Stem Cell Reports
Report
OPEN ACCESS

Transcriptional Profiling of Hypoxic Neural Stem Cells Identifies Calcineurin-NFATc4 Signaling as a Major Regulator of Neural Stem Cell Biology

Marta Moreno,[1] Virginia Fernández,[2] Josep M. Monllau,[1] Víctor Borrell,[2] Carles Lerin,[3] and Núria de la Iglesia[1,*]

[1]Clinical and Experimental Neurosciences, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), 08036 Barcelona, Spain
[2]Instituto de Neurociencias, Consejo Superior de Investigaciones Científicas and Universidad Miguel Hernández, 03550 Sant Joan d'Alacant, Spain
[3]Endocrinology, Hospital Sant Joan de Déu, 08950 Barcelona, Spain
*Correspondence: niglesia@clinic.ub.es
http://dx.doi.org/10.1016/j.stemcr.2015.06.008

SUMMARY

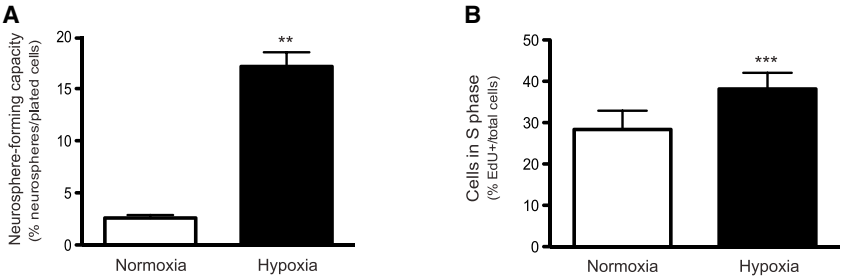
Neural stem cells (NSCs) reside in a hypoxic microenvironment within the brain. However, the crucial transcription factors (TFs) that regulate NSC biology under physiologic hypoxia are poorly understood. Here we have performed gene set enrichment analysis (GSEA) of microarray datasets from hypoxic versus normoxic NSCs with the aim of identifying pathways and TFs that are activated under oxygen concentrations mimicking normal brain tissue microenvironment. Integration of TF target (TFT) and pathway enrichment analysis identified the calcium-regulated TF NFATc4 as a major candidate to regulate hypoxic NSC functions. *Nfatc4* expression was coordinately upregulated by top hypoxia-activated TFs, while NFATc4 target genes were enriched in hypoxic NSCs. Loss-of-function analyses further revealed that the calcineurin-NFATc4 signaling axis acts as a major regulator of NSC self-renewal and proliferation in vitro and in vivo by promoting the expression of TFs, including Id2, that contribute to the maintenance of the NSC state.

INTRODUCTION

Stem cells, including neural stem cells (NSCs), reside in specialized compartments that contribute to their maintenance. In the embryonic and adult mammalian brains, signals emanating from cells in the niche or the cerebrospinal fluid promote NSC self-renewal and proliferation (Lehtinen et al., 2011; Shen et al., 2004). Therefore, the NSC microenvironment has a strong influence on NSC behavior. Most cells in multicellular organisms live in an environment within tissues where oxygen levels are usually far below atmospheric oxygen concentrations. Physiologic oxygen levels may vary depending on cell type. Within the brain, physiologic oxygen concentrations are at least one order of magnitude lower than in the atmosphere (from 2%–5% in the cortex to 0.4% in pons) (Ereciń-ska and Silver, 2001; Mohyeldin et al., 2010; Panchision, 2009). In the hippocampus, where a specific population of adult NSCs resides, oxygen partial pressure is 3%–4%, a condition that persists from embryonic development. In other words, NSCs reside in a hypoxic environment. Physiologic hypoxia has been shown to promote cell population growth, survival, proliferation, and multipotency (Chen et al., 2007; Pistollato et al., 2007; Studer et al., 2000). However, the molecular mechanisms by which low oxygen levels impact on normal NSC behavior are poorly understood. This is probably due to the fact that most functional studies are performed under non-physiologic experimental conditions (21% oxygen), thereby underestimating crucial factors that are active under physiologic hypoxic conditions.

Hypoxia is known to affect the transcriptional status of the cell by activating important transcription factor (TF) families, such as hypoxia-inducible factor (HIF) or necrosis factor κB (NF-κB, nuclear factor of kappa light polypeptide gene enhancer in B cells), among others (Cummins et al., 2006; Wang and Semenza, 1993). These pivotal factors control a wide range of vital cell functions, such as cell fate, proliferation, and survival in different cell types. As a master regulator of the hypoxic response, HIF is one of the primary candidates to regulate NSC biology at low oxygen levels. Up until now, only a candidate approach-driven study had identified a couple of HIF targets that are, at least in part, involved in mediating the NSC response to hypoxia (Studer et al., 2000). Another study had reported that low oxygen levels repress BMP-dependent SMAD activation, thereby inhibiting gliogenic differentiation of NSC (Pistollato et al., 2007). However, the molecular mechanisms by which physiologic hypoxia controls NSC behavior remained poorly understood.

The NSC response to hypoxia may go beyond the sole upregulation of HIF-dependent gene targets. It also may require the activation of other TFs. Most probably, it may involve the coordinated response of several TFs at the same time. The goal of this study was to identify the crucial TFs that control NSC functions under physiologic oxygen levels. Using gene set enrichment analysis (GSEA), we have shown that physiologically low oxygen levels activate a large number of TFs, well beyond the HIF family of TFs. Furthermore, integration of transcription factor target (TFT) and pathway enrichment analysis allowed us to uncover the calcium-regulated calcineurin-NFATc4 axis as a



C

GENE SET NAME	NES	FDR q-val
KEGG_GLYCOLYSIS_GLUCONEOGENESIS	1.95	0.027
KEGG_GALACTOSE_METABOLISM	1.90	0.026
KEGG_RIBOSOME	1.89	0.020
KEGG_STARCH_AND_SUCROSE_METABOLISM	1.80	0.044
KEGG_FRUCTOSE_AND_MANNOSE_METABOLISM	1.79	0.040
KEGG_ARGININE_AND_PROLINE_METABOLISM	1.77	0.042
KEGG_PEROXISOME	1.74	0.048
KEGG_ECM_RECEPTOR_INTERACTION	1.74	0.044
KEGG_ARACHIDONIC_ACID_METABOLISM	1.73	0.042
KEGG_PYRUVATE_METABOLISM	1.72	0.041
KEGG_COMPLEMENT_AND_COAGULATION_CASCADES	1.69	0.048
KEGG_GLYCEROPHOSPHOLIPID_METABOLISM	1.66	0.061
KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	1.61	0.086
KEGG_TASTE_TRANSDUCTION	1.61	0.087
KEGG_AMINO_SUGAR_AND_NUCLEOTIDE_SUGAR_METABOLISM	1.60	0.088
KEGG_ETHER_LIPID_METABOLISM	1.58	0.091
KEGG_OLFACTORY_TRANSDUCTION	1.57	0.100
KEGG_CALCIUM_SIGNALING_PATHWAY	1.52	0.133
KEGG_GLYCEROLIPID_METABOLISM	1.52	0.128
KEGG_DILATED_CARDIOMYOPATHY	1.51	0.139

D

GENE SET NAME	NES	FDR q-val
V$E12_Q6	1.81	0.109
V$GATA_Q6	1.79	0.072
V$AP1_Q2_01	1.73	0.103
YTAAYNGCT_UNKNOWN	1.72	0.086
YATGNWAAT_V$OCT_C	1.68	0.113
V$OCT1_01	1.66	0.115
GATAAGR_V$GATA_C	1.65	0.113
V$STAT1_01	1.61	0.145
V$CEBP_Q2	1.60	0.148
V$AP1_Q4_01	1.59	0.146
V$OCT1_B	1.59	0.136
V$MYOD_01	1.59	0.125
V$CP2_02	1.59	0.120
V$NFKAPPAB_01	1.58	0.122
V$AML_Q6	1.57	0.128
V$FXR_Q3	1.57	0.122
V$MAF_Q6	1.56	0.120
V$OCT_Q6	1.56	0.116
V$STAT5A_01	1.55	0.124
TTCYNRGAA_V$STAT5B_01	1.55	0.119

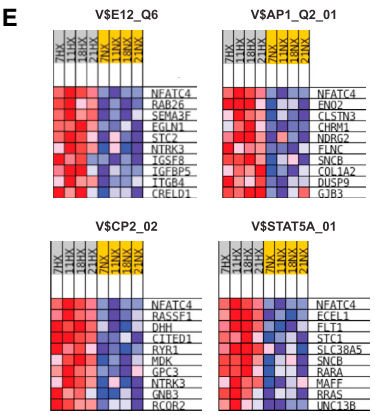


Figure 1. GSEA of Microarray Datasets from Hypoxic versus Normoxic NSCs Identifies NFATc4 as a Candidate TF to Regulate Hypoxic NSC Functions

(A) Self-renewal of mouse E13.5 NSCs maintained in 5% oxygen (hypoxia) or 21% oxygen (normoxia) was measured as the efficiency of single cells to give rise to neurospheres at low density. Data are presented as mean ± SEM (n = 4 independent experiments, paired t test, $^{**}p < 0.005$).

(B) Cell proliferation of NSCs maintained in hypoxia or normoxia as in (A) was assessed by EdU incorporation. Data are presented as mean ± SEM (n = 8 independent experiments, paired t test, $^{***}p < 0.001$).

(C) GSEA of microarray datasets using the gene sets of the KEGG pathway database. The top 20 KEGG gene sets that correlate with hypoxic NSCs are shown. GSEA statistical significance: FDR $\leq$ 0.25.

(D) GSEA of microarray datasets using the TFT gene sets. The top 20 TFT gene sets that correlate with hypoxic NSCs are shown. GSEA statistical significance: FDR $\leq$ 0.25.

(E) Enrichment profiles of E12, AP1, CP2, and STAT5A TFT gene sets are shown.

See also Figure S1 and Table S1.

key transcriptional program that controls NSC biology in vitro and in vivo.

RESULTS AND DISCUSSION

Physiologic Hypoxia Promotes Neurosphere Formation and Proliferation of Mouse Cortical NSCs

Culturing NSCs at low oxygen levels has been shown to promote cell population growth, survival, proliferation, and multicompetency of NSCs of different origins (Chen et al., 2007; Pistollato et al., 2007; Studer et al., 2000). However, the effect of hypoxia on NSC self-renewal through a neurosphere formation assay had not been tested. We thus measured the neurosphere formation capacity at low density of embryonic day (E) 13.5 mouse cortical NSCs, and we observed that culturing NSCs in hypoxic conditions dramatically increased neurosphere numbers (Figure 1A). Moreover, we confirmed that mouse cortical NSCs displayed increased proliferation at 5% versus 21% oxygen, as measured by EdU incorporation (Figure 1B).

Transcriptomic Analysis of Hypoxic NSCs

To identify the molecular mechanisms mediating the effect of low oxygen levels on NSC biology, we profiled the global effect of sustained, physiologic hypoxia on NSC gene expression using an unbiased approach by genome-wide microarray analysis. Unsupervised principal component analysis of microarray data showed reasonable separation

between the hypoxia and normoxia groups (Figure S1A). Comparative analysis of the transcriptomes revealed 374 significantly deregulated genes (Table S1). Microarray expression changes were validated using qRT-PCR for a subset of genes of interest (Figure S1B). Notably, *Ndufa4l2* (NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, 4-like 2) was strongly induced by hypoxia, as previously reported. Upregulation of this gene is part of the cell's metabolic adaptation to hypoxia (Tello et al., 2011). We also observed an increase in the expression of *Gabra3* (subunit alpha 3 of the gamma-aminobutyric acid [GABA] A receptor). Interestingly, $GABA_A$ receptors have been described to regulate stem cell proliferation (Andäng et al., 2008). We also determined by qRT-PCR the upregulation of two well-described players in the cell's response to hypoxia, namely, *Hif3a*, member of the HIF family of TFs, and *Vegfa*, a potent pro-angiogenic factor. These results suggest that low oxygen levels induce changes in the transcriptome of NSCs that might affect important NSC functions, such as metabolism and proliferation.

To gain more insight into the biological significance of our results, we next applied GSEA to evaluate our microarray data at the level of gene sets. This approach was aimed at identifying pathways and TFs that are activated in NSCs cultured under oxygen concentrations that more closely mimic their normal tissue microenvironment. First, we interrogated for pathway enrichment by using the gene sets from the KEGG pathway database. GSEA-KEGG pathway analysis revealed an enrichment of genes within both the glycolytic and glycogen synthesis pathways in hypoxic NSCs (Figure 1C), which is consistent with a metabolic switch in response to hypoxia (Pescador et al., 2010; Seagroves et al., 2001). Remarkably, several members of the aldehyde dehydrogenase family (*Aldh9a1*, *Aldh1a3*, *Aldh1b1*, *Aldh7a1*, and *Aldh2*) significantly contributed to the enrichment signal in the glycolysis gene set (Figure S2B). Interestingly, the ALDH family of enzymes has been described to be enriched in different types of stem and cancer stem-like cells (Armstrong et al., 2004; Corti et al., 2006; Mao et al., 2013), supporting the notion that physiologic hypoxia might favor a stem cell phenotype. Taken together, these results suggest that the naturally hypoxic microenvironment of NSCs might physiologically support a non-oxidative metabolism that promotes stemness.

Identification of TFs Activated under Physiologic Hypoxic Conditions

The primary goal of the present study was to identify TFs that are active in physiologic hypoxic conditions and thus might be controlling hypoxic NSC behavior. To this end, we performed GSEA using the TFT gene sets. GSEA-TFT revealed that more than 110 TFT gene sets were enriched in hypoxic NSCs, which suggests that TFs controlling expression of these genes might be activated in these conditions. As expected, genes with HIF1-binding sites were enriched in hypoxic NSCs (V$HIF1_Q5, normalized enrichment score [NES] = 1.33, false discovery rate [FDR] = 0.22). We also identified other TFs previously described to be activated by hypoxia in other cell types, such as NF-κB (Rius et al., 2008). Importantly, there is a large number of TFT gene sets with a higher NES than HIF1, thus highlighting their potential role as important regulators of hypoxic NSCs and suggesting that the hypoxic transcriptional response goes far beyond the sole activation of the HIF family of TFs. Such TFs included E2A immunoglobulin enhancer binding factor E12 (E12), GATA binding protein 1 (GATA-1), and activator protein 1 (AP-1) (Figure 1D). Strikingly, E12 is a basic helix-loop-helix (bHLH) TF with a prominent role in the development of the nervous system (Ross et al., 2003). These results robustly validate our transcriptional profiling approach to identify factors that are important in NSC biology by searching for TFT enriched in hypoxic NSCs.

Remarkably, in four of the top 20 TFT gene sets from our GSEA-TFT (E12, AP-1, CP2, and STAT5A), *Nfatc4* was the gene with the highest score, indicating that it was the gene that better correlated with the hypoxic phenotype in these gene sets (Figure 1E). Accordingly, the NFATc4 TFT gene set itself (V$NFAT_Q6) was enriched in hypoxic NSCs (NES = 1.30, FDR = 0.25), indicating that NFATc4 targets also were upregulated by hypoxia (Figure S2C). Taken together, the results from the GSEA-TFT analysis point to NFATc4 as a good candidate to regulate NSC functions.

Functional Characterization of Calcineurin-NFATc4 Signaling in NSC Biology

The activity of the nuclear factor of activated T cell (NFAT) family of TFs is regulated by calcium signaling via calcineurin, a calcium/calmodulin-dependent serine phosphatase. Upon dephosphorylation by calcineurin, NFATs (NFATc1 through NFATc4) shuttle to the nucleus and bind to their target promoters (Crabtree and Olson, 2002; Hogan et al., 2003). In agreement with our GSEA-TFT results regarding NFATc4 (Figure 1E), the GSEA-KEGG analysis identified a positive enrichment of calcium-signaling pathway members in NSCs cultured at 5% oxygen, suggesting that this pathway is activated in hypoxic NSCs (Figure 1C). Therefore, we hypothesized that calcium-calcineurin-NFAT signaling might be activated under physiologic hypoxic conditions and have a relevant role in NSC functions.

To test our hypothesis, we first functionally characterized the role of calcineurin in the neurosphere formation capacity and proliferation of hypoxic NSCs. Using the pharmacological calcineurin inhibitors cyclosporin A (CsA) and

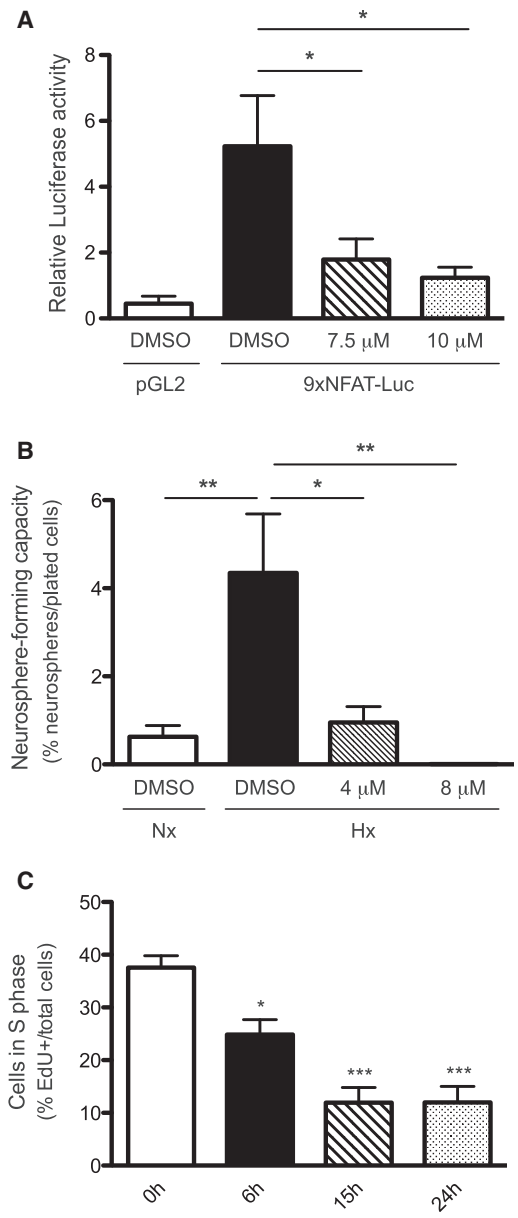


Figure 2. Calcineurin Promotes Neurosphere Formation and Proliferation

(A) NSCs were electroporated with a luciferase reporter gene controlled by nine NFAT-binding sites, together with a renilla expression plasmid to serve as an internal control, and treated with increasing concentrations of CsA plus FK-506 or vehicle control (DMSO). After O/N drug treatment, cells were subjected to a dual luciferase assay. Data are presented as mean ± SEM (n = 3 independent experiments, ANOVA, *p < 0.05).

(B) NSC self-renewal was assessed by neurosphere formation assay in the presence of different concentrations of CsA+FK-506 or vehicle control. Data are presented as mean ± SEM (n = 3 independent experiments, ANOVA, *p < 0.05, **p < 0.01).

(C) NSCs maintained in hypoxia were treated with either CsA+FK-506 (7.5 μM) or vehicle control. Cell proliferation was assessed by EdU incorporation at the indicated time points after drug treatment. Data are presented as mean ± SEM (n = 3 independent experiments, ANOVA, *p < 0.05, ***p < 0.005).

FK-506 (tacrolimus), we inhibited NFAT-dependent transcriptional activity in a dose-dependent manner (Figure 2A). Importantly, CsA plus FK-506 decreased both the neurosphere-forming capacity and proliferation of hypoxic NSCs (Figures 2B and 2C).

Next, we set out to identify what NFAT family member might be controlling NSC properties downstream of calcineurin. We started out by analyzing the subcellular localization of the four NFAT family members that are regulated by calcium: NFATc1 through NFATc4 (Figure 3A). The subcellular localization of NFATc1 was cytoplasmic. NFATc2 was present mainly in the cytoplasm, although it also would faintly stain the nuclei of some cells. NFATc3 displayed both nuclear and cytoplasmic localization. NFATc4 was expressed at high levels and was concentrated heavily in the nuclei of hypoxic NSCs. The specificity of the antibody was tested by peptide competition assay (Figure S3A).

In agreement with the results described in Figure 1E, qRT-PCR data revealed that *NFATc4* mRNA expression was upregulated in hypoxic NSCs (Figure 3B). Western blotting analysis using the NFATc4 antibody also showed an increase in NFATc4 protein levels in hypoxic NSCs (Figure S3B). Expression of the rest of NFAT family members (*Nfatc1* to *Nfatc3*) didn't change between normoxia and hypoxia as assessed by microarray analysis (data not shown) or qRT-PCR (Figure 3B). The latter results were corroborated by our GSEA-TFT analysis, where NFATc1, NFATc2, and NFATc3 TFT gene sets were not significantly enriched in hypoxic NSCs (data not shown). We also validated by qRT-PCR the upregulation in hypoxic NSCs of three NFATc4 target genes, namely, *Trim47*, *Foxp2*, and *Id2* (Figure 3C). Its nuclear localization, together with its upregulation in hypoxia and our GSEA-TFT results (Figure 1E), pointed to NFATc4 as the NFAT family member that might mostly contribute to NSC properties under physiologic hypoxia.

The above results suggest that NFATc4 upregulation by hypoxia depends on transcriptional activation of the *Nfatc4* gene. However, oxygen availability also has been shown to regulate HIF-1 protein levels by promoting its proteasomal degradation in the presence of high oxygen levels and inducing protein stabilization under low oxygen conditions (Semenza, 2001). Therefore, we asked whether NFATc4 also was regulated via this mechanism. To test this, we incubated NSCs maintained under normoxia at 5% oxygen during different periods of time. Western blot analysis revealed that NFATc4 protein levels remained

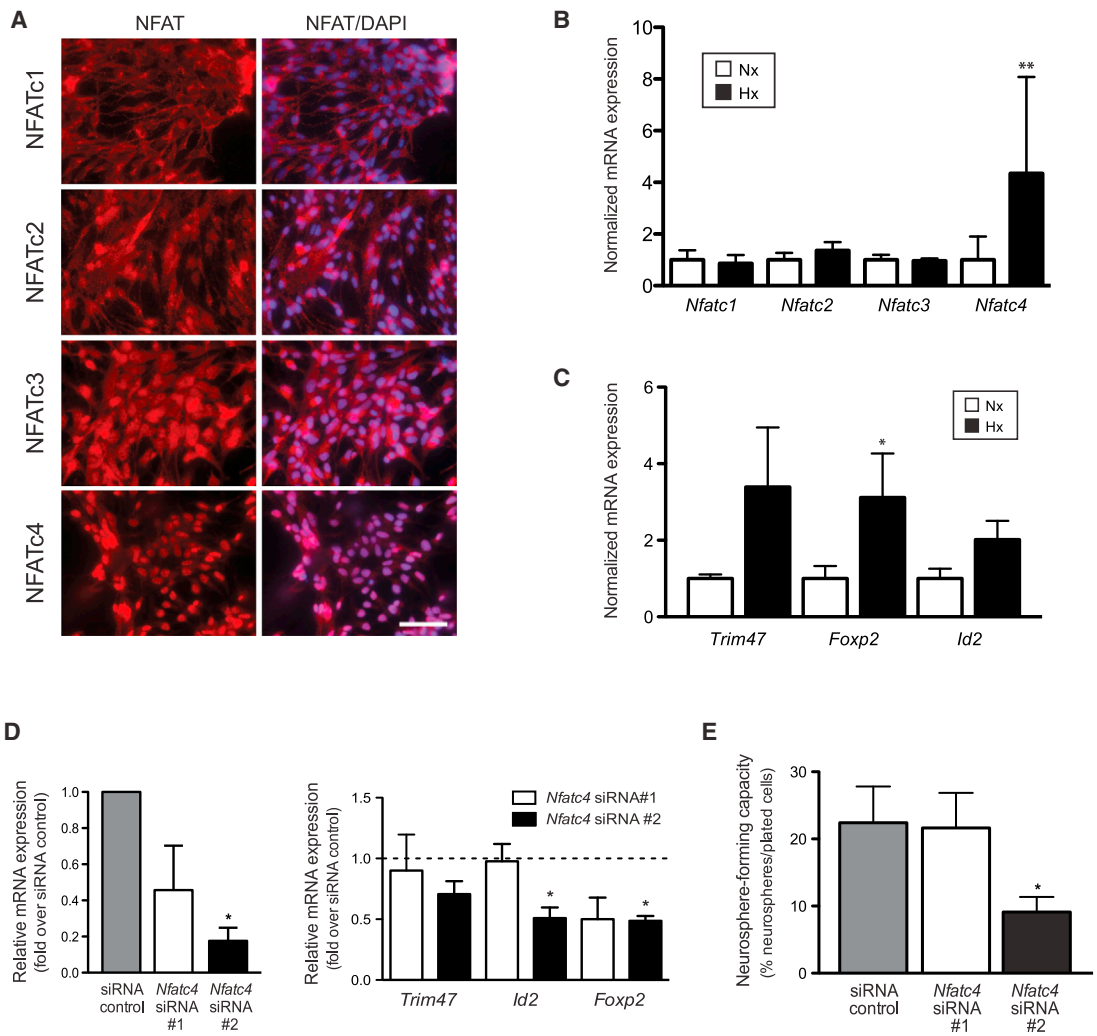


Figure 3. NFATc4 Promotes the Neurosphere-Forming Capacity of NSCs

(A) Immunocytochemistry of NFATc1 through NFATc4 in hypoxic NSCs. Nuclei were stained with a DNA dye (DAPI). Scale bar, 50 μm. See also Figure S3A.

(B) Normalized mRNA expression of *Nfatc1* through *Nfatc4* in hypoxic versus normoxic NSCs. Data are presented as mean ± SEM (n = 4 independent experiments, paired t test, **p < 0.005).

(C) Normalized mRNA expression of NFATc4 target genes in hypoxic versus normoxic NSCs. Data are presented as mean ± SEM (n = 4 independent experiments, paired t test, *p < 0.05).

(D) (Left) *Nfatc4* mRNA expression in hypoxic NSCs electroporated with two different siRNA directed against *Nfatc4* or a control siRNA. (Right) NFATc4 target gene expression in *Nfatc4*-knockdown NSCs (relative to siRNA control-electroporated NSCs). Data are presented as mean ± SEM (n = 3 independent experiments, paired t test, *p < 0.05).

(E) NSCs maintained in hypoxia were electroporated with an siRNA directed against *Nfatc4* or a control siRNA. Viable cells were immediately sorted and plated at low density. Neurosphere formation was assessed 7 days after plating. Data are presented as mean ± SEM (n = 3 independent experiments, paired t test, *p < 0.05).

See also Figures S2 and S3.

unaltered at short time points after the switch to hypoxia. NFATc4 protein was only upregulated after long-term exposure to hypoxia (Figures S3B and S3C), which would better correlate with transcriptional activation of the *Nfatc4* gene rather than an increase in NFATc4 protein stability. To further confirm that NFATc4 protein was not regulated by proteasomal degradation, we treated normoxic cells with the proteasome inhibitor MG132. Inhibition of the proteasome didn't affect NFATc4 protein levels, thereby suggesting that oxygen levels are not regulating NFATc4

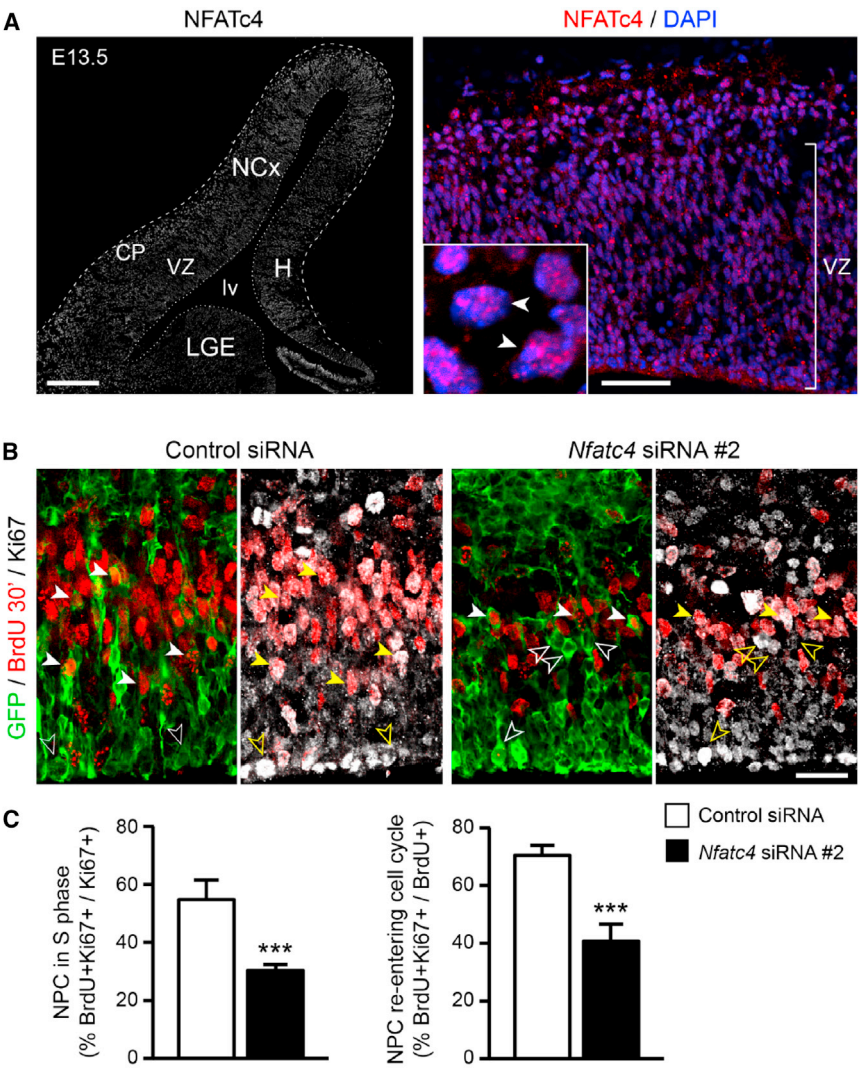


Figure 4. NFATc4 Promotes Self-Renewal and Proliferation of NPCs in the VZ of the Mouse Embryonic Brain

(A) Immunohistochemistry of NFATc4 in the E13.5 mouse telencephalon demonstrating in vivo expression in the neocortex (NCx) and hippocampus (H). Higher-magnification image (right) is counterstained with DAPI and demonstrates strong expression in the germinal layer ventricular zone (VZ), where NFATc4 protein accumulates inside cell nuclei (arrowheads in inset). LGE, lateral ganglionic eminence. Scale bar, 100 μm.

(B) Images of the VZ of E14.5 embryos electroporated at E13.5 with control siRNA or *Nfatc4* siRNA 2, together with *Gfp*-encoding plasmids, and receiving a single pulse of BrdU 30 min prior to analysis. Solid arrowheads point to electroporated (GFP^+, green) NPCs ($Ki67^+$, white) found in S phase of the cell cycle ($BrdU^+$, red). Open arrowheads point to $GFP^+/Ki67^+/BrdU^-$ cells. Scale bar, 20 μm.

(C) (Left) Quantification of proliferating NPCs (cells in S phase) in the VZ of control- or *Nfatc4* siRNA-electroporated mouse brains is shown. (Right) Quantification of NPCs re-entering the cell cycle in the VZ of control- or *Nfatc4* siRNA-electroporated mouse brains is shown. Data are presented as mean + SEM (n = 3 animals and 732–945 cells per group, X^2 test, ***p < 0.001).

See also Figure S4.

expression by controlling protein stability (Figure S3D). Therefore, our results suggest that NFATc4 is upregulated in hypoxic NSCs by transcriptional activation.

To test whether NFATc4 is functionally relevant in the control of NSC functions, we performed NFATc4-silencing experiments using two small interfering RNA (siRNA) directed against different sequences in *Nfatc4* mRNA. *Nfatc4* siRNA 1 achieved a 50% knockdown efficiency, while *Nfatc4* siRNA 2 induced an 80% reduction in *Nfatc4* mRNA expression (Figure 3D, left). Accordingly, some of the NFATc4 target genes also were reduced in *Nfatc4* siRNA-transfected cells. *Foxp2* and *Id2* were two of the NFATc4 target genes most robustly and significantly downregulated (Figure 3D, right). Importantly, *Nfatc4* siRNA 2-transfected NSCs displayed a robust reduction in their neurosphere-forming capacity (Figure 3E), thereby suggesting that NFATc4 promotes NSC self-renewal.

To validate the above results in an in vivo setting, we first studied the expression pattern of NFATc4 in the mouse embryonic brain by immunohistochemistry. We confirmed that NFATc4 protein is present in the nucleus of neural progenitor cells (NPCs) in the ventricular zone (VZ) of E13.5 and E15.5 mouse brains (Figures 4A and S4). It should be noted that NFATc4 expression is not limited to NPCs, but it also is expressed by cells in the cortical plate, where post-mitotic neurons reside. This would be in agreement with previous studies describing a function for NFATc4 in neurons, including survival, dendritic/axonal growth, and synaptogenesis (Benedito et al., 2005; Graef et al., 2003; Schwartz et al., 2009). Next, we further tested the function of NFATc4 in NPCs residing in the neurogenic VZ of the embryonic brain by performing in utero electroporation of *Nfatc4* siRNA 2 into the lateral ventricle of E13.5 mouse brains (Figures 4B and 4C). To analyze the

effect of *Nfatc4* knockdown on NPC proliferation, we measured the number of NPCs in the S phase of the cell cycle (Figure 4B). Control siRNA-electroporated NPCs displayed a higher proliferative rate than *Nfatc4* siRNA-electroporated cells (Figure 4C, left). Furthermore, self-renewal of NPCs was markedly reduced by the knockdown of *Nfatc4*, since the percentage of NPCs that re-entered the cell cycle was significantly higher in siRNA control-electroporated progenitors than in *Nfatc4* siRNA-electroporated cells (Figure 4C, right). These results are in agreement with our results in cultured NSCs, thus indicating that NFATc4 controls NSC proliferation and self-renewal both in vitro and in vivo.

It is worth noting that only *Nfatc4* siRNA 2 had a significant effect on the neurosphere-forming capacity of hypoxic NSCs (Figure 3E). Interestingly, this phenotype correlated very well with the mRNA levels of the NFATc4 target gene *Id2* (Figure 3D, right). Therefore, we might speculate that inhibitor of differentiation/inhibitor of DNA binding 2 (ID2) is a major mediator of the effect of NFATc4 on NSC biology. ID2 is a bHLH TF that preserves the NSC state by promoting self-renewal, proliferation, and preventing differentiation (Jung et al., 2010; Ross et al., 2003; Wang et al., 2001). Accordingly, downregulation of *Id2* might explain the inhibition of neurosphere formation in *Nfatc4*-silencing experiments. We might thus speculate that NFATc4 controls NSC biology by promoting the expression of genes (including *Id2*) that contribute to the maintenance of the NSC phenotype. It is worth noting that, despite its pro-stemness role, ID2 is still present in differentiated cells where it is required for axonal growth of post-mitotic neurons (Lasorella et al., 2006). Importantly, ID2 levels are crucial to determine its function: ID2 levels are very high in stem cells but downregulated in neurons where it no longer contributes to self-renewal and proliferation (Iavarone and Lasorella, 2006). In this scenario, hypoxia-activated NFATc4 might promote *Id2* gene expression to ensure that ID2 levels are high enough to keep NSC stemness and prevent differentiation. Therefore, even a moderate but consistent change in *Id2* levels, as observed in hypoxic NSCs, might have a profound effect on NSC self-renewal. Furthermore, this might provide a plausible explanation to the fact that NSCs cultured in normoxia have an increased tendency to spontaneously differentiate.

Taken together, our results suggest that the calcium-activated calcineurin-NFATc4 pathway promotes NSC proliferation and self-renewal. Along the same lines, there are evidences in the literature suggesting that calcium signaling might promote NSC properties. Purinergic signaling, which activates P2Y receptors and induces intracellular calcium transients, promotes the expansion of NSCs of the same embryonic day as the NSCs in this study (Lin et al., 2007). In this context, we might speculate that P2Y-induced calcium transients activate calcineurin-NFATc4, thereby promoting NSC proliferation. In contrast, an opposed role for NFATc4 has been suggested in adult mouse hippocampal NPCs, where it promotes neuronal differentiation downstream of $GABA_A$ receptor signaling (Quadrato et al., 2014), while not affecting NPC proliferation (Quadrato et al., 2012). NFATs are pleiotropic TFs that may elicit different responses depending on cell type and upstream activators, thus providing a plausible explanation for this difference.

Interestingly, another member of the NFAT family of TFs, NFATc1, has been shown to suppress Wnt signaling, thereby inducing cell-cycle exit and differentiation of NSCs in the neural tube of the chick embryo (Huang et al., 2011). Although NFAT family members often play redundant functions, these results suggest that NFATc1 and NFATc4 play opposed roles in the control of NSC functions. The differential subcellular localization of NFATc1 and NFATc4 in E13.5 mouse NSCs (Figure 3A) might provide an additional layer of regulation to their opposed functions.

Beyond the identification of the calcineurin-NFATc4 axis as a major regulator of NSC biology under physiologic oxygen concentrations, we have undertaken a hypothesis-generating genomic study that might be useful to identify other regulators of NSC biology. Furthermore, our results may have an impact beyond the NSC field, since other stem cell types, including embryonic and somatic stem cells, also reside in hypoxic microenvironments.

EXPERIMENTAL PROCEDURES

Mouse Cortical NSC Culture

All animal procedures were conducted in accordance with the European Union Directive (2010/63/EU) and were approved by the ethical committee for the use of laboratory animals at the University of Barcelona. NSCs were isolated from E13.5 mouse embryos (OF-1 strain) as previously described (Johe et al., 1996). After isolation, cells were immediately cultured at 37°C, 5% CO_2, and either 5% or atmospheric (≈21%) oxygen and maintained in these conditions for two to three passages.

Neurosphere Formation Assay

NSCs maintained in normoxia (21% oxygen) or hypoxia (5% oxygen) were mechanically dissociated at a single-cell level and plated at low density using a cell sorter. In the *Nfatc4*-silencing experiments, NSCs were electroporated with the different siRNAs before sorting and plating. Neurosphere formation was assessed 7 days after plating.

In Utero Electroporation

In utero electroporation was performed as in Borrell et al., (2005). In brief, DNA was injected in the lateral telencephalic ventricle of

E13.5 mouse embryos. BrdU was injected intraperitoneally (i.p.) in electroporated embryos 30 min or 24 hr prior to analysis. Embryos were then fixed, cryosectioned, and stained.

Microarray Analysis

Normalized microarray data were analyzed using paired t test Limma to detect hypoxia differentially expressed transcripts. Principal component analysis was performed with STATA software. Microarray data also were examined by GSEA, using the KEGG pathway gene sets or the TFT gene sets.

ACCESSION NUMBERS

The accession number for the microarray experiments is GEO: GSE68572.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.06.008.

ACKNOWLEDGMENTS

The authors thank Dr. Aryaman Shalizi for his invaluable guidance in the NFAT field and Dr. Jose Aramburu for generously providing the 9xNFAT-Luc reporter plasmid. This work was supported by grants from the Spanish Ministry of Economy and Competitiveness (MINECO) to N.d.l.I. (BFU2009-14616 and RyC-09-389-10-01), V.B. (BFU2012-33473), and C.L. (RYC2010-06789); support from Fundación Ramón Areces to N.d.l.I. (CIVP16A1850); a Marie Curie grant to C.L. (FP7-PEOPLE-2011-CIG); and predoctoral fellowships (FPI, MINECO) to M.M. and V.F. The Instituto de Neurociencias is a "Centre of Excellence Severo Ochoa." This work was co-funded by the European Regional Development Fund (ERDF).

Received: May 29, 2014
Revised: June 29, 2015
Accepted: June 29, 2015
Published: July 30, 2015

REFERENCES

Andäng, M., Hjerling-Leffler, J., Moliner, A., Lundgren, T.K., Castelo-Branco, G., Nanou, E., Pozas, E., Bryja, V., Halliez, S., Nishimaru, H., et al. (2008). Histone H2AX-dependent GABA(A) receptor regulation of stem cell proliferation. Nature *451*, 460–464.

Armstrong, L., Stojkovic, M., Dimmick, I., Ahmad, S., Stojkovic, P., Hole, N., and Lako, M. (2004). Phenotypic characterization of murine primitive hematopoietic progenitor cells isolated on basis of aldehyde dehydrogenase activity. Stem Cells *22*, 1142–1151.

Benedito, A.B., Lehtinen, M., Massol, R., Lopes, U.G., Kirchhausen, T., Rao, A., and Bonni, A. (2005). The transcription factor NFAT3 mediates neuronal survival. J. Biol. Chem. *280*, 2818–2825.

Borrell, V., Yoshimura, Y., and Callaway, E.M. (2005). Targeted gene delivery to telencephalic inhibitory neurons by directional in utero electroporation. J. Neurosci. Methods *143*, 151–158.

Chen, H.L., Pistollato, F., Hoeppner, D.J., Ni, H.T., McKay, R.D., and Panchision, D.M. (2007). Oxygen tension regulates survival and fate of mouse central nervous system precursors at multiple levels. Stem Cells *25*, 2291–2301.

Corti, S., Locatelli, F., Papadimitriou, D., Donadoni, C., Salani, S., Del Bo, R., Strazzer, S., Bresolin, N., and Comi, G.P. (2006). Identification of a primitive brain-derived neural stem cell population based on aldehyde dehydrogenase activity. Stem Cells *24*, 975–985.

Crabtree, G.R., and Olson, E.N. (2002). NFAT signaling: choreographing the social lives of cells. Cell *109*(Suppl), S67–S79.

Cummins, E.P., Berra, E., Comerford, K.M., Ginouves, A., Fitzgerald, K.T., Seeballuck, F., Godson, C., Nielsen, J.E., Moynagh, P., Pouyssegur, J., and Taylor, C.T. (2006). Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. Proc. Natl. Acad. Sci. USA *103*, 18154–18159.

Erecińska, M., and Silver, I.A. (2001). Tissue oxygen tension and brain sensitivity to hypoxia. Respir. Physiol. *128*, 263–276.

Graef, I.A., Wang, F., Charron, F., Chen, L., Neilson, J., Tessier-Lavigne, M., and Crabtree, G.R. (2003). Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. Cell *113*, 657–670.

Hogan, P.G., Chen, L., Nardone, J., and Rao, A. (2003). Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev. *17*, 2205–2232.

Huang, T., Xie, Z., Wang, J., Li, M., Jing, N., and Li, L. (2011). Nuclear factor of activated T cells (NFAT) proteins repress canonical Wnt signaling via its interaction with Dishevelled (Dvl) protein and participate in regulating neural progenitor cell proliferation and differentiation. J. Biol. Chem. *286*, 37399–37405.

Iavarone, A., and Lasorella, A. (2006). ID proteins as targets in cancer and tools in neurobiology. Trends Mol. Med. *12*, 588–594.

Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., and McKay, R.D. (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev. *10*, 3129–3140.

Jung, S., Park, R.H., Kim, S., Jeon, Y.J., Ham, D.S., Jung, M.Y., Kim, S.S., Lee, Y.D., Park, C.H., and Suh-Kim, H. (2010). Id proteins facilitate self-renewal and proliferation of neural stem cells. Stem Cells Dev. *19*, 831–841.

Lasorella, A., Stegmüller, J., Guardavaccaro, D., Liu, G., Carro, M.S., Rothschild, G., de la Torre-Ubieta, L., Pagano, M., Bonni, A., and Iavarone, A. (2006). Degradation of Id2 by the anaphase-promoting complex couples cell cycle exit and axonal growth. Nature *442*, 471–474.

Lehtinen, M.K., Zappaterra, M.W., Chen, X., Yang, Y.J., Hill, A.D., Lun, M., Maynard, T., Gonzalez, D., Kim, S., Ye, P., et al. (2011). The cerebrospinal fluid provides a proliferative niche for neural progenitor cells. Neuron *69*, 893–905.

Lin, J.H., Takano, T., Arcuino, G., Wang, X., Hu, F., Darzynkiewicz, Z., Nunes, M., Goldman, S.A., and Nedergaard, M. (2007). Purinergic signaling regulates neural progenitor cell expansion and neurogenesis. Dev. Biol. *302*, 356–366.

Mao, P., Joshi, K., Li, J., Kim, S.H., Li, P., Santana-Santos, L., Luthra, S., Chandran, U.R., Benos, P.V., Smith, L., et al. (2013). Mesenchymal glioma stem cells are maintained by activated glycolytic metabolism involving aldehyde dehydrogenase 1A3. Proc. Natl. Acad. Sci. USA *110*, 8644–8649.

Mohyeldin, A., Garzón-Muvdi, T., and Quiñones-Hinojosa, A. (2010). Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell *7*, 150–161.

Panchision, D.M. (2009). The role of oxygen in regulating neural stem cells in development and disease. J. Cell. Physiol. *220*, 562–568.

Pescador, N., Villar, D., Cifuentes, D., Garcia-Rocha, M., Ortiz-Barahona, A., Vazquez, S., Ordoñez, A., Cuevas, Y., Saez-Morales, D., Garcia-Bermejo, M.L., et al. (2010). Hypoxia promotes glycogen accumulation through hypoxia inducible factor (HIF)-mediated induction of glycogen synthase 1. PLoS ONE *5*, e9644.

Pistollato, F., Chen, H.L., Schwartz, P.H., Basso, G., and Panchision, D.M. (2007). Oxygen tension controls the expansion of human CNS precursors and the generation of astrocytes and oligodendrocytes. Mol. Cell. Neurosci. *35*, 424–435.

Quadrato, G., Benevento, M., Alber, S., Jacob, C., Floriddia, E.M., Nguyen, T., Elnaggar, M.Y., Pedroarena, C.M., Molkentin, J.D., and Di Giovanni, S. (2012). Nuclear factor of activated T cells (NFATc4) is required for BDNF-dependent survival of adult-born neurons and spatial memory formation in the hippocampus. Proc. Natl. Acad. Sci. USA *109*, E1499–E1508.

Quadrato, G., Elnaggar, M.Y., Duman, C., Sabino, A., Forsberg, K., and Di Giovanni, S. (2014). Modulation of GABAA receptor signaling increases neurogenesis and suppresses anxiety through NFATc4. J. Neurosci. *34*, 8630–8645.

Rius, J., Guma, M., Schachtrup, C., Akassoglou, K., Zinkernagel, A.S., Nizet, V., Johnson, R.S., Haddad, G.G., and Karin, M. (2008). NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. Nature *453*, 807–811.

Ross, S.E., Greenberg, M.E., and Stiles, C.D. (2003). Basic helix-loop-helix factors in cortical development. Neuron *39*, 13–25.

Schwartz, N., Schohl, A., and Ruthazer, E.S. (2009). Neural activity regulates synaptic properties and dendritic structure in vivo through calcineurin/NFAT signaling. Neuron *62*, 655–669.

Seagroves, T.N., Ryan, H.E., Lu, H., Wouters, B.G., Knapp, M., Thibault, P., Laderoute, K., and Johnson, R.S. (2001). Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. Mol. Cell. Biol. *21*, 3436–3444.

Semenza, G.L. (2001). HIF-1 and mechanisms of hypoxia sensing. Curr. Opin. Cell Biol. *13*, 167–171.

Shen, Q., Goderie, S.K., Jin, L., Karanth, N., Sun, Y., Abramova, N., Vincent, P., Pumiglia, K., and Temple, S. (2004). Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science *304*, 1338–1340.

Studer, L., Csete, M., Lee, S.H., Kabbani, N., Walikonis, J., Wold, B., and McKay, R. (2000). Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. J. Neurosci. *20*, 7377–7383.

Tello, D., Balsa, E., Acosta-Iborra, B., Fuertes-Yebra, E., Elorza, A., Ordóñez, Á., Corral-Escariz, M., Soro, I., López-Bernardo, E., Perales-Clemente, E., et al. (2011). Induction of the mitochondrial NDUFA4L2 protein by HIF-1α decreases oxygen consumption by inhibiting Complex I activity. Cell Metab. *14*, 768–779.

Wang, G.L., and Semenza, G.L. (1993). General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc. Natl. Acad. Sci. USA *90*, 4304–4308.

Wang, S., Sdrulla, A., Johnson, J.E., Yokota, Y., and Barres, B.A. (2001). A role for the helix-loop-helix protein Id2 in the control of oligodendrocyte development. Neuron *29*, 603–614.