Oxygen Tension Within the Neurogenic Niche Regulates Dopaminergic Neurogenesis in the Developing Midbrain

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Oxygen tension is an important factor controlling stem cell proliferation and maintenance in various stem cell populations with a particular relevance in midbrain dopaminergic progenitors. Further studies have shown that the oxygen-dependent transcription factor hypoxia-inducible factor 1 α (HIF-1 α) is involved in these processes. However, all available studies on oxygen effects in dopaminergic neuroprogenitors were performed in vitro and thus it remains unclear whether tissue oxygen tension in the embryonic midbrain is also relevant for the regulation of dopaminergic neurogenesis in vivo. We thus dissect here the effects of oxygen tension in combination with HIF-1 α conditional knockout on dopaminergic neurogenesis by using a novel experimental design allowing for the control of oxygen tension within the microenvironment of the neurogenic niche of the murine fetal midbrain in vivo. The microenvironment of the midbrain dopaminergic neurogenic niche was detected as hypoxic with oxygen tensions below 1.1%. Maternal oxygen treatment of 10%, 21%, and 75% atmospheric oxygen tension for 48 h translates into robust changes in fetal midbrain oxygenation. Fetal midbrain hypoxia hampered the generation of dopaminergic neurons and is accompanied with restricted fetal midbrain development. In contrast, induced hyperoxia stimulated proliferation and differentiation of dopaminergic progenitors during early and late embryogenesis. Oxygen effects were not directly mediated through HIF-1 α signaling. These data—in agreement with in vitro data—indicate that oxygen is a crucial regulator of developmental dopaminergic neurogenesis. Our study provides the initial framework for future studies on molecular mechanisms mediating oxygen regulation of dopaminergic neurogenesis within the fetal midbrain as its natural environment.

Introduction

THE CENTRAL NERVOUS SYSTEM originates from a small number of cells proliferating during early development from the neural plate [1], which reaches a number of hundreds of billions neurons in higher mammals [2]. This process named neurogenesis involves cell division, migration, differentiation, and interaction of cells to form an extremely dynamic, functional network. Focusing on the dopaminergic neurogenesis, midbrain dopaminergic neurons develop from proliferating dopaminergic neural progenitor cells (NPCs) at the ventral midbrain starting at around E10.5 in the mouse brain [3,4]. This process depends on the proper formation of two signaling centers, the isthmus organizer defining the midbrain–hindbrain boundary [5], and the floor plate, which controls the ventral identities [6,7].

A distinct network composed of various morphogens [eg, fibroblast growth factor 8, Wnts, and sonic hedgehog (Shh)] and transcription factors (eg, Nurr1, Lmx1a, Pitx3, En1/2, and Ngn2) controls the process of dopaminergic neurogenesis, including the specification and proliferation of dopaminergic NPCs, as well as dopaminergic neurogenesis and maturation and survival of dopaminergic neurons [8–14].

Besides morphogens and transcription factors, oxygen is another important factor controlling stem cell proliferation and maintenance in various stem cell populations [15–17], with a particular relevance in midbrain dopaminergic progenitors. Low atmospheric oxygen levels of 3% confer long-term proliferation and stem cell maintenance of midbrain dopaminergic NPCs in vitro, including the conservation of their dopaminergic differentiation potential, and avoid senescence in various species, including mouse [18–24]. Further in vitro studies have shown that the oxygen-dependent transcription factor hypoxia-inducible factor 1 α (HIF-1 α) is involved in these processes [15,25,26] and is responsible for the initial adaptive response of cells to oxygen variations [27,28].

HIFs are dimeric transcription factors belonging to a family of environmental sensors known as bHLH-PAS (basic helix-loop-helix-Per-Arnt-Sim) and regulate a large variety of biological processes induced by hypoxic circumstances. In particular, the HIF-1 heterodimer consists of an α subunit (HIF-1 α) produced in the cytoplasm, and the β subunit (HIF-1 β , also known as the aryl hydrocarbon receptor nuclear translocator) is localized in the nucleus. The corresponding genes are normally transcribed and translated

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at high rate, but the deriving proteins are rapidly polyubiquitinated and degraded by the proteasome in the presence of sufficient O₂ levels [29]. Under hypoxic conditions (3%–5%), the HIF-1 α subunit accumulates in the cytoplasm of O₂-starved cells and translocates to the nucleus, where they dimerize with HIF-1 β and then bind to HIF-responsive elements on the DNA, exerting their effect on a large number of genes, including glucose transporters, glycolytic enzymes, and angiogenic factors [30,31].

Dopaminergic progenitor cells reside in a neurogenic niche at the ventral midbrain, where conditions are believed to be hypoxic [32–34]. However, since all studies on oxygen effects in dopaminergic NPCs were performed *in vitro*, it remains unclear whether tissue oxygen tension in the embryonic midbrain is also relevant for the regulation of dopaminergic neurogenesis *in vivo*. To address this issue, we modulated oxygenation of the fetal midbrain by exposing timed pregnant mice with or without HIF-1 α conditional knockout (CKO) to various oxygen tensions during critical phases of dopaminergic neurogenesis. Immunofluorescence staining against pimonidazole as an oxygen-sensing molecule showed that by modulating maternal oxygenation, fetal ventral midbrain oxygen levels are robustly affected, subsequently leading to a modification in dopaminergic neurogenesis: midbrain hypoxia reduced dopaminergic progenitor cell proliferation and is accompanied by restricted dopaminergic neurogenesis. Interestingly, hyperoxic *in vivo* conditions within the neurogenic niche of the ventral midbrain promote proliferation of dopaminergic progenitors and increased dopaminergic neuron output. The absence of HIF-1 α expression does not seem to affect neuronal proliferation directly, but conceivably influences the outcome by controlling angiogenesis, which, in turn, regulates oxygen supply to the tissue.

Materials and Methods

Animals and breeding

For HIF-1 α studies, the Hif-1 α gene was knocked out by crossing HIF-1 $\alpha^{\text{lox/lox}}$ mice [35] (generously provided by Shuhei Tomita, MD, PhD) with a conditional transgenic mouse strain expressing the *Cre* recombinase gene under the control of the promoter of the *Nestin* gene, which is typically upregulated in all neural precursors during embryonic development. Further details on the generation and genotyping of Hif-1 α CKO embryos have been described previously [36]. HIF-1 α CKO embryos and their control littermates (HIF-1 $\alpha^{\text{lox/lox}}$) were used in this study. All animals were maintained on C57BL/6 background. All animal experiments were performed according to the International and Institutional Guidelines for Animal Care (Committee on Animal Experimentation of the TU Dresden) and approved by the Landesdirektion Sachsen (Germany) as the governmental authority.

Oxygen treatment

To investigate the role of oxygen on different stages of dopaminergic neurogenesis in mammals, brains of HIF-1 α CKO and control littermates were examined after exposing pregnant mice to hypoxic (10%) or hyperoxic (75%) conditions. To this end, E10 and E14 timed-pregnant mice were housed in a closed oxygen chamber (InerTec AG, Grenchen, Switzerland) and treated with different atmospheric oxygen

concentrations (10% or 75%) for 48 h. For the normoxic control, pregnant animals were kept at normoxic condition (21%). During the simulation, the oxygen level was controlled by an integrated oxygen probe. Pimonidazole hydrochloride (hypoxypoint; hpi, Burlington, VT) was injected 1 h before sacrificing the animals to detect the hypoxic cells with an oxygen level of <1.1% O₂ within the fetal midbrain.

Bromodeoxyuridine administration

E14 pregnant mice received a single dose of 50 mg/kg bromodeoxyuridine (BrdU; SERVA Electrophoresis GmbH, Heidelberg, Germany) in 0.9% NaCl before oxygen treatment and were sacrificed 48 h later.

Tissue preparation

E12 and E16 embryos were dissected from pregnant mice after oxygen treatment. The embryos or isolated fetal brains were fixed overnight at 4°C with 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4. In view of cryosectioning, the samples were further equilibrated in 30% sucrose in PBS (Carl Roth GmbH, Karlsruhe, Germany) and subsequently snap-frozen and stored at –80°C. Coronal sections of the embryonic brains as well as sagittal sections of the whole embryos were obtained with a cryostat (Leica Microsystems GmbH, Wetzlar, Germany). All sections were cut at 14 μ m and collected on Superfrost Ultra Plus-coated glass slides (Menzel GmbH, Braunschweig, Germany), which were stored at 4°C before proceeding with immunohistochemical analysis.

Immunofluorescence

For immunodetection of midbrain-related neuronal markers in tissue sections, slides were incubated in blocking solution, consisting of 8% donkey serum (Jackson ImmunoResearch, West Grove, IA), 2% Triton X-100 (Thermo Scientific, Rockford, IL), and 1 $\times$ Tris-buffered saline (Dako Real, Glostrup, Denmark), and subsequently treated with the following primary antibodies at 4°C overnight: 1:100 phosphohistone 3 (pH3; Cell Signaling Technology, Boston, MA) produced in mouse, 1:200 tyrosine hydroxylase (TH; Chemicon/Millipore, Billerica, MA) from rabbit, 1:100 Sox2 (Santa Cruz, Dallas, TX) from goat, 1:800 NeuN (Chemicon/Millipore) from rabbit, and 1:100 Nurr1 (R&D Systems, Minneapolis, MN) from goat. Incubation with secondary antibodies was executed the next day with Alexa Fluor antimouse 555, Alexa Fluor antirabbit, Alexa Fluor antirabbit 647, Alexa Fluor antigoat-555, and Alexa Fluor antirat-488 (all from Molecular Probes, Eugene, OR), incubated for 90 min at room temperature. In addition, cell nuclei were stained with Hoechst staining (Invitrogen, Eugene, OR) in PBS. Finally, sections were mounted with Fluoromount G mounting medium (Biozol, Eching, Germany) and stored at 4°C before imaging. For BrdU immunostaining, sections were permeabilized in 1.5 N HCl at 37°C for 30 min and, after the blocking step, incubated overnight at 4°C with 1:200 BrdU (Abcam, Cambridge, UK) from rat. Another type of staining was performed to investigate the connection between oxygen tension and angiogenesis. Therefore, sections were incubated with the primary antibody hypoxypoint-FITC (hpi, Burlington, VT) diluted 1:150 and 1:100 von Willebrand factor antibody (Chemicon/Millipore, Billerica, MA) from rabbit for 2 h followed by incubation for

90 min with 1:500 secondary antibody Alexa Fluor antirabbit 555 (Molecular Probes).

Cell counting

Quantifications of cells were performed from confocal images acquired with a spinning disc confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and processed using Fiji [National Institutes of Health (NIH), Bethesda, MD]. Hypoxic regions were assessed by measuring the area of positive pixels for hypoxypoint using the Fiji plug-in “3D cell counter” and normalized to total midbrain area. Angiogenesis was investigated at medial midbrain sections by counting all positively stained blood vessels in the field of view relative to the determined area of the midbrain (without the area of aqueduct/ventricles).

For the evaluation of the dopaminergic system, Nurr1 positive area was divided into three zones: ventricular zone (VZ), subventricular zone (SVZ), and mantle zone (MZ). The quantification of BrdU⁺ proliferating dopaminergic progenitor cells at the VZ was done in every sixth medial midbrain section. BrdU⁺/Nurr1⁺ double-immunofluorescence cells were calculated to determine the number of recently generated dopaminergic neurons within the SVZ and MZ. All double stainings were additionally confirmed by parallel viewing of the different optic channels using the Fiji software (NIH). For E12 embryos, the number of mitotic (pH3⁺) cells was obtained by counting cells along the Sox2 expressing VZ from a medial midbrain section.

The total number of dopaminergic TH⁺ cells in E12 and E16 midbrain sections was obtained by counting cells of every six sections throughout the entire midbrain using the unbiased optical fractionation method. The analysis was performed using the Zeiss Axioplan 2 microscope with StereoInvestigator software (NIH). The outline of the ventral tegmental area (VTA) and substantia nigra pars compacta (SNpc) (separation was only possible in E16 midbrain) was carried out at 10× magnification and the counting of TH⁺ cells occurred at 40× magnification with oil using a 50×50 μm counting frame. Cells were counted from four age-matched embryos per oxygen condition. For measurement of the heart size, sagittal sections of the whole embryo were stained with hematoxylin and eosin and every seventh section was taken for measurement, starting from the left lateral part of the heart throughout to the right lateral part. The outline of every heart section was carried out at 5× magnification.

Image acquisition and statistical analysis

Data were analyzed by unpaired two-sided *t*-test, one-way analysis of variance (ANOVA), and post hoc *t*-test with Games-Howell correction or two-way ANOVA with Bonferroni's post hoc *t*-test as appropriate using IBM SPSS Statistic software (version 23.0). Values are expressed as mean ± standard error of the mean. All experiments were independently replicated at least four times. Statistical significance was set at *P* < 0.05.

Results

Maternal oxygenation affects fetal brain development without changing heart size

Before we address the function of oxygen on dopaminergic neurogenesis, we initially analyzed the general mid-

brain development in normal E16 embryos, which were previously exposed to different atmospheric oxygen conditions. Therefore, coronal midbrain sections were stained with hematoxylin and eosin for determining the area of medial midbrain sections by stereological measurement (Fig. 1A–C). Interestingly, absolute fetal midbrain area of 10% treated embryos was significantly reduced, whereas a significant increase in midbrain area was observed after treatment with 75% oxygen tension when compared to 21% (Fig. 1B). To address the potential bias of the general size of the embryos, which might depend not only on maternal oxygen tension but also mainly on the actual time point of fertilization (within the 12-h period of mating) and number of embryos per gravidity, we normalized the midbrain size to embryo body weight (Fig. 1C). There was not only an increase in relative midbrain size in the 75% oxygen condition compared to 21% oxygen condition, but also an enlargement of the midbrain in 10% oxygen condition compared to 21% and 75% oxygen conditions. Analyses of the whole brain volume revealed similar results (Fig. 1D, E), indicating that midbrain development reacted similar to alterations of oxygen tension when compared to general brain development [36].

To investigate whether these observed oxygen effects on midbrain development are attributed to a hypotrophy/hypertrophy of the fetal heart or because of differences in blood oxygen saturation, we determined the heart volume and normalized it to the embryonic body weight of all three oxygen groups (Fig. 1A). Interestingly, no differences in heart size were detectable (Fig. 1F), indicating that the oxygen effects are rather local and not systemic.

Different external oxygen environments affect the oxygen levels of the fetal developing ventral midbrain

Oxygen is one of the main regulators of proliferation and maintenance of stem cells, including midbrain dopaminergic NPCs in vitro [15,18,19]. To understand the role of oxygen in dopaminergic neurogenesis, we first investigated the possibility of transmission of maternal hypoxia and hyperoxia toward the developing brains in an in vivo mouse model. To this end, we treated E14 timed pregnant mice with 10%, room air (21%), and 75% of atmospheric oxygen tension for 48 h (until E16) in an airtight plexiglass chamber. We first looked for the presence and distribution of hypoxic regions in normal and HIF-1α CKO medial midbrains by using a specific hypoxic marker (pimonidazole hydrochloride). Hypoxic tissue was detected through immunofluorescence against adducts of pimonidazole hydrochloride, a molecule capable of binding peptide thiols specifically when pO₂ < 10 mmHg (1.1% O₂) [37]. Our data thereby showed that at developmental stage E16, the midbrains exhibited different levels of hypoxic tissue depending on the investigated atmospheric oxygen conditions and HIF-1α CKO genotype (Fig. 2A, B). Using this tool, we were able to further analyze the tissue distribution of hypoxic regions on a subregional level (Fig. 2A, insets): Hypoxic regions of the normal midbrains were concentrated at the level of the VZ surrounding the aqueduct with maternal oxygen treatment affecting mainly the oxygenation of the ventral midbrain VZ, where the proliferative dopaminergic cells reside. Maternal hyperoxia led to less hypoxic tissue within the VZ in comparison to normoxic

FIG. 1. Morphological analysis of the fetal midbrain and heart size after maternal hypoxia or hyperoxia. **(A)** Hematoxylin and eosin staining coronal midbrain sections highlight the altered size of the midbrain areas in 10% and 75% mice at E16. Sagittal sections of E16 hypoxic, normoxic, and hyperoxic fetuses revealed no apparent differences in heart size in consequence of different maternal oxygenations. Scale bar = 500 μm . **(B, C)** Quantification of midbrain area in mice after maternal hypoxia/hyperoxia compared to normoxia. Absolute midbrain size **(B)** and midbrain size normalized to embryo body weight **(C)** are displayed. Statistical significance was evaluated by one-way analysis of variance (ANOVA) and post hoc *t*-test with Games-Howell correction (absolute size: $P < 0.001$, $F = 32.2$; normalized size: $P < 0.001$, $F = 109.1$). $^{\#}P < 0.05$, $^{###}P < 0.001$, data are presented as mean $\pm$ standard error of the mean (SEM) ($n = 4$). **(D, E)** Whole brain volume in mice after maternal hypoxia/hyperoxia compared to normoxia. Absolute brain volume size **(D)** and brain volume normalized to embryo body weight **(E)** are displayed. Statistical significance was evaluated by one-way ANOVA and post hoc *t*-test with Games-Howell correction (absolute volume: $P < 0.001$, $F = 23.4$; normalized volume: $P < 0.001$, $F = 205.3$). $^{\#}P < 0.05$, $^{###}P < 0.001$, data are presented as mean $\pm$ SEM ($n = 4$). **(F)** Quantitative determination of heart volume using stereological technique relativized to the embryonic body weight indicates no significant differences in heart development after different maternal oxygen exposures. One-way ANOVA revealed no significant differences between atmospheric oxygen tensions ($P = 0.549$, $F = 0.6$). Data are presented as mean $\pm$ SEM ($n = 4$).

FIG. 2. Distribution of the oxygen tension within E16 fetal ventral midbrain after several maternal oxygen treatments. **(A)** Representative photographs of hypoxyprobe staining (green) showed the distribution of hypoxic tissue within the E16 normal and hypoxia-inducible factor 1 α (HIF-1 α) conditional knockout (CKO) midbrains at three different levels of atmospheric oxygen tension. Vessels were observed using von Willebrand factor staining (vWF). Scale bar = 500 μ m. *Insets* display the presence of hypoxic tissue within the ventral midbrain after oxygen treatment at higher magnification. Aq, aqueduct; VZ, ventricular zone. **(B)** Quantification of the hypoxic regions of the fetal normal and HIF-1 α CKO medial midbrain by fluorescence intensity measurement of the hypoxyprobe staining. Hypoxic regions were normalized to total midbrain area. Statistical significance was evaluated by two-way ANOVA with atmospheric oxygen concentrations and Hif-1 α genotype as fixed factors (Supplementary Table S1A, B; Supplementary Data are available online at www.liebertpub.com/scd). Displayed are Bonferroni-adjusted *P* values. ###*P* < 0.01, ####*P* < 0.001, and ****P* < 0.001 when compared to control. Data are presented as mean $\pm$ SEM (*n* = 4). **(C)** Proportion of vessels counted in normal and HIF-1 α CKO midbrain sections of mice subjected to 10%, 21%, and 75% atmospheric oxygen treatment, respectively. Statistical significance was evaluated by two-way ANOVA with atmospheric oxygen concentrations and Hif-1 α genotype as fixed factors (Supplementary Table S2A, B). Displayed are Bonferroni-adjusted *P* values. ####*P* < 0.001 and ****P* < 0.001 when compared to control. Data are presented as mean $\pm$ SEM (*n* = 4). Color images available online at www.liebertpub.com/scd

brains. Hypoxic condition, on the contrary, revealed an increase of hypoxic tissue within the dopaminergic neurogenic zone of the ventral midbrain. Analysis of the HIF-1 α CKO samples revealed a dramatic increase of hypoxic tissue throughout the fetal midbrain (Fig. 2A, B). However, on the subregional level, hypoxia within the ventral midbrain VZ

appears to be unchanged by the various atmospheric oxygen conditions in HIF-1 α CKO midbrain. In addition, description of the van Willebrand factor (vWF) immunoreaction highlighted whether oxygen also regulated vascularization (Fig. 2A). vWF binds, in fact, to many blood particles, including coagulation factor VIII, platelets, and collagen exposed in

endothelial cells after blood vessel damage [38]. We measured the vessel density in medial midbrain sections of hypoxic, normoxic, and hyperoxic fetal E16 normal and HIF-1 α CKO mice brains. Under normal conditions, hypoxia induces angiogenesis through activation of HIF-1 α signaling [39]. Therefore, the vessel density in hypoxic normal brains was significantly higher than in normoxic samples. Moreover, we found a significant reduction of angiogenesis in the hyperoxic experimental group most likely because of less activity of the HIF-1 α pathway (Fig. 2C). Our previous *in vitro* studies had shown that the loss of HIF-1 α is accompanied by a significant decrease of vascular endothelial growth factor (VEGF) expression [28]. VEGF is a target gene of HIF-1 and represents an inducer of angiogenesis. Here we show that vascularization of the midbrain is restricted because of the Nestin-Cre-driven CKO of HIF-1 α , which is consistent with the *in vitro* data (Fig. 2C). Indeed, maternal hypoxia and the oxygen deficiency of the fetal midbrain promote angiogenesis in this transgenic mouse model, indicating a HIF-1 α -independent component in blood vessel formation.

Collectively, our observations suggest that maternal oxygen treatment affects the oxygenation of the neurogenic niches of the developing ventral midbrain and that HIF-1 α is a positive regulator of angiogenesis in the fetal midbrain.

Increased progenitor proliferation and generation of dopaminergic neurons in hyperoxic normal midbrain

We then analyzed whether the altered tissue oxygen tension within the low-oxygen environment of the fetal ventral midbrain particularly controls the generation of dopaminergic neurons and whether HIF-1 α promotes this process. Using TH staining, a catecholaminergic neuron marker generally used for the identification of mature dopaminergic neurons, we revealed a significant increase in the total number of TH⁺ cells within the hyperoxic normal midbrain (Fig. 3A, B). Itemization into the two major populations, namely the medially located VTA and the laterally located SNpc, displayed that TH⁺ cells was enhanced in hyperoxia within the VTA only (Fig. 3C, D). In contrast, reduced oxygen tension significantly restricted the generation of dopaminergic neurons in both regions (Fig. 3C, D). Moreover, the dopaminergic system revealed no alterations in the distribution and quantity of TH⁺ neuron by inactivation of HIF-1 α , neither in the hypoxia nor in the normoxia experimental group (Fig. 3B–D). Exclusively, the hyperoxic transgenic mice possessed a similar abundance of TH⁺ neurons within the VTA as the normoxic normal midbrain (Fig. 3C).

These results suggested that not a decrease but an increase of tissue oxygen tension within the neurogenic niche of the ventral midbrain promotes the development of dopaminergic neurons. Due to this interesting finding, we continued our investigations of the dopaminergic neurogenesis exclusively in the hyperoxic fetal midbrain.

To dissect whether oxygen is a stimulus for proliferation and/or differentiation in dopaminergic neurogenesis, we initially performed a birth-dating study using BrdU labeling. BrdU (50 mg/kg) was injected before starting the oxygen therapy (E14) to observe on the one hand proliferative dopaminergic progenitors within the neurogenic VZ of the

ventral midbrain and on the other hand to detect dopaminergic cells within several regions of the dopaminergic system, which were generated during the oxygen challenge (Fig. 4A). First, we quantified the number of BrdU⁺ cells within the VZ of normal and HIF-1 α -deficient fetal midbrains to identify the effect of maternal hyperoxia on the proliferation of dopaminergic progenitor cells and the involvement of HIF-1 α in this process. We found significantly more BrdU⁺ cells resided in the VZ of hyperoxic normal midbrain than in the normoxic normal ventral midbrain (Fig. 4B). HIF-1 α signaling is mainly activated under hypoxic conditions [39], and an increase of tissue oxygen tension by maternal hyperoxia rather inhibits this pathway. The HIF-1 α deletion in all neuroprogenitors of the normoxic fetal midbrain revealed no differences in their proliferation properties in comparison to the normal normoxic midbrain (Fig. 4A, B), indicating that HIF-1 α is not essential for the dopaminergic neurogenesis and that other oxygen-dependent pathway(s) might be more relevant for this process. An increase of tissue oxygen tension using maternal hyperoxia is not ensured because of the lack of vessels in the ventral midbrain of the HIF-1 α CKO brain, and thus, the observed effects of hyperoxia on dopaminergic neurogenesis in the normal midbrain are not reproducible in the HIF-1 α transgenic mouse model (Figs. 3B, C, and 4B).

To identify whether hyperoxia enhanced proliferation that resulted in an increase of the generation of dopaminergic neurons, we investigated the localization of newly generated cells within the dopaminergic domain using BrdU staining (Fig. 5). The dopaminergic system was observed using Nurr1 staining (marker of postmitotic dopaminergic cells) and subdivided it into SVZ comprising Nurr1⁺/TH[−] postmitotic immature dopaminergic cells and the MZ containing mature Nurr1⁺/TH⁺ expressing neurons [32]. Double staining of BrdU and Nurr1 was used to evaluate the number of newly generated dopaminergic cells in both regions. Interestingly, we could demonstrate that maternal hyperoxia led to a significant increase of recently generated immature and mature neurons in the fetal midbrain, which should have originated from the dopaminergic progenitor cells of the VZ during oxygen challenge (Fig. 5).

Together, these data suggest that higher oxygen tension within the hypoxic microenvironment of the VZ induces elevated neurogenesis of dopaminergic cells.

Hyperoxia induces increased dopaminergic neurogenesis at early developmental stages

To elucidate whether dopaminergic neurogenesis at earlier developmental stages is also susceptible to manipulation by hyperoxia, we placed E10 timed pregnant mice in an oxygen chamber with 75% oxygen for 48 h (until E12). We used pH3 as a proliferation marker that revealed a significantly elevated number of Sox2⁺ progenitor cells in M-phase in the VZ of the hyperoxic ventral midbrain (Fig. 6A, B). Analysis of TH expression by immunohistochemistry showed a significant increase in the total number of TH⁺ dopaminergic cells in hyperoxic embryos at E12 (Fig. 6C).

Thus, we can conclude that maternal hyperoxia also enhances dopaminergic neurogenesis during early stage of brain development, and dopaminergic progenitor cells are sensitive to alterations of tissue oxygen tension within the VZ of the fetal midbrain.

FIG. 3. Maternal hyperoxia promotes the generation of dopaminergic neurons in E16 mouse ventral midbrain. **(A)** Expression of tyrosine hydroxylase (TH) in E16 normal and HIF-1 α CKO medial midbrain sections at different oxygen exposures. The TH⁺ area was subdivided into ventral tegmental area (VTA) and substantia nigra pars compacta (SNpc) (dotted lines). Scale bar=200 μ m. **(B)** Bar plot represents the total number of TH⁺ cells in the fetal midbrain of normal and HIF-1 α CKO mice subjected to 10%, 21%, and 75% atmospheric oxygen treatment, respectively. Statistical significance was evaluated by two-way ANOVA with atmospheric oxygen concentrations and Hif-1 α genotype as fixed factors (Supplementary Table S3A, B). Displayed are Bonferroni-adjusted *P* values. [#]*P*<0.05, ^{###}*P*<0.001, and ^{**}*P*<0.01 when compared to control. Data are presented as mean $\pm$ SEM (*n*=4). **(C, D)** TH expressing area was split into VTA (C) and SNpc (D) and the distribution of the TH⁺ cells was quantitatively estimated in these regions of both genotypes. Statistical significance was evaluated by two-way ANOVA with atmospheric oxygen concentrations and Hif-1 α genotype as fixed factors (Supplementary Tables S4A, B and S5A, B). Displayed are Bonferroni-adjusted *P* values. ^{##}*P*<0.01, ^{###}*P*<0.001, and ^{**}*P*<0.01 when compared to control. Data are presented as mean $\pm$ SEM (*n*=4). Color images available online at www.liebertpub.com/scd

Discussion

Normal development of the midbrain central dopaminergic system is controlled by an orchestrated network of transcription factors and morphogens [8–14]. Besides this network, oxygen signaling emerged as another crucial factor controlling stem cell proliferation and maintenance in various stem and progenitor cell populations, including dopaminergic

NPCs [15–17]. Since oxygen effects on dopaminergic NPCs were studied exclusively *in vitro*, it is still unclear whether tissue oxygen tension in the embryonic midbrain is also relevant for the regulation of dopaminergic neurogenesis *in vivo*. The detailed understanding of the factors regulating proliferation and dopaminergic differentiation of NPCs in their natural environment is not only of general interest but also resembles an important prerequisite for improving

FIG. 4. Hyperoxia results in an increase of proliferating dopaminergic progenitors at E16. **(A)** Immunohistochemistry for bromodeoxyuridine (BrdU) (white), Nurr1 (green), and NeuN (red) in normoxic and hyperoxic ventral midbrains of normal and HIF-1 α CKO mice at E16 to observe dopaminergic progenitor cells within the VZ surrounding the aqueduct (Aq). Scale bar = 50 μ m. **(B)** Quantification of BrdU⁺ cells in the VZ of the fetal normal and HIF-1 α CKO ventral midbrain after different oxygen exposures for 48 h. Statistical significance was evaluated by two-way ANOVA with atmospheric oxygen concentrations and Hif-1 α genotype as fixed factors (Supplementary Table S6A, B). Displayed are Bonferroni-adjusted P values. ### P < 0.001 and *** P < 0.001 when compared to control. Data are presented as mean $\pm$ SEM (n = 4). Color images available online at www.liebertpub.com/scd

FIG. 5. Increased cell production in the subventricular zone (SVZ) and mantle zone (MZ) of the dopaminergic system during maternal hyperoxic treatment. **(A)** BrdU labeled immature dopaminergic cells in SVZ and mature dopaminergic cells of the MZ are presented using BrdU (green) and Nurr1 (red) double staining of E16 medial midbrain sections. Scale bar = 50 μ m. **(B, C)** Quantification of BrdU⁺/Nurr1⁺ cells in the SVZ and MZ of hyperoxic and normoxic groups. Statistical significance was evaluated by unpaired two-sided t -test. # P < 0.05, data are presented as mean $\pm$ SEM (n = 4). Color images available online at www.liebertpub.com/scd

FIG. 6. Hyperoxia affects the development of the dopaminergic system at an earlier stage of dopaminergic neurogenesis (E12). **(A)** Representative images show the expression pattern of phosphohistone 3 (pH3)/Sox2/TH in hyperoxic and normoxic ventral midbrain at E12 after 48 h of maternal oxygen treatment. Scale bar = 100 μ m. **(B)** The number of pH3⁺ cells was counted along the Sox2⁺ VZ and illustrated in a histogram. Statistical significance was evaluated by unpaired two-sided *t*-test. ^{##}*P* < 0.01, data are presented as mean $\pm$ SEM (*n* = 4). **(C)** Quantitative analysis of newly generated dopaminergic (TH⁺) cells in response to the hyperoxic treatment. Statistical significance was evaluated by unpaired two-sided *t*-test. ^{##}*P* < 0.01, data are presented as mean $\pm$ SEM (*n* = 4). Color images available online at www.liebertpub.com/scd

transplantation and/or future regenerative approaches by enhancing/initiating endogenous dopaminergic neurogenesis for restoration in Parkinson's disease and other disorders of the dopaminergic system.

Numerous *in vitro* studies have previously shown that the oxygen condition is the main regulator for the proliferation and differentiation of isolated NPCs. In particular, low oxygen promotes their stemness and potential to differentiate into dopaminergic lineage [15,18,19]. Therefore, the majority of *in vivo* oxygen studies were performed under hypoxia and in adult rodents to stimulate the neurogenesis in the SVZ of the lateral ventricles and the subgranular zone in the hippocampal dentate gyrus, which represent the remaining neurogenic regions within the adult brain [40–43]. One part of the present work was to evaluate also the hypoxic effect on the dopaminergic neurogenesis of the fetal midbrain *in vivo*. Interestingly, we showed that external hypoxia application hampered the generation of dopaminergic neurons in the fetal midbrain and is accompanied with a restricted midbrain development, indicating that decreased tissue oxygenation is not beneficial for the neurogenic potential of the fetal midbrain.

Recently, Porzionato et al. have shown that hyperoxic exposure also affects the proliferation and survival of NPCs in

the main neurogenic sites of the postnatal brain [44]. However, specific analysis of hyperoxia on dopaminergic neurogenesis in prenatal brain is still lacking. The present study was the first to address the effect of maternal hyperoxia on fetal midbrain development. We showed that maternal exposure to hyperoxia increases the oxygen tension within the neurogenic niche of the developing ventral midbrain and stimulates the proliferation of dopaminergic progenitors during early and late embryogenesis. It is certain that dopaminergic NPCs generate immature dopaminergic cells, which migrate further into the SVZ and subsequently differentiate into mature dopaminergic neurons of the MZ [32]. Our hyperoxic embryos displayed increased number of immature and mature dopaminergic cells, which were generated during the oxygen challenge. This resulted in a significant increase of total dopaminergic TH⁺ neurons within the VTA. Thus, these findings document that relatively increased oxygen tension within the developing ventral midbrain is a stimulator of dopaminergic progenitor proliferation and neurogenesis.

We further demonstrated that maternal oxygenation affects the size of the fetal midbrain similar to that of the whole brain. The absolute fetal midbrain and whole brain size with its continuous enlargement from 10% to 75%

maternal oxygen condition correlate with the gain of cell proliferation with increasing maternal oxygen tension [36]. However, from the quantification of the dopaminergic cell counts (refer to Fig. 3), it is clear that the changes in dopaminergic cell counts are not only responsible for the increased midbrain size and other yet unknown factors but also cell types must contribute to the oxygen effects on midbrain size. In contrast, the analyses of relative organ size revealed that maternal oxygenation again affects the size of fetal midbrain similar to whole brain volume but at constant heart volume, indicating differential sensitivity to oxygen effects of brain compared to other tissues. Interestingly, not only the relative (mid)brain size of the 75% oxygen condition but also that of 10% oxygen-treated animals is enlarged compared to those of the normal 21% oxygen condition. The factors mediating these differential effects remain enigmatic and need future investigations.

HIF-1 α is an important regulator in normal embryonic brain development as homozygous HIF-1 α knockouts led to embryonic lethality at E11 resulting from defects in angiogenesis and neural-fold closure [45,46]. CKO of HIF-1 α exon 2, which is required for heterodimerization of HIF-1 α and HIF-1 β subunits and DNA binding, resulted in decreased dopaminergic cell counts in the adult midbrain (*substantia nigra*) as well as reduced proliferation and dopaminergic differentiation capacities of NPCs in vitro [25,47]. These data in conjunction with our results showing no direct involvement of HIF-1 α in the development of the central dopaminergic system in all oxygen conditions in vivo strongly suggest a role of HIF-1 α in mediating postdevelopmental regeneration and/or survival of dopaminergic neurons [25]. However, other reasons for the discrepancies in the morphology of the dopaminergic system between the two HIF-1 α CKO models could be the different genetic manipulation (knock out of exon 2 as used by Milosevic et al. [25] and exon 13–15 in the present study [35]) or differences in the genetic background.

Our in vivo data support the conclusion that the low oxygen atmospheric culture (3%) represents rather the physiological environment of the nervous tissue (average 4.6% O₂) than the so called hypoxic condition, because the observation that hyperoxia stimulates dopaminergic neurogenesis fits to previous in vitro studies, showing that proliferation and dopaminergic differentiation capacity of isolated midbrain dopaminergic NPCs depend on optimized oxygen levels: oxygen conditions of 3%–5% enhance long-term expansion and reduce spontaneous differentiation and cell senescence of dopaminergic progenitors in vitro [18–20,48]. However, severe hypoxia (<1% O₂) as well as normoxia (21% O₂) represses their proliferation and promotes spontaneous differentiation [17,34]. By using an oxygen tension marker (hypoxyprobe), we showed that maternal hyperoxygenation led to an increase of oxygen tension in the dopaminergic neurogenic niche of the midbrain VZ from below 1.1% in maternal normoxic and hypoxic conditions (tissue positive for hypoxyprobe staining) toward levels above this threshold in hyperoxic animals (tissue negative for hypoxyprobe staining). This higher oxygen tension likely represents the in vitro condition of 3%–5% O₂ and thus stimulates the proliferation and differentiation of dopaminergic progenitors during early and late embryogenesis in vivo. There are no in vivo oxygen markers available that are applicable in small laboratory animals to detect changes in oxygen levels in the range of 5%–10%.

After showing that mild hyperoxia regulates cell proliferation and differentiation of dopaminergic neural progenitors, we further elucidate the regulatory role of oxygen in the early stages of mammalian dopaminergic neurogenesis. Maternal hyperoxia and subsequent hyperoxia within the midbrain tissue stimulate the proliferation of NPCs along the ventral midbrain and affect the subsequent dopaminergic neuron development from E10 to E12. This finding suggests that oxygen tension within the ventral midbrain at earlier stages is one major regulator of the quantity of dopaminergic neurogenesis.

Together, our results strongly support the notion that increased oxygen levels within the midbrain neurogenic niche induce dopaminergic neurogenesis during midbrain development. Thereby, these data indicate that oxygen is a crucial regulator of developmental neurogenesis. Interestingly, we have further shown that maternal oxygenation also affects the relative size of fetal (mid)brain at constant heart volume, indicating differential sensitivity of brain tissue toward oxygen effects compared to other tissues. Longer oxygen maternal administration (>3 days) was not tolerated by the animals. Maternal and fetal susceptibility to hyperoxia for more than 3 days is known also for other rodents [49]. Analysis of the effects of various oxygen tensions on midbrain neurogenesis in long-term experiments seems thus not possible.

In the present study, we aim to dissect the direct effects of oxygen on dopaminergic neurogenesis without effects of reoxygenation. However, further studies are warranted to investigate whether the observed changes in dopaminergic neurogenesis translate into functional changes of the dopaminergic system in the postnatal and/or adult brain. Detailed knowledge of the composition of the midbrain neurogenic niche harboring dopaminergic NPC capacity will eventually help to develop restorative strategies for neurodegenerative diseases such as Parkinson's disease by both recruitment of endogenous progenitor cells from the niche [50–52] and improvement of dopaminergic cell graft. Our study, therefore, provides the initial framework for future studies on the molecular mechanisms mediating oxygen regulation of dopaminergic neurogenesis within the midbrain neurogenesis as its natural environment.

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Author Disclosure Statement

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