Differential effects of glucose deprivation on the survival of fetal versus adult neural stem cells-derived oligodendrocyte precursor cells

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Running title: OPCs metabolism drives OGD vulnerability

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Main points:

- fetal OPCs are selectively vulnerabile to hypoxia/ischemia
- fetal NSCs-derived mixed cultures are glycolytic, while adult mainly use OXPHOS
- fetal and adult OPCs show different vulnerability to metabolic challenges in mixed cultures containing astrocytes

Abstract

Impaired myelination is a key feature in neonatal hypoxia/ischemia (HI), the most common perinatal/neonatal cause of death and permanent disabilities, which is triggered by the establishment of an inflammatory and hypoxic environment during the most critical period of myelin development. This process is dependent on oligodendrocyte precursor cells (OPCs) and their capability to differentiate into mature oligodendrocytes.

In this study, we investigated the vulnerability of fetal and adult OPCs derived from neural stem cells (NSCs) to inflammatory and HI insults. The resulting OPCs/astrocytes cultures were exposed to cytokines to mimic inflammation, or to oxygen-glucose deprivation (OGD) to mimic an HI condition. The differentiation of both fetal and adult OPCs is completely abolished following exposure to inflammatory cytokines, while only fetal-derived OPCs degenerate when exposed to OGD.

We then investigated possible mechanisms involved in OGD-mediated toxicity: i) T3-mediated maturation induction; ii) glutamate excitotoxicity; iii) glucose metabolism. We found that while no substantial differences were observed in T3 intracellular content regulation and glutamate-mediated toxicity, glucose deprivation lead to selective OPC cell death and impaired differentiation in fetal cultures only.

These results indicate that the biological response of OPCs to inflammation and demyelination is different in fetal and adult cells, and that the glucose metabolism perturbation in fetal central nervous system (CNS) may significantly contribute to neonatal pathologies. An understanding of the underlying molecular mechanism will contribute greatly to differentiating myelination enhancing and neuroprotective therapies for neonatal and adult CNS white matter lesions.

Keywords

oligodendrocyte precursor cells, inflammation, hypoxia/ischemia, oxygen-glucose deprivation, white matter lesion

1. *Introduction*

Myelin depletion is a major pathological event in inflammatory diseases, vascular and traumatic lesions of the central nervous system (CNS), and a major cause of consequent neurodegeneration and chronic disabilities in both infants and adults (Verden & Macklin, 2016). Oligodendrocyte precursors (OPCs) are the cells responsible for myelin formation during development, and for myelin turnover and repair during the adult life, thanks to their ability to differentiate into myelinating oligodendrocytes (OLs) (Alizadeh, Dyck, & Karimi-Abdolrezaee, 2015). During gestational development, OPCs progenitors migrate in three regionally and temporally distinct waves to populate the white and grey matters, partially differentiating into myelinating OLs which guarantee proper white matter formation, and partially remaining at undifferentiated stage (resident OPCs) (Michalski & Kothary, 2015). During adult life, and under appropriate conditions, resident and newly generated OPCs from neural stem cells (NSCs) of the subventricular zone (SVZ) can proliferate, migrate to the nude axons, and differentiate into mature oligodendrocytes (OLs) to provide myelin turnover and repair following lesion (Crawford, Chambers, & Franklin, 2013; Trotter, Karram, & Nishiyama, 2010).

Both OPCs and mature OLs are highly vulnerable to inflammation and hypoxia/ischemia (HI), the main pathogenic mechanisms determining demyelination through a number of downstream cellular and molecular mechanisms (Calzà et al., 2018). In particular, neonatal HI is the most common cause of death, as well as of motor, sensory and cognitive disability, during the perinatal/neonatal period (Millar, Shi, Hoerder-Suabedissen, & Molnár, 2017). Clinical conditions leading to neonatal HI generally occur in the time window when OPCs should switch to myelinating OLs to guarantee proper myelination, i.e. from late embryonic gestation throughout early post-natal life (Fancy, Chan, Baranzini, Franklin, & Rowitch, 2011). HI and associated inflammation induces OPC proliferation through Cdk2 pathway activation, but also delayed/impaired maturation and apoptosis (Back et al., 2002; Jablonska et al., 2012; Janowska and Sypecka, 2014), finally resulting in an impairment of myelination (Rocha-Ferreira & Hristova, 2016). The overproduction of cytokines/chemokines itself also promotes OPCs proliferation and inhibits their differentiation into myelinating OLs (Patel & Klein, 2011; Tognatta & Miller, 2016).

As well as in neonatal injuries, the OPC differentiation block is considered a major cause of myelin repair impairment during adult life, as demonstrated in multiple sclerosis (MS), spinal

cord injury and related animal models (Irvine & Blakemore, 2008; Mekhail, Almazan, & Tabrizian, 2012; van Tilborg et al., 2018). In these conditions, OPCs proliferate (Picard-Riera et al., 2002), and part of them differentiate into astrocytes, while the remaining cells are mainly blocked in an OL progenitor phenotype (Picard-Riera et al., 2002; Sozmen et al., 2016). Moreover, both inflammation and HI interfere with the thyroid hormone- (T3) dependent mechanism of OPC cell cycle arrest (Billon, Jolicoeur, Tokumoto, Vennström, & Raff, 2002) and differentiation induction (Fernández, Baldassarro, Sivilia, Giardino, & Calzà, 2016).

Since OPCs are the cells responsible for both developmental myelination and myelin turnover and repair during adult life, it has been suggested that remyelination in adulthood resembles the developmental myelination mechanism (Franklin & Hinks, 1999), and this hypothesis has driven research for remyelination enhancing therapies. However, various studies indicate a number of differences in mechanisms responsible for myelin formation during development and myelin repair during adulthood, leading to the view that this process is differentially regulated depending on age (Baldassarro, Marchesini, Giardino, & Calzà, 2017; Miron, Kuhlmann, & Antel, 2011).

To investigate the vulnerability of fetal and adult OPCs to inflammation and HI in terms of cell death and differentiation, we analyzed the *in vitro* response of OPCs derived from both the fetal and the adult brain to stimuli responsible for myelin loss *in vivo*. To explore the impact of these challenges on maturation, from stem cells to myelinating OLs, we differentiated OPCs from NSCs obtained from the fetal forebrain and the SVZ of adult mice. We preferred this model respect to primary OPCs, because we intended generate a spontaneous mixed culture also containing astrocytes, to better mimic the *in vivo* environment. In order to dissect the mixed cell population, we monitored the GFAP-positive cells (astrocytes) in all the tested conditions.

In the first part of the study, we exposed these cell systems to experimental conditions mimicking *in vitro* inflammation (a cytokine cocktail composed of TGF-b1, TNF-a, IL-1b, IL-6, IL-17 and IFN-γ) or to an HI model (oxygen-glucose deprivation, OGD). All the noxious stimuli were applied to target the precursor phase of the OLs, when cells actively proliferate, to mimic the inflammatory/HI challenge during developmental myelination. We demonstrated that T3-induced differentiation is blocked by the cytokine cocktail in both fetal and adult OPCs. In contrast, OGD impairs differentiation and induces cell death in fetal but

not adult OPCs. We then explored possible mechanisms underlying vulnerability in fetal OPCs, focusing on glutamate toxicity, apoptosis, and metabolic profile, finally finding a different basal and induced metabolism between fetal and adult mixed cultures responsible for driving the differing vulnerability to OGD. Results from this study may have important implications for developing myelin-enhancing therapies and neuroprotection strategies in adult and fetal demyelination diseases and CNS lesions.

2. *Materials and methods*

2.1 *Cell preparation and cultures*

All animal protocols described herein were carried out according to European Community Council Directives (86/609/EEC) and comply with the guidelines published in the *NIH Guide for the Care and Use of Laboratory Animals*.

Fetal and adult NSCs were isolated from E.13.5 fetal forebrain or 2.5 month old adult sub-ventricular zone (SVZ), following the Ahlenius and Kokaia protocol (Ahlenius & Kokaia, 2010). Oligodendrocyte differentiation was performed following the Chen protocol (Chen et al., 2007) with some modifications. Tissues were enzymatically dissociated using trypsin (Sigma-Aldrich, Saint Louis, MO, USA), hyaluronidase (Sigma-Aldrich) and DNase (Sigma-Aldrich), then mechanically dissociated by pipetting. The solution was filtered, centrifuged and the resulting pellet was washed twice in HBSS. Following 7 minutes centrifugation at 400 × *g*, the cellular pellet was resuspended in serum-free medium (DMEM/F12 GlutaMAX 1 x; 8 mmol/L HEPES; 100 U/100 µg Penicillin/Streptomycin; 0.1 x B27; 1 x N-2; 20 ng/mL bFGF; 20 ng/mL EGF; Thermo Fisher Scientific, Waltham, MA, USA), and following cell count, cells were plated in suspension at a density of 10 cells/µl in a final volume of 3 mL in low-attachment 6-well plates (Nunc, Roskilde, DK). The half medium was changed every three days, centrifuging the cell suspension at 300 × *g* for 5 minutes and gently resuspending the cellular pellet in fresh medium. Neurospheres were allowed to proliferate until they attained a diameter of about 100µm.

To obtain oligospheres, primary neurospheres were centrifuged at 300 x *g* for 5 minutes. The pellet was mechanically dissociated by pipetting and cells were counted and plated again at a density of 10 cells/µl in a final volume of 3 mL of OPC medium (DMEM/F12 GlutaMAX 1 x; 8 mmol/L HEPES; 100 U/100 µg Penicillin/Streptomycin; 0.1 × B27; 1 × N-2; 20 ng/mL bFGF; 20 ng/mL PDGF; Thermo Fisher Scientific) in low-attachment 6-well plates (Nunc).

Oligospheres were centrifuged and the pellet was mechanically dissociated to obtain a single cell suspension. Following cell count, cells were plated at a density of 3000 cells/cm^2 on poly-D,L-ornithine (50µg/ml)/laminin (5µg/ml; Sigma-Aldrich) coating, in OPC medium.
To induce oligodendrocyte differentiation and maturation, the OPC medium was replaced with the oligodendrocyte differentiation medium (DMEM/F12 GlutaMAX 1 x; 8 mmol/L HEPES; 100 U/100 µg Penicillin/Streptomycin; 0.1 x B27; 1 x N-2; 50 nM T3; 10 ng/ml CNTF; 1x *N*-acetyl-L-cysteine – NAC; Thermo Fisher Scientific) following 3 DIVs.

2.2 *Cytokine exposure*

Following primary neurosphere splitting, fetal and adult cultures were divided in two groups: one treated with a mix of six different cytokines (TGF-β1, TNF-α, IL-1β, IL-6, IL-17 and IFN-γ; 20 ng/mL each; Thermo Fisher Scientific), the other treated with vehicle (0.04% of the cytokine solvent: 10% glycerol/100 nM glycine/25 nM Tris, pH 7.3). Following oligosphere expansion, the medium was changed to the standard medium, without cytokines, in both groups.

2.3 *Oxygen-glucose deprivation exposure*

OGD was performed using an air-tight hypoxia chamber (Billups-Rothenberg Inc., Del Mar., CA) saturated with 95% N_2 and 5% CO_2 (Goldberg & Choi, 1993). Glucose deprivation was achieved using a glucose-free complete medium (Thermo Fisher Scientific). Oxygen was removed by flushing the hypoxia chamber with N_2-CO_2 mixture for 6-8 min at 25 l/min. The flushing was repeated after half of the incubation time. The OGD condition was maintained for 3 h, after which plates were re-oxygenated in the old glucose-containing medium in the incubator.

2.4 Iopanoic acid treatments

To study the role of deiodinase 3 (D3) activation following OGD-exposure, the effect of deiodinase inhibition was analyzed in fetal cultures. Iopanoic acid (IOP, 10 µM) treatment was performed starting from 24 hours prior to OGD exposure (-1 DIV) and continuing throughout the entire differentiation phase (12 DIV; Figure 4 A). The percentage of D3-positive cells was quantified both at 0 and 12 DIVs. Moreover, at the end of the

differentiation phase (12 DIV), the mature/myelinating OLs (CNPase/MBP-positive cells) were quantified.

2.5 Glutamate/NMDA treatments

To study the role of glutamate excitotoxicity in fetal OGD-induced cell death, another set of experiments was set up.
Cells were exposed in parallel to normoxia or OGD, or treated with Glutamate or NMDA (1 mM) (Matute, 2009). Moreover, to inhibit the glutamate action, all groups were treated with vehicle, MK801 or NBQX in a concentration (10 μM) already proven to rescue primary neurons from excitotoxicity (Baldassarro et al., 2018) from 1h prior to the challenge exposure throughout the entire differentiation phase (12 DIV).
At the end of the differentiation phase, cell number and cell death were analyzed in all groups using cell-based HCS.

2.6 Glucose deprivation exposure, 2-DG and Oligomycin treatments

To study the role of glucose metabolism perturbation in fetal OGD-induced cell death, in another set of experiments, cells were exposed to 24 or 3 hours of glucose deprivation, 2-deoxy-D-glucose (2-DG; 50 mM; Cayman Chemical, Ann Arbor, MI, USA) or oligomycin (1 μM; Cayman Chemical) treatment, 24 hours prior to T3-mediated differentiation induction.
Cells exposed for 24 hours were analyzed for lactate production, while cells exposed for 3 hours, were analyzed for caspase activation (0 DIV) and cell death and differentiation (0 and 6 DIV) by using specific markers for OPCs (PDGFαR-positive cells), mature OLs (CNPase-positive cells) and myelinating OLs (MBP-positive cells).

2.7 Lactate quantification

Lactate production was quantified in fetal and adult cultures 24 hours following GD exposure, 2-DG or oligomycin treatments, using the colorimetric glycolysis cell-based assay kit (Cayman Chemical) and normalizing the absorbance on the control group (standard culture conditions treated with vehicle).

2.8 Immunocytochemistry

Indirect immunofluorescence was used to identify OPCs (NG2 or PDGFαR-positive cells), mature (CNPase-positive cells) and myelinating (MBP-positive cells) oligodendrocytes, neurons (β-III-tubulin-positive cells), astrocytes (GFAP-positive cells) and deiodinase 3 expressing cells (D3). The following primary antisera were used: mouse anti-β-III-tubulin (R&D system, Trento, IT) 1:3000; rabbit anti-GFAP (Glial Fibrillary Acidic Protein, Dako, Santa Clara, CA, USA) 1:1000; rabbit anti-NG2 (chondroitin sulphate proteoglycan, neural/glial antigen 2, Millipore, Merck S.p.a., Milan, IT) 1:350; mouse anti-CNPase (2', 3'-cyclic nucleotide 3'-phosphodiesterase, Millipore) 1:500; rabbit anti-MBP (Myelin Basic Protein, Dako) 1:500; goat anti-D3 (deiodinase type 3, Santa Cruz Biotechnology, Dallas, TX, USA) 1:100; rabbit anti-cleaved caspase 3 (BD Pharmingen, San Jose, CA, USA) 1:200 and rabbit anti-PDGFαR (Platelet derived growth factor alpha receptor; Santa Cruz Biotechnologies) 1:300. Donkey Alexa 488-conjugated anti mouse IgG donkey Alexa 568-conjugated anti-rabbit IgG, 1:500 (Invitrogen, Carlsbad, CA, USA) and DyLight488-conjugated affinity-pure donkey anti-Goat IgG (Jackson Immunoresearch, Cambridge, UK) 1:100 were used as secondary antisera. Following immunofluorescence staining, cells were incubated with the nuclear dye Hoechst 33258 (1 μg/mL in PBS, 0.3% Triton-X 100) for 20 min at RT. Finally, cells were washed in PBS and mounted in glycerol and PBS (3:1, v/v) containing 0.1% paraphenylendiamine.

2.9 *Fluorescence microscopy*

Fluorescence microscopy was used to quantify the percentage of D3-expressing cells and cleaved-caspase 3 – positive cells in fetal cultures 24 hours following OGD or GD exposure.

Images were captured using a Nikon Eclipse E600 microscope equipped with a digital CCD camera Q Imaging Retiga-2000RV (Q Imaging, Surrey, BC, CA). Measurements were performed using Nis-Elements AR 3.2 software.

Five random fields per glass (duplicate per group) were analyzed. Marker-positive cell percentage was calculated on the total number of cells per field, identified by the nuclear staining.

2.10 *High Content Screening*

For HCS analysis, cells were grown in 96 flat-bottom well HCS plates (Nunc). Analysis of condensed nuclei, cell number and lineage/differentiation markers were performed with Cell Insight™ CX5 High Content Screening (HCS; Thermo Fisher Scientific), using the *Compartmental analysis* BioApplication. Based on nuclear staining, the software is able to recognize the nuclei and calculate the percentage of high intensity/small-sized condensed nuclei. Moreover, based on the identification of the nuclei, the software is able to detect the presence of the marker-specific staining in the cell body, calculating the percentage of immunoreactive cells. Lineage/differentiation marker analysis was performed only on cells showing intact nuclei, excluding condensed nuclei from the percentage calculation. The HCS system permits analysis of the whole culture, avoiding the bias in choosing random fields. A mean of 10000 cells/wells was included in this analysis.

2.11 *RNA extraction, reverse transcription and PCR arrays*

The RNeasy Micro Kit (Qiagen, Venlo, NL) was used for total RNA extraction, and 500 ng was retrotranscribed using the RT^2 First Strand Kit (Qiagen) following the manufacturer's instructions. Cells were lysed 24 hours following normoxia or OGD exposure, and three samples from independent experiments were used in the analysis. For the study of glucose-related gene expression, PCR array for mouse glucose metabolism (Table S1; PAMM-006ZA; Qiagen) was used in combination with the RT^2 SYBR Green qPCR Mastermix (Qiagen).

2.12 *Study design and statistical analysis*

Experiments were performed in triplicate (three independent experiments) for cell number and viability, and in duplicate for cell marker analysis. Data is reported as mean ± SEM. Prism software (GraphPad Software, San Diego, CA, USA) was used for statistical analyses and graph generation. Student's t-test or one-way ANOVA were used to analyze data. Results were considered significant when the probability of their occurrence as a result of chance alone was less than 5% ($p < 0.05$).

3. *Results*

3.1 The cell systems

OPCs were obtained by driving NSCs differentiation using specific factors (Figure 1 A). Primary neurospheres were obtained in the presence of EGF and bFGF. Following neurosphere splitting, EGF was replaced by PDGF in the culture medium, starting the OPC differentiation (oligospheres). To obtain the OPCs, oligospheres were seeded as single cell, and the bFGF/PDGF medium replaced with T3 containing medium after 3 days, allowing the OLs to mature for 12 DIVs.

NSCs-derived cultures are mixed cultures composed of OPCs, OLs and astrocytes, with a small percentage of neurons (Baldassarro et al., 2017). Lineage progression from NG2-positive OPCs (DIV0) through CNPase-positive, mature OLs as far as myelinating CNPase/MBP-positive OL is illustrated in Fig. 1 B-D (representative images). The culture composition changes during the 12 DIV differentiation period, starting from 0 DIV, where the majority of cells are NG2 positive (NG2-positive cells 80%; CNPase-positive cells 5%), passing thorough DIV 6, where CNPase cells are growing in number (NG2-positive cells 60%; CNPase-positive cells 30%), reaching the end of the differentiation with CNPase/MBP positive cells representing the majority of cells (CNPase-positive cells; 70% in fetal and 45% in adult cultures; MBP-positive cells, 40% in fetal and 30% in adult cultures). Both cultures show around 40% of GFAP-positive cells at 12 DIVs, and a small percentage (6-8%) of beta-III-tubulin positive cells (neurons).

The experimental design used in all experiments aims to expose the cell system to the challenge during the T3-mediated cell cycle arrest and maturation induction of OPCs (Billon et al., 2002), keeping a mixed environment also containing astrocytes.

3.2 Inflammatory cytokines block OPC differentiation in both fetal and adult OPCs

In a first set of experiments, we tested fetal and adult OPCs in the presence of inflammatory stimuli. Fetal and adult NSCs-derived OPCs were exposed to an inflammatory cytokine mix composed of TGF-β1, TNF-α, IL-1β, IL-6, IL-17 and IFN-γ. This cocktail blocks adult OPC differentiation when applied in the proliferation phase of the culture, while exposure during the differentiation phase results in cytotoxic effects (Fernández et al., 2016). Thus, the cytokine mix was applied to the oligosphere phase, during replication, and in the absence of differentiating stimuli (Figure 2A). Three days after the OPC cell seeding, cells were triggered to differentiate into OPCs by T3. At the end of the differentiation phase (12 DIV), cell number, cell death and differentiation were analyzed using HCS technology.

Fetal cultures treated with the cytokine mix show an increase in total cell number (Student's t-test: $p < 0.0001$) and a reduction in the percentage of condensed nuclei ($p = 0.0007$; Figure 2 B). Adult cells also show an increase in cell number ($p = 0.0009$), while no changes in cell death was detected (Figure 2 C).

At the end of the differentiation period, cytokine-treated cells show a different cell composition compared to vehicle-treated cells. Fetal cultures show no changes in the percentage of precursors (NG2-positive cells), but a drastic decrease in mature/myelinating OLs (Student's t-test: CNPase, $p < 0.0001$; MBP, $p < 0.0001$). Moreover, a reduction in astrocyte population was also observed (GFAP-positive cells, $p = 0.0030$; Figure 2 D).

As already described (Fernández et al., 2016), cytokine treatment in adult cultures causes an increase in the percentage of NG2-positive cells (Student's t-test, $p = 0.0025$). Moreover, as per the fetal cultures, adult cell differentiation/maturation is drastically reduced in the cytokine- compared to the vehicle-treated cells (Student's t-test, CNPase, $p = 0.0273$; MBP, $p = 0.0036$). As well as in fetal cultures, astrocyte differentiation is also impaired in cytokine treated cells ($p = 0.0001$; Figure 2 E). As already described for adult cultures (Fernández et al., 2016), this perturbation during differentiation/maturation maintains cells at undifferentiated state, as indicated by negativity to all tested lineage-specific markers Representative images of CNPase and MPB-immunofluorescence staining in fetal and adult cultures treated and untreated with the cytokines mix are included in Figure 2 (F-M).

3.3 *Fetal but not adult OPCs are vulnerable to OGD-*

In these experiments, we tested fetal and adult OPC vulnerability to OGD, the most widely used *in vitro* model for HI. OGD was applied for 3 hours, followed by 24 hours of reperfusion/reoxygenation during the proliferation phase of seeded OPCs. T3-induced differentiation was triggered immediately following the reperfusion/reoxygenation phase (Figure 3 A). We decided to use this OGD schema, which has been widely used on neuronal cultures, where it causes 50 % and 20 % cell death in pure and mixed neonatal fetal cortical neurons respectively (Baldassarro et al., 2018). Here we analyzed OPCs survival and lineage specification and maturation at the end of the differentiation phase (12 DIV) by HCS technology.

OGD induces a reduction in cell number (Student's t-test, $p < 0.0001$) and an increase in cell death ($p < 0.0001$; Figure 3 B) at the end of the differentiation phase in fetal cultures. In

contrast, adult cultures exposed to OGD show an increase in cell number ($p < 0.0001$) without affecting cell death ($p = 0.1247$; Figure 3 C).

As already indicated, the preparation of OPCs derived from NSCs also contains astrocytes, and few neurons. To establish the cell population affected by OGD, we used Cell Insight™ CX5 HCS. This software identifies nuclear size and morphology (normal nucleus for living cells; condensed nuclei for dead cells), and the associated cytoplasm immunostaining for astrocytes (GFAP), neurons (b-III-tubulin), OPCs (NG2), mature OLs (CNPase) and myelinating OLs (MBP), thus allowing the identification of degenerating cell types.

We first quantified the percentage of the different cell-specific markers in cells showing normal nuclear morphology, considered live cells. In fetal cultures, OPCs and mature/myelinating OL populations are affected by OGD exposure, as shown by the decrease in NG2- ($p = 0.0158$), CNPase- ($p < 0.0001$) and MBP- ($p = 0.0002$) positive cells, while no effects are present in astrocyte (GFAP, $p = 0.3137$) or neuron (b-III-tubulin, $p = 0.2998$) populations (Figure 3 D). In contrast, adult cultures show an increase in NG2-positive cells ($p = 0.0025$), while no effects on all the other analyzed markers are observed (CNPase, $p = 0.6283$; MBP, $p = 0.3606$; GFAP, $p = 0.1530$; b-III-tubulin, $p = 0.9914$; Figure 3 E). The specificity of OGD-induced cell death in fetal OPCs/OLs was also confirmed by the analysis of the cells showing condensed nuclei. Fetal-derived cultures show an increase in cell death in NG2, CNPase and MBP-positive cells (Student's t-test, NG2, $p = 0.0002$; CNPase, $p = 0.0028$; MBP, $p = 0.0095$), without affecting astrocytes and the small percentage of neurons (GFAP, $p = 0.0880$; beta-III-tubulin, $p = 0.1272$; Figure 3 F). Adult-derived cultures, as expected from the analysis of the whole culture, do not show cell death in any population (NG2, $p = 0.4690$; CNPase, $p = 0.2321$; MBP, $p = 0.3568$; GFAP, $p = 0.8302$; beta-III-tubulin, $p = 0.4015$, Figure 3 G). Representative images of the OGD-exposed cultures are included in the figure (Figure 3 H-O).

To investigate the mechanisms underlying the OGD-induced cell death and impaired differentiation of surviving cells observed in fetal but not adult cells, we examined the three main players in the process: i) the T3-dependent differentiation; ii) glutamate excitotoxicity and iii) glucose metabolism.

3.4 Deiodinase inhibition is not effective in rescuing OGD-induced cell death or the differentiation impairment of OPCs derived from fetal NSCs.

We have already demonstrated that under cytokine exposure, and coupled with the OPC differentiation block, the expression of the T3-inactivating enzyme (D3) increases in the cytoplasm of OPCs, while its inhibition by IOP (10 μM) restores proper OPC differentiation (Fernandez et al., 2016). We then explored whether this mechanism also supports OPC differentiation block in fetal preparations exposed to OGD. First, we demonstrated that following the reperfusion/reoxygenation phase of the OGD model (0 DIV, Figure 4 A), fetal-derived cultures show an increase in D3-positive cell percentage (Student's t-test, $p = 0.0009$; Figure 5 B-D). However, the treatment with IOP is not able to rescue the decrease in mature/myelinating OLs in the culture induced by OGD exposure (Figure 4 E). In fact, the IOP treatment is able to decrease D3-positive cells in OGD-exposed cultures at the end of the differentiation phase (one-way ANOVA, $F(3, 17) = 0.7360$, $p = 0.0337$; Tukey post-test $p = 0.0213$) and, as expected, is also able to increase the percentage of myelinating MBP-positive OLs in normoxia-exposed control cultures (Dunnett's post-test, $p = 0.0162$). Following OGD exposure, however, the composition of the culture shows a decrease in mature/myelinating OPCs in both vehicle and IOP-treated cells after 12 DIVs (one-way ANOVA, CNPase, $F(2, 28) = 1.099$, $p < 0.0001$, Dunnett's post-test, vehicle, $p < 0.0001$, IOP $p < 0.0001$; MBP, $F(3, 32) = 1.438$, $p < 0.0001$, Dunnett's post-test, vehicle $p < 0.0001$, IOP $p < 0.0001$).

3.5 Glutamate excitotoxicity is not involved in the OGD-induced cell death of OPCs derived from fetal NSCs

Glutamate-triggered cell death is a mechanism proposed in several *in vitro* and *in vivo* conditions, including HI characterized by OL and OPC cell death (Káradóttir & Attwell, 2007); (Simonishvili, Jain, Li, Levison, & Wood, 2013); (Deng, Rosenberg, Volpe, & Jensen, 2003). To study the possible role of glutamate excitotoxicity in OGD-induced cell death in fetal NSCs-derived OPCs, we compared different treatment schemas with glutamate and glutamate receptor antagonists in normoxia and OGD conditions. In particular, we exposed fetal cultures to normoxia, or OGD and glutamate or NMDA (1mM) to confirm glutamate excitotoxicity in our experimental conditions. Moreover, to test a protective treatment by glutamate receptor antagonists, cells were treated with vehicle, MK801 (NMDA-inhibitor) or NBQX (AMPA-inhibitor), starting one hour prior to the exposures and continuing throughout the entire differentiation phase (Fig. 5 A).

We first examined the effect of glutamate in our cell system in normoxia. At the end of the differentiation phase (12 DIV), glutamate-treated cultures showed a reduction in cell number,

while glutamate and NMDA treatment had no effects on the percentage of condensed nuclei. The treatment with the two glutamate antagonists was also ineffective in normoxia (Fig. 5 B, one-way ANOVA, $F(10, 75) = 0.4191$, $p < 0.0001$; Sydak's post-test, glut, $p < 0.0198$; glut-MK801, $p = 0.0054$; glut-NBQX, $p = 0.0033$).

We then tested the effect of glutamate receptor antagonists in OGD. As described above, in the OGD exposed cultures, we observed a decrease in cell number (Sydak's post-test, OGD, $p = 0.0375$) and an increase in condensed nuclei (one-way ANOVA, $F(10, 77) = 13.37$, $p < 0.0001$; Sydak's post-test, $p < 0.0001$), while the exposure to the two glutamate antagonists was ineffective in both cell number (Sydak's post-test, MK801, $p = 0.0133$; NBQX, $p = 0.0002$) and cell death (Sydak's post-test, MK801, $p < 0.0001$; NBQX, $p < 0.0001$) rescues.

3.6 Glucose metabolism perturbation is responsible for the OGD-induced cell death of OPCs derived from fetal NSCs

Finally, we investigated the contribution of glucose metabolism to OGD-induced cell death in OPCs derived from fetal NSCs. We first attempted to establish the glucose metabolic state in both fetal and adult cells, by using tools able to differentiate glycolysis and oxidative phosphorylation (OXPHOS). We exposed cultures to glucose deprivation-only (GD), and to metabolic path interfering agents (2-DG and oligomycin), before measuring glycolytic activity by lactate production following 24 hours of exposure (0 DIV; Figure 6 A, red arrow). 2-DG contains the 2-hydroxyl group replaced by hydrogen, thus blocking glycolysis at early stages, resulting as toxic to highly glycolytic cells (Coleman et al., 2008); oligomycin inhibits ATP synthase, blocks the proton channel and consequently OXPHOS, resulting in a compensatory increase in glycolysis activity (Bertina, Steenstra, & Slater, 1974).

Fetal and adult NSCs-derived OPCs/astrocytes mixed cultures show a different metabolic state in basal conditions. In particular, fetal cultures metabolism resulted as being completely dependent on glycolysis (Figure 6 B). In fact, the relative quantification of lactate production showed a decrease in this parameter following 24 hours of GD exposure or 2-DG treatment compared to the standard condition (100%) (one-way ANOVA, $F(3, 11) = 40.14$, $p < 0.0001$; Dunnett's post-test, GD, $p < 0.0001$; 2-DG, $p < 0.0001$), but oligomycin treatment showed no changes in lactate levels, proving that the entire metabolism of these cells is based on glycolysis.

On the other hand, adult cell metabolism is not strictly glycolytic (Figure 6 C). In fact, even when lactate production breaks down during GD or 2-DG exposure, these cultures show a

compensatory activation of glycolysis, as indicated by increased lactate production, when exposed to oligomycin (one-way ANOVA, $F(3, 8) = 97.57$, $p < 0.0001$; Dunnett's post-test, GD, $p = 0.0029$; 2-DG, $p = 0.0030$; oligomycin, $p < 0.0001$)

The absence of a compensatory overproduction of lactate in oligomycin treated fetal cultures suggests that no compensatory glycolytic activity is needed when OXPHOS is blocked, meaning that the entire metabolism of these cells is based on glycolysis in standard conditions. By contrast, adult cells need to compensate when treated with oligomycin, with increasing glycolytic activity, indicating that their metabolism in basal conditions contemplates OXPHOS.

To investigate the role of glucose metabolism block as a component of OGD action and downstream effects (cell death and differentiation), we used the same tools of the OGD experiments (3 hours followed by 24 hours of reperfusion). The effect of the different treatments on cell metabolism was analyzed at intermediate stages of the differentiation/maturation process, i.e. after the reperfusion phase (0 DIV; Figure 6 A, red arrow) or in the middle of the differentiation phase (6 DIV; Figure 6 A, black arrow). Glucose metabolism perturbation induced a reduction of cell number and an increase of cell death in fetal cultures only. In particular, cell number was reduced at both time points in fetal cultures (Figure 6 D; one-way ANOVA, 0 DIV, $F(3, 8) = 109.4$, $p < 0.0001$; 6 DIV, $F(3, 8) = 54.05$, $p < 0.0001$) in cells exposed to GD (Sidak's post-test, 0 and 6 DIV, $p < 0.0001$) and 2-GD (0 and 6 DIV, $p < 0.0001$), while no effect was detected in fetal cultures treated with oligomycin and in adult cultures in all the tested conditions (Figure 6 E).

Moreover, we detected cell death, expressed as percentage of condensed nuclei, in fetal cultures only, at both time points (Figure 6 F; one-way ANOVA, 0 DIV, $F(2, 14) = 6.718$, $p = 0.0005$; 6 DIV, $F(2, 14) = 0.3273$, $p = 0.0003$), but at 0 DIV only 2-DG treatment (i.e. the glycolysis block) resulted in an increase in cell death (Sidak's post-test, $p = 0.0004$), while at a late stage, both GD exposure and 2-DG treatment showed strong cell death induction (Sidak's post-test, GD $p = 0.0107$, 2-DG $p = 0.0001$). As expected, treatments had no effect on adult cultures (Figure 6 G).

To explore the down-stream effects of glucose metabolism perturbation in fetal cultures, we analyzed apoptosis activation and OPC differentiation (Figure 7 A).

First, we analyzed the effect of GD on apoptosis by quantifying the percentage of cells positive for cleaved caspase-3 following reperfusion (0 DIV). Cultures show an increase in

the percentage of cleaved-caspase 3 positive cells (Student's t-test, $p < 0.0001$; Figure 6 C-E), proving that the absence of glucose induces apoptosis as soon as 24 hours later.

Keeping the focus on the mixed composition of the NSCs-derived OPCs cultures, we already showed that only OPCs/OLs lineages are vulnerable to OGD insult. Thus, we quantified the lineage-specific cell death also in GD, 2-DG and oligomycin exposed fetal cultures, showing that at an intermediate time point in the differentiation phase (6 DIV) only OPCs are vulnerable to glucose metabolism perturbation (Figure 7 E). In particular, only PDGFαR-positive cells show vulnerability at 6 DIV (one-way ANOVA, $F(9, 32) = 52.87$, $p < 0.0001$) only after GD and 2-DG exposure (Sidak's post-test, GD, $p < 0.0001$; 2-DG, $p < 0.0001$).
Finally, we analyzed the composition of the culture in the middle of the differentiation phase (6 DIV; Figure 7 F-O) for OPCs (PDGFαR-positive cells), mature OLs (CNPase-positive cells) and myelinating OLs (MBP-positive cells), in both GD-exposed and 2-DG treated cultures. A decrease in the three cell populations (one-way ANOVA, PDGFαR, $F(2, 6)$, $p < 0.0001$; CNPase, $F(2, 6) = 0.4218$, $p = 0.0003$; MBP, $F(2, 6) = 4.602$, $p = 0.0021$) was observed. In particular, the exposure to both challengers strongly decreases the PDGFαR population (Sidak's post-test, GD, $p < 0.0001$; 2-DG, $p < 0.0001$; Figure 6 G-I), but also the CNPase-positive (Sidak's post-test, GD, $p = 0.0237$; 2-DG, $p = 0.0002$; Figure 6 J-L) and myelinating MBP-positive OL populations (Sidak's post-test; GD, $p = 0.0262$; 2-DG, $p = 0.0015$; Figure 6 M-O).

3.7 OPCs derived from fetal NSCs are not able to react to OGD

Once we had established that the different response to glucose metabolism perturbation of mixed OPCs/astrocytes cultures may drive the selective vulnerability of fetal OPCs to OGD, we investigated how fetal and adult cells respond to this challenge in terms of metabolic re-adaptation. To do so, we used a PCR array® to analyze the main genes involved in glucose metabolism in basal conditions and 24 hours after OGD exposure (0 DIV; Figure 8 A). In particular, the array allows the analysis of 86 genes involved in different glucose-related processes: glycolysis, gluconeogenesis, glucose metabolism regulation, the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway (PPP), glycogen synthesis, glycogen degradation, and glycogen metabolism regulation (the full list of genes is reported in the supplementary data, Table S1).

Following OGD and reperfusion/reoxygenation, the gene expression of fetal cells does not change (Figure 8 B). On the other hand, adult cultures respond to OGD, increasing the expression of 37 genes (Figure 8 C) related to glucose metabolism (Table 1).

4. *Discussion*

White matter injury is recognized as a major determinant in long-term outcome following traumatic CNS and vascular lesions in both infancy and adulthood, impacting on axonal integrity, function, and retrograde neuronal degeneration (Fern, Matute, & Stys, 2014), making hypomyelinating and demyelinating white matter pathology a recognized target for pharmacological intervention (Chew & DeBoy, 2016). OPCs, when differentiating into mature/myelinating OLs in a T3-drived, synchronized process (Raff, Lillien, Richardson, Burne, & Noble, 1988), are the cells responsible for both developmental myelination and myelin turnover and repair during adulthood, leading to the hypothesis that myelin repair "recapitulates" developmental myelination (Fancy et al., 2011).

The ability of OPCs to correctly establish or repair the myelin sheaths is severely impaired during pathological conditions characterized by HI and severe inflammation both in development and adulthood, OPCs and OLs being the most vulnerable cells in the CNS (Bradl & Lassmann, 2010; Y. Lee et al., 2012). Moreover, oligodendroglial lineage susceptibility to adverse stimuli is also dependent on developmental and maturation stage, from early precursors to mature OLs (Amaral, Hadera, Tavares, Kotter, & Sonnewald, 2016), and drug development strategy should be undertaken accordingly.

To investigate the vulnerability of fetal and adult OPCs to inflammation and HI, and the effects of these challenges on cell survival, maturation and developmental myelination and/or myelin repair, in this study we exposed fetal and adult OPCs to *in vitro* models of inflammation and HI. To shed light on the entire differentiation/maturation window of these cells, from multipotent NSCs through precursor (OPCs) to mature and myelinating OLs, we differentiate OPCs from fetal and adult NSCs. This system allows the establishment of a mixed cellular environment enriched in OPCs/OLs but keeping astrocytes (30-40%) throughout the whole differentiation period, thus avoiding the bias of isolated OPCs. Moreover, we monitored the effect of the tested stimuli also in the astrocytes.

We first demonstrated that no substantial differences are observed in vulnerability to cytokine exposure in fetal and adult NSCs-derived OPCs. In particular, cytokine exposure during the

proliferative phase leads to an increased proliferation and a differentiation block in both fetal and adult OPCs, as indicated by lineage progression marker analysis. Numerous *in vitro* and *in vivo* studies have indicated that inflammatory cytokines promote OPC proliferation, involving, among others, the VEGFR2 (Choi et al., 2018) and Notch pathways (Wang et al., 2017). Inflammatory cytokines are also proved to be involved specifically in the differentiation block of OPCs derived from the adult brain, affecting cell cycle exit and the expression of genes encoding for proteins that regulate and determine remyelination (Chew, King, Kennedy, & Gallo, 2005; Falahati et al., 2013; Kang et al., 2013; Su et al., 2011; Tanner, Cherry, & Mayer-Pröschel, 2011). The most investigated example of the role of inflammation in remyelination failure is MS, the most diffuse inflammatory/demyelinating disease in humans (Goodin, 2014). In the acute focal phase of the disease, remyelination is efficient (Rodgers, Robinson, & Miller, 2013), however this process fails progressively (Boyd, Zhang, & Williams, 2013; Lassmann, van Horssen, & Mahad, 2012). The most accredited hypothesis for remyelination failure is the block of OPC differentiation and maturation into myelinating OLs (Kuhlmann et al., 2008; Miller & Mi, 2007), leading to inefficient myelin repair (Zhang, Luo, Wu, & Xu, 2015). We have previously demonstrated that a complex dysregulation of TH tissue signaling is present in experimental CNS inflammation/demyelination models (experimental allergic encephalomyelitis in rodents and non-human primates) (D'Intino et al., 2011; Dell'Acqua et al., 2012; Fernández et al., 2016), which correlates with the rise of inflammatory cytokines released by peripheral cells and resident microglia (Borjini, Fernández, Giardino, & Calzà, 2016). This pathological feature is causally linked to an increase in D3-positive cells and D3 expression both *in vivo* and *in vitro*, causing the inactivation of T3 and subsequent OPC differentiation block. This hypothesis has been confirmed by *in vitro* studies of our group, in which we demonstrated that the inhibition of D3 restores OPC differentiation during exposure to the inflammatory cytokine cocktail (Fernández et al., 2016). An increase in D3 activity and mRNA expression level has been also described in other neurological conditions characterized by inflammation and demyelination, e.g. ischemia-induced hypoxic brain damage (Jo et al., 2012) and nerve lesion (Li et al., 2001).

While inflammation evokes the same cellular outcome in both fetal and adult OPCs, blocking the differentiation process, here we demonstrated that fetal and adult OPCs substantially differ in vulnerability to OGD. In adult cultures, 3 hours of OGD applied 24 hours prior to the T3-mediated differentiation does not affect the cell death (nuclear condensation) or

differentiation process. In contrast, in fetal cultures we observed an increase in cell death, a reduction in cell number, and impaired differentiation of surviving cells. Notably, OGD-induced cell death is selective for OPC/OL lineage, not affecting the astrocyte population. The effect of OGD on OPC maturation seems to be stage-dependent, PreOLs (late OPCs) being the most susceptible cells, followed by early OPCs and mature OLs. This *in vitro* result corresponds to *in vivo* studies, where the death of PreOLs and the OPC differentiation block in the pre-myelinating and immature state are indicated as responsible for the subsequent failure of myelination in neonatal encephalopathy and related animal models (Back et al., 2002).

Notably, in both inflammation and OGD-induced differentiation block, the whole lineage is perturbed, as indicated by the presence of cells that are negative to all the tested lineage markers, and cells that co-express OPCs/OLs markers. This is probably due to the fact that OLs-specific markers identification is not a black-or-white system, but lineage antigens expression and disappearance overlaps in the maturation steps (Silbereis, Huang, Back, & Rowitch, 2010).

Having described the fetal-selective vulnerability of OPCs to OGD, we then investigated the main components that may be implicated in this selective cell death and differentiation impairment.

First, since OPC differentiation is a T3-driven process, we investigated intracellular T3 metabolism. In fact, as already described for inflammation mediated-differentiation block, an appropriate T3 intracellular concentration and signaling in OPCs is mandatory for proper OPC differentiation into myelinating OLs (Calzà et al., 2018; J. Y. Lee & Petratos, 2016). The most remarkable demonstration of this is observed in Allan-Herndon-Dudley syndrome, due to a mutation of the TH cell membrane transporter MCT8, characterized by impaired OL maturation, hypo/dis-myelination and mental retardation (La Piana, Vanasse, Brais, & Bernard, 2015). The causal role of the inappropriate intracellular T3 content on OPC differentiation block has been also demonstrated by the *in vitro* manipulation of the MCT8 transporter in human embryonic stem cell-derived OLs (J. Y. Lee et al., 2017). In this study, we demonstrated that as well as inflammatory cytokine exposure, OGD also generates an increase in the percentage of D3-positive cells, suggesting a perturbation of T3-mediated OPC differentiation. As expected, the pharmacological inhibition of D3 by IOP favors OPC maturation in normoxia, as indicated by the increased percentage of MBP-positive cells, but is

unable to restore OL differentiation following OGD, in contrast to the positive results obtained in adult OPCs exposed to cytokines (Fernández et al., 2016).

These results indicated that, even if a perturbation of T3-mediated differentiation may occur in OPCs exposed to OGD, the restoration of a suitable T3 level by D3 inhibition is not enough to rescue OPC differentiation. However, we cannot exclude that a long-lasting TR perturbation also occurs in the experimental conditions used in this study.

We then explored alternative mechanisms to explain the selective sensitivity of fetal OPCs to OGD. Extensive literature points to the role of glutamate receptor overactivation in HI-induced cell death. In particular, AMPA/kainite, but not NMDA receptors, have been considered the mediator of glutamate excitotoxicity in glial cells (Tekkök & Goldberg, 2001), in particular in the glutamate-mediated cell death of preOLs during HI (Deng, Yue, Rosenberg, Volpe, & Jensen, 2006).

We then tested glutamate and NMDA treatment in fetal OPCs in normoxia. The cell system resulted as being partially sensitive to glutamate, which reduces cell number without inducing cell death. On the contrary, exposure to NMDA is ineffective. We then used specific NMDA and AMPA inhibitor (MK801 and NBQX respectively) treatments to interfere with potential glutamate excitotoxicity during OGD, showing that OGD-mediated cell death is not rescued by MK801 and NBQX treatments. This data suggests that in a complex system, consisting of OPCs and astrocytes, OGD is able to evoke oligodendroglial specific cell death, which is not mediated by glutamate release and excitotoxicity. A different expression of NMDA receptors during the differentiation process may explain the different sensitivity to glutamate at different stages. In particular, OPCs show a high expression of the AMPA/kainite receptor mGluR, while postmitotic OLs have high NMDA receptor expression (Butts, Houde, & Mehmet, 2008). Therefore, we cannot exclude that a different exposure scheme targeting OPCs at a different maturation stage might result in a different sensitivity to glutamate excitotoxicity.

Finally, we hypothesized that a different metabolic profile might drive the different vulnerability to OGD of fetal compared to adult OPCs/astrocytes mixed cultures. In fact, previous studies from other groups have described that OLs at late differentiation stages avidly metabolize glucose and pyruvate derived from glucose, also demonstrating a high degree of cellular independence (Amaral et al., 2016). We decided to perform also these experiments using OPCs/astrocytes mixed cultures, and not isolated OPCs and astrocytes,

because of the importance of the metabolic cross-talk between OPCs and astrocytes (Kıray, Lindsay, Hosseinzadeh, & Barnett, 2016). In the balance between losing the cell-specificity of the metabolism information, and the bias derived from isolating a single cell type which implies the risk of producing inconsistent results because of the metabolic modifications induced by the procedure itself, we considered that mixed cultures derived from neurospheres were more appropriate for the aim of the study.

Here we first demonstrated that fetal and adult NSC-derived OPCs/astrocytes mixed cultures differ in their metabolism in basal conditions. In particular, glycolysis within fetal mixed OPC-astrocyte preparations is crucial to ensure OPC survival, which is not the case in adult cultures. In fact, even if we cannot discern the cell type producing or not lactate in the mixed cultures, fetal cells do not increase lactate levels when exposed to OXPHOS block, showing that both fetal OPCs and astrocytes are at the maximum level of glycolysis activity. This may explain the death of fetal OPCs during glucose depletion, while adult OPCs are not sensitive to this insult. A limitation of this result is that we cannot identify if the fetal OPCs death is directly linked to glucose deprivation, or indirectly related to the astrocytes metabolism perturbation. However, the goal of the study is to investigate the vulnerability of the OPC/OL lineage cells, always taking into account the astrocytic component of the culture. In fact, using the cell-based HCS, and as already described for OGD experiments, we are able to discriminate the lineage-specific cell death and differentiation. As we described in long term cultures (12 DIV) after OGD exposure, both OPCs and OLs are sensitive to the noxious stimulus, as indicated by increased cell death. In the middle term cultures (6 DIV) cell death is restricted to OPCs after glucose metabolism perturbation, probably for the low percentage of mature OLs at this time point.

Interestingly the astrocytic component of the culture is sensitive to cytokine mix in term of differentiation, while OGD or glucose metabolism are ineffective neither in cell survival nor differentiation. Due to the complex metabolic crosstalk between astrocytes, OPCs and OLs (Kıray et al., 2016) future studies are needed to discern the respective roles during metabolic disturbance.

Moreover, glucose metabolism perturbation in the OPCs/astrocytes mixed cultures, when cells are still precursors, results in impaired differentiation of fetal OPCs, something which could potentially lead to long-term effects. Even if in our model we cannot selectively study the OPCs metabolism, the fundamental role of glycolysis has been already observed in human primary OPCs/OLs, with a higher susceptibility of precursors to glucose metabolism perturbation (Rone et al., 2016). Not only energy metabolism, but also the significant

reduction in lactate production itself, by OPCs and/or astrocytes, observed following GD or in response to 2-DG, may underlie the vulnerability of OPCs, particularly in terms of differentiation. In fact, lactate itself induces the cell cycle, differentiation/myelination, reduces cell death in OPCs (Ichihara et al., 2017), and has been described as fundamental for developmental myelination (Rinholm et al., 2011). More generally, it is well-known that low oxygen tension, by shifting the cell metabolism towards anaerobic glycolysis, enhances pluripotency-related gene expression, including Wnt (De Miguel, Alcaina, de la Maza, & Lopez-Iglesias, 2015), which arrests OPC differentiation (Yuen et al., 2014).

All these experiments from other groups contribute to indicating a primary role of glucose metabolism in fetal-derived OPCs, pointing to this intrinsic characteristic as the main culprit behind the selective OGD-induced noxious effect. Given that the different basal metabolism of the mixed system may result in the different vulnerability to OGD of OPCs, finally we analyzed the expression of the main genes related to glucose metabolism in fetal and adult OPCs/astrocytes mixed cultures in normoxia or following OGD. Notably, OGD alters the gene expression profile in adult cultures only, increasing the expression level of genes involved in glycolysis, the regulation of glucose metabolism, the TCA cycle, PPP, glycogen synthesis, glycogen degradation, and glycogen metabolism. This gene expression regulation indicates that adult OPCs/astrocytes mixed cultures exposed to OGDs are able to regulate glycolysis, as was expected, but that they also fuel production from alternative substrates, such as glycogen, necessary for OPC survival and differentiation (Ichihara et al., 2017).

In conclusion, we demonstrated that OPCs derived from NSCs from the fetal forebrain and from the adult SVZ are differentially vulnerable to challenges mimicking pathogenic mechanisms in inflammatory and vascular diseases, thus supporting the view that drug development strategies for myelination/remyelination enhancing therapies must be selectively designed for neonatal and adult demyelinating diseases and lesions.

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752 **References**

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754 Ahlenius, H., & Kokaia, Z. (2010). Isolation and Generation of Neurosphere Cultures from
755 Embryonic and Adult Mouse Brain (pp. 241–252). Humana Press.
756 https://doi.org/10.1007/978-1-59745-019-5_18

757 Alizadeh, A., Dyck, S. M., & Karimi-Abdolrezaee, S. (2015). Myelin damage and repair in
758 pathologic CNS: challenges and prospects. *Frontiers in Molecular Neuroscience*, *8*, 35.
759 https://doi.org/10.3389/fnmol.2015.00035

760 Amaral, A. I., Hadera, M. G., Tavares, J. M., Kotter, M. R. N., & Sonnewald, U. (2016).
761 Characterization of glucose-related metabolic pathways in differentiated rat
762 oligodendrocyte lineage cells. *GLIA*, *64*(1), 21–34. https://doi.org/10.1002/glia.22900

763 Back, S. A., Han, B. H., Ning, *, Luo, L., Chricton, C. A., Xanthoudakis, S., … Holtzman, D.
764 M. (2002). *Selective Vulnerability of Late Oligodendrocyte Progenitors to Hypoxia-*
765 *Ischemia*. Retrieved from http://www.jneurosci.org/content/jneuro/22/2/455.full.pdf

766 Baldassarro, V. A., Marchesini, A., Facchinetti, F., Villetti, G., Calzà, L., & Giardino, L.
767 (2018). Cell death in pure-neuronal and neuron-astrocyte mixed primary culture
768 subjected to oxygen-glucose deprivation: The contribution of poly(ADP-ribose)
769 polymerases and caspases. *Microchemical Journal*, *136*, 215–222.
770 https://doi.org/10.1016/J.MICROC.2016.11.008

771 Baldassarro, V. A., Marchesini, A., Giardino, L., & Calzà, L. (2017). PARP activity and
772 inhibition in fetal and adult oligodendrocyte precursor cells: Effect on cell survival and
773 differentiation. *Stem Cell Research*, *22*, 54–60.
774 https://doi.org/10.1016/J.SCR.2017.05.011

775 Bertina, R. M., Steenstra, J. A., & Slater, E. C. (1974). The mechanism of inhibition by
776 oligomycin of oxidative phosphorylation in mitochondria. *Biochimica et Biophysica*
777 *Acta*, *368*(3), 279–297. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4451653

778 Billon, N., Jolicoeur, C., Tokumoto, Y., Vennström, B., & Raff, M. (2002). Normal timing of
779 oligodendrocyte development depends on thyroid hormone receptor alpha 1 (TRalpha1).
780 *The EMBO Journal*, *21*(23), 6452–6460. https://doi.org/10.1093/EMBOJ/CDF662

781 Borjini, N., Fernández, M., Giardino, L., & Calzà, L. (2016). Cytokine and chemokine
782 alterations in tissue, CSF, and plasma in early presymptomatic phase of experimental
783 allergic encephalomyelitis (EAE), in a rat model of multiple sclerosis. *Journal of*
784 *Neuroinflammation*, *13*(1). https://doi.org/10.1186/s12974-016-0757-6

785 Boyd, A., Zhang, H., & Williams, A. (2013). Insufficient OPC migration into demyelinated

lesions is a cause of poor remyelination in MS and mouse models. *Acta Neuropathol*, *3*, 841–859. https://doi.org/10.1007/s00401-013-1112-y

Bradl, M., & Lassmann, H. (2010). Oligodendrocytes: Biology and pathology. *Acta Neuropathologica*. https://doi.org/10.1007/s00401-009-0601-5

Butts, B. D., Houde, C., & Mehmet, H. (2008). Maturation-dependent sensitivity of oligodendrocyte lineage cells to apoptosis: implications for normal development and disease. *Cell Death and Differentiation*, *15*, 1178–1186. https://doi.org/10.1038/cdd.2008.70

Calzà, L., Baldassarro, V. A., Fernandez, M., Giuliani, A., Lorenzini, L., & Giardino, L. (2018). Thyroid Hormone and the White Matter of the Central Nervous System: From Development to Repair. *Vitamins and Hormones*, *106*, 253–281. https://doi.org/10.1016/BS.VH.2017.04.003

Chen, Y., Balasubramaniyan, V., Peng, J., Hurlock, E. C., Tallquist, M., Li, J., & Lu, R. (2007). Isolation and culture of rat and mouse oligodendrocyte precursor cells. https://doi.org/10.1038/nprot.2007.149

Chew, L.-J., & DeBoy, C. A. (2016). Pharmacological approaches to intervention in hypomyelinating and demyelinating white matter pathology. *Neuropharmacology*, *110*(Pt B), 605–625. https://doi.org/10.1016/j.neuropharm.2015.06.008

Chew, L.-J., King, W. C., Kennedy, A., & Gallo, V. (2005). Interferon-γ inhibits cell cycle exit in differentiating oligodendrocyte progenitor cells. *Glia*, *52*(2), 127–143. https://doi.org/10.1002/glia.20232

Choi, E. H., Xu, Y., Medynets, M., Monaco, M. C. G., Major, E. O., Nath, A., & Wang, T. (2018). Activated T cells induce proliferation of oligodendrocyte progenitor cells via release of vascular endothelial cell growth factor-A. *Glia*, *66*(11), 2503–2513. https://doi.org/10.1002/glia.23501

Coleman, M. C., Asbury, C. R., Daniels, D., Du, J., Aykin-Burns, N., Smith, B. J., … Cullen, J. J. (2008). 2-Deoxy-d-glucose causes cytotoxicity, oxidative stress, and radiosensitization in pancreatic cancer. *Free Radical Biology and Medicine*, *44*(3), 322–331. https://doi.org/10.1016/j.freeradbiomed.2007.08.032

Crawford, A. H., Chambers, C., & Franklin, R. J. M. (2013). Remyelination: The True Regeneration of the Central Nervous System. *Journal of Comparative Pathology*, *149*, 242–254. https://doi.org/10.1016/j.jcpa.2013.05.004

D'Intino, G., Lorenzini, L., Fernandez, M., Taglioni, A., Perretta, G., Del Vecchio, G., … Calzà, L. (2011). Triiodothyronine Administration Ameliorates the

Demyelination/Remyelination Ratio in a Non-Human Primate Model of Multiple Sclerosis by Correcting Tissue Hypothyroidism. *Journal of Neuroendocrinology*, *23*(9), 778–790. https://doi.org/10.1111/j.1365-2826.2011.02181.x

De Miguel, M. P., Alcaina, Y., de la Maza, D. S., & Lopez-Iglesias, P. (2015). Cell metabolism under microenvironmental low oxygen tension levels in stemness, proliferation and pluripotency. *Current Molecular Medicine*, *15*(4), 343–359. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/25941818

Dell'Acqua, M. L., Lorenzini, L., D'Intino, G., Sivilia, S., Pasqualetti, P., Panetta, V., … Calzà, L. (2012). Functional and molecular evidence of myelin- and neuroprotection by thyroid hormone administration in experimental allergic encephalomyelitis. *Neuropathology and Applied Neurobiology*, *38*(5), 454–470. https://doi.org/10.1111/j.1365-2990.2011.01228.x

Deng, W., Rosenberg, P. A., Volpe, J. J., & Jensen, F. E. (2003). Calcium-permeable AMPA/kainate receptors mediate toxicity and preconditioning by oxygen-glucose deprivation in oligodendrocyte precursors. *Proceedings of the National Academy of Sciences*, *100*(11), 6801–6806. https://doi.org/10.1073/pnas.1136624100

Deng, W., Yue, Q., Rosenberg, P. A., Volpe, J. J., & Jensen, F. E. (2006). Oligodendrocyte excitotoxicity determined by local glutamate accumulation and mitochondrial function. *Journal of Neurochemistry*, *98*(1), 213–222. https://doi.org/10.1111/j.1471-4159.2006.03861.x

Falahati, S., Breu, M., Waickman, A. T., Phillips, A. W., Arauz, E. J., Snyder, S., … Krieger, K. (2013). Ischemia Induced Neuroinflammation is Associated with Disrupted Development of Oligodendrocyte Progenitors in a Model of Periventricular Leukomalacia. *Dev Neurosci*, *35*(0), 182–196. https://doi.org/10.1159/000346682

Fancy, S. P. J., Chan, J. R., Baranzini, S. E., Franklin, R. J. M., & Rowitch, D. H. (2011). Myelin Regeneration: A Recapitulation of Development? *Annual Review of Neuroscience*, *34*(1), 21–43. https://doi.org/10.1146/annurev-neuro-061010-113629

Fern, R. F., Matute, C., & Stys, P. K. (2014). White matter injury: Ischemic and nonischemic. *Glia*, *62*(11), 1780–1789. https://doi.org/10.1002/glia.22722

Fernández, M., Baldassarro, V. A., Sivilia, S., Giardino, L., & Calzà, L. (2016). Inflammation severely alters thyroid hormone signaling in the central nervous system during experimental allergic encephalomyelitis in rat: Direct impact on OPCs differentiation failure. *Glia*, *64*(9), 1573–1589. https://doi.org/10.1002/glia.23025

Franklin, R. J. M., & Hinks, G. L. (1999). Understanding CNS remyelination: Clues from

developmental and regeneration biology. *Journal of Neuroscience Research*, *58*(2), 207–213. https://doi.org/10.1002/(SICI)1097-4547(19991015)58:2<207::AID-JNR1>3.0.CO;2-1

Goldberg, M. P., & Choi, D. W. (1993). Combined oxygen and glucose deprivation in cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *13*(8), 3510–3524. https://doi.org/10.1523/JNEUROSCI.13-08-03510.1993

Goodin, D. S. (2014). The epidemiology of multiple sclerosis. insights to disease pathogenesis. *Handbook of Clinical Neurology*, *122*, 231–266. https://doi.org/10.1016/B978-0-444-52001-2.00010-8

Ichihara, Y., Doi, T., Ryu, Y., Nagao, M., Sawada, Y., & Ogata, T. (2017). Oligodendrocyte Progenitor Cells Directly Utilize Lactate for Promoting Cell Cycling and Differentiation. *Journal of Cellular Physiology*, *232*(5), 986–995. https://doi.org/10.1002/jcp.25690

Irvine, K. A., & Blakemore, W. F. (2008). Remyelination protects axons from demyelination-associated axon degeneration. *Brain*, *131*(6), 1464–1477. https://doi.org/10.1093/brain/awn080

Jablonska, B., Scafidi, J., Aguirre, A., Vaccarino, F., Nguyen, V., Borok, E., … Gallo, V. (2012). Oligodendrocyte Regeneration after Neonatal Hypoxia Requires FoxO1-Mediated p27Kip1 Expression. *Journal of Neuroscience*, *32*(42), 14775–14793. https://doi.org/10.1523/JNEUROSCI.2060-12.2012

Jo, S., Kalló, I., Bardóczi, Z., Arrojo Drigo, R., Zeöld, A., Liposits, Z., … Bianco, A. C. (2012). Cellular/Molecular Neuronal Hypoxia Induces Hsp40-Mediated Nuclear Import of Type 3 Deiodinase As an Adaptive Mechanism to Reduce Cellular Metabolism. https://doi.org/10.1523/JNEUROSCI.6514-11.2012

Kang, Z., Wang, C., Zepp, J., Wu, L., Sun, K., Zhao, J., … Li, X. (2013). Act1 mediates IL-17-induced EAE pathogenesis selectively in NG2 + glial cells. *Nat Neurosci*, *16*(10), 1401–1408. https://doi.org/10.1038/nn.3505

Káradóttir, R., & Attwell, D. (2007). Neurotransmitter receptors in the life and death of oligodendrocytes. *Neuroscience*, *145*(4), 1426–1438. https://doi.org/10.1016/j.neuroscience.2006.08.070

Kıray, H., Lindsay, S. L., Hosseinzadeh, S., & Barnett, S. C. (2016). The multifaceted role of astrocytes in regulating myelination. *Experimental Neurology*, *283*(Pt B), 541–549. https://doi.org/10.1016/j.expneurol.2016.03.009

Kuhlmann, T., Miron, V., Cuo, Q., Wegner, C., Antel, J., & Bruck, W. (2008). Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. *Brain*, *131*(7), 1749–1758. https://doi.org/10.1093/brain/awn096

La Piana, R., Vanasse, M., Brais, B., & Bernard, G. (2015). Myelination Delay and Allan-Herndon-Dudley Syndrome Caused by a Novel Mutation in the *SLC16A2* Gene. *Journal of Child Neurology*, *30*(10), 1371–1374. https://doi.org/10.1177/0883073814555189

Lassmann, H., van Horssen, J., & Mahad, D. (2012). Progressive multiple sclerosis: pathology and pathogenesis. *Nature Reviews Neurology*, *8*(11), 647–656. https://doi.org/10.1038/nrneurol.2012.168

Lee, J. Y., Kim, M. J., Deliyanti, D., Azari, M. F., Rossello, F., Costin, A., … Petratos, S. (2017). Overcoming Monocarboxylate Transporter 8 (MCT8)-Deficiency to Promote Human Oligodendrocyte Differentiation and Myelination. *EBioMedicine*, *25*, 122–135. https://doi.org/10.1016/j.ebiom.2017.10.016

Lee, J. Y., & Petratos, S. (2016, November 18). Thyroid Hormone Signaling in Oligodendrocytes: from Extracellular Transport to Intracellular Signal. *Molecular Neurobiology*. https://doi.org/10.1007/s12035-016-0013-1

Lee, Y., Morrison, B. M., Li, Y., Lengacher, S., Farah, M. H., Hoffman, P. N., … Rothstein, J. D. (2012). Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature*, *487*(7408), 443–448. https://doi.org/10.1038/nature11314

Li, W. W., Le Goascogne, C., Ramaugé, M., Schumacher, M., Pierre, M., & Courtin, F. (2001). Induction of Type 3 Iodothyronine Deiodinase by Nerve Injury in the Rat Peripheral Nervous System. *Endocrinology*, *142*(12), 5190–5197. https://doi.org/10.1210/endo.142.12.8532

Matute, C. (2009). Glutamate-Mediated Injury to White Matter: Mechanisms and Clinical Relevance. In *Encyclopedia of Neuroscience* (pp. 1750–1753). Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-29678-2_2036

Mekhail, M., Almazan, G., & Tabrizian, M. (2012). Oligodendrocyte-protection and remyelination post-spinal cord injuries: A review. *Progress in Neurobiology*, *96*, 322–339. https://doi.org/10.1016/j.pneurobio.2012.01.008

Michalski, J.-P., & Kothary, R. (2015). Oligodendrocytes in a Nutshell. *Frontiers in Cellular Neuroscience*, *9*, 340. https://doi.org/10.3389/fncel.2015.00340

Millar, L. J., Shi, L., Hoerder-Suabedissen, A., & Molnár, Z. (2017). Neonatal Hypoxia Ischaemia: Mechanisms, Models, and Therapeutic Challenges. *Frontiers in Cellular Neuroscience*, *11*. https://doi.org/10.3389/fncel.2017.00078

Miller, R. H., & Mi, S. (2007). Dissecting demyelination. https://doi.org/10.1038/nn1995

Miron, V. E., Kuhlmann, T., & Antel, J. P. (2011). Cells of the oligodendroglial lineage, myelination, and remyelination. *BBA - Molecular Basis of Disease*, *1812*, 184–193. https://doi.org/10.1016/j.bbadis.2010.09.010

Patel, J. R., & Klein, R. S. (2011). Mediators of oligodendrocyte differentiation during remyelination. *FEBS Letters*, *585*(23), 3730–3737. https://doi.org/10.1016/j.febslet.2011.04.037

Picard-Riera, N., Decker, L., Delarasse, C., Goude, K., Nait-Oumesmar, B., Liblau, R., … Evercooren, A. B.-V. (2002). Experimental autoimmune encephalomyelitis mobilizes neural progenitors from the subventricular zone to undergo oligodendrogenesis in adult mice. *Proceedings of the National Academy of Sciences*, *99*(20), 13211–13216. https://doi.org/10.1073/pnas.192314199

Raff, M. C., Lillien, L. E., Richardson, W. D., Burne, J. F., & Noble, M. D. (1988). Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. *Nature*, *333*(6173), 562–565. https://doi.org/10.1038/333562a0

Rinholm, J. E., Hamilton, N. B., Kessaris, N., Richardson, W. D., Bergersen, L. H., & Attwell, D. (2011). Regulation of oligodendrocyte development and myelination by glucose and lactate. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(2), 538–548. https://doi.org/10.1523/JNEUROSCI.3516-10.2011

Rocha-Ferreira, E., & Hristova, M. (2016). Plasticity in the Neonatal Brain following Hypoxic-Ischaemic Injury. *Neural Plasticity*, *2016*, 1–16. https://doi.org/10.1155/2016/4901014

Rodgers, J. M., Robinson, A. P., & Miller, S. D. (2013). Strategies for protecting oligodendrocytes and enhancing remyelination in multiple sclerosis. *Discovery Medicine*, *16*(86), 53–63. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23911232

Rone, M. B., Cui, Q.-L., Fang, J., Wang, X. L.-C., Zhang, J., Khan, D., … Antel, J. P. (2016). Neurobiology of Disease Oligodendrogliopathy in Multiple Sclerosis: Low Glycolytic Metabolic Rate Promotes Oligodendrocyte Survival. https://doi.org/10.1523/JNEUROSCI.4077-15.2016

Silbereis, J. C., Huang, E. J., Back, S. A., & Rowitch, D. H. (2010). Towards improved animal models of neonatal white matter injury associated with cerebral palsy. *Disease Models & Mechanisms*, *3*(11–12), 678–688. https://doi.org/10.1242/dmm.002915

Simonishvili, S., Jain, M. R., Li, H., Levison, S. W., & Wood, T. L. (2013). Identification of

Bax-interacting proteins in oligodendrocyte progenitors during glutamate excitotoxicity and perinatal hypoxia-ischemia. *ASN Neuro*, *5*(5), e00131. https://doi.org/10.1042/AN20130027

Sozmen, E. G., Rosenzweig, S., Llorente, I. L., DiTullio, D. J., Machnicki, M., Vinters, H. V, … Carmichael, S. T. (2016). Nogo receptor blockade overcomes remyelination failure after white matter stroke and stimulates functional recovery in aged mice. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(52), E8453–E8462. https://doi.org/10.1073/pnas.1615322113

Su, Z., Yuan, Y., Chen, J., Zhu, Y., Qiu, Y., Zhu, F., … He, C. (2011). Reactive Astrocytes Inhibit the Survival and Differentiation of Oligodendrocyte Precursor Cells by Secreted TNF-α. *Journal of Neurotrauma*, *28*(6), 1089–1100. https://doi.org/10.1089/neu.2010.1597

Tanner, D. C., Cherry, J. D., & Mayer-Pröschel, M. (2011). Oligodendrocyte progenitors reversibly exit the cell cycle and give rise to astrocytes in response to interferon-γ. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(16), 6235–6246. https://doi.org/10.1523/JNEUROSCI.5905-10.2011

Tekkök, S. B., & Goldberg, M. P. (2001). Ampa/kainate receptor activation mediates hypoxic oligodendrocyte death and axonal injury in cerebral white matter. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *21*(12), 4237–4248. https://doi.org/10.1523/JNEUROSCI.21-12-04237.2001

Tognatta, R., & Miller, R. H. (2016). Contribution of the oligodendrocyte lineage to CNS repair and neurodegenerative pathologies. *Neuropharmacology*, *110*(Pt B), 539–547. https://doi.org/10.1016/j.neuropharm.2016.04.026

Trotter, J., Karram, K., & Nishiyama, A. (2010). NG2 cells: Properties, progeny and origin. *Brain Research Reviews*, *63*(1–2), 72–82. https://doi.org/10.1016/j.brainresrev.2009.12.006

van Tilborg, E., de Theije, C. G. M., van Hal, M., Wagenaar, N., de Vries, L. S., Benders, M. J., … Nijboer, C. H. (2018). Origin and dynamics of oligodendrocytes in the developing brain: Implications for perinatal white matter injury. *Glia*, *66*(2), 221–238. https://doi.org/10.1002/glia.23256

Verden, D., & Macklin, W. B. (2016). Neuroprotection by central nervous system remyelination: Molecular, cellular, and functional considerations. *Journal of Neuroscience Research*, *94*(12), 1411–1420. https://doi.org/10.1002/jnr.23923

Wang, C., Zhang, C.-J., Martin, B. N., Bulek, K., Kang, Z., Zhao, J., … Li, X. (2017). IL-17

induced NOTCH1 activation in oligodendrocyte progenitor cells enhances proliferation and inflammatory gene expression. *Nature Communications*, *8*(1), 15508. https://doi.org/10.1038/ncomms15508

Yuen, T. J., Silbereis, J. C., Griveau, A., Chang, S. M., Daneman, R., Fancy, S. P. J., … Rowitch, D. H. (2014). Oligodendrocyte-encoded HIF function couples postnatal myelination and white matter angiogenesis. *Cell*, *158*(2), 383–396. https://doi.org/10.1016/j.cell.2014.04.052

Zhang, Y., Luo, X., Wu, D., & Xu, Y. (2015). ROR nuclear receptors: structures, related diseases, and drug discovery. *Acta Pharmacologica Sinica*, *36*(1), 71–87. https://doi.org/10.1038/aps.2014.120

Legend to table and figures.

Table 1. Mouse Glucose Metabolism PCR array analysis

The table shows the Gene Bank code, gene symbols, gene names and fold of increase in OGD vs normoxia of all modified genes in adult OPC cultures.

Figure 1. Experimental design

(A) Following NSC isolation and neurosphere expansion, in the presence of bFGF and EGF, oligospheres were generated by spheroid disaggregation and bFGF/PDGF exposure. Oligospheres were than dissociated and single cell cultures seeded in the same medium. After 3 DIVs, the medium was changed and the cells were exposed to T3 for 12 DIVs to induce differentiation.
(B-D) Representative images of culture progress from OPCs (NG2-positive cells, 0 DIV, B), through mature OLs (CNPase-positive cells, 6 DIV, C) to mature/myelinating OLs (CNPase/MBP-positive cells, 12 DIV, D).
Abbreviations: CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; MBP, myelin basic protein; NG2, chondroitin sulphate proteoglycan, neural/glial antigen 2.

Figure 2. The effect of cytokine exposure effect on viability and differentiation in fetal and adult NSCs-derived OPCs.

(A) Experimental design. Cells were exposed to cytokine mix during the oligosphere proliferation phase. Three days after cell seeding, differentiation was induced by T3 exposure. Cells were analyzed at the end of the differentiation phase (black arrow, 12 DIV).
(B) Graph shows the cell number (total cell number per well) and cell death (percentage of cells showing condensed nuclei on total number of cells per well) measured at the end of the differentiation phase (12 DIV), in fetal cultures exposed to vehicle or cytokine mix.
(C) Graph shows the cell number (total cell number per well) and cell death (percentage of cells showing condensed nuclei on total number of cells per well) measured at the end of the differentiation phase (12 DIV), in adult cultures exposed to vehicle or cytokine mix.

(D) Graph shows the percentage of lineage-specific positive cells at the end of the differentiation phase (12 DIV) in fetal cultures, exposed to vehicle or cytokine mix. The percentage of OPCs (NG2-positive cells), mature OLs (CNPase-positive cells), myelinating OLs (MBP-positive cells), and astrocytes (GFAP-positive cells) was calculated on the total cell number per well, showing a normal nuclear morphology.
(E) Graph shows the percentage of lineage-specific positive cells at the end of the differentiation phase (12 DIV) in adult cultures, exposed to vehicle or cytokine mix. The percentage of OPCs (NG2-positive cells), mature OLs (CNPase-positive cells), myelinating OLs (MBP-positive cells), and astrocytes (GFAP-positive cells) was calculated on the total cell number per well, showing a normal nuclear morphology.
(F-M) Images show CNPase-positive (F, J, H, L) and MBP-positive cells (G, K, I, M) detected in fetal (F, G, J, K) and adult (H, I, L, M) cultures exposed to vehicle (F-I) or cytokine mix (J-M). White bar in image L: 50 μm.
Bars represent mean value + SEM. Statistical analysis: Student's t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.
Abbreviations: CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; GFAP, Glial fibrillary acidic protein; MBP, myelin basic protein; NG2, chondroitin sulphate proteoglycan, neural/glial antigen 2.

Figure 3. OGD effect on viability of fetal and adult NSCs-derived OPCs

(A) Experimental design. Cells were exposed to 3 hours OGD, followed by 24 hours of reoxygenation, one day prior to T3-mediated differentiation induction.
(B) Graph shows the cell number (total cell number per well) and cell death (percentage of cells showing condensed nuclei on total number of cells per well) measured at the end of the differentiation phase (12 DIV), in fetal cultures exposed to normoxia or OGD.
(C) Graph shows the cell number (total cell number per well) and cell death (percentage of cells showing condensed nuclei on total number of cells per well) measured at the end of the differentiation phase (12 DIV), in adult cultures exposed to normoxia or OGD.
(D) Graph shows the percentage of lineage specific positive cells at the end of the differentiation phase (12 DIV), in fetal cultures exposed to normoxia or OGD. The percentage of OPCs (NG2-positive cells), mature OLs (CNPase-positive cells), myelinating OLs (MBP-positive cells), astrocytes (GFAP-positive cells) and neurons (beta-III-tubulin), was calculated on the total cell number per well, showing a normal nuclear morphology.

(E) Graph shows the percentage of lineage-specific positive cells at the end of the differentiation phase (12 DIV), in adult cultures exposed to normoxia or OGD. The percentage of OPCs (NG2-positive cells), mature OLs (CNPase-positive cells), myelinating OLs (MBP-positive cells), astrocytes (GFAP-positive cells) and neurons (beta-III-tubulin), was calculated on the total cell number per well, showing a normal nuclear morphology.
(F) Graph shows the percentage of lineage-specific positive cells which also show condensed nuclei, at the end of the differentiation phase (12 DIV), in fetal cultures exposed to normoxia or OGD. The percentage of OPCs (NG2-positive cells), mature OLs (CNPase-positive cells), myelinating OLs (MBP-positive cells), astrocytes (GFAP-positive cells) and neurons (beta-III-tubulin), was calculated on the total cell number per well, showing a normal nuclear morphology.
(G) Graph shows the percentage of lineage-specific positive cells which also show condensed nuclei, at the end of the differentiation phase (12 DIV), in adult cultures exposed to normoxia or OGD. The percentage of OPCs (NG2-positive cells), mature OLs (CNPase-positive cells), myelinating OLs (MBP-positive cells), astrocytes (GFAP-positive cells) and neurons (beta-III-tubulin), was calculated on the total cell number per well, showing a normal nuclear morphology.
(H-O) Images show NG2-positive (H, L, J, N) and CNPase/MBP-positive cells (I, M, K, O) detected in fetal (H, I, L, M) and adult (J, K, N, O) cultures exposed to normoxia (H-K) or OGD (L-O). White bar in image J: 50 μm.
Bars represent mean value + SEM. Statistical analysis: Student's t-test; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$.
Abbreviations: CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; GFAP, Glial fibrillary acidic protein; MBP, myelin basic protein; NG2, chondroitin sulphate proteoglycan, neural/glial antigen 2; OGD, oxygen-glucose deprivation.

Figure 4. Role of D3 induction in OGD-mediated differentiation impairment in fetal NSC-derived OPCs

(A) Experimental design. Fetal cultures were exposed to 3 hours OGD, followed by 24 hours of reoxygenation, one day prior to T3-mediated differentiation induction. During OGD exposure and the entire differentiation phase, cells were exposed to vehicle or IOP.
(B) Graph shows the percentage of D3-positive cells following OGD/reoxygenation exposure (DIV 0).

(C-D) Images show D3-positive cells following normoxia (C) or OGD/reoxygenation (D) exposure (DIV 0). White bar in image H: 50µm.

(E) Graph shows the percentage of D3, CNPase and MBP-positive cells at the end of the differentiation phase (12 DIV).

Bars represent mean value + SEM. Statistical analysis: One-way ANOVA followed by Tukey's post-test * $p < 0.05$; **** $p < 0.0001$.

Abbreviations: CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; D3, deiodinases 3; MBP, myelin basic protein; OGD, oxygen-glucose deprivation.

Figure 5. Role of glutamate excitotoxicity in OGD-mediated cell death in fetal NSCs-derived OPCs

(A) Experimental design. Fetal cultures were exposed to 3 hours OGD, followed by 24 hours of reoxygenation or to 3 hours of glutamate (1mM) or NMDA (1mM), prior to T3-mediated differentiation induction. During OGD, glutamate or NMDA exposure and the entire differentiation phase, cells were exposed to vehicle, MK801 or NBQX.

(B) Graph shows the cell number at the end of the differentiation phase (12 DIV), in cells exposed to normoxia, OGD, glutamate and NMDA and treated or not with MK801 or NBQX. Bars represent the total cell number per well.

(C) Graph shows the cell death at the end of the differentiation phase (12 DIV), in cells exposed to normoxia, OGD, glutamate and NMDA, and treated or untreated with MK801 or NBQX. Bars represent the percentage of cells showing condensed nuclei, on the total number of cells per well.

Bars represent mean value + SEM. Statistical analysis: One-way ANOVA followed by Tukey's post-test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Abbreviations: Glut, glutamate; OGD, oxygen-glucose deprivation.

Figure 6. Effect of glucose metabolism perturbation in fetal and adult NSC-derived OPCs

(A) Experimental design. Fetal and adult cultures were exposed to 24 hours of GD, 2-DG or oligomycin treatment to analyze lactate production. In another set of experiments, cells were exposed to 3 hours GD, 2-DG or oligomycin treatment, followed by 24 hours of reperfusion, prior to T3-mediated differentiation induction.

(B-C) Graph shows the relative quantification of lactate production following 24 hours of GD, 2-DG or oligomycin treatment, compared to standard culture conditions (100%) in fetal (B) and adult (C) cultures.
(D-E) Graph shows the cell number at the beginning (0 DIV) and the middle (6 DIV) of the differentiation phase, in fetal (D) and adult € cultures exposed to standard conditions, GD, 2-DG or oligomycin treatment.
(F-G) Graph shows the cell death at the beginning (0 DIV) and the middle (6 DIV) of the differentiation phase, in fetal (F) and adult (G) cultures exposed to standard conditions, GD, 2-DG or oligomycin treatment. Bars represent the percentage of cells showing condensed nuclei, on the total number of cells per well.
Bars represent mean value + SEM. Statistical analysis: One-way ANOVA followed by Dunnet's post-test * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.
Abbreviations: 2-DG, 2-deoxy-D-glucose*; GD, glucose deprivation; olig, oligomycin.*

Figure 7. Effect of glucose metabolism perturbation in fetal NSC-derived OPC differentiation

(A) Experimental design. Fetal cultures were exposed to 3 hours GD or 2-DG treatment, followed by 24 hours of reperfusion, prior to T3-mediated differentiation induction.
(B) Graph shows the percentage of cleaved-caspase 3 positive cells following GD exposure (0 DIV).
(C-D) Images show cleaved-caspase 3 staining in cultures exposed to standard conditions (Ctrl; C) or GD (D). White bar in image E: 50µm.
(E) Graph shows the percentage of lineage-specific positive cells which also show condensed nuclei, at the middle of the differentiation phase (6 DIV), in fetal cultures exposed to standard condition, GD, 2-DG or oligomycin treatment. The percentage of OPCs (PDGFαR-positive cells), mature OLs (CNPase-positive cells), myelinating OLs (MBP-positive cells) and astrocytes (GFAP-positive cells), was calculated on the total cell number per well showing a normal nuclear morphology.
(F) Graph shows the percentage of lineage-specific positive cells in the middle of the differentiation phase (6 DIV) in cultures exposed to standard conditions, GD or 2-DG treatment. The percentage of OPCs (PDGFαR-positive cells), mature OLs (CNPase-positive cells) and myelinating OLs (MBP-positive cells) was calculated on the total cell number per well, showing a normal nuclear morphology.

(G-O) Images show PDGFαR (G-I), CNPase (J-L) and MBP-positive (M-O) cells detected in fetal cultures exposed to standard conditions (Ctrl; G, J, M), GD (H, K, N) or 2-DG treatment (I, L, O). White bar in image J: 50 µm.

Bars represent mean value + SEM. Statistical analysis: Student t-test (B) or one-way ANOVA followed by Sidak's post-test (E, F) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Abbreviations: 2-DG, 2-deoxy-D-glucose*; CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; GD, glucose deprivation; MBP, myelin basic protein; PDGFαR, platelet derived growth factor alpha receptor.*

Figure 8. Effect of OGD on glucose metabolism-related gene expression in fetal and adult NSC-derived OPCs.

(A) Experimental design. Cells were exposed to 3 hours OGD, followed by 24 hours of reperfusion/reoxygenation, one day prior to T3-mediated differentiation induction.

(B-C) Representation of gene expression changes in fetal (B) and adult (C) OPC cultures. Graphs represent the fold of change of expression of all the analyzed genes in OGD vs. normoxia groups.

Abbreviations: OGD, oxygen-glucose deprivation.