Cell. Mol. Life Sci. (2011) 68:2831–2844
DOI 10.1007/s00018-011-0723-5

Cellular and Molecular Life Sciences

REVIEW

Hypoxia in the regulation of neural stem cells

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Received: 28 December 2010 / Revised: 8 April 2011 / Accepted: 3 May 2011 / Published online: 17 May 2011

Abstract In aerobic organisms, oxygen is a critical factor in tissue and organ morphogenesis from embryonic development throughout post-natal life, as it regulates various intracellular pathways involved in cellular metabolism, proliferation, survival and fate. In the mammalian central nervous system, oxygen plays a critical role in regulating the growth and differentiation state of neural stem cells (NSCs), multipotent neuronal precursor cells that reside in a particular microenvironment called the neural stem cell niche and that, under certain physiological and pathological conditions, differentiate into fully functional mature neurons, even in adults. In both experimental and clinical settings, oxygen is one of the main factors influencing NSCs. In particular, the physiological condition of mild hypoxia (2.5–5.0% O_2) typical of neural tissues promotes NSC self-renewal; it also favors the success of engraftment when in vitro-expanded NSCs are transplanted into brain of experimental animals. In this review, we analyze how O_2 and specifically hypoxia impact on NSC self-renewal, differentiation, maturation, and homing in various in vitro and in vivo settings, including cerebral ischemia, so as to define the O_2 conditions for successful cell replacement therapy in the treatment of brain injury and neurodegenerative diseases.

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Keywords Neural stem cells · Hypoxia · Neurodegenerative diseases · Ischemia · Neurovascular niche · Oxygen · Human stem cells

Oxygen tension in the central nervous system

In aerobic organisms, oxygen plays an essential role in oxidative metabolism. Oxygen also serves important functions in signal transduction pathways controlling cell proliferation, fate and survival, and in tissue and organ morphogenesis. Since even minor changes in oxygen tension can impact upon cellular physiology and survival, aerobic organisms must maintain their tissues in a state of dynamic equilibrium, called oxygen homeostasis.

Physiological oxygen concentration in tissues is substantially lower than the 20% found in the atmosphere. Tissue normoxia, however, varies widely—from 0.002 to 10%—and is tightly regulated. For example, in human brain, there is a physiological gradient of oxygen which is highest in the alveolar space (14%) and lowest in the tissues, with values ranging from 8% (19–40 mmHg) in the pia down to 0.55% (4.1 mmHg) in the midbrain [1–3]. Large variations in oxygen concentration have been measured over small distances in the dura: 3% (23.2 mmHg) at a depth of 22–27 mm, but 4% (33 mm Hg) at 7–12 mm [1]; these variations illustrate the remarkable specialization of different tissue compartments in the central nervous system (CNS). Moreover, these results illustrate how hypoxia, i.e. a state of reduced tissue oxygenation, is not only a pathological event but also a physiological condition.

In this review, we focus on the role of oxygen tension in the regulation of neural stem cells (NSCs), in both physiological and pathological conditions. We use the term

normoxia to refer to tissue oxygen levels around 8%, mild hypoxia for oxygen levels between 2.5 and 5%, and severe hypoxia for oxygen levels <1%.

NSCs and hypoxia

NSCs are multipotent precursor cells that reside in specialized regions of the fetal and adult CNS; they possess life-long self-renewal ability and, as needed, generate neurons, astrocytes and oligodendrocytes. In vivo, NSCs contribute to tissue homeostasis and repair throughout adulthood [4–6]. When propagated in culture, they form free-floating aggregates called neurospheres and demonstrate the same characteristics of self-renewal and potential to differentiate into functionally mature neural cells. Cultured NSCs represent an important model system for studying neurogenic processes during development and neurobiological mechanisms for maintaining cellular complexity and plasticity. Moreover, these cells are the focus of active study for their potential use in novel cell-based therapies for brain injury and neurodegenerative disease. Indeed, although in physiological conditions NSCs play an important role in CNS cellular homeostasis, there is limited spontaneous recovery after brain damage [7, 8], so the integration of new functional neurons following injury can be achieved by transplanting exogenous cells.

Interesting, though limited, evidence suggests that transplanted NSCs sustain CNS repair through massive cell replacement [9]. Functional recovery by NSC transplantation, however, does not necessarily correlate with the number of transplant-derived terminally differentiated neural cells [10]. Indeed, the transplanted cells may act through alternative mechanisms, with cell replacement playing a minor role. These mechanisms include neuroprotection and reduction of host cell death [11], enhancement of endogenous angiogenesis after stroke [12], immunomodulatory effects on inflammatory damage [10, 13, 14] and scavenging of neurotoxic molecules [15].

Thus far, cells with stem-like properties have been identified in the mammalian CNS, including that of humans, throughout development and adulthood [16–18]. In particular, NSCs have been derived from germinative zones of the brain such as the hippocampal dentate gyrus, olfactory bulb, subventricular zone, subcallosal zone and spinal cord of embryonic, neonatal, and adult rodents [5, 19, 20]. NSCs have also been isolated from the developing and mature human CNS and propagated in vitro in the presence of mitogenic factors or after immortalization with the oncogenes v-myc and large T-antigen [21–23]. These cells retain the capacity to differentiate into neurons, astrocytes and oligodendrocytes [21, 24, 25], and are of particular clinical interest owing to their capacity to follow physiological neurogenic pathways [25]. Experimentation with intracerebral transplantation of NSCs into animal models has helped to individuate strategies to develop pharmacological and cell replacement therapies for different neurodegenerative pathologies [26], including both genetic diseases like metachromatic leukodystrophy, Huntington's disease and sporadic Alzheimer's disease (AD), and idiopathic diseases like Parkinson's disease (PD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and stroke [27].

In the last few years, various studies have shown that culturing NSCs under physiological oxygen tension (mild hypoxia), instead of at atmospheric oxygen, strongly enhances both self-renewal and neurogenic abilities (Table 1). In fact, in different stem cell systems, mild hypoxia activates molecular pathways that regulate Wnt/beta-catenin, Oct4 and Notch signaling, all positive regulators of self-renewal and proliferation [28–32]. Under mild hypoxia, embryonic stem cells (ESCs) [33] as well as somatic adult stem cells, e.g., mesenchymal stem cells [34, 35], hematopoietic stem cells [36] and NSCs [37, 38], all tend to proliferate and self-maintain. Interestingly, severe hypoxia (<1% O_2), which occurs under pathological conditions such as stroke, ischemia or tumor growth [39], has been shown to arrest the proliferation of stem cells and to drive them into quiescence and even apoptosis [33, 37, 40]. Differently, atmospheric culture conditions (20% O_2) lead stem cells into mitotic arrest and differentiation [33, 37, 40].

Hypoxic conditioning of NSC differentiation

In the absence of mitogens, in vitro-cultured NSCs spontaneously differentiate into neurons, astrocytes and oligodendrocytes in proportions that mirror their physiological distribution. In the perspective of using NSCs for cell-mediated therapy of neurodegenerative diseases, it is of paramount importance to be able to direct NSC differentiation toward specific phenotypes. Commitment of NSCs toward specific phenotypes is strongly conditioned by oxygen tension. In particular, 2.5–5% oxygen (mild hypoxia) promotes embryonic rat CNS precursors to generate tyrosine-hydroxylase-positive (TH+) dopaminergic neurons, a neuronal subtype that is lost in PD [41]. Mild hypoxia also promotes the generation and survival of TH+ sympatoadrenal cells from neural crest stem cells [42] as well as the proliferation of ventral midbrain precursors and their differentiation into dopaminergic neurons [38, 43]. On the other hand, severe hypoxia represses neuronal differentiation [44]. Interestingly, even atmospheric oxygen (20%) drives rat cortical neuronal precursors [44] and human post-natal NSCs and oligodendroglial progenitor cells (OPCs) to terminal differentiation [40], while it drives

Table 1 Effects of lowered O_2 on proliferation and differentiation of human neural stem and precursor cells compared to atmospheric O_2 conditions

References	Human cells	Oxygen tension (%)	Effect on proliferation/ self-renewal	Effect on differentiation	Supplemental treatment
Storch et al. [38]	Fetal mesencephalic precursors	3	Increase	Expression of dopaminergic markers (TH, DAT)	±IL1-b/IL11/LIF/GDNF
	Forebrain precursors	3	Modest increase	Not inducible to dopaminergic phenotype	±IL1-b/IL11/LIF/GDNF
Pistollato et al. [40]	Postnatal brain CD133+ nestin+ precursors	5	Increase	17-fold increase of oligodendrocytes	
				Decrease of astroglia	BMP2 gliogenic effect is repressed
				Increase of neurons	
Santilli et al. [37]	Fetal NSC	1	Decrease	Precocious differentiation of neurons and slight increase of astroglial cells	–
		2.5	Increase	Increase of neurons and oligodendrocytes	–
		5	Increase	Increase of neurons and oligodendrocytes	–
Giese et al. [142]	Fetal neural progenitor cells	3	Increase	Differentiation is enhanced and increase of neurons	Epo reproduces the effects of lowered oxygen (increase of metabolic activity and protection from apoptosis)
Pistollato et al. [31]	Medulloblastoma (MDB) cancer stem cells	5	Low oxygen is permissive for MDB-SC expansion	Acute exposure to 20%O2 induces tumor cell death and differentiation	

mouse fetal cortical NSCs and OPCs to apoptosis [45]. Indeed, both severe hypoxia (<1% O_2) and atmospheric oxygen (20% O_2) represent limiting conditions that lead to cell cycle arrest followed by terminal differentiation or apoptosis. We recently demonstrated that, in vitro, mild hypoxia (2.5–5% O_2) enhanced human NSC proliferation and differentiation into neuronal and oligodendroglial cells (Fig. 1), while lower oxygen levels, such as those occurring in cerebral ischemia (1% O_2), caused cell death and enhanced the fraction of quiescent NSCs [37]. In this view, apoptosis and quiescence are physiological states that could represent alternative but complementary strategies adopted by cells as a defense against oxidative damage.

Multiple intracellular pathways are involved in determining cell fate in response to changes in oxygen tension. One of the most important pathways leads to the phosphorylation of the tumor suppressor protein p53 [40, 45]. While 5% O_2 stands in the range permissive for clonal, long-term expansion of mouse cortical progenitors, 20% O_2 is a stringent condition leading to rapid decrease of hypoxia inducible factor 1α (HIF-1α) followed by the activation of p53. Similarly to NSCs, OPCs are sensitive to high levels of oxygen and at 20% O_2 never appear during differentiation of mouse CNS precursors [45]. Two other parameters, namely the intracellular reduction–oxidation (redox) balance and the activity of ataxia telangiectasia mutated (ATM) protein, a kinase that participates in the DNA-damage response, have been implicated in controlling oligodendroglial survival and proliferation. Pharmacologically induced redox variations have been associated with a modulation of the responses elicited by mitogens or differentiating factors in neural precursors [46]. Consistently, human NSCs cultured under 5% O_2 generated 17-fold more oligodendrocytes than they did at 20% O_2, but the dynamic shift from expansion in 5% O_2 to differentiation in 20% O_2 led to a further enhancement of oligodendroglial maturation [40]. We also showed that under mild hypoxia the differentiation-associated apoptosis of human NSCs is ATM-

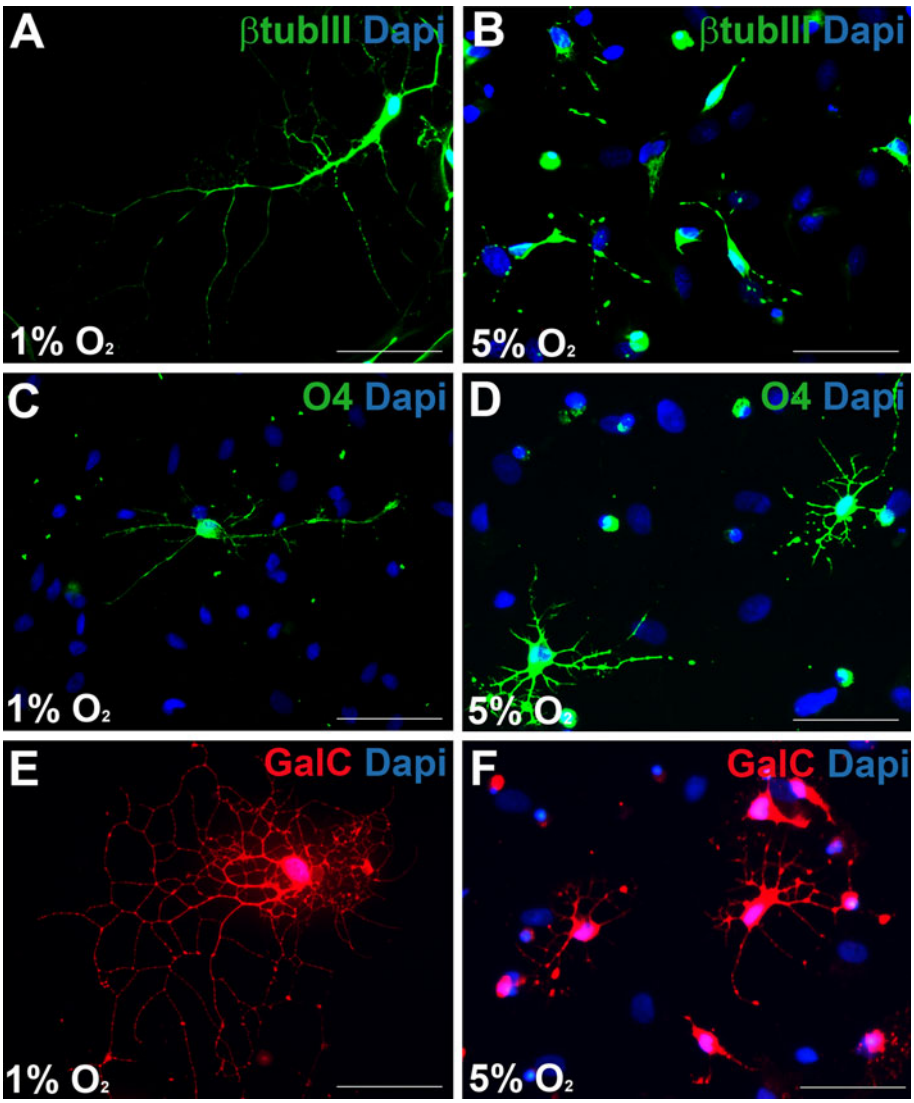

Fig. 1 Hypoxic conditioning of human NSC differentiation Precocious differentiation of human NSCs into neurons and oligodendrocytes in vitro after 17 days of severe hypoxia (1% O_2; **a**, **c**, **e**) and mild hypoxia (5% O_2; **b**, **d**, **f**), as shown by immunocytochemical detection of neuronal markers. **a**, **b** *β*-Tubulin III-positive neuronal cells; **c**, **d** O4-positive oligodendroglial cells; **e**, **f** Galactocerebroside C (*GalC*)-positive oligodendroglial cells (*red*) *Scale bars* 50 μm

dependent, being attenuated by ATM RNA interference [24]. ATM deficiency also appeared to affect the differentiation of oligodendroglial cells and to enhance their sensitivity to increased oxidative stress [47]. Hypoxic preconditioning, consisting of a brief exposure of human neural precursor cells to hypoxic conditions, has been found to markedly enhance survival of human ES cell-derived neural progenitors, a neuroprotective event important especially during the critical acute phase after transplantation in ischemic brains [48, 49]. This strategy thus provides an effective way to optimize cell transplantation therapy.

Altogether, these findings highlight the importance of oxygen homeostasis in regulating different steps in cell fate commitment and maturation and suggest that oxygen tension control may be crucial for treating neurodegenerative diseases. In fact, rapid oscillations in oxygen tension result in severe oxidative damage in a wide array of brain injuries [3, 40, 45, 50, 51].

Recovery from CNS injury: role of the neurovascular niche

Neuroinflammation is one of the main ways the brain responds to infection, disease and injury [52–54]. This complex process, which is a hallmark of neurological diseases including PD, AD, ALS and MS [55], alters the permeability of the blood–brain barrier (BBB), thereby allowing cells from the hematopoietic system to come into contact with diseased neurological tissue [56]. The immune cells, in particular lymphocytes, monocytes and macrophages, respond by eliminating debris and releasing powerful regulatory substances such as complement proteins, cytokines, chemokines, glutamate, interleukins, nitric oxide, reactive oxygen species (ROS) and transforming growth factors [57–61]. These substances have both beneficial and harmful effects on the cellular environment, and can cause further damage [62].

Mature astrocytes are also activated following CNS injury [63, 64], and this is believed to help control the immune response by restoring BBB impermeability and preventing the death of additional neurons [56, 65]. In parallel, NSCs are able to respond to local injury-induced expression of trophic factors and cytokines. Hence, both cell-autonomous and non-cell-autonomous effects are involved in regulating the endogenous response to neurological damage. These responses occur in what is now called the neurovascular niche, i.e. a microenvironment in which the processes of neovascularization and neuroregeneration share dynamic regulatory pathways, both contributing to tissue repair [66, 67].

Of note, following stroke or traumatic brain injury, both angiogenesis and neurogenesis are involved in mobilizing NSCs from the stem cell niche and in inducing them to generate amplifying progenitors that migrate to and differentiate in the lesioned areas. The neurogenic stem cell niches currently recognized in mammalian adult brain, e.g., the subventricular zone and dentate gyrus, are intimately associated with local microvascular terminals and this interplay appears to be finely regulated through oxygen tension and diffusible molecules (e.g., neurotrophins, vascular growth factors, cytokines and nitric oxide) secreted by NSCs and endothelial cells [66, 68–72]. The latter cells, in particular, modulate neurogenesis by secreting nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) [66, 68, 72], ensuring maintenance of the neurovascular niche. Among the different regulatory molecules, VEGF has emerged as determinant for both the recruitment of circulating cells to angiogenic sites [73] and the activation of NSC-mediated neurogenesis [67].

Schmidt et al. [74] observed the accumulation of human NSCs in the proximity of cerebral microvessels upon induction of angiogenesis by infusion of VEGF into murine brain. To further investigate this issue, the researchers evaluated the chemotactic migration of human NSCs after being cultured in vitro with media conditioned by human endothelial cells: NSC chemotaxis was dramatically enhanced by medium from VEGF-stimulated cells compared with that from unstimulated cells. Interestingly, in adult rat brain lesioned by global ischemia, transplanted human NSCs were highly migratory and displayed a remarkable tropism for the lesioned areas and for local microvessels; in contrast, human NSCs transplanted into healthy rat brain remained near the injection site (Fig. 2) [13].

Most mechanisms involved in the regulation of neurogenesis and angiogenesis during inflammatory responses to neurological injury remain to be elucidated. In the complex neurological environment, the gradient of oxygen plays an essential role in regulating the balance between degeneration and regeneration. In a model proposed by Madri [67], after acute hypoxia, NSCs produce nitric oxide which induces endothelial cells to secrete BDNF and VEGF. These two molecules, in turn, stimulate angiogenesis by endothelial cells and activate endothelial nitric oxide synthase, thus amplifying the production of nitric oxide. This cross-talk among endothelial cells and NSCs generates a persistent loop of nitric oxide production that perpetuates the secretion of VEGF and BDNF and, as direct consequence, promotes NSC self-renewal and neurogenesis [68, 70].

Analysis of gene expression profiles in mice revealed that sublethal hypoxia enhances the transcription of genes involved in the development of presynaptic machinery and in angiogenesis, but suppresses the transcription of genes involved in glial and synaptic maturation [75]. These findings suggest the importance that both oxygen and NSC homeostasis have in the maintenance of synaptic plasticity and circuitry in the brain and of the reciprocal signaling between the two components; but, in many instances, the endogenous supply of NSCs is insufficient for complete recovery from hypoxic injury. Hence, the transplantation of exogenous neural progenitor and stem cells is a valid approach for promoting structural and functional recovery, but the pathological inflammation and the transplantation procedure itself can have adverse effects on the success of an NSC graft. Several studies have revealed that adult-derived neural progenitor and stem cells offer neuroprotection by an immunomodulatory mechanism [10, 76, 77]. We recently reported that transplantation of human NSC-derived progenitors into adult rat brain lesioned by global ischemia decreased the reactive astrogliosis and dampened microglial activation in the injured areas [13]. These effects occurred exclusively in the transplanted regions and were most prominent when the inflammatory reaction had reached its nadir. NSC transplantation altered the state of activation of microglia, which shifted from a macrophagic–amoeboid phenotype to a resting stellate one. Also evident was a concomitant shift of astrocytes from a fibrotic and globular appearance to a star-shaped, long-branching morphology in the transplanted areas. Following this immunomodulatory effect, we also observed that transplanted human NSCs were longevous and able to re-establish the disrupted synaptic contacts in the rat hippocampal CA1 layer of lesioned brains. These results documented that grafted neural progenitor and stem cells interact with the host to promote functional recovery; this interaction may have clinical value in the development of NSC-based therapy.

Cerebral ischemia: role of NSCs in recovery

After a cerebral infarct, the interruption of blood flow causes cerebral ischemia and neuronal death, leading to a

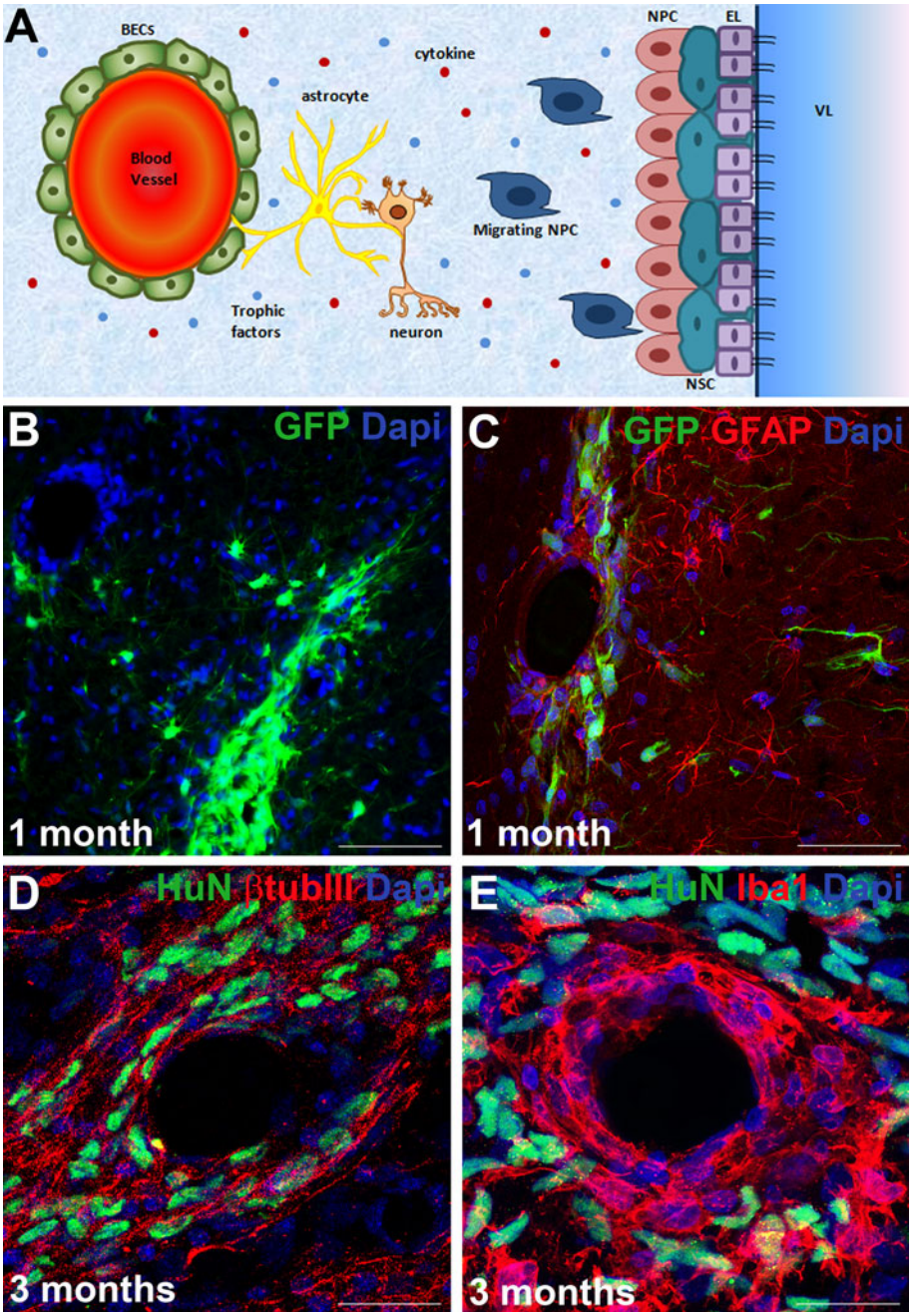

Fig. 2 Homing of neural stem cells in the neurovascular niche. **a** The neurovascular niche. From the neural stem cell niche, NSC-derived progenitor cells (*NPC*) migrate towards blood vessels. Here, differentiated astrocytes come in contact with blood endothelial cells (*BECs*) and neuronal cells: cytokines and trophic factors are important mediators of these multiple interactions. *VL* ventricular lumen, *EL* ependymal layer **b**–**e**). Confocal microscopy analysis showing human NSCs [transduced with a lentiviral vector carrying green fluorescent protein (*GFP*) gene or immunolabeled with anti-human nuclei antibody, (*HuN*), *green*] 1 and 3 months after transplantation into the hippocampal fissure of an adult rat brain lesioned by transient global ischemia. **b** At 1 month, GFP+ human NSCs (*green*) have migrated from the injection site to a small blood vessel. **c** After 3 months, they have reached the endothelial wall and have integrated around the blood vessel, where they come in contact with astroglial cells (stained for glial fibrillary acidic protein, GFAP+, *red*). **d** In proximity to the blood vessel, human NSCs (HuN+, *green*) have differentiated into *β*-tubulin III+ neuronal cells (*red*). **e** Note the high density of microglial Iba1+ cells nested in the endothelial wall and the surrounding area, showing the persistence of an inflammatory environment. *Scale bars* (**b**) 70 μm, (**c**) 63 μm, (**d**) 25 μm, (**e**) 20 μm

series of acute and delayed neurological defects. Currently, no effective treatments are available to delay the progression of injury or restore lost neurons. Both in rodents and humans [37, 78, 79, 80], acute and delayed neuronal death following ischemia occurs through the activation of a complex series of events such as a rapid decrease in adenosine triphosphate levels, calcium release from intracellular stores, loss of calcium homeostasis, excitotoxicity, activation of proteases, arachidonic acid release and metabolism, mitochondrial dysfunction, acidosis and edema [81]. These result in an overproduction of ROS and lead to oxidative damage of cellular components [82–84]). In this toxic environment, the primary endogenous response to free radical-induced injury is the enzymatic defense, consisting in the cooperative action of two classes of intracellular antioxidant enzymes: (1) cytosolic CuZn superoxide dismutase (SOD) and mitochondrial Mn-SOD, which metabolize superoxide anion to hydrogen peroxide; and (2) cytosolic glutathione peroxidase and peroxisomal catalase, which break down hydrogen peroxide [50]. During the blood reperfusion that follows ischemic injury, these endogenous antioxidative defenses as well as the mobilization of NSCs from endogenous pools are likely to be insufficient or, at least, scarcely efficacious to dampen the

neurological damage [85]. Homi et al. [82] reported that, in the early phase of ischemia–reperfusion in rat brain, the decay in catalase and SOD activities was related to the high susceptibility of the hippocampus and striatum to oxidative damage. In the adult rodent brain, global and focal ischemia increases the proliferation of NSCs residing in the subgranular zone of the dentate gyrus, the anterior subventricular zone and the posterior periventricular zone adjacent to the hippocampus [50, 51]. A parallel increase of the migration of NSCs along neurogenic pathways has also been observed [83], but the mechanisms involved are still unknown. Numerous studies have shown that stem cell grafting into ischemic brain can reduce the neurological deficits in experimental models of ischemia [86–90]. Furthermore, studies experimenting with the intravenous injection of immortalized human neural precursors in rat models of focal ischemia [91, 92] and cerebral hemorrhage [93–96] have also shown that NSCs cross the BBB and migrate to damaged brain areas according to preferential "pathotropism" pathways. Transplanted cells differentiated at the lesion site within 8 weeks and thereafter supported progressive functional improvement not observed in non-grafted control animals [91, 92].

More recently, Daadi et al. [86] showed that human ESCs, transplanted in neonatal rodent forebrain 24 h after ischemia, induced axonal sprouting and increased the effectiveness of endogenous recovery mechanisms.

A different yet more robust modulatory action is obtained when NSC transplantation is performed during the subacute phase of ischemia, e.g., within 3–7 days after injury. In this window, a complex series of signals involved in NSC-mediated regeneration is transiently elaborated in situ by the damaged tissue and terminates once the chronic phase ensues, sending NSCs into a quiescent state [51]. Ishibashi et al. [87] showed that, in gerbils, human fetal NSC-derived progenitors transplanted into the caudate nucleus 4 days after ischemia gave rise to neurons, astrocytes and oligodendroglial cells, thereby contributing to restoring synaptic plasticity and improving sensorimotor functions.

The choice of injection site is crucial in correctly targeting NSCs to specific migratory routes. Englund et al. [97, 98] showed that, 14 weeks after injection into the subventricular zone of non-lesioned adult or neonatal rat brain, fetal NSCs had migrated through the rostral migratory stream and to the striatum and hippocampus; yet, when NSCs were injected into the striatum, they differentiated into neuronal cells with no particular migration pattern, and when they were injected into the hippocampus, they generated interneurons and granule-like neurons. Subsequently, Wong et al. [90] reported that, in rodent brain lesioned by global cerebral ischemia, immortalized ESCs transplanted into the striatum 3 days after injury reached the corpus callosum and caudate nucleus within 4 weeks. Moreover, Olstorn et al. [88] showed that human neural progenitors derived from temporal lobe specimens reached the CA1 hippocampal layer when injected above the hippocampus, in the periventricular region of adult rat brain.

We recently showed that, in an adult rat model of global ischemic injury, human NSC-derived progenitors migrated from the transplantation site in the hippocampal fissure to the dentate gyrus and then to the subventricular zone within as few as 14 days, and finally reached the CA3 and CA1 hippocampal layers 4 months later [13]. In these regions, grafted cells generated functionally mature neurons with normal efferent projections and made heterotypic synaptic junctions with host cells. Alternatively, when transplanted above the CA1 layer in the periventricular region, the cells migrated along the corpus callosum and reached the striatal area within 3 months, giving rise to GABAergic and GLUTAmatergic neuronal subtypes according to the appropriate regional specifications [13].

Together, these studies support attempts to develop NSC-based therapy for hypoxic neurological damage. It is believed that this type of therapy represents the most viable approach for rescuing damaged tissue, replacing lost cells, and restoring neurological function after cerebral hypoxic ischemia.

Hypoxia and gene regulation

Exposure of cells to hypoxia initiates a transcriptional response that compensates the metabolic demand with the reduced oxygen availability. The key player of this adaptive response is hypoxia inducible factor (HIF), a protein whose expression and activity are regulated in an oxygen-dependent manner. HIF-1 is a dimeric transcription factor consisting of one constitutively expressed β subunit [99, 100] and one of three O_2-labile α subunits (HIF-1α, HIF2α, HIF3α). HIF α subunits are substrates of a family of prolyl hydroxylase domain enzymes (PHDs) that use oxygen as a cofactor; these enzymes are believed to be the true sensors linking oxygen tension to HIF activity. In the presence of oxygen, PHDs hydroxylate two proline residues in HIF α subunits making them substrates for E3 ubiquitin ligase and targeting them for proteasomal degradation. In severely hypoxic cells (<1% O_2), PHDs are inactive, so HIF α subunits can accumulate and dimerize with HIF-1β, translocate to the nucleus, and bind to hypoxia response elements; this results in the activation of more than 60 genes involved in glycolysis, angiogenesis, cell cycle and survival [99, 100]. Some of these genes code for proteins (e.g., VEGF and erythropoietin) that promote NSC proliferation following ischemic insults to the brain [99, 100]. Erythropoietin, in particular, increases NSC proliferation through activation of NF-κB, a signaling pathway which

also directs NSC fate by upregulating the expression of the transcription factor Mash1 [101].

HIF-1 upregulation and activation have been observed after focal brain ischemia [102], neonatal hypoxia–ischemia [99] and global brain ischemia [143, 144]. HIF-1 has been implicated in mediating neuroprotective gene expression after hypoxic preconditioning and in inducing tolerance to transient and permanent focal cerebral ischemia in mice [100, 103].

The HIF complex also modulates signaling by Notch, a critical regulator of undifferentiated stem and progenitor cells. Accordingly, under conditions of hypoxia, HIF-1α interacts with the intracellular domain of activated Notch, augmenting the Notch downstream response and precluding the terminal differentiation of NSCs [44]. Indeed, a recent study by Pistollato et al. [31] showed that viability and expansion of medulloblastoma stem cells are preserved by hypoxia, through the maintenance of Notch1 in its active form. The molecular link between HIF and Notch thus offers an explanation of how a relatively hypoxic microenvironment like the neural stem cell niche, which promotes cell cycle arrest while avoiding the oxidative stress associated with more well-oxygenated tissue [104–107], facilitates the maintenance of a stem-like state and prevents NSCs from differentiating [108].

The control of "stemness" by oxygen-regulated HIF activity appears to also be related to the capacity of HIF2α and HIF3α to regulate the levels of pluripotency-associated transcription factors NANOG, OCT4 and SOX2 [109, 110].

Oxygen deprivation also paradoxically increases mitochondrial production of ROS, which cause additional intracellular stress on proteins and DNA. Mitochondrial generation of superoxide, which occurs in hypoxic conditions, is currently believed to be the key cytosolic signal that induces the accumulation of HIF-1α subunits [111] by inhibiting PHD activity through an unknown mechanism [112, 113].

HIF-1 complex is also able to inhibit phosphorylation of p53, and thereby inhibit its activity in cell-cycle arrest and apoptosis. Low oxygen tension also promotes phosphorylation of JNK, which upregulates cyclin D1, the latter involved in cell-cycle progression and self-renewal of NSCs. Moreover, stabilization of HIF-1α through pretreatment with the hypoxia-mimic deferoxamine, is neuroprotective against ischemic damage and strongly enhances the therapeutic efficacy of transplanted NSCs in the striatum of adult rats [114].

Effects of hypoxia on mitochondria and cellular metabolism

When oxygen delivery to cells is insufficient for the demand, a limited provision of energy is rapidly offered by anaerobic metabolism, leading to the production of lactic acid. Accumulation of lactic acid in tissues and blood is sign of inadequate mitochondrial oxygenation, which may be due to hypoxemia, poor blood flow (e.g., shock) or a combination of both. If severe or prolonged, hypoxia leads to cell death. In the nervous system, hypoxia is caused by injuries such as stroke and serial microischemic events (as in vascular dementia), which trigger massive acute and progressive neurodegeneration. When oxidative phosphorylation becomes limited, neurons—unlike other cell types—are not able to switch to glycolysis: in this context, neuronal mitochondrial function is essential both for delaying the neurodegenerative process and for re-establishing the metabolic and bioenergetic homeostasis. Mitochondria are responsible not only for energy production but also for Ca^{2+} buffering, maintenance of plasma membrane potential, protein folding by chaperones, axonal and dendritic transport and modulate the release and re-uptake of neurotransmitters at synapses [115–117]. Furthermore, mitochondrial transport, together with the dynamic processes of mitochondrial fission and fusion, facilitate the transmission of energy across long distances, which is particularly important in motor neurons given that axons can extend up to one meter.

Under physiological conditions, where mitochondrial fission allows for mitochondrial renewal, redistribution and proliferation into synapses [116–118], the competing process, mitochondrial fusion, allows mitochondria to interact and communicate with each other, facilitating mitochondrial movement and distribution across long distances and to the synapses in order to provide the appropriate bioenergetic balance [118, 119]. However, excessive mitochondrial fission induces ultrastructural changes, including cristae fragmentation, dilation or vesiculation, and the disappearance of the cristae membranes, leading to the collapse of mitochondria into spherical organelles. Cells constantly adjust the rate of mitochondrial fission and fusion in response to changing energy demands and to facilitate the distribution of mitochondria [120]. We showed that, in post-mitotic differentiated human NSCs under severe hypoxic conditions (<1% O_2) [37], this dynamic equilibrium shifted definitely toward mitochondrial fission; this was correlated with cell death, even if this issue has not been totally resolved [121]. The reason for mitochondrial fission could be that the differentiation of human NSCs into mature neuronal cells requires amounts of energy not available under low oxygen conditions; evidence from our research supports this hypothesis, showing that, at 1% oxygen, differentiating human NSCs finally undergo apoptosis or quiescence, the latter as an ultimate effort to survive (Fig. 3) [37].

In the past few years, several studies have suggested that disruption of mitochondrial function and dynamics

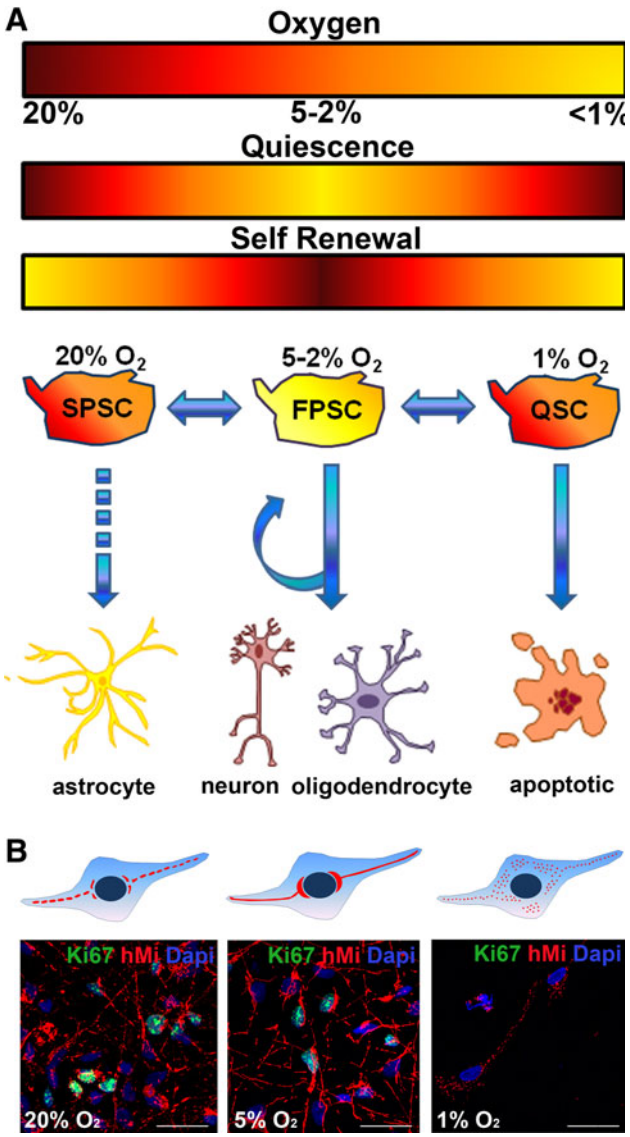

Fig. 3 Mild hypoxia enhances self-renewal of human NSCs and their ability to generate neurons and oligodendrocytes. **a** Model of how changes of oxygen tension from atmospheric levels to severe hypoxia may regulate proliferation, self-renewal and differentiation of NSCs. Under conditions of atmospheric oxygen, human NSCs have a slow proliferation rate (*SPSC*, slowly proliferating stem cells) and a preferential commitment to the astroglial lineage. Mild hypoxia increases the self-renewal of human NSCs (*FPSC*, fast proliferating stem cells) and leads to an enrichment of neuronal and oligodendroglial progenitors. In contrast, severe hypoxia drives human NSCs toward a state of quiescence (*QSC*, quiescent stem cells), characterized by a very slow proliferation rate accompanied by tendency to exit from the cell cycle and to undergo precocious differentiation or apoptosis. **b** Confocal microscopy images of human NSCs at 17 days of differentiation under 20, 5 and 1% oxygen. Note proliferating cells (Ki67+, *green*) and the distribution of mitochondria (HuMi, *red*) at 1% oxygen, where they are aggregated and fixed in small clusters. Conversely, in mild hypoxia (5% O_2), mitochondria appear fused in long homogeneous chains running along the processes and nested in correspondence of the nuclei. In 20% O_2, mitochondria are still fused, but their chains appear shortened and more disorganized than in 5% O_2. *Scale bars* 30 μm

contributes to neurodegenerative diseases [122–124]. In humans, free radicals generated from mitochondria have been implicated in acute brain injuries such as stroke and in neurodegeneration [125, 126].

The CNS has a high rate of oxygen metabolism and, due to its strict aerobic glucose metabolism, is totally dependent on arterial blood flow. Even under physiological conditions, neuronal and glial metabolism produces ROS (2–5% of the electron flow of the respiratory chain in isolated brain mitochondria produces superoxide anion and hydrogen peroxide). A main scavenging enzyme of the superoxide anion is SOD1. Studies in genetically modified mice or rats that overexpress or are deficient in SOD support the role of mitochondrial dysfunction and oxidative stress in neuronal death after stroke and in neurodegenerative disease, including ALS [127]. ALS is a multifactorial disease characterized by a progressive and massive degeneration of motor neurons, likely due to the oxidation and nitration of membrane lipids and neuronal proteins which enhances their sensitivity to the damaging effects of glutamate [128]. Consistently, stroke-induced mitochondrial failure mediates glutamate excitotoxicity at both presynaptic and postsynaptic levels [129]. Mitochondrial dysfunction has been observed in the skeletal muscle of ALS patients [130] and is considered among the possible determinants of ALS progression. In a vicious cycle of events, the mitochondrial membrane potential, fundamental for synthesizing ATP, is perturbed by free radical injury; the ensuing release of cytochrome C into the neuronal cytoplasm activates a cascade of caspases leading to apoptosis [131]. Mitochondria are also involved in mediating neuronal necrosis, since they participate in the dysregulation of cytosolic Ca2+ homeostasis followed by an overproduction of free radicals.

Hypoxia enhances the self-renewal of induced pluripotent stem cells

In the many studies that investigated approaches for cell-mediated therapy of neurodegenerative diseases [132–134], the possibility of graft rejection has emerged as a dramatic limit to the use of exogenous somatic stem cells. Moreover, the generation of teratomas after stem cell transplantation in animal models [135, 136], together with ethical issues, has blocked the use of ESCs in clinical trials [134]. An alternative to using heterologous cell sources is to obtain autologous cells through minimally invasive surgery. Indeed, in the past few years, induced pluripotent stem cells (iPSCs) have been generated from fibroblasts and other somatic cells through the co-expression of four master regulatory genes, namely Oct3/4, c-myc, Klf4 and Sox2 [137–139].

Remarkably, the efficiency of this genetic "reprogramming" of mouse embryonic fibroblasts and human dermal fibroblasts has been shown to be quite low under normoxic culture conditions, but significantly greater under mild hypoxia (5% O_2) [140]. This enhancement was associated with changes in the activity of HIF through the differential activation of transcriptional networks regulating the self-renewal and maintenance of stem cells [109]. Indeed, stabilization of HIF-2α and HIF-3α may increase endogenous expression of transcription factors, such as Oct4, used to generate iPSCs. In human ESCs, knockdown of HIF-2α or HIF-3α, which are stably expressed under 5% oxygen, decreases the expression of Oct4, Nanog and Sox2 pluripotency markers [109]. The same effect is not induced by the knockdown of HIF-1α, which has only a transient nuclear localization under 5% oxygen and which seems likely involved in the initial adaptation of human ESCs to the hypoxic environment. A similar pattern was observed in ESC-derived tumors [141], thus suggesting that HIF-2α and HIF-3α are determinant in the maintenance of stem cell self-renewal and pluripotency under hypoxic conditions.

Conclusions

Oxygen plays an essential role in the maintenance and regulation of NSCs. Severe hypoxia (<1%), which occurs in neurodegenerative conditions like cerebral ischemia, induces human NSCs to go into apoptosis or a quiescent state. Conversely, mild hypoxia (2.5–5% O_2), which represents the in vivo situation of the neural stem cell niche, is optimal for proliferation and neuronal differentiation. The studies performed during the last few years on human NSCs and the optimization of culture conditions under low oxygen tension are important for testing new therapies for neurodegenerative diseases and brain injury. These model systems may ultimately permit high-throughput drug discovery.

Acknowledgements This work was supported by the Italian Telethon grant GGP 10066 (to D.D.) and by the no-profit Foundations Neurothon, Cellule Staminali (Terni, Italy) and Borgonovo, the research Institute Casa Sollievo della Sofferenza (S.Giovanni Rotondo, Italy), Stem Cell Factory (Ospedale S.Maria, Terni), which are funding and providing the necessary support to start up the clinical trial for ALS patients in Italy (information available at the site: http://www.adottaunacellula.org). A special thank you to Luigi Carlessi, Cristina Zalfa, Elena Fusar Poli and Guido Santilli for their precious technical and scientific support. Prof. Angelo Vescovi supported us with important suggestions and critical discussions.

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