Mechanisms Involved in the Remyelinating Effect of Sildenafil

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Abstract

Remyelination occurs in demyelinated lesions in multiple sclerosis (MS)

and pharmacological treatments that enhance this process will critically impact the long term functional outcome in the disease. Sildenafil, a cyclic GMP (cGMP)-specific phosphodiesterase 5 inhibitor (PDE5-I), is an oral vasodilator drug extensively used in humans for treatment of erectile dysfunction and pulmonary arterial hypertension. PDE5 is expressed in central nervous system (CNS) neuronal and glial populations and in endothelial cells and numerous studies in rodent models of neurological disease have evidenced the neuroprotective potential of PDE5-Is. Using myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) as a MS model, we previously showed that daily administration of sildenafil starting at peak disease rapidly ameliorates clinical symptoms while administration at symptoms onset prevents disease progression. These beneficial effects of the drug involved down-regulation of adaptive and innate immune responses, protection of axons and oligodendrocytes (OLs) and promotion of remyelination. In this work we have investigated mechanisms involved in the remyelinating effect of sildenafil. Using demyelinated organotypic cerebellar slice cultures we demonstrate that sildenafil stimulates remyelination by direct effects on CNS cells in a nitric oxide (NO)-cGMP-protein kinase G (PKG)-dependent manner. We also show that sildenafil treatment enhances OL maturation and induces expression of the promyelinating factor ciliary neurotrophic factor (CNTF) in spinal cord of EAE mice and in cerebellar slice cultures. Furthermore, we demonstrate that sildenafil promotes a M2 phenotype in bone marrow derived macrophages (BMDM) and increases myelin phagocytosis in these cells and in M2 microglia/macrophages in the spinal cord of EAE mice. Taken together these data indicate that promotion of OL maturation directly or through induction of growth factor expression, regulation of microglia/macrophage inflammatory phenotype and clearance of myelin debris may be relevant mechanisms involved in sildenafil enhancement of remyelination in demyelinated tissue and further support the contention that this well tolerated drug could be useful for ameliorating MS pathology.

Keywords

Sildenafil
remyelination
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CNTF

macrophage phenotypes
myelin phagocytosis

Daniela Díaz-Lucena and María Gutierrez-Mecinas Equal contribution.

Introduction

Multiple Sclerosis (MS) is a chronic autoimmune demyelinating disease characterized by an inflammatory attack on the central nervous system (CNS) myelin sheaths that causes damage to the underlying axons and leads to functional impairments and clinical disability (Lassmann 2008; Trapp and Nave 2008). Neuropathological observations indicate that re-ensheathment of demyelinated axons, the process termed remyelination, occurs in demyelinated lesions in MS and may contribute to clinical remittance (Franklin and Ffrench-Constant 2008). Studies in experimental animal models of CNS demyelination indicate that remyelination is mediated by oligodendrocytes (OLs) generated from oligodendrocyte progenitor cells (OPCs) that proliferate in the germinal zones and are recruited to the lesion site where they differentiate into mature OLs (Fancy et al. 2011; Maki et al. 2013). Increasing evidence supports the capacity of subsets of activated resident microglia and infiltrating monocyte-derived macrophages for promoting remyelination in inflammatory demyelinating disease. In the experimental autoimmune encephalomyelitis (EAE) model of MS, a shift toward an anti-inflammatory M2 phenotype is associated with milder disease symptoms (Mikita et al. 2011) and administration of M2-polarized monocytes into the cerebrospinal fluid enhances differentiation of OLs (Butovsky et al. 2006). After focal demyelination in the adult mouse brain, the initial activation of microglia/macrophages to a pro-inflammatory M1 phenotype appears to drive OPC proliferation but a later switch to a pro-regenerative M2 phenotype is required for progenitors to differentiate into mature OLs and remyelination to take place (Lloyd and Miron 2016). M2 cells have higher myelin phagocytic activity than M1 cells (Durafourt et al. 2012). This property of M2 cells can critically contribute to remyelination since myelin debris inhibits OPC differentiation (Kotter et al. 2006). Release of trophic factors and cytokines by M2 microglia/macrophages and reactive astrocytes will also contribute to create a permissive environment for remyelination (Li et al. 2016; Rawji et al. 2016). The generation of a nonpermissive environment or deficits in remyelinating cells may result in the remyelination failure that occurs as MS progresses. Therapeutic agents that combine anti-

inflammatory, neuroprotective and remyelinating effects will critically determine the long-term functional outcome in the disease.

Phosphodiesterase 5 (PDE5), a member of the large family of cyclic nucleotide hydrolyzing enzymes, is specific for cyclic GMP (cGMP) and its inhibition will potentiate the actions of this second messenger. Specific inhibitors of this enzyme (PDE5-Is) such as sildenafil (Viagra®) are oral vasodilator drugs extensively used in humans for treatment of erectile dysfunction and pulmonary arterial hypertension, and long-term studies of daily administration have shown that these drugs are well tolerated and have a favorable side-effect profile (Frey and Lang 2012; Fusco et al. 2010). In the CNS, cGMP can be formed by stimulation of nitric oxide-dependent guanylyl cyclase (NO-GC) that is expressed in neurones, astrocytes, OLs and vascular endothelial cells (Baltrons and Garcia 2001; Garthwaite et al. 2015; Garthwaite 1991; Zhang et al. 2003). Astroglial and microglial cells can also produce cGMP by stimulation of natriuretic peptide receptors (Prado et al. 2010). PDE5 expression has been described in neuronal and glial populations and cerebrovascular endothelial cells (Bender and Beavo 2004; Prado et al. 2010; Zhang et al. 2003) and PDE5-Is have been shown to exert neuroprotective actions in rodent models of neurological disease through different mechanisms. In rats with embolic stroke administration of sildenafil improves functional recovery in association to enhanced angiogenesis, neurogenesis, synaptogenesis, axonal remodeling and oligodendrogenesis (Ding et al. 2008; Menniti et al. 2009; Wang et al. 2005; Zhang et al. 2005; Zhang et al. 2002; Zhang et al. 2012). PDE5-Is also protect against different neurotoxins by activating survival pathways (Barros-Minones et al. 2013; Puerta et al. 2009) and after focal brain lesion by regulating the innate immune response and promoting antioxidant mechanisms (Pifarre et al. 2010; Prado et al. 2013). In models of Alzheimer’s disease PDE5-Is ameliorate synaptic and memory abnormalities by reducing Tau phosphorylation, amyloid burden and neuroinflammation (Cuadrado-Tejedor et al. 2011; Puzzo et al. 2009). Reduction of neuroinflammation has been also shown in rats with hepatic encephalopathy (Hernandez-Rabaza et al. 2015). Interestingly, sildenafil amelioration of peripheral neuropathy in diabetic mice was reported to occur concomitantly with increases in myelin sheath thickness and subcutaneous nerve fibers (Wang et al. 2011). In light of these pleiotropic effects of PDE5-Is some years ago we hypothesized that treatment with these drugs could be beneficial in MS. Using EAE induced by immunization with myelin oligodendrocyte glycoprotein peptide 35–55 ($MOG_{35–55}$) as a model

mimicking many aspects of progressive MS, we initially showed that daily administration of sildenafil starting at peak disease rapidly ameliorates clinical symptoms by preventing axonal damage and promoting remyelination in the spinal cord (SC). These effects were associated with decreased T-cell infiltration and microglial/macrophage activation and increased presence of T regulatory cells (Tregs) (Pifarre et al. 2011). The presence of reactive astrocytes forming scar-like structures around infiltrates was enhanced by sildenafil suggesting a possible mechanism for restriction of leukocyte spread into healthy parenchyma. The myelin and axon protective effects of sildenafil together with reduced inflammatory gene expression have been also observed in the cuprizone demyelination model (Nunes et al. 2012). In a more recent work, we showed that administration of the drug at the onset of EAE symptoms prevents disease progression (Pifarre et al. 2014). Ultrastructural and immunocytochemical analysis of SCs evidenced that early sildenafil treatment preserves axons and myelin, protects immature and mature myelinating OLs and increases the number of axons with remyelinating appearance. Up-regulation of brain-derived neurotrophic factor (BDNF) in immune and neural cells suggested its implication in the neuroprotective effects of the drug. Increased expression of YM-1, a marker of the macrophage/microglial M2 phenotype was also induced by the treatment. Furthermore, sildenafil down-regulated the adaptive immune response (Pifarre et al. 2014). In this work we have further investigated mechanisms involved in the remyelinating effect of this drug. Using organotypic cerebellar cultures demyelinated with lysophosphatidylcholine (LPC) we demonstrate that sildenafil induces remyelination by direct effects on CNS cells in a NO-cGMP-protein kinase G (PKG) dependent manner. We also show that sildenafil-treatment enhances OL maturation and ciliary neurotrophic factor (CNTF) expression in the SC of EAE mice and in demyelinated slice cultures, regulates the inflammatory phenotype of macrophages and promotes myelin debris phagocytosis.

Materials and Methods

Animals

All procedures used in studies involving animals were approved by the Autonomous University of Barcelona Animal and Human Experimentation Ethics Committee in accordance to the ethical standards of the Federation of European Laboratory Animal Science Associations (FELASA).

EAE Induction and Treatments

Two-month old female C57BL/6 mice (Charles River) were housed in the animal facility of the Universitat Autonoma de Barcelona (UAB) under constant temperature and provided food and water *ad libitum*. Chronic EAE was induced by immunization with $MOG_{35–55}$ peptide (Scientific Technical Service, Universitat Pompeu Fabra, Barcelona, Spain) and clinically evaluated for EAE progression as previously described (Pifarre et al. 2011). As controls naïve mice were used. After immunization animals were randomly divided in 4 groups before treatment by subcutaneous (s.c) injection with vehicle (purified water) or sildenafil (10 mg/kg; Sigma-Aldrich) once a day starting at the onset of clinical symptoms (mean score ≈ 1, loss of tail tonus) or at peak disease (around 18 days post-immunization; mean score ≈ 2.5, severe hind limb paraparesis) for 15 days. We previously demonstrated that these treatment regimens are effective at preventing and ameliorating disease progression (Pifarre et al. 2014; Pifarre et al. 2011). The sildenafil dose used is equivalent to 57 mg/day in a 70 kg human, according to Reagan-Shaw et al. (2008). Mice were sacrificed under pentobarbital anesthesia and SCs were removed.

LPC-demyelinated Cerebellar Organotypic Cultures

Organotypic cultures were established from cerebellum of 7-day-old C57BL/6 mice (Harlan Inc.) as described by Birgbauer et al. (2004). Mice were sacrificed by decapitation and cerebella dissected. Sagittal 300 μm cerebellum slices were obtained with a McIlwain Tissue Chopper. Three or four slices were seeded per well on culture inserts (Millipore) and incubated at 37 °C and 5% CO_2 in 50% basal medium Eagle, 25% Hanks' balanced salt solution, 25% horse serum, 5 mg/ml glucose, 0.25 mM L-glutamine and 25 μg/ml penicillin/streptomycin (pen/strep) from Sigma-Aldrich. Medium was changed every 2–3 days. After 7 days in vitro (DIV), slices were demyelinated by incubation with L-α-lysophosphatidylcholine from egg yolk (LPC, 0.5 mg/ml; Sigma-Aldrich) for 14 h and afterwards medium was replaced with LPC-free medium. Twenty-four hours after removing LPC (9 DIV), demyelinated slices were treated with vehicle or cGMP increasing compounds: sildenafil (1 μM; Sigma-Aldrich) alone or combined with the NO-GC direct stimulator BAY 41–2272 (3 μM; Tocris), or the glutamate receptor agonist N-methyl-D-aspartate (NMDA, 30 μM; Sigma-Aldrich). Treatments were repeated at 12, 14 and 16 DIV and cultured slices were harvested at 19 DIV. Inhibitors of NO-GC (1H-[1,2,4]oxadiazolo[4,3-

a]quinoxalin-1-one, ODQ, 10 μM; Sigma-Aldrich), NO synthase (NOS) (L-nitroarginine, L-NNA, 100 μM; Sigma-Aldrich) or PKG (Rp-8-(para-chlorophenylthio) guanosine-3′,5′-cyclic monophosphorothioate, Rp-8pCPTcGMP, 10 μM; Biolog) were added 24 h before the initiation of treatments (8 DIV) and combined with treatments thereafter. Concentrations of drugs used have been previous demonstrated by us and others to be effective in CNS cell cultures and slices (Boran and Garcia 2007; Ferrero and Torres 2001; Garthwaite et al. 2015; Poppe et al. 2008; Stasch et al. 2001).

Isolation, Culture and Polarization of Mouse Bone Marrow-derived Macrophages (BMDM)

BMDM were generated from C57BL/6 mice (Harlan Inc.) as previously described (Classen et al. 2009). Briefly, bone marrow was extracted and plated in 150 mm diameter culture dishes and incubated in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 10 ng/ml recombinant mouse macrophage colony stimulating factor (M-CSF; Immunotools), 20% fetal bovine serum (FBS; Gibco) and 1% pen/strep for 7 days. A second boost of 5 ng/ml M-CSF was given on the 4th day. After, cells were re-seeded at the appropriate density and incubated for 18 h in DMEM-10% FBS-1% pen/strep at 37 °C in 5% CO_2. Polarization to M1 phenotype was induced by incubation with 10 ng/ml lipopolysaccharide from *Salmonella typhimurium* (LPS; Sigma-Aldrich) plus 20 ng/ml interferon gamma (IFNγ; Millipore), and to M2 phenotype with 20 ng/ml interleukin 4 (IL-4; Immunotools). Cells were treated with sildenafil (1–100 μM), or cGMP analogues 8-Br-cGMP (500 μM; Tocris) or 8-(4-chlorophenylthio)-cGMP (8pCPTcGMP, 50 μM; Sigma-Aldrich) alone or with the phenotype polarization stimuli. PKG inhibitors Rp-8pCPTcGMP (1 μM; Biolog) or Rp-8-Br-β-phenyl-1,N2-ethenoguanosine 3′,5′-cyclic monophosphorothioate sodium salt (Rp-8Br-PET-cGMP; 0,5 μM; Sigma) were added 30 min before. Cells were incubated for 24 h for morphology examination, arginase activity, nitrite measurement and phagocytosis determination, 18 h for Luminex cytokine assay and 48 h for flow cytometry.

Histological Methods

Cervical-thoracic SCs and cultured cerebellar slices were fixed in 4% paraformadehyde (PFA). SCs sections were cut into coronal or longitudinal 16 μm-thick slices (Microtome Cryostat Thermoshandon). For intracellular staining tissue was permeabilized with Triton X-100 (Sigma-Aldrich) 0.1%

for SCs and 0.3% for cultured cerebellar slices. After blocking with phosphate-buffered saline (PBS)-10% normal goat serum (NGS), samples were incubated with the appropriate dilution of the following primary antibodies: rat anti-myelin basic protein (MBP, Abcam; 1:200) and rabbit anti-contactin-associated protein (Caspr, Abcam; 1:1000), mouse anti-NF-200 (Sigma-Aldrich; 1:400), rabbit anti-Olig2 (Millipore; 1:500), mouse anti-NOS-2 (BD; 1:100), goat anti-CNTF (RD Systems; 1:75), rabbit anti-glial fibrillary acidic protein (GFAP, Dako; 1:900), mouse anti-Nkx2.2 (DSHB 75.5A5, developed by Jessell, T.M. and Brenner-Morton, S.; 1:200), mouse anti-CD68 (Serotec; 1:100) and rabbit anti-YM-1 (StemCell Tech; 1:75). For colorimetric assays biotin-conjugated secondary antibodies that were detected by streptavidin/horseradish peroxidase and the peroxidase substrate DAB kit were used (Vector). For fluorescence staining Alexa-488 and 568-conjugated secondary antibodies (Invitrogen Molecular Probes) were used. Nuclei were stained with DAPI (0.25 μg/ml). Control sections were incubated in the absence of primary antibodies. Oil-Red-O (ORO) staining was performed in SC longitudinal sections as previously described with slight modifications (Koopman et al. 2001). Briefly, a solution of 0.5% *w*/*v* ORO (Sigma-Aldrich) in 60% triethyl-phosphate was diluted to 60% in deionized water. Sections were incubated at room temperature in ORO solution, washed twice and embedded in 10% glycerol containing DAPI.

Bright field images were acquired using an Eclipse 90i microscope with Nikon Digital camera DXM 1200F and Nikon Act-1 software. Confocal images were acquired using a microscope Zeiss LSM 700 with ZEN 2010 software. Colocalization of MBP/NF200 and Caspr/NF200 were obtained by Fiji ImageJ software. 3D reconstruction of YM1-ORO staining was performed with Imaris software. Quantification of CNTF, MBP and ORO staining intensity was performed with Scion Image (NIH) software in 4–8 areas (1 mm^2) randomly chosen along the length of 2–3 longitudinal SC sections separated at least 300 μm. Quantification of single (Nkx2.2) and double (MBP-Olig2) stained cells was performed manually using Fiji ImageJ software in 2–3 SC sections separated at least 300 μm or in 5–6 areas of organotypic cultures. All images were analyzed by two independent investigators blinded to treatment and clinical score.

Western Blot Analysis

Four cerebellar slices per condition (in triplicates) were homogenized using RIPA buffer (Merk-Millipore) with anti-phosphatase (Sigma-Aldrich) and

anti-protease cocktails (Roche). Thirty μg of protein were subjected to 4–12% NuPAGE Novex Bis-Tris Midi-Gels (Thermo Fisher Scientific). Transference was performed with iBlot system (Invitrogen) into PVDF membranes. For blot staining, membranes were blocked at 4 °C overnight in 5% *w*/*v* non-fat dry milk in PBS and were further incubated with the appropriate dilution of primary antibodies: rat anti-MBP (1:200) and mouse anti-2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase; 1:200) from Abcam and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:20,000) from Ambion. Horseradish peroxidase (HRP) coupled anti-rat (Thermo Fisher) and anti-mouse (Sigma-Aldrich) secondary antibodies were used and staining was revealed using Luminata Forte Western HRP substrate (Millipore) in a molecular imager ® VersaDoc ™ MP Imaging System (Bio-Rad). The intensity was quantified in 2–3 independent experiments using Quantity One software (BioRad) and expressed relative to LPC-treated cultures. Values were normalized to GAPDH levels.

mRNA Extraction and qPCR

mRNA extraction was performed in cultured cerebellar slices (4 slices per condition) using Maxwell® RSC simplyRNA Tissue Kit (Promega) following user's manual instructions. Reverse transcription was carried out by iScript (BioRad) following the supplier's instructions. qPCR was performed by iTaq Super Mix (BioRad). To determine changes in CNTF gene expression the sense TCGTTCAGACCTGACTGCTC and anti-sense ACTCCAGCGATCAGTGCTTG primers (NM_170786.2) and Bio-Rad CFX Touch™ Real-Time PCR Detection System were used. Further analysis was performed by Bio-Rad CFX Manager™ 3.1 Software. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as reference gene, sense GATTAGCGATGATGAACCAGGTT and anti-sense CCTCCCATCTCCTTCATGACA (NM_013556.2). Relative gene expression was assessed using the ΔΔCq Method (Livak and Schmittgen 2001).

Determination of Arginase Activity and Nitrites in BMDM Cultures

BMDM were seeded in 96-well plates at a density of 2×10^5 cells/ml. Arginase activity was determined as previously described (Classen et al. 2009) with slight modifications. Briefly, cells were lysed and arginine hydrolysis was performed by incubating the lysate with L-arginine at 37 °C for 60 min. Afterwards lysates were combined with 6%

α-isonitropropiophenone and the concentration of urea produced was determined by reading the absorbance at 540 nm in a multilabel plate reader VICTOR 3 (PerkinElmer). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol urea per minute. Data was expressed as units of arginase activity per 1×10^6 cells. Results are presented as percentage of activity respect to untreated controls. Nitrite concentration in the media was measured with the Greiss reagent.

M1/M2 Phenotype Analysis of BMDM by Flow Cytometry

After stimulation, BMDM were washed and harvested in Ca^{2+}/Mg^{2+}-free PBS by softly scraping. Cells were centrifuged and resuspended in the same buffer at a concentration of 1×10^6 cells/ml. For M2 cell staining the cell membrane marker CD206 was used. After blocking with TruStain FcX anti–mouse CD16/32 (Biolegend; 1:100), cells were stained with Alexa Fluor 488 coupled anti-CD206 antibody (BiolegendM; 1:100), washed and fixed in 1% PFA. For M1 cell staining the intracellular marker NOS-2 was used. Cells were fixed in 1% PFA, blocked and permeabilized with PBS-0.5% Tween-20 and 10% NGS. Cells were resuspended in primary anti-NOS-2 antibody (BD; 1:100) and incubated for 2 h at RT. The appropriate Alexa-coupled secondary antibody was used. Cells incubated in the absence of primary antibody were used as controls. Flow cytometry was performed using a FACScanto flow cytometer (BD Biosciences; 10,000 events per sample). Cell population was first gated by cell size (forward scatter, FSC) and complexity (side scatter, SCC) and were afterwards excited with the 488 nm laser and fluorescence emission measured with 530 nm filter detector. Positive gates were set with negative controls. Data analysis was performed with the BD FACSDiva Software.

Measurement of TNFα and IL-1β Release

BMDM seeded at 1×10^6 cells/ml in six-well plates were used. TNFα and IL-1β cytokine levels were measured in supernatants of treated cells using Luminex technology. The MILLIPLEX® MAP Mouse Cytokine/Chemokine Magnetic 96-well plate Bead Panel Millipore (MCYTOMAG-70 K) assay was used following the manufacturer's instructions. Results were analyzed with the Milliplex Analyte program version 5.1.0.0.

Myelin Phagocytosis by BMDM

Myelin was isolated from SC and cerebella of naïve mice as previously

described (Larocca and Norton 2007). A concentration of 1 mg/ml of isolated myelin was labelled with 12.5 μg/ml of 1,1″-diotadecyl-3,3,3′,3′-tetramethyl-lindocarbocyanide perchlorate (DiI, Sigma-Aldrich) for 30 min at 37 °C and the phagocytosis assay was performed as previous described (Hendriks et al. 2008). Briefly, BMDM were plated in 24-well plates at a density of 5×10^5 cells/ml un-polarized and M2- polarized cells were treated with sildenafil 30–100 μM or the cGMP analogue 8Br-cGMP (500 μM) for 24 h. Afterwards cells were incubated with 25 μg/ml Dil-labelled myelin for 90 min at 37 °C. Afterwards cells were detached and fluorescence was determined using a BD FACScalibur cytometer. Mean intensity times number of Dil-positive cells was used to quantify myelin phagocytosis.

Statistical Analysis

Differences were analyzed by one- or two-way ANOVA followed by the appropriate post-hoc test as indicated using GraphPad Prism v5 software. Student's t-test was used for two group comparisons. Results shown are means ± SEM of the indicated number of animals (EAE) or independent cultures (organotypic and BMDM) or means ± SD of different measures in a representative experiment. Differences were considered significant at *P* values lower than 0.05 ($p < 0.05$).

Results

Sildenafil Promotes Remyelination in LPC-demyelinated Mouse Cerebellar Organotypic Cultures

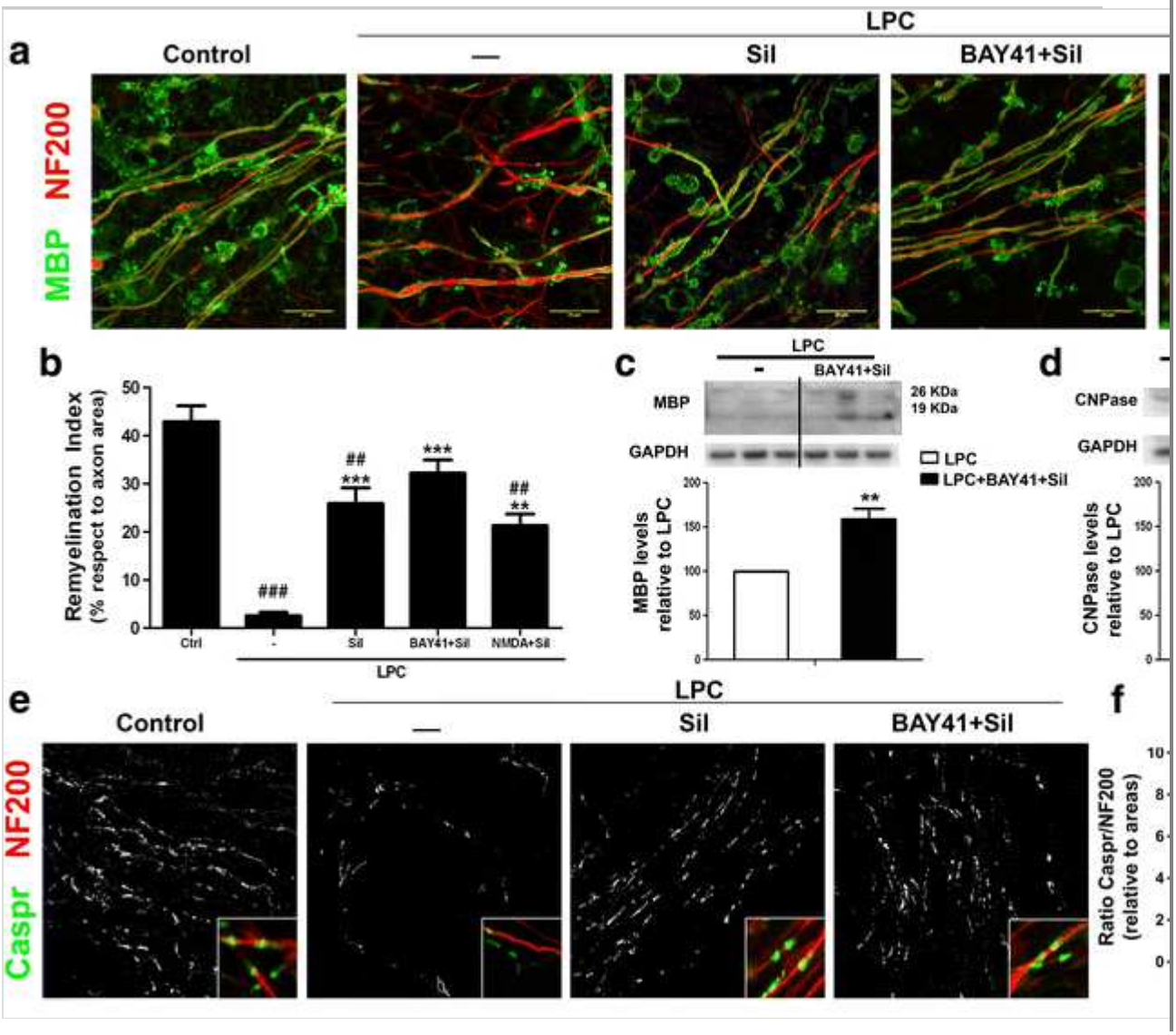
To investigate if the remyelinating effect of sildenafil we previously observed in EAE mice involved effects on CNS cells or also required peripheral immunoregulatory actions, we used the *in vitro* model of mouse cerebellar organotypic cultures (ORG) demyelinated with LPC. Cultures were established from cerebella of 7-day-old C57BL/6 mice as described in Methods. After 7 DIV, cerebellar slices were exposed to LPC (0.5 mg/ml) for 14 h, a treatment that causes a striking demyelination without neuronal death (Birgbauer et al. 2004). Twenty-four hours after removing LPC, cultures were treated with sildenafil (1 μM every 2–3 days) alone, or in combination with the NO-GC allosteric enhancer BAY 41–2272 (BAY41; 3 μM), or the glutamate receptor agonist NMDA (30 μM) that stimulates NO formation by the constitutive Ca^{2+}-dependent NO synthase in granule cells and this NO can subsequently target NO-GC in astrocytes, oligodendrocytes and endothelial cells (Garthwaite 1991). At 19 DIV, cerebellar slices were harvested, double

immunofluorescence-stained for axon neurofilament NF200 and for the major myelin protein MBP and analyzed by confocal microscopy. As shown in Fig. 1a, exposure to LPC produced a great reduction in MBP staining around axons and cell bodies compared to control slices. Treatment of LPC-demyelinated slices with cGMP-increasing compounds notably enhanced MBP staining (50–70%). Quantification of the remyelination index (RI; area stained for MBP relative to area stained for NF200) showed that remyelination induced by sildenafil alone was not significantly different from that attained in combination with BAY41 (BAY41 + Sil) or NMDA (NMDA + Sil), although the combination with the NO-GC activator appeared to be somewhat more effective (Fig. 1b). Remyelination attained with BAY41 alone was 40% lower than that induced by BAY41 + Sil (not shown), indicating that PDE5 inhibition by sildenafil is sufficient to enhance cGMP accumulation and suggesting that an endogenous source of NO is responsible for NO-GC stimulation. In agreement with the immunostaining data, analysis by WB of levels of MBP and of CNPase showed a significant increase of both myelin proteins in the demyelinated slices after treatment with BAY41 + Sil (Fig. 1c,d). The presence of Caspr clusters in the paranodes delimiting myelin sheaths correlates with a proper remyelination of axons (Arancibia-Carcamo and Attwell 2014). To investigate the remyelination status of the cerebellar cultures, double immunostaining for Caspr and NF200 was performed. As expected, Caspr clusters associated with axons were drastically reduced in LPC-demyelinated cultures (Fig. 1e). Treatment with sildenafil or BAY41 + Sil largely restored Caspr staining levels and distribution (Fig. 1e,f).

Fig. 1

Sildenafil promotes remyelination in LPC-demyelinated cerebellar organotypic cultures. (**a**) Representative images of MBP (green) and NF200 (red) immunostaining in control and LPC (0.5 mg/ml)-demyelinated cerebellar slices treated or not for 10 days with sildenafil (1 μM) alone or in combination with the NO-GC activator BAY41–2272 (3 μM) or the glutamate receptor agonist NMDA (30 μM); scale bar = 20 μm; (**b**) Quantification of MBP and NF200 staining overlap (RI, remyelination index) reveals a drastic reduction in LPC-demyelinated cultures and recovery in cultures treated with sildenafil alone or in combination with cGMP synthesis stimuli. Values are means ± SEM of three independent experiments. One-way ANOVA revealed statistically significant differences among treatments ($p < 0.001$). Bonferroni's post-hoc analysis indicated significant differences between control and LPC-demyelinated slices ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$) and between LPC-demyelinated slices treated or not with cGMP-increasing compounds ($^{**}p < 0.01$, $^{***}p < 0.001$); (**c**) and (**d**)

Representative WBs for MBP and CNPase in triplicate samples of LPC-demyelinated slices treated with vehicle or BAY41 + Sil in one experiment. Protein levels were normalized to GAPDH. Results ~~from three independent experiments are~~ presented in the graph relative to levels in vehicle-treated samples ~~and~~ are means ±SEM of three independent experiments for MBP and two for CNPase. The treatment significantly increased myelin proteins (Student's t-test, $^{**}p < 0.01$); (**e**) Double immunostaining of paranodal protein Caspr (green) and NF200 (red) in LPC-demyelinated slices treated or not with sildenafil or BAY41 + Sil. Representative images of Caspr mascara (white); small panels show Caspr clusters over axons. (**f**) Quantification of area immunostained for Caspr relative to the area stained for NF-200. Values are means ± SD (5–6 images per condition) in a representative experiment that was replicated with similar results. One-way ANOVA revealed significant differences among treatments ($p < 0.01$). Bonferroni's post-hoc analysis revealed significant differences between control and vehicle-treated LPC-demyelinated slices ($^{\#}p < 0.05$) and between vehicle- and sildenafil- or BAY41 + Sil-treated slices ($^{*}p < 0.05$)

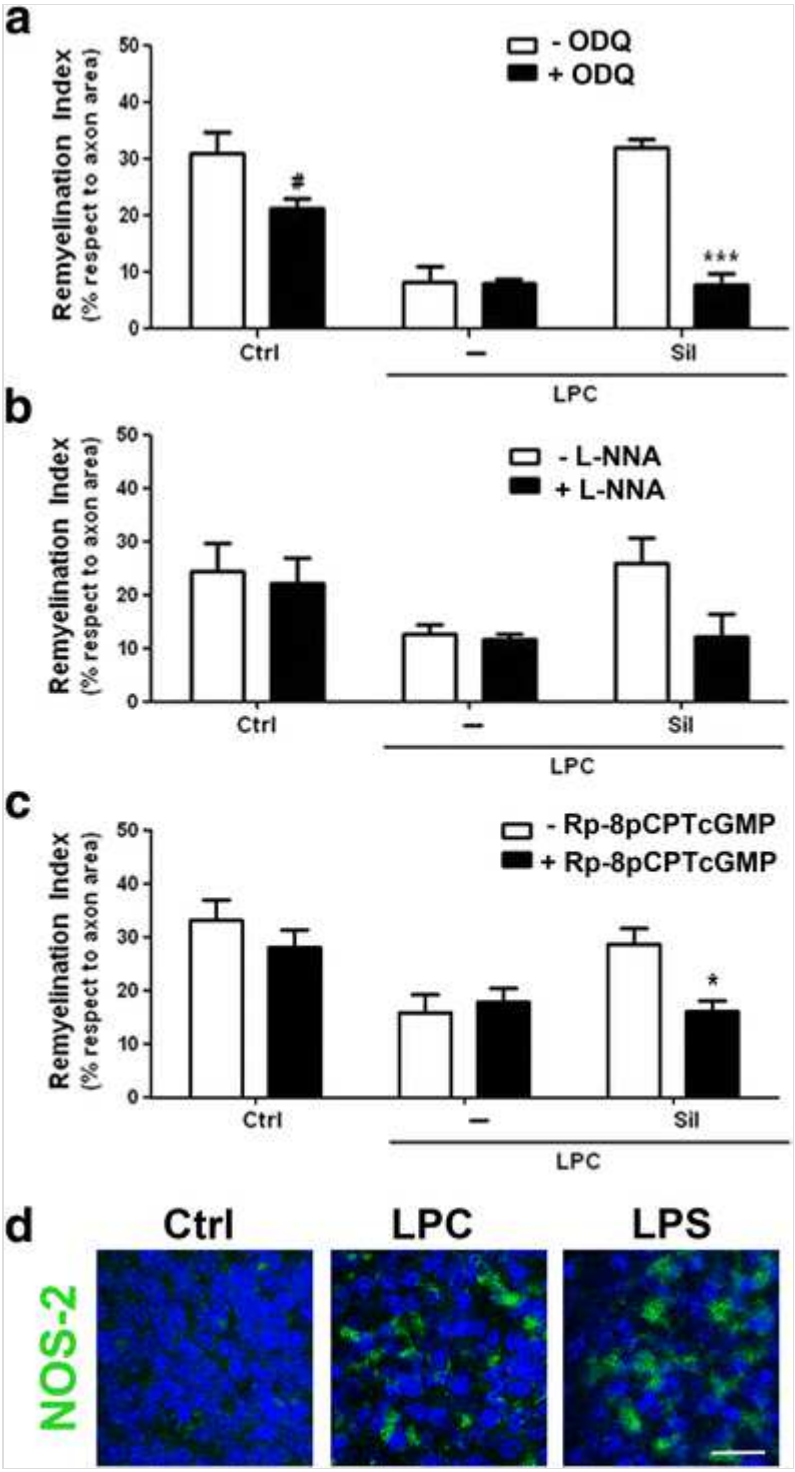


The NO-cGMP-PKG Pathway is Involved in the Remyelinating Effect of Sildenafil in LPC-demyelinated Cerebellar Cultures

As mentioned above, the observation that treatment of demyelinated cerebellar slices with sildenafil alone produced a similar remyelinating effect than its combination with BAY41 or NMDA, stimuli that increase cGMP production via NO-GC, suggested that endogenous activation of this enzyme was involved in the sildenafil effect. In agreement with this assumption sildenafil-induced remyelination was prevented by the NO-GC inhibitor ODQ (10 μM; Fig. 2a), as well as by the NOS inhibitor L-NNA (100 μM; Fig. 2b). The remyelinating effect of sildenafil also required PKG activity since it was prevented by the specific inhibitor Rp-8pCPTcGMP (10 μM) (Fig. 2c). Treatment with ODQ, but not L-NNA or Rp-8pCPTcGMP, significantly decreased (30%) the amount of myelin associated with axons in control cultures which may be due to some toxic effect in the immature cerebellar slices under prolonged exposure, as reported to occur in cultured rat brain OLs (Garthwaite et al. 2015) and other cells types (Haramis et al. 2008). A potential source of NO in LPC-demyelinated slices is the induction of NOS-2 in reactive glial cells, predominantly in microglia (Miron et al. 2010). Immunofluorescence staining for NOS-2 in the cerebellar cultures showed that while only a few cells stained for NOS-2 in control cultures, numerous cells showing a rounded morphology more typical of activated microglia than of activated astrocytes were NOS-2 positive in cultures exposed for 24 h to LPC, or to LPS (1 μg/ml) used as a positive control (Fig. 2d).

Fig. 2

The remyelinating effect of sildenafil in LPC-demyelinated cerebellar cultures is prevented by inhibitors of NO-GC, NOS-2 and PKG activities. The remyelination index was quantified in control or LPC-demyelinated slices treated or not with sildenafil (1 μM) in the presence of: (**a**) the NO-GC inhibitor ODQ (10 μM; $n = 2$); (**b**) the NOS-2 inhibitor L-NNA (100 μM; $n = 3$); (**c**) the PKG inhibitor Rp-8pCPT-cGMP (10 μM; $n = 3$). Results are means ± SEM of 2–3 independent experiments. Two-way ANOVA showed significant differences between treatments in all cases (**a**, $p < 0.001$; **b**, $p < 0.05$; **c**, $p < 0.01$). Bonferroni's post-hoc analysis revealed significant effects of ODQ in vehicle ($^{*}p < 0.05$) and sildenafil-treated slices ($^{***}p < 0.001$) and of Rp-8pCPTcGMP in sildenafil-treated slices ($^{*}p < 0.05$); (d) NOS-2 induction shown by immunostaining (green) in cerebellar slices 24 h after LPC or LPS treatment. DAPI-stained nuclei (blue). Scale bar = 20 μm



Sildenafil Promotes OL Maturation in the SCs of EAE Mice and in LPC-demyelinated Cerebellar Cultures

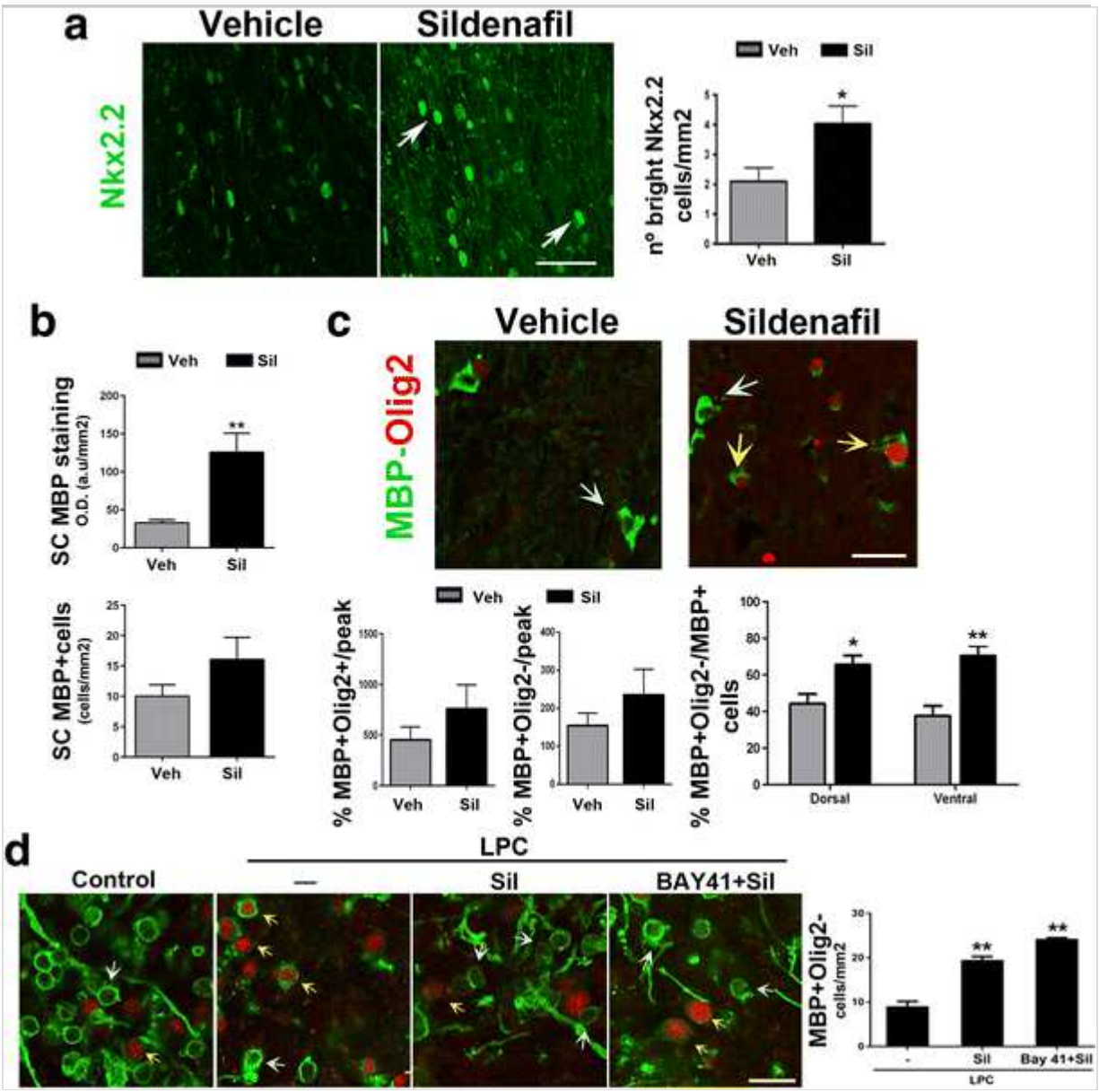
Effects of sildenafil treatment on OL maturation were examined in the SCs of EAE animals treated for 15 days at peak disease by immunostaining for Nkx2.2 and Olig2, transcription factors involved in OL linage specification and differentiation (Fancy et al. 2004; Takebayashi et al. 2000; Zhu et al. 2014), and for the mature OL marker MBP. As shown in Fig. 3a, staining intensity of Nkx2.2+ cells (green) in thoracic longitudinal sections of EAE mice SCs was increased after sildenafil treatment. Quantification of bright

Nkx2.2+ cells (high expression) confirmed a significant increase respect to vehicle-treated animals (Fig. 3a). In agreement with our previous results using Luxol Fast Blue staining (Pifarre et al. 2011), total MBP immunostaining in SC longitudinal sections showed a 3-fold increase in sildenafil-treated animals (Fig. 3b, upper graph). A tendency to increase was also observed in the total number of MBP+ cells/mm^2 (Fig. 3b, lower graph). Double-immunostaining for MBP (green) and Olig2 (red) showed that both MBP+/Olig2+ cells (yellow arrows; left graph) and MBP+/Olig2- cells (white arrows; middle graph) increased respect to the time of initiation of treatment (peak) in vehicle-treated as well as sildenafil-treated animals (Fig. 3c). Further analysis of the percentage of MBP+/Olig2- cells (mature OLs) respect to total MBP+ cells showed a significant increase in dorsal and ventral thoracic SC sections of sildenafil-treated animals compared to vehicle-treated controls (Fig. 3c, right graph). A significant increase in MBP+/Olig2− cells/mm^2 ~~cells~~ was also observed in sildenafil- and BAY41 + Sil-treated LPC-demyelinated cerebellar slices (Fig. 3d, white arrows). Taken together these results support the role of cGMP in OL maturation, in agreement with recent observations in brain of ischemic mice (Zhang et al. 2012) and in OL cultures (Garthwaite et al. 2015), and implicate this mechanism in the remyelinating effect of sildenafil.

Fig. 3

Sildenafil treatment promotes OL maturation in the SC of EAE mice and in LPC-demyelinated cerebellar cultures. (**a**) Representative images of immunostaining for Nkx2.2 (left) and quantification of bright Nkx2.2 cells (high expression, white arrows) (right) showing a significant increase after sildenafil treatment. Values are means ± SEM (n = 3–6 mice/group; *p < 0.05, Student's t-test). (**b**) Total MBP immunostaining in cervical-thoracic longitudinal SC sections of EAE mice treated with sildenafil for 15 days starting at peak disease showed a significant increase (upper graph) and the number of MBP+ cells showed a tendency to increase (lower graph). Values are means ± SEM (n = 3–6 mice/group; **p < 0.01, Student's t-test). (**c**) Double immunostaining for MBP (green) and Olig2 (red) in longitudinal SC sections of EAE mice showed that both MBP+/Olig2+ cells (yellow arrows, left graph) and MBP+/Olig2- cells (white arrows, middle graph) increase respect to the time of initiation of treatment (peak) in both vehicle- and sildenafil-treated animals. Further quantification of MPB+/Olig2- cells relative to total MBP+ cells in dorsal and ventral areas showed a significant increase after sildenafil treatment (right graph) analyzed by two-way ANOVA (p < 0.001) followed by Bonferroni's post-hoc test (*p < 0.05, **p < 0.01). Values are means ± SEM

(*n* = 3–5 mice/group). (**d**) Representative images of double immunostaining for MBP (green) and Olig2 (red) in control and LPC-demyelinated cerebellar cultures treated or not with sildenafil (1 μM) or BAY41 (3 μM) + Silsildenafil (1 μM); MPB+/Olig2+ cells (yellow arrows) and MPB+/Olig2- cells (white arrows); Quantification of total MBP+/Olig2- cells/mm^2 (right) showed a significant increase in LPC-demyelinated slices after cGMP increasing treatments using a one-way ANOVA ($p < 0.01$) followed by Bonferroni's post-hoc test (**$p < 0.01$). Values are means ± SEM of two independent experiments (*n* = 2). Scale bars = 20 μm



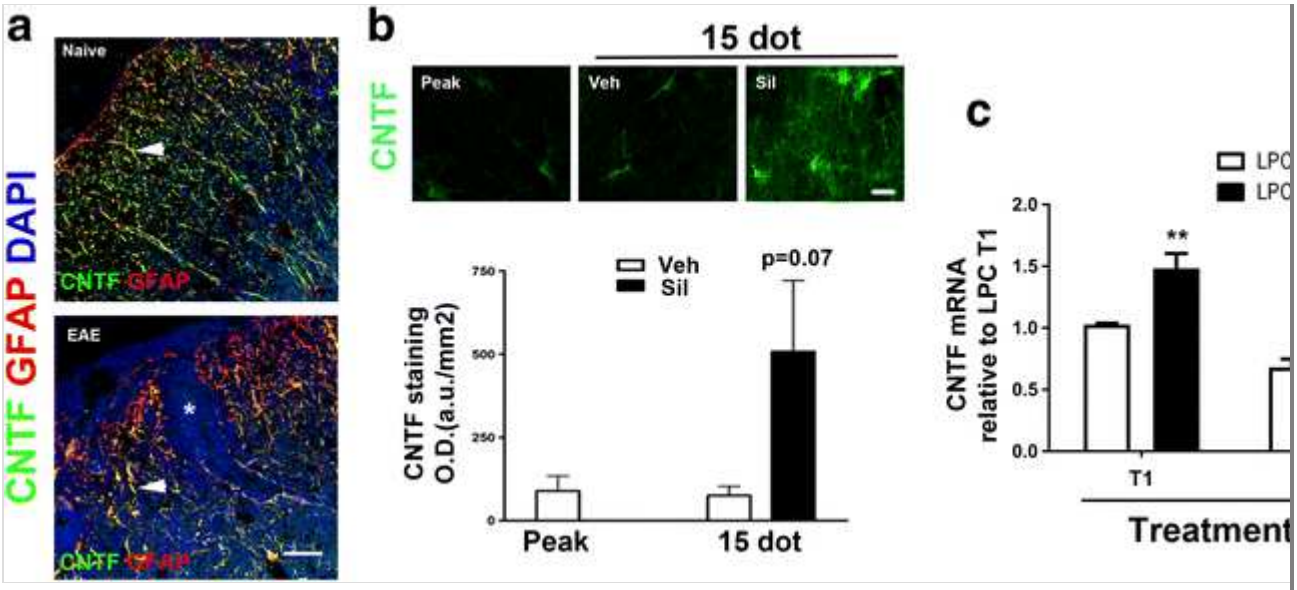
Sildenafil Increases CNTF Expression in SC of EAE Mice and in LPC-demyelinated Cerebellar Cultures

CNTF has a recognized influence on OL differentation (Stankoff et al. 2002). Recent in vitro studies have demonstrated that the NO-cGMP-PKG pathway

up-regulates CNTF expression in astrocytes and that astrocyte-derived CNTF enhances OL differentiation (Paintlia et al. 2013). Using double immunostaining for CNTF and the astrocyte protein GFAP we have observed CNTF expression in resting astrocytes in the SC of naïve animals, as well as in reactive astrocytes in the SC of EAE animals (Fig. 4a, arrowhead). Quantification of CNTF staining intensity in longitudinal slices of SC showed that treatment with sildenafil for 15 days (15 dot) at peak EAE notably enhanced CNTF immunostaining in cells with morphological appearance of astrocytes (Fig. 4b). A significant increase in CNTF mRNA expression was also observed in LPC-demyelinated cerebellar slices 24 h after the first treatment (T1, 9DIV) with BAY41 + Sil but not after the third treatment (T3, 14 DIV) (Fig. 4c). These results suggest that induction and release of CNTF may be a factor contributing to the effect of cGMP-increasing compounds on OL maturation and remyelination.

Fig. 4

Sildenafil treatment increases CNTF expression in the SC of EAE mice and in LPC-demyelinated cerebellar cultures. (**a**) Double immunostaining for GFAP (red) and CNTF (green) in coronal SC cervical sections showing colocalization (arrowheads) in resting astrocytes in naïve animals and in reactive astrocytes surrounding a leukocyte infiltrate (asterisk) in EAE animals. Scale bar = 50 μm; (**b**) Representative images of CNTF immunostaining in longitudinal SC cervical sections of EAE animals treated with vehicle or sildenafil for 15 days (15 dot) starting at peak disease showing increased staining in cells with morphological appearance of astrocytes (scale bar = 20 μm). Quantification of staining intensity values are means ± SEM (n = 5 mice per group, p = 0.07, Student t-Test); (**c**) CNTF mRNA levels assessed by qPCR in LPC-demyelinated cerebellar cultures 24 h after the first (T1, 9 DIV) and third (T3, 14 DIV) treatments with BAY41 (3 μM) + Silsildenafil (1 μM), showing a significant increase in CNTF expression after the first treatment. Values are means ± SEM fold-change in three independent experiments. Two-way ANOVA showed significant differences respect to treatment ($p < 0.05$) and treatment time ($p < 0.001$). Bonferroni's post-hoc analysis revealed significant differences between LPC-demyelinated cultures treated or not with BAY41 + sSil for 24 h after removing LPC (**$p < 0.01$)



Treatments that Increase cGMP Induce a M2-Phenotype in BMDM

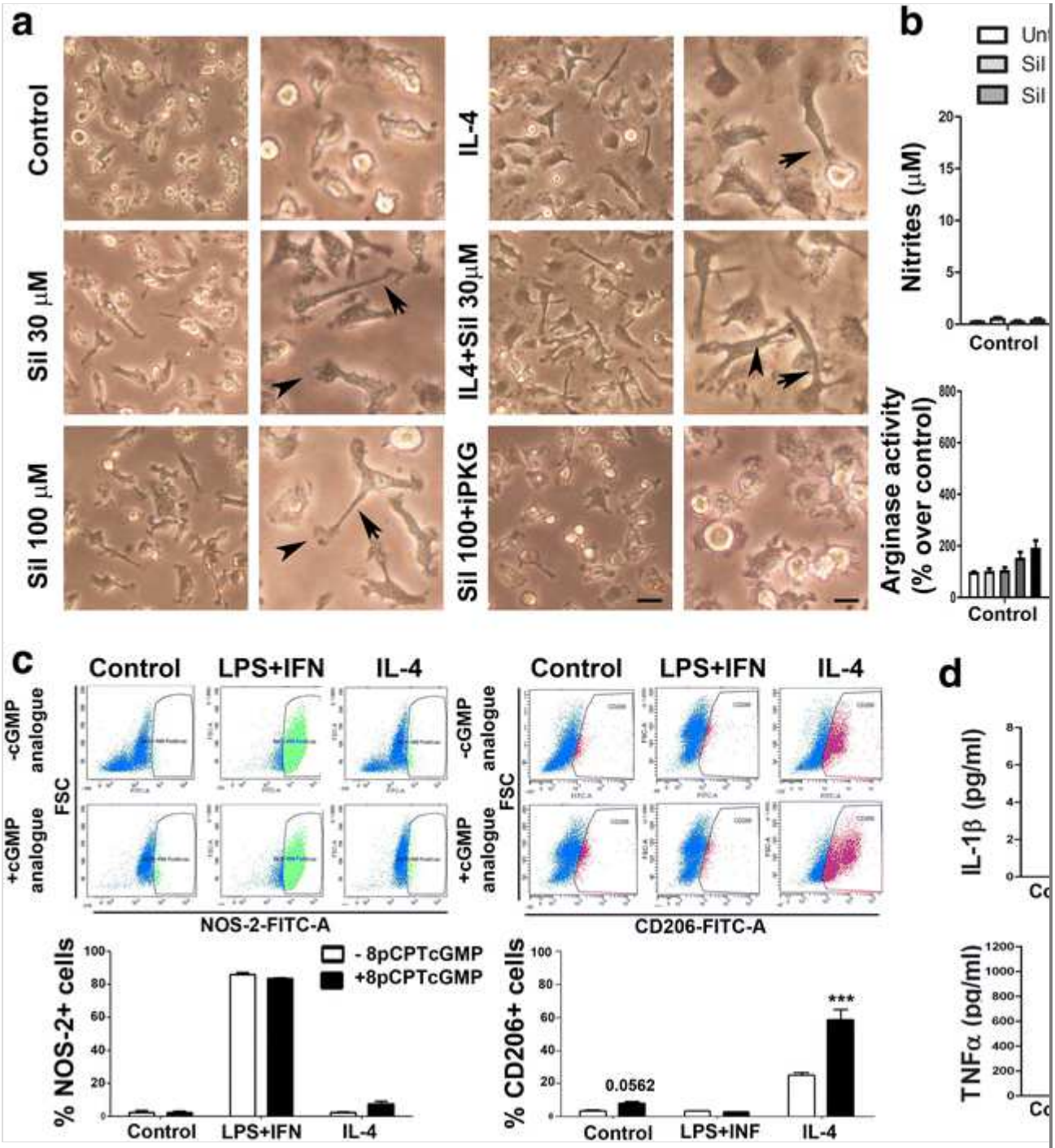
Our previous studies in EAE mice have shown that after sildenafil treatment expression of the classical microglia/macrophage activation marker Iba-1 in the SC was decreased while that of the alternative activation marker YM-1 was increased (Pifarre et al. 2014; Pifarre et al. 2011). In order to directly examine the potential of cGMP-increasing compounds to modulate the inflammatory phenotype in macrophages, we used mice BMDM in primary culture. These cells are readily polarized to a M1 phenotype by incubation with LPS (10 ng/ml) + IFNγ (20 ng/ml) and to a M2 phenotype by incubation with IL-4 (20 ng/ml) (Classen et al. 2009). These conditions were used as controls of phenotypic changes. As shown in Fig. 5a, treatment of BMDM with sildenafil (30–100 μM, 24 h) induced a change in cell morphology from a round/amoeboid shape to an elongated shape (left panels) similar to that induced by treatment with IL-4 (upper right panel). Moreover, the combination of sildenafil (30 μM) with IL-4 (20 ng/ml) appeared to induced further process elongation (middle right panels). The morphological change induced by sildenafil required PKG activity since it was prevented by the PKG inhibitor Rp-8pCPTcGMP (1 μM) (Fig. 5a, lower right panels). We further investigated if sildenafil could promote a shift from a M1 to a M2 phenotype in BMDM by analyzing nitrite concentration in the media as an index of NOS-2 induction (M1 effect), and arginase activity as an index of Arg1 gene expression (M2 effect), in cultures treated with sildenafil alone (1–100 μM) or in combination (added 60 min before) with the inflammatory stimuli for 24 h. As expected, LPS + IFNγ induced a large increase in nitrite concentration in the media while IL-4 or sildenafil had no effect. Sildenafil

did no significantly affect LPS + IFNγ-induced nitrite accumulation (Fig. 5b, upper graph). In contrast, arginase activity showed a tendency to increase at high sildenafil concentrations (30–100 μM) in control and LPS + IFNγ-treated cells and a significant 2–3-fold increase in IL-4-treated cells (Fig. 5b, lower left graph). This later effect was prevented by preincubation with the PKG inhibitor Rp-8Br-PET-cGMP (0,5 μM) (Fig. 5b, lower right graph). These results suggested that sildenafil was promoting a M2 phenotype but not properly inducing a shift from a M1 to a M2 phenotype. In order to confirm the induction of these phenotype changes by cGMP we analyzed by flow cytometry the percentage of cells expressing NOS-2 or the M2 marker CD206 in BMDM treated with the cGMP analogue 8pCPTcGMP (50 μM) alone, or in combination with LPS + IFNγ or IL-4. As shown in Fig. 5c (left), more than 80% percent of the cells expressed NOS-2 in LPS + IFNγ-treated BMDM, whereas the number of cells expressing this M1 marker was minimal in control cells or in cells treated with IL-4. In agreement with the nitrite data, 8pCPTcGMP did not affect the percentage of cells expressing NOS-2 in LPS + IFNγ-treated BMDM (Fig. 5c, left graph). As expected, the number of cells expressing the M2 marker CD206 was increased by treatment with IL-4 but not with LPS + IFNγ (Fig. 5c, right). The cGMP analogue alone had a small effect to the limit of significance in control cells and notably increased the effect of IL-4 (Fig. 5c, right graph). We further analyzed the levels of pro-inflammatory cytokines (IL-1β and TNFα) in the media of BMDM 18 h after treatment with the same stimuli. As expected, an increase in both pro-inflammatory cytokines was detected in the media of LPS + IFNγ-treated- but not IL-4-treated BMDM (Fig. 5d). The cGMP analogue significantly reduced the release of both cytokines induced by the pro-inflammatory stimuli indicating that it has an anti-inflammatory effect. Surprisingly, while the cGMP analogue alone did not significantly increase basal levels of IL-1β, it did significantly induce the release of TNFα in control cells as well as in cells treated with IL-4 (Fig. 5d).

Fig. 5

Sildenafil alters the inflammatory phenotype of mouse BMDM. (**a**, left panels) Treatment of C57BL/6 mice BMDM with sildenafil (30–100 μM) for 24 h induced process elongation (arrow) and lamellipodia formation (arrowheads). This morphological change was similar to that produced by IL-4 (20 ng/ml), a M2 phenotype inducer (upper right panels). Combination of sildenafil with IL-4 appeared to induce further process elongation (middle right panels). The specific protein kinase G (PKG) inhibitor Rp-8pCPTcGMP (1 μM; added 30 min before) prevented the effect of sildenafil (lower right panels); Scale bar:

100 μm left panels and 50 μm right panels; (~~b~~**b**, upper graph) Nitrite accumulation in the media was induced by the M1 phenotype stimuli LPS (10 ng/ml) + INFγ (20 ng/ml) but was not affected by IL-4 (20 ng/ml) or sildenafil (1–100 μM); (**b**, lower graph) Arginase activity showed a tendency to increase at high sildenafil concentrations in control cells and LPS + INF-treated cells and a significant increase at 30–100 μM in IL-4-treated cells. Results are means ± SEM, $n = 3$ (two-way ANOVA $p < 0.001$; Bonferroni's post-hoc analysis $^{***}p < 0.001$). Treatment with the PKG inhibitor Rp-8Br-PET-cGMP (0,5 μM; added 30 min before) prevented the increase in arginase activity induced by sildenafil in IL-4-treated BMDM (lower right). Values are means ± SD (triplicates per condition) in a representative experiment that was replicated with similar results. One-way ANOVA ($p < 0.001$) followed by Bonferroni's post-hoc analysis showed significant differences between IL-4 alone and combined with sildenafil ($^{***}p < 0.001$) and between sildenafil alone and combined with the PKG inhibitor ($^{###}p < 0.001$). (**c**) FACS analysis of BMDM positive for the M1 phenotype marker NOS-2 (left) or the M2 phenotype marker CD206 (right) in untreated (control) or treated with LPS + IFNγ or IL-4 alone, or in combination with the cGMP analogue 8pCPTcGMP (50 μM, added 30 min before); (lower left graph) the significant increase in NOS-2 positive cells induced by LPS + IFNγ ($p < 0.001$, two-way ANOVA) was not affected by 8pCPTcGMP; (lower right graph) CD206 positive cells were significantly increased by IL-4 (two-way ANOVA $p < 0.001$). 8pCPTcGMP alone had a small effect to the limit of significance ($p = 0.056$) and notably potentiated the effect of IL-4 (Bonferroni's post-hoc analysis, $^{***}p < 0.001$). Values are means ± SEM of two (NOS-2) or three (CD206) independent experiments; (**d**) TNFα and IL-1β measured by Luminex technology in the media of BMDM treated for 18 h with LPS + IFNγ, IL-4 or 8pCPTcGMP alone or in combination. Cytokine release was significantly induced by LPS + IFNγ (two-way ANOVA; TNFα, $p < 0.001$, IL-1 β, $p < 0.01$). The cGMP analogue significantly increased TNF release in control and IL-4-treated cells and decreased the induction by LPS + IFNγ of both cytokines (Bonferroni's post-hoc analysis $^{*}p < 0.05$; $^{**}p < 0.01$) Results are means ± SEM, $n = 2$



Sildenafil Increases Myelin Phagocytosis in Microglia/Macrophages in SC of EAE Animals and in BMDM

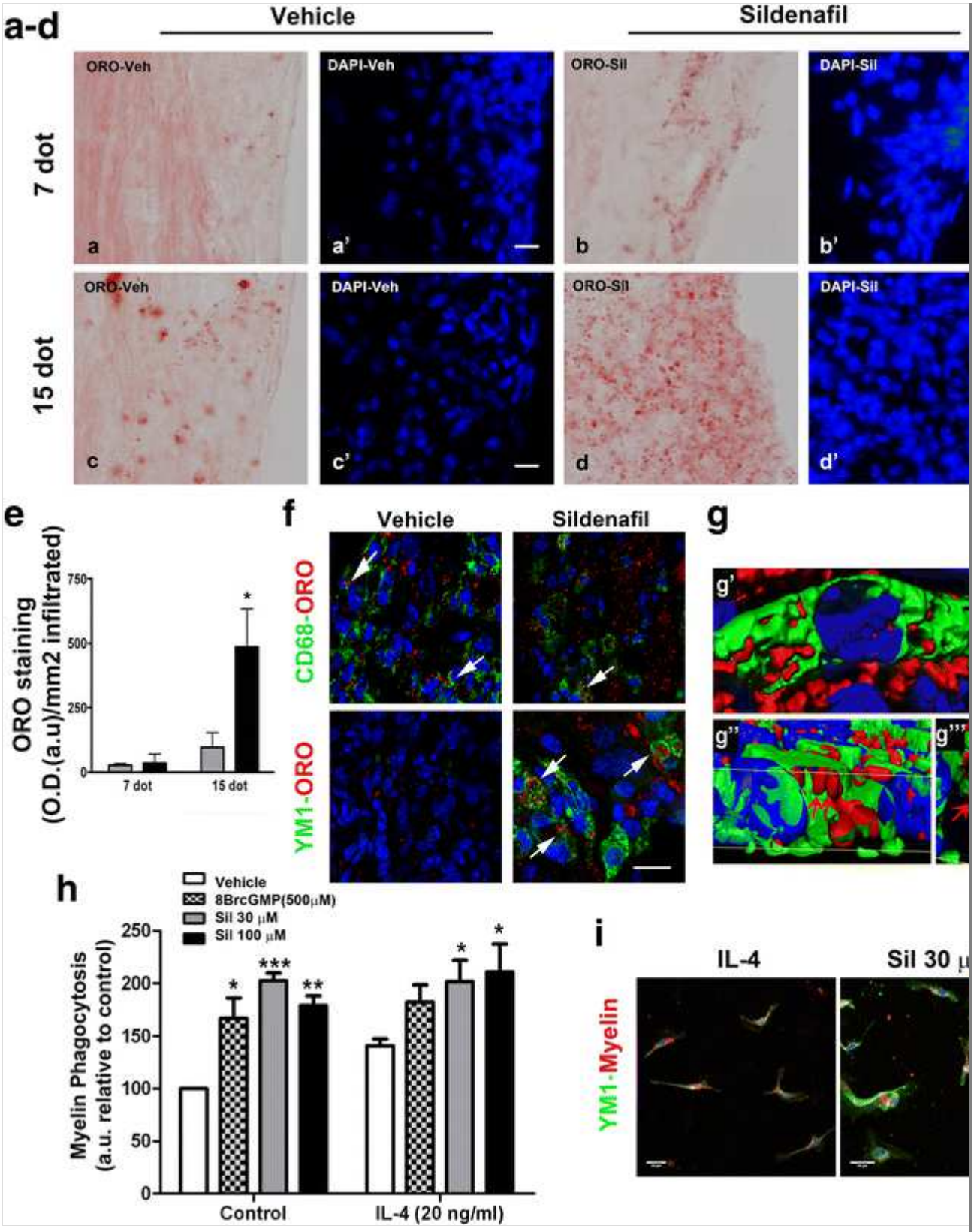
It has been reported that myelin clearance correlates with increased differentiation of OPCs and remyelination (Kotter et al. 2006) and that M2 cells have higher myelin phagocytic activity than M1 cells (Durafourt et al. 2012). To investigate if sildenafil treatment was affecting myelin debris phagocytosis we performed ORO staining of myelin in longitudinal SC sections of EAE mice treated from disease onset when immune cell infiltration and inflammation are maximal. Figure 6a-d show representative

images of ORO staining in infiltrates (cell nuclei stained with DAPI) in animals treated with vehicle (a,c) or sildenafil (b,d) for 7 (a,b) or 15 days (c,d). Quantification revealed that sildenafil significantly enhances ORO staining in an area of similar density of infiltration. A significant increase in vesicular ORO-staining was shown in mice treated with sildenafil for 15 days, but not for 7 days, respect to vehicle-treated controls (Fig. 6e). Double staining for ORO and the macrophage/microglial activation marker CD68 or the M2-phenotype marker YM-1, followed by confocal analysis, showed that ORO staining was low in CD68+ cells present in the SC of vehicle- and sildenafil-treated EAE mice (Fig. 6f, upper images). In contrast, YM-1 + −cells that were only present in sildenafil-treated animals contained numerous ORO stained vesicles (Fig. 6f, lower images, and Fig. 6g). To examine if sildenafil has a direct effect on the myelin phagocytic capacity of macrophages, an *in vitro* myelin phagocytosis assay was performed in mouse BMDM. Cells were treated with IL-4 (20 ng/ml), sildenafil (30–100 μM) or the cGMP analogue 8Br-cGMP (500 μM) alone or in combination. After 24 h, cells were incubated with Dil-labelled myelin (25 μg/ml, 90 min) and myelin uptake was analyzed by flow cytometry. Sildenafil and the cGMP analogue significantly increased myelin phagocytosis both in vehicle- and IL-4-treated cells (Fig. 6h,i). The effect of cGMP-increasing stimuli was of higher magnitude than that of IL-4 and when added in combination with the cytokine their effects were less than additive suggesting that they may share mechanisms.

Fig. 6

Sildenafil treatment enhances myelin phagocytosis by microglia/macrophages in the SC of EAE animals and by BMDM in culture. (**a**–**d**) Representative images of ORO staining in infiltrates (cell nuclei stained with DAPI) of different size in animals treated with vehicle (**a,c**) or sildenafil (10 mg/kg) (**b, d**) for 7 (**a,b**) or 15 (**c,d**) days (dot) starting at disease onset. Scale bar = 20 μm; (**e**) Quantification of ORO staining revealed significant difference between 7 and 15 dot ~~a~~ (two-way ANOVA ##$p < 0.01$) and a significant 5-fold increase of ORO staining in sildenafil-treated compared to vehicle-treated animals at 15 dot (Bonferroni's test, *$p < 0.05$). ~~Values are means ± SEM (*n* = 4–6)~~. Values are means ± SEM (n = 4–6 animals); (**f**) Double staining for ORO (red) and the microglia/macrophage classical activation marker CD68 (green; upper panels) or the alternative activation marker YM-1 (green; lower panels) in longitudinal SC sections showing low ORO staining (arrows) in CD68+ cells in vehicle- and sildenafil-treated mice and strong ORO staining in YM-1+ cells that were only present in sildenafil-treated mice (right lower panel). Scale bar = 20 μm; (**g**) 3D

rendering of microgia/macrophages positive for the M2-phenotype marker YM-1 (green) containing ORO-stained vesicles (red arrows); (**h**) Phagocytosis of Dil-labeled myelin analyzed by flow-cytometry in BMDM treated for 24 h with 8Br-cGMP (500 μM) or sildenafil (30 μM and 100 μM) alone or in combination with IL-4 (20 ng/ml). Values are means ± SEM of three independent experiments. Two-way ANOVA revealed statistically significant differences between treatments ($^{###}p < 0.001$). Bonferroni's post-hoc analysis indicated significant differences of cGMP-increasing treatments respect to control or IL-4-treated cells ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$); (**i**) Double immunostaining for YM-1 and Dil-labelled myelin showing colocalization in IL-4 and sildenafil-treated BMDM



Discussion

Our previous studies in the MOG-induced EAE model showed that daily administration of the cGMP PDE5-I sildenafil at peak disease markedly ameliorates clinical symptoms by preventing axonal loss and promoting remyelination (Pifarre et al. 2011). We additionally showed that

administration of sildenafil from disease onset prevents disease progression, preserves axons and myelin, protects OLs at different stages of differentiation and increases the proportion of axons presenting thinner than normal but compact myelin sheaths suggestive of remyelination (Pifarre et al. 2014; Pifarre et al. 2011). Beneficial effects of sildenafil have been also described in cuprizone-induced demyelination in C57BL/6 mice. In this model, animals receiving sildenafil simultaneously with cuprizone for a 30 day period, did not present the motor limitations shown by the cuprizone control group and their cerebellar myelin and axon ultrastructure was preserved (Nunes et al. 2012). More recently the same group showed that the treatment increases MBP levels and improves myelin structure (Nunes et al. 2016). However, in contrast to our results in the EAE mice these effects were not observed in the cuprizone model when sildenafil treatment was initiated 15 days after inducing neurodegeneration. Demyelination in the cuprizone model is caused by depletion of mature OLs in the brain without involvement of the immune system that occurs in the EAE model (Matsushima and Morell 2001). Thus a possible explanation for the difference in therapeutic effect of sildenafil administration in the two models could be that the protective and remyelinating effects observed in EAE were secondary to sildenafil immunoregulatory actions. In this context, we previously demonstrated that therapeutic administration of sildenafil decreases T cell infiltration and microglial/macrophage activation in the SC while increasing the presence of Tregs (Pifarre et al. 2011). Moreover, analysis of splenocytes from animals treated with sildenafil from disease onset that were re-exposed to MOG in vitro showed down-regulation of Th1/Th2/Th17 responses while Tregs were up-regulated. Sildenafil additionally prevented MOG-specific IgG2b accumulation in serum indicating that it also decreases the B cell response (Pifarre et al. 2014). Altogether, these results demonstrated that sildenafil treatment regulates innate and adaptive immune responses. To investigate if peripheral immunoregulatory effects were determinant in the remyelinating effect of sildenafil in the present work we used cerebellar organotypic cultures demyelinated with LPC, a preparation proven to be an excellent in vitro model to study demyelination-remyelination processes without the interference of the immune system (Birgbauer et al. 2004; Zhang et al. 2011). As previously reported, confocal analysis of control cultured cerebellar slices showed a significant colocalization of immunostaining for myelin (MBP+) and axons (NF200) with a regular distribution of nodes of Ranvier (~~Carsp~~ Caspr+ pairs) and after LPC exposure, myelin debris and naked axons with occasional signs of damage evidenced by the presence of axonal bulbs

could be observed. Furthermore, Caspr clusters associated with axons were drastically reduced indicating disruption of nodes of Ranvier and loss of functionality in nerve impulse (Coman et al. 2006). However, when LPC-demyelinated cultures were treated with sildenafil for 10 days starting 24 h after LPC removal myelin sheaths reappeared and the remyelination index was notably increased. This effect was accompanied by a significant increase in myelin protein expression and recovery of Caspr distribution over axons. These results indicate that sildenafil promotes potentially functional remyelination through direct actions on CNS cells. The combination of sildenafil with a direct activator of NO-GC (BAY41) or with the ionotropic glutamate receptor agonist NMDA that stimulates NO production by the Ca^{2+}-dependent NOS-1 activity in cerebellar granule cells (Garthwaite 1991), induced a similar remyelination as sildenafil alone suggesting the participation of endogenously generated NO in the action of the PDE5-I. This was confirmed by the observation that the remyelinating effect of sildenafil was prevented by inhibitors of NO-GC (ODQ) and NOS (L-NNA) activities. OLs, the cells responsible for myelin production, have been shown to generate cGMP by activation of NO-GC in culture (Benjamins and Nedelkoska 2007; Garthwaite et al. 2015) and in brain slices (Garthwaite et al. 2015; Tanaka et al. 1997; Teunissen et al. 2000). The small amounts of NO produced under physiological conditions by the constitutive Ca^{2+}-dependent NOS-1 in neurons and to a smaller extend in astrocytes (Murphy et al. 1993), have been implicated in physiological processes such as synaptic plasticity, brain development, visual and sensory processing and cerebral blood flow (Contestabile et al. 2012). We hypothesised that in LPC-demyelinated cerebellar slices an aditional source of NO could be NOS-2 induction in reactive glial cells (Murphy 2000). Reactive gliosis is a characteristic feature of neuroinflammation (Aloisi 2001; Dong and Benveniste 2001) and in cerebellar organotypic cultures soon after LPC-induced demyelination maximal reactivity of both microglia and astroglia has been observed (Birgbauer et al. 2004; Miron et al. 2010). Using NOS-2 immunostaining, we showed 24 h after LPC-induced demyelination a notable increase in NOS-2+ cells presenting a rounded morphology more typical of reactive microglia than of reactive astrocytes that are highly ramified. An excess NO production after NOS-2 induction has been traditionally associated to cell damage (Brown and Neher 2010), however our results suggest that NO produced by NOS-2 in demyelinated cerebellar slices may have a beneficial effect by stimulating cGMP-dependent remyelination. In support of this assumption, recent studies in the cuprizone-induced demyelination model have shown that

NOS-2$^{-/-}$ mice suffer more severe inflammation and damaged myelin structure than wild type mice (Raposo et al. 2013).
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Previous studies have demonstrated that the NO-cGMP pathway promotes protection against OL apoptotic death *in vitro* (Benjamins and Nedelkoska 2007). In agreement with this, we previously demonstrated protection of immature and mature OLs from apoptotic cell death in the SC of EAE mice treated with sildenafil from disease onset (Pifarre et al. 2014). Here we have additionally shown that treatment with sildenafil to EAE mice for 15 days starting at peak disease increased immunostaining for the transcription factor Nkx2.2, a marker of activated and proliferating OPCs (Fancy et al. 2004). Furthermore, double immunostaining for the transcription factor Olig2, expressed from early to advanced OL maturation stages (Fancy et al. 2004), and for MBP, a mature OL marker, indicated that sildenafil promotes the final OL differentiation stage (MBP+/Olig2- cells) both in SC of EAE mice and in demyelinated cerebellar cultures. These results support the implication of OL maturation in the remyelinating effect of the PDE5-I. Increased levels of the mature OL marker GST-pi have been also observed in the cuprizone demyelinating model after sildenafil treatment (Nunes et al. 2016). A role for cGMP-mediated pathways in OL differentiation and maturation is also supported by the increase in the number of nestin-neural stem cells differentiating into OPCs and mature OLs (CNPAse + and CC1+) observed in the corpus callosum and striatum of ischemic mice treated with sildenafil (Zhang et al. 2012). Furthermore, recent studies in cultures of rat cortex OLs have shown that increasing cGMP by exposure to NO in the presence of a PDE-I promotes the morphological differentiation of these cells (Garthwaite et al. 2015).

PKG is a major target of cGMP and the two isoforms of this enzyme, PKGI and PKGII, are expressed in the CNS and mediate important regulatory actions of cGMP in neurons, astrocytes and OLs (Feil et al. 2005). Our results using the specific PKG inhibitor Rp-8pCPTcGMP indicate that this activity is mediating sildenafil-induced remyelination in cerebellar demyelinated cultures. Importantly, PKG is known to phosphorylate and activate ERK and Akt (Das et al. 2008; Mejia-Garcia et al. 2013), two kinases involved in OL protection and differentiation. Phosphorylation by Akt inactivates GSK3β, an effect reported to promote survival, proliferation and maturation of OPCs and remyelination *in vivo* (Azim and Butt 2011; Luo et al. 2014). Furthermore,

Akt downstream effector mTOR appears to be critically important for OPC differentiation and myelination both *in vitro* and *in vivo* (Gaesser and Fyffe-Maricich 2016; Narayanan et al. 2009; Tyler et al. 2009). Apart from direct effects on OL maturation by potentiating cGMP-mediated pathways, sildenafil could also exert indirect effects by inducing release of neurotrophic factors in neighbor cells. We have previously shown that sildenafil treatment from disease onset potentiates expression of BDNF in immune and CNS cells in the SC of EAE mice (Pifarre et al. 2014). BDNF is involved in neuronal survival, differentiation, plasticity and axonal growth, and also supports OL proliferation and axon remyelination (De Santi et al. 2009; Weishaupt et al. 2012). Furthermore, studies in mice with selective removal of BDNF expression in astroglial or immune cells have demonstrated the importance of BDNF expression in these cells as a protective mechanism in EAE (Lee et al. 2012; Linker et al. 2010). CNTF is another promyelinating factor with a recognized influence on OL maturation (Stankoff et al. 2002). *In vitro* studies have demonstrated that CNTF expression in astrocytes is up-regulated by the NO-cGMP-PKG pathway and that astrocyte-derived CNTF enhances OL protection, differentiation and myelin protein production (Modi et al. 2013; Paintlia et al. 2013). In agreement with this, we have shown here that CNTF immunoreactivity notably increases in cells with astrocyte morphology in the SC of EAE animals treated with sildenafil. We have also observed that cGMP increasing treatments induce CNTF mRNA expression, but not that of BDNF (not shown) in LPC-demyelinated cerebellar cultures. Taken together these results suggest that enhancement of CNTF production in astrocytes together with BDNF production in infiltrating immune cells may contribute to sildenafil-induced OL protection and maturation in EAE mice.

Recent studies have shown that after LPC-induced demyelination in mice corpus callosum, a switch from a M1 to a M2 phenotype occurs in resident microglia and peripherally-derived macrophages at the initiation of remyelination (Miron et al. 2013). In the same work it was demonstrated that OL differentiation was impaired *in vivo* following M2 cell depletion at the lesion site and was enhanced *in vitro* by the addition of M2 cell conditioned media, suggesting that polarization of microglia/macrophages to the M2 phenotype promotes remyelination. Our previous results showed that treatment with sildenafil to EAE mice from disease onset increased the expression of the alternative activation marker YM-1 in the SC (Pifarre et al. 2014). Here we have confirmed that cGMP stimuli alter the inflammatory phenotype of BMDM. In these cells, sildenafil induced a morphological

change from a rounded/amoeboid type to an elongated shape similar to that induced by IL-4 and notably potentiated the increase in arginase activity induced by the cytokine, suggesting that it had a pro-M2 phenotype effect. In contrast, sildenafil did not significantly inhibit the accumulation of nitrites in the media of LPS + INFγ-treated cells. In agreement with these results, a cGMP analogue potentiated the IL-4-induced increase in the number of cells expressing the M2 marker CD206 but had no effect on the number of cells expressing the M1 marker NOS-2 in response to LPS + INFγ. However, it did have an inhibitory effect on the release of the pro-inflammatory cytokines IL-1 β and TNF-α. Decreased pro-inflammatory cytokine release has been previously demonstrated in different populations of rodent macrophages including BMDM in response to the particulate GC agonist atrial natriuretic peptide A (ANP) (Kiemer and Vollmar 2001). However, in contrast to our results NOS-2 expression was also inhibited by ANP-stimulation of cGMP formation. Increasing cGMP by inhibiting its degradation was also found to decrease the release of TNF-α induced by LPS in isolated human monocytes (von Bulow et al. 2005). Unexpectedly, we also observed that the cGMP analogue alone induced a significant TNF-α production in unpolarized and M2-polarized BMDM. Up-regulation of TNF-α synthesis by cGMP has been reported in other macrophage populations (Arias-Diaz et al. 1995; Renz et al. 1988), but the mechanism and significance of this effect is unknown at present. Taken together our results in BMDM indicate that an increase in cGMP can induce or potentiate a M2 anti-inflammatory phenotype but not properly induce a shift M1-M2.

An important function of microglia/macrophages in neurodegenerative diseases is the clearance by phagocytosis of apoptotic cells and of myelin debris produced as a consequence of the damage (Ransohoff and Perry 2009). In demyelinating diseases, OL differentiation into mature myelinating cells is not observed in the demyelinated areas (Wolswijk 2000) and *in vitro* and *in vivo* studies have shown that the presence of myelin impairs OLs maturation (Robinson and Miller 1999). Our studies using ORO staining in the SC of EAE mice demonstrate that sildenafil treatment induces a notable increase of lipid vesicles in infiltrates compared to vehicle-treated controls indicating that the drug promotes myelin phagocytosis. Furthermore, confocal analysis of double staining for ORO and M1 or M2 markers indicated that while in vehicle-treated animal’s cells engulfing ORO-stained vesicles were largely M1 microglia/macrophages, in sildenafil-treated animals a much larger proportion of lipid-rich vesicles were inside M2 cells. In agreement with this,

treatments with sildenafil or a cGMP analogue promoted myelin phagocytosis by BMDM. Our results in EAE animals and in BMDM are in line with recent reports indicating that M2 cells have higher myelin phagocytic activity than M1 cells (Durafourt et al. 2012) and that M2 cell polarization contributes to efficient remyelination (Miron et al. 2013).

Effects of sildenafil on the blood-brain barrier (BBB) and on cerebral blood flow could also contribute to its neuroprotective and regenerative actions in EAE animals. Using an in vitro BBB model (Wong et al. 2004) showed that increasing cGMP enhanced trans endothelial electrical resistance and reversed the increased permeability of the monolayers induced by IL-1β, IFN-γ, and LPS. Moreover, it has been reported that PDE5 inhibitors enhance endothelial cell migration, growth, and organization into capillary-like structures in vitro, as well as angiogenesis in vivo (Pifarre et al. 2010; Pyriochou et al. 2007; Zhang et al. 2003). Increased vascularization together with vasodilation has been correlated with sildenafil-induced reduction in neurological damage and enhanced functional recovery in a stroke model (Ding et al. 2008). MS patients show evidence of reduced cerebral blood flow in grey and white matter and seem to have an increased risk for ischemic stroke (D'Haeseleer et al. 2011). Interestingly, an fMRI study in a small cohort of secondary progressive MS patients that had been prescribed sildenafil as a treatment for erectile dysfunction exhibited increases in grey and white matter perfusion (Manson et al. 2005).

In summary, results shown here indicate that treatment with the PDE5-I inhibitor sildenafil promotes remyelination in CNS demyelinated tissue by potentiating the cGMP-PKG pathway in different cell types. We previously demonstrated that sildenafil treatment to EAE animals at disease onset protects immature and mature myelinating OLs, we now additionally show that the sildeanfil promotes OL maturation in the SC of EAE animals and in LPC-demyelinated organotypic cultures. This effect can be exerted by directly increasing cGMP in OLs (Garthwaite et al. 2015) or indirectly by the induction and release of the oligodendrocyte differentiation factor CNTF in neighboring astrocytes shown here. Sildenafil induction of phenotypic changes in microglia/ macrophages can also be an important mechanism contributing to cell protection and regeneration of demyelinated tissue. We previously showed that sildenafil treatment increased the expression of the microglial/macrophage M2 phenotype YM1 in spinal cord of EAE animals (Pifarre et al. 2014). We have now confirmed in cultured macrophages that

increasing cGMP promotes a M2 anti-inflammatory phenotype and we further show in vitro and in SC of EAE animals that sildenafil treatment increases the myelin phagocytic activity of these cells, an effect recognized to contribute to OL precursor differentiation (Kotter et al., 2016) and efficient remyelination (Miron et al. 2013). Results presented here together with our previous demonstration in the EAE model that sildenafil, at a dose within the range of those demonstrated to be safe in clinical assays for chronic administration in humans (Potter et al. 2012; Sommer et al. 2007), down-regulates the adaptive immune responses and induces BDNF expression in immune and neural cells in parallel to axon and OL protection, and also that human T regulatory cell activity is potentiated by sildenafil in vitro (Pifarre et al. 2014), further support the therapeutic potential of PDE5-Is for treatment of MS.

AQ2

AQ3

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving animals were approved by the Autonomous University of Barcelona Animal and Human Experimentation Ethics Committee in accordance to the ethical standards of the Federation of European Laboratory Animal Science Associations (FELASA).

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