Redox Biology 13 (2017) 82–93

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

Decreased neural precursor cell pool in NADPH oxidase 2-deficiency: From mouse brain to neural differentiation of patient derived iPSC

Zeynab Nayernia[a], Marilena Colaianna[a], Natalia Robledinos-Antón[e], Eveline Gutzwiller[a], Frédérique Sloan-Béna[f], Elisavet Stathaki[f], Yousef Hibaoui[b], Antonio Cuadrado[e], Jürgen Hescheler[c], Marie-José Stasia[d], Tomo Saric[c], Vincent Jaquet[a], Karl-Heinz Krause[a,*]

[a] Department of Pathology and Immunology, University of Geneva Medical School, 1-rue Michel Servet, 1211 Geneva, Switzerland
[b] Department of Genetic Medicine and Development, University of Geneva Medical School, 1 rue Michel Servet, 1211 Geneva, Switzerland
[c] Center for Physiology and Pathophysiology, Institute for Neurophysiology, Medical Faculty, University of Cologne, Cologne 50931, Germany
[d] Université Grenoble Alpes, Techniques de l'Ingénierie Médicale et de la Complexité- Grenoble, F38000 Grenoble, France
[e] Instituto de Investigaciones Biomédicas "Alberto Sols", Faculty of Medicine, Autonomous University of Madrid (UAM), Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain
[f] Hôpitaux Universitaires de Genève HUG, Laboratoires de Cytogénétique Constitutionnelle, Service de Médecine Génétique, Geneva, Switzerland

ARTICLE INFO

Key words:
NOX2
Reactive oxygen species
Adult neurogenesis
in vitro neural differentiation
Neural stem/progenitor cells
Induced pluripotent stem cells (iPSC)

ABSTRACT

There is emerging evidence for the involvement of reactive oxygen species (ROS) in the regulation of stem cells and cellular differentiation. Absence of the ROS-generating NADPH oxidase NOX2 in chronic granulomatous disease (CGD) patients, predominantly manifests as immune deficiency, but has also been associated with decreased cognition. Here, we investigate the role of NOX enzymes in neuronal homeostasis in adult mouse brain and in neural cells derived from human induced pluripotent stem cells (iPSC). High levels of NOX2 were found in mouse adult neurogenic regions. In NOX2-deficient mice, neurogenic regions showed diminished redox modifications, as well as decrease in neuroprecursor numbers and in expression of genes involved in neural differentiation including *NES*, *BDNF* and *OTX2*. iPSC from healthy subjects and patients with CGD were used to study the role of NOX2 in human in vitro neuronal development. Expression of NOX2 was low in undifferentiated iPSC, upregulated upon neural induction, and disappeared during neuronal differentiation. In human neurospheres, NOX2 protein and ROS generation were polarized within the inner cell layer of rosette structures. NOX2 deficiency in CGD-iPSCs resulted in an abnormal neural induction in vitro, as revealed by a reduced expression of neuroprogenitor markers (*NES*, *BDNF*, *OTX2*, *NRSF/REST*), and a decreased generation of mature neurons. Vector-mediated NOX2 expression in NOX2-deficient iPSCs rescued neurogenesis. Taken together, our study provides novel evidence for a regulatory role of NOX2 during early stages of neurogenesis in mouse and human.

1. Introduction

There is increasing evidence for the role of reactive oxygen species (ROS) in developmental processes, such as cardiomyogenesis [33,7,8], hematopoiesis [48,49], and neurogenesis [14,30,42,54,59,66,67]. This role of ROS might at least in part be due to a regulatory role in stem cell homeostasis and differentiation. Several redox-sensitive signaling pathways have been described, including H_2O_2-dependent protein phosphatase inhibition, as well as redox-regulation of transcription factors and ion channels [19].

There is a wide range of possible cellular sources of ROS, including mitochondria, xanthine oxidase, lipoxygenase, cyclooxygenases, cytochrome P450, nitric oxide synthase and peroxisomes [23]. In addition to these systems that produce ROS as a side reaction, there is the NOX family of NADPH oxidases (NOXs), for which production of ROS is the main biological function [3,32]. Enzymes of the NOX family include the phagocyte NAPDH oxidase NOX2, as well as more recently discovered isoforms (NOX1, NOX3-5, and DUOX1-2). NOXs are highly regulated enzymes, with some of the isoforms (NOX1-NOX4) functioning in a subunit-dependent manner. For example, NOX2 functions as a complex containing at least five subunits ($p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$, and small Rac GTPases) [29]. Loss of function mutations in any of the constituents of the NOX2 complex leads to the genetic disorder chronic granulomatous disease (CGD). CGD patients predominantly show an

* Correspondence to: Dept. of Pathology and Immunology, Centre Médical Universitaire, 1, rue Michel-Servet, CH-1211 Geneva 4, Switzerland.
E-mail address: Karl-Heinz.Krause@unige.ch (K.-H. Krause).

http://dx.doi.org/10.1016/j.redox.2017.04.026
Received 8 February 2017; Received in revised form 19 April 2017; Accepted 20 April 2017
Available online 24 April 2017
2213-2317/ © 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).

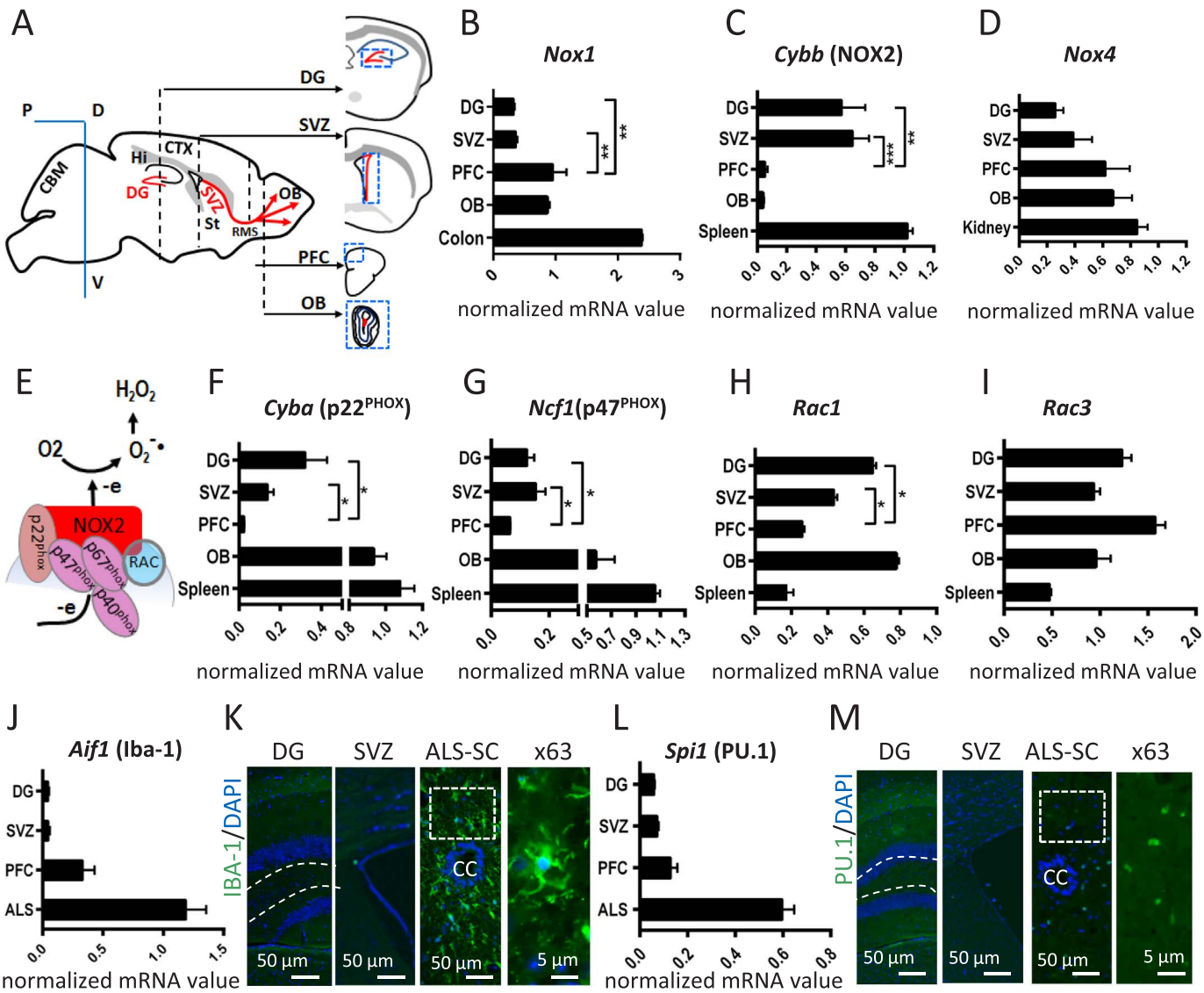


Fig. 1. NOX2 and subunits, but not microglia markers, are expressed in neurogenic regions of the mouse brain. (A) Schematic representation of micro-dissected adult mouse brain areas (dashed blue lines) including the neurogenic regions dentate gyrus (DG) and subventricular zone (SVZ) and the non-neurogenic regions olfactory bulb (OB) and prefrontal cortex (PFC). CBM: cerebellum, Hi: hippocampus, St: striatum, RMS: rostral migratory stream. (B-D, F-I) mRNA expression of NOX isoforms and NOX subunits in micro-dissected brain regions. The following murine tissues were used as positive control; colon (*Nox1*), spleen (*Nox2*), and kidney (*Nox4*). (E) Schematic representation of the *Nox2* complex including its subunits *Cyba* ($p22^{phox}$), *Ncf1* ($p47^{phox}$), *Ncf2* ($p67^{phox}$), *Ncf4* ($p40^{phox}$) and *Rac*. (J, L): expression of microglia markers *Aif-1* (IBA-1) and *Spi-1* (PU.1), in different brain regions as assessed by RT-qPCR (J, L) or by immunofluorescence (K, M). ALS = spinal cord of mice overexpressing $SOD1^{G93A}$ mutant (a model for amyotrophic lateral sclerosis, ALS) served as a positive control for high levels of microglial gene expression [51]. For RT-qPCR, expression values were normalized to housekeeping genes and data are shown as mean ± SEM. Statistics were done by Student's *t*-test from $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***} < 0.001$.

immunological phenotype, including susceptibility to infection, hyper inflammation and autoimmunity [55]. However, there is also evidence for a role of NOX2 in the physiological function of the central nervous system (CNS): NOX2-deficient mice [26] and CGD patients [45] have a discreet, but consistently observed cognitive impairment. Thus, the question to which extent NOX2 plays a role in the physiological function of the CNS is pertinent.

Basically there are two main concepts to explain how ROS might be involved in the physiological function of the CNS: modulation of the function of mature neurons [12,24,25,27,31,43,4,52,58,61] versus impact on neural development and neurogenesis [14,30,31,59]. These two concepts are not mutually exclusive and the nature of ROS regulation might depend on the brain area and neural cell types. For instance, RNAseq studies in the murine cerebral cortex demonstrate that under physiological conditions, NOX2 is mostly expressed in microglia, while NOX4 is mostly found in endothelial cells [68]. However, several recent publications suggest that NOX2 is highly expressed in neural progenitor cells (NPCs) in murine adult neurogenic regions: the subventricular zone (SVZ) of the lateral ventricle and the dentate gyrus (DG) of the mouse hippocampus [30,60,67]. These regions contain adult neural stem cells (NSCs) and differentiate into neural progenitors which migrate, mature and are finally integrated into various brain regions. Adult neurogenesis is not only involved in brain repair, but regulates several physiological functions, in particular memory [13].

A recent review on the role of redox environment in neurogenic development [44] summarized the present knowledge as follows. The cellular redox state during neurogenesis and neural differentiation controls specific mechanism of lineage progression through control of signal transduction and transcriptional regulation. More specifically, redox-dependent mechanisms regulate NSC proliferation, cell cycle exit, and differentiation into mature neurons and glia. Oxidizing species accumulate during early stages of differentiation, while a reductive environment persists during terminally differentiated neural cells. The

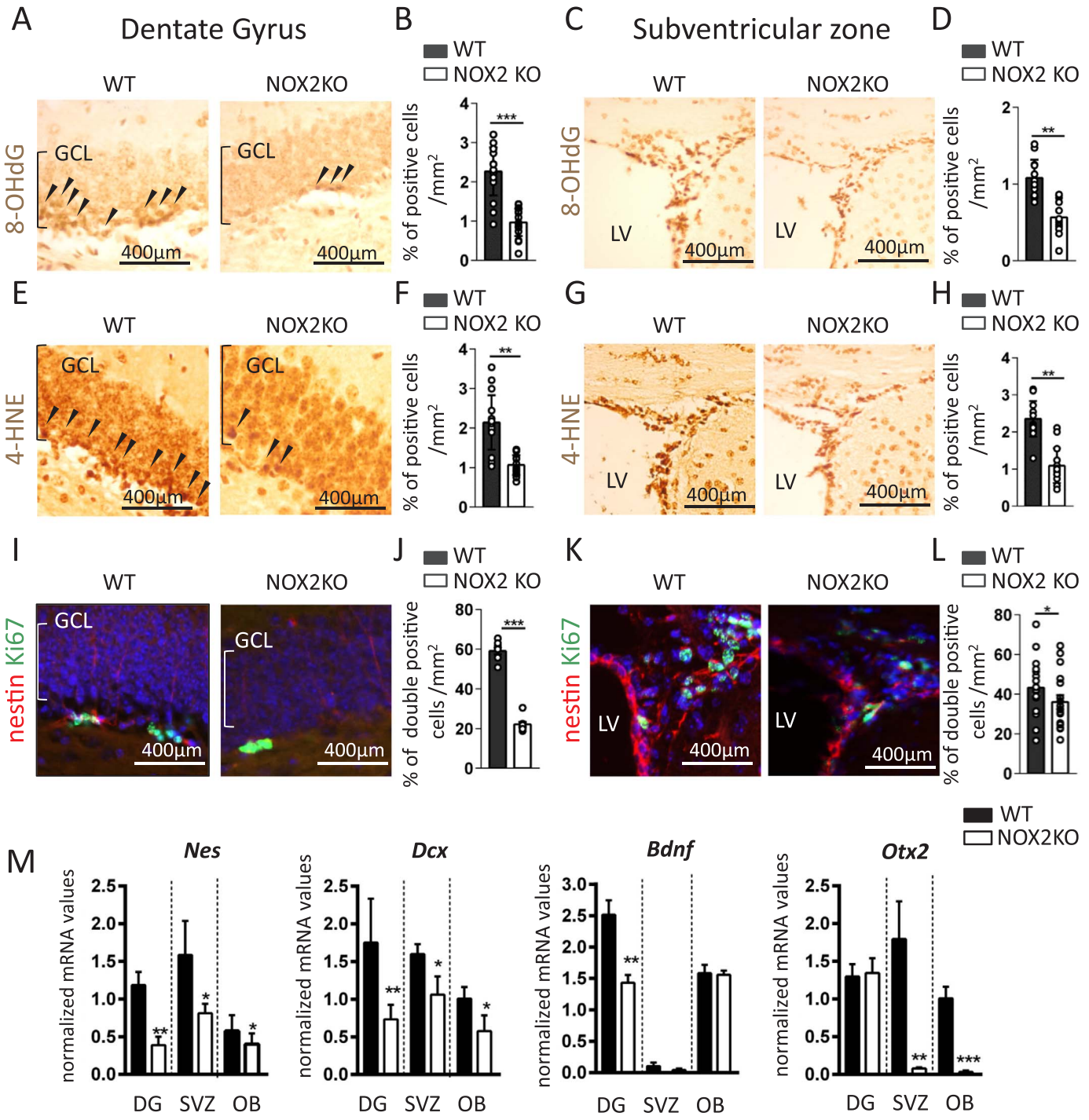


Fig. 2. Decreased oxidative modifications and proliferating neuroprecursor cells in neurogenic brain regions of NOX2-deficient mice. Oxidative modifications of DNA (8-hydroxydeoxyguanosine, 8-OHdG in Figure A, C) and lipids (4-hydroxynonenal; 4-HNE in Figure E, G) were detected using DAB immunohistochemistry in sections of the neurogenic mouse brain regions. The SVZ and DG from wild type (WT) and NOX2-knock-out mice were analyzed. Arrows in panel A and E indicate intensely stained cells in the subgranular zone (SGZ) of the dentate gyrus. (B, D, F and H) Quantification of 8-OHdG and 4-HNE DAB staining are shown as bar graphs as % of positive cells per analyzed area as analyzed by Metamorph software (see Material and Methods). Immunofluorescence staining of proliferating progenitor cells in DG (I) and SVZ (K) of wild type and NOX2 KO mice. IF markers: Nestin (red); Ki67 (green); DAPI (blue). Quantification of immunofluorescence staining (% of double-positive cells/total cells) in the DG (J) and SVZ (L). Data are shown as mean ± SEM. Statistics were performed using Student's *t*-test from 8 animals per condition. (M) RT-qPCR analysis of *Nes* (nestin), neuroblast marker *Dcx* (doublecortin), *Bdnf* (brain derived neurotrophic factor) and *Otx2* transcription factor in neural stem/progenitor cells of DG and SVZ. Data are shown as mean ± SEM. Statistics done by Student's *t*-test from n =7. *P < 0.05, **P < 0.01, *** < 0.001.

generation of certain neurotrophic growth factors also appears to be under redox control [47]. For example, there is a redox-regulation of brain-derived neurotrophic factor BDNF [39,53], which contributes to the self-renewal and survival of neural stem/progenitor cells during neurogenesis, influencing memory and cognition [46,5,64]. These findings on the role of redox-dependent processes in neurogenesis raise the question whether NOX enzymes play a role during early stages of neural differentiation.

While hitherto the study of neurodevelopmental processes was mostly restricted to animal models, the availability of human pluripotent stem cells provides now powerful tools to study the mechanisms underlying cellular decisions during neural differentiation in a human

system [15,56]. And, while there are many fundamental developmental mechanisms shared between mice and humans, there are also considerable differences. For example, preclinical mouse studies did not predict the teratogenicity of thalidomide while transcriptomic and proteomic investigation of the compound using human embryonic stem cells (hESCs) clearly indicated that thalidomide impacts several key developmental human genes at clinically relevant concentrations [40]. This could be due to differences in the metabolism of the compounds.

In this study we have investigated the role of NOX2 during neurogenesis in mice and in human induced pluripotent stem cells (hiPSCs). For this purpose, we investigated brains from NOX2-deficient mice, as well as in NPCs and neurons differentiated from iPSCs derived from NOX2-deficient and $p22^{phox}$-deficient CGD patients. We show that NOX2 is highly expressed in neurogenic regions of the mouse brain, as well as in NPCs derived from hiPSCs. NOX2 deficiency or inhibition decreases oxidative modifications in neural stem/precursor cells, reduces the size of the neuroprogenitor pool, and impacts down-stream neural differentiation. Correction of NOX2 expression in CGD-hiPSCs was associated with the restoration of the expression to near normal levels of crucial genes involved in neurogenesis. Our results provide further evidence for a role of NOX2 in neurogenesis, unrelated to the well-known function of the enzyme in inflammation and host defense.

2. Results

2.1. NOX expression and activity in neurogenic regions of the adult mouse brain

To examine the expression and function of NOX enzymes and their subunits in neurogenic regions [1], we first investigated the subventricular zone (SVZ) and the dentate gyrus (DG) isolated from adult mouse brain (see Material and methods). In the SVZ, proliferating NPCs are lining the lateral ventricles from where they migrate towards the olfactory bulb and differentiate into granular interneuron cells [35,36,38]. In the DG of the hippocampus, the sub-granular zone (SGZ) contains precursor cells, which proliferate and migrate along the granular cell layer to differentiate into hippocampal granule cells [9,2,28] (Fig. 1A).

We studied the expression of the different NOX isoforms in microdissected neurogenic regions (see *Methods*) (Fig. 1B-D and Fig. S1D and S1E). NOX1 and NOX4 expression was relatively low in neurogenic brain regions, but showed higher levels in the prefrontal cortex (PFC) and the olfactory bulb (OB). No relevant expression in neurogenic regions was observed for *Nox3* and the two *Duox* genes (Fig. S1D). In contrast, *Cybb* (NOX2) was markedly higher in both neurogenic regions as compared to PFC and OB (Fig. 1C). Similarly, *Cyba* ($p22^{phox}$) and *Ncf1* ($p47^{phox}$) as well as GTPase Rac1were significantly higher expressed in DG, SVZ and the olfactory bulb (Figs. 1F, 1G and 1H), while there was no distinct pattern for *Ncf4* ($p40^{phox}$) and *Ncf2* ($p67^{phox}$) or for other GTPase Rac isoforms including Rac2 and Rac3 (Figs. 1H and 1I, and Fig. S1E). Note that the NOX2 enrichment in the neurogenic regions is considerable as it approaches levels comparable to those observed in spleen.

The CNS phagocyte microglia is the most relevant NOX2 expressing cells in the brain and its presence might provide an explanation for the high NOX2 levels observed in neurogenic regions [50]. We therefore investigated microglia markers in the SVZ and the DG. The microglia marker *Aif1* (IBA-1) was expressed only at very low levels in neurogenic regions (Figs. 1J and 1K). Similarly, the transcription factor *Spi1* (PU.1) which is highly expressed in microglia and other types of phagocytes [3] was found at only very low levels in neurogenic regions (Figs. 1L and 1M).

To assess the relative contribution of NOX2-derived ROS in murine neurogenic areas, we investigated the levels and localization of oxidative modifications of DNA (8-OHdG; 8-hydroxyguanosine) and of lipids (4-HNE; 4-hydroxynonenal). Analysis of both markers revealed elevated staining localizing to cells within SVZ and DG (Fig. 2A-H). In the granular cell layer of the DG, the staining intensity of 8-OHdG was strongest in the lower layer, i.e. the SGZ, which is enriched in proliferating NPCs (Figs. 2A and 2B, arrow heads).

Staining for 8-OHdG and 4-HNE was markedly decreased in NOX2-deficient mice as compared to wild-type DG (Figs. 2A and 2E, left vs. right panels) and SVZ (Figs. 2C and 2G, left vs. right panels). Quantification of staining showed lower levels of 8-OHdG (Figs. 2B and 2D) and 4-HNE (Figs. 2F and 2H) in NOX2-deficient mice suggesting that NOX2 contributes to oxidative modifications in specific subsets of cells of SVZ and DG. Thus, a significant fraction of oxidative modifications in the neurogenic areas can most likely be attributed to NOX2-derived ROS. The residual oxidative modifications might be due to low levels of other NOX isoforms (see Fig. S1D) or to distinct cellular sources, such as mitochondria and xanthine oxidase.

In order to assess proliferation in NOX2 deficient mice, we analyzed the proportion of nestin and Ki-67 double positive cells in the DG (Figs. 2I and 2J) and the SVZ (Figs. 2K and 2L). As shown in Fig. 2I-L, the proportion of nestin-Ki67 positive cells is significantly lower in the DG and the SVZ from NOX2-deficient mice suggesting that NOX2 deficiency is associated with a reduced proliferation of NPCs in adult neurogenic regions.

We next analyzed mRNA levels of a panel of neuronal and neural stem/progenitor markers [41] using qPCR in the two neurogenic regions as well as in the OB, which is the projection zone of SVZ. These results showed a clear impact of NOX2-deficiency: mRNA levels for nestin (expressed in NSCs and NPCs), doublecortin (*Dcx*) (expressed in young migrating neurons), *BDNF* (a soluble factor regulating neurogenesis), *Otx2* (neurogenesis transcription factor), as well as *NeuN* (marker of mature neurons) were significantly decreased in NOX2-deficient regions (Fig. 2M and S1F)[34]. Note, however, that there were regional differences. While *Otx2* was massively decreased in the SVZ of NOX2-deficient mice, there was no impact on expression in the DG. Conversely, while there was a significant decrease of BDNF in the DG of NOX2-deficient mice, BDNF was below detection levels in the SVZ (as described previously [20] (Fig. 2M). No impact of NOX2 deficiency was observed on mRNA levels of the glutamatergic marker *mGluR*, as well as the GABAergic markers *Calb1* (*Calbindin)* and *Gad67* (Fig. 2G). Interestingly, in the OB global neuronal and NPC markers (*Nestin*, *Otx2*, *Dcx*, *NeuN*) were also decreased, despite the fact that NOX2 levels in this region are low (Fig. 2M and S1F). Taken together these results demonstrate a functional impact of NOX2 deficiency not only on neural stem/precursor cells as well but also on the olfactory bulb, a mature neural region.

2.2. Proliferation deficit of NPCs derived from the neurogenic niche of NOX2 deficient mice

To asses further whether NOX2 affects the stemness of NPCs, we isolated NPCs from the neurogenic niches of WT and NOX2-deficient pups (P0 to P4). Relatively high level expression of NOX2 was observed in the dissected regions (Fig. S2A) but, unlike the adult neurogenic niches, the microglia markers IBA-1 and PU.1 were present (Fig. S2B; note, however, that the microglia markers disappeared during neurosphere formation, Fig. S2C). The dissected tissues were dissociated into single cells and allowed to proliferate and form primary neurospheres [33] (Fig. 3A, B). A serial dilution assay [11] indicated a decrease in the number of formed neurospheres after 7 days at every dilution tested in the NOX2-deficient cultures, (Fig. 3C). To analyze the cellular composition of the neurospheres, we performed immunocytochemical staining for nestin. The number of nestin-positive cells was strikingly reduced in NOX2-deficient neurospheres (Fig. 3D, E), strengthening the idea of a decreased NSC pool in the absence of NOX2. The proliferation rate of NSCs was evaluated using EdU pulse labeling assay. A decrease in the percentage of EdU-positive cells in NOX2-deficient neurospheres was observed at 2, 4 and 8 h after incubation with EdU compared to WT

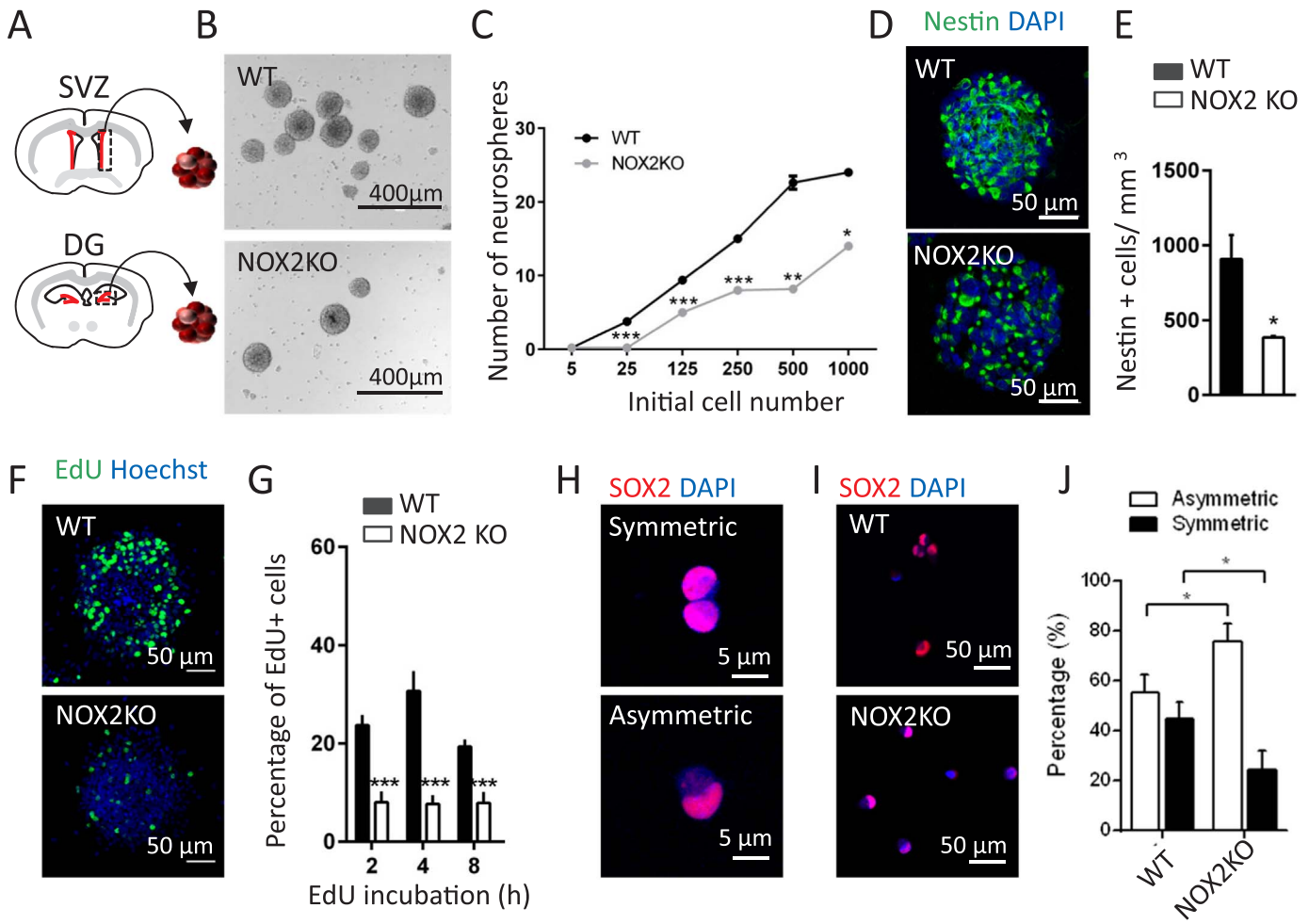


Fig. 3. Impaired proliferation and altered balance in symmetric/asymmetric divisions of NSCs from NOX2-deficient mice. (A) Schematic representation of generation of neurospheres from subventricular zone (SVZ) and dentate gyrus (DG). (B) Light microscope pictures of the neurospheres derived from the SVZ of WT and NOX2-deficient mice (NOX2-KO). (C) Dilution assay and quantification of neurosphere generation as a function of the initial number of seeded cells demonstrates decreased proliferation of NOX2-deficient neural stem/precursor cells. (D) Immunostaining of nestin within neurospheres. (E) Quantification of nestin-staining in neurospheres (n = 6) from D. (F) Representative image of EdU-labeled cells in neurospheres after 8 h of incubation from WT and NOX-deficient mice; (G) Quantification of EdU incorporation after incubation of neurospheres during 2, 4 and 8 h. (H) Cell pair assay with SOX2 staining allows visualizing symmetric and asymmetric division of the cells; (I) representative image of SOX2 staining of WT and NOX2-deficient neurospheres (J). Quantification of symmetric and asymmetric divisions in neurospheres from WT and NOX2-KO as percentage of dividing cells. Data were analyzed by Student's *t*-test and shown as mean values ± SEM.*P < 0.05, **P < 0.01, *** < 0.001.

neurospheres (Figs. 3F and 3G). The decreased number of proliferative cells could be caused by an imbalance in the symmetric/asymmetric divisions. An increase of asymmetric divisions would lead to a decrease in the pool of undifferentiated NSCs and to an increase in the number of differentiated cells. To analyze this, we performed the cell-pair assay, showing that asymmetric divisions (Fig. 3H-J) are more prominent in NOX2-deficient cultures suggesting that lack of NOX2 leads to a bias towards asymmetric cell divisions.

Taken together, these results suggest that NOX2 deficiency diminishes self-renewal of NPCs through symmetric divisions, affecting the maintenance of stem cells pool.

2.3. Immunolocalization of NOX2 in Macaca fascicularis brain

To our knowledge, there are no useful antibodies to perform immunohistochemistry of NOX2 in mouse tissues (available antibodies lack specificity and sensitivity). However, there are very good immunohistochemistry-compatible antibodies against human NOX2. In particular, there is the monoclonal 7D5 antibody, which has also been shown to specifically recognize NOX2 in other primates [65]. We therefore performed immunohistochemistry of the DG in the Macaca fascicularis (macaque) brain (Fig. S3). DCX immunostaining (young migrating neurons) showed a moderately intense, but clearly discernable staining in the DG, with a preferential staining of the SGZ (Fig. S3A). Immunostaining of NOX2 revealed a remarkably intense staining throughout the granular cell layer (GCL). A weaker staining of NOX2 could also be found in some adjacent areas. Although the exact nature of these NOX2-expressing cells is unknown, immunohistochemistry in the macaque brain confirms that the high expression of NOX2 in the DG is not limited to rodents, but is also observed in primates.

2.4. Role of NOX2 during neural differentiation of human iPSCs

To study the effect of NOX2 deficiency on human neurogenesis, we generated iPSCs from two different types of CGD patients, X-linked NOX2-deficient patient (X°-CGD) and an autosomal-recessive $p22^{phox}$-deficient patient (AR-22°-CGD) [6]. Two iPSC lines from healthy donors (control-iPSCs) and the human ESC line H1 (H1-hESCs) were used as control lines. As expected for cells that have acquired a pluripotent state, these iPSCs showed typical morphology of hPSC colonies, expressed markers of pluripotent cells and differentiated in vivo and in vitro into the lineages derived from three embryonic germ-layers mesoderm, ectoderm and endoderm (Fig. S4). We also confirmed that, like the parental fibroblasts, the X°-CGD-iPSCs harbored the nonsense

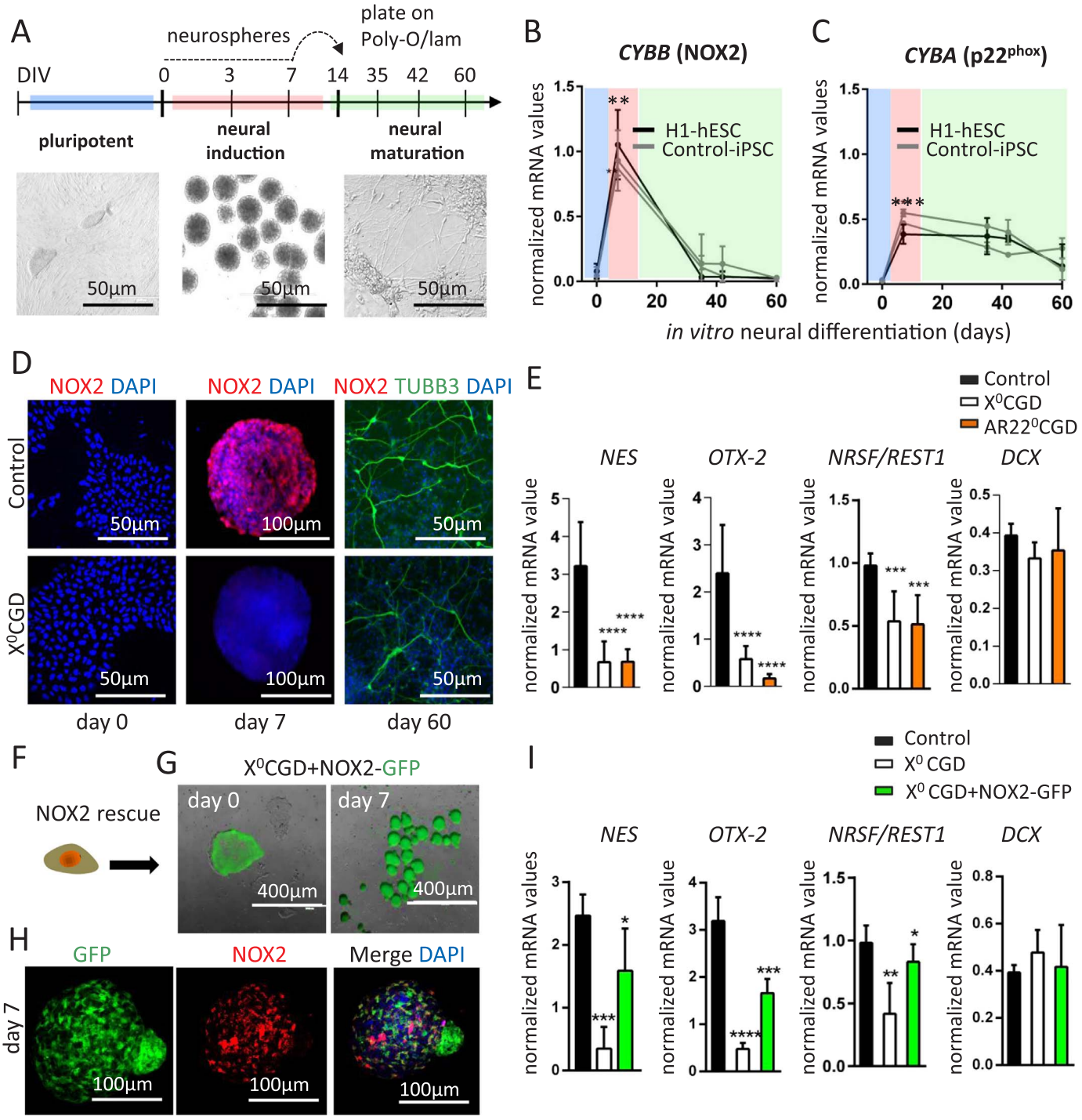


Fig. 4. NOX2 impacts early human neural differentiation **in vitro.** (A) Schematic representation of in vitro neural differentiation of human PSCs: undifferentiated pluripotent stage (blue), neural induction in neurosphere culture (pink) neural maturation on poly L-ornithine/laminin (green). DIV = days of in vitro. (B) Expression of *CYBB* (NOX2; B) and its subunit *CYBA* ($p22^{phox}$; C) was assessed in control hiPSCs (C3, C4 lines) and human ESC line H1 (H1-hESCs) at the undifferentiated pluripotent stage (blue bar: day 0), after neural induction (red bar: day 7) and during neuronal maturation (green bar: day 7–60). (D) Immunostaining demonstrates the absence of NOX2 protein in the pluripotent stage (day 0), the presence of NOX2 protein during the neural induction phase (day 7), and the absence of NOX2 protein in beta 3 tubulin-positive neurons (day 60). (E) Quantitative RT-PCR expression of a NPC marker nestin (*NES)*, transcription factors involved in neural fate and differentiation (*OTX2* and *NRSF/REST1)* and a gene involved in neurodevelopment (*DCX)* in neurospheres derived from control-iPSCs, X°-CGD-iPSCs and AR-22°-iPSCs (n = 8). (F) Schematic representation of NOX2 rescue in the X°-CGD-iPSC line using a lentiviral vector expressing *NOX2* and GFP. (G) High expression of GFP in pluripotent colonies (day 0) and in neurospheres (day 7) derived from X°-CGD + NOX2-GFP-iPSC lines. (H) In neurospheres derived from the X°-CGD + NOX2-GFP-iPSC line (day 7), the GFP fluorescence (green) and positive NOX2 immunostaining (red) are observed. (I) Expression of *NES*, *OTX2*, *NRSF/REST* and *DCX* in neurospheres derived from X°-CGD + NOX2-GFP-iPSCs. Data are shown as mean ± SEM, $n = 9$. Statistical analyses were done by one-way ANOVA followed by multiple comparisons Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***} < 0.001$, $^{****} < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mutation (**C**gA→**T**gA) at the position 469 of exon 5 of the *CYBB* (*NOX2)* gene leading to the absence of NOX2 mRNA and protein. The mutation was absent in the iPSC control lines and present in all the X°CGD-iPSC lines (Fig. S6A). Among the three CGD iPSC lines, one had large copy number variations detected by CGH array and was therefore excluded from further experiments (Fig. S5).

Based on the mouse data obtained in the first part of this study, we examined a possible role of NOX2 in neural differentiation of the

described iPSC lines, as well as the human embryonic pluripotent stem cell line H1-hESCs (for protocol see Fig. 4A). We first investigated the temporal expression pattern of *CYBB* (NOX2*)* and *CYBA* ($p22^{phox}$) by quantitative RT-PCR (qRT-PCR) upon neural induction and neuronal differentiation of control-iPSCs and H1-hESCs (Figs. 4B and 4C). As shown in Fig. 4B, *CYBB* and *CYBA* expression was very low in undifferentiated control-iPSCs and H1-hESCs. Upon neural induction into NPCs, the expression of both genes was strongly increased. In contrast to *CYBB* which expression returns to baseline levels in neurons (Fig. 4B), *CYBA* expression remains high (Fig. 4C). These results were also confirmed at the protein level showing (i) high expression of NOX2 in the NPCs and (ii) t absence of NOX2 protein in undifferentiated iPSC and in neurons. As expected, NOX2 protein was undetectable in X°-CGD-iPSC-derived NPCs (Fig. 4D).

2.5. Neurogenesis impairment in NPCs derived from X°-CGD-iPSCs and AR-22°-iPSCs

To investigate a possible functional impact of NOX2 deficiency on early stage of neural induction, we measured by q-RT-PCR the expression of neural progenitor markers in neurospheres derived from control-iPSCs, X°-CGD-iPSCs and AR-22°-iPSCs. Corroborating our observations in the mouse brain, NOX2 deficiency was associated with a 4-fold decrease of *NES* (Nestin) in NPCs (Fig. 4E). This was associated with a decrease of the expression of transcription factors involved in neural fate and differentiation such as *OTX2* and *NRSF/REST1* (Fig. 4E). However, no difference in the expression of *DCX* was found between NPCs derived from control-iPSCs, X°-CGD-iPSCs and AR-22°-iPSCs (Fig. 4E).

To provide additional support for NOX2 involvement in the defective neurogenesis of X°-CGD-iPSCs, we performed a rescue of NOX2 expression in X°-CGD-iPSCs providing an isogenic iPSC-based model. For this, X°-CGD-iPSCs were transduced with a lentiviral vector carrying human *CYBB (NOX2)* cDNA and GFP as selection marker (Fig. S6A). GFP-positive cells were selected by flow cytometry sorting and induced to differentiate into neurospheres (Fig. 4G). The obtained rescue line will be referred to as X°-CGD + hNOX2. At the undifferentiated pluripotent stage, X°-CGD + hNOX2 expressed mRNA for *CYBB* (*NOX2)* (Fig. S6B), but no NOX2 immunoreactivity was observed (not shown). This is possibly explained by the absence of $p22^{phox}$ in undifferentiated human PSCs (Fig. 4C), and the fact that NOX2 requires $p22^{phox}$ for protein stability. However, upon differentiation of PSCs towards neurospheres, $p22^{phox}$ expression increases (Fig. 4C), which stabilizes the NOX2 protein and hence allows its detection (Fig. 4D). Accordingly, a clear NOX2 staining could be observed in neurospheres derived from the X°-CGD + hNOX2-iPSCs (Fig. 4H). Importantly, NOX2 rescue in neurospheres derived from X°-CGD-C6 + hNOX2-iPSCs increased the expression of the neural markers *NES*, *OTX2*, and *NRSF/REST1* (Fig. 4I), indicative of an improved neurogenesis.

2.6. Localization and activity of NOX2 in neural rosettes derived from PSCs

In order to identify which type of cells are expressing NOX2 during neural induction, we used a co-immunostaining for NOX2 and the NPC marker nestin in neurospheres derived from control and X°-CGD-iPSCs [21]. Within neurospheres, we observed a clear population of cells with nuclei-rich regions positive for both nestin and NOX2 (Fig. 5A), with NOX2 staining occurring mostly in cell bodies, and nestin staining extending to neurites (Fig. 5A, right panel). As expected, NOX2 was not expressed in the X°-CGD neurospheres (Fig. 4A, lower panel, and Fig. 5B). Interestingly, the number of nestin and Ki67 double positive cells was lower in X°-CGD than in control neurospheres (Figs. 5C and 5D) while the fraction of young migratory -DCX-positive neurons was similar in both groups (Figs. 5E and 5F).

To further investigate cellular localization of NOX2, we generated neurospheres from H1-hESCs. Basically we observed two different types of rosettes in neurospheres derived from H1-hESCs: poly-rosette neurospheres (Fig. 5G) and mono-rosette neurospheres (Fig. 5I). NOX2 staining was observed in inner cell layers of these rosettes (Figs. 5H and 5J), which represent the in vitro analogue of the neural tube, where neurogenesis is initiated [10,16]. To verify whether NOX2 is functional, we used two different probes to detect ROS, namely CellROX and nitroblue tetrazolium (NBT). Fig. 5K shows an example of CellROX visualization in a poly-rosette neurosphere. Intense red fluorescence indicates ROS generation, which was observed in the center of the rosettes (Fig. 5K, left panel). After addition of the NOX inhibitor diphenyleneiodonium (DPI), the red fluorescence of CellROX decreased (Figs. 5K and 5L), compatible with the concept that NOX2 is the source of ROS generation. A blue formazan precipitate corresponding to NBT reduction was observed in two different mono-rosette neurospheres, and was absent in rosettes pre-treated with DPI (Figs. 5M and 5N). Note that addition of DPI to neurospheres would not remove already established NBT precipitates.

2.7. Altered maturation of NPCs derived from NOX2-deficient iPSCs into neurons

Given the impact of NOX2 deficiency on differentiation of iPSCs into NPCs, we further studied the effect of NOX2 deficiency on later stages of neural differentiation (Fig. 6). For this purpose, NPCs were plated on polyornithin/laminin-coated dishes (at day 0) and allowed to mature for 60 days. The expression of neuronal markers (*TUBB3*, *MAP2* and *BDNF*) was analyzed by RT-qPCR at different time points of neural differentiation (Figs. 6A, 6C and 6E) and by immunofluorescence staining at day 60 of neuronal differentiation (Figs. 6F, 6J, 6M, and 6P). Interestingly, neurons derived from X°-CGD-iPSCs and AR-22°-CGD-iPSCs expressed lower levels of neuronal markers (*TUBB3*, *MAP2* and *BDNF*) than those derived from control-iPSCs (Figs. 6A, 6C and 6E). Consistent with these results, upon neuronal differentiation, X°-CGD-iPSCs and AR-22°-CGD-iPSCs generated less β3-tubulin positive cells and MAP2-positive cells (Figs. 6B and 6D). Quantification of neurite branching from soma of MAP2-positive neurons at day 60 of differentiation showed that the mean number of branches was lower in neurons derived from X°-CGD-iPSCs and AR-22°-CGD-iPSCs compared to those derived from control-iPSCs (Figs. 6F and 6G), which translates into a reduced length of neurites in neurons derived from X°-CGD-iPSCs and AR-22°-CGD-iPSCs (Fig. 6H).

Considering that an impaired maturation could explain the cognitive impairment observed in CGD patients, we investigated the expression of both pre- and post-synaptic markers of neurons by RT-qPCR and by immunofluorescence analysis. As shown, no difference in *SYN1*, *SNAP25* and *PSD95* expression was found in neurons derived from X°-CGD-iPSCs, AR-22°-CGD-iPSCs and control-iPSCs (Fig. 6I). In line with this, the density of synapsin punctae was similar in neurons derived from X°-CGD-iPSCs, AR-22°-CGD-iPSCs and control-iPSCs (Figs. 6J and 6K).

In order to determine if the reduced number of neurons generated from X°-CGD-iPSCs, AR-22°-CGD-iPSCs is associated with a gliogenic shift, we analyzed the expression of astroglial (*S100B* and *GFAP*) and oligodendroglial markers (*OLIG1* and *OLIG2*) by RT-qPCR upon neuronal differentiation of these iPSCs. Interestingly, no difference in the expression of *GFAP* and *S100B* as well as in the number of GFAP-positive cells was observed upon neuronal differentiation of X°-CGD- and AR-22°-CGD-iPSCs (Figs. 6L, 6M and 6N). By contrast, upon neuronal differentiation both X°-CGD- and AR-22°-CGD-iPSCs showed a reduced expression of *OLIG1* and *OLIG2* as well as a reduced number of OLIG2-positive cells (Figs. 6O, 6P and 6Q). Collectively, our results suggest that NOX2 deficiency is associated with a reduced generation of neurons and oligodendroglial cells with no impact in the number of astroglial cells.

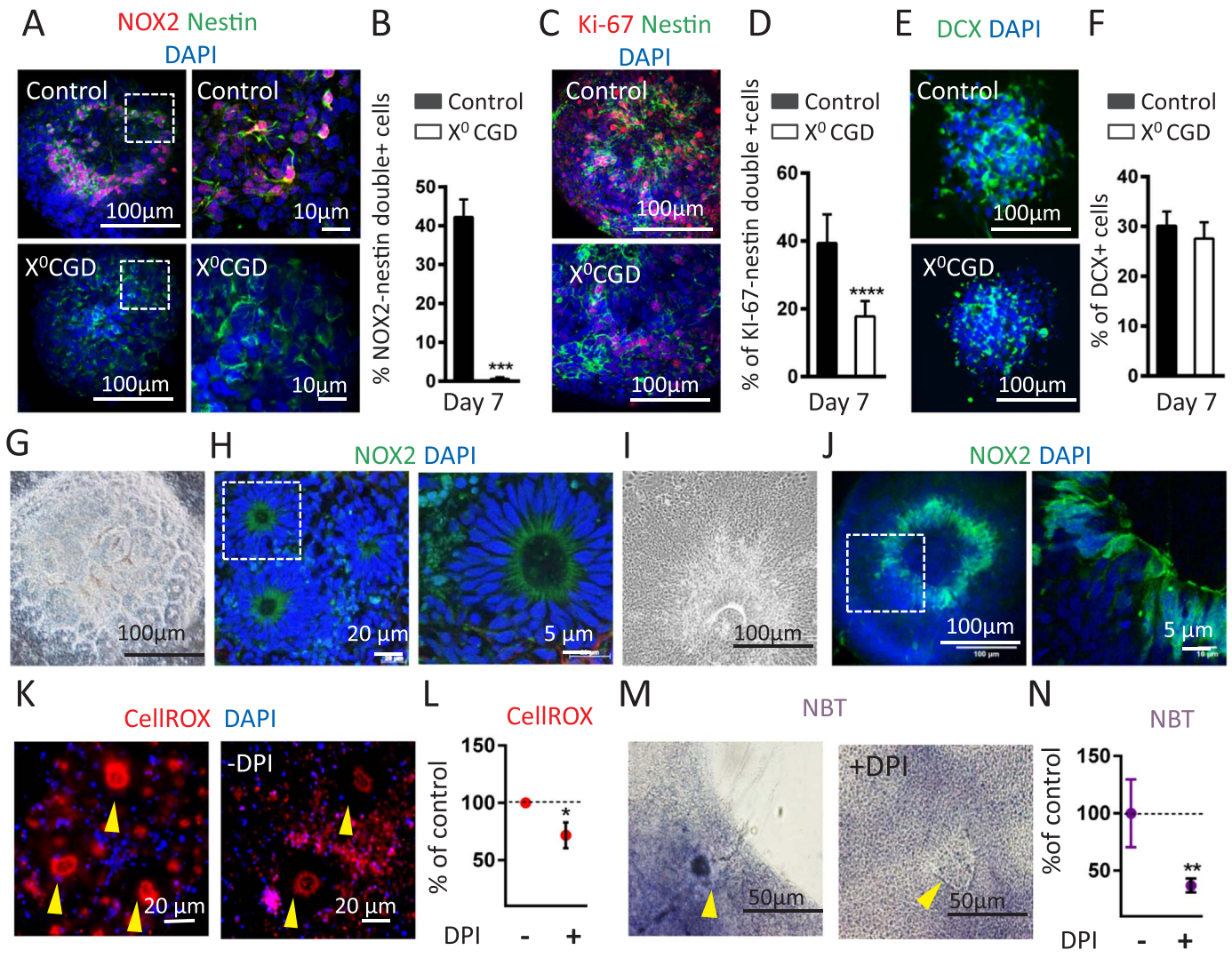


Fig. 5. NOX2 localization and activity in NPCs derived from iPSCs Immunofluorescence images (A) and quantification (B) of NOX2 (red) and the NPC marker nestin (green) in neurospheres (day7) of control and X°-CGD-iPSCs. Images in panel (A) are shown at lower (left) and higher (right) magnifications. (C) Co-staining of neurospheres with the proliferation marker Ki-67 and the NPC marker nestin. (D) Quantification of percentage of Ki67-nestin double positive cells. Immunostaining images (E) and quantification (F) of the early neuronal marker DCX (n = 6). Formation of multiple small rosettes (G) and single large rosettes (I) in neurospheres (day 7) derived from H1-hESCs (light microscopy). (H, J) NOX2 immunostaining in the two distinct types of rosette structures (right panels: higher magnification). (K, M) ROS production was visualized in neural rosette structures by using CellROX and NBT (yellow arrows). Generation of ROS was decreased in presence of 10uM DPI (flavoprotein inhibitor), arrows in yellow, suggesting NOX2 as a main source of ROS generation in rosettes (right panels in K and M). (L and M) Dot plot graph demonstrating ROS measurement in neural rosettes using DPI (flavoprotein inhibitor) (n = 5). Statistics were done Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, *** < 0.001, **** < 0.0001. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

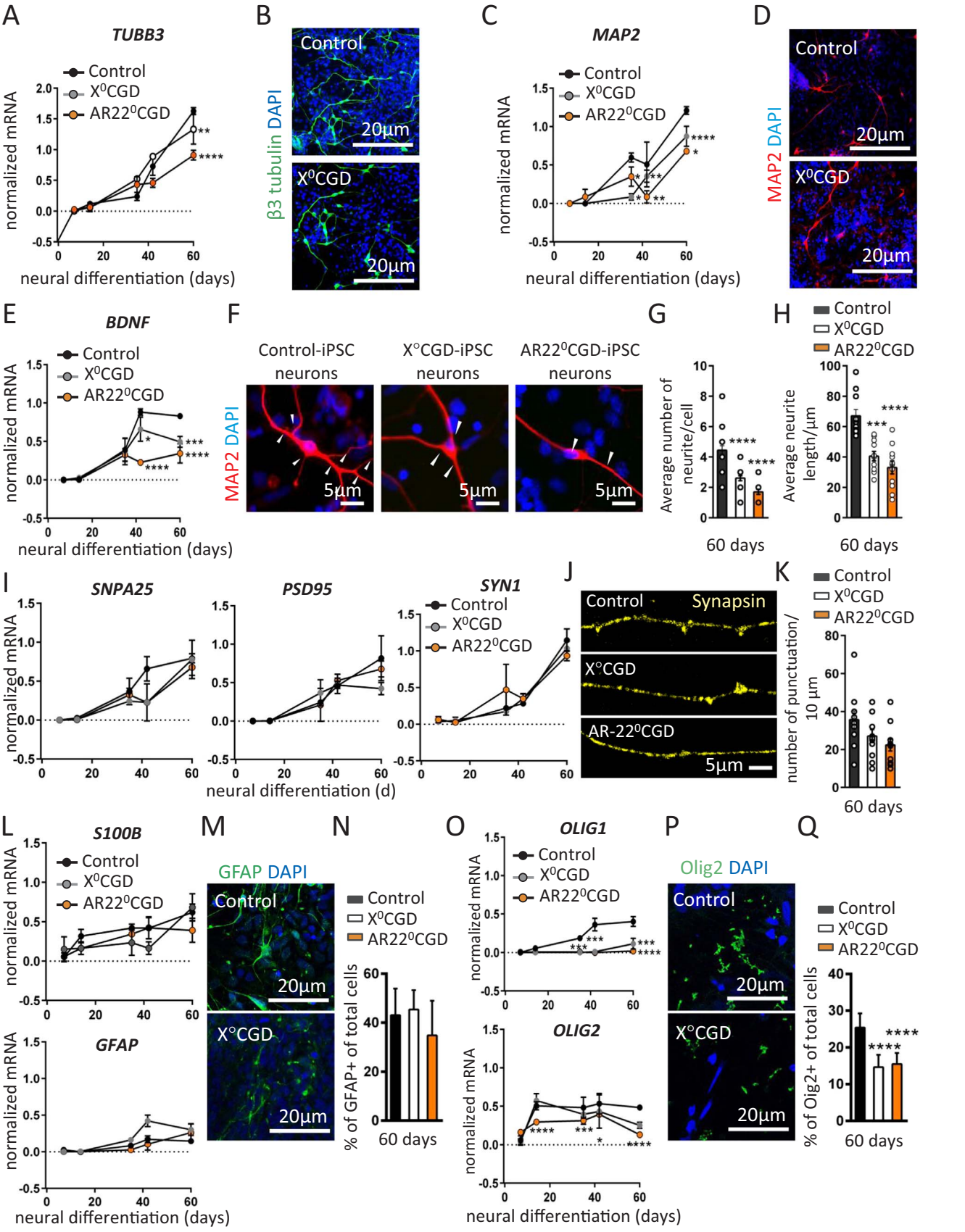
3. Discussion

In this study, we show that the ROS-generating enzyme NOX2 is highly expressed in neurogenic regions of the mouse and macaque brain, as well as in neurally differentiated human iPS cells. Using NOX2-deficient mice and iPSC from NOX2-deficient patients, we demonstrate that NOX2 is functionally relevant in nestin positive NPCs and their proliferation.

To our knowledge, this is the first article that presents the role of NOX2 during neural differentiation by using human NOX2 deficient-iPSC lines. Two previous studies have investigated the role of ROS, and to some extent NOX, in NPCs. Le belle et al. has described the presence of NOX2 in the SVZ and a role of ROS in the self-renewal and multipotency of mouse NSCs. However, the conclusions of this study was mostly based on the use of non-specific inhibitors [14,30]. have shown NOX2-dependent regulation of proliferation and signaling in adult mouse hippocampal precursor (AHP) cells and decrease NPC proliferation NOX2-deficient adult mouse dentate gyrus [14,30].

Our study provides several novel and relevant elements: i) we investigated all NOX isoforms and demonstrate that only NOX2 mRNA is specifically enriched in the DG and SVZ; ii) we excluded the possibility that the presence of NOX2 in neurogenic regions is due to microglia; iii) by demonstrating decreased oxidative modifications in neurogenic regions of NOX2-deficient animals, we demonstrated that NOX2 is functional in neurogenic regions; iv) we detected region-specific role of NOX2, which mediates BDNF generation in the dentate gyrus, but Otx2 expression in the subventricular zone and olfactory bulb.

The role of human NOX2 was studied using iPSC from two different patients, one with X-linked *CYBB* (NOX2) deficiency, and one with autosomal *CYBA* ($p22^{phox}$) deficiency. As discussed in the introduction, $p22^{phox}$ is an obligatory subunit of the NOX2 complex, but in addition, it is also part of the NOX1, NOX3, and NOX4 complex [3]. Globally speaking, the results obtained with the two different types of CGD iPSCs were similar. However, the results obtained with the $p22^{phox}$ deficient cells (AR-22°) were more pronounced than the one observed with the NOX2-deficient cells. Thus, we cannot exclude a small contribution of NOX1, NOX3, or NOX4 to ROS generation in neurogenesis. Note, that in



(caption on next page)

Fig. 6. *In vitro* neuronal maturation of control and CGD iPSC. Control iPSCs, X°-CGD-iPSCs and AR-22°-iPSCs were assessed by RT-qPCR analysis and immunofluorescent staining. Expression of the neuronal marker, β3-tubulin (A, B), and the mature neuronal marker *MAP2* (C, D), and brain derived neurotrophic factor *BDNF* (E). (F) Representative images and quantitative analysis of neurite number (G) and neurite length (H) from MAP2 positive neurons at day 60 of differentiation (n = 7). (I) Analysis of synaptic markers *SNPA25*, *PSD95* and *SYN1* by RT-qPCR in neurons derived from control and CGD-iPSCs, and immunostaining of synapsin (J) and quantification of the number of synapsin punctuations/10 μm (K, n = 4) at day 60 of differentiation. Analysis of glial markers S100B and GFAP (astrocytes, L, M, N), Olig1 and Olig2 (oligodendrocytes, O, P,Q) by RT-qPCR and immunostaining after 60 days of neural differentiation of control, X^0-CGD- and AR-22^0-CGD iPSC lines (n = 4 for RT-qPCR and n = 5 for immunofluorescence). Data are shown as mean ± SEM. Statistics were done by one-way ANOVA followed by multiple comparison Tukey's *test.* *P < 0.05, **P < 0.01, *** < 0.001, **** < 0.000.

contrast to the rapid down-regulation of NOX2 during neuronal differentiation of human ESCs and iPSCs, there was a persistence of $p22^{phox}$ expression, possibly compatible with a role of other NOX enzymes during later stages of neuronal differentiation.

Also, a role for microglia during neurogenesis is emerging [50]. Thus a likely explanation for the presence of NOX2 in neurogenic zones would be the presence of microglia, a CNS phagocyte which typically expresses the phagocyte NADPH oxidase NOX2 [12,18,31,42,54,63]. However, the following arguments plead against a microglial localization of NOX2 in neurogenic regions: i) NOX2-rich neurogenic regions of the adult mouse brain contained only very low amounts of microglia; ii) in vitro neuroectodermal differentiation of iPSCs is NOX2-regulated, but no microglia (of mesodermal origin) is present under these conditions; and iii) immunofluorescence staining shows NOX2 within rosette-like structures, which are analogous to the neural tube [16,17], and it co-localization with the NPC marker nestin. Interestingly, the presence of NOX2 appears to be required for symmetric divisions, which might not be an independent effect, but rather a mechanism that contributes to the maintenance of the physiological number of proliferating NSC. In the absence of this NOX2-driven oxidizing environment there is a decrease in the neuroprecursor pool.

Eventually, at the final stage of neural maturation, NOX2 appears to be strongly down-regulated. This corroborates RNAseq data from a mature neuronal region (cortex) where NOX2 is almost exclusively detected in microglia and completely absent in mature neurons [68]. However our data do not formally exclude the possibility that low level NOX2 expression may be present in neuronal subpopulations or at specific developmental stages (for review see [42].

We observed particularities associated with the NOX2 expression in neurogenic regions which merit discussion: i) NOX2 expression is not accompanied by a detectable expression of the transcription factors PU.1, which is one of the main known transcription factors driving NOX2 expression in leukocytic cells [57]. This suggests a PU.1-independent NOX2 expression in NPCs and the presence of alternative transcriptional regulation of NOX2. ii) The expression levels of several cytoplasmic NOX2 subunits (in particular $p67^{phox}$, $p40^{phox}$) were below detection threshold. Yet, our data show that NOX2 is functional in mouse and human NSCs/NPCs: NOX2 deficiency has a marked impact on NSC/NPC proliferation and gene expression and NOX2 expression is associated with ROS generation in neurogenic regions. Based on our present knowledge of NOX2 biochemistry, it seems unlikely that NSCs/NPCs exhibit a subunit-independent ROS generation by NOX2, but rather that low level and/or intermittent expression of subunits suffices to activate NOX2 in NSCs/NPCs; iii) the expression of the Rac GTPase isoforms in the neurogenic regions of the mouse brain is also unique as compared with other NOX2 expressing tissues. As expected, the neutrophil-typical Rac2 is virtually absent. However, in addition to Rac1, which is typically associated with NOX2 in macrophages, neurogenic regions also express high levels of the neuronal Rac3 [37]. Thus, it is tempting to speculate that Rac3 might be implicated in NOX2 activity in NSC/NPC.

In human phagocytes, activation of NOX2 is a tightly regulated system which responds to specific stimuli and rapidly deactivates once the relevant stimulus has disappeared. For the time being, we do not have evidence that NOX2 in NSCs behaves in a similar way. Indeed, under our experimental conditions, NOX2 appears to be active without addition of a specific stimulus (data not shown). However, we observed NOX2 activity in NSCs/NPCs within the neurogenic niche, where various growth factors are released which contributes to the control of the NSC/NPC system. Indeed, there is evidence for activation and/or enhanced expression of NOX2 by neural growth factors such as FGF2 [14], BDNF [53] and VEGF [31]. For example, NOX2 expression is decreased in neurogenic regions of VEGF-deficient mice [31]. In addition, there appears to be a feed-forward mechanism through a redox-sensitive activation of the respective growth factor receptors by NOX2 [62]. Thus, although we do not have experimental evidence for a receptor-dependent activation of NOX2 in neural precursors, our data certainly do not exclude this possibility.

Deficiency of NOX2 leads to a moderate decrease in cognitive function in man [45] and mice [26]. Given recent results that connect adult neurogenesis and learning [46,5,64], it is possible that the defective neurogenesis caused by NOX2 deficiency plays a causal role in the decreased cognition observed in CGD. This is also particularly relevant with respect to BDNF, which is thought to play a role in learning [22] and was found in our study to be decreased in human and mouse carrying NOX2-loss of function mutations. Future studies are needed to address whether learning associated neurogenesis is indeed decreased in CGD mice.

These results likely explain a hitherto poorly understood phenotype of NOX2-deficient CGD patients. It also adds a new level of complexity to the concept of NOX2 as a drug target for the treatment of oxidative stress diseases of the CNS.

Author contributions

Z.N. conceived the study, designed and performed experiments, analyzed the data and wrote the manuscript. M.C. performed the dissection of the neurogenic regions of adult mouse. N.R.A. performed mouse neurospheres experiments under A.C supervision. E.G. performed several immunohistochemical analyses on human iPSCs. F.S.B and E.S performed the array-CGH for iPSC characterization. Y.H. provided the human primers as well as useful experience in handling and experimenting with iPSC. M.J.S. diagnosed and characterized the mutation of CGD patients. T.S. and J.H. generated the iPSC lines from CGD fibroblasts. KHK, VJ, conceived the study, wrote and edited the manuscript. All authors read and approved the final manuscript.

Acknowledgment

This study was supported by the Swiss National Foundation (SNF) grant number 31003A-160220/1, European Community's Framework Programme (FP7/2007–2013) under grant 278611 (Neurinox), Swiss State Secretariat for Education and Innovation C13.0142, German Federal Ministry for Education and Research (Bundesministerium(für Bildung und Forschung, BMBF; grant number 01GN0824), Ministry for Innovation, Science und Research of the State of North Rhine-Westphalia (323-400-010-13), and Köln Fortune Programs of the Medical Faculty of the University of Cologne, Germany. Natalia Robledinos-Anton was supported by a grant from the COST action EUROS BM1203 and the program for training university lecturers (FPU14/01073) from Spanish Ministry of Education. The authors would like to thank Eric Rouiller from the University of Fribourg (Laboratory of Neurophysiology, Fribourg, Switzerland) for kindly donating *Macaca fascicularis* brain samples for NOX2 analysis. The authors would also like to thank the collaborators in human genetics, genomic platform, flow cytometry and bioimaging core facility, Yves Alexandre Cambet at

READS units at the University of Geneva. We are grateful to Olivier Plastre, Stéphanie Sgroi and Christophe Delgado for technical help. The authors would also like to thank Michel Dubois-Dauphin for discussions.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.04.026.

References

[1] J. Altman, G.D. Das, Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats, J. Comp. Neurol. 124 (1965) 319–335.
[2] J. Altman, G.D. Das, Autoradiographic and histological studies of postnatal neurogenesis. I. A longitudinal investigation of the kinetics, migration and transformation of cells incorporating tritiated thymidine in neonate rats, with special reference to postnatal neurogenesis in some brain regions, J. Comp. Neurol. 126 (1966) 337–389.
[3] K. Bedard, K.H. Krause, The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology, Physiol. Rev. 87 (2007) 245–313.
[4] C. Betzen, R. White, C.M. Zehendner, E. Pietrowski, B. Bender, H.J. Luhmann, C.R. Kuhlmann, Oxidative stress upregulates the NMDA receptor on cerebrovascular endothelium, Free Radic. Biol. Med. 47 (2009) 1212–1220.
[5] M. Blurton-Jones, M. Kitazawa, H. Martinez-Coria, N.A. Castello, F.J. Muller, J.F. Loring, T.R. Yamasaki, W.W. Poon, K.N. Green, F.M. LaFerla, Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease, Proc. Natl. Acad. Sci. USA 106 (2009) 13594–13599.
[6] J. Brault, E. Goutagny, N. Telugu, K. Shao, M. Baquie, V. Satre, C. Coutton, D. Grunwald, J.P. Brion, V. Barlogis. et al. Optimized Generation of Functional Neutrophils and Macrophages from Patient-Specific Induced Pluripotent Stem Cells: Ex Vivo Models of X(0)-Linked, AR22(0)- and AR47(0)- Chronic Granulomatous Diseases. BioResearch open access 3, 2014, pp. 311–326.
[7] M. Buggisch, B. Ateghang, C. Ruhe, C. Strobel, S. Lange, M. Wartenberg, H. Sauer, Stimulation of ES-cell-derived cardiomyogenesis and neonatal cardiac cell proliferation by reactive oxygen species and NADPH oxidase, J. Cell Sci. 120 (2007) 885–894.
[8] J.R. Burgoyne, H. Mongue-Din, P. Eaton, A.M. Shah, Redox signaling in cardiac physiology and pathology, Circ. Res. 111 (2012) 1091–1106.
[9] H.A. Cameron, R.D. McKay, Restoring production of hippocampal neurons in old age, Nat. Neurosci. 2 (1999) 894–897.
[10] S.M. Chambers, C.A. Fasano, E.P. Papapetrou, M. Tomishima, M. Sadelain, L. Studer, Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling, Nat. Biotechnol. 27 (2009) 275–280.
[11] M.J. Corenblum, S. Ray, Q.W. Remley, M. Long, B. Harder, D.D. Zhang, C.A. Barnes, L. Madhavan, Reduced Nrf2 expression mediates the decline in neural stem cell function during a critical middle-age period, Aging Cell 15 (2016) 725–736.
[12] A. Coyoy, M. Olguin-Albuerne, P. Martinez-Briseno, J. Moran, Role of reactive oxygen species and NADPH-oxidase in the development of rat cerebellum, Neurochem. Int. 62 (2013) 998–1011.
[13] W. Deng, J.B. Aimone, F.H. Gage, New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat. Rev. Neurosci. 11 (2010) 339–350.
[14] B.C. Dickinson, J. Peltier, D. Stone, D.V. Schaffer, C.J. Chang, Nox2 redox signaling maintains essential cell populations in the brain, Nat. Chem. Biol. 7 (2011) 106–112.
[15] R. Dolmetsch, D.H. Geschwind, The human brain in a dish: the promise of iPSC-derived neurons, Cell 145 (2011) 831–834.
[16] Y. Elkabetz, G. Panagiotakos, G. Al Shamy, N.D. Socci, V. Tabar, L. Studer, Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage, Genes Dev. 22 (2008) 152–165.
[17] Y. Elkabetz, L. Studer, Human ESC-derived neural rosettes and neural stem cell progression, Cold Spring Harb. Symp. Quant. Biol. 73 (2008) 377–387.
[18] M.T. Fischer, R. Sharma, J.L. Lim, L. Haider, J.M. Frischer, J. Drexhage, D. Mahad, M. Bradl, J. van Horssen, H. Lassmann, NADPH oxidase expression in active multiple sclerosis lesions in relation to oxidative tissue damage and mitochondrial injury, Brain 135 (2012) 886–899.
[19] H.J. Forman, F. Ursini, M. Maiorino, An overview of mechanisms of redox signaling, J. Mol. Cell. Cardiol. (2014).
[20] R.P. Galvao, J.M. Garcia-Verdugo, A. Alvarez-Buylla, Brain-derived neurotrophic factor signaling does not stimulate subventricular zone neurogenesis in adult mice and rats, J. Neurosci. 28 (2008) 13368–13383.
[21] A.V. Gilyarov, Nestin in central nervous system cells, Neurosci. Behav. Physiol. 38 (2008) 165–169.
[22] A. Gomez-Palacio Schjetnan, M.L. Escobar-Rodriguez, [Memory coding and retention: brain-derived neurotrophic factor (BDNF) in synaptic plasticity], Rev. De. Neurol. 45 (2007) 409–417.
[23] K.M. Holmstrom, T. Finkel, Cellular mechanisms and physiological consequences of redox-dependent signalling, Nat. Rev. Mol. Cell Biol. 15 (2014) 411–421 (LID −410.1038/nrm3801)(doi).
[24] S.K. Hota, K.B. Hota, D. Prasad, G. Ilavazhagan, S.B. Singh, Oxidative-stress-induced alterations in Sp factors mediate transcriptional regulation of the NR1 subunit in hippocampus during hypoxia, Free Radic. Biol. Med. 49 (2010) 178–191.
[25] A.T. Huddleston, W. Tang, H. Takeshima, S.L. Hamilton, E. Klann, Superoxide-induced potentiation in the hippocampus requires activation of ryanodine receptor type 3 and ERK, J. Neurophysiol. 99 (2008) 1565–1571.
[26] K.T. Kishida, C.A. Hoeffer, D. Hu, M. Pao, S.M. Holland, E. Klann, Synaptic plasticity deficits and mild memory impairments in mouse models of chronic granulomatous disease, Mol. Cell. Biol. 26 (2006) 5908–5920.
[27] K.T. Kishida, M. Pao, S.M. Holland, E. Klann, NADPH oxidase is required for NMDA receptor-dependent activation of ERK in hippocampal area CA1, J. Neurochem. 94 (2005) 299–306.
[28] H.G. Kuhn, H. Dickinson-Anson, F.H. Gage, Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation, J. Neurosci. 16 (1996) 2027–2033.
[29] J.D. Lambeth, T. Kawahara, B. Diebold, Regulation of Nox and Duox enzymatic activity and expression, Free Radic. Biol. Med. 43 (2007) 319–331.
[30] J.E. Le Belle, N.M. Orozco, A.A. Paucar, J.P. Saxe, J. Mottahedeh, A.D. Pyle, H. Wu, H.I. Kornblum, Proliferative neural stem cells have high endogenous ROS levels that regulate self-renewal and neurogenesis in a PI3K/Akt-dependant manner, Cell Stem Cell 8 (2011) 59–71.
[31] A. Lelli, A. Gervais, C. Colin, C. Cheret, C. Ruiz de Almodovar, P. Carmeliet, K.H. Krause, S. Boillee, M. Mallat, The NADPH oxidase Nox2 regulates VEGFR1/CSF-1R-mediated microglial chemotaxis and promotes early postnatal infiltration of phagocytes in the subventricular zone of the mouse cerebral cortex, Glia 61 (2013) 1542–1555.
[32] T.L. Leto, S. Morand, D. Hurt, T. Ueyama, Targeting and regulation of reactive oxygen species generation by Nox family NADPH oxidases, Antioxid. Redox Signal 11 (2009) 2607–2619.
[33] J. Li, M. Stouffs, L. Serrander, B. Banfi, E. Bettiol, Y. Charnay, K. Steger, K.H. Krause, M.E. Jaconi, The NADPH oxidase NOX4 drives cardiac differentiation: role in regulating cardiac transcription factors and MAP kinase activation, Mol. Biol. Cell 17 (2006) 3978–3988.
[34] P.M. Lledo, M. Alonso, M.S. Grubb, Adult neurogenesis and functional plasticity in neuronal circuits, Nat. Rev. Neurosci. 7 (2006) 179–193.
[35] C. Lois, A. Alvarez-Buylla, Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia, Proc. Natl. Acad. Sci. USA 90 (1993) 2074–2077.
[36] C. Lois, A. Alvarez-Buylla, Long-distance neuronal migration in the adult mammalian brain, Science 264 (1994) 1145–1148.
[37] A.M. Lorincz, G. Szarvas, S.M. Smith, E. Ligeti, Role of Rac GTPase activating proteins in regulation of NADPH oxidase in human neutrophils, Free Radic. Biol. Med. 68C (2013) 65–71.
[38] M.B. Luskin, Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone, Neuron 11 (1993) 173–189.
[39] M.P. Mattson, S. Maudsley, B. Martin, A neural signaling triumvirate that influences ageing and age-related disease: insulin/igf-1, BDNF and serotonin, Ageing Res. Rev. 3 (2004) 445–464.
[40] K. Meganathan, S. Jagtap, V. Wagh, J. Winkler, J.A. Gaspar, D. Hildebrand, M. Trusch, K. Lehmann, J. Hescheler, H. Schluter, et al., Identification of thalidomide-specific transcriptomics and proteomics signatures during differentiation of human embryonic stem cells, PloS One 7 (2012) e44228.
[41] G.L. Ming, H. Song, Adult neurogenesis in the mammalian brain: significant answers and significant questions, Neuron 70 (2011) 687–702.
[42] Z. Nayernia, V. Jaquet, K.H. Krause, New Insights on NOX Enzymes in the Central Nervous System, Antioxid. Redox Signal. (2014).
[43] M. Olguin-Albuerne, J. Moran, ROS produced by NOX2 control in vitro development of cerebellar granule neurons development, ASN Neuro 7 (2015).
[44] E.A. Ostrakhovitch, O.A. Semenikhin, The role of redox environment in neurogenic development, Arch. Biochem. Biophys. 534 (2013) 44–54 (LID -10.1016/j.abb.2012.1008.1002)(doi) (LID - S0003-9861)(1012)(00301-00303)(pii).
[45] M. Pao, E.A. Wiggs, M.M. Anastacio, J. Hyun, E.S. DeCarlo, J.T. Miller, V.L. Anderson, H.L. Malech, J.I. Gallin, S.M. Holland, Cognitive function in patients with chronic granulomatous disease: a preliminary report, Psychosomatics 45 (2004) 230–234.
[46] D. Park, A.P. Xiang, F.F. Mao, L. Zhang, C.G. Di, X.M. Liu, Y. Shao, B.F. Ma, J.H. Lee, K.S. Ha, et al., Nestin is required for the proper self-renewal of neural stem cells, Stem Cells 28 (2010) 2162–2171.
[47] A. Petry, M. Weitnauer, A. Gorlach, Receptor activation of NADPH oxidases, Antioxid. Redox Signal. 13 (2010) 467–487.
[48] C. Piccoli, A. D'Aprile, M. Ripoli, R. Scrima, L. Lecce, D. Boffoli, A. Tabilio, N. Capitanio, Bone-marrow derived hematopoietic stem/progenitor cells express multiple isoforms of NADPH oxidase and produce constitutively reactive oxygen species, Biochem. Biophys. Res. Commun. 353 (2007) 965–972.
[49] C. Piccoli, R. Ria, R. Scrima, O. Cela, A. D'Aprile, D. Boffoli, F. Falzetti, A. Tabilio, N. Capitanio, Characterization of mitochondrial and extra-mitochondrial oxygen consuming reactions in human hematopoietic stem cells. Novel evidence of the occurrence of NAD(P)H oxidase activity, J. Biol. Chem. 280 (2005) 26467–26476.
[50] K. Sato, Effects of Microglia on Neurogenesis, Glia 63 (2015) 1394–1405.
[51] T. Seredenina, Z. Nayernia, S. Sorce, G.J. Maghzal, A. Filippova, S.C. Ling, O. Basset, O. Plastre, Y. Daali, E.J. Rushing, et al., Evaluation of NADPH oxidases as drug targets in a mouse model of familial amyotrophic lateral sclerosis, Free Radic. Biol. Med. 97 (2016) 95–108.
[52] F. Serrano, N.S. Kolluri, F.B. Wientjes, J.P. Card, E. Klann, NADPH oxidase immunoreactivity in the mouse brain, Brain Res. 988 (2003) 193–198.
[53] X. Song, B. Zhou, P. Zhang, D. Lei, Y. Wang, G. Yao, T. Hayashi, M. Xia, S. Tashiro, S. Onodera, et al., Protective Effect of Silibinin on Learning and Memory

Impairment in LPS-Treated Rats via ROS-BDNF-TrkB Pathway, Neurochem. Res. 41 (2016) 1662–1672.
[54] S. Sorce, K.H. Krause, NOX enzymes in the central nervous system: from signaling to disease, Antioxid. Redox Signal 11 (2009) 2481–2504.
[55] M.J. Stasia, X.J. Li, Genetics and immunopathology of chronic granulomatous disease, Semin. Immunopathol. 30 (2008) 209–235.
[56] J.L. Stein, L. de la Torre-Ubieta, Y. Tian, N.N. Parikshak, I.A. Hernandez, M.C. Marchetto, D.K. Baker, D. Lu, C.R. Hinman, J.K. Lowe, et al., A quantitative framework to evaluate modeling of cortical development by neural stem cells, Neuron 83 (2014) 69–86.
[57] S. Suzuki, A. Kumatori, I.A. Haagen, Y. Fujii, M.A. Sadat, H.L. Jun, Y. Tsuji, D. Roos, M. Nakamura, PU.1 as an essential activator for the expression of gp91(phox) gene in human peripheral neutrophils, monocytes, and B lymphocytes, Proc. Natl. Acad. Sci. USA 95 (1998) 6085–6090.
[58] M.V. Tejada-Simon, F. Serrano, L.E. Villasana, B.I. Kanterewicz, G.Y. Wu, M.T. Quinn, E. Klann, Synaptic localization of a functional NADPH oxidase in the mouse hippocampus, Mol. Cell. Neurosci. 29 (2005) 97–106.
[59] M. Tsatmali, E.C. Walcott, K.L. Crossin, Newborn neurons acquire high levels of reactive oxygen species and increased mitochondrial proteins upon differentiation from progenitors, Brain Res. 1040 (2005) 137–150.
[60] H.L. Vieira, P.M. Alves, A. Vercelli, Modulation of neuronal stem cell differentiation by hypoxia and reactive oxygen species, Progress. Neurobiol. 93 (2011) 444–455.
[61] X. Wang, E.K. Michaelis, Selective neuronal vulnerability to oxidative stress in the brain, Front. Aging Neurosci. 2 (2010) 12.
[62] J.F. Woolley, A. Corcoran, G. Groeger, W.D. Landry, T.G. Cotter, Redox-regulated growth factor survival signaling, Antioxid. Redox Signal. 19 (2013) 1815–1827.
[63] D.C. Wu, D.B. Re, M. Nagai, H. Ischiropoulos, S. Przedborski, The inflammatory NADPH oxidase enzyme modulates motor neuron degeneration in amyotrophic lateral sclerosis mice, Proc. Natl. Acad. Sci. USA 103 (2006) 12132–12137.
[64] A.G. Xuan, D.H. Long, H.G. Gu, D.D. Yang, L.P. Hong, S.L. Leng, BDNF improves the effects of neural stem cells on the rat model of Alzheimer's disease with unilateral lesion of fimbria-fornix, Neurosci. Lett. 440 (2008) 331–335.
[65] A. Yamauchi, L. Yu, A.J. Potgens, F. Kuribayashi, H. Nunoi, S. Kanegasaki, D. Roos, H.L. Malech, M.C. Dinauer, M. Nakamura, Location of the epitope for 7D5, a monoclonal antibody raised against human flavocytochrome b558, to the extracellular peptide portion of primate gp91phox, Microbiol. Immunol. 45 (2001) 249–257.
[66] M. Yoneyama, K. Kawada, Y. Gotoh, T. Shiba, K. Ogita, Endogenous reactive oxygen species are essential for proliferation of neural stem/progenitor cells, Neurochem. Int. 56 (2010) 740–746.
[67] T.F. Yuan, S. Gu, C. Shan, S. Marchado, O. Arias-Carrion, Oxidative Stress and Adult Neurogenesis, Stem Cell Rev. 11 (2015) 706–709.
[68 Y. Zhang, K. Chen, S.A. Sloan, M.L. Bennett, A.R. Scholze, S. O'Keeffe, H.P. Phatnani, P. Guarnieri, C. Caneda, N. Ruderisch, et al., An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex, J. Neurosci.: Off. J. Soc. Neurosci. 34 (2014) 11929–11947.