Severe Hypoxia: Consequences to Neural Stem Cells and Neurons

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Abstract

Background: Multiple neurological diseases result from a pathological hypoxia in the brain, resulting in various motor, sensory or cognitive sequelae. Understanding the response of neural stem cells (NSCs) and differentiated neurons to hypoxia will help better treat such diseases.

Methods: We exposed mouse embryonic primary neurons (PN) and neural stem cells to 1% O_2 in *vitro*.

Results: Both cell types survived and retained their immunocytochemical markers, and neurons showed no obvious morphological changes. Microarray analysis showed that the number of genes with significantly altered expression levels was almost five-fold higher in NSCs compared to PN. NSCs displayed a clear block in G1/S phase of the cell cycle and a number of down-regulated cytokine genes. Various growth factors (e.g. neural growth factor, prolactin), involved in survival and proliferation, genes of the Notch pathway, and genes involved in glial differentiation, and cell-matrix adhesion were up-regulated. PN displayed a down-regulation of a number of genes involved in neuron-specific functions, in particular, transmitter-related (e.g. synaptic transmission, neurotransmitter transport and release, learning, adult behavior).

Conclusions: We conclude that hypoxia 1-down-regulates genes involved in multiple neuronal functions which can negatively impact learning and memory; 2-induces a cell cycle block in NSCs; 3-can precondition NSC towards a particular differentiation potential while maintaining them fully undifferentiated.

Keywords: Hypoxia; Neural stem cells; Cell cycle; Primary neuron; Neurotransmitter; Neuronal function

Introduction

Neural stem cells (NSCs) are first generated during embryonic gastrulation and then persist throughout the mammalian lifespan to help preserve equipoise in the face of perturbations [1]. The division and differentiation of these endogenous NSCs is regulated by both physiological stimuli and pathological conditions [2]. Most tissue culture protocols maintain NSCs at an oxygen tension that is equivalent to that of room air at sea level - 21% O_2. However, the natural in vivo environment where NSCs reside at various stages of life is actually hypoxic relative to atmospheric oxygen levels. Placental development [3], rat embryo at 9.5 days post coitum [4], human embryo [5] and interstitial tissues [6, 7, 8] are examples of tissues with low O_2. Oxygen levels have been shown to vary by brain regions too [9]. These data demonstrate that the embryonic, fetal, and adult brain tissues naturally exist in a relatively hypoxic environment [10].

Understanding the response of NSC and neurons to pathological hypoxia first requires profiling the expression pattern of key genes under hypoxic versus normoxic conditions (relative to room air) using a population of NSCs and neurons Therefore, for this study, we performed microarray analysis on a clone of NSCs that could be efficiently grown as essentially homogeneous monolayers in quantities sufficiently large and healthy to ensure reproducible patterns of growth and differentiation, making comparisons between cultures feasible while eliminating confounders such as vari-

Figure 1. Phenotypic characterization of C17.2 NSCs and PN in 21% O_2 and 1% O_2. A-NSCs were cultured either in normoxia for 4 days (a, b, c, g, h) or in normoxia for 2 days then transferred to 1% O_2 for 2 days (d, e, f, i, j). Cells were stained using antibodies directed against mouse stem cell marker Nestin and neuronal markers MAP2 and NeuN (a-f). Under both normoxia and hypoxia, all cells expressed nestin (c, f), but there was no detectable MAP2 or NeuN (a, b, d, e). Phase contrast (Ph. Contr.) microscopy shows the cells in normoxia (g) and 1% O_2 (i). Propidium Iodide (P.I.) staining revealed a very low cell death similarly in normoxia (h) and 1% O_2 (j). Magnification ×100; inset × 400. Western blot analysis confirmed the absence of MAP2 and NeuN from NSCs, and the presence of equivalent amounts of nestin in normoxia (N) and hypoxia (H, 1% O_2) (k); B-Primary neurons were cultured form Embryonic (E) day 17 of mouse cortex in normoxia for 8 days (a, b, c, g, h) or in normoxia for 6 days then transferred to 1% O_2 for 2 days (d, e, f, i, j). Immunocytochemistry was performed for Nestin, MAP2 and NeuN. There was a complete absence of Nestin both in normoxia (c) and 1% O_2 (f). MAP2 and NeuN were detected in all cells both in normoxia (a, b) and 1% O_2 (d, e). Phase contrast (Ph. Contr.) microscopy revealed typical neuronal shape with processes and synapses both in normoxia (g) and 1% O_2 (i). PI staining showed a comparable cell death between normoxia (h) and 1% O_2 (j). Magnification ×100; inset × 400. Western blot confirmed the absence of Nestin, and the presence of MAP2 and NeuN (k). Quantifications revealed ~two-fold decrease in the protein level of MAP2 and a significant ($P \leq 0.05$) ~five-fold decrease in protein level of NeuN under hypoxia (l).

ability in migration, differentiation, cell number, and survival. These NSCs were compared to neurons obtained from the same strain for their response to hypoxia.

Our data suggested that, while both NSC and neurons tolerate 1% O_2 well, primary neurons exhibited a suppression of genes involved in neuronal-specific functions (e.g. neuron differentiation, neurotransmitter transport, synaptic transmission, and learning), while NSCs exhibited down-regulation of many inflammatory genes (e.g., interferons

and tumor necrosis factors) and an up-regulation of multiple growth factors. The expression pattern of genes involved in cell cycle control suggested a tendency not to proceed from G1 to S, which correlated with a clear G1/S block.

Materials and Methods

Murine NSCs (mNSCs)
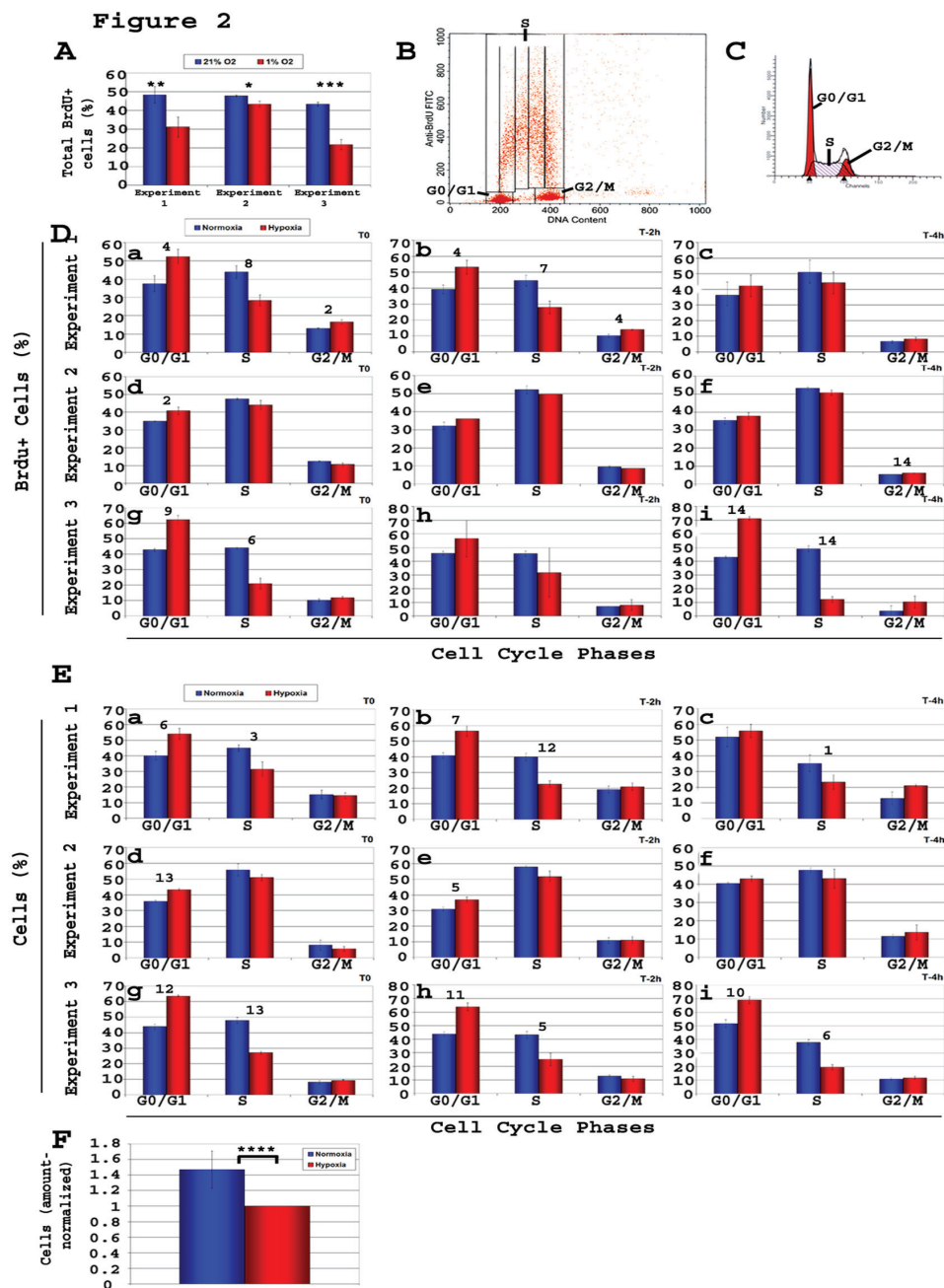
Figure 2. Kinetic analysis of cell cycle of C17.2 neural stem cells (NSCs)
C17.2 cells were incubated in either 21% O_2 or 1% O_2. Brdu pulse of 20 minutes was followed by cell harvest either immediately (T0) or after a chase time of either 2 h or 4 h (T-2 h, T-4 h). Brdu staining and flow cytometry analysis was performed on all samples, and the percentage of Brdu+ cells relative to cell cycle was determined. Shown here are three experiments of n = 3 each (total n = 9). Two types of flow cytometry analyses were performed, one using total cells (Brdu+ and Brdu-), and one gated on the Brdu+ population. A-Total percentage of Brdu+ cells in each of the 3 experiments both in 1% O_2 and 21% O_2. T-test, normoxia vs. hypoxia: *, P < 0.03; **, P < 0.01; ***, P < 0.002; B-Gates used to calculate the percentage of Brdu+ cells in each of the phases G0/G1, S, and G2/M of the cell cycle; C-Graphics used to calculate the percentage of total cells in the various phases of the cycle (G0/G1, S, G2/M). Values obtained were used to draw the histograms in panel E; D-Brdu+ cells, and their progression in cell cycle phases. Under hypoxia, the percentage of Brdu+ cells showed a consistent increase in G1 and a decrease S relative to normoxia showing a clear G1/S block; E-There is a consistent increase in the percentage of cells in G1 and a decrease in S phase, in hypoxia compared to normoxia, as analyzed based on their DNA content; F-Cell counts reflect fewer cells in hypoxia. T-test, value in normoxia vs. hypoxia of same cell cycle phase: Panel A: *, P < 0.03; **, P < 0.02; ***, P < 0.0002. Panels D and E: 1, P < 0.05; 2, P < 0.04; 3, P < 0.03; 4, P < 0.02; 5, P < 0.009; 6, P < 0.007; 7, P < 0.006; 8, P < 0.005; 9, P < 0.004; 10, P < 0.003; 11, P < 0.002; 12, P < 0.0008; 13, P < 0.0004; 14, P < 3 ×10^{-5}. Panel F: ****, P < 0.03.
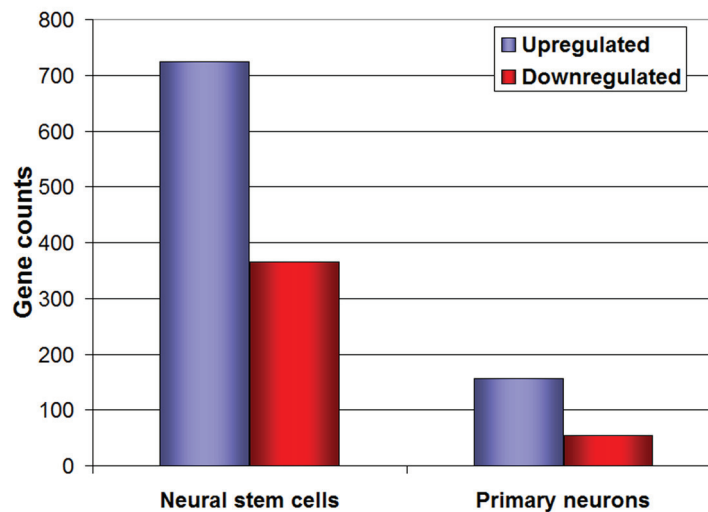
Figure 3. Microarray analysis of neural stem cells and neurons. Representation of the number of genes up-regulated (blue bars) and down-regulated (red bars) under 1% O_2, both in neural stem cells and primary embryonic neurons.

We began the study using a stable, clonal population of dependably engraftable *lacZ*-expressing mNSCs taken from a well-characterized clone, C17.2, because its enhanced yet constitutively self-regulated expression of stemness genes [11, 12] permits its cells to be efficiently grown in sufficiently large, homogenous, and viable quantities to ensure reproducible patterns of growth and integration. The mNSCs were cultured and passaged as previously described [11, 12]. The C17.2 line was graciously provided by Dr Evan Snyder.

Primary embryonic neuronal culture

C57BL/6J female mice were obtained from the Jackson Laboratories with timed pregnancy (Jackson Laboratories, Bar Harbor, ME, USA). Embryos were extracted at embryonic day (E) 17, cortexes were incubated with 0.25% trypsin (Gibco) then triturated, centrifuged and pellets resuspended in plating medium consisting of Neurobasal Medium (Gibco), B27 1X final (Gibco), Glutamine 500 µM (Gibco), penicillin/streptomycin 100 U/ml and 100 µg/ml respectively (Gibco), and glutamic acid 25 µM (Sigma), 1ml of 10^6 cells/ml suspension was added onto a 35 mm plate previously coated with Poly-D-Lysine > 300 KDa (Sigma) at 0.15 mg/ml, and cultured in 5% CO_2 at 37 ºC (day 0). After 24 h (day 1), 1ml plating medium was added. Then every 4 days, 1ml of the plating medium was replaced with an equal volume of maintaining medium (same composition as plating medium without glutamic acid). Cells were grown for 6 days before experimental use.

Hypoxia studies

To study the effect of hypoxia on NSC and primary neurons

(PN), 1% O_2 was used with 5% CO_2 and 37 ºC. Unless otherwise indicated, all cells used were incubated in normoxia for a certain time, then transferred to hypoxia, while controls were maintained in parallel at 21% O_2. Cells were incubated in normoxia (2 days for NSCs, until they reach ~ 20% confluency; 6 days for PN), then transferred to the 1% O_2 incubator for 2 days, while control counterparts were maintained in normoxia all the duration (total 4 days for NSCs, 8 days for PN). Unless otherwise indicated, gene expression studies and immunocytochemistry analyses were performed on cells after 2 days in 1% O_2, as well as on the normoxic counterparts.

Phase contrast microscopy and cell death

Using phase contrast microscopy, the morphology of all cultured cells was monitored, and cell death was assessed by propidium iodide staining (5 µg/µl) (Sigma). Images were acquired with Zeiss microscope with AxioCam MRm camera (Zeiss) using the Axiovision Rel 4.5 software.

Immunocytochemistry (ICC) staining

ICC was performed on NSC and primary neurons to detect the neural stem cell marker Nestin, the neuronal marker MAP2, and late neuronal marker NeuN using unlabelled primary antibodies and fluorescent secondary antibodies (Chemicon). Briefly, cells were fixed, permeabilized, blocked, then incubated with primary antibodies at 4 ºC overnight, followed by secondary antibodies for 2.5 h at RT. DAPI was added and cells observed under fluorescent microscope (Zeiss). Images were acquired with AxioCan MRm camera (Zeiss) using the Axiovision Rel 4.5 software. Magnifications used were × 100 and × 400.
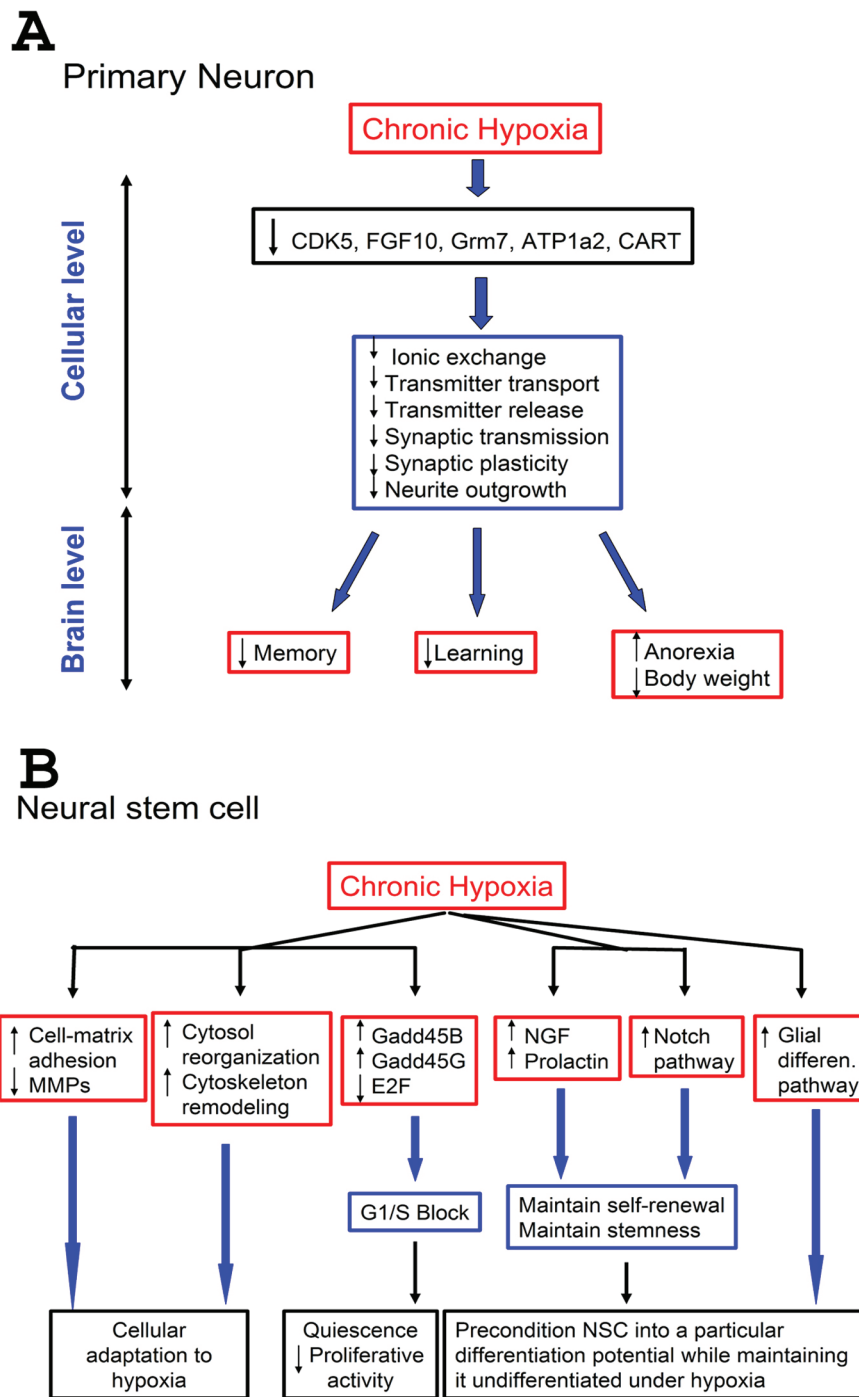
Figure 4. Effect of hypoxia on neurons and neural stem cells: a proposed model. A-Effect of hypoxia on neurons. A low (1%) level of oxygen for prolonged periods down-regulates certain genes that control pathways important for neuronal physiology.This results in suppression of neuronal functions at cellular level related to neurotransmitter processes, such as transmitter transport and release, and synaptic transmission. This suppression can negatively impact vital brain functions that depend on proper neurotransmission, such as memory and learning. Since some pathways (CART) are involved in controlling appetite, this suppression also results in anorexia, leading to weight loss; B-Effect of hypoxia on neural stem cells (NSCs). Prolonged exposure of NSCs to 1% O_2 up-regulates cell-matrix adhesion molecules and down-regulates matrix metallo-proteases (MMPs). It also up-regulates genes involved in cytoskeleton remodeling, and causes changes in cell-cycle components resulting in G1/S block. Furthermore, hypoxia up-regulates some growth factors (NGF, neural growth factor, and prolactin), known to stimulate survival, and the Notch pathways as well. Also genes responsible for glial differentiation were up-regulated. Combined, these changes help maintaining the self-renewal of NSCs under hypoxia, keep them undifferentiated but pre-condition them into a particular differentiation potential.

Table 1. Major Cellular Functions With Genes Changed in Primary Neurons at 1% O_2 Relative to 21% O_2 . Down-regulated

GO Type	GO Name	No Changed	No Measured	No in GO	Adjusted P
P	Feeding behavior	3	41	43	< 0.0001
F	Glycosaminoglycan binding	4	74	79	< 0.0001
P	Adult behavior	4	78	88	< 0.0001
P	Learning	3	46	52	< 0.0001
P	Polyol transport	3	47	57	< 0.0001
P	Cellular chemical homeostasis	6	186	207	< 0.0001
P	Reflex	3	50	63	< 0.0001
P	Mechanoreceptor differentiation	3	67	74	< 0.0001
P	Synaptic transmission	5	188	205	< 0.0001
P	Epidermal cell differentiation	3	93	101	0.001
P	Neurotransmitter transport	3	98	105	0.0005
P	Cellular morphogenesis during differentiation	4	198	217	0.001
C	Extracellular region part	7	553	612	< 0.0001
F	Transmembrane transporter activity	8	710	777	< 0.0001
P	Neuron differentiation	5	343	371	< 0.0001

Abbreviations: GO-gene ontology; P-biological process; C- cellular component; F-molecular function; No, number.

Analysis of neural stem cell division and cell cycle

To quantify NSC division, pulse-chase BrdU incorporation experiment was performed followed by flow cytometry to detect BrdU+ cells and DNA content, using EZ-BrdU kit (Phoenix Flow Systems) according to the manufacturer recommendations, with a 20 min BrdU pulse, and 2 h and 4 h chase. Nine independent BrdU incorporation experiments were performed. BrdU+ cells and DNA content were assessed for each time point using CellQuest Pro Software. Cell proliferation was also assessed using total cell counts.

Protein quantification

Proteins were extracted using HEPES buffer and 30 μg protein per sample were loaded on gel. Membranes were probed for MAP2, NeuN, Nestin, actin (Chemicon), then incubated with HRP-conjugated secondary antibodies (ZYMED).

Microarray analysis

Total RNA was extracted from NSC and PN, (n = 3 each) using the RNeasy kit (Qiagen) according to the manufacturer instructions. Spectrophotometer readings were taken with the NanoDrop ND-1000. The RNA integrity was checked with the Agilent 2100 BioAnalyzer system (Agilent Technologies, Santa Clara, CA). Sample processing for microarray data collection was performed as previously described [13].

For analysis, intensities were normalized using "modified LOESS" as previously described [14]. Pairwise fold changes were computed for the hypoxia and normoxia cultured primary neurons and NSCs. Genes with a fold change > 2 and P < 0.05 were considered significant and comparison groups were annotated with statistically significant GeneOntology term overrepresentation using the MappFinder algorithm and GO-Elite software packages [15]. Functional categorization of gene alterations was created with Ingenuity Pathway Analysis (IPA) program (Ingenuity Systems, Redwood city, CA) [13]. The P-value for each network or function was calculated with a right-tailed Fisher's exact test. The score for each network of function was shown as $-\log 10$ (P value), which indicates the likelihood of finding a set of focus genes in the network or function by random chance. The significance threshold was set to a score of 1.3 (i.e. P ≤ 0.05). All microarray data is MIAME compliant and the raw data has been deposited in the Gene Expression Ominibus database at http://www.ncbi.nlm.nih.gov/geo and can be re-

Table 2. Major Cellular Functions With Genes Changed in Primary Neurons at 1% O_2 Relative to 21% O_2, Up-regulated

GO Type	GO Name	No Changed	No Measured	No in GO	Adjusted P
P	Glycolysis	12	39	45	< 0.0001
C	MHC protein complex	6	24	29	< 0.0001
P	Antigen processing and presentation of peptide antigen	6	32	36	< 0.0001
F	L-ascorbic acid binding	4	17	19	< 0.0001
F	Dioxygenase activity	6	60	62	< 0.0001
F	Oxidoreductase activity	4	28	29	< 0.0001
P	Gluconeogenesis	3	17	19	< 0.0001
F	Protein kinase inhibitor activity	3	17	18	< 0.0001
F	Carbohydrate kinase activity	3	18	20	< 0.0001
P	Cell cycle arrest	4	43	46	0.0005
P	Vascular endothelial growth factor receptor signaling	4	61	67	0.0005
P	Programmed cell death	14	648	703	< 0.0001
P	Negative regulation of catalytic activity	3	55	58	0.0045
P	Hepatocyte metabolic process	3	63	70	0.006
F	Iron ion binding	7	284	305	0.001

Abbreviations: GO-gene ontology; P-biological process; C- cellular component; F-molecular function; No, number.

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Research Ethics

The experimentations requiring animal use were submitted to the Institutional Animal Care and Use Committee (IA-CUC) at the University of California San Diego (UCSD) and approval was obtained (S05534).

Statistical analysis

Statistical analysis was performed using the Student T-test available from Microsoft Excel software. Values were considered significant for $P \leq 0.05$.

Results

Survival of NSCs and primary neurons under hypoxic vs. normoxic conditions

All NSCs expressed the stem cell marker Nestin (Fig. 1A, c) in normoxia, and expressed no mature neuronal markers,

e.g., MAP2 (Fig. 1A, a, k) and NeuN (Fig. 1A, b, k) and showed little cell death (Fig. 1A, h). When NSCs were transferred to 1% O_2, the expression profiles of Nestin, MAP2 and NeuN were similar to those in normoxia (Fig. 1B, e, f). Interestingly, NSCs tolerated well the 1% O_2 for 2 days and there was no increase in cell death (Fig. 1B, i). Western blot confirmed the absence of MAP2 and NeuN and showed similar levels of Nestin in normoxia and 1% O_2 (Fig. 1A, k).

Cortical primary neurons (PN) cultured in normoxia for 8 days displayed typical morphology (Fig. 1B, g), little cell death (Fig. 1B, h), and expressed MAP2 and NeuN but not Nestin (Fig. 1B, a, b, c). When incubated for 2 days in hypoxia (days 7 & 8 in culture), PN continued a normal developmental pattern as in normoxia (Fig. 1B, i). They also had equivalent survival (Fig. 1B, j) and a similar pattern of marker gene expression (Fig. 1B, d, e, f). There was, however, a significant ($P \leq 0.05$) decrease in NeuN (more than 3-4 fold) and MAP2 (about twice) protein levels at 1% O_2 compared to normoxia (Fig. 1B, k, l).

NSCs induced to become more quiescent in hypoxia than in normoxia

Table 3. Major Genes With Expression Level Changes Under Hypoxia. Major Neuronal-Specific Functions and the Genes Which are Down-Regulated, in Primary Embryonic Neurons

Function	Genes down-regulated
Adult behavior	Atp1a2, Cartpt, Cdk5, Grm7
Learning	Atp1a2, Cdk5, Grm7
Reflex	Cartpt, Fgf10, Satb1
Mechanoreceptor differentiation	Fgf10, Hes5, Ntrk2
Synaptic transmission	Atp1a2, Cartpt, Cdk5, Grm7, Ntrk2
Neurotransmitter transport	Atp1a2, Ntrk2, Slc6a11
Cellular morphogenesis during differentiation	Apoe, Cdk5, Ntrk2, Rtn4rl1
Transmembrane transporter activity	Aqp4, Atp1a2, Grm7, Kcnd2, Slc25a10, Slc25a18, Slc6a11, Tfrc
Neuron Differentiation	Apoe, Cdk5, Fgf10, Hes5, Rtn4rl1

Table 4. Major Cellular Functions With Genes Changed in Neural Stem Cells at 1% O_2 Relative to 21% O_2, Down-Regulated

GO Type	GO Name	No Changed	No Measured	No in GO	Adjusted P
P	Actin filament bundle formation	3	14	15	0.001
P	Interferon-gamma biosynthetic process	3	16	16	0.0015
C	Mitochondrial part	15	298	331	< 0.0001
P	Interleukin-2 biosynthetic process	3	19	20	0.002
F	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amidines	3	22	25	0.002
P	Nucleoside phosphate metabolic process	9	154	169	< 0.0001
P	Synaptic vesicle transport	3	23	24	0.0035
F	Oxidoreductase activity, acting on the CH-CH group of donors	4	41	47	0.001
P	Myelination	3	27	32	0.0035
P	Carboxylic acid metabolic process	15	392	421	< 0.0001
P	tRNA metabolic process	6	94	97	0.0005
P	Amino acid derivative metabolic process	5	82	92	0.003
P	Exocytosis	5	90	97	0.0025
C	Anchored to membrane	6	121	129	0.002

AbbrAbbeviations: GO-gene ontology; P-biological process; C- cellular component; F-molecular function; No, number.

Table 5. Major Cellular Functions With Genes Changed in Neural Stem Cells at 1% O_2 Relative to 21% O_2, Up-Regulated

GO Type	GO Name	No Changed	No Measured	No in GO	Adjusted P
F	Protein binding	203	4602	5018	< 0.0001
F	L-ascorbic acid binding	6	17	19	< 0.0001
P	Blood vessel morphogenesis	21	165	182	< 0.0001
F	Transcription regulator activity	62	944	1013	< 0.0001
P	ER overload response	3	6	7	< 0.0001
P	Positive regulation of cell motility	6	22	25	< 0.0001
C	Nucleus	153	3642	3941	< 0.0001
F	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors	6	28	29	< 0.0001
F	MAP kinase phosphatase activity	3	8	9	0.0005
P	Cytoplasm organization and biogenesis	3	8	9	< 0.0001
P	Death	43	681	740	< 0.0001
P	Glial cell differentiation	10	72	79	< 0.0001
P	Cellular component assembly	15	145	159	< 0.0001
P	Regulation of cell-matrix adhesion	3	9	11	0.001
P	Purine salvage	82	1717	1843	< 0.0001

AbbrAbbeviations: GO-gene ontology; P-biological process; C- cellular component; F-molecular function; No, number.

A high proliferation rate was observed in NSCs in normoxia, as 45 - 48% (n = 9) (Fig. 2A) of cells were BrdU+ after a 20 min pulse. In 1% O_2, the total percentage of BrdU+ cells was 31 - 43% (n = 9) (Fig. 2A), significantly lower from that in normoxia (P < 0.02 to P < 0.002), indicating that hypoxia is affecting cell cycle, prompting us to perform a more in-depth analysis of cell proliferation rates. Kinetic analysis of cell cycle progression (Fig. 2B, D) showed significantly more BrdU+ cells in G1 (P < 0.04 - 3 ×10^{-5}) and significantly (P < 0.006) less BrdU+ cells in S phase in hypoxia as compared to normoxia, in 3 independent experiments of n = 3 each, at all time points (Fig. 2D, a-i), concordant with DNA content analysis (Fig. 2 C, E). This is consistent with a G1/S block with hypoxia. The percentage of cells in the S phase averaged 45.5 ± 7.7% (n = 9) in normoxia and significantly (P < 0.03) less in hypoxia (average 32.6 ± 12.6%, n = 9).

After seeding a similar amount of cells, plates incubated in hypoxia for 2 days showed significantly (P < 10^{-4}) less cells (Fig. 2F) than normoxia counterparts (ratio normoxia-to-hypoxia = 1.48 ± 0.2). Altogether, these data confirm a greater number of quiescent NSCs (i.e., decreased progression into the cell cycle) in 1% compared to 21% O_2 attributable to predominantly a G1/S block. Altogether, these data indicate that under hypoxia, NSCs have become more quiescent.

Microarray analysis reveals a major difference between PN and NSCs in response to hypoxia

Under 1% O_2, only 210 genes changed more than two-fold (P < 0.05) in PN, but about 5 × more in NSCs (i.e., 1090 genes). In PN and NSCs, most genes were up-regulated (156 genes in PN and 725 genes in NSC (Fig. 3). Through GO analysis, we also found that, in hypoxia, PN had a large array of genes involved in neuronal specific functions that were down-regulated, including adult locomotory and fear/anxiety behavior (atp1a2 [16]) , feeding behavior and cellular response to starvation (Cartpt) [17], behavioral response

Table 6. Major Genes With Expression Level Changes Under Hypoxia. Changes in Expression Level of Genes Involved in Cell Cycle Block in NSCs

Function	Gene change
Arrest in G1/decrease cell cycle progression	↑AKAP12, ↑BHLHE40, ↓CAMKK2, ↓CCND2, ↑CSF1, ↑CYR61, ↓DDIT3, ↑DGKZ, ↑DUSP1, ↑E2F2, ↑FN1, ↑GHR, ↑KLF4, ↑NGF, ↑PTPN22, ↑RASSF1, ↑SIRT1, ↑SKP2, ↓TYMS, ↑ARID3A, ↓CHN2, ↓E2F2, ↑GADD45B, ↑GADD45G, ↑HMOX1, ↑ID1, ↑ID2, ↓IGFBP5, ↑SNAI1, ↑THBS1, ↑VEGFA

to coccain, and cerebral cortex development (CDK5) [18, 19], behavioral fear response and memory (Grm7), learning [20], chemotaxis (FGF10), mechanoreceptor differentiation (FGF10, hes5, ntrk2), synaptic transmission (atp1a2, Cartpt, Cdk5, Grm7, Ntrk2), neurotransmitter transport (Atp1a2, Ntrk2, Slc6a11), and neuronal differentiation (Apoe, Cdk5, Fgf10, Hes5, Rtn4rl1) (Table 1, 2, 3). This gene expression profile is consistent with a suppression of neuronal function under hypoxic conditions. There was also up-regulation of genes related to various metabolic processes, including oxidation/reduction, gluconeogenesis, glycolysis, ascorbic acid binding, and lipid biosynthetic process (Table 1, 2), pointing towards a metabolic adaptation; a well-established role for hypoxic gene responses.

NSCs down-regulated, in hypoxia, multiple members of the interferon (IFN) family and tumor necrosis factors (TNF) (Table 4, 5). They displayed an up-regulation of multiple growth factors, such as insulin, VEGF, NGF, prolactin, GH-

Rc (data available at the Ominibus database, materials & methods), factors involved in cell survival and proliferation. They also up-regulated genes involved in glial cell differentiation and Notch signaling pathway (data not shown). Furthermore, there was an up-regulation of genes involved in cellular component assembly (Fn1, Itga5, Lox), cell-matrix adhesion genes (Bcl6, csf1, Pik3cb), cytoplasm organization and biogenesis (Fn1, Itga5, Tcfcp2l1), and genes involved in positive regulation of cell motility (Bcl6, Csf1, Hbegf, Irs2, Prl2c2, Rras2).

Significant changes in transcript levels of genes affecting many functions in PN and NSCs were also detected through gene categorization using the Ingenuity Pathway Analysis (IPA) software (data not shown). Since the NSC cultures showed a cell cycle block and a change in morphology, genes involved in these two functions were among the genes we focused our attention on. There was a significant change in expression levels of genes involved in favoring G1/S arrest,

Table 7. Major Genes With Expression Level Changes Under Hypoxia. Changes in Expression Levels of Genes Involved in Cytoskeleton Rearrangements in NSCs

Effect	Genes
Increase formation of actin stress fibers	↓ARKGEF3, ↑CTGF, ↑EDN1, ↑FN1, ↓GNG7, ↓LPAR4, ↓S1PR2, ↑VEGFA
Decrease formation of actin stress fibers	↑RND3, ↓S1PR2, ↓STARD13, ↑TNC
Increase rearrangement of actin cytoskeleton	↑EDN1, ↑PALLD, ↑PDGF1
Increase rearrangement of cytoskeleton	↑CTGF, ↑EDN1, ↑ELMO1, ↑PALLD, ↑PDGFB, ↓SPP1, ↑VEGFA
Increases reorganization of actin	↓ARFIP2, ↑FN1, ↑PAUR, ↑VEGFA
Increase reorganization of cytoskeleton	↑CSF1, ↑EDN1, ↑FN1, ↓GAS1, ↑GDNF, ↑VEGFA

such as up-regulation of GADD45B and GADD45G and down-regulation of E2F (Table 6). There were also changes in expression levels of genes involved in cytoskeletal remodeling, such as biogenesis of cytoskeleton, depolymerization of microtubules, formation of actin stress fibers, formation of lamellopodia (Table 7), the combination of which can be associated with change in proliferative status.

Detailed analysis of PN expression profile using the IPA software also showed changes in expression levels of multiple genes. These changes, based on the IPA database, favor neuro-degeneration, correlating with the GO analysis.

Discussion

In this study, our goal was to investigate the effect of hypoxia on gene expression of NSCs and primary neurons to gain more insight about their response to hypoxia. We have shown that hypoxia: a) down-regulates genes involved in multiple neuronal functions, including neuron differentiation, neurotransmitter transport, synaptic transmission, and learning b) affects cell cycle progression of neural stem cells, c) up-regulates genes involved in glial cell differentiation and Notch pathway in NSCs, d) up-regulates many growth factors and IFN and TNF in NSCs.

To start with we can deduce from the microarray data how PN adapt, at least in part, to a hypoxic stress: neurons did not die after 2-day exposure to 1% O_2, yet, we see for the first time that they down-regulated a large array of genes involved in various neuronal functions (Table 1, 2, 3), a change that can impact the physiology of neurons at a cellular level, and can potentially have a broad effect on brain functioning in an in vivo setting.

Most of the functions affected by these gene expression changes (such as learning, memory, synaptic transmission, neurotransmitter transport) are related to neurotransmitter biology. These findings point towards a hypoxia-induced neurotransmitter physiology alterations. We thus speculate that hypoxia can negatively affect learning and memory, and induce anorexia, by down-regulation of molecules involved in transport and release of neurotransmitters (Fig. 4A). Hypoxia has been previously known to impair learning and memory [21, 22], as well as body weight [23, 24], and our study thus provides potential mechanisms for these phenotypic changes.

When NSCs were exposed to 1% O_2 for 2 days, they displayed a predominantly clear G1/S block (Fig. 2C, D). It is known that most stem cells enter quiescence in their natural environment. Under hypoxia, the NSCs might therefore be acting like stem cells do in their natural niche. This was demonstrated by 3 different experimental approaches: 1) total DNA content showing more cells in G1 and less cells in S phase under hypoxia; 2) pulse-chase BrdU labeling coupled to cell cycle which showed the same result; 3) microarray

data showing up-regulation of genes involved in DNA damage-induced cell cycle arrest (Gadd45B, Gadd45G) [25], and down-regulation of a transcription factor needed for the G1-to-S transition (E2F) [26]. These data demonstrate that cell G1/S block is a mechanism through which hypoxia decelerates cell division. However, we cannot exclude the possibility that there are other mechanisms operating in parallel to decelerate cell division in hypoxia (Fig. 4B).

Previous studies related to cell cycle of various stem cells, including neural stem cells, have indicated that hypoxia can either decelerate or accelerate the cell cycle, based on cell type, level of oxygen, and culture conditions. [18, 27, 28, 10, 29, 30], providing evidence that every stem cell line of any particular stem cell type can react differently to hypoxia. Here we show that CNS NSCs mitosis is negatively regulated by 1% O_2 hypoxia.

The GO analysis showed an up-regulation of genes of the Notch pathway which is known to maintain the undifferentiated stem cell phenotype [31]. It has been shown that under hypoxia, HIF1-alpha interacts with activated Notch to repress terminal differentiation in NSCs [31]. This is concordant with the lack of spontaneous differentiation of NSCs in our study (Fig. 1A, Fig. 4B).

Finally, we have shown that under hypoxia, NSCs have up-regulated many growth factors and down-regulated IFN and TNF. This finding complements a recent study demonstrating that IFN-γ promotes the entry of hematopoietic stem cells into cell cycle [32]. Hence, the down-regulation of IFN in NSC would imply that their down-regulation correlates with decreased cell cycling or entry into quiescence. Altogether, these findings point to a global effect of hypoxia on NSC: a decrease in proliferation and promotion of quiescence, and maintenance of self-renewal/stemness, metabolic/cellular adaptation to hypoxia (Fig. 4B).

In summary, our study has revealed for the first time that hypoxia suppresses the expression of genes involved in neuronal processes in primary neurons. It also showed that this suppression is not associated with cell death since cell viability was similar between normoxia and hypoxia. We also showed that hypoxia supports the maintenance of NSCs in an undifferentiated and quiescent state, actually preserving their health, while more differentiated, primary neurons may have their function inhibited, slowed down, or even impaired under hypoxic conditions that persist for more than a few hours.

Grants

Disclosures

The authors declare that they have no conflict of interest.

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