouse strains, wherein the hypoxemic insult activates HIF-1α, which in turn upregulates expression of BDNF, VEGF, and SDF-1. These three signaling cascades all converge on the PI3K/AKT signal transduction pathway, which serves to activate endothelial nitric oxide synthase (eNOS). Increased NO levels then further upregulate HIF-1α expression, closing the positive feedback loop, and serve to stimulate NSC proliferation and survival (Fig 5) (9, 37-43). As a result, fetal or neonatal expression levels of BDNF, VEGF, and SDF-1 could be prime candidates for potential molecular biomarkers that could predict a neonate's ability to return to baseline after experiencing prolonged hypoxia as a consequence of prematurity.

In addition, recent data has shown that expression levels of BDNF and SDF-1 could be also affected by the presence or absence of specific single nucleotide polymorphisms (SNPs) within the genome. The BDNF val66met polymorphism (rs6265) is a G to A variant of the BDNF gene at nucleotide 196, which produces an amino acid substitution in the 5' pro-BDNF region that affects the protein's intracellular processing and secretion. One study showed hippocampal neurons transfected with metBDNF-GFP recombinant proteins exhibited abnormal localization of the protein to the neuronal cell bodies in the peri-nuclear region versus a more diffuse distribution throughout the cell body and extending into dendritic processes that occurred with the valBDNF-GFP variant. Moreover, BDNF secretion studies in these neurons showed a significant decrease in the activity-dependent secretion of BDNF in neurons transfected with the metBDNF gene compared to valBDNF variant, upon nerve stimulation (19). The mechanism responsible for this difference is thought to be rooted in the disruption of interaction between pro-BDNF and the sortilin protein, which is responsible for sorting BDNF into the regulated secretory pathway (20).

A less-well established link between the presence of a polymorphism and its effect on protein expression exists in SDF-1. The G801A (rs1801157) polymorphism is located in the 3' untranslated region of the SDF-1 (also known as CXCL12) gene that has been linked to a change in a variety of different disease susceptibilities among individuals (21-23). A genetic association analysis of 2857 of patients enrolled in five AIDS cohort studies revealed that individuals homozygous for the G801A polymorphism infected with HIV had a significant delay in the onset of AIDS. This observation is

thought to be rooted in increased SDF-1 expression levels, which bind SDF-1's cognate receptor, CXCR4, thereby decreasing the ability of HIV viral particles from infecting new CD4 T-cells (which also utilize the CXCR4 receptor) (22). A later study measuring plasma SDF-1 levels in HIV positive and HIV negative patients showed significantly decreased levels in patients homozygous for the G801A SNP (23). Similarly, Florence et al. found that patients positive for the G801A polymorphism on either one or both alleles had an increased rate of extramedullary metastasis in acute myeloid leukemia—a process which is thought to be linked with decreased expression levels of SDF-1 (21). Consequently, though the effects of the G801A SNP on SDF-1 expression are not well established, its presence appears to play a role in a variety of disease processes. As a result, both the G196A SNP in BDNF and the G801A SNP in SDF-1 have the potential to be genetic biomarkers that would be predictive of an individual's ability to recover from hypoxia induced damage, and should be investigated further.

To assess the utility of BDNF expression levels and the presence of the G196A BDNF and G801A SDF-1 SNPs in predicting neonatal outcomes, our group analyzed the cord blood of 53 premature singleton neonatal patients born to mothers at Yale New Haven Hospital. BDNF expression levels in a subset of these patients was determined using a quantitative ELISA assay, which were then compared to the presence or absence of a variety of neonatal complications associated with premature birth and hypoxia (Table F). Of the complications analyzed, the only one to yield statistical significance was death, which was surprisingly associated with an increased average BDNF level in neonates who died versus those who survived. This seemingly paradoxical outcome is

likely a statistical anomaly that is due to the fact that out of the 23 patients that were analyzed, only 3 died. Moreover, one of these three patients exhibited BDNF levels more than two-fold higher than the other two, thereby significantly skewing the average for the entire group.

Both presumed early onset neonatal sepsis and clinical early onset neonatal sepsis (EONS) trended towards significance, with p-values <0.10. The average BDNF expression levels for patients experiencing either presumed or clinical EONS was 30% lower than in patients who did not experience these complications. EONS is associated with a variety of multi-systemic complications including respiratory distress, dehydration secondary to diarrhea and vomiting, and apnea, all of which have the potential to decrease perfusion and/or oxygenation of tissues (44). Though poorly understood, members of the neurotrophin family, including nerve growth factor (NGF) and BDNF have been shown to be expressed in a variety of non-neuronal systems throughout development, including the cardiovascular, endocrine, reproductive, and immune systems, with the highest levels of BDNF mRNA expressed in the heart, spleen, and lung tissues (45, 46). Given the key role BDNF plays in protecting against hypoxia-induced cellular damage as part of the HIF-1α response, and the fact that the diagnosis of neonatal sepsis most often occurs based on clinical symptoms (rather than positive blood cultures), it would be reasonable to extrapolate that premature infants who exhibit higher baseline levels of BDNF would be better protected against hypoxia induced damage and would therefore be less likely to acquire the diagnosis of sepsis.

Analysis of the development of bronchopulmonary dysplasia (BPD) also showed a trend towards significance with regards to BDNF expression levels. Neonates who developed BPD had on average nearly a 40% lower BDNF expression level than those who did not (69.2 vs 114.36 pg/mg, $p<0.08$). As would be expected, this trend is consistent with BDNF's protective properties against hypoxia-induced damage. The pathogenesis of BPD is the result of direct airway and parenchymal damage prior to the maturation of the lungs, and is associated, among other causes, with a variety of medical interventions such ventilator-induced lung injury, oxygen toxicity secondary to O_2 supplementation, and exposure to steroids (47). Moreover, as described above, developing lung tissue is one of the most active areas of transcription of BDNF mRNA outside of the nervous system, suggesting the importance BDNF may play in proper lung development. Consequently, premature infants who exhibit higher levels of BDNF at baseline would be more resilient to the deleterious effects of low O_2 associated with premature birth, and would therefore be less likely to receive potentially harmful interventions, which would in turn decrease their chances of developing BPD.

The last of the neonatal complications that were analyzed with regards to baseline BDNF expression levels to show a trend towards significance was the development of necrotizing enterocolitis (NEC). The average BDNF expression levels of neonates who developed NEC was less than 60% then that of newborns that did not develop this complication (74.48 pg/mg versus 124.16 pg/mg, $p < 0.09$). The pathogenesis of NEC is primarily due to changes from intestinal infarction, with the resulting inflammation and reactive tissue repair playing key roles in impairment of

proper gastrointestinal function (48). Hoehner et al. have shown that BDNF plays a key role in the development of the enteric nervous system, and is actively expressed in enteric ganglion cells (EGCs) throughout different stages of development (49). More recently, Steinkamp et al. have also shown that BDNF secreted by EGCs under inflammatory conditions plays a significant role in attenuating the EGCs apoptotic response (50). Thus, once again, it appears that increased baseline levels of BDNF may serve a protective role in infants from developing one of the complications associated with prematurity.

Analysis of the presence of possible placental infections and their association with BDNF levels yielded two statistically significant results (Table F). The development of both amnionitis and funisitis during the pregnancy was associated with significantly lower levels of cord blood BDNF detected than was found in the cord blood of patients who did not develop these infections (92.54 vs 154.92 pg/mg for amnionitis, p-value <0.02; 93.83 vs 147.14 pg/mg for funisitis, p-value 0.03). Moreover, it should be noted, that although not statistically significant, the average cord blood BDNF levels in patients who suffered from development of chorionic plate inflammation or choriodeciduitis was also lower than in patients whose placentas did not show signs of infection on histological analysis. A recent study on the differential expression of BDNF in the human placenta during the third trimester showed that BDNF and its cognate receptors are expressed throughout the membranous chorion, trophoblast, and endothelium. Moreover, this expression was higher in placentas of mothers experiencing preeclampsia, normotensive small for gestational age placentas, and the placentas of

the small twin of discordant twin pregnancies. Fujita et al. went on demonstrate that BDNF had anti-apoptotic effects against oxidative stress in the choriocarcinoma cell line (JEG-3), suggesting that BDNF plays a protective role in human villous tissue under a variety of unfavorable conditions in utero (51). These findings are compatible with our observation that the development and progression of more severe inflammatory processes in the placenta is associated with lower BDNF expression levels.

Surprisingly, of the complications analyzed, the three dealing with the developing nervous system (development of interventricular hemorrhage, retinopathy of prematurity, and periventricular leukomalacia) did not reveal statistically significant associations with cord blood BDNF levels. One possible explanation for this phenomenon is the relatively high amount of comorbidities that are present in the premature infants analyzed, the effects of which may be obscuring the link between BDNF levels and the development of complications. Upon more careful analysis of infants who developed IVH, for instance, we are able to tease out the link by separating the patients into three distinct groups: (1) infants who did not develop IVH, (2) infants who did not develop IVH, but did have EONS, and lastly (3) infants who had IVH. Analysis of these three groups revealed that infants who were IVH neg/EONS neg had a nearly two fold higher baseline BDNF expression level than infants who were IVH neg/EONS pos ($p = 0.021$), and infants who were IVH neg/EONS neg had a 44% higher BDNF expression levels than children who were IVH pos ($p = 0.054$, Fig 3). As previously described, BDNF may play a role in the body's response to the development of EONS. Thus, given the limited number of patients whose BDNF levels were able to be assessed,

outside factors such as the presence EONS in the patient may obscure less robust associations between baseline BDNF levels and development of certain complications. As a result, further studies with increased number of subjects are necessary to better elucidate the presence of these links.

Next, we analyzed for the presence of the G196A BDNF SNP in the cord blood of fifty three premature neonates using both RFLP assays and direct Sanger sequencing of fetal DNA. The prevalence of the G196A SNP did not significantly differ among patients with respect to maternal age, race, or gender, and was not found to be associated with any statistically significant changes in neonatal outcomes (Tables B and C). Pregnancy outcomes that appeared to trend towards significance included median gestational age at delivery ($p = 0.065$) and median birthweight ($p = 0.099$). However, even among these two outcomes, the interquartile range between neonates with the G-allele vs the A-allele, had substantial overlap. Of the neonatal characteristics analyzed, both median cord blood arterial pH and culture positive EONS appeared to trend towards significance, with p-values of 0.096 and 0.077, respectively. In light of the previously discussed protective roles that BDNF may play as part of the HIF-1α signaling pathway, which serves to blunt the negative effects of hypoxia, our observation of lower levels of acidosis associated with the G-allele is logical. However, given that our analysis for the presence of the G196A SNP did not yield any statistically significant correlation with baseline cord blood BDNF expression levels, any such results should be interpreted with caution.

There are many possible reasons for the discordance between the presence of the G196A SNP in a patient and its lack of correlation to the detected cord blood BDNF levels. The path from gene transcription to protein expression is riddled with many different regulatory pathways, and the SNP's effect on secretion is just one of them. Recently, Verhmehren-Schmaedick et al. have shown that loss of the methy-CpG-binding protein 2 (MeCP2) transcription factor, that occurs in Rett syndrome, results in the blunting of the hypoxia-induced increase in BDNF transcript and protein expression levels in the pons and medulla of mice lacking MeCP2 (52). This finding suggests that a variety of unaccounted for factors affecting BDNF expression levels, which may vary among the patients studied, may be obscuring the effect of G196A SNP on BDNF expression. Alternatively, the study by Egan et al. has shown that the presence of the G196A SNP affects nerve depolarization-induced secretion in cultured hippocampal neurons, but does not have any effect on the constitutive secretion of BDNF (19). Since the nerve-stimulatory pathway of BDNF secretion differs from that of the hypoxia-induced pathway, the effects of the G196A SNP on BDNF expression under hypoxic conditions may in fact be different than we hypothesized. Lastly, Chen et al. have shown that the effects of the G196A polymorphism on BDNF secretion appears to be specific to neural tissue, and while endothelial cells and vascular smooth muscle cells secrete BDNF at levels comparable with the CNS at baseline (53-55), the secretion of BDNF by these cell types was not affected by the presence of the G196A SNP (56). Consequently, since we are determining BDNF protein levels as they are detected in samples of cord blood, it is likely that the BDNF levels that are present overwhelmingly represent active secretion

by the developing cardiovascular system, which would mask any variations in BDNF expression localized to the nervous system.

The presence of the G801A SDF-1 SNP was also analyzed in duplicate using both RFLP and direct Sanger sequencing analysis, in the same fifty three premature neonates as the G196A SNP. Once again, the prevalence of the G801A SNP did not significantly differ among patients with respect to maternal age, race, or gender (Table D). Analysis of neonatal outcomes and placental inflammation yielded a significantly greater percentage of premature neonates with the G allele developing clinical EONS ($p<0.04$), and choriodeciduitis in the fetal placenta ($p<0.04$), with a trend towards significance in the development of chorionic plate inflammation ($p<0.09$) (Table E). All other outcome measures did not yield any statistically significant results. The importance of the role SDF-1 plays in the immune response through its effects on leukocyte recruitment, migration, and adhesion has been well established (57). Moreover, SDF-1/CXCR4 signaling has been implicated in modulating the materno-fetal immunological tolerance in all three semesters of gestation that is essential for fetal survival (58). Consequently, differences in SDF-1 expression levels that result from the presence or absence of the G801A SNP may be responsible for the correlation between the presence of the A-allele and a decreased inflammatory response. Furthermore, such an association would be consistent with previous reports of the G801A SNP being linked to lower SDF-1 expression levels (21, 23). However, direct measurement of SDF-1 serum protein levels would be necessary to further validate this link.

There are number of factors in this study that limit the generalizability of our results, and thus require further investigation. First, the study was limited by the number of patients that were used to assess the links between presence of SNPs, BDNF protein expression levels, and neonatal outcomes. The number of neonates examined served well as a preliminary study to support the link between higher BDNF protein expression levels and more positive neonatal incomes. However, more patients would need to be analyzed to move the results from trends to statistical significance. Moreover, although the available literature supports a possible link between the presence of particular SNPs and outcomes, this relationship is affected by a multitude of outside factors, which would require several thousand more patients to be examined before sufficient power can be reached to confidently support or reject such a relationship. Another important limitation to this study is the non-specific nature of the source of blood from which protein expression levels were determined. Cord blood was obtained from the umbilical veins of patients upon delivery. While easily accessible, this source represents a mixture of proteins secreted throughout the body, with perhaps an overrepresentation of secretions from the fetal vasculature and the placenta, thus obscuring localized changes in expression that may be occurring in more isolated tissues like the nervous system. It would be interesting to examine differences in protein expression levels obtained from a variety of sites, such as both the arterial and venous systems, and see if any significant differences result. Lastly, multiple studies of the G801A SDF-1 SNP have suggested that the presence of the A-allele has no effect on SDF-1 protein expression levels, but it is in fact the presence of a number of other SNPs that

are in linkage disequilibrium with the G801A SNP, which affect protein expression levels and disease outcomes (59, 60). Consequently, it important to consider the effects of other known SNPs in both BDNF and SDF-1 genes that may be in linkage disequilibrium with the SNPs examined in this study, as it is they that might ultimately be responsible for (and be most useful in predicting) neonatal response to hypoxia induced damage.

In conclusion, the ability to be able to accurately predict both short term and long term clinical outcomes for premature infants promises to hold much benefit. From rationing out limited resources to patients who would benefit from them the most, to avoiding unnecessary long term and serious complications, being able to separate infants who need medical intervention from those who will recover on their own would result in millions of dollars in saving and greatly decrease morbidity. Moreover, such a tool would also allow us to evaluate the efficacy of novel therapies by helping us separate the benefits gained through a given intervention from the natural progression of disease that would have occurred even without clinical interference. Genetic and molecular biomarkers in cord blood, which is easily obtainable at birth, are prime candidates to enable us to accomplish this task.

Here we report preliminary exploration of three such potential biomarkers: the presence of G196A SNP in the BDNF gene, the presence of G801A SNP in the SDF-1 gene, and BDNF protein expression levels present in umbilical cord venous system. Of these, direct measurement of protein levels in cord blood appear to be most promising, at least in the short term. The effects of an individual SNP on neonatal outcomes do not

appear strong enough to discern any clinically useful stratification among patients on their own. However, there are many different candidates that are yet to be examined, that together have the potential to be treasure trove of prognostic biomarkers. Our initial attempts at characterizing a number of SNPs associated with the VEGF gene (data not shown) proved unsuccessful due to technical difficulties in amplifying the gene's G-C rich promoter sequence in time for this manuscript. However, further investigation of both VEGF associated SNPs and cord blood protein levels is necessary, given the key role VEGF plays in stabilizing the neurovascular niche and overall vascular development/ homeostasis. Moreover, given the disproportionate costs associated with long-term complications of prematurity, further studies with long term follow up over a period of decades would also be useful. Nevertheless, despite the limitations of the current study, this preliminary foray into improving our abilities at predicting neonatal recovery from hypoxia induced damage associated with prematurity helps pave the way into a line of inquiry that has the potential to revolutionize neonatal medicine

SNP	PCR Primer (5' → 3')	Amplicon size (bp)	SNP Sequence	SNP Position	MAF
BDNF (rs6265)	Forward: GCGCAAACATCCGAGGACAA†§ Reverse: GCTACTGAGCATCACCCTGG† Reverse: GCCTTCATTGGGCCGAACTTTCTGGT§	223	CGAACAC[A/G]TGATAGA	196	0.229
SDF-1 (rs1801157)	Forward: GCTGATGAGCAGAACGTGGAGGATGT†§ Reverse: GCCAGGCATTGCCAAGGGCTTTGTTT†§	543	GCAGACC[C/T]GGCTCCC	801	0.207

Table A. Single Nucleotide Polymorphism location and primers. MAF Minimal Allele Frequency.
† Primer used in PCR amplification reaction.
§ Primer used in gene sequencing reaction.

	Presence of BDNF G to A SNP		*P*
	G (n=43)	A (n=10)	
Maternal demographic and clinical characteristics at enrollment (amniocentesis)			
Maternal Age, years†	27 [23-33]	31 [23.75-33.25	0.459
Gravidity†	3 [2-4]	3 [2-3.25]	0.898
Parity†	1 [0-2]	1 [0.75-2]	0.426
Race‡			0.876
Caucasian	14 (32)	4 (40)	
African American	19 (44)	3 (30)	
Hispanic	7 (16)	2 (20)	
Other	3 (7)	1 (10)	
Non-Causasian race§	29 (67)	6 (60)	0.719
Ruptured Membranes§	30 (70)	6 (60)	0.709
Clinical chorioamnionitis§	3 (7)	1 (10)	0.579
Antenatal Drug treatments/exposure			
Steroids§	42 (98)	10 (100)	1.000
Antibiotics§	40 (93)	8 (80)	0.235
Tocolysis§	20 (47)	3 (30)	0.484
Magnesium sulfate§	16 (37)	1 (10)	0.140
Progesterone during pregnancy§	9 (21)	4 (40)	0.237
Pregnancy outcome characteristics			
Indicated preterm delivery§	31 (72)	4 (40)	0.071
Gestational age at delivery, *weeks*†	26.2 [25.1-30]	30.15 [27.43-32.7]	0.065*
Birthweight, *grams*†	930 [780-1450]	1460 [1114-1800]	0.099*
Apgar score at 1 minute†	6 [4-8]	6.5 [4.75-8.25]	0.646
Apgar score at 5 minutes†	8 [7-9]	8 [8-9]	0.756
1 min Apgar <7§	22 (51)	5 (50)	1.000
5 min Apgar <7§	8 (19)	1 (10)	1.000
Newborn male gender§	19 (45)	3 (30)	0.488

Table B. Pre-natal maternal and peri-natal infant characteristics. Data analyzed with respect to presence of either the G- or the A-allele in the BDNF gene amplified from venous cord blood.

† Data analyzed by Mann Whitney Rank Sum test and presented as median [interquartile range].
‡ Data analyzed by Chi Square Analysis and presented as n (%).
§ Data analyzed by Fischer's exact tests and presented as n (%).
* Data trending towards significance ($p < 0.10$).

	Presence of BDNF G to A SNP		*P*
	G (n=43)	A (n=10)	
Umbilical cord blood analysis			
Arterial pH†	7.32 [7.29-7.35]	7.30 [7.28-7.31]	0.096*
Arterial base deficit†	4.55 [3.05-6.08	4.8 [3.35-6.80]	0.747
Venous pH†	7.38 [7.34-7.40]	7.35 [7.33-7.39]	0.159
Venous base deficit†	3.9 [2.1-7.25]	3.5 [1.9-8.0]	1.000
Placental histological analysis			
Chorionic plate inflammation, *stages II-III*§	33 (79)	5 (50)	0.109
Amnionitis, *stages 2-4*§	25 (60)	4 (40)	0.307
Choriodeciduitis, *stages 2-4*§	36 (86)	7 (70)	0.349
Funisitis, *stages 1-4*§	22 (52)	5 (50)	1.000
Neonatal Complications			
SDF-1 T-allele§	9 (21)	4 (40)	0.237
Innterventricular Hemorrhage§	10 (24)	1 (11)	0.663
Brochopulmonary Dysplasia§	7 (18)	1 (10)	1.000
Necrotizing Enterocolitis§	5 (12)	1 (10)	1.000
Retinopathy of Prematurity§	15 (36)	2 (22)	0.699
Periventricular Leukomalacia§	4 (10)	1 (11)	1.000
Presumed EONS§	28 (65)	6 (60)	1.000
Culture positive (confirmed) EONS§	3 (7)	3 (30)	0.077*
Clinical EONS§	14 (33)	5 (50)	0.467
Death§	4 (10)	0 (0)	0.576
Any Adverse Outcome§	17 (40)	3 (30)	0.722

Table C. Post-natal clinical characteristics of the newborn infants. Data analyzed with respect to presence of either the G- or the A-allele in the BDNF gene amplified from venous cord blood.

† Data analyzed by Mann Whitney Rank Sum tests and presented as median [interquartile range].
§ Data analyzed by Fischer's exact tests and presented as n (%).
* Data trending towards significance ($p < 0.10$).

	Presence of SDF-1 G to A SNP		*P*
	G (n=40)	A (n=13)	
Maternal demographic and clinical characteristics at enrollment (amniocentesis)			
Maternal Age, years†	29.5 [23.25-34]	25 [22.5-31.5]	0.246
Gravidity†	3 [2-4]	2 [1.5-3.5]	0.440
Parity†	1 [0-2]	1 [0-2]	0.596
Race‡			0.165
Caucasian	11 (27.5)	7 (53.8)	
African American	19 (47.5)	3 (23.1)	
Hispanic	6 (15)	3 (23)	
Other	4 (10)	0 (0)	
Non-Causasian race§	29 (72.5)	6 (46.15)	0.101
Ruptured Membranes§	28 (70)	8 (61.5)	0.734
Clinical chorioamnionitis§	3 (7.5)	1 (7.7)	1.000
Antenatal Drug treatments/exposure			
Steroids§	40 (100)	12 (92.3)	0.245
Antibiotics§	37 (92.5)	11 (84.6)	0.586
Tocolysis§	16 (40)	7 (53.8)	0.522
Magnesium sulfate§	12 (30)	5 (38.5)	0.734
Progesterone during pregnancy§	10 (25)	3 (23.1)	1.000
Pregnancy outcome characteristics			
Indicated preterm delivery§	26 (65)	9 (69.2)	1.000
Gestational age at delivery, *weeks*†	26.8 [25.3-30.2]	26.2 [25.1-33.2]	0.764
Birthweight, *grams*†	1025 [785-1487.5]	920 [805-2000]	0.577
Apgar score at 1 minute†	6 [4-8]	8 [3.5-8.5]	0.699
Apgar score at 5 minutes†	8 [7-9]	8 [6.5-9]	0.811
1 min Apgar <7§	21 (52.5)	6 (46.2)	0.757
5 min Apgar <7§	6 (15)	3 (23)	0.672
Newborn male gender§	17 (42.5)	5 (41.7)	1.000

Table D. Pre-natal maternal and peri-natal infant characteristics. Data analyzed with respect to presence of either the G- or the A-allele in the SDF-1 gene amplified from venous cord blood.

† Data analyzed by Mann Whitney Rank Sum test and presented as median [interquartile range].
‡ Data analyzed by Chi Square Analysis and presented as n (%).
§ Data analyzed by Fischer's exact tests and presented as n (%).

	Presence of SDF-1 G to A SNP		*P*
	G (n=40)	A (n=13)	
Umbilical cord blood analysis			
Arterial pH†	7.315 [7.293-7.350]	7.31 [7.285-7.327]	0.449
Arterial base deficit†	4.2 [3.05-6.05]	5.1 [3.325-6.825]	0.683
Venous pH†	7.365 [7.338-7.40]	7.380 [7.367-7.39]	0.406
Venous base deficit†	3.9 [1.9-7.8]	3.45 [2.3-5.25]	0.688
Placental histological analysis			
Chorionic plate inflammation, *stages II-III*§	31 (79.5)	7 (53.8)	0.086*
Amnionitis, *stages 2-4*§	24 (61.5)	5 (38.5)	0.201
Choriodeciduitis, *stages 2-4*§	35 (89.7)	8 (61.5)	0.033**
Funisitis, *stages 1-4*§	22 (56.4)	5 (38.5)	0.343
Neonatal Complications			
BDNF A-Allele§	6 (15)	4 (30.8)	0.237
Innterventricular Hemorrhage§	9 (22.5)	2 (18.2)	1.000
Brochopulmonary Dysplasia§	5 (13.5)	3 (27.3)	0.361
Necrotizing Enterocolitis§	5 (12.5)	1 (8.3)	1.000
Retinopathy of Prematurity§	12 (30)	5 (45.5)	0.472
Periventricular Leukomalacia§	4 (10)	1 (9.1)	1.000
Presumed EONS§	26 (65)	8 (61.5)	1.000
Culture positive (confirmed) EONS§	6 (15)	0 (0)	0.316
Clinical EONS§	18 (45)	1 (8.3)	0.037**
Death§	3 (7.5)	1 (8.3)	1.000
Any Adverse Outcome§	14 (35)	6 (50)	0.500

Table E. Post-natal clinical characteristics of the newborn infants. Data analyzed with respect to presence of either the G- or the A-allele in the SDF-1 gene amplified from venous cord blood.

† Data analyzed by Mann Whitney Rank Sum tests and presented as median [interquartile range].
§ Data analyzed by Fischer's exact tests and presented as n (%).
* Data trending towards significance ($p < 0.10$).
** Data statistically significance ($p < 0.05$).

	Presence of Complication		*P*
	YES	NO	
Placental histological analysis			
Chorionic plate inflammation, stages II-III‡	112.06 ± 16.62	145.08 ± 20.94	0.192
Amnionitis, stages 2-4‡	92.54 ± 15.95	154.92 ± 21.41	0.014**
Choriodeciduitis, stages 2-4‡	114.50 ± 15.75	140.63 ± 33.65	0.271
Funisitis, stages 1-4‡	93.83 ± 16.14	147.14 ± 21.98	0.030**
Neonatal Complications			
Presence of G196A SNP‡	116.31 ± 15.62	110.23 ± 25.19	0.443
Innterventricular Hemorrhage‡	109.46 ± 29.56	118.75 ± 15.0	0.379
Brochopulmonary Dysplasia‡	69.2 ± 8.45	114.36 ± 14.89	0.079*
Necrotizing Enterocolitis‡	74.48 ± 8.88	124.16 ± 16	0.089*
Retinopathy of Prematurity‡	108.83 ± 16.38	122.82 ± 23.38	0.312
Periventricular Leukomalacia‡	149.8 ± 54.08	108.3 ± 12.78	0.132
Presumed EONS‡	104.29 ± 17.05	147.33 ± 17.7	0.089*
Culture positive (confirmed) EONS‡	118.16 ± 45.13	114.78 ± 13.51	0.461
Clinical EONS‡	95.66 ± 19.78	141.33 ± 16.28	0.051*
Death‡	183.43 ± 57.93	105.33 ± 12.63	0.027**
Any Adverse Outcome‡	120.58 ±18.16	107.64 ± 22.27	0.329

Table F. BDNF protein expression levels. Average protein levels given with respect to presence or absence of complication.

‡ Data analyzed by 1-tailed student t-tests and presented as mean BDNF ± SEM.
* Data trending towards significance ($p < 0.10$).
** Data statistically significance ($p < 0.05$).

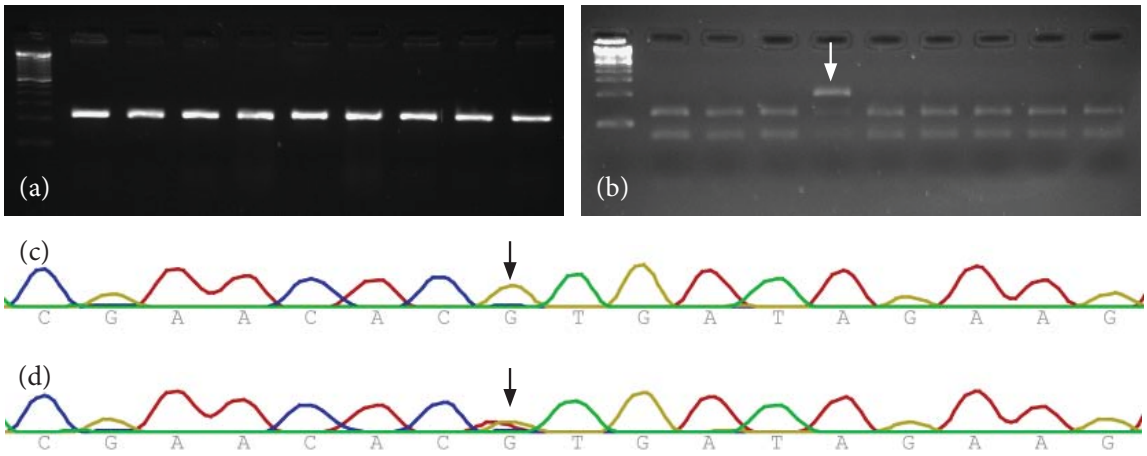

Figure 1. Identifcation of G196A SNP in the BDNF gene. (a) Representative gel demonstrating BDNF PCR product. (b) Representative gel demonstrating cleavage products following digestion of PCR product by AflIII and PmlI restriction endonucleases. Sample heterozygous for the A-allele is marked with an arrow. (c) Representative trace sequence of BDNF gene homozygous for the G-allele at position 196 (arrow). (d) Representative trace sequence of the BDNF gene heterozygous for the A-allele at position 196 (arrow).

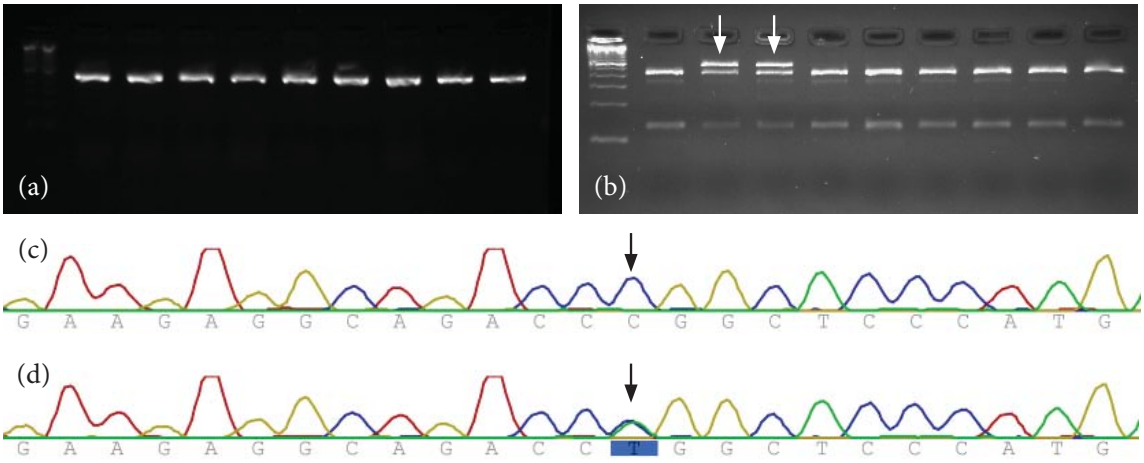

Figure 2. Identifcation of G801A SNP in the SDF-1 gene. (a) Representative gel demonstrating SDF-1 PCR product. (b) Representative gel demonstrating cleavage products following digestion of PCR product by NciI restriction endonuclease. Samples heterozygous for the A-allele are marked with arrows. (c) Representative trace sequence of SDF-1 gene homozygous for the G-allele at position 801 (arrow). (d) Representative trace sequence of the SDF-1 gene heterozygous for the A-allele at position 801 (arrow).

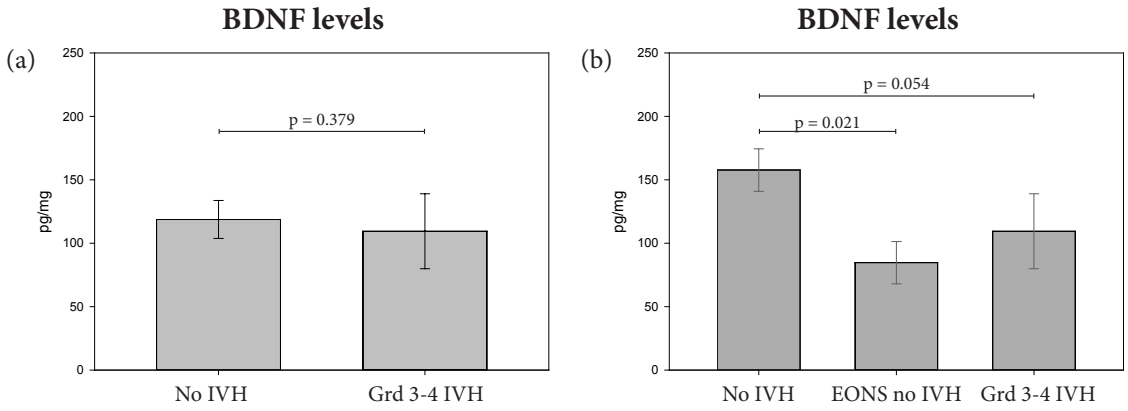

Figure 3. Average BDNF expression levels with respect to presence or absence of grade 3 or 4 intraventricular hemorrhage (IVH). (a) BDNF levels in patients seperated into two groups, without regard for presence or absence of other co-morbidities. (b) BDNF levels in patients seperated into three groups, demonstrating the development of early onset neonatal sepsis (EONS) as a possible confounder for analyzing the relationship between BDNF expression levels and development of IVH (vertical bars represent standard error).

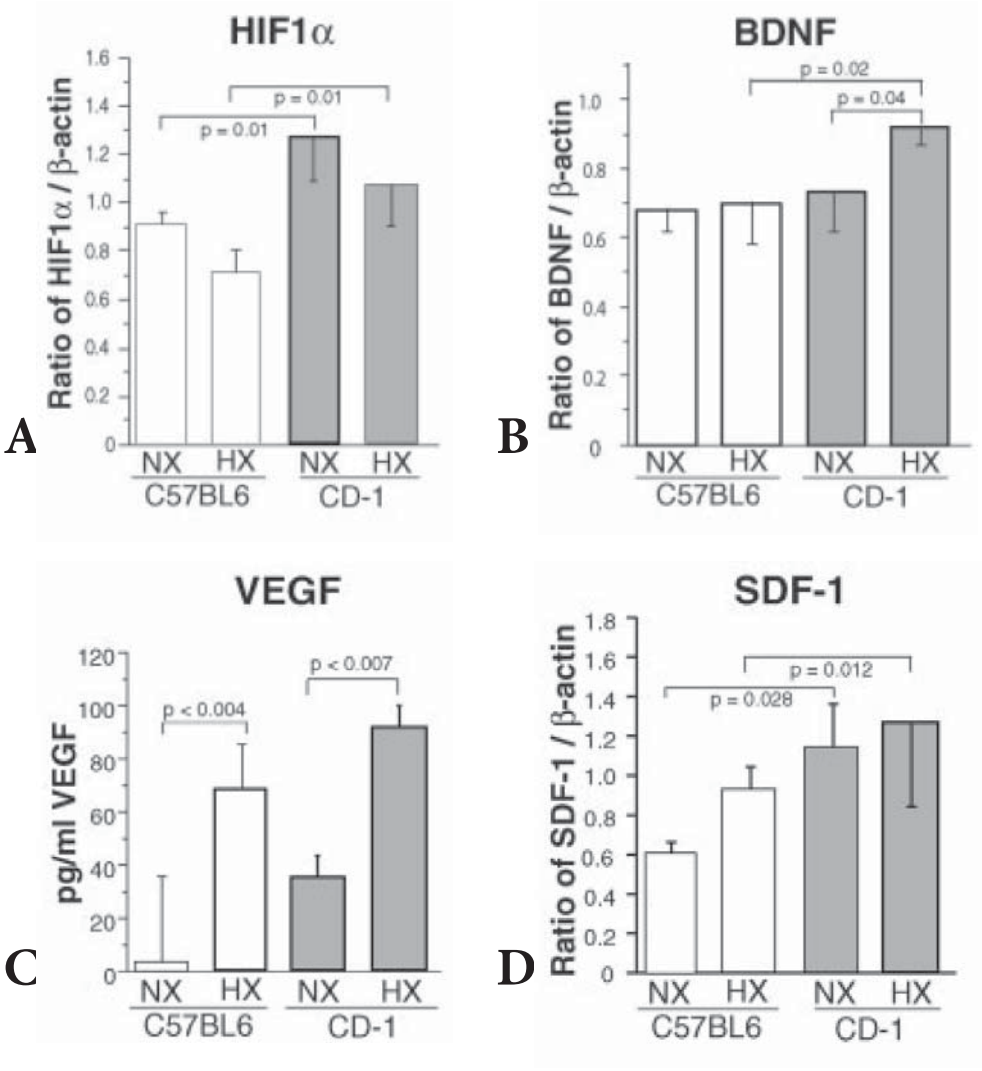

Figure 4. Adapted with permission from [9]. P11 C57BL/6 brain homogenates exhibit distinct normoxic and hypoxic selected transcription factor and growth factor expression levels compared with CD-1 pups. Analysis of expression levels of (A) HIF1α, (B) BDNF, (C) VEGF, and (D) SDF-1 in C57BL/6 (open boxes) and CD-1 (shaded boxes) brain homogenates under normoxic (NX) and hypoxic (HX) conditions (vertical bars represent standard deviations; n = 3).

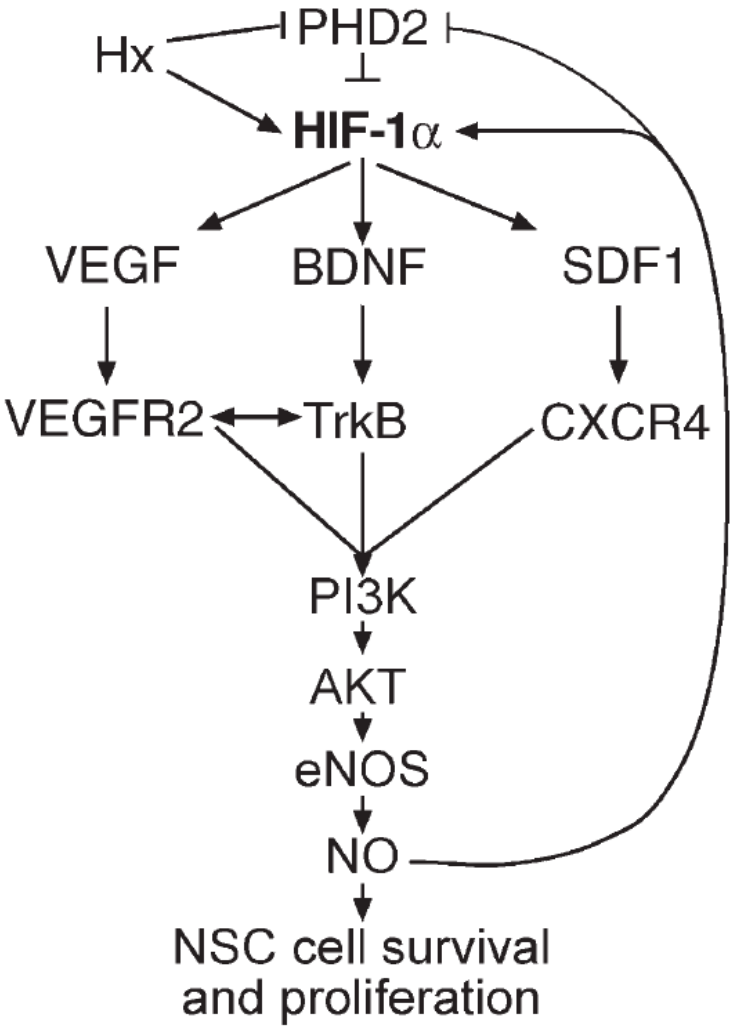

Figure 5. Reproduced with permission from [9]. Working model of the signaling pathway components that are differentially regulated in C57BL/6 and CD-1 pup brain tissues and cultured NPCs under normoxic and hypoxic conditions.

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