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Fingolimod protects against neonatal white matter damage and long-term cognitive deficits caused by hyperoxia

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ABSTRACT

Cerebral white matter injury is a leading cause of adverse neurodevelopmental outcome in prematurely born infants involving cognitive deficits in later life. Despite increasing knowledge about the pathophysiology of perinatal brain injury, therapeutic options are limited. In the adult demyelinating disease multiple sclerosis the sphingosine-1-phosphate (S1P) receptor modulating substance fingolimod (FTY720) has beneficial effects. Herein, we evaluated the neuroprotective potential of FTY720 in a neonatal model of oxygen-toxicity, which is associated with hypomyelination and impaired neuro-cognitive outcome.

A single dose of FTY720 (1 mg/kg) at the onset of neonatal hyperoxia (24 h 80% oxygen on postnatal day 6) resulted in improvement of neuro-cognitive development persisting into adulthood. This was associated with reduced microstructural white matter abnormalities 4 months after the insult. In search of the underlying mechanisms potential non-classical (i.e. lymphocyte-independent) pathways were analysed shortly after the insult, comprising modulation of oxidative stress and local inflammatory responses as well as myelination, oligodendrocyte degeneration and maturation. Treatment with FTY720 reduced hyperoxia-induced oxidative stress, microglia activation and associated pro-inflammatory cytokine expression. *In vivo* and *in vitro* analyses further revealed that oxygen-induced hypomyelination is restored to control levels, which was accompanied by reduced oligodendrocyte degeneration and enhanced maturation. Furthermore, hyperoxia-induced elevation of S1P receptor 1 (S1P1) protein expression on *in vitro* cultured oligodendrocyte precursor cells was reduced by activated FTY720 and protection from degeneration is abrogated after selective S1P1 blockade. Finally, FTY720s' classical mode of action (i.e. retention of immune cells within peripheral lymphoid organs) was analysed demonstrating that FTY720 diminished circulating lymphocyte counts independent from hyperoxia. Cerebral immune cell counts remained unchanged by hyperoxia and by FTY720 treatment.

Taken together, these results suggest that beneficial effects of FTY720 in neonatal oxygen-induced brain injury may be rather attributed to its anti-oxidative and anti-inflammatory capacity acting in concert with a direct protection of developing oligodendrocytes than to a modulation of peripheral lymphocyte trafficking. Thus, FTY720 might be a potential new therapeutic option for the treatment of neonatal brain injury through reduction of white matter damage.

1. Introduction

Preterm birth represents an international health problem affecting up to 10% of newborns in the western world (Howson et al., 2013). Major advances in neonatal intensive care have led

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to substantially improved survival rates of very low-birth weight infants (<1500 g). However, these patients are at risk to develop cerebral white and grey matter injury making prematurity the leading cause of neurodevelopmental disability in childhood (Lindstrom et al., 2007; Volpe, 2009). Functional consequences may involve permanent motor and cognitive deficits in later life (Johnson et al., 2011; Wilson-Costello et al., 2005).

Cerebral oxygenation disturbances, like hypoxia–ischemia and premature exposure to high oxygen levels are likely to contribute to the pathogenesis of brain lesions. Aberrant responses to injury may disrupt maturation of glial progenitors and neurons, which fail to generate neuronal networks resulting in a specific preterm brain phenotype (Back and Rosenberg, 2014; Ferriero, 2004). Based on clinical observations (Collins et al., 2001), experimental work confirmed the role of oxygen toxicity in preterm brain injury (Felderhoff-Mueser et al., 2004; Sirinyan et al., 2006). Neonatal hyperoxia-triggered neurodegeneration correlates with inflammatory responses and induction of reactive oxygen species inducing oligodendroglial cell death and hypomyelination associated with ultrastructural changes of the developing white matter and motor-cognitive deficits (Brehmer et al., 2012; Dzietko et al., 2008; Gerstner et al., 2008; Pham et al., 2014; Ritter et al., 2013; Schmitz et al., 2012, 2014; Sifringer et al., 2012, 2010, 2009; Vottier et al., 2011).

Sphingosine-1-phosphate (S1P) is a phosphorylated sphingolipid regulating growth, survival, proliferation, and differentiation of cells (Spiegel and Milstien, 2011). The S1P analogue and receptor modulator fingolimod (FTY720) is approved for the treatment of relapsing-remitting multiple sclerosis. Due its pleiotropic effects, the detailed mechanisms of action are still unclear. Previous work suggests that degradation of S1P receptors by FTY720 leads to inhibition of immune cell migration from the peripheral lymphoid organs into the circulation and thus into the CNS in classical neuro-immunological disorders such as experimental autoimmune encephalomyelitis (Aktas et al., 2010; Brunkhorst et al., 2014). However, in addition to this classical role in lymphocyte trafficking, FTY720 crosses the blood–brain barrier, gets activated by sphingosine kinase 2 in the CNS and may directly modulate CNS cells expressing S1P receptors, such as microglia and oligodendrocytes (Foster et al., 2007).

Beyond multiple sclerosis, several studies using adult neurodegenerative disease models, e.g. cerebral ischemia and Alzheimer's disease demonstrated a protective role of FTY720 (Brunkhorst et al., 2014). Beneficial effects of FTY720 have been mainly linked to immunomodulation and protection or trophic support of neuronal cells. Additional cell-protective effects have been observed in oligodendrocyte progenitor cell cultures. However, *in vivo* effects of FTY720 on oligodendrocyte differentiation and associated myelination are controversially discussed (Coelho et al., 2007; Jung et al., 2007; Miron et al., 2008).

The present study was designed to investigate potential neuroprotective effects of FTY720 in an *in vivo* model of preterm brain injury (i.e. neonatal hyperoxia) and in primary pre-oligodendrocyte cultures. The primary endpoint analysis was the assessment of long-term, motor-cognitive development and associated structural white matter alterations. To identify the potential underlying mechanisms we further investigated hyperoxia-mediated hypomyelination, oligodendrocyte degeneration, and differentiation as well as oxidative stress and local inflammation. A selective S1P receptor 1 antagonist was applied on *in vitro* cultured pre-oligodendrocytes to verify specific interactions between FTY720 and oligodendrocyte precursor cells. In addition to these non-classical pathways FTY720s' classical mode of action involving alterations of leukocyte migratory pathways were investigated.

2. Materials and methods

2.1. Pharmaceutical compounds

FTY720 and biologically active, phosphorylated FTY720 (pFTY720) were kindly provided by Novartis Pharma (Switzerland). FTY720 was dissolved in PBS and applied at 1 mg/kg. PBS served as vehicle control. pFTY720 was reconstituted in dimethylsulfoxide/50 mM HCl as a 1 mM stock solution and was further diluted in basal defined medium (10–1000 nM), appropriate vehicle served as control. The selective S1P receptor 1 antagonist W146 (Sigma–Aldrich, Germany) was dissolved in 0.1% trifluoroacetic acid in methanol according to the manufacturers' instructions.

2.2. Animals and experimental procedures

All animal experiments were approved and performed in accordance with the guidelines of the University Hospital Essen, Germany and with permission of the local animal welfare committee.

Six-day-old Wistar rat pups were placed in an oxygen chamber containing 80% oxygen (OxyCycler, BioSpherix, USA, 80% O_2, 24 h; HO) together with their lactating dams. Control pups were kept under normoxic conditions (21% O_2, NO). At the beginning of hyperoxia animals received a single intraperitoneal injection of 1 mg/kg FTY720 or vehicle (four study groups: hyperoxia/PBS (HO); hyperoxia/FTY720 (HO + FTY720); normoxia/PBS (NO); normoxia/FTY720 (NO + FTY720). The dose of FTY720 was selected according to previous reports in adult models of stroke (Czech et al., 2009; Liesz et al., 2011). Bodyweight was recorded at P6, P7, P9, P10, P11 and weekly after the weaning period. There were no changes in weight gain between experimental groups. Pups were sacrificed at P7, P11 and P125 under deep anaesthesia.

In accordance with our previous observations (Brehmer et al., 2012; Felderhoff-Mueser et al., 2004) myelin basic protein (MBP) expression and cellular degeneration were determined at P11 and P7, respectively. Functional deficits were analysed at P30 and additionally at P90; and diffusion tensor imaging was carried out at P125 to evaluate long term microstructural white matter and behavioural abnormalities at the fully adult developmental stage which has not been assessed in this specific model before. For protein and mRNA analysis, rats were transcardially perfused with PBS and brain hemispheres or dissected white matter fractions (including corpus callosum, deep cortical white matter and external capsule) were snap-frozen in liquid nitrogen. For histological and MR imaging studies, pups were transcardially perfused with PBS followed by 4% paraformaldehyde (Sigma–Aldrich). Brains were postfixed in 4% paraformaldehyde overnight at 4 °C and embedded in paraffin or sent for diffusion tensor imaging. Based on previous experience (Brehmer et al., 2012) whole hemispheres (excluding cerebellum) were used for Western blot for overall evaluation of MBP expression. To correlate specific effects on myelination with local inflammatory reactions and oxidative stress induction we used dissected white matter enriched preparations of single hemispheres for evaluation of ionised calcium binding adapter molecule 1 (Iba1) protein expression, IL-1β and TNFα protein and mRNA expression and for analysis of oxidative stress related parameters. For lymphocyte cell count analysis via flow cytometry, whole brains (excluding cerebellum) had to be used due to very few immune cells which can be isolated from individual brains.

A total of 285 (138 female and 147 male) rat pups were enrolled in the study and randomly assigned to the treatment groups. Gender distribution is depicted as female/male (f/m) in the following

paragraph. In the first set of experiments 36 rats (17/19) were used for behavioural testing. Out of these brains of 6 animals (3/3) of each experimental group were subjected to diffusion tensor imaging. A second cohort was used for western blot and immunohistochemistry analysis of MBP expression (NO: $n = 10$ (5/5), NO + FTY720: $n = 10$ (6/4), HO: $n = 11$ (4/7), HO + FTY720: $n = 12$ (5/7)) and (NO: $n = 10$ (6/4), NO + FTY720: $n = 10$ (6/4), HO: $n = 12$ (6/6), HO + FTY720: $n = 12$ (5/7)) respectively. A third set was used to assess oxidative stress parameters (NO: $n = 8$ (5/3), NO + FTY720: $n = 9$ (4/5), HO: $n = 9$ (5/4), HO + FTY720: $n = 9$ (4/5)). For Western blot analysis of Iba1, IL-1β and TNFα protein and mRNA expression another cohort was generated (NO: $n = 10$ (4/6), NO + FTY720: $n = 10$ (5/5), HO: $n = 12$ (6/6), HO + FTY720: $n = 10$ (5/5)). These animals were also used for quantification of pro-inflammatory plasma cytokine levels. For immunohistochemical detection of oligodendrocyte degeneration/maturation and microglia activation a fifth set was evaluated (NO: $n = 10$ (4/6), NO + FTY720: $n = 10$ (4/6), HO: $n = 12$ (6/6), HO + FTY720: $n = 10$ (5/5)). Another set of animals was used for flow cytometry analysis of blood and brain (NO: $n = 11$ (6/5), NO + FTY720: $n = 11$ (5/6), HO: $n = 10$ (4/6), HO + FTY720: $n = 11$ (6/5)).

2.3. Immunoblotting

Western blotting was performed as described previously (Brehmer et al., 2012), with adaptions in epitope detection. Membranes were incubated overnight (4 °C) with a primary monoclonal mouse anti-MBP antibody (1:10,000, Abcam, UK), detecting two classical isoforms at 18.5 kDa and 21.5 kDa. The 21.5 kDa isoform, known to be increased during active early myelination (Harauz and Boggs, 2013) was quantified. Further epitopes were detected by polyclonal rabbit anti-Iba1 antibody (17 kDa, 1:1000, Wako, Japan), polyclonal rabbit anti-rat IL-1β (17 kDa; 1:500), polyclonal rabbit anti-rat TNFα (17 kDa; 1:500, both from PromoKine, Germany), polyclonal rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (37 kDa; 1:1000, Santa Cruz, Germany) or monoclonal mouse anti-β-actin antibody (42 kDa; 1:500; Sigma–Aldrich). Antibodies were diluted in 5% non-fat dry milk in Tris buffered saline, 0.05% Tween-20 (TBST, Sigma–Aldrich). Horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody (1:5000 Dako, Denmark) was diluted in 5% non-fat dry milk in TBST. Antibody binding was detected by using enhanced chemiluminescence (GE healthcare Life Sciences, Germany). For visualisation and densitometric analysis ChemiDoc XRS+ imaging system and ImageLab software (Bio-Rad, Germany) were used.

2.4. Immunohistochemistry and confocal microscopy

After deparaffinization, 10 µm coronal sections (−3.72 ± 0.7 mm from bregma) were rehydrated. Antigen-retrieval was performed in a pre-heated 10 mM sodium-citrate buffer (pH 6.0) for 30 min. After blocking with 1% bovine serum albumin, 0.3% cold fish skin gelatine in 0.1% Tween-20 TBS (all Sigma–Aldrich) slides were incubated with primary antibodies overnight at 4 °C followed by appropriate secondary antibody incubation for 1 h at room temperature. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (1 µg/ml, Invitrogen, Germany).

Myelination was evaluated at P11 using the primary mouse anti-rat MBP antibody (1:100, SMI-99, Sternberger Monoclonals, USA). Microglia activation was detected by Iba1 (1:1000, rabbit polyclonal anti-Iba1, Wako) staining on section of P7 old rats. Degeneration of oligodendrocytes or neurons were evaluated at P7 via co-labelling with Olig2 (1:100, polyclonal rabbit anti-Olig2, Millipore, Germany) or NeuN (1:200, polyclonal rabbit anti-NeuN, Millipore) and terminal deoxynucleotidyltransferase-mediated biotinylated dUTP nick end labelling (TUNEL, In Situ cell death detection kit, FITC; Roche, Germany), performed according to the manufacturers' instructions. Mature oligodendrocytes were identified by adenomatous polyposis coli, clone CC1 (referred as CC1; 1:100, monoclonal mouse anti-APC, Calbiochem, Germany) and Olig2 (1:100; polyclonal rabbit anti-Olig2, Millipore) co-stainings. All primary antibodies were followed by appropriate secondary antibody staining (anti-rabbit Alexa Fluor 594 (1:300), anti-mouse Alexa Fluor 488 (1:500); Invitrogen).

Stained brain sections were analysed by confocal microscopy (A1plus, Eclipse Ti, with NIS Elements AR software, Nikon, Germany) using 10× or 20× objectives. Two laser lines (laser diode, 405 nm; Ar laser, 514 nm; G-HeNe laser, 543 nm) and three different filters (450/50-405 LP, 515/20-540 LP, 585/65-640 LP) were used for image acquisition. Confocal z-stacks of 10 µm thickness (z-plane distance 1 µm) were converted into 2-dimensional images using maximum intensity projections. Image acquisition and analysis were performed by an observer blinded to treatment. The quantification of each staining was performed with the NIS Elements AR software. For MBP stained sections, large scale images (stitching) of complete hemispheres from two sections per animal were acquired followed by measurement of mean pixel intensities. Morphometric changes of microglia (Iba1), were determined in a total of 12 images (each 396,900 µm^2) derived from 2 sections per animal within 3 regions of interest (ROI) in the white matter (cingulate, deep cortical, external capsule). The circularity of Iba1 positive cells was measured on single demarcated cells. A total of 2897 cells ($n = 715$ NO, $n = 681$ NO + FTY720, $n = 771$ HO, $n = 730$ HO + FTY720) was analysed. Degenerating oligodendrocytes or neurons and maturating oligodendrocytes were analysed by counting triple positive cells in 6 ROIs derived from 2 sections per animal (each ROI: 396,900 µm^2, cortex, deep cortical white matter, thalamus).

2.5. Real-time PCR

Gene expression analysis was performed as previously described using the PCR ABI Prism 7500 (Brehmer et al., 2012). The PCR products of *IL-1β* (NM_031512), *TNFα* (NM_012675), *S1P receptor 1* (Rn02758712_s1; life technologies, Germany), *S1P receptor 3* (Rn02758880_s1; life technologies, Germany), *S1P receptor 5* (Rn00572952_s1; life technologies, Germany) and *β-actin* (as internal standard, NM_031144) were quantified by fluorogenic reporter oligonucleotide probes (Sifringer et al., 2013). Real-time PCR and detection were performed in triplicate, measurements were repeated 3 times for each sample. Target gene expression was quantified according to the $2^{-\Delta\Delta CT}$ method.

2.6. Evaluation of oxidative stress parameters

Oxidative stress response was measured in white matter homogenates. Total glutathione (GSH and GSSH) was assessed by using the thiol reagent 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and 4-vinylpyridine, and lipid peroxidation was determined by the reaction of thiobarbituric acid with malondialdehyde (MDA) as previously described (Sifringer et al., 2010).

2.7. Behavioural studies

Behavioural testing was initiated in adolescent animals on postnatal day 30 (P30) and repeated on P90 in adult animals. From P20 on animals were familiarised with the investigator through

every-other-day-handling during the active phase under an inversed 12 h light–dark cycle. Testing was started with 1 day of open field followed by 4 days of Barnes maze. Data were recorded using an automatic tracking system (Video-Mot2, TSE Systems, Germany) and exported for statistical analysis.

For the open field test (DeFries et al., 1966), animals were placed into the centre of a dimly lit open field arena ($50 \times 50 \times 40$ cm, or $75 \times 75 \times 40$ cm for adult animals), placed upon an infrared-box (850 nm, TSE Systems). Movements were recorded by the tracking system for 5 min. Activity and anxiety, i.e. travelled distance, velocity and the percentage of time the animal stayed in the central area of the box in relation to the total time spent in the arena were analysed. For Barnes maze (Barnes, 1979) animals were placed into the centre of the maze (1.22 m width, 0.8 m height, 20 holes at the border, TSE Systems) under red light followed by bright light to allow the animal to recognise extra-maze cues. The animal was allowed to explore the maze and find the escape box within 120 s, animals were left in the escape box for 1 min. Animals who did not find the escape box were gently placed into the escape box for 1 min. To avoid intra-maze cues due to odour the escape box was clockwise rotated for every other animal, with the same escape location for each animal at the three training days. The latency to find the trained escape box was assessed on the fourth day of each experiment when all holes were closed (O'Leary et al., 2011).

2.8. Diffusion tensor imaging

At P125, *ex vivo* brains from the behaviour tested animals were subjected to diffusion tensor MR imaging. All experiments were performed on an actively-shielded 9.4T/31 cm magnet (Varian/Magnex Scientific, UK) equipped with 12 cm gradient coils (400 mT/m, 120 μs) with a transceiver 25 mm birdcage volume RF coil. First and second order shims were adjusted manually; with a water bandwidth ranged between 20 and 30 Hz. Diffusion gradients were applied along six spatial directions in a spin echo sequence (Basser and Pierpaoli, 1998). The intensity (G_d), duration (δ) and separation time of the pulsed diffusion gradients were set to 22 G/cm, 3 ms and 20 ms, respectively (b-value of 1185 s/mm^2). A field of view of 27×27 mm^2 was sampled on a 128×128 cartesian grid and 12 slices of 0.8 mm thickness were acquired in the axial plane with 20 averages. The echo time was set to 35 ms and the repetition time between consecutive measurements was 2000 ms. Using homemade Matlab (Mathworks, MA) software, the radial diffusivity ($D_\perp$), the axial diffusivity ($D_{//}$), the mean diffusivity (MD) and the fractional anisotropy (FA) were derived from the tensor. The program allows manual delineation of region of interest (ROI) on the direction encoded colour maps. On three different structures of the brain (cortex (Cx), corpus callosum (CC) and external capsule (EC)), ROIs were carefully manually delimited at six different image-planes of the brain from the genu to the splenium of the corpus callosum.

2.9. Primary oligodendrocyte cultures and immunocytochemistry

Primary oligodendrocytes were isolated from brains of Sprague Dawley rats at P1–P2 as previously described (Brehmer et al., 2012). After 3 to 5 days (O4-maturity level), activated FTY720 (pFTY720) was added at different concentrations (10–1000 nM) at the onset of hyperoxic (80% oxygen, CB 150 incubator, Binder, Germany) or standard culture conditions for 24 h. The selected concentration range for studying *in vitro* effects of pFTY720 treatment was based on previous reports in rat oligodendrocyte precursor cells (Coelho et al., 2007; Jung et al., 2007).

Immunocytochemical analysis was conducted to determine S1P receptor 1 (S1P1) protein expression on pre-oligodendrocytes. Briefly, cells were blocked with 5% normal goat serum in 0.1% Triton X-100 (Sigma–Aldrich) in PBS for 1 h at room temperature followed by incubation with anti-S1P1 (polyclonal rabbit anti-Edg1, 1:300, abcam) and anti-Olig2 (monoclonal mouse anti-Olig2, 1:500, Millipore) at 4 °C overnight. Antibody binding was visualised by incubation with the appropriate secondary antibodies (1:1000 anti-rabbit Alexa Fluor 488, 1:1000 anti-mouse Alexa Fluor 594; both Invitrogen). Per group and experiment 5–6 random fields (each 45,500 μm^2) were visualised by fluorescence microscopy (Axioplan; Zeiss, Germany) connected to a CCD camera (Microfire; AVT Horn, Germany). The mean signal intensity of S1P1 was measured on single Olig-2 positive cells using Image J. A total of 1437 cells (NO $n = 380$, NO+: $n = 411$, HO: $n = 375$, HO+: $n = 271$) were analysed while being blinded to the experimental groups.

The differentiation capacity of premature oligodendrocytes in response to a single dose of activated FTY720 was evaluated in isolated premature oligodendrocytes that were treated with pFTY720 or vehicle and cultured as above. Cells were further cultivated under differentiating conditions (basal defined medium supplemented with 10 ng/ml triiodothyronine (Sigma–Aldrich) and 10 ng/ml ciliary neurotrophic growth factor (Pan-Biotech, Germany) for another 3 days in the absence of FTY720. MBP expression of oligodendrocytes was evaluated by MBP/Olig2 co-staining. After fixation cells were blocked with 5% normal goat serum in 0.1% Triton X-100 (Sigma–Aldrich) in PBS and incubated overnight with anti-rat MBP (1:500, SMI-99, Monoclonals, USA) and anti-Olig2 (monoclonal mouse anti-Olig2, 1:500, Millipore) at 4 °C, followed by secondary antibody (1:1000 anti-mouse, Alexa Fluor 488; 1:1000 anti-rabbit, Alexa Fluor 594) incubation for 1 h at room temperature. Immature oligodendrocytes were stained for O4 (1:750, MO15002, Neuromics, USA) and Olig2 (1:200) overnight at 4 °C and incubated with appropriate secondary antibodies (1:1000 anti-mouse Alexa Fluor 488; 1:1000 anti-rabbit Alexa Fluor 594, Invitrogen) for 1 h at room temperature. Nuclei were counterstained with DAPI (100 ng/ml). Five random fields (each 189,140 μm^2) were visualised by fluorescence microscopy and triple-positive cells were counted for each condition.

To specifically block the S1P receptor 1 premature oligodendrocytes were incubated with 10 μM of a selective pharmacological S1P receptor 1 receptor antagonist (W146) or vehicle for 30 min prior to the start of hyperoxia/normoxia and addition of 50 nM pFTY720. The W146 dose was chosen based on previous reports demonstrating efficient blockade of protection by pFTY720 in a model of excitotoxicity (Di Menna et al., 2013).

2.10. Processing of peripheral blood and brain tissues

Isolation of single cell suspension for flow cytometry analysis was performed as previously described (Herz et al., 2014). Briefly, animals were euthanised by i.p. injections of chloralhydrate. Blood specimens were collected into ethylenediaminetetraacetate (EDTA) coated collection tubes by puncture of the inferior *vena cava* followed by transcardial perfusion with ice-cold PBS and removal of brains. Erythrocytes were lysed by incubation with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 3 mM EDTA) for 5 min followed by two washing steps with PBS prior to staining. Brains were dissected, cerebellum removed followed by homogenisation through a 40 μm cell strainer (BD Biosciences, Germany) during continuous rinsing with 15 mL of cold HEPES-buffered RPMI1640. Samples were centrifuged at $400 \times g$ for 5 min at 18 °C. The supernatants were discarded and the pellets were re-suspended in 37% Percoll in 0.01 N HCl/PBS and centrifuged at $2800 \times g$ for 20 min. Myelin was removed and the remaining cell pellet was washed twice in PBS.

2.11. Flow cytometry

Isolated cells were incubated with the following antibodies: anti-CD45 PE-Cy7 (leukocytes, clone OX-1, BD Biosciences), anti-CD3 APC (T cells, clone: REA223, Miltenyi Biotec, Germany), anti-CD161a Fitc (NK cells, clone 10/78, BD Biosciences) and anti-CD45RA APC-Cy7 (B cells, clone: OX-33, BD Biosciences) for 30 min at 4 °C followed by two washing steps prior to analysis Total cell counts of blood- and brain-derived leukocytes were determined using BD TrueCount beads on the basis of CD45 positive events.

Isolated oligodendrocytes were harvested by trypsinization (0.05% Trypsin–EDTA, Gibco, Germany) for 2-3 min. A2B5-positive oligodendrocytes (biotinylated anti-A2B5, 1:50, Miltenyi Biotec; followed streptavidin-coupled Alexa Fluor 488, 1:1000; Invitrogen) were co-labelled with the PE Apoptosis Detection Kit (BD Biosciences) according to the manufactures' instructions. Data acquisition and analysis were performed were using the BD FACS LSRII and FACS Diva software (BD Biosciences).

2.12. Analysis of plasma cytokines

Blood from the vena cava was collected in ethylenediaminetetraacetate (EDTA) coated tubes. Samples were centrifuged at 1000×*g* for 10 min and the supernatant was frozen at −80 °C until further analysis. Plasma samples were assayed for the presence of IL-1α, IL-1β, TNFα and IFNγ using a Luminex Screening assay (R&D Systems, Germany) according to the manufacturers' instructions. Samples were analysed by Luminex 200 technology using Luminex IS software (Luminex Corporation, USA).

2.13. Statistical analysis

Data are presented as mean ± standard deviation (SD) with Prism 6 (GraphPad Software, USA). Statistical analyses were performed with SPSS 22 (IBM, USA), differences between groups were determined by two-way analysis of variance (two-way ANOVA) followed by Bonferroni post hoc test for multiple comparison. For MRI results, nonparametric Mann–Whitney test was used. *p*-values less than 0.05 were considered as statistically significant.

3. Results

3.1. FTY720 improves long-term cognitive development and reduces white matter injury after neonatal hyperoxia

To assess the effect of FTY720 treatment on motor activity and cognitive function after neonatal (P6) exposure to hyperoxia (24 h 80% oxygen (HO), (Brehmer et al., 2012; Schmitz et al., 2014), we performed behavioural tests in adolescent (P30) and adult (P90) rats. General motor activity assessed in the open field was not affected by hyperoxia or FTY720 treatment (Supplemental Fig. S1). However, spatio-temporal learning evaluated in the Barnes maze was significantly decreased after neonatal hyperoxia persisting into adulthood (P90) (Fig. 1A and B). Interestingly, FTY720 treatment improved hyperoxia-induced memory deficits as shown by decreased latency to control levels at P30 and P90 (Fig. 1A and B). To determine whether these functional deficits were associated with white matter structure development diffusion tensor imaging (DTI) was performed on brains of P125 old rats (Fig. 1C–E, Supplemental Fig. S2). Fractional anisotropy (FA) measured in the corpus callosum and the external capsule was significantly reduced in the HO group compared to normoxic controls (Fig. 1D and E). Mostly, this FA decrease was related to a significant increase of radial diffusivity ($D_{\perp}$) in the white matter of HO rats (Supplemental Fig. S2). Of note, single FTY720 treatment

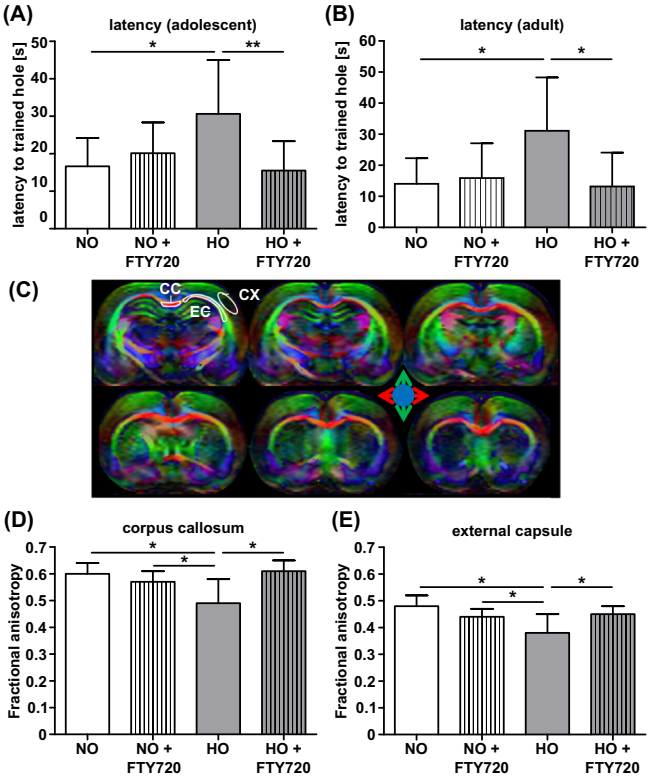

Fig. 1. FTY720 improves memory function and white matter microstructure development in adult rats after neonatal hyperoxia-mediated brain injury. (A and B) Cognitive development was assessed in the Barnes maze test starting at P31 and P91 after exposure to 24 h normoxia (21% O_2; (NO)) or hyperoxia (24 h 80% O_2; (HO)) at P6 and treated with PBS or 1 mg/kg FTY720. Memory function is expressed as the latency to find the trained escape hole after a 3-day-training period. n = 8–10 rats/group. (C) Representative direction-encoded colour maps of a P125 NO rat derived from diffusion tensor imaging showing the different levels used for quantitative analysis. ROIs: corpus callosum (CC), external capsule (EC) and cortex (CX) are displayed on the maps. (D and E) Quantification of fractional anisotropy in corpus callosum and external capsule determined by diffusion tensor imaging. n = 6 rats/group. $^{*}p < 0.05$, $^{**}p < 0.01$.

ameliorates neonatal hyperoxia-induced white matter injury demonstrated by a sustained increase of FA values in the hyperoxic brain (Fig. 1D and E). In the cortex, no significant differences were observed between the analysed groups (data not shown).

3.2. Hyperoxia-mediated hypomyelination in the developing brain is ameliorated by FTY720 treatment

Cognitive deficits have been associated with white matter changes in preterm born children (Soria-Pastor et al., 2008), and are supposed to be related to several adult neurological diseases (Nave and Ehrenreich, 2014). Therefore, we investigated the myelination capacity of developing oligodendrocytes after exposure to hyperoxia by MBP protein expression at P11 via immunohistochemistry and Western blotting. A significant decrease of MBP protein expression was detected in hyperoxia-exposed animals as compared to normoxic controls (Fig. 2). Hyperoxia-induced hypomyelination was partially reversed by FTY720 treatment leading to significantly increased levels of MBP expression for the combined treatment of FTY720 and hyperoxia when compared to hyperoxia only (Fig. 2B and C).

3.3. FTY720 ameliorates hyperoxia-triggered oxidative stress response in the developing white matter

In search of underlying mechanisms for the observed protective effects to hypomyelination by FTY720 treatment we evaluated the

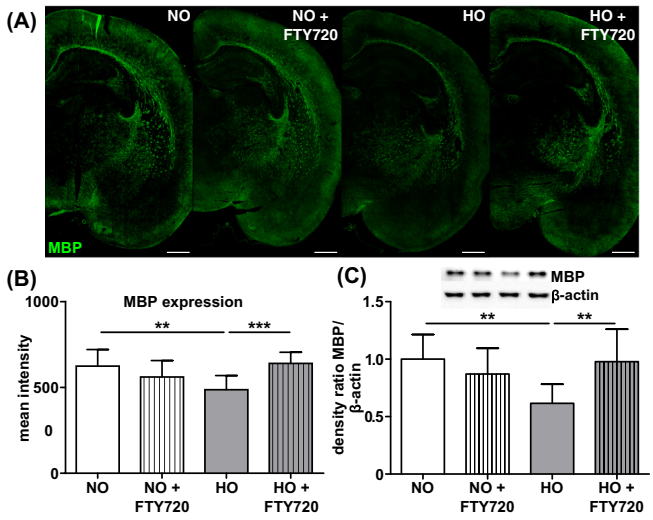

Fig. 2. FTY720 restores neonatal hyperoxia-induced hypomyelination *in vivo*. Myelin basic protein (MBP) expression was analysed 4 days post hyperoxia. (A and B) Immunohistochemical stainings were analysed via confocal microscopy (large scale confocal images, scale bar = 1000 μm) and mean fluorescence intensities per hemisphere were quantified at the level of −3.72 ± 0.7 mm from bregma as indicated in (A), n = 10–12 rats/group. (C) Ratio of MBP/β-actin protein expression was analysed in protein lysates of complete hemispheres (excluding cerebellum). n = 10–12 rats/group. $^{**}p < 0.01$, $^{***}p < 0.001$.

regulation of oxidative stress parameters known to be involved in hyperoxia-mediated neonatal brain injury (Schmitz et al., 2014; Sifringer et al., 2010). The measurement of reduced and oxidised glutathione revealed a decrease in reduced and an increase in oxidised glutathione in hyperoxic rats (Fig. 3A–C). However, these hyperoxia-triggered imbalances were neither detectable in control nor in FTY720 treatment animals (Fig. 3A–C). Furthermore, by measurement of malondialdehyde (MDA), a marker for lipid peroxidation, we detected an increase in MDA concentration in the white matter of hyperoxic rats, which is significantly reduced upon FTY720 treatment (Fig. 3D).

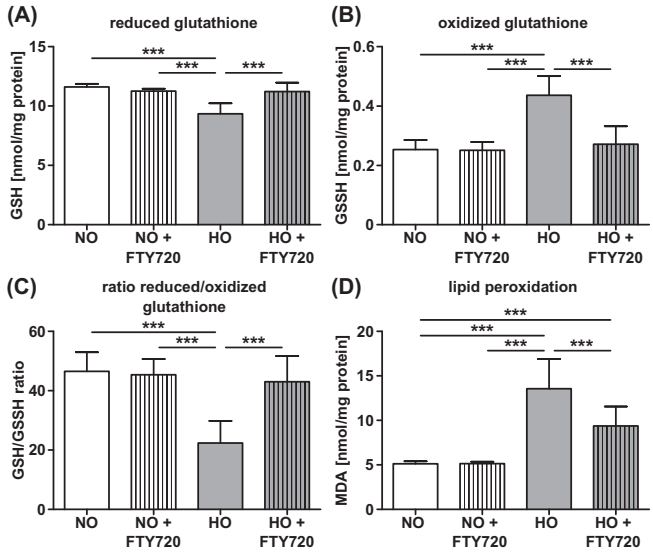

Fig. 3. Augmented hyperoxia-triggered oxidative stress response in the developing white matter is prevented by FTY720 treatment. White matter fractions of P7 rats exposed to 24 h NO or HO combined with PBS or FTY720 treatment at P6 were used for measurement of the oxidative stress response. (A–C) Reduced (GSH) and oxidised glutathione (GSSH) levels was measured by spectrophotometric analysis at 412 nm. (D) Lipid peroxidation was determined by HPLC measurement of malondialdehyde (MDA). n = 8–9 rats/group. $^{***}p < 0.001$.

3.4. Hyperoxia-induced local inflammatory response and microglia activation is prevented by FTY720 treatment

Since an increase in oxidative stress response after neonatal hyperoxia coincides with induction of inflammatory cytokines and microglia activation (Schmitz et al., 2014; Sifringer et al., 2010) we analysed FTY720's potential immunomodulatory effect. We detected an eightfold increase in *IL-1β* mRNA and a tenfold increase in *TNFα* mRNA expression in the hyperoxic white matter whereas in FTY720 treated animals expression decreased almost comparable to control levels (Fig. 4A and C). To corroborate these findings on the protein level we performed Western blot analyses confirming significant regulations on the transcriptional level (Fig. 4B and D). Immunohistochemical analysis of microglia activation revealed a shift of Iba1-positive cells from a resting, ramified to a more activated, amoeboid microglia phenotype in hyperoxic samples, not observed in FTY720-treated hyperoxic rats (Fig. 4E). By assessing the total amount of Iba1 positive cells we did not observe marked alterations in total microglia numbers (Fig. 4F), however, microglia circularity was increased in the hyperoxic group which was undetectable in the other groups (Fig. 4G). Furthermore, using Western blot analysis we observed an increase in Iba1 protein expression after hyperoxia, which is ameliorated by FTY720 administration (Fig. 4H). These results combined with morphological changes suggest a more activated microglia phenotype in response to hyperoxia that is decreased by FTY720.

3.5. FTY720 induces reduced circulating lymphocyte cell counts independent of hyperoxia

Considering FTY720s' classical immunomodulatory role associated with retention of lymphocytes in peripheral lymphoid organs resulting in reduced circulating and brain-infiltrating numbers in autoimmune mediated neuroinflammation (Fujino et al., 2003; Kataoka et al., 2005) we performed flow cytometry on different lymphocyte subsets in the blood and brain differentiated by their antigen expression using multichannel flow cytometry. Absolute cell counts of circulating leukocytes were reduced by FTY720 both under normoxic and under hyperoxic conditions (Fig. 5A). FTY720-induced reduction of circulating white blood cells can be mainly attributed to a reduced number of lymphocytes particularly T and B cells (Fig. 5A). These results might support the concept that FTY720 restrains lymphocytes within the lymphoid organs resulting in reduced circulating lymphocyte counts. In spite of reduced peripheral lymphocyte counts after FTY720 administration, which was independent of hyperoxia, there were no significant changes in the level of circulating cytokines, i.e. IL-1β, IL-1α, TNFα, IFNγ (Supplemental Fig. S3). Of note, brain lymphocyte counts were modulated neither by FTY720 nor by hyperoxia (Fig. 5B). Taken together, these data suggest that peripheral immunmodulatory effects of FTY720 are independent of FTY720s' local anti-inflammatory and anti-oxidative capacity in the hyperoxic brain and might therefore not explain the neuroprotective effect in the present model.

3.6. Hyperoxia-induced oligodendrocyte degeneration and impaired maturation is attenuated by FTY720

To get deeper insight into the underlying causes of FTY720s' protective effect on hyperoxia-induced hypomyelination and cognitive deficits we investigated oligodendrocyte degeneration and maturation by performing TUNEL and CC1 staining in combination with the pan-oligodendrocyte marker Olig2. We

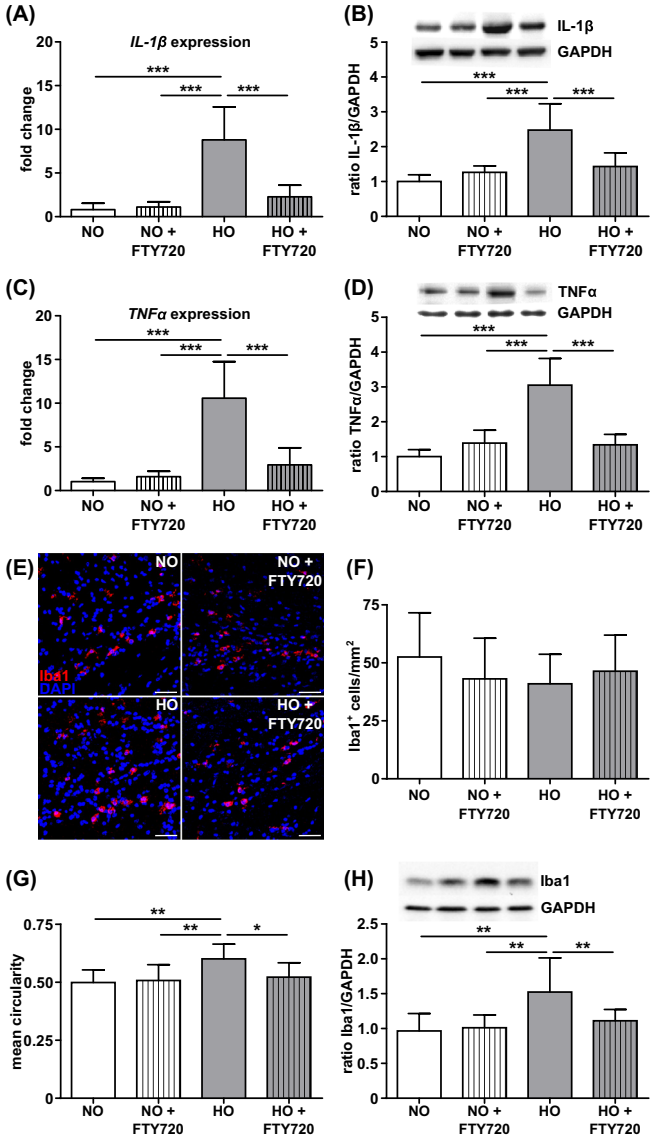

Fig. 4. Hyperoxia-associated inflammatory response and microglia activation in the developing white matter is prevented by FTY720. Local inflammatory response assessed by *IL-1β* and *TNFα* mRNA (A and C) and protein (B and D) expression in isolated white matter fractions of P7 rats exposed to 24 h room-air (NO) or hyperoxia (HO) combined with PBS or FTY720 treatment, $n = 10–12$ rats/group. (E) Representative images of immunohistochemical staining of activated microglia (Iba1) in the cingulate white matter (scale bar = 100 μm). (F and G) Quantification of microglia cell number and circularity. (H) Protein expression analysis of Iba1 in isolated white matter fractions. $n = 10–12$ rats/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

detected a significant increase in oligodendrocyte death in the developing white matter after 24 h of hyperoxia, which was abolished in FTY720 treated animals (Fig. 6A and B). Neither hyperoxia nor FTY720 treatment affected neuronal degeneration in the white matter (WM) and hippocampus (HC) (WM: NeuN/TUNEL^{+} cells/mm^2: NO = 1.11 + 0.69, NO + FTY720 = 0.92 + 0.92, HO = 1.33 + 0.78, HO + FTY720 = 1.17 + 0.64 cell/mm^2, $p = 0.89$ and HC: NeuN/TUNEL^{+} cells/mm^2, NO: 5.39 + 1.37, NO + FTY720: 4.82 + 0.71, HO: 5.5 + 1.45, HO + FTY720: 5.79 + 1.30; $p = 0.35$). Oligodendrocytes (Olig2) co-labelled with CC1, a marker for mature oligodendrocytes showed a significant drop of CC1 positive oligodendrocytes in the developing white matter in rats exposed to hyperoxia, which was counterbalanced by FTY720 treatment (Fig. 6C and D). Similar effects of oligodendrocyte degeneration and impaired maturation were observed in the thalamus and cortex (Supplemental Fig. S4A–D) whereas the amount of degenerating oligodendrocytes in the hippocampus remained unchanged (Olig2/TUNEL^{+} cells/mm^2, NO: 2.41 + 0.72, NO + FTY720: 2.66 + 0.52, HO: 2.33 + 0.73, HO + FTY720: 2.46 + 0.48, $p = 0.68$).

3.7. pFTY720 reduces hyperoxia-induced cell death and improves differentiation of primary oligodendrocytes

To test whether FTY720-induced hypomyelination is a consequence of reduced oxidative stress and local inflammation or whether FTY720 might also directly target immature oligodendrocytes we used a primary immature oligodendrocyte culture model. Immature oligodendrocytes (O4-maturity level) were cultured in the presence or absence of biological active FTY720 (pFTY720). pFTY720 does not affect morphology or number of cells under standard culture conditions (NO). However, hyperoxia markedly decreased the total amount of oligodendrocytes and their processes in the absence of pFTY720 whereas the number of primary O4 positive oligodendrocytes and their processes are increased in the presence of FTY720 (Fig. 7A). We confirmed this protective effect on cell degeneration by flow cytometry demonstrating that hyperoxia leads to a shift from vital to apoptotic cells (0 nM, NO vs. HO) which decreases upon pFTY720 treatment (Fig. 7B). Quantification of three independent experiments

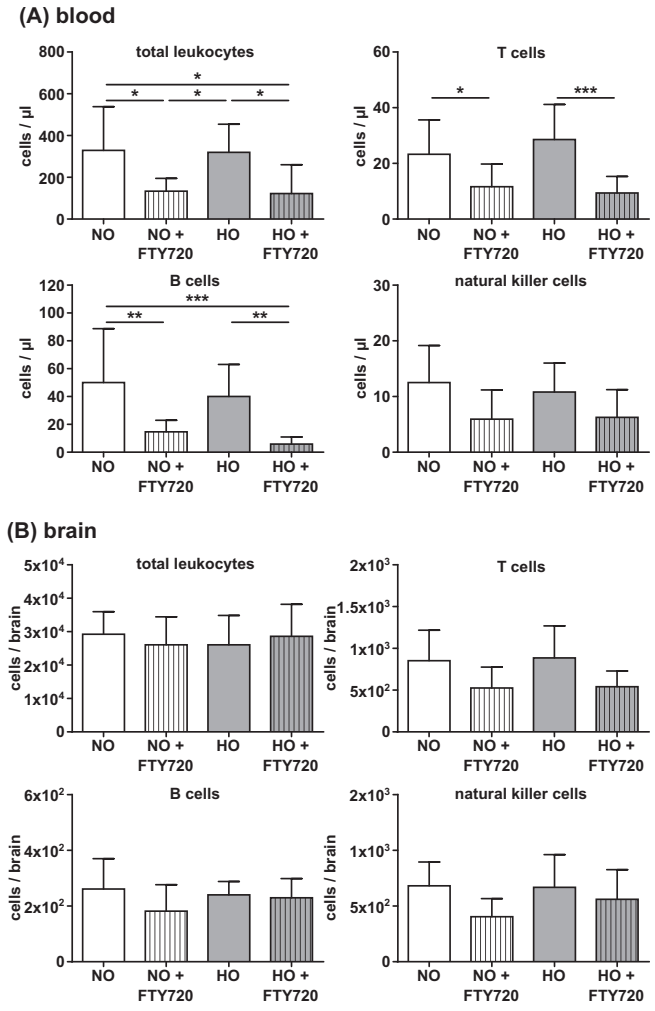

Fig. 5. FTY720 induces peripheral blood lymphopenia without affecting cerebral lymphocyte counts which is independent of hyperoxia. Modulations of total immune cell counts in the blood and brain were assessed by flow cytometry of P7 rats exposed to 24 h room-air (NO) or hyperoxia (HO) combined with PBS or FTY720 (1 mg/kg) treatment at P6. Isolated white blood cells were differentiated by their antigen expression using multichannel flow cytometry. Lymphocyte subsets were identified by gating for CD45 positive cells in the blood (A) and for $CD45^{high}$ cells in the brain (B). These cells were further divided into lymphoid subsets distinguishing T cells, B cells and natural killer cells. Specifications of leukocyte subset identification according to their specific antigen expression are given in Section 2.11. n = 10–11 rats/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

revealed a significant induction of pro-apoptotic but not late apoptotic cells by hyperoxia which is partially reversed by pFTY720 treatment (Fig. 7C and D). In addition to enhanced oligodendrocyte survival pFTY720 restores oligodendrocyte differentiation as demonstrated by an increased MBP expression and a higher number of differentiated oligodendrocytes in pFTY720-treated hyperoxic oligodendrocytes. Quantification of myelinating oligodendrocytes revealed no significant differences under normoxic culture conditions for all pFTY720 concentrations, tested (Fig. 7E–G).

3.8. Cellular protection by activated FTY720 is abrogated following pharmacological S1P receptor 1 blockade

To get deeper insights into pFTY720s' mode of action on oligodendrocyte precursor cells we determined mRNA expression levels of S1P receptors, namely *S1P1*, *S1P3* and *S1P5* on *in vitro* cultured pre-oligodendrocytes. In agreement with previous reports (Miron et al., 2008) basal *S1P1* expression on oligodendrocyte precursor

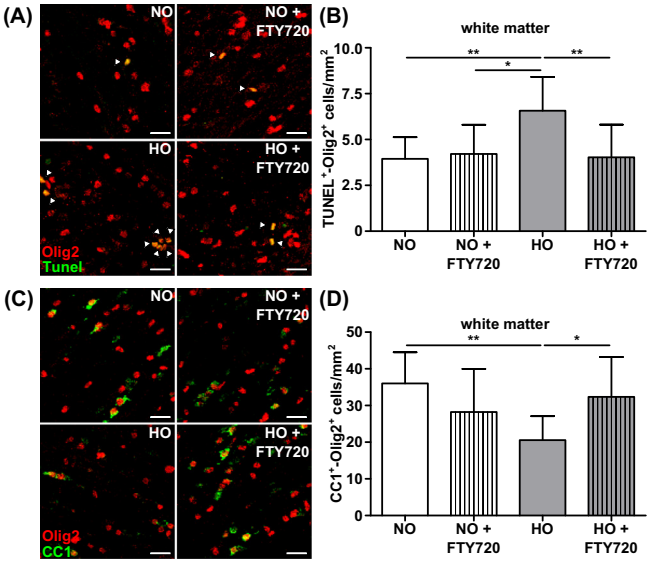

Fig. 6. Hyperoxia-induced oligodendrocyte degeneration and disturbed maturation in the developing white matter is ameliorated by FTY720 treatment. Oligodendrocyte degeneration and maturation were determined in deep cortical white matter structures of brain sections from P7 rats that were exposed either to 24 h normoxia (NO) or hyperoxia (HO) combined with PBS or FTY720 treatment at P6. (A and B) Oligodendrocyte degeneration was determined by immunohistochemical TUNEL/Olig2 co-staining (double positive cells are indicated by arrowheads). (C and D) Maturation was assessed by CC1/Olig2 co-staining Scale bar = 20 µm, n = 10–12 rats/group. $^{*}p < 0.05$, $^{**}p < 0.01$.

cells was increased compared to *S1P5* and *S1P3* expression under normoxia (Fig. 8A). We further focused on the modulation of S1P1 by hyperoxia and 50 nM pFTY720 since this concentration was efficient to protect pre-oligodendrocytes from hyperoxia-induced apoptosis and differentiation arrest (Fig. 7C and D). Hyperoxia increases S1P1 expression on protein and mRNA level (Fig. 8B). Whereas pFTY720 did not alter *S1P1* mRNA we detected significantly decreased S1P1 protein expression at the single cell level (Fig. 8C and D). To verify the functional relevance we specifically inhibited the S1P1 prior to hyperoxia and pFTY720 treatment demonstrating that pFTY720s' cell protective effect was diminished through this selective pharmacological blockade (Fig. 8E).

4. Discussion

The present study demonstrates that FTY720 modulates the hyperoxia-exposed neonatal brains' microenvironment through reduction of inflammation and oxidative stress accompanied by a direct action on oligodendrocyte precursor cell development resulting in long-term improvement of white matter structures and cognitive function in later life.

Cerebral grey and white matter injury are the leading cause of motor and cognitive impairment in later life of preterm infants (Volpe, 2009). Inflammation and disturbed oxygenation have been identified as major causes for acute and chronic white matter injury (Back and Rosenberg, 2014). Myelination is the key to the functional activity of axons, allowing them to connect to neurons and strengthen circuitry throughout the nervous system not only during development but also in the adult CNS (de Hoz and Simons, 2015). Clinical studies revealed that cognitive and motor deficits in preterm born infants can be directly related to microstructural abnormalities in particular white matter regions (Counsell et al., 2008). In contrast to human brain development, there is a delay in rodent brain maturation, i.e. oligodendrogenesis

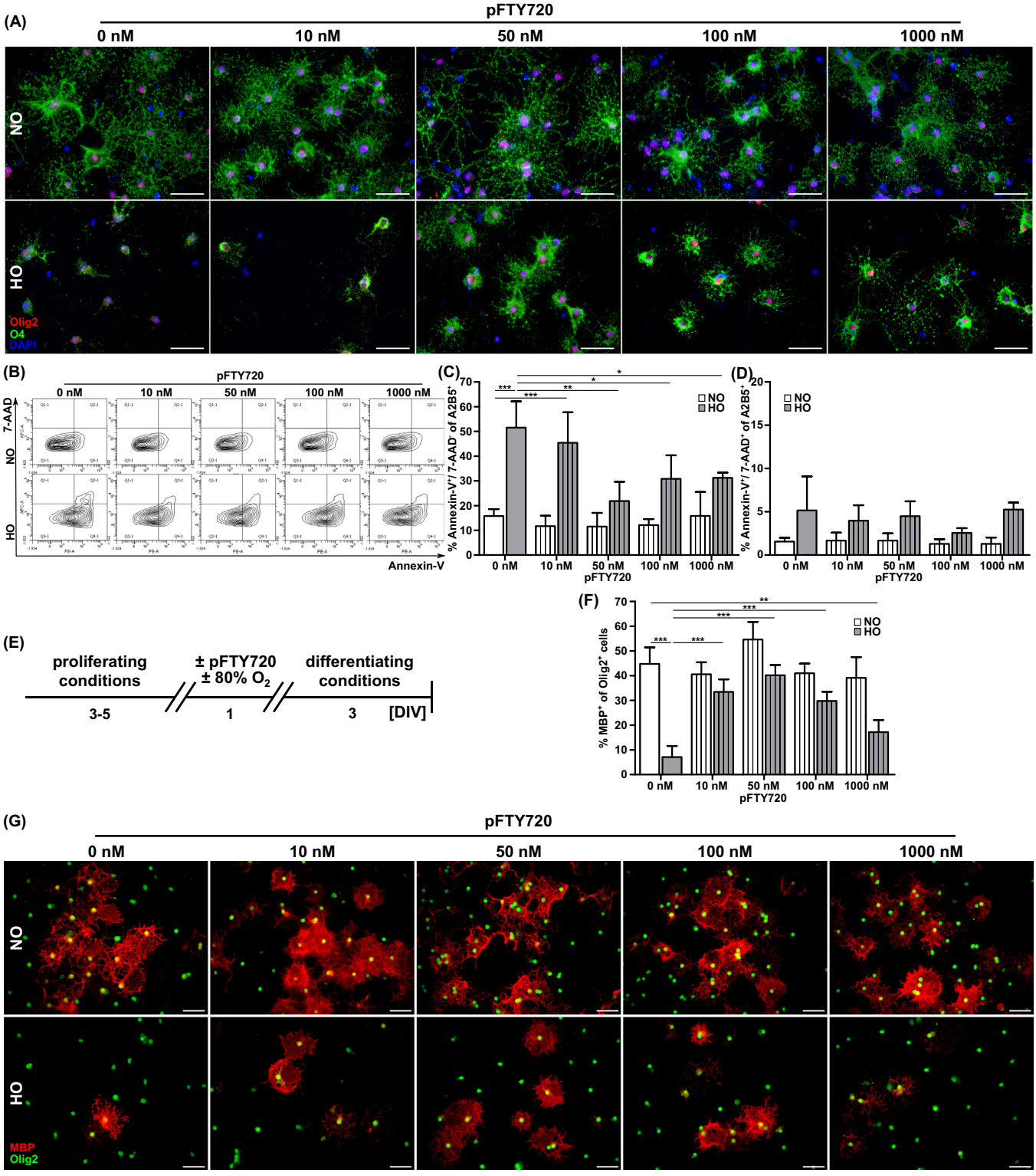

Fig. 7. Ameliorated hyperoxia-mediated pre-oligodendrocyte death is accompanied by improved oligodendrocyte differentiation after FTY720 treatment. Immature oligodendrocytes were treated with indicated concentrations of pFTY720 (or vehicle) prior to hyperoxia, normoxic controls were cultured under standard conditions. (A) Representative immunofluorescence images of immature oligodendrocytes, Olig2 (red), O4 (green), DAPI (blue), scale bar = 50 μm. (B) Cell death was evaluated by flow cytometry. Therefore, cells were stained for A2B5 and co-labelled with Annexin-V (pro-apoptotic) and 7-AAD (dead); representative contour plots are shown. (C and D) Analysis of pro-apoptotic (A2B5$^+$ Annexin V$^+$ 7AAD, (C)) and late apoptotic (A2B5$^+$ Annexin V$^+$ 7AAD, (D)) oligodendrocytes via flow cytometry; $n = 3$ independent experiments. (E) Effects of FTY720 on oligodendrocyte differentiation were investigated in primary oligodendrocyte precursor cell cultures that were enriched under proliferating conditions. Cells were treated with or without the indicated concentrations of pFTY720 prior to 24 h hyperoxia (HO) or normoxia (NO) followed by media exchange to differentiating conditions for another 3 days). (F) Quantification of immunocytochemical staining. Data are derived from 3 independent experiments and are represented as percentage of MBP$^+$ cells from all Olig2$^+$ cells. (G) Representative fluorescence images of oligodendrocyte differentiation evaluated by immunocytochemistry for MBP (red) and Olig2 (green). Scale bar = 50 μm. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

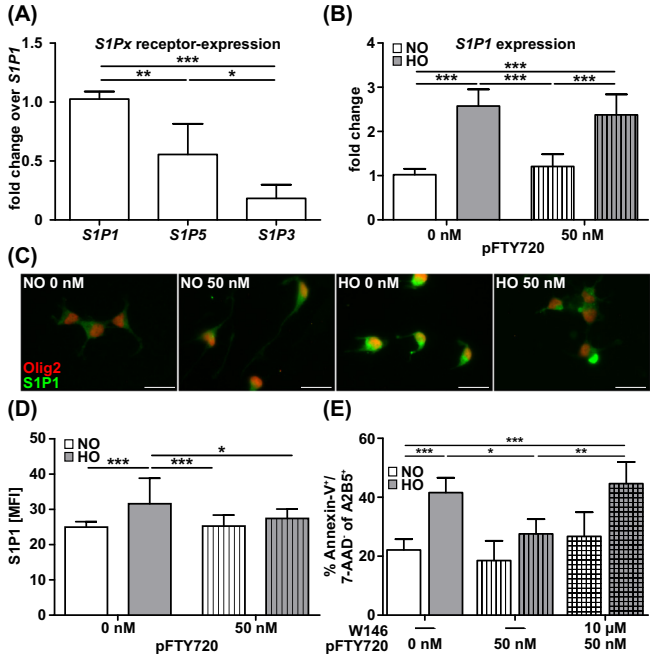

Fig. 8. FTY720s' protective effects on oligodendrocytes involve interactions with the S1P receptor 1. (A) Pre-oligodendrocytes harvested from standard culture conditions derived from 5 independent cell preparations were used for analysis of basal S1P receptor 1, 3, and 5 (*S1P1*, *S1P3*, *S1P5*) mRNA expression. (B) Immature oligodendrocytes treated with 50 nM of pFTY720 (or vehicle) with the onset of hyperoxia or normoxia were used for quantification of *S1P1* mRNA expression ($n = 5$ independent experiments). (C) Immunocytochemical co-stainings for Olig2 (red) and S1P1 (green) were used for quantification of S1P1 protein expression on single immature oligodendrocytes. Scale bar = 20 µm. (D) Mean fluorescence intensities per cell were normalised to the normoxia control group. $n = 16$ regions of interest derived from 3 independent experiments. (E) Premature oligodendrocytes were incubated with the selective S1P receptor 1 antagonist W146 (10 µM) or vehicle for 30 min prior to the beginning of hyperoxia/normoxia and addition of 50 nM pFTY720 or vehicle. Cell death was assessed by flow cytometry on A2B5 positive cells co-labelled with Annexin-V (pro-apoptotic) and 7-AAD (dead). The percentage of pro-apoptotic cells ($A2B5^+$ Annexin-V^+ 7-AAD^-) was quantified. $n = 5$ independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and maturation starts in the first postnatal week which corresponds to preterm born human infants (23–36 weeks of gestation) (Semple et al., 2013). Previous experimental studies revealed that neonatal hyperoxia-triggered neural cell degeneration leads to a transient hypomyelination with long-lasting microstructural changes in the white matter (Brehmer et al., 2012; Dzietko et al., 2008; Felderhoff-Mueser et al., 2004; Schmitz et al., 2014; Sifringer et al., 2012). We observed similar changes in white matter microstructures on diffusion tensor imaging scans, i.e. reduced fractional anisotropy and increased diffusivity of adult rat brains following neonatal hyperoxia that is restored by a single FTY720 treatment. The present study shows that FTY720 significantly increases MBP expression in the developing rat brain following neonatal hyperoxia. Impaired myelination is associated with significant cognitive deficits of hyperoxic rats in later life. These data support a recent report showing that disrupted axon-oligodendrocyte integrity in a hyperoxic model of premature brain injury results in long-lasting impairment of conduction properties in the adult white matter (Ritter et al., 2013). Of note, hyperoxia-mediated hypomyelination is a transient phenomenon, since significant changes in MBP expression that become evident at P10–P11 are normalised to control levels 9–15 days following hyperoxia exposure. Moreover, hyperoxia-induced apoptosis and loss of CC1+ cells occur immediately after hyperoxia (P7) and last for maximal 48 h (Brehmer et al., 2012; Felderhoff-Mueser et al., 2004; Schmitz et al., 2014). However, these transient effects directly translate into long-term alterations of white matter brain structures, which can be detected as early as at P21 and persist up to P60 (Brehmer et al., 2012; Schmitz et al., 2014). Considering the brains plasticity in this developmental stage and the fact that axons continue to myelinate into the second and third decade one limitation of previous pre-clinical studies is the lack of data from P60 onwards which can provide insight into maturation of white matter structures throughout adulthood (Semple et al., 2013). Therefore, we included analyses of functional deficits and microstructural white matter abnormalities at the fully adult developmental stage (>P90) demonstrating that FTY720-mediated acute neuroprotection by prevention of oligodendrocyte degeneration and maturation arrest results in long-term protection. These findings emphasise the need of acute therapies during the very vulnerable phase of brain maturation to enhance the developmental and regenerative potential of the preterm brain. Since premature infants are exposed to partial oxygen concentrations that are fourfold higher compared to intrauterine conditions, any therapy can be applied immediately at onset of hyperoxia/birth. The present study was carried out as a first proof-of-principal analysis to investigate FTY720s' effects on acute brain injury and corresponding long-term developmental processes. Nevertheless, further studies applying delayed treatment paradigms might uncover additional target mechanisms promoting regenerative capacities in response to neonatal brain injury.

Recent work in models of cerebral ischemia and Alzheimer's disease have demonstrated that FTY720 improves neurological outcome in adult rodents (Brunkhorst et al., 2014). However, aetiology, underlying pathology and timing of the insult significantly differ from the pathogenesis of neonatal white matter damage, and functional outcome measures largely concentrated on motor function as direct consequences of injury (e.g. d3–d15 post injury). Our study focused on long-term analysis of cognitive capacities up to 3 months following the insult. Only sparse data exists on long-term cognitive function after FTY720 treatment which mainly focuses on adult hippocampal learning and memory. Intra-hippocampal injection of Aβ1–42 followed by 14 days of FTY720 treatment ameliorated spatial learning in Morris water maze at the end of treatment (Asle-Rousta et al., 2013). Whereas these effects were attributed to reduced hippocampal damage, we do not find cell degeneration to a large extend in our model. Therefore, the development of neuronal networks through restoration of disturbed myelination rather than inhibition of direct neurodegeneration in the hippocampus might be the underlying mechanism explaining the present observations.

Proposed lymphocyte-independent mechanisms of FTY720 include reduced brain inflammation and nitrosative stress and direct neuroprotective effects (Brunkhorst et al., 2014). Although we detected the suggested reduction of circulating lymphocyte counts after FTY720 treatment, this effect was independent of hyperoxia. Therefore, we hypothesise that the beneficial effects of FTY720 in neonatal oxygen-induced brain injury may be rather attributed to inhibition of microglia activation than to modulation of lymphocyte trafficking between peripheral lymphoid organs, the circulation and the CNS. This is further supported by the fact, that the relative mild neonatal hyperoxia model (24 h) used in the present study does not involve increased infiltration of peripheral immune cells to brain. However, hyperoxia is strongly associated with an increased level of microglia activation. A microglial response to FTY720 has been postulated in a model of adult cerebral ischemia and excitotoxicity showing that it decreases total microglia counts (Cipriani et al., 2015; Czech et al., 2009). However, in brain slices microglial numbers were increased by FTY720 treatment whereas their activation status remains unchanged (Miron et al., 2010). *In vivo* effects of FTY720 on

activation and effector mechanisms of microglia have been rarely addressed. Instead, *in vitro* studies revealed reduction of pro-inflammatory cytokine production including TNFα and IL-1β (Noda et al., 2013). We did not observe a modulation of total microglia cell counts but a reduced level of microglia activation suggesting reduced IL-1β and TNFα production after FTY720 treatment as a consequence of inhibited microglia activation. In addition to activated microglia other glial cell types (e.g. astrocytes) are known to release pro-inflammatory cytokines under pathological conditions. Given that astrocytes express different S1P receptors these cells might have also contributed to the observed decline in cerebral cytokine levels following FTY720 treatment. Astrocytes react with a biphasic response to hyperoxia, i.e. GFAP and glutamate uptake transporter expression is down-regulated immediately after hyperoxia whereas GFAP significantly increases on P12 (Schmitz et al., 2011). These data suggest that astrocytes rather protect oligodendrocyte precursor cells from glutamate-induced toxicity after hyperoxia than contribute to acute induction of pro-inflammatory cytokines (e.g. IL-1β and TNFα). Nevertheless, direct effects of FTY720 on astrocytes cannot be excluded. This is supported by the elementary work of Choi et al. showing that treatment efficacy of FTY720 in experimental autoimmune encephalomyelitis (EAE) is dependent on S1P1 expression on astrocytes (Choi et al., 2011). However, EAE involves autoimmune inflammation which is different from local inflammatory reactions after hyperoxia in the neonatal brain. Molecular mechanisms of myelin maturation disturbances differ with respect to its cause and to developmental factors. Moreover, local inflammatory responses (i.e. cytokine production) were assessed in Nestin-S1P1 knockout mice (Choi et al., 2011). Taken into account that Nestin is also supposed to be expressed by oligodendrocyte progenitor cells (Walker et al., 2010) and is a marker for neural precursor cells which can differentiate into neuronal and glial CNS cell types (Wiese et al., 2004), direct effects of astrocyte specific S1P1 modulation in neonatal brain injury and hyperoxia-induced inflammation remain to be clarified.

Perinatal exposure to IL-1β alters the developmental program of the white matter resulting in magnet resonance imaging abnormalities and cognitive deficits (Favrais et al., 2011). Therefore FTY720 might improve memory function and restoration of long-term structural white matter development by reduction of microglia activation and cytokine production. Furthermore, FTY720 ameliorates hyperoxia-induced oxidative stress levels. Nitric oxide production is a key target mechanism of FTY720's beneficial effects in experimental autoimmune encephalomyelitis (Colombo et al., 2014). Since oxidative stress is a major hallmark of hyperoxia-mediated brain injury involving lipid peroxidation, glutathione oxidation and reduction (Felderhoff-Mueser et al., 2004; Sifringer et al., 2010), the anti-oxidative capacity of FTY720 demonstrated in the present study might act in concert with reduced brain inflammation to create a beneficial microenvironment for increased survival, maturation and differentiation of immature oligodendrocytes.

Whereas neuroprotective effects of FTY720 were mainly attributed to immunomodulatory and neurotrophic effects, (re)-myelination has rarely been addressed in relevant neurodegenerative experimental models. The present study emphasises that protection of oligodendrocyte development and restoration of white matter structures after neonatal hyperoxia might be an important mode of action of FTY720. Analysing specific brain regions commonly affected in preterm infants (Volpe, 2009), we demonstrate that FTY720 treatment decreases oligodendrocyte degeneration which is accompanied by an improved maturation of Olig2 positive cells at P7 culminating in increased myelination at P11. Noteworthy, these acute protective effects on oligodendrocytes were not associated with general tissue atrophy (data not shown) or neuronal degeneration in white matter and hippocampus supporting the hypothesis that long term hyperoxia-induced cognitive deficits which were improved by FTY720 may be rather attributed to specific alterations in oligodendrocyte cellularity and structures of the white matter than to overall tissue loss or hippocampal neurodegeneration.

Studies focusing on the *in vivo* evaluation of FTY720 on myelination reveal contradicting results. In an adult model of cuprizone-induced demyelination FTY720 treatment decreased cell degeneration, astro- and microgliosis and normalised levels of oligodendrocyte maturation and myelination, probably due to reduced inflammatory cytokine expression, such as IL-1β (Kim et al., 2011). However, recent findings in the same model did not confirm these striking effects (Slowik et al., 2015). It must be further taken into account that oligodendrogenesis and myelination differ between adult and postnatal brains, with a specific growth factor and gene expression pattern and also different proliferation and differentiation capacities (Greenwood and Butt, 2003; Kang et al., 2010). In accordance FTY720 improves brain development in a neonatal model of endotoxin-sensitised hypoxia–ischemia (Yang et al., 2014). Whereas this study identifies immunomodulation and protection of degenerating neurons as target mechanisms, direct effects on oligodendrocyte development and myelination have been recently addressed in human oligodendrocyte progenitors under normoxic cell culture conditions (Cui et al., 2014). We did not observe any modulation of myelination by pFTY720 under normoxic conditions which is most probably due to species differences and another treatment paradigm. As such, we performed analysis immediately after a single treatment for 24 h whereas Cui et al. used a repetitive (daily) treatment regime to observe increased myelination after 4 weeks. Nevertheless, this study is a fundamental report on the molecular targets of pFTY720 demonstrating that a single dose of pFTY720 results in rapid Erk1/2 activation which is reversed to control levels at 4 h whereas repeated treatment blocks Erk1/2 activation (Cui et al., 2014). We did not observe significant alterations of Erk1/2 activation by pFTY720 in our treatment regime (data not shown) which might be explained by the chosen late time point of analysis (24 h) after a single treatment. A report from a spheroid cell culture system suggested effects of FTY720 on remyelination via modulation of microglial responses (Jackson et al., 2011). Therefore we wondered whether improved myelination might be a consequence of a preserved microenvironment involving reduction of inflammation and oxidative stress or whether oligodendrocytes are directly targeted. By the use of *in vitro* cultures we show that a single treatment with activated FTY720 exerts cytoprotective effects on pre-oligodendrocytes and increases oligodendrocyte maturation after hyperoxia. Similar results have been obtained in studies investigating serum withdrawal or growth factor deprivation even though culture conditions and the duration of pFTY720 treatment differed (Coelho et al., 2007; Jung et al., 2007). Further evaluation of S1P1 expression demonstrated that the hyperoxia-induced increase in S1P1 expression was downregulated by pFTY720. Since differences were evident on protein but not on mRNA level pFTY720s' protective effect might be mediated through S1P1 modulation on pre-oligodendrocytes most likely through receptor internalization as previously described for other cell types (Mullershausen et al., 2009). Interestingly, selective pharmacological S1P1 blockade diminished pFTY720s' protection suggesting direct interactions of FTY720 with the S1P1 resulting in decreased hyperoxia-induced pre-oligodendrocyte degeneration.

Except from blood lymphocyte counts we did not observe significant *in vivo* baseline effects of FTY720 (i.e. NO vs. NO + FTY720) and neither the percentage of apoptotic cells nor MBP

positive cells were significantly altered by different concentrations up to 1000 nM of pFTY720 in the normoxia control group *in vitro*. However, potential undesired effects cannot be excluded since continuous treatment regimes and/or higher doses might result in harmful effects with regard to differentiation and cell survival (Jung et al., 2007; Kohne et al., 2012). As such, we observed cell loss after prolonged treatment with higher doses (data not shown). Potential toxic effects elicited by other pharmacological regimes need to be further investigated which is beyond of the scope of the current study.

Since a large number of very immature infants suffer from white matter injury associated with cognitive and behavioural deficits in later life there is an urgent need for treatment strategies preventing tissue loss and/or promoting repair of white matter injury. FTY720 is already in clinical use in adult multiple sclerosis and studies in childhood multiple sclerosis are currently under way. We show that it confers white matter protection and provides the features of a promising neuroprotectant also for developmental brain injury. When considering an immunomodulatory agent as a neuroprotectant for the developing organism one has to take the immaturity of the immune system of preterm infants into account. There are strong distinctions between the newborn infants' and the adults' immune system with relatively few T cells in the preterm organism. It has recently been shown that preterm infants physiologically display a strong tendency towards T_H2 responses upon a viral or bacterial stimulus and a markedly reduced T_H1 response including production of T_H17 cytokines (Gibbons et al., 2014), which might be modulated by FTY720. Although a single application of FTY720 conferred neuroprotection without detectable side effects as shown by regular weight gain of animals we observed a hyperoxia-independent acute reduction of circulating lymphocyte counts after FTY720 treatment. However, diffusible inflammatory mediators were not modulated and thus may not have interfered with brain pathology in this specific setting. This is further supported by the fact that lymphocyte counts in the brain were not modulated by FTY720 and hyperoxia. Nevertheless, peripheral immune responses in a clinical setting cannot be excluded and should be carefully considered in future longitudinal studies.

Taken together, we show the pleiotropic action of FTY720 on different neural cell types by reduction of inflammation and oxidative stress and a direct action on oligodendrocyte precursor cells leads to improved functional outcome following neonatal hyperoxia. Our findings add important knowledge to FTY720's mode of action in the developing CNS in addition to its established effects on the peripheral immune system and particularly emphasises the need for further pre-clinical research also in other models of perinatal brain injury.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2015.10.004.

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