Long-Lasting Changes in DNA Methylation Following Short-Term Hypoxic Exposure in Primary Hippocampal Neuronal Cultures

Iain Hartley1*, Fuad F. Elkhoury1*, Joo Heon Shin2, Bin Xie2, Xiangqun Gu1, Yuan Gao2, Dan Zhou1, Gabriel G. Haddad1,3,4*

1 Division of Respiratory Medicine, Department of Pediatrics and Biomedical Sciences Graduate Program, University of California San Diego, La Jolla, California, United States of America, 2 Division of Genomics, Epigenomics and Bioinformatics, Lieber Institute for Brain Development, Baltimore, Maryland, United States of America, 3 Department of Neurosciences, University of California San Diego, La Jolla, California, United States of America, 4 Rady Children’s Hospital, San Diego, California, United States of America

Abstract

While the effects of hypoxia on gene expression have been investigated in the CNS to some extent, we currently do not know what role epigenetics plays in the transcription of many genes during such hypoxic stress. To start understanding the role of epigenetic changes during hypoxia, we investigated the long-term effect of hypoxia on gene expression and DNA methylation in hippocampal neuronal cells. Primary murine hippocampal neuronal cells were cultured for 7 days. Hypoxic stress of 1% O2, 5% CO2 for 24 hours was applied on Day 3, conditions we found to maximize cellular hypoxic stress response without inducing cell death. Cells were returned to normoxia for 4 days following the period of hypoxic stress. On Day 7, Methyl-Sensitive Cut Counting (MSCC) was used to identify a genome-wide methylation profile of the hippocampal cell lines to assess methylation changes resulting from hypoxia. RNA-Seq was also done on Day 7 to analyze changes in gene transcription. Phenotypic analysis showed that neuronal processes were significantly shorter after 1 day of hypoxia, but there was a catch-up growth of these processes after return to normoxia. Transcriptome profiling using RNA-Seq revealed 369 differentially expressed genes with 225 being upregulated, many of which form networks shown to affect CNS development and function. Importantly, the expression level of 59 genes could be correlated to the changes in DNA methylation in their promoter regions. CpG islands, in particular, had a strong tendency to remain hypomethylated long after hypoxic stress was removed. From this study, we conclude that short-term, sub-lethal hypoxia results in long-lasting changes to genome wide DNA methylation status and that some of these changes can be highly correlated with transcriptional modulation in a number of genes involved in functional pathways that have been previously implicated in neural growth and development.

Introduction

During early mammalian development, oxygen plays a vital role in the growth and maturation of every organ system. The central nervous system is particularly dependent on oxygen for proper development and function. Hence, a hypoxic insult during development may cause significant cognitive and behavioral impediments at a later stage in life. For example, in the United States, perinatal asphyxia has an incidence of up to 8 per 1000 live births with results ranging from mild neurological difficulties to severe neonatal encephalopathy, including cerebral palsy [1]. Interestingly, these neurological diseases are not always easily correlated to hypoxia-induced neuronal injury [2]. This raises the question of how such temporary hypoxia influences long-term function of cells still viable after hypoxic stress.

While the detrimental effect of short-term hypoxia on neurological development has been phenotypically characterized, the underlying mechanisms responsible for the deleterious effects continue to be elucidated. Furthermore, although it is possible that severe hypoxia in early life can cause cell death, it is also possible that hypoxia can induce
Changes in DNA Methylation after Hypoxic Exposure

sub-lethal biochemical changes, including long-lasting alterations in gene expression. It has been shown that hypoxia activates certain genes necessary for cellular survival and adaptation in hypoxic conditions as well as genes for inducing cell injury [3-5]. For example, acute modest hypoxia, in particular that lasting for minutes or hours, is known to upregulate genes coding for neuroprotective endoplasmic reticulum proteins, proteins involved in ubiquitinylation, and those involved in hypoxic neuronal death[4,6]. Concordantly, genes involved in cellular protection, neurogenesis, and neuronal regeneration were strongly repressed following such a stress [7-9]. Whether these gene expression changes persist many days after the short-term hypoxic stress has yet to be determined.

Epigenetic mechanisms, including DNA methylation and histone modification, result in long-term changes in gene expression [10]. Epigenetic changes are central in controlling longer term effects of stresses such as in hypoxia-mediated gene expression [11,12]. How acute hypoxic stress influences gene expression through modifications of DNA methylation and histone acetylation has been studied in the context of tumor suppressor genes and cancer progression [13]. Other research has demonstrated that intermittent hypoxia may initiate epigenetic changes leading to long-lasting increases in persistent oxidative stress and manifestations of cardiovascular disease in adult rats [14]. However, the enduring effects and the extent of short-term hypoxia on methylation and subsequent gene expression in the central nervous system have not been explored. Thus, in this present study, we investigate the long-lasting impact of acute sub-lethal hypoxic stress on gene expression in hippocampal neuronal cells and hypoxia-induced changes in DNA methylation.

Materials & Methods

Ethics Statement
This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. The mice were anaesthetized with Isoflurane by inhalation and sacrificed. All efforts were made to minimize suffering.

Primary Hippocampal Cell Culture
Time-pregnant female mice were obtained from Charles River (CD-1 strain). Dissection and culturing protocol was adapted from Fath et. al., 2008 [15]. Briefly, hippocampi were carefully dissected from E16.5 mouse embryo brain. The neuronal cells were dissociated with trypsin (Gibco) digestion. The dissociated cells were re-suspended in neurobasal medium (Gibco) (B27 1× final, Gibco) containing 2mM GlutaMAX. Cells were diluted to 106 cells/ml. 3 milliliters (about 300 cells) of cell suspension were added to each 35-mm poly-d-lysine coated plate and cultured for 2 hours in 5% CO2 at 37°C. The plating medium was then replaced with 3 ml of neurobasal/B27 medium, and the culture was continued in 5% CO2 and 21% O2. The primarily cultured cells were allowed to grow for 48 hours before treatment.

The morphology of all cultured cells was assessed with microscopy, and cell death was evaluated by trypan blue exclusion assay. Images were acquired with a Zeiss microscope with AxioCam MRm camera using the Axiosvision Rel 4.5 software, which was also used to determine the dendrite length and cell area before and after hypoxia treatment.

Experimental Design
The primary neuronal cells were cultured in room air condition for 48 hours, and then the cultured cells were treated with 1% O2 for 24 hours and returned to room air condition for continuing culture for another 4 days. Cells were harvested at this time point. Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) to survey CpG methylation using MSCC assay. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Maryland) for RNA-Seq based transcriptome profiling.

The duration and severity of hypoxia was determined prior to the experiment by exposure of different hippocampal cell cultures to varying levels of oxygen during cell incubation. After 48 hours of growing at room air condition with 21% O2 and 5% O2, the cells were transferred to hypoxia chambers and treated with 1%, 2%, or 3% O2 for 24 or 48 hours, respectively. Cell death and morphological changes were assayed immediately after each treatment with trypan blue exclusion assay and microscope imaging. In addition, cellular hypoxic response was monitored by measuring transcriptional changes of hypoxia responding genes (i.e., Vegf and Epo) using quantitative real-time PCR.

Sequenced reads for both RNA-Seq and MSCC assays can be accessed via the NCBI Sequence Read Archive under accession number SRP028757.

Genomic wide CpG methylation profiling with Methyl-Sensitive Cut Counting (MSCC)
Genome-wide cysteine methylation profiling was carried out using methyl sensitive cut counting (MSCC) assay as previously described [16,17]. Briefly, the sequencing libraries were generated using 0.65μg of genomic DNA for each library. A methylation sensitive library and an inverse methylation insensitive library were generated for each sample. The methylation sensitive and inverse insensitive libraries were each digested with HpaII and MspI (NEB, Ipswich, MA). An internal CpG methylation standard was created by mixing methylated and unmethylated PCR products of E. coli DNA and included in each library generation to represent 0% methylation, 33% methylated, 67% methylated and 100% methylated CpG sites. The CpG tag libraries were sequenced using Illumina Genome Analyzer IIX and returned ~1 GB of sequence partitioned into 36-nt long reads per library. The resulting sequence files were mapped to the Mus musculus reference genome (version mm9), and the data were analyzed by creating frequency histograms showing the number of times reads identified at any given CpG site. The CpG sites with at least 30 reads for both methylation sensitive and insensitive
libraries combined were included for further analyses to minimize technical variations in each sample, a list of which may be found in Table S1.

Transcriptome profiling with RNA-Seq

Each RNA-seq library was generated using 200ng of rRNA-depleted RNA with the Illumina®-compatible ScriptSeq™ mRNA-Seq Library Preparation Kit (Epicentre Biotechnologies, Madison, WI) according to manufacturer’s instruction with 200-250bp insertion fragments. The quality of the libraries was checked on the Bioanalyzer using a High Sensitivity DNA Chip (Agilent, Waldbronn, Germany) and quantified using the Illumina Genome Analyzer DNA library quantification kit (Kapa Biosystems, Woburn, MA). Two libraries for each condition (control and hypoxia-treated) were sequenced using a 2x36 cycle paired-end run on Illumina Genome Analyzer IIx. The resulting reads were mapped using the RUM alignment package [18] with default settings to the mouse reference genome mm9. The aligned reads were then processed with htsq-count to count the number of reads mapped to genes defined by Illumina’s iGenome GTF annotation for mm9. The differently expressed genes were determined by DeSeq [19] wherein variation in the raw read counts was estimated using a technique based on the negative binomial distribution. Results produced by DeSeq were filtered to select genes with a p-value less than 0.05 and an absolute fold change greater than 1.5, a list of which may be found in Table S2.

Association between changes of CpG methylation in the promoter regions and gene expression

The promoter region of a gene was defined as 1500bp upstream to 500bp downstream of the gene’s transcription starting site (TSS). The differentially expressed genes and the changes in CpG methylation that were identified with aforementioned filtering criteria were merged to determine the association between CpG methylation and gene expression. Further filtering was imposed to exclude all the genes with promoter regions containing less than two CpG sites measured to reduce the incidence of noise. The promoter regions of the remaining genes were profiled to determine the direction of methylation change at each site, whether positive, indicating hyper-methylation relative to control, or negative, indicating hypo-methylation relative to control.

Results

We first present the morphologic changes that occur with hypoxia; we then present the effect of hypoxia on gene expression, and finally the DNA methylation results.

The hypoxic condition of 1% O2 for 24 hours was found, after a number of trials at various concentrations and durations, to induce a cellular stress response with no or very little cell death (see Methods). It was found that after 24 hours of hypoxic stress, the neuronal dendritic lengths were significantly shorter than the dendritic lengths of the control cells (Hypoxic cells: 54.57 +/-7.78 μm; Normoxic Cells: 74.92 +/-6.85 μm) (Figure 1a,b). However, when the cell cultures initially exposed to hypoxia were later subjected to a normal oxygen environment for 4 days, their dendritic lengths grew rapidly to catch up with the dendritic lengths of the control cells (Hypoxic cells: 72.20 +/-18.18 μm; Normoxic cells: 76.52 +/-15.00 μm).

Comparing cell body area between the hypoxic and control cells revealed no difference between these two cell populations, both immediately (1 day after exposure to hypoxia) and after 4 days in normoxia (Figure 1b).

We next investigated the effects of such short-term hypoxia at the transcriptional level. RNA-Seq revealed that 369 genes were differentially expressed (>1.5-fold change) in the hypoxic cells 4 days after hypoxic exposure was removed compared to control cells. Of these 369 genes, 225 genes were upregulated and 144 genes were downregulated (Figure 2).

Analysis of the global methylation profile of all observed CpG sites with greater than 30 reads reveals that out of 268,479 reads (Figure 3a), there is an almost even split between hyper- and hypo-methylated sites with 147,052 exhibiting hypermethylation and the remaining 121,427 displaying hypomethylation. However, when setting a cutoff to exclude all sites with less than 20% methylation change, 65,653 sites were observed to be hypermethylated and about half of these (30,412 sites) were hypomethylated in hypoxic cells compared to control. Additionally, when changes within gene promoter regions were analyzed, we found that more sites within these regions were hypomethylated following hypoxia with 17,675 of 23,552 observed in gene promoters displaying hypomethylation (Figure 3b). The level of methylation within CpG islands throughout the genome also showed a greater level of hypomethylation in the hypoxic cells compared to control with 50,686 out of 57,976 observed sites displaying hypomethylation (Figure 3c). Importantly, when comparing the data within Figure 3(a-c) it is evident that the majority of hypomethylated sites are localized in gene promoter regions and CpG islands, both regions shown to have significant regulatory impact [20-24]. In contrast, while there are more hypermethylated sites observed to have substantial changes, there appears to be no localization to well-known regulatory regions (Figure 3d).

When we determined the proximity of the methylated CpG sites to the transcription start sites (TSS) of the differentially expressed genes, it was clear that long-term methylation changes due to hypoxia occur predominantly near the transcription start site (Figure 4). Additionally, of the CpG islands undergoing methylation change within 1500bp of a gene TSS, 44,625 of 50,842 observed sites were hypomethylated following acute hypoxic stress.

We next determined whether the changes in DNA methylation correlated with the changes in gene expression, as expected. It is known that a decrease in DNA methylation in the promoter region is correlated with an up-regulation of the related gene, while an increase in DNA methylation induces the opposite [25,26]. Analysis of the methylation and gene expression changes revealed that 59 genes and their respective changes in DNA methylation followed the aforementioned expected paradigm. More specifically, 53 genes were up-regulated and had hypomethylated promoter regions, and 6 genes were downregulated with
Figure 1. Dendrite growth blunted following 24 hour hypoxic exposure mounts recovery following return to room air. (a) Microscopy of cell cultures immediately following removal of hypoxic sample from the hypoxic incubator in addition to 4 days after return to room air. (b) Quantification of dendritic length and cell area changes both immediately after 24 hours of 1% O2 hypoxic stress (Culture Day 3) and 4 days post return to room air (Culture Day 7).

doi: 10.1371/journal.pone.0077859.g001
hypermethylated promoter regions (using >90% hypermethylated or hypomethylated promoters compared to control as the threshold) (Figure 5). However, there were an additional 22 genes that exhibited the opposite of what was expected, with 12 genes showing up-regulation coupled with hyper-methylated promoters and 10 displaying the down-regulation and hypo-methylated promoters. Pathway analysis of the 59 genes revealed that the top two functional groups characterized contribute to neuronal growth, neurological development, and developmental disorders (p<0.01). Additionally, 4 of the 59 genes with expected correlation between transcript regulation and promoter methylation were found to be involved in the Wnt signaling pathway with the use of the DAVID functional analysis tool (p=1.8e-2) and 3 of the 59

Figure 2. Number of genes with significant modification in expression as determined by RNASeq analysis. RNA isolated from post-hypoxic and control cell cultures was sequenced, the sequence was aligned and the alignment quantified as described in the methods. Each range of fold-change values includes only genes with a p-value less than 0.05.
doi: 10.1371/journal.pone.0077859.g002
were found to be involved in the mTOR signaling pathway (p=1.8e-2), both of which are implicated in regulation of cell growth.

Discussion

We have made several major observations in this study. First, short-term acute sublethal hypoxia caused inhibition of
process and dendritic growth of hippocampal neurons, and after return to normal oxygen conditions, cells exhibited a rapid recovery of this growth. Second, this acute hypoxic exposure caused long-term differential expression of 369 genes, of which 225 genes were up-regulated. Third, 59 of the 369 differentially expressed genes displayed modified promoter methylation consistent with the previously observed relation, namely, that decreased promoter methylation correlated with increased RNA transcription and increased promoter methylation correlated to a decrease in transcription. Finally, a whole genomic view of methylation revealed a strong tendency for CpG islands to remain hypomethylated long term after the hypoxic stress was removed. Indeed, short-term, sub-lethal hypoxia results in long-lasting gene expression changes in addition to long lasting and substantial changes to DNA methylation.

Analysis of hippocampal cells exposed to hypoxia revealed blunted growth during stress but accelerated growth following return to normoxia. Previously, other studies have shown that hypoxia indeed suppresses genes involved in neuronal maturation and growth [27,28] but the subsequent phase of catch-up growth observed in this study has never been...
described in the literature. Short-term hypoxia has been shown to activate genes responsible for hypoxia-induced protection against brain ischemia, though the mechanism through which such hypoxia results in long-lasting tolerance to future hypoxic stress has yet to be elucidated [29-32]. Our results show that DNA methylation plays a lasting role in hypoxia-induced gene expression changes, though the exact nature of this regulatory action is still uncertain.

In this study, hypoxia resulted in more upregulated (225) than downregulated (144) genes. Also, a greater proportion of promoter and CpG islands were hypomethylated rather than hypermethylated, several days after hypoxic exposure. These findings indicate that there is a correlation between whole genome changes in methylation and gene expression, days after sub-lethal hypoxic stress. When investigating the site of methylation in the 59 genes, it was evident that methylation as an epigenetic mechanism was strongly responsible for the observed lasting changes in gene expression. While these 59 genes fit the widely-accepted paradigm of the influence of methylation on gene expression, the majority of genes that had altered expression either did not have observed methylation changes within their promoter or displayed methylation modification that were contrary to the expected paradigm. This suggests that, while methylation plays a potential role in gene regulation, it is not the only mediator, and there is still much to

Figure 5. Fold change of differentially expressed genes relative to promoter methylation profile. Each point represents one gene with significant change in expression (p < 0.05) in addition to having at least two measured methylation sites within its promoter region. Vertical axis is log2(fold change), horizontal axis is the number of sites displaying hyper-methylation, in the hypoxic sample relative to control, within the promoter region divided by the total number of sites measured expressed as a percentage. Genes exhibiting increased expression with decreased promoter methylation or decreased expression coupled with increased promoter methylation conform to previously described methylation mediated expression regulation while all other genes presumably are regulated by additional epigenetic factors.

doi: 10.1371/journal.pone.0077859.g005

Changes in DNA Methylation after Hypoxic Exposure
be discerned regarding the role of methylation in gene expression regulation. 

Transcription regulatory mechanisms are diverse and influenced by various environmental and chemical conditions. Histone acetyltransferases and deacetylases have been found to have a role in HIF-1-dependent transcriptional induction [33-37]. Hypoxia-related microRNAs have also surfaced as important in processes such as apoptosis and proliferation [38,39]. Ostensibly, epigenetic regulation of gene expression is a uni-factorial mechanism influencing transcription. While one epigenetic mechanism may play a dominant role in regulating gene expression, there are many other epigenetic influences that may impact transcription. We found that variable expression of 59 genes was predominantly impacted by DNA methylation changes due to sublethal hypoxia. However, there were many other genes whose expression changes are not solely explained by methylation changes. Other epigenetic mechanisms are likely involved in these cases.

Of the 59 genes predominantly impacted by DNA methylation, 4 of them that displayed upregulation of RNA and demethylated promoter regions (APC, CTNNB1, CCND2, PLCB1) were found to be involved in the Wnt signaling pathway. APC and CTNNBP1 act as negative regulators of the canonical pathway through disruption of β-catenin nuclear localization [40,41]. PLCB1 interacts with the Ca2+ pathway and is involved in the development of normal cortical circuitry; mice without PLCB1 display changes in synaptic and dendritic morphology [42]. CCND2 encodes the Cyclin D2 protein and is involved in cell cycle regulation. Furthermore, Wnt is involved in the up-regulation of neuronal stem cell growth through HIF-1α signaling [43].

We found that RICTOR, a subunit of the mTORC2 complex in the mTOR pathway, was found to have undergone both transcriptional up-regulation and substantial promoter demethylation in the culture subjected to hypoxia (Table 1). However, Igf1, which has been characterized as an upstream regulator of the mTOR pathway, was found to be down-regulated with increased promoter methylation. While the functional characteristics of mTORC2 have yet to be fully elucidated, loss of mTORC2 has been implicated in impairment of long term memory and long term potentiation and plays a role in cytoskeleton organization [44]. We hypothesize here that this up-regulation of mTORC2 is associated with the increased growth of dendrites with the return to room air after the hypoxic stress.

We conclude that acute sublethal hypoxia has a lasting impact on DNA methylation that persists days after normoxia is restored and that this modulation of methylation status, particularly in promoter regions and CpG islands, is correlated with the expression of neuronal genes involved in networks such as Wnt and mTOR that have been linked to roles regulating growth and development. The clinical significance of hypoxia to cell viability and functionality cannot be understated, and a deeper understanding of the mechanism of how hypoxia leads to changes in gene expression through epigenetic mechanisms will shed some light on the effect of hypoxia on

### Table 1. Genes with significant changes in expression in addition to having significant methylation changes in their associated promoter regions.

| Chr | Name   | Fold Change | Sites | % Methylated |
|-----|--------|-------------|-------|-------------|
| chr7 | Lira5  | 0.057094225 | 2     | 100.0000    |
| chr3 | Casq2  | 0.377452087 | 3     | 100.0000    |
| chr8 | Car14  | 0.397536108 | 2     | 100.0000    |
| chr17| Rps10  | 0.476757358 | 2     | 100.0000    |
| chr1 | Rab7f1 | 0.493967019 | 4     | 100.0000    |
| chr10| Igf1   | 0.546404576 | 2     | 100.0000    |
| chr11| Snf1   | 1.839627401 | 18    | 0.0000      |
| chr16| Dlg1   | 1.843121706 | 19    | 5.2632      |
| chr15| Ricor  | 1.850269418 | 31    | 9.6774      |
| chr10| Ppa1   | 1.856936562 | 17    | 5.8824      |
| chr10| Cs     | 1.871982757 | 13    | 0.0000      |
| chr11| Psmd12 | 1.872473838 | 7     | 0.0000      |
| chr1 | Dst    | 1.893206535 | 15    | 0.0000      |
| chr4 | Ctnnb1 | 1.901794587 | 20    | 5.0000      |
| chr19| Smc3   | 1.906543381 | 23    | 4.3478      |
| chr6 | Nafl   | 1.915112216 | 13    | 0.0000      |
| chr5 | Limch1 | 1.935521552 | 15    | 0.0000      |
| chr4 | Hspg2  | 1.948056828 | 15    | 6.6667      |
| chr17| Lclat1 | 1.980329242 | 15    | 0.0000      |
| chr4 | Sh3gl2 | 1.99214433  | 8     | 0.0000      |
| chr3 | Igsf3  | 2.016889197 | 22    | 0.0000      |
| chr6 | Aebp2  | 2.019027499 | 17    | 0.0000      |
| chr7 | Tsh23  | 2.030247358 | 48    | 4.1667      |
| chr14| Hsds1t | 2.04849538  | 37    | 0.0000      |
| chr2 | Mettl8 | 2.04718557  | 2     | 0.0000      |
| chr16| Elf4g1 | 2.084264604 | 32    | 3.1250      |
| chrX| Cdkt5  | 2.095452345 | 2     | 0.0000      |
| chr13| Dip2c  | 2.10943596  | 11    | 0.0000      |
| chr11| Ccdc88a| 2.116618436 | 18    | 5.5556      |
| chr1 | Ncna2  | 2.129957531 | 3     | 0.0000      |
| chr5 | Abnx2  | 2.171412892 | 35    | 5.7143      |
| chr2 | Tanc1  | 2.180687145 | 25    | 8.0000      |
| chr4 | E130308a19Rik | 2.198178177 | 25    | 4.0000      |
| chr17| Smcd1  | 2.209599962 | 3     | 0.0000      |
| chr9 | Peli   | 2.216518007 | 6     | 0.0000      |
| chr5 | Tc28b  | 2.221237478 | 13    | 0.0000      |
| chr5 | Lrcl8d | 2.246920303 | 31    | 3.2258      |
| chr18| Apc    | 2.24916978  | 17    | 0.0000      |
| chr2 | Pldc1  | 2.260103731 | 22    | 0.0000      |
| chr9 | Csgg4  | 2.272506555 | 4     | 0.0000      |
| chr2 | Rbgap1 | 2.280957971 | 12    | 8.3333      |
| chr12| Snp54a | 2.281532794 | 6     | 0.0000      |
| chr17| Ppg    | 2.289821387 | 23    | 4.3478      |
| chr16| Dopey2 | 2.345032587 | 17    | 5.8824      |
| chr3 | Fx1    | 2.37585388  | 26    | 3.8462      |
| chr12| Smoc1  | 2.416242757 | 18    | 5.5556      |
| chr2 | Cybrd1 | 2.475468964 | 14    | 0.0000      |
| chr11| Pldc3  | 2.565426619 | 3     | 0.0000      |
| chr12| Glrx5  | 2.611097466 | 26    | 7.6923      |
| chr5 | Pppl12 | 2.64924452  | 4     | 0.0000      |
| chr3 | Sec62  | 2.700480763 | 5     | 0.0000      |
| chr6 | Gse1   | 2.747996289 | 36    | 8.3333      |
gene expression and thus the phenotypic consequence of a stress like hypoxia.

Supporting Information

Table S1. Observed genome methylation status after filtering. A listing of CCGG sites observed with MSCC assay after being filtered for reads and percentage change as described in Methods.

| Chr | Name          | Fold Change | Sites | % Methylated |
|-----|---------------|-------------|-------|--------------|
| chr3| 2810046L04Rik | 2.79091895  | 11    | 0.0000       |
| chr18| Mbp         | 2.791553334 | 2     | 0.0000       |
| chr1 | Cab39        | 2.843285092 | 21    | 0.0000       |
| chr6 | Ccnd2        | 2.853125022 | 8     | 0.0000       |
| chr8 | 2310036O22Rik| 3.340649265 | 14    | 7.1429       |
| chr10| Ppp1r12a     | 3.800728332 | 28    | 3.5714       |
| chr4 | CK137956     | 3.848546303 | 4     | 0.0000       |

A final listing of genes with correlated changes to methylation and promoter methylation as described in Methods.

Note: Genes exhibiting both significant (p<0.05) and substantial (>1.5 fold change) change in expression in addition to more than methylation site observed within its promoter region with no more than 10% of observed sites differing in direction of methylation change. Genes with bold names are involved in the WNT or mTor pathway.

doi:10.1371/journal.pone.0077859.t001

Table S2. Genes with significant expression changes. A listing of genes with significant changes in expression based on RNASeq reads processed with the RUM aligner and DESeq differential expression analysis as described in Methods.

Acknowledgements

We wish to thank Orit Poulsen, Travis Smith, Yu-hsin Muller for their technical support.

Author Contributions

Conceived and designed the experiments: FFE DZ GGH. Performed the experiments: FFE XG ISH DZ JHS BX YG. Analyzed the data: ISH JHS BX YG. Contributed reagents/materials/analysis tools: JHS BX YG. Wrote the manuscript: ISH FFE DZ GGH.

References

1. Obstetricians ACo, Encephalopathy GTFoN, Palsy C, Pediatrics AAo (2003) Neonatal encephalopathy and cerebral palsy: defining the pathogenesis and pathophysiology. American College of Obstetricians and Gynecologists.
2. MacLennan A (1999) A template for defining a causal relation between acute intrapartum events and cerebral palsy: international consensus statement. BMJ 319: 1054-1059. doi:10.1136/bmj.319.7195.1054. PubMed: 10521205.
3. Jögi A, Øra I, Nilsson H, Lindeheim A, Makino Y et al. (2002) Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. Proc Natl Acad Sci USA 99: 7021-7026. doi:10.1073/pnas.102660199. PubMed: 12011461.
Changes in DNA Methylation after Hypoxic Exposure

4. Baggett, W.T., Cudworth, W.W., Gillespie, D.L., Jensen, R.L. (2007) Identification of hypoxia-induced genes in a malignant glioma cell line (U-251) by cDNA microarray analysis. Neurosurg Rev 30: 181-187; discussion: 10.1007/s10143-007-0070-z. PubMed: 17486380.

5. Ralph, G.S., Parham, S., Lee, S.R., Beard, G.L., Craigon, M.H. et al. (2004) Identification of potential stroke targets by lentiviral vector mediated overexpression of HIF-1 alpha and HIF-2 alpha in a primary neuronal model of hypoxia. J Cereb Blood Flow Metab 24: 245-258. PubMed: 14747751.

6. Jin, K., Mao, X.O., Estoh, M.W., Del Rio, G., Rao, R. et al. (2002) cDNA microarray analysis of changes in gene expression induced by hypoxia in vitro. Neurochem Res 27: 1105-1112. doi: 10.1023/A:1020913123054. PubMed: 12469360.

7. Amicarelli, F., Colafarina, S., Cattani, F., Cimini, A., Di Ilio, C. et al. (2003) Scavenging system efficiency is crucial for cell resistance to ROS-mediated mitochondrial injury. Free Radic Biol Med 35: 856-871. doi:10.1016/S0891-5849(03)00438-6. PubMed: 14556850.

8. Burgula, S., Medisetti, R., Jampanamadaka, N., Musturi, S., Ilavazhahan, G. et al. (2010) Downregulation of PEPBP1 in rat brain cortex in hypoxia. J Mol Neurosci 41: 36-47. doi:10.1007/s12031-009-9275-7. PubMed: 19705086.

9. Zhou, D., Wang, J., Zapala, M.A., Xue, J., Schork, N.J. et al. (2008) Gene expression in mouse brain following chronic hypoxia: role of sarscopin in glial cell death. Physiol Genomics 32: 370-379. PubMed: 18056785.

10. Feinberg AP (2007) Phenotypic plasticity and the epigenetics of human disease. Nature 443: 433-440. doi:10.1038/nature05199. PubMed: 17522677.

11. Okano, M., Bell, D.W., Haber, D.A., Li, E. (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99: 247-257. doi:10.1016/S0092-8674(00)81656-6. PubMed: 10555141.

12. Yoder, J.A., Soman, N.S., Verdin, G., Bestor, T.H. (1997) DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. J Mol Biol 270: 385-395. doi:10.1006/jmbi.1997.1125. PubMed: 9237905.

13. Liu, Y., Chu, A., Turker, M.S., Glazer, P.M. (2011) Hypoxia-induced epigenetic regulation and silencing of the BRCAl promoter. Mol Cell Biol 31: 3339-3350. doi:10.1128/MCB.01121-10. PubMed: 21670155.

14. Nanduri, M., Makarenko, V., Reddy, V.D., Gnan, P.A. et al. (2012) Epigenetic regulation of hypoxia sensing disrupts cardiorespiratory homeostasis. Proc Natl Acad Sci USA 109: 2515-2520. doi:10.1073/pnas.1105713109. PubMed: 21602498.

15. Fath, T., Ke, Y.D., Gunning, P., Götz, J., Ittner, L.M. (2009) Primary support cultures of hippocampal and substantia nigra neurons. Nat Protoc 4: 153-161. doi:10.1038/jcb.20133. PubMed: 15352172.

16. Arany, Z., Zhang, H., Ecker, R., Bhattacharya, S., Jiang, C. et al. (1996) An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci USA 93: 12969-12973. doi:10.1073/pnas.93.23.12969. PubMed: 8917528.

17. Carrero, P., Okamoto, K., Counailleau, P., O'Brien, S., Tanaka, H. et al. (2000) Redox-regulated recruitment of the transcriptional activators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha. Mol Cell Biol 20: 402-415. doi:10.1128/MCB.20.1.402-415.2000. PubMed: 10594042.

18. Ema, M., Hirota, K., Mimura, J., Abe, H., Yodoi, J. et al. (1999) Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J 18: 1905-1914. doi:10.1093/emboj/18.7.1905. PubMed: 10202154.

19. Ruas JL, Poellinger L, Pereira T (2002) Functional analysis of hypoxia-inducible factor-1 alpha-mediated transactivation. Identification of amino acid residues critical for transcriptional activation and/or interaction with CREB-binding protein. J Biol Chem 277: 38723-38730. doi:10.1074/jbc.M205051200. PubMed: 12133832.

20. Ruas JL, Poellinger L, Pereira T (2005) Role of CBP in regulating HIF-1-mediated activation of transcription. J Cell Sci 118: 301-311. doi:10.1242/jcs.01617. PubMed: 15617575.

21. Donker RB, Mulliett JF, Nelson DM, Sadowsky Y (2007) The expression of Argonaute2 and related microRNA biogenesis proteins in normal and hypoxic trophoblasts. Mol Hum Reprod 13: 273-279. doi:10.1093/molehr/gam006. PubMed: 17237265.

22. Hua, Z., Lv, Q., Ye, W., Wong, C.K., Cai, G. et al. (2006) miRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. PLOS ONE 1: e161. doi:10.1371/journal.pone.0001116. PubMed: 17205120.

23. Nakamura T, Hamada F, Isahata T, Anai K, Kawahara K et al. (1998) Axin, an inhibitor of the Wnt signalling pathway, interacts with beta-catenin, GSK-3beta and APC and reduces the beta-catenin level. Genes Cells 3: 395-403. doi:10.1046/j.1365-2443.1998.00118.x. PubMed: 9734785.

24. Sekiya, T., Nakamura, T., Kuzuyi, Y., Oshimura, M., Kuhu K et al. (2002) Overexpression of Icat induces G(2) arrest and cell death in tumor cell mutants for adenomatous polyposis coli, beta-catenin, or Axin. Cancer Res 62: 3322-3326. PubMed: 12036951.

25. Sjulmann T, Molnar Z, Martinelli K, Johnson RA, Compton PM, Upton AL et al. (2005) Activity-dependent regulation of synapse and dendritic spine morphology in developing barrel cortex requires phospholipase C-beta1 signalling. Cereb Cortex 15: 385-393. doi:10.1093/cercor/bhh141. PubMed: 15749982.

26. Mazumdar J, O'Brien WT, Johnson RS, LaManna JC, Chavez JC et al. (2010) O2 regulates stem cells through Wnt/[beta]-catenin signalling. Nat Cell Biol 12: 1007-1013. doi:10.1038/nclb2102. PubMed: 20852629.

27. Huang W, Zhu P, Zhang S, Zhou H, Staica L et al. (2013) mTORC2 controls actin polymerization required for consolidation of long-term
Changes in DNA Methylation after Hypoxic Exposure

memory. Nat Neurosci 16: 441-448. doi:10.1038/nn.3351. PubMed:
23455608.