Universitat Autònoma de Barcelona

Mechanisms involved in the remyelinating effect of sildenafil

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Dedicada mi familia

Mis padres, mi hermano y mi tía

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ABSTRACT

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Multiple Sclerosis (MS) is a chronic autoimmune demyelinating disease of the central nervous system characterized by a coordinated inflammatory attack on the myelin sheaths with ensuing damage to the underlying axons. Using myelin oligodendrocyte glycoprotein (MOG)-induced chronic experimental autoimmune encephalomyelitis (EAE) as a MS model, it has been previously demonstrated that daily administration of the PDE-5 inhibitor sildenafil starting at peak disease rapidly ameliorates clinical symptoms whereas administration at the onset of symptoms prevents disease progression. These beneficial effects involved down-regulation of adaptive and innate immune responses and protection of axons and oligodendrocytes and promotion of remyelination. The aim of this work was confirm the remyelinating potential of sildenafil treatment and investigate mechanisms involved in this effect in CNS cells. Results show that sildenafil induces remyelination in EAE mice even when the administration of the drug starts during the chronic stage of the disease. Sildenafil also stimulates remyelination in cerebellar organotypic cultures demyelinated with lysophosphatidylcholine and this effect is prevented by inhibitors of nitric oxide-dependent guanylyl cyclase (NO-GC), NO synthase type 2 (NOS-2) and cGMP-dependent protein kinase (PKG), indicating the involvement of the endogenous NO-cGMP-PKG pathway. Maturation of oligodendrocytes as a potential mechanism implicated in the remyelinating effect of sildenafil was investigated by immunostaining for transcription factors involved in different stages of oligodendrocyte development. Results in the EAE model show that sildenafil treatment increases oligodendrocyte precursor cells (OPCs; Nkx2.2+ cells) and promotes the final stage of oligodendrocyte maturation ($olig2^{-}/MBP^{+}$ cells). These later result was confirmed in LPC-demyelinated cerebellar slices treated with cGMP increasing compounds. This work also shows that expression of the neurotrophic factor CNTF, that has been implicated in oligodendrocyte maturation, is increased in astroytes of sildenafil-treated EAE mice spinal cord, as well as in cerebellar slice cultures. Results also show that cGMP-increasing treatments alter expression of inflammatory phenotype markers (COX-2 and Arg-1) in microglia in demyelinated slice cultures. The potential of cGMP-increasing treatments for regulating the inflammatory phenotype of monocytes was confirmed in bone marrow derived macrophages (BMDM). In these cells sildenafil treatment induces arginase activity and potentiates the effect of IL-4 suggesting the promotion of an M2 phenotype. Analysis by flow cytometry of BMDM confirmed that cGMP augments the number of cells expressing an M2 phenotype marker (CD206). This work further demonstrates that sildenafil significantly increases the myelin phagocytic capacity of microglia/macrophages in EAE mice and in BMDM. Taken together these data suggest that promotion of oligodendrocyte maturation, growth factor expression, modulation of the inflammatory process and clearance of myelin debris may be relevant mechanisms involved in sildenafil enhancement of remyelination in demyelinated tissue.

ABREVIATIONS

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Abreviation	Name
8BrcGMP	8-Bromoguanosine 3′,5′-cyclic monophosphate sodium salt
8pCPTcGMP	8-(4-Chlorophenylthio)-guanosine 3´,5´-cyclic monophosphate sodium salt
ANP	Atrial natriuretic peptide
BAY41	BAY 41-2272
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BME	Basal medium containing Earle's salt
BNP	Brain natriuretic peptide
BSA	Neonatal Goat serum
cGMP	Cyclic Guanosine Monophospate
CNP	C-type natriuretic peptide
CNPase	Cyclic Nucleotide Phosphodiesterase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CPB	Cytometry permeabilization buffer
dbcGMP	dibutyryl-cGMP
Dil	1,1"-diotadecyl-3,3,3',3'-tetramethyl-lindocarbocyanide perchlorate
DMEM	Dubelco's Modified Eagle medium GlutaMax

Abreviation	Name
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	TRIS buffer solution
FBS	Fetal Bovine Serum
GC	Guanylyl cyclase enzymes
GFAP	Glial fibrillary acidic protein
Glucose	Glucose
HBSS	Hank's buffered salt solution
HS	Heat Inactivated horse serum
Iba-1	ionized calcium-binding adaptor protein-1
IFNγ	Recombinant Mouse Interferon γ
IGF-1	Insuline-like growth factor
IL-4	Recombinant Murine Interleukin-4
INFγ	Interferon gamma
IS	Immune system
L-Glutamine	L-Glutamine 200mM
L-NNA	L-NNA
LPC	L-α-Lysophosphatidylcholine from egg yolk
LPS	Lipopolysaccharide
LPS	Lipopolysaccharides from Salmonella enterica

Abreviation	Name
MAG	Myelin Associated Glycoprotein
MBP	Myelin Basic Protein
M-CSF	Recombinant Macrophage Colony Stimulating Factor
MHC	Major histocompatibility complex
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
NGF	Nerve growth factor
NGS	Neonatal Goat serum
NMDA	N-methyl-d-aspartate
NMDA	N-methyl-D-Aspartate
NO	Nitric oxide
NO-GC	NO-sensitive guanylyl cyclase
NOS	Nitric oxide synthases
NOS-1	Neuronal NOS
NOS-2	Inducible NOS
NOS-3	Endothelial NOS
NP	Natriuretic peptide
NPR	Natriuretic peptide receptor
ODQ	ODQ
Ols	Oligodendrocytes
ON	overnight
OPCs	Oligodendroglial precursor cells
ORO	Oil Red-O
PBS	Bovine serum albumim

Abreviation	Name
PDE5	Phosphodiesterase type 5
PDEs	Phosphodiesterases
PDGFαR	Platlet Derived Growth Factor α Receptor
Pen/Strep	Penicillin-Streptomycin
PFA	Paraformaldehyde
pGC	Particulate guanylyl cyclases
PKG	cGMP dependent protein kinases
PLP	Proteolipid Protein
PP-MS	Primary Progressive Multiple Sclerosis
RI	Remyelination index
RIPA	RIPA Lysis Buffer
ROCK	Rho-Associated-Kinase
ROS	Reactive oxygen species
Rp-8pCPT-cGMP	8-[(4-Chlorophenyl)thio]-guanosine-cyclic 3',5' monophosphate
RR-MS	Relapsing-Remitting Multiple Sclerosis
s.c	sub-cutaneous
SC	Spinal cord
Sil	Sildenafil
TGF-β	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
Tris	Phosphate buffer saline
U0126	1,4-Diamino-2,3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene
WB	Western Blot
Ym-1	Chitinase-like 3

INTRODUCTION

INTRODUCTION

1. Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic, inflammatory and demyelinating disease of the CNS (Herz et al. 2010). MS etiology and the initiation of autoimmunity that comes with it, are still unknown (Nave 2010). Symptoms of MS include limb weakness, numbness, blurred or double vision and ataxia. Some advanced cases present cognitive impairments and memory loss (Hauser and Oksenberg 2006). In MS the immune system reacts against central nervous system (CNS) myelin components, initiating a detrimental inflammatory cascade that leads to demyelination and axonal and neuronal degeneration which is the principal anatomical correlate of progressive clinical deterioration. Axon protection can be achieved directly by intervention on the mechanisms by which axons are injured or degenerate and can also be achieved by immune-modulatory therapies and by promotion of remyelination. MS research has mainly focused on the immunological aspects of the disease and has translated into the development of highly effective immune-modulatory therapies to control the initial relapsing-remitting phase of MS, however the secondary progressive phase, in which there is continual atrophy of demyelinated axons, remains largely untreatable. (Garg and Smith 2015, Nave 2010, Peterson and Fujinami 2007).

2. Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) has been extensively used as an animal model of MS since it shares with the human autoimmune disease the presence of inflammatory infiltrates in the CNS parenchyma, demyelination and axonal loss, predominantly in the spinal cord, and paralysis (Friese et al. 2006). Like MS, EAE seems to be initiated by myelin antigen-

specific CD4+ T-lymphocyte infiltration into the CNS. CD4+ cells together with infiltrated macrophages, dendritic cells, and resident microglia constitute the ultimate effector cells of neuroinflammation, progression of demyelination, and axonal damage (Bynoe et al. 2007, Friese et al. 2006). In contrast, accumulating evidence indicates that local astroglial activation is neuroprotective in EAE. Using transgenic mice with targeted ablation of proliferating scar-forming astrocytes it has been shown that severity of EAE is enhanced and that leukocyte and macrophage entry into the CNS parenchyma is significantly increased (Voskuhl et al. 2009). Recent evidence additionally indicates that demyelination and oligodendrocyte degeneration follows inflammation-induced astrocyte dysfunction (Sharma et al. 2010). High levels of inflammatory mediators are secreted by infiltrating immune cells and resident CNS cells, which is characteristic of the inflammatory environment during disease (Raivich and Banati 2004).

EAE can be classified as passive (when induced by introducing MBP-reactive T-cells into the animal) or active (when induced by immunization with CNS tissue or myelin peptides). Active EAE can also be divided into two different types according to the mouse strain and peptide used to induce it. In the SJL/J mouse strain active induction with PLP develops a relapsing–remitting form, while in the C57BL/6 mouse, immunization with $MOG_{35–55}$ in CFA induces a chronic-sustained form of EAE (Constantinescu et al. 2011, McCarthy et al. 2012). Symptoms are characterized by ascending hind limb paralysis that is associated with inflammation and demyelination of axonal tracks.

3. Neuroinflammation

3.1. Immunity in the Central Nervous System

The CNS has been classically considered an immunologically "privileged" organ because it was thought to be isolated from the immune system (IS) and excluded from its surveillance. Nowadays, it is well accepted that the "immune privilege" of the CNS is an active process, the immune response is present but restricted and involves a closely regulated inter-communication between CNS resident cells and the IS which allows control of immune-mediated inflammation and related secretion of potentially damaging molecules that could have devastating consequences in this essential organ with limited capacity for regeneration (Nakamizo et al. 2003).

The Blood Brain Barrier

The most important feature in CNS immune privilege is the presence of the Blood-Brain Barrier (BBB), a structure formed by blood vessel endothelial cells joined by tight junctions, the basal lamina in which pericytes are embedded and the end-feet of astrocytes, in conjunction with intra- and extracellular enzymes that represent a metabolic barrier. The BBB exerts bi-directional control over the transcellular passage of substances such as regulatory proteins, nutrients and electrolytes, maintaining the optimal ionic composition for axonal transmission and synaptic signaling. The BBB also avoids the entry of potentially harmful or toxic substances and supervises the infiltration of IS cells by expression of adhesion molecules in endothelial cells. However, in neuropathological conditions activating signals are produced by resident (glial) cells that can facilitate inflammation or promote recovery, but uncontrolled neuroinflammation can induce secondary injury (Abbott et al. 2010).

In addition to the BBB, other factors also contribute to restricting the immune response in the CNS, like the absence of a conventional lymphatic system and the failure of antigen presenting cells (APCs) to migrate to lymph nodes along perivascular lymphatic drainage pathways in the healthy brain, although they might do so in the inflamed CNS. In addition, the highly regulated entrance of immune cells by the presence of immune-inhibitory factors constitutively produced by neurons, and the very low expression of activating and co-stimulatory molecules, are also important factors that contribute to minimize inflammatory responses in the CNS (Griffiths et al. 2009, Weller et al. 2010). Nevertheless, immunological reactions do occur in the CNS in response to infections and in immune-mediated disorders such as multiple sclerosis (MS).

Resident immune effector cells in the CNS

The functions of immune surveillance and differentiation between "self" and "non-self" antigens in non-CNS tissue, provided by neutrophils, dendritic cells, macrophages and natural killer cells in the periphery, are in the CNS attributed to resident glial cells, astrocytes and microglia (Veerhuis et al. 2011). After a CNS injury, glial cells show phenotypic changes referred to as reactive gliosis, one of the most important features in neuroinflammation (Aloisi 2001, Dong and Benveniste 2001). Glial activation plays a crucial role in acute and chronic inflammatory responses and reactive glia has been found in brains after traumatic injury, ischemia, infections, neuropathic pain, seizures, autoimmune inflammatory diseases, glaucoma, leukodystrophies, edema, psychiatric disorders, brain tumors and also in neurodegenerative diseases (Ghafouri et al. 2006, Kim Y. S. and Joh 2006, Sofroniew and Vinters 2010, Streit et al. 2005, Wyss-Coray 2006)

Astrocytes

Astrocytes are the glial cells responsible of the CNS structure support, regulate blood flow, form and maintain the BBB and also mediate its permeability. Astrocytes regulate transport of ions, nutrients and toxins. These cells also regulate synapses and nerve impulse transmission (Ludwin et al. 2016). After a brain injury, astrocytes are recruited to the lesion site and help forming a glial scar that promotes healing after the lesion (Lee et al. 2015). Astrocytes express neurotrophic and growth factors such as brain derived neurotrophic factor (BDNF), ciliary neurotrofic factor (CNTF), transforming growth factor beta (TGF-β) and insulin-like growth factor (IGF-1). Astrocytes are also able to express pro and anti-inflammatory cytokines (Ludwin et al. 2016, Peterson and Fujinami 2007).

Microglia

Microglial cells are considered the resident macrophages of the CNS, descendants of the monocytic lineage invade the central nervous system early during ontogenesis (Fedoroff and Hao 1991, Hailer 2008). In resting conditions, microglial cells present a ramified morphology with an elaborate tertiary and quaternary branch structure. Their prolongations cover a space of 30-50 µm not overlapping each other, expanding throughout the CNS (Raivich et al. 1999). Although in their apparent resting state, microglial cells are greatly active, continually surveying their microenvironment with extremely motile processes and protrusions poised to rapidly respond to environmental changes (Nimmerjahn et al. 2005). CNS injury provokes immediate and focal activation of microglia, which is thought to occur before astrocyte activation, switching their behavior from resting to defending cells in the injured site, with a different response depending on the stimulation provided (Town et al. 2005).

Reactive microglial cells proliferate, retract its ramifications and acquire amoeboid morphology, extend new pseudopodia that enable active migration and phagocytic capacity for removing dead cells and cell debris. These characteristics together with their antigen presenting capacity in MHC-II, make them the antigen-presenting cells of the CNS initiating the adaptive immune response (Fig.2) (Aloisi 2001, Hailer 2008, Kim S. U. and de Vellis 2005).

Microglia/macrophage activation phenotype

Activation of microglia in response to brain injury involves a continuum spectrum of phenotypes that share characteristics with their homologue IS cells, the macrophages. The reactive microglia/macrophage phenotype oscillates between two end-points depending on the stimulatory environment, one corresponds to the adaptive activation with release of pro-inflammatory cytokines that is associated with capacity for stimulation of Th1 and Th2 subpopulations (M1 or classically activated phenotype), and the other to innate activation with a phagocytic phenotype and anti-inflammatory cytokine secretion (M2 o alternativelly activated). In vitro, the M1 phenotype is induced in the presence of pro-inflammatory cytokines and LPS and the M2 phenotype in the presence of anti-inflammatory cytokines such as IL-4. Recent studies have identified M2 phenotype subtypes that have been classified according to specific protein expression and possible functions. M2a has been associated with regeneration and repair, M2b is considered immunoregulatory and M2c immunosuppressive (Chhor et al. 2013, Miron and Franklin 2014, Mosser and Edwards 2008, Ransohoff and Perry 2009). Microglia/macrophage phenotypes can be recognized by changes in the expression of specific molecules. NOS-2, TNFα, COX-2, and IL-1β are characteristic molecules of a M1 phenotype while arginase-1, CD206, Ym-1 and FIZZ-1 are considered M2-phenotype markers

(Chhor et al. 2013, Fernando et al. 2014, Gensel and Zhang 2015). However, recent studies *in vitro* and *in vivo* have revealed that reactive microglia/macrophages are able to co-express M1 and M2 markers. *In vivo* experiments have shown that treatent of mice with LPS increases pro-inflammatory cytokines including IL-1β, but also IL-4R and further treatment with IL-4 induced the expression of Arg-1 as well as IL-1β, and this unique phenotype was able to promote neurite growth and spinal cord injury recovery (Fenn et al. 2014). Furthermore, macrophages treated with IL-4 *in vitro,* were shown to produce IL-6 while co-expressing the M2 characteristic molecule CD206, and the co-expression of IL-6 did not affect immunosuppressive properties of the M2 phenotype macrophages (Casella et al. 2016). Other studies aimed at characterizing specific molecules to delineate and identify microglia /macrophage phenotypes have also shown co-expression of the anti- and pro-inflammatory phenotype markers (Chhor et al. 2013, Gensel and Zhang 2015).

Myelination/Remyelination

Myelination is an ordered and rapid process the uninjured, healthy adult brain. New myelin is continually generated along with new oligodendrocytes in active myelination, which perform myelin maintenance with subtle remodeling of specific areas (Bercury and Macklin 2015).

Demyelination is the pathological process where myelin sheaths are lost from around axons impairing nerve impulse conduction (Franklin and Ffrench-Constant 2008). There are two major causes of primary demyelination in the CNS, the first is due to genetic abnormalities that affect glia, such as leukodystrophies, and the second is due to inflammatory damage to myelin and oligodendrocytes, which can be observed in neurological diseases such as

MS and other neuroinflammatory disorders (Franklin and Ffrench-Constant 2008).

Remyelination is the process responsible of restoring entired myelin sheaths around demyelinated axons, reinstating saltatory conduction and ameliorating functional deficits (Franklin and Ffrench-Constant 2008). Extensive data indicate that, after demyelination, remyelinated internodes are thinner and shorter than, but after late time of recovery remyelinated fibers may have similar internode length and thickness to developmentally myelinated axons (Bercury and Macklin 2015).

Oligodendrocytes

Oligodendrocytes (OLs) are the CNS glial cells responsible for the production of the myelin sheath that surrounds axons isolating the electrical impulse in order to gain speed and efficiency (Nave 2010). OLs can have over 50 extensions reaching axons, and can myelinate them simultaneously (Copray et al. 2006, Liu et al. 2007, Nave 2010). OLs can interact with surrounding cells that influence OLs migration, differentiation and myelination (Kimelberg 2010, Li et al. 2016, Talbott et al. 2005). OL proliferation and differentiation is regulated by cytokines and growth factors (Nave 2010), such as BDNF (De Santi et al. 2009, Weishaupt et al. 2012) and CNTF (Stankoff et al. 2002). OLs express characteristic transcription factors that allow determination of their differentiation or maturation stage, such as Nkx.2, an early marker of OL precursor differentiation, and Olig1 and Olig2, expressed during early to late differentiation stages. Mature OLs express myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), myelin associated glycoprotein (MAG) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) among others (Copray et al. 2006, Liu et al. 2007, Meffre et al. 2015, Miron et al. 2010, Nishiyama et al. 2009, Zhang H. et al. 2011).

4. Cyclic GMP

Cyclic guanosine monophospate (cGMP) is a second messenger in signal transduction. It is generated by guanylyl cyclase enzymes (GC) that catalyze the conversion of GTP into cGMP. There are two main classes of GCs: nitric oxide-sensitive guanylyl cyclase (NO-GC) that is mainly soluble, and particulate guanylyl cyclases (pGC) represented by the natriuretic peptide (NP)-receptor (NPR) group (Tsai and Kass 2009) (Fig 1).

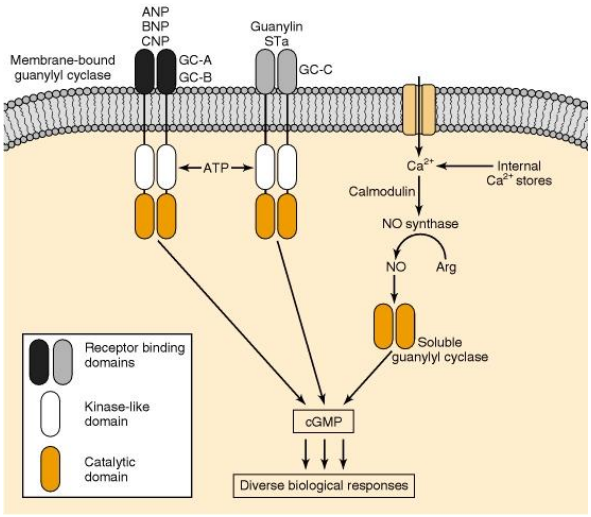

Figure 1: Schematic representation of cyclic GMP synthesizing pathways. GC activation mechanisms. GC-A, GC-B and GC are membrane bound GC and NO-GC is cytoplasmic. Scheme from Duman and Nestler, Guanylyl Ciclase, 1999 (Duman RS 1999).

5. The NO – cGMP pathway

Nitric oxide (NO) is a small, gaseous, highly diffusible, and reactive molecule with a short half-life, rapidly oxidized to the stable and inactive end-products, nitrite and nitrate (Zhang J. and Snyder 1995). In the CNS, NO has been associated with the modulation of synaptic plasticity, brain development, visual and sensory processing, neuro-endocrine secretion and cerebral blood flow (Garthwaite J. 2000, Guix et al. 2005). It can act as a

neurotransmitter/neuromodulator or as an inflammatory response mediator, low concentrations of NO mediate physiological signaling and can also be neuroprotective and it has shown to be essential for synaptic plasticity, control of cerebral blood flow, neurogenesis and synaptogenesis (Manucha 2016, Zhang J. and Snyder 1995). However, in high concentrations is a neuropathological agent responsible for excitotoxic cell death and neuroinflammatory cell damage in many neurological disorders (Duncan and Heales 2005, Manucha 2016, Murphy 2000) .

5.1. Nitric oxide synthases

Nitric oxide synthases (NOS) are a group of enzymes that produce NO by oxidation of one of the guanidine nitrogens of L-arginine, using O_2 and NADPH as co-substrates and forming L-citrulline as product, a reaction that requires O_2 and NADPH as co-substrates (Zhang J. and Snyder 1995). There are three isoforms of NOS, two of them NOS-1 and NOS-3 are expressed constitutively while NOS-2 is an inducible enzyme. NOS-1 is found in the nervous system (primarily in neurons and astrocytes) and NOS-3 is expressed in vascular endothelial cells. Both, NOS-1 and NOS-3, are calcium-calmodulin-dependent and produce nanomolar concentrations of NO in response to transient elevations in intracellular calcium. In neuronal populations this generally occurs by N-methyl-D-aspartate (NMDA) type glutamate receptor stimulation that allows calcium entry (Maarsingh et al. 2009, Manucha 2016). NOS-2 is inducible at the transcriptional level and its activity is calcium-independent since it has tightly-bound calmodulin. NOS-2 produces higher and longer-lasting amounts of NO (micromolar concentrations) after induction by inflammatory compounds such as LPS, pro-inflammatory cytokines (IL-1β, TNF-α and IFN-γ) or Aβ peptides, that can cause neurotoxicity. In peripheral tissues this enzyme is mainly, but not exclusively, expressed in macrophages. In the CNS, NOS-2 is

expressed in microglia and astroglia, and its generation of NO has been implicated in the pathogenesis of various insults as well as in neurological disorders (Brown and Neher 2010, Murphy 2000, Steinert et al. 2011). Up-regulation of iNOS has been implicated in tissue damage in MS and EAE (Raivich and Banati 2004). However, iNOS-deficient mice develop more severe EAE (Willenborg et al. 2007), suggesting that NO may be neuroprotective.

5.2 . Guanylyl cyclises

NO-sensitive guanylyl cyclases

NO-GC is recognized as the major receptor for NO, and mediates numerous of its physiological functions. NO-GC was initially thought to be entirely cytosolic and was thus named “soluble GC”, however it has also been found associated to membranes (Zabel et al. 2002). NO-GC is composed of an α and a β subunit and both subunits are required for catalytic activity (Koesling et al. 2004). Each subunit has a regulatory domain which contains a prosthetic heme group that is the NO-binding site, a catalytic domain that shares sequence homology with the corresponding domains in pGC and adenylyl cyclases and a central domain which allows subunit dimerization (Foster et al. 1999) (Fig. 4).

In contrast to the ubiquitous NO formation in CNS parenchymal cells, NO-dependent cGMP synthesis by activation of NO-GC appears to occur mainly in neurons and astrocytes. Not much is known about the regulation and function of cGMP formation in astrocytes during neuroinflammation, when an excess of NO production occurs (Baltrons et al. 2008). Inflammatory compounds known to induce astroglial reactivity and NOS-2 expression, such as LPS, Aβs or IL-1β have been shown to down-regulate NO-GC at the protein and mRNA level in rat brain astroglial cultures and after intracerebral

administration in adult rat brain (Baltrons and Garcia 1999, Baltrons et al. 2002, Pedraza et al. 2003). In addition, decreased astroglial expression of the NO-GCβ subunit was observed in post-mortem brains of Alzheimer's disease (AD), MS and Creutzfeld-Jacob disease patients, suggesting that an impairment of the astroglial NO/cGMP system may bear some relation to neuronal dysfunction (Baltrons et al. 2004).

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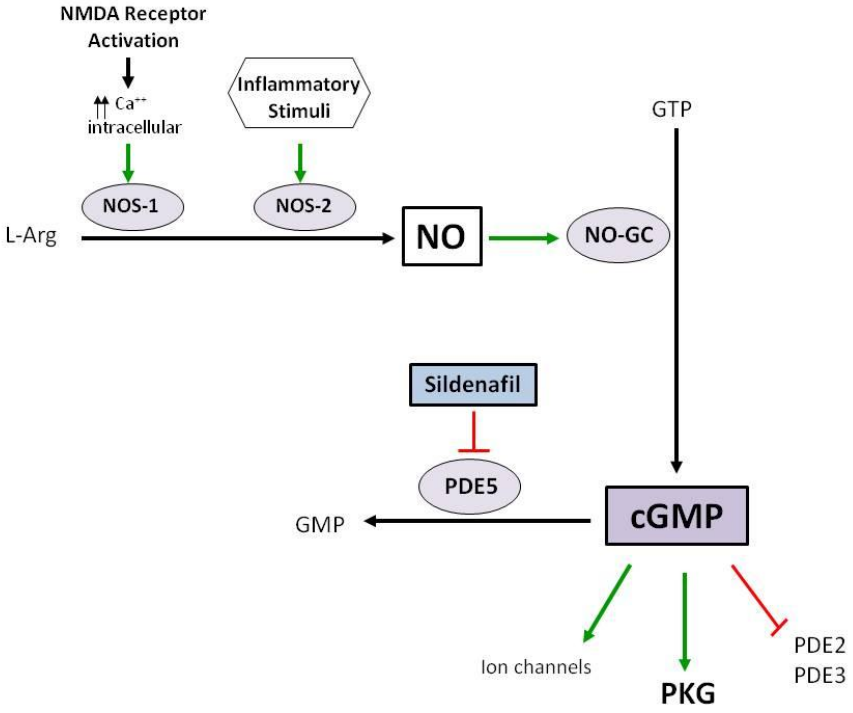

Figure 2: Schematic representation of NO-dependent cGMP formation, cGMP targets and site of action of sildenafil in the pathway

Natriuretic peptide receptor- guanylyl ciclases

Particulate guanylyl cyclases (pGC) are membrane bound with extracellular receptors (Zabel et al. 2002). The pGC has three isoforms, two of them are membrane NPR that have GC activity: NPR-A or GC-A that binds atrial (ANP) and brain natriuretic peptide (BNP), NPR-B or GC-B binds C-type natriuretic peptide (CNP) and is expressed in neuronal. The third isotype of GC is GC-C binds the endogenous peptide guanylin and can be activated by a bacterial enterotoxin and localized in the intestine (Fig 3) (Duman RS 1999). Functional natriuretic peptide receptors are mainly expressed un neuron; however they can also be expressed in astroglia and microglia regulating important physiological responses (Prado et al. 2010).

5.3 cGMP targets

Downstream targets of cGMP are cyclic nucleotide regulated ion channels, PKGs and cGMP-regulated PDEs (Fig 2).

Cyclic nucleotide regulated ion channels.

Cyclic nucleotide regulated ion channels are non-selective cation channels activated by cGMP and/or cAMP binding and have calcium permeability under physiological conditions (Bradley et al. 2005). Cyclic nucleotide regulated ion channels form heterotetrameric complexes consisting of two or three different types of subunits (Kaupp and Seifert 2002). In the CNS, this channels have been implicated in the regulation of synaptic plasticity, these channels are widely expressed in central and peripheral neurons, they control a variety of fundamental processes including signal transduction in retinal photoreceptors and in olfactory neurons (Biel et al. 1996, Bradley et al. 2005).

PKGs.

PKGs are serine/threonine-kinases that are activated by cGMP binding. Two subtypes of PKG exists PKGI and PKGII, both homodimers that regulate multiple signaling pathways, phosphorylating ion channels, G proteins and phosphorylating downstream signaling pathways. PKGI can be found as a soluble protein and has two isoforms PKGIα and PKGIβ, both of them expressed predominantly in the CNS, especially in cerebellum and hippocampus. PKGII is membrane-bound and can also be found in CNS. PKGI/II mediates most of the cGMP effects, regulating multiple signaling pathways by phosphorylation of ion channels, G proteins and associated regulators and cytoskeleton-associated proteins, among others. (Hofmann et al. 2009, Tsai and Kass 2009). PKG is the main target of cGMP, its binding to PKG regulates transcription factors such as CREB, NF-κB and c-Fos (Vollmar 2005); more over PKG activation increases ErkI/II, Akt y GSK3β phosphorylation, among other molecules (Das et al. 2008). Erk and Akt pathways have been related with oligodendrocyte protection and differentiation (Guardiola-Diaz et al. 2012).

cGMP-regulated PDEs.

cGMP selectively regulates the activity of PDE2,PDE3 and PDE5 by binding to their regulatory domains. In PDE5, cGMP binding into its regulatory domain induces cGMP hydrolysis into GTP. In the case of PDE2 that has high affinity for cAMP and low affinity for cGMP, the binding of cGMP to the regulatory site provokes a conformational change which increases its enzymatic activity towards cAMP. PDE3 also has affinity for both, cAMP and cGMP, with a higher hydrolysis rate towards cAMP; however, cGMP binding to the catalytic site and inhibits cAMP breackdown, thereby regulating cAMP and cGMP signaling (Francis et al. 2011, Tsai and Kass 2009).

5.4. cGMP inactivation

Cyclic GMP PDEs

PDEs are the enzymes responsible for the hydrolysis of the phosphodiester bond of cyclic nucleotides such as cAMP and cGMP, controlling the duration and reach of the cyclic nucleotide signaling. Eleven different families of PDEs have been identified (PDE1 to PDE11), with different expression, substrate specificity, regulation or sensitivity to inhibitors. PDEs can be divided in three groups depending on the specificity for the nucleotide. PDEs specific for cGMP are:PDE5, PDE6 and PDE9. cGMP-specific PDE5 has 3 isoforms (PDE5A1-3) widely distributed and having no difference in activity between them (Tsai and Kass 2009).

PDE inhibitors were initially developed as potential therapeutic tools, recently PDE inhibitors are being used according to their specificity, to treat different disorders and potentiate the effect of the cyclic nucleotide signaling (Bell and Palmer 2011, Hertz and Beavo 2011). PDE5 inhibitors were initially developed because of the important role of this PDE in controlling cGMP levels in vascular smooth muscle and its effects on vascular tone. Three PDE5 inhibitors (vardenafil, tadalafil y sildenafil) are used worldwide for the treatment of different pathologies. Sildenafil (Viagra) is currently approved for the treatment of erectile dysfunction and pulmonary arterial hypertension (Bell and Palmer 2011, Tsai and Kass 2009).

Numerous studies in animal models have shown beneficial effects of PDE5 inhibition in the CNS. In focal brain injury, PDE5 inhibition promotes astrogliosis and angiogenesis, decreases microglia/macrophage activation and oxidative stress contributing with neuroprotection (Pifarre et al. 2010). Moreover, it has been described that sildenafil treatment ameliorates clinical symptoms of EAE,

an *in vivo* model that mimics neuropathological and neuroinflammatory features of MS, inducing almost full recovery after 8 days of sildenafil treatment starting at peak of disease, effect associated with an increase in myelin staining and reduced axonal damage (Pifarre et al. 2011). In addition, sildenafil treatment after cuprizone induced demyelination *in vivo*, showed protective effects in cerebellum by preserving myelin and axons ultra-structure and reducing pro-inflammatory cytokines IFN-γ, TNF-α, IL-1β, IL-2 and COX-2 (Nunes et al. 2012). Furthermore, starting treatment with sildenafil at the onset on EAE prevents disease progression, showed oligodendrocyte protection against apoptotic death, reduced microglia/macrophage reactivity while increased the expression of Ym-1 anti-inflammatory microglia/macrophage phenotype (M2) protein, increased in BDNF expression in splenocytes accompanied by an increase immune-reactivity of this neurotrophic factor in the SC of sildenafil treated mice; the augment of BDNF expression in SC of sildenafil treated mice was associated with increased expression in infiltrating leukocytes and axons and decreased pro-inflammatory cytokine expression in splenocytes, modulating adaptative immune response (Pifarre et al. 2014).

5.5. cGMP and remyelination.

cGMP and oligodendrocytes

Studies in immature rat brain slices have shown that increasing NO levels with a NO donor combined with a PDE inhibitor generated an increase in cGMP accumulation in oligodendrocytes (Tanaka et al. 1997). Furthermore, increasing cGMP levels by inhibiting PDE5 with sildenafil have been reported to increase neural stem cells and their neuronal and oligodendrocyte progeny in the mouse ischemic brain model (Bibollet-Bahena and Almazan 2009). Additionally, it has been reported that low NO concentrations or a cGMP analogue protect

differentiated murine oligodendrocytes from caspase-mediated death via PKG (Benjamins and Nedelkoska 2007). Protection of mature and immature oligodendrocytes has been also reported in sildenafil-treated EAE mice (Pifarré et al. 2014) and very recent studies have shown that stimulation of the NO-cGMP pathway promotes oligodendrocyte morphological development in vitro (Garthwaite G. et al. 2015).

As above mentioned, sildenafil treatment after cuprizone induced demyelination preserved myelin and axon integrity (Nunes et al. 2012, Raposo et al. 2014) and previous studies developed in the laboratory where this thesis was developed have shown in significant amelioration of EAE clinical symptoms after sildenafil treatment, suggesting a possible role of sildenafil in remyelination due to an increase in myelin stainings the protection exerted against apoptotic death of oligodendrocytes, the decrease of reactive gliosis and increase in neurotrophic factor BDNF (Pifarre et al. 2011, Pifarre et al. 2014).

AIMS

AIMS

Studies conducted in the laboratory where this thesis was developed showed that in a model of multiple sclerosis (MS), MOG induced experimental autoimmune encephalomyelitis (EAE), that the administration of the PDE5 inhibitor sildenafil in the acute phase of the disease rapidly ameliorates clinical symptoms, prevents axonal, decreased CD3+-leukocyte infiltration and microglial/macrophage activation in the spinal cord and suggests to restore myelin content (Pifarre et al. 2011). Additionally, early administration of sildenafil have shown to delay EAE progression, preserves axons and myelin sheath integrity, prevents the death of oligodendrocytes and enhances expression of BDNF neurotrofic factor in chronic EAE, it was also observed reduced microglia/macrophage reactivity while increased the expression of Ym-1 anti-inflammatory microglia/macrophage phenotype (M2) protein and modulating adaptative immune response (Pifarre et al. 2014).

1. General aim.

The general aim of this thesis was to study mechanisms involved in the remyelinating effect of sildenafil in CNS cells.

2. Specific aims.

1. To confirm the remyelinating potential of sildenafil in the spinal cord of EAE mice and in demyelinated cerebellar organotypic cultures

2. To investigate the implication of the NO-cGMP-PKG pathway in the remyelinating effect of sildenafil

3. To investigate if sildenafil induces CNTF in CNS cells

4. To investigate if sildenafil induces changes in inflammatory phenotype of microglia in demyelinated slice cultures and in bone marrow-derived macrophages (BMDM).

5. To investigate if sildenafil increases myelin phagocytosis in spinal cord of EAE mice and in BMDM

MATERIALS AND METHODS

MATERIALS AND METHODS

1. MATERIALS

1.1. Reagents

Table 1: Tissue Culture Material.

Product	Abbreviation	Supplier
Dubelco's Modified Eagle medium GlutaMax	DMEM	Gibco
Fetal Bovine Serum	FBS	Gibco
Basal medium containing Earle's salt	BME	Gibco, Invitrogen
Hank's buffered salt solution	HBSS	Gibco, Invitrogen
Heat Inactivated horse serum	HS	Gibco, Invitrogen
Glucose	Glucose	Sigma-Aldrich
L-Glutamine 200mM	L-Glutamine	Sigma-Aldrich
Penicillin-Streptomycin	Pen/Strep	Sigma-Aldrich
Recombinant Macrophage Colony Stimulating Factor	M-CSF	Immunotools
Cell Culture Inserts		Millipore

Table 2: Specific Reagents

Product	Abbreviation	Use	Supplier
cGMP analogues			
8-Bromoguanosine 3′,5′-cyclic monophosphate sodium salt	8BrcGMP	Cell-permeable hydrolysis resistant cGMP analog. Activates PKG.	Sigma-Aldrich
8-(4-Chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate sodium salt	8pCPTcGMP	Membrane-permeable analog of cGMP, selective activator of PKG.	Sigma-Aldrich
cGMP increasing agents			
N-methyl-D-Aspartate	NMDA	NMDA receptor agonist	Sigma-Aldrich
BAY 41-2272	BAY41	NO-GC Activator	TOCRIS

Sildenafil	Sil	PDE-5 Inhibtor	Sigma-Aldrich
cGMP production inhibitors			
1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one	ODQ	Selective inhibitor of NO-GC	Sigma-Aldrich
N_{ω}-Nitro-L-arginine	L-NNA	Inhibitor of NOS	Sigma-Aldrich
Signaling pathways inhibitors			
8-[(4-Chlorophenyl)thio]-guanosine-cyclic 3',5' monophosphate	Rp-8pCPT-cGMP	PKG inhibitor	Biolog
Rapamycin		mTOR inhibitor	Calbiochem
1,4-Diamino-2,3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene	U0126	Selective non-competitive inhibitor of the MAP kinase kinase pathway. Inhibits MEK 1/2	Calbiochem
Inflammatory stimuli			
Recombinant Murine Interleukin-4	IL-4	Induces M2 phenotype	Immunotools
Recombinant Mouse Interferon γ	IFNγ	Induces M1 phenotype	Millipore
Lipopolysaccharides from Salmonella enterica	LPS	Induces M1 phenotype	Sigma-Aldrich
Other specific reagents			
L-α-Lysophosphatidylcholine from egg yolk	LPC	Demyelinates axonal fibers	Sigma-Aldrich
1,1”-diotadecyl-3,3,3’,3’-tetramethyl-lindocarbocyanide perchlorate	DiI	Lipophilic membrane stain	Sigma-Aldrich
Oil Red-O	ORO	Fat-soluble dye for lipid staining	Sigma
FluoPrep		Mounting medium	Biomeireux S.A.
RIPA Lysis Buffer	RIPA	Lysis of cells to perform analysis on proteins.	Millipore
Anti phosphatases cocktail		Improves the yields of intact proteins inhibiting enzymes that modify proteins in cell extracts	Sigma-Aldrich

Anti proteases		Inhibits degradation of proteins by endogenous proteases	Roche
Ethylenediaminetetraacetic acid	EDTA	Ligand and chelating agent. Preventing cells aggregates and detaching adherent cells	Fluka
TRIS buffer solution	Tris	Buffer use in protein and nucleic acid extraction and purification.	Sigma
Phosphate buffer saline	PBS		Gibco
Bovine serum albumin	BSA	Blocking non-specific antibody binding	Sigma
Neonatal Goat serum	NGS	Blocking non-specific secondary antibody binding	Gibco
TruStain FcX anti–mouse CD16/32		Blocking Fc receptors in cell membranes	Biolegend

1.2. Antibodies.

Table 3: Primary Antibodies

Antibodies	Origin	Supplier and Reference
anti-MBP	Rat	Abcam ab7349
anti- CNPase	Mouse	Abcam Ab6319
anti-Caspr	Rabbit	Abcam ab34151
anti-Olig2	Rabbit	Millipore ab9610
anti-NF-200	Mouse	Sigma-Aldrich N0142
anti- βActin	Mouse	Sigma-Aldrich A5316
anti-GAPDH	Mouse	Ambion AM4300
anti-NOS-2	Mouse	BD 610329
Primary antibodies coupled to fluorescent dyes		
Alexa Fluor 488-anti-CD206	Rat	Biolegend 141710 (Rat IgG2a,k)
Alexa Fluor 488-Rat IgG2a,k Isotype	Rat	Biolegend 400525 (Rat IgG2a,k)

Table 4: Secondary antibodies

Antibody	Made in	Supplier
Alexa coupled to anti-IgG		
Alexa Fluor anti Rat 488	Goat	Invitrogen
Alexa Fluor anti Mouse 568	Goat	Invitrogen
Alexa Fluor anti Rabbit 488	Goat	Invitrogen
Alexa Fluor anti Rabbit 568	Goat	Invitrogen
***Horseradish peroxidase* (*HRP*) conjugated anti-IgG**		
anti -Rat-HRP	Goat	Thermo Fisher
anti -Mouse-HRP	Goat	Sigma

2. EAE

Two-month old female C57BL/6 mice (Charles River) were housed in the animal facility of Universitat Autonoma de Barcelona (UAB) under constant temperature and provided food and water *ad libitum*. Chronic Experimental Autoimmune Encephalomyelitis (EAE) was induced by immunization with MOG_{35-55} peptide (Scientific Technical Service, Universitat Pompeu Fabra Barcelona, Spain) as a model of MS. Mice were injected subcutaneously (sc) into the hind flanks with an emulsion of 100 µl $MOG_{35–55}$ (3 mg/ml) and 100 µl Complete Freund's Adjuvant (CFA) supplemented with 4 mg/ml *Mycobacterium tuberculosis* H37RA (Difco). Additionally, animals received an intraperitoneal injection of 500 ng pertussis toxin that was repeated 2 days after immunization. Control mice were immunized only with CFA supplemented with mycobacterium and compared with Naïve group (Pifarre et al. 2011).

2.1. EAE clinical score evaluation

Mice were weighted daily and clinically evaluated for EAE-ch progression according to the following scores: 0 no disease sings, 0.5 partial loss of tail tonus, 1 loss of tail tonus, 2 moderate hind limb paraparesis, 2.5 severe hind limb paraparesis, 3 partial hind limb paralysis, 3.5 hind limb paralysis, 4 tetraplegy and 5 death.

2.2. Treatments and Sacrifice

2.2.1. Acute phase treatment

Immunized animals were randomly divided into two groups before being treated by injection (s.c) with vehicle (water) or sildenafil (10 mg/kg) once a day starting near peak of the disease (16 days post-immunization (dpi) score close to 2). Mice were sacrificed under pentobarbital anesthesia after 15 days of treatment (dot), spinal cords (SC) were collected (Pifarre et al. 2014).

2.2.2. Chronic phase treatment

Once immunized, the animals were randomly divided into two groups before starting treatments. Clinical symptoms peaked at 18 dpi and stabilized thereafter at a mean score of 2. Mice were treated by injection (s.c) with vehicle (water) or sildenafil (10mg/kg) once a day starting at 41 dpi. After 17 days of treatment, mice were sacrificed 2 h after the last administration under pentobarbital anesthesia and SC were removed.

Experiments were approved by the UAB Animal and Human Experimentation Ethics Committee.

3. Culture procedures.

3.1. Cerebellar Organotypic Cultures

Organotypic slice cultures were stablished from cerebellum of 7-day-old C57BL/6 mice (Harlan). Mice were killed by decapitation and cerebella were dissected. Sagital 300 µm cerebellum slices were obtained by a McIlwain Tissue Chopper (Mickle Laboratory). Three or four slices per well were seeded on Cell Culture Inserts in six-well plates and incubated at 37 ºC with 5 % CO_2 in medium containing 50 % BME, 25% HBSS, 25% HS, 5 mg/ml glucose, 0.25 mM L-glutamine and 25 ug/ml penicillin/streptomycin. Medium was changed every 2–3 days. After 7 days in vitro (DIV), the slices were demyelinated using L-α-lysophosphatidylcholine (LPC) from egg yolk (0.5 mg/ml) for 14 h. After that time medium was replaced with LPC-free medium (Birgbauer et al. 2004).

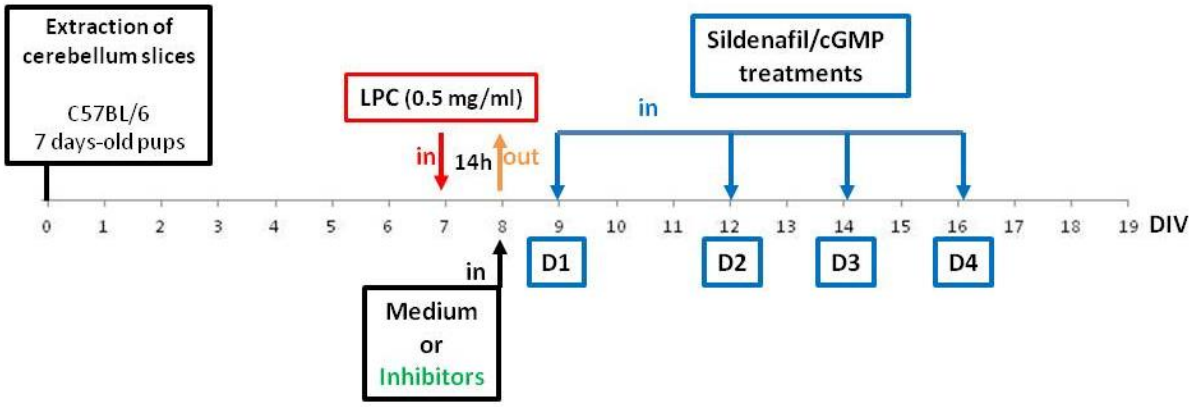

Figure 3: Scheme of mouse organotypic cerebellar culture. Treatment time points.

Treatments with specific activators or inhibitors of cGMP mediated pathways and kinases (Fig 2) were started at 9 DIV (Fig 1), and medium were replaced every 2-3 days until 19 DIV. Inhibitors of the NO-cGMP pathway were added 24h before (8 DIV) (Fig 1).

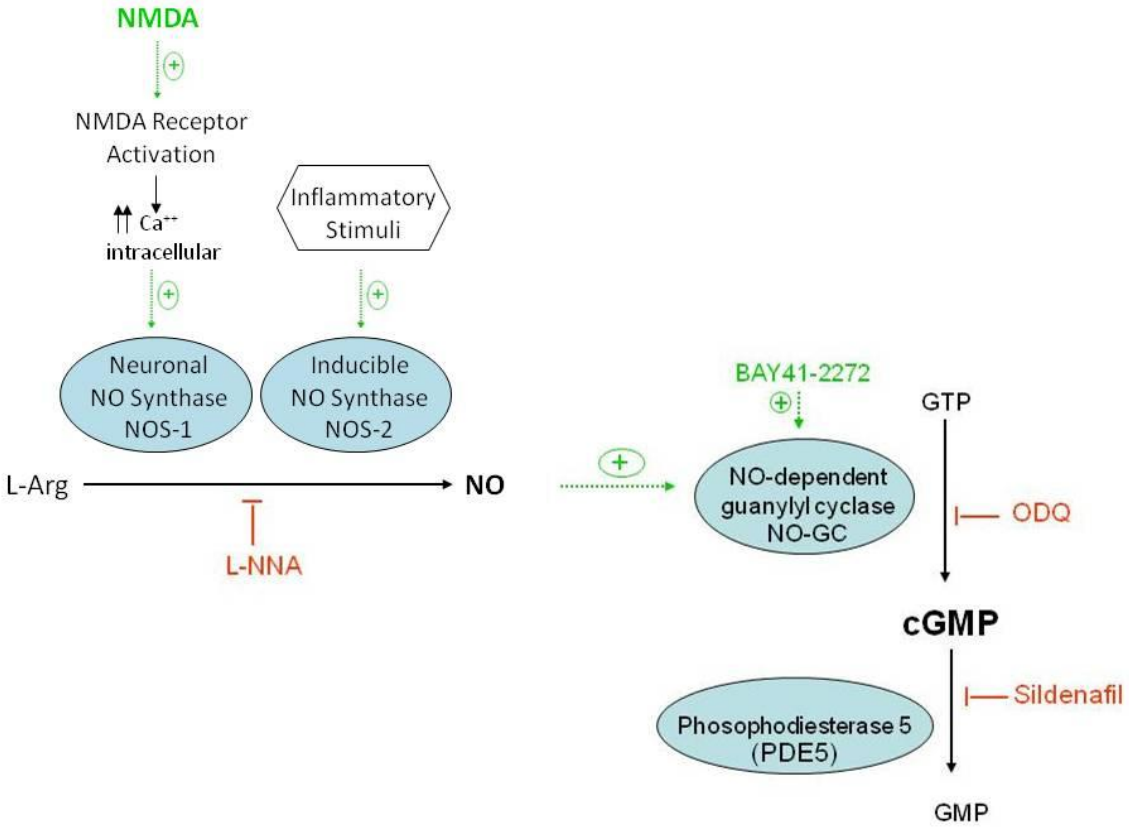

Figure 4: Treatments with specific activators or inhibitors of cGMP mediated pathways

3.2. Bone Marrow derived Macrophage Culture

Bone marrow derived macrophage cultures were obtained as previously describe by Classen, *et al.,* 2009 (Classen et al. 2009) with some modifications. Briefly C57BL/6 naïve 8 to 12 week-old mice were used. Mice were euthanized by decapitation and hind legs were removed. Tibia and femur were exposed and epiphyses were cut and the medullary cavity of the bones was left visible. A 25G needle was introduced in the cavity, and the content was washed off with DMEM medium. Bone marrow was seeded in 140 mm plates in DMEM+20%FBS+1%peniciline/streptomycin containing 10 ng/ml M-CSF and

incubated at 37 ºC at 5% CO_2. After 4 days, a boost of 5 ng/ml M-CSF was added.

Cells were harvested at day 7 and isolated BMDM were seeded in DMEM+10%FBS+1%peniciline/streptomycin. For arginase activity and nitrite concentration 96-well plates were used, containing $2x10^5$ cells/well. For contrast phase examination 35 mm plates were used, containing $1.5x10^6$ cells/well. For mRNA and flow cytometry studies 6-well plates were used with a density of $1.5x10^6$ cells/well. Myelin phagocytosis experiments were carried out in 24-well plates with $3x10^5$ cells/well. After treatments, all culture media were collected and stored at -80ºC until use.

4. Myelin phagocytosis.

4.1. Myelin Isolation and labelling.

Myelin isolation was performed as previously describe by Larocca *et al.,* 2007 with some modifications (Larocca and Norton 2007). Briefly, two C57BL/6 mice were euthanized by decapitation. Brains and SC were collected, weighted and homogenized in 0.3 M sucrose containing anti-protease (1:25 dilution) to achieve to a final concentration of 5 % w/v, with a Potter-Elvehjem homogenizer mechanically driven at 800 rpm (5 strokes). The homogenate was added into a tube containing sucrose 0.83 M (carefully through the tube wall) to achieve an equal volume and generate a two-phase discontinuous sucrose gradient. The suspension was centrifuged at 75.000xg for 30 min at 4 ºC. Myelin was isolated from the interface. Ten milliliters of Tris-HCl 1 M was added to the myelin extract that was homogenized in Potter at 800 rpm. The suspension was brought to a final volume of 114 ml with Tris-HCl 1 M. After centrifugation the pellet was resuspended in Tris-HCl 1 M. The procedure was repeated twice times with a first centrifugation at 75.000xg and a second at

12.000xg for 15 min at 4 ºC. The pellet was resuspended in MilliQ water and lyophilized in SPD Speed Vac (Thermo). Lyophilized myelin was weighted and stored at -20 ºC until use (Larocca and Norton 2007).

For myelin labeling, lyophilized myelin was resuspended in MiliQ water to a final concentration of 1 mg/ml and one volume of 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine perchlorate (Dil) was added to reach a final concentration of 12 µg/ml. After incubating at 37 ºC for 30 min, the myelin-Dil suspension was centrifuged at 24.000xg for 20 min and 4 ºC. Supernatant was discarded and labeled myelin was further resuspended in sterile PBS to a final concentration of 1mg/ml and stored at -20ºC (Hendriks et al. 2008, van der Laan et al. 1996).

4.2. Myelin phagocytosis assay.

Myelin phagocytosis assay was performed as previously described (van der Laan et al. 1996). BMDM seeded in 24-well plates and stimulated for 24h, were incubated with Dil-labeled myelin (25 µg/ml) for 1.5 h at 37ºC. Cells were detached by adding PBS/5 mM EDTA for 15 min at 37ºC and cell fluorescence was determined by flow cytometry using a FACScalibur cytometer. Intensity and percentage of positive cells were used to quatify Dil-labeled myelin uptake after treatments (Hendriks et al. 2008).

5. Nitrite concentration determination

NO production was measured as nitrite using the Griess reagent (Classen et al. 2009) 24h after BMDM stimulation. Briefly, the volume of 100 µl culture supernatant was mixed with 100 µl 1% sulfanilamide 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 2.5 % H3PO4. Absorbance was measured at 540 nm in a multilabel plate reader VICTOR 3 (PerkinElmer).

6. Arginase activity assay.

Arginase activity was measured in as previously described (Classen et al. 2009). Briefly, cells were lysed with 100 µl 0.1% Triton X-100. After 15 min on a shaker, 100 µl 50 mM Tris-HCl and 10 µl 100 mM MnCl2 were added. The enzyme activativation was performed by heating for 7 min at 56 ºC. The lysate was incubated with 100 µl 0.5 M L-arginine (pH 9.7) at 37ºC for 60 min. Reaction was stopped with 900 µl 96% H2SO4/85% H3PO4/H2O2 (1:3:7). Urea concentration was measured by adding 40 µl α-isonitrosopropiophenone (dissolved in100% ethanol) followed by heating at 95C for 30 min and read at 540 nm in a multilabel plate reader VICTOR 3 (PerkinElmer).

7. Determination of mRNA levels by q-PCR.

7.1. RNA extraction

RNA was extracted using Maxwell® RSC simply RNA Tissue Kit Promega following the manufacturer's instructions. In organotypic cerebellar cultures, RNA was extracted from 4 slices per condition, 24h after treatments. In BMDM cultures, RNA extraction was performed 6 h after treatments.

7.2. RNA Reverse Transcription

RNA was reverse-transcribed using i-Script de Bio-Rad following the manufacturer's protocol. RNA was quantified using NanoDrop 1000 Thermo Scientific. Reverse transcription of 500 ng RNA from BMDM and 100 ng RNA from organotypic slices was performed. The reaction was done 5 min at 25 ºC, 30 min at 42ºC and finally samples were heated at 95 ºC for 5 min using MJ Research PTC-150 MiniCycler.

7.3. q-PCR

The cDNA obtained was appropriately diluted to 100ng/ml cDNA, and qPCR was performed using i-Taq de Bio-Rad. Master-mix for q-PCR contained 5 µl Syber green, 0.3 µl of each forward and reverse primers and 1.9 µl nuclease-free water, for a total of 7.5 µl of master-mix per reaction well. The primers used can be found in table 5. For amplification Bio-Rad CFX96 Touch™ Real-Time PCR Detection System was used. Further analysis was performed by Bio-Rad CFX Manager™ 3.1 Software. HPRT was chosen as reference gene. Relative gene expression was assessed using the ΔΔCq Method (Livak and Schmittgen 2001).

Table 5: Primer Sequences

Primers	Sequence 5'-3'	Size	NCBI Ref
HPRT-Fw HPRT-Rv	GATTAGCGATGATGAACCAGGTT CCTCCCATCTCCTTCATGACA	150bp	NM_013556.2
MBP-Fw MBP-Rv	CACACACGAGAACTACCCA GGTGTTCGAGGTGTCACAA	115bp	NM_001025258.2
PLP-FW PLP-Rv	AGCAAAGTCAGCCGCAAAAC CCAGGGAAGCAAAGGGGG	121bp	NM_001290562.1
CNTF-Fw CNTF-Rv	TCGTTCAGACCTGACTGCTC ACTCCAGCGATCAGTGCTTG	115bp	NM_170786.2
BDNF-Fw BDNF-Rv	ATCCACTGAGCAAAGCCGAAC GCCTTCATGCAACCGAAGTAT	198bp	NM_007540.4
Arg 1-Fw Arg 1-Rv	TTGCGAGACGTAGACCCTGG CAAAGCTCAGGTGAATCGGC	160bp	NM_007482.3
CD206-Fw	CTTCGGGCCTTTGGAATAAT	149bp	NM_008625.2

CD206-Rv	TAGAAGAGCCCTTGGGTTGA		
NOS-2-Fw NOS-2-Rv	CTGCATGGACCAGTAATAAGGCAAAC CAGACAGCTTCTGGTCGATGTCATGA	231bp	NM_001313922.1
COX-2-Fw COX-2-Fw	TCATTCACCAGACAGATTGCT AAGCGTTTGCGGTACTCATT	137bp	NM_011198.4
IL-10-Fw IL-10-Rv	GCTCTTGCACTACCAAAGCC CTGCTGATCCTCATGCCAGT	112bp	NM_010548.2
IL-6-Fw IL-6-Rv	GCTTAATTACACATGTTCTCTGGGAAA CAAGTGCATCATCGTTGTTCATAC	93bp	NM_031168.2
IL-1 β-Fw IL-1β-Rv	CCTGGGCTGTCCTGATGAGAG TCCACGGGAAAGACACAGGTA	131bp	NM_008361.4
TNFα-Fw TNFα-Rv	CTTCTCATTCCTGCTTGTG ACTTGGTGGTTTGCTACG	198bp	NM_013693.3

8. Analysis of BMDM phenotype by Flow Cytometry.

After stimulation, BMDM were washed with medium and detached using cold PBS Ca^{++} and Mg^{++} free, scraping softly. Cells were centrifuged at 500xg for 5 min at 4 ºC and distributed in tubes at a concentration of $5x10^5$ cells per tube.

For cellular membrane marker (CD206) staining, cells were centrifuged and pellets were resuspended in 100 µl of TruStain FcX anti–mouse CD16/32 diluted 1:100 v/v in DMEM 1% FBS and incubated for 15 min at 4 ºC, in order to block non-specific binding to Fc receptors. Cells were washed in DMEM+1%FBS and centrifuged at 500xg for 5 min, at 4 ºC. Pellets were resuspended in fluorescence-labelled antibody (table 3) at the proper dilution and incubated for 1 h at 4 ºC in the darkness. After washing, pellets were fixed in 1%

Paraformaldehyde (PFA) for 30 min at room temperature (RT). Cells were washed and pellets resuspended in PBS.

For intracellular marker (NOS-2) staining, cell pellets were fixed in 1% PFA 30min at RT prior to staining. Cells were washed in PBS and centrifuged at 500g for 5 min, at 4ºC. Fc blocking and permeabilization were performed at the same time with TruStain FcX anti–mouse CD16/32 diluted 1:100 v/v in cytometry permeabilization buffer (CPB) prepared with PBS, 0.5% Tween-20 and 10% NGS, 100 µl of blocking-permeabilization solution was added and incubated 1 h at RT. Cells were washed by adding CPB and centrifuged at 500xg for 5 min, at 4ºC. Pellets were resuspended in 100 µl primary antibody (table 3) proper diluted in CPB and incubated for 2 h at RT. As secondary antibody control, the primary antibody was replaced by CPB. Cells were washed and pellets were resuspended in 100 µl of secondary Alexa-coupled antibody diluted 1:400 v/v in CPB (table 4) and incubated for 1 h at RT in the darkness. After washing, cells were resuspended in PBS and were analysed using a BD FACScanto (10,000 events per sample).

9. Stainings

9.1. Immunofluorescence staining.

Cervical–thoracic regions of the SC were fixed in 4% PFA overnight at 4 °C, cryopreserved by immersion in 30% sucrose in PBS and fresh-frozen cryostat-cut into 16 µm-thick sections (Microtome Cryostat Thermoshandon). Immunofluorescence staining (IF) was performed in all sections. After blocking with PBS, 3% BSA, 10% NGS, 0.2 M Glycine for 2 h, sections were incubated overnight (ON), at 4 °C with primary antibodies (table 3). Tissue was washed and incubated with the appropriated secondary Alexa-conjugated antibodies

(table 4) diluted 1:1000 v/v. Nuclei were stained with DAPI (0.25 µg/ml). Control sections were incubated in the absence of primary antibodies.

Cerebellar organotypic slices were fixed with 4% PFA for 60 min at RT. Slices were rinsed 10 min in PBS and further blocked with PBS, 10% NGS, 0.5% Triton X-100 for 2 h. Afterwards slices were incubated with primary antibody (table 3) dilution overnight (ON), at 4ºC. Slices were washed twice prior to incubation with the appropriate secondary Alexa-conjugated antibody (table 4) diluted 1:1000 v/v in PBS+10%NGS+0.3%Triton X-100. Slices were washed twice with PBS for 10 min, and incubated with Dapi for 10 min, washed and mounted with FluoPrep.

9.2. Oil-Red-O Staining.

SC longitudinal sections were washed in PBS. ORO stainig was performed, slightly modified, as previously described (Koopman et al. 2001). Brieffly, 0.5mg ORO was dissolved in 100ml of 60% triethyl-phospate, creating a stock solution. To stain SC sections a working solution (6 stock solution : 4 deionized water) was used. Sections were rinsed in PBS and then incubated 1h at RT in de ORO working solution. Afterwards sections were rinsed in deionized water three times and 10 min in tap water. Sections were flat embedded in 10% glycerol containing Dapi.

10. Determination of protein levels by Western Blot.

10.1 Protein collection

SC from EAE mice were homogenized in in 50 mM Tris–HCl and 1 mM EDTA, pH 7.4 containing RIPA with antiprotease and antiphosphatases cocktail at a 10% w/v proportion in a glass–teflon Potter-Elvehjeim homogenizer

mechanically with 20 strokes at 800 rpm as previously described (Pifarre et al. 2011).

Cerebellar slices, 4 per condition, were homogenized in 300 µl of the previous mentioned homogenizing solution, by pipetting up and down 10 times. Homogenates were centrifuged at 700xg and supernatants were aliquot and frozen at -80 ºC until use.

Protein levels in all samples were measured by Pierce BCA Protein Assay Kit (Thermo Scientific), following manufacturer's instructions.

10.2. Western Blot assay

Samples from SC or organotypic cultures homogenates, containing 20 µg and 30 µg of protein respectively, were subjected to Novex NuPAGE® 4-12% Bis-Tris Midi Gels Invitrogen electrophoresis carried out with the XCell SureLock™ Electrophoresis Cell. Transference was carried out using iBlot™ Gel Transfer Device with the iBLot® Gel Transfer Stacks PVDF membrane, following manufacturer's instructions, during 7 minutes. The protein transference was confirmed by Ponceau staining. To avoid non-specific binding, membranes were blocked with 5% w/v powder milk in PBS ON at 4 ºC. Membranes were incubated ON at 4ºC with the appropriated primary antibody (table 3). After washingwith PBS, 0,05% Tween-20, membranes were incubated for 1 hour with secondary HRP-conjugated antibodies (table 4). Detection was performed with Millipore Luminata Forte Western HRP substrate. Results were analyzed with QuantityOne software.

11. Cytokine Detection

Mouse TNFα, IL-1β and IL-10 cytokines were detected using Luminex technology. Briefly, MILLIPLEX® MAP Mouse Cytokine/Chemokine Magnetic 96-well plate Bead Panel Millipore (MCYTOMAG-70K) assay was used, following the manufacturer's instructions and with program Milliplex Analyte version 5.1.0.0.

RESULTS

RESULTS

1. Sildenafil administration to animals with EAE ameliorates symptoms and restores myelin protein levels.

Using MOG-induced EAE as a model of MS, the laboratory were this thesis was developed has previously shown that daily treatment with sildenafil at peak disease rapidly ameliorates clinical symptoms and neuropathology (Pifarre et al. 2011). An increase in LFB staining of myelin after 8 days of treatment suggested that sildenafil was promoting remyelination. Furthermore, administration of sildenafil from the onset of EAE symptoms when the immune response prevails, prevented disease progression (Pifarre et al. 2011, Pifarre et al. 2014). Ultrastructural analysis of spinal cord evidenced that sildenafil treatment was preserving axons and myelin and increasing the number of remyelinating axons. In order to confirm the remyelinating potential of sildenafil in EAE we have analyzed the effect of the drug on myelin proteins levels in the spinal cord of EAE animals treated from peak disease o and during the chronic phase of the disease. As shown in Fig 1A, C57BL/6 mice immunized with $MOG_{35–55}$ developed clinical symptoms of EAE around 8 days post-immunization (dpi). Symptoms rapidly increased up to day 16 (clinical score around 2.5; moderate hind limb paraparesis) and slightly increased thereafter until the last day examined (30 dpi). Treatments with vehicle (water) or sildenafil (10mg/kg) started at 16 dpi (black arrow). A rapid amelioration was observed in the sildenafil-treated group (n=17) compared with the vehicle-treated group (n=11). The clinical score stabilized close to 1 (loss of tail tonus) after 5 days of treatment and no further improvement was observed even when the treatment was extended for 10 more days (Fig 1A). Immune-staining for the major myelin protein MBP was performed in SC sections at the end of the treatment. As can be observed in the representative images shown in Fig

1B, MBP staining was stronger in sildenafil-treated mice. Quantification of staining intensity showed than sildenafil treatment significantly increased MBP immune-reactivity respect to the vehicle-treated group.

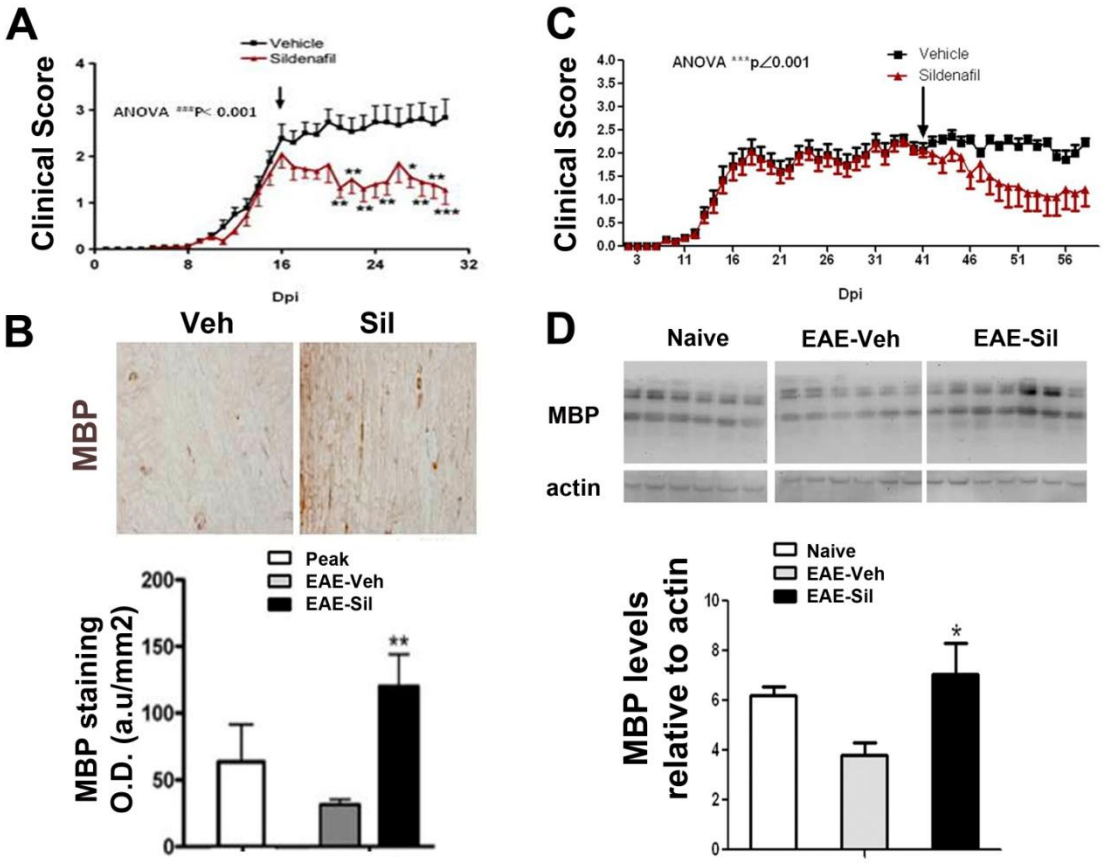

Figure 1: Sildenafil administration to animals with EAE ameliorates symptoms and restores myelin protein levels. Clinical evolution of MOG-induced EAE in mice treated with vehicle (VH; water) or sildenafil (Sil; 10 mg/kg) daily starting at peak disease (16 dpi, arrow in **A**) or during the chronic phase (41 dpi, arrow in **C**). Sildenafil significantly ameliorates EAE severity with both treatments. Values are means±SEM (n = 11 Vh and 17 Sil, acute phase; 8 Vh-8 Sil, chronic phase). Two-way ANOVA reveals statistically significant differences between vehicle- and sildenafil-treated animals with both treatments protocols (***p < 0.001); Bonferroni´s post hoc analysis reveals significant differences after 5 days of treatment (dot) from peak disease (*p < 0.05; **p < 0.01; ***p < 0.001). **(B)** MBP immune-staining in the SC of EAE mice treated with sildenafil from peak disease is significantly increased after 15 dot. Values are means±SEM (n=3-5 mice per group). Student's t-Test **p<0.01 vs vehicle-treated; **(D)** MBP protein levels analyzed by WB in SC of EAE mice treated during the chronic phase. In EAE vehicle-treated animals, MBP levels decrease respect to naïve group and recover after sildenafil treatment. Values are means±SEM (n=6), one-way ANOVA followed by Bonferroni's post-hoc (*p<0.05).

In a parallel experiment (Fig 1C), treatment was initiated during the chronic phase of the disease (41 dpi, arrow). Ten days after the initiation of sildenafil-treatment the clinical score was significantly decreased from values around 2 to values close to 1, and stabilized at that level until the animals were sacrificed (58 dpi). WB analysis of MBP protein in SC homogenates (Fig 1D) showed that levels in EAE vehicle-treated animals were 40% lower than in naïve animals and that sildenafil treatment restored MBP levels. Taken together these results show that sildenafil stimulates myelin protein recovery when administered both in the acute phase of the disease and when the disease has become chronic.

2. Sildenafil promotes remyelination in LPC-demyelinated mouse cerebellar organotypic cultures.

To investigate if the remyelinating effect of sildenafil observed in EAE mice involved effects only in CNS cells or also required peripheral immune-regulatory actions, we used the *in vitro* model of mouse cerebellar organotypic cultures (ORG) demyelinated with LPC (Zhang H. et al. 2011). Cultures were established from cerebella of 7-day-old C57BL/6 mice as described in Methods (section 3.1). After 7 days in vitro (DIV), cerebellar slices were exposed to LPC (0.5 mg/ml) for 14-17h, a treatment that causes a dramatic demyelination without axonal or cell death (Birgbauer et al. 2004). Twenty-four hours after removing LPC, cultures were treated with four doses of sildenafil (1 µM) alone or in combination with the cGMP-increasing agents BAY 41-2272 (BAY41; 3 µM), a NO-GC activator, or the glutamate receptor agonist NMDA (30 µM), as indicated in Methods (section 3.1). At 19 DIV, cerebellar slices were harvested and double immune fluorescence-stained for neurofilament (NF200, red) and myelin (MBP, green) and analyzed by confocal microscopy and Image-J

software. Quantification of the area stained for MBP relative to the area stained for NF200 gave a remyelination index (RI). As shown in Fig 2A, LPC-treated slices show a significant reduction of MBP staining compared to control slices, whereas in slices exposed to cGMP-increasing compounds the amount of MBP surrounding neurofilaments notably increased. Quantification of the RI shows that the remyelination induced by sildenafil alone is similar to that attained in combination with BAY41 (BAY41+Sil) or NMDA (NMDA+Sil) (Fig 2B). Remyelination by BAY41 alone was 40% lower than that induced by BAY41+Sil (not shown). To confirm the remyelinating effect of cGMP-increasing treatments in cerebellar slices, we analyzed by WB the levels of MBP, a major myelin protein, and of 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase), a minor CNS myelin-protein that is only expressed in CNS oligodendrocytes. Results show that BAY41+Sil treatment significantly increased both MBP and CNPase (Fig 2C) in LPC-demyelinated cultures. We further analyzed mRNA expression of MBP by qPCR and showed that BAY41+Sil tendency to induced MBP gene expression (Fig 2D).

The presence of contactin-associated protein (Caspr) clusters in the paranodes delimiting myelin sheaths correlates with a proper remyelination of the axons (Fancy et al. 2011, Meffre et al. 2015). To investigate the remyelination status in sildenafil-treated cerebellar slices, double immune-staining for Caspr and NF200 was performed. As shown in Fig 2E, Caspr clusters associated with axons were drastically reduced in LPC-demyelinated cultures. Treatment with BAY41+Sil largely restored Caspr staining levels and distribution (Fig 2E,F).

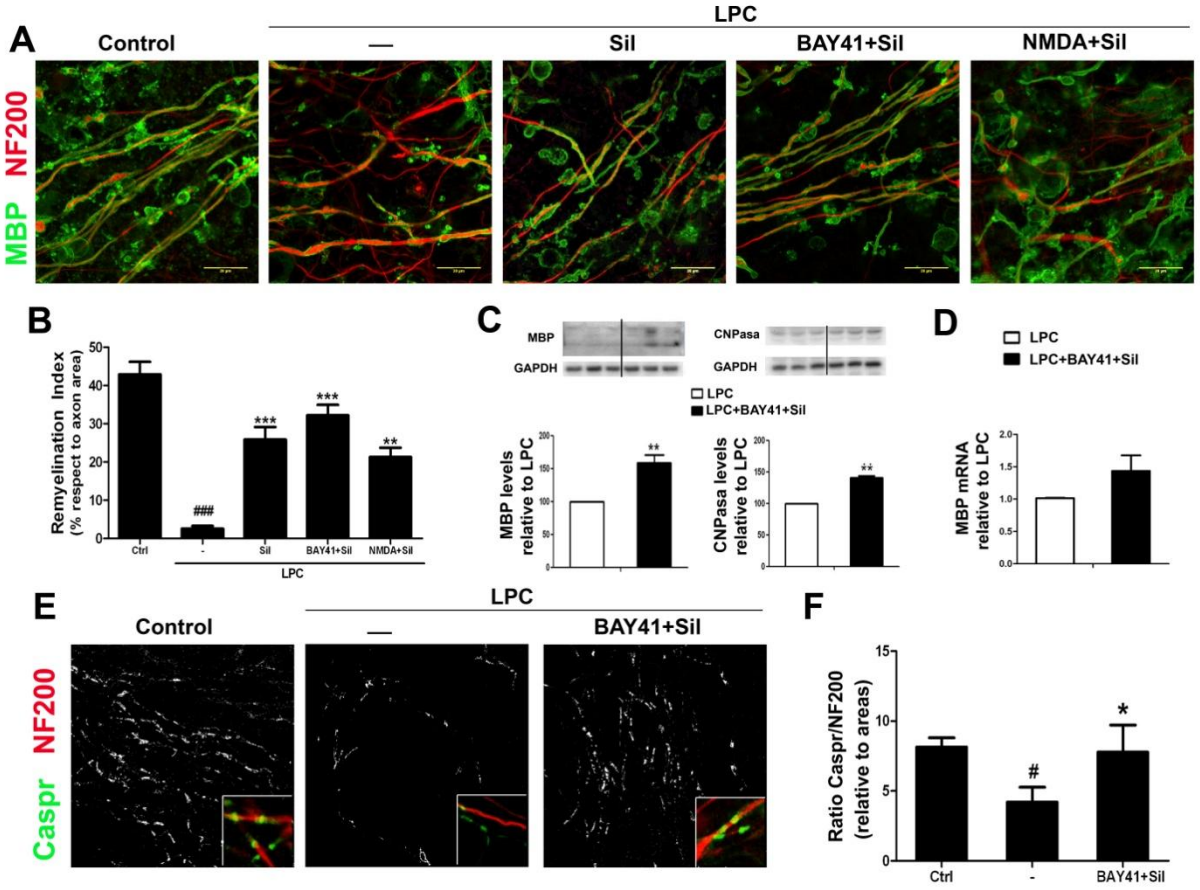

Figure 2: Treatments that increase cGMP promote remyelination in LPC-demyelinated organotypic cultures. (A) Representative images of MBP (green) and NF200 (red) staining in control or LPC (0.5mg/ml)-demyelinated mouse organotypic cerebellar cultures treated or not for 10 days with sildenafil (1 µM) alone or in combination with BAY41 (3 µM) or NMDA (30 µM); Scale bar=20µm. **(B)** Quantification of MBP and NF200 immune-staining overlap (remyelination index) reveals a drastic reduction in LPC-demyelinated cultures and recovery to almost control levels in cultures treated with sildenafil alone or in combination with cGMP synthesis stimuli. Values are means±SEM of three independent experiments. One-way ANOVA followed by Bonferroni's post-hoc analysis reveals statistically significant differences between control and LPC-demyelinated slices ($^{###}p<0.001$) and between LPC-demyelinated slices treated or not with cGMP-increasing compounds (**p<0.01, ***p<0.001). **(C)** Representative WBs and quantification of MBP (n=3) and CNPase (n=2) in LPC-demyelinated slices treated for 10 days with vehicle (LPC) or BAY41+Sil. Protein levels were normalized to GAPDH. The treatment significantly increased the amount of MBP and CNPase protein in LPC-demyelinated cultures; **(D)** MBP mRNA levels, analyzed by qPCR, showed a tendency to increase in LPC-demyelinated slices 24h after the first dose (D1) of BAY41+Sil. Values are means±SEM of three independent experiments. Data is presented relative to LPC. Student's t-test shows no significant differences in BAY41+Sil-treated LPC-demyelinated cultures respect to untreated. **(E)** Colocalization analysis of paranodal protein Caspr (green) and NF200 (red) immune-staining. Representative images of Caspr mascara (white); small panels show Caspr clusters over axons. **(F)** Quantification of area immune-stained for Caspr relative to area stained for NF-200 shows a decrease in LPC-demyelinated slices and restoration to control levels after BAY41+Sil treatment. Values are means±SD (n=5-6 images per condition) in a representative experiment that was replicated with similar results. One-way ANOVA

followed by Bonferroni's post-hoc analysis reveals statistically significant differences between vehicle- and BAY41+sildenafil-treated slices (*p<0.05 vs LPC; #p<0.05 vs control).

3. Mechanisms implicated in the remyelinating effect of sildenafil

3.1. Involvement of the NO-cGMP-PKG pathway in the remyelinating effect of sildenafil in LPC-demyelinated cerebellar cultures

The observation that treatment of LPC-demyelinated cerebellar slices with sildenafil alone produced a similar remyelinating effect than its combination with BAY41 or NMDA, both of which increase cGMP production via NO-GC, suggested that activation of this enzyme was involved in the sildenafil effect. That this was in fact the case was demonstrated by the observation that sildenafil-induced remyelination was prevented in the presence of the NO-GC inhibitor ODQ (10 µM; Fig 3A). Moreover, ODQ treatment decreased the amount of myelin associated with axons in control cultures, suggesting that NO-GC activity may be also implicated in the myelination process that occurs in the immature cerebellar slices during culture (Fig 3A). To investigate if induction of NOS-2 in LPC-demyelinated slices was responsible for the generation of the NO that stimulates NO-GC activity we first examined the effect of L-NNA, a predominant NOS-2 inhibitor, in sildenafil-induced remyelination. As shown in Fig 3B, L-NNA prevented the sildenafil effect. To further examine if NOS-2 was actually induced in the LPC-demyelinated slices we performed immune-fluorescence staining for NOS-2 (green) in cerebellar cultures exposed for 24h to LPC, or to LPS (1 µg/ml) as a positive control. Fig 3C shows that while only a few cells were stained for NOS-2 in control cultures, numerous cells were NOS-2+ in cultures exposed to LPC or LPS.

Cyclic GMP-dependent kinase (PKG) is the main target of cGMP. To examine if PKG was involved in sildenafil-induced remyelination we examined this effect in the presence or absence of the PKG inhibitor Rp-8pCPTcGMP (10 µM). Results showed that the PKG inhibitor prevented the sildenafil effect (Fig 3D) implicating the kinase in the NO-cGMP signaling cascade.

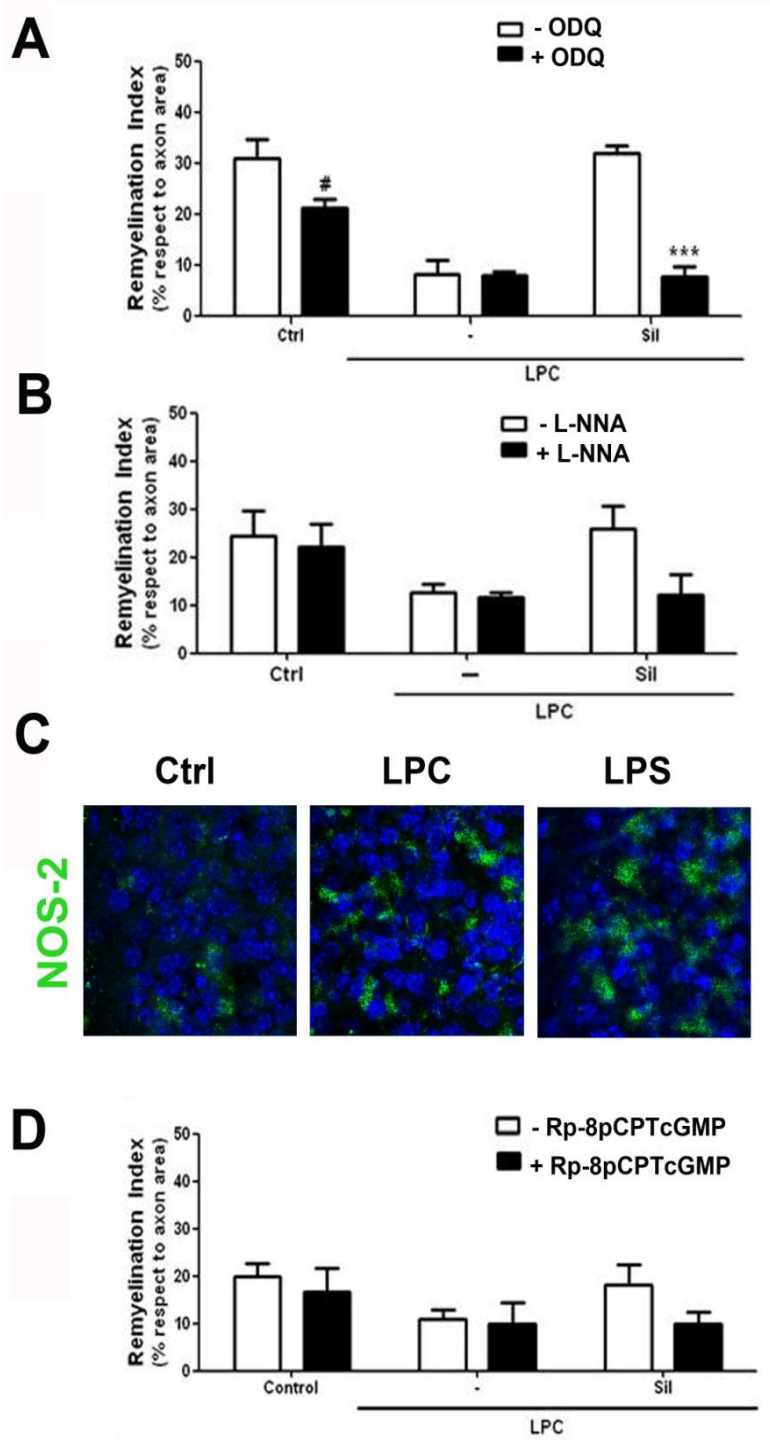

Figure 3: The remyelinating effect of sildenafil in LPC-demyelinated cultures is prevented by inhibitors of NO-GC, NOS-2 and PKG activities. The remyelination index pre- and post-LPC was quantified in cerebellar slices treated 10 DIV post-LPC with sildenafil (1 µM) in the presence of: **(A)** NO-GC inhibitor ODQ (10 µM), n=2; **(B)** NOS-2 inhibitor L-NNA (100 µM), n=3. Values are means±SEM. Two-way ANOVA followed by Bonferroni's post-hoc analysis reveals statistically significant differences in the absence or presence of ODQ in controls ($^{\#}p<0.05$) and in sildenafil-treated (***P<0.001) slices. **(C)** NOS-2 immune-staining (green) reveals similar levels of induction in LPC-demyelinated slices and in 24h LPS-treated slices. Cell nuclei stained with DAPI (blue). **(D)** PKG inhibitor Rp-8pCPT-cGMP (10 µM). Representative figure replicated with similar results. Values are means±SD (n=5-6 images per condition).

3.2. Increasing cGMP promotes oligodendrocyte differentiation in the spinal cord of EAE mice and in LPC-demyelinated cerebellar cultures

Previous results have shown that treatment with sildenafil to MOG-immunized mice at the initiation of EAE symptoms prevents disease progression, preserves axons and myelin and protects immature and mature OLs (Pifarre et al. 2014). We have now examined if sildenafil affects OL maturation in the spinal cord of EAE animals treated with sildenafil for 15 days at peak disease by immune-staining for transcription factors involved in early (Nkx2.2) and late stages of OL differentiation (Olig2) and for the mature OL marker MBP. As shown in Fig 4A, staining intensity of Nkx2.2+-cells (green) is increased after sildenafil treatment. Quantification of bright Nkx2.2 cells (high expression) confirms a significant increase respect to vehicle-treated animals (Fig 4A). Double immune-staining for olig2 (red) and MBP (green) shows that after 15 days of treatment from peak disease olig2+/MBP+-cells (yellow arrows) and olig2-/MBP+-cells (green arrows) significantly increase in both vehicle-treated and sildenafil-treated animals (Fig 4B). In both cases the number of positive cells was higher in sildenafil-treated animal compared to vehicle-treated animals. The percentage of Olig2-/MBP+ cells respect to total MBP+ cells was significantly increased in thoracic SC sections after 15 days of sildenafil treatment respect to vehicle (Fig 4B, right panel). A significant increase in the number olig2-/MBP+cells relative to total MBP+cells was also observed in LPC-demyelinated cerebellar cultures after 10 days of treatment with BAY41+Sil or NMDA+Sil (Fig 4C). Taken together these results indicate that stimulation of OL maturation may be an important mechanism contributing to the remyelinating effect of sildeanfil.

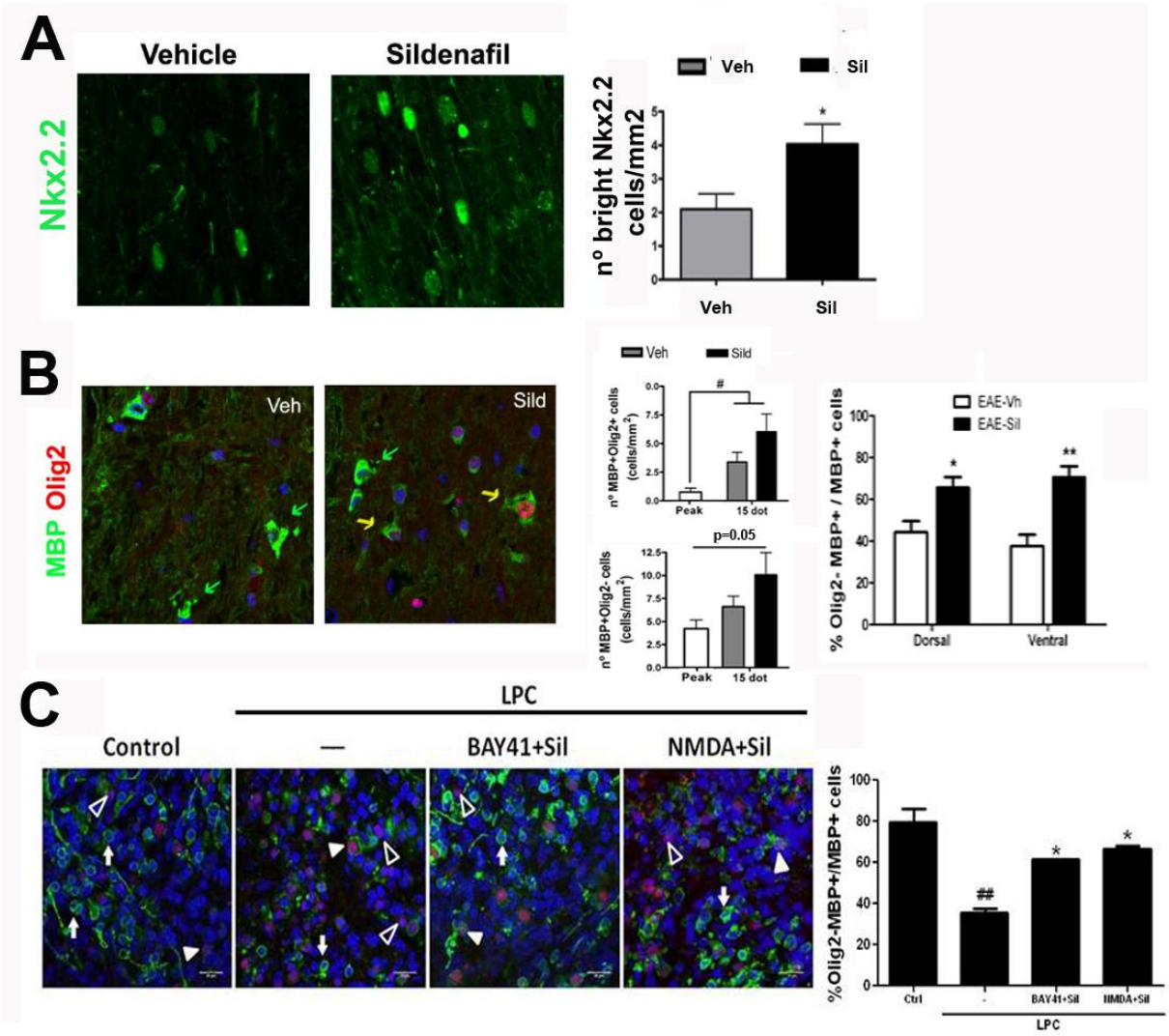

Figure 4: Increasing cGMP promotes oligodendrocyte differentiation in the spinal cord of EAE mice and in LPC-demyelinated cerebellar cultures. (A) Representative images of immune-staining for Nkx2.2, a transcription factor involved in early stages of OL differentiation, in the SC of EAE mice treated with vehicle or sildenafil for 15 days starting at peak disease. Quantification of bright Nkx2.2 cells (high expression) shows a significant increase after sildenafil treatment. Values are mean±SEM (n=3-6 mice per group) analyzed by Student's t-test (*p<0.05); **(B)** Immune-staining for MBP (green) and Olig2 (red), a transcription factor expressed by differentiating oligodendrocyte lineage cells, images taken at 63X (left panel). Quantification of the number of MPB+/Olig2+ (yellow arrow) and MPB+/Olig2- (green arrow) is shown in the center panel. The percentage of Olig2-/MBP+ cells respect to total MBP+ cells in thoracic SC sections is shown in the right panel. Values are mean±SEM (n=3-5 mice per group) and were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test (*p<0.05, **p<0.01). **(C)** MBP and Olig2 staining in LPC-demyelinated cerebellar cultures treated or not with Sil (1 µM)+BAY41 (3 µM) or NMDA (30 µM). Open arrow heads: Olig2+/MBP- (immature OLs; open arrow heads); Olig2+/MBP+ (early mature OLs; arrow heads); Olig2-/MBP+ (mature myelinating OLs; arrow). Scale bar=

20µm. The percentage of Olig2-/MBP+ respect to total MBP+ cells is significantly increased by cGMP-increasing treatments (right graph). Values are mean±SEM of two independent experiments, analyzed by one-way ANOVA followed by Bonferroni's post hoc (*$p<0.05$ respect to LPC; $^{\#\#}p<0.01$ respect to control).

3.3. Sildenafil increases CNTF expression in spinal cord of EAE mice and in cerebellar organotypic cultures.

Increased expression of trophic factors is associated with neuroprotective effects, oligodendrocyte support and proliferation and axon remyelination (De Santi et al. 2009, Weishaupt et al. 2012). CNTF has a recognized influence on OL differentation (Stankoff et al. 2002). Moreover, it has been shown that this growth factor acts as a quemoatractant controling OL progenitor migration during (Vernerey et al. 2013). Interestingly, recent in vitro studies demonstrated that the NO-cGMP-protein kinase G pathway up-regulates CNTF expression in astrocytes and that astrocyte-derived CNTF enhances oligodendrocyte (OL) differentiation (Paintlia et al. 2013). In this work, using double immune-staining for CNTF and the astrocyte protein GFAP, we have observed a strong CNTF-staining in highly reactive astrocytes in the spinal cord of EAE mice (Fig 5A). We further show that treatment with sildenafil for 15 days at peak disease strongly enhances CNTF immune-staining compared to vehicle-treated animals (Fig 5B). In agreement with these results, we have also observed a significant increase in CNTF mRNA expression in LPC-demyelinated cerebellar slices 24h after the first dose (D1) of Sil (1µM)+ BAY41 (3 µM), but not after the third dose, suggesting that CNTF induction may be is an early event contributing to the remyelinating effect of cGMP-increasing compounds (Fig 5C).

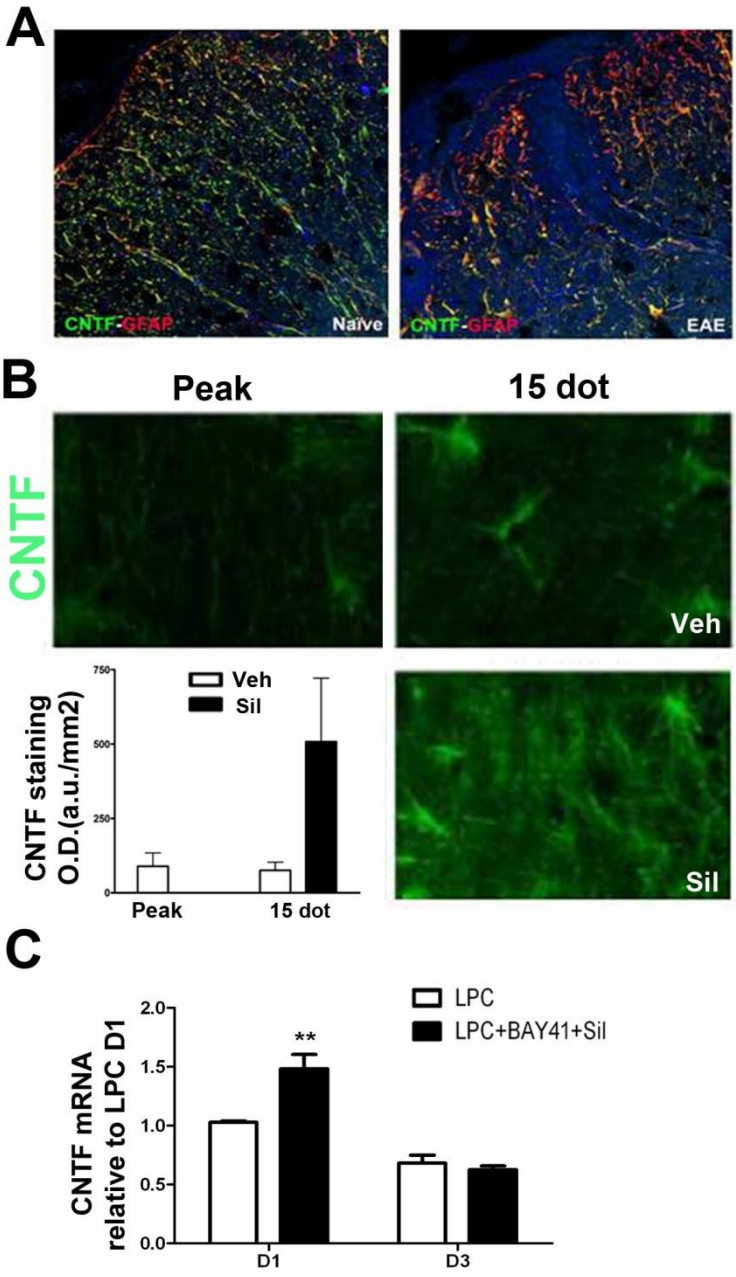

Figure 5: Sildenafil treatment increases CNTF expression in the spinal cord of EAE mice and in LPC-demyelinated cerebellar cultures. (A) Colocalization of GFAP (red) and CNTF (green) immune-staining in coronal sections of cervical spinal cord from naïve and EAE animals showing CNTF increased expression in reactive astrocytes; **(B)** Treatment with sildenafil for 15 days at peak EAE further increases CTNF levels when compared with vehicle-treated EAE animals Values are mean±SEM (n=5 per group); **(C)** CNTF mRNA levels assessed by qPCR in LPC-demyelinated cerebellar cultures 24h after the first (D1) and third (D3) doses of BAY41 (3 µM)+Sil (1 µM), showing a significant increase of CNTF mRNA expression after the first dose. Values are mean±SEM of three independent experiments. Data shown is relative to LPC. Two-way ANOVA followed by Bonferroni's post-hoc analysis reveals statistically significant differences respect to LPC(**$p<0.01$).

3.4. Treatments that increase cGMP induce changes in the inflammatory microglial phenotype in LPC-demyelinated cerebellar cultures.

It has been reported that in the cuprizone-induced demyelinization mouse model that treatment with sildenafil reduces expression of the inflammatory cytokines IL-1β and TNFα and increases the expression of the anti-inflammatory cytokine IL-10 in cerebella (Nunes et al. 2015). Additionally it has been shown that treatment with sildenafil to EAE mice for 7 days starting at disease onset induced a decrease in the release of the pro-inflammatory cytokines IL-2, IFNγ and TNFα in splenocytes. Furthermore, RNA microarray analysis of spinal cord and immune-staining revealed that sildenafil up-regulates YM-1 (Pifarré et al. 2014), a marker of the alternative macrophage/microglial M2 phenotype that has neuroprotective and regenerative properties. To gain insight into the possible implication of a microglial phenotype change in the remyelinating effect of cGMP-increasing compounds, we have examined by qPCR mRNA expression of M1 (NOS-2 and COX-2) and M2 phenotype markers (Arg-1 and CD206) and of pro-inflammatory (IL-1β, TNFα, IL-6) and anti-inflammatory (IL-10) cytokines in LPS-demyelinated cerebellar slices 24h after the first and third doses of Sil (1 µM)+ BAY41 (3 µM). Results showed that mRNA expression of phenotype marker molecules in LPC-demyelinated slices do not significantly change between dose one (D1) and three (D3) of vehicle (Fig 6). In BAY41+Sil-treated slices there was a tendency to increase in Arg1 and COX-2 mRNA, but not in CD206 or NOS-2 mRNA after D1 and no change was observed after D3, except for a tendency to decrease in NOS-2 expression (Fig 6).

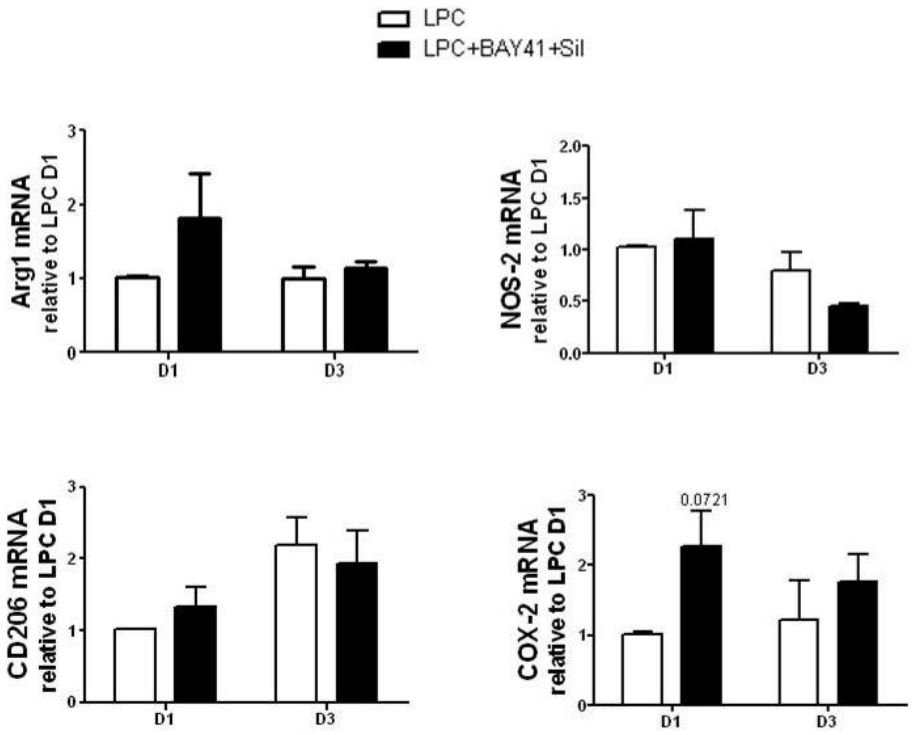

Figure 6: Effect of sildenafil treatment on microglia inflammatory phenotype markers in LPC-demyelinated cerebellar cultures. mRNA levels of M1 (NOS-2 and COX-2) and M2 (Arg-1 and CD206) phenotypic markers determined by qPCR in LPC-demyelinated slices treated or not with BAY41(3µM)+Sil(1µM). mRNA was collected 24h after first (D1) and third (D3) dose. Values are mean fold change±SEM of three independent experiments. Two-way ANOVA followed by Bonferroni's post-hoc analysis.

Regarding cytokines (Fig 7), again no change was observed in any of the cytokines from D1 to D3 in vehicle-treated slices. BAY41+Sil treatment induced a significant increase in IL-10 and IL-6 after D1, the larger increase corresponding to IL-6 (2-fold). After D3, no significant changes were observed in any of the cytokines, although a tendency to decrease was apparent in IL-1β, TNFα and IL-10. Taken together these results suggest that a M2-like phenotype is induced after the first dose of cGMP stimuli (increases in Arg1 and IL-10) that also shows expression of IL-6 and COX-2.

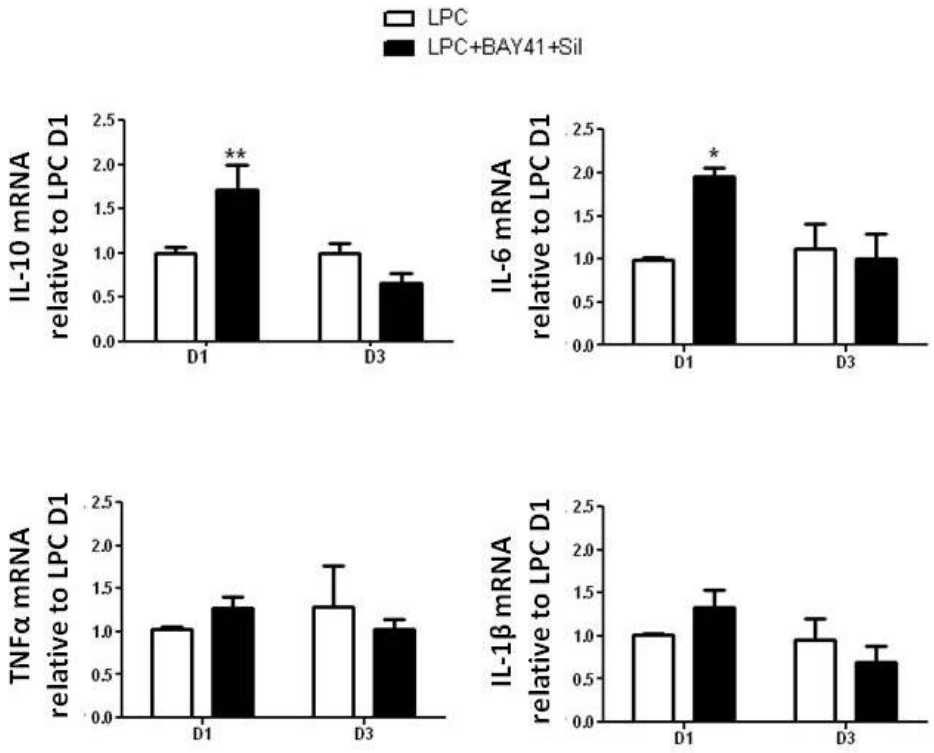

Figure 7: Sildenafil treatment induces changes in cytokine expression in LPC-demyelinated cerebellar cultures. mRNA levels of inflammatory cytokines assessed by qPCR in LPC-demyelinated cerebellar cultures 24h after the first (D1) and third (D3) doses of BAY41 (3µM)+Sil (1µM), showing a significant increase in IL-6 and IL-10 cytokines after the first dose. IL-6, IL-1β and TNFα are values are mean fold change±SEM of three independent experiments. IL-10 is a representative figure replicated with similar results; values are mean fold change±SD of triplicates. Two-way ANOVA followed by Bonferroni's post hoc analysis reveals statistically significant differences respect to LPC (*p<0.05, **p<0.01).

3.5 Treatments that increase cGMP induce phenotype changes in BMDM

LPS-demyelinated cerebellar cultures are a complex system to study inflammatory phenotypic changes induced in microglia and the results presented above on the effect of treatment with cGMP-increasing stimuli are not easy to interpret. In order to directly examine the potential of cGMP-increasing compounds to modulate the inflammatory phenotype in monocytes, we used as model mice bone marrow derived macrophage (BMDM) primary cultures. These cells are readily polarized to a M1 phenotype by incubation

with LPS (10-100 ng/ml)+IFNγ (20 ng/ml) and to a M2 phenotype by incubation with IL-4 (10-20 ng/ml) (Classen et al. 2009). These conditions were used as controls of phenotypic changes

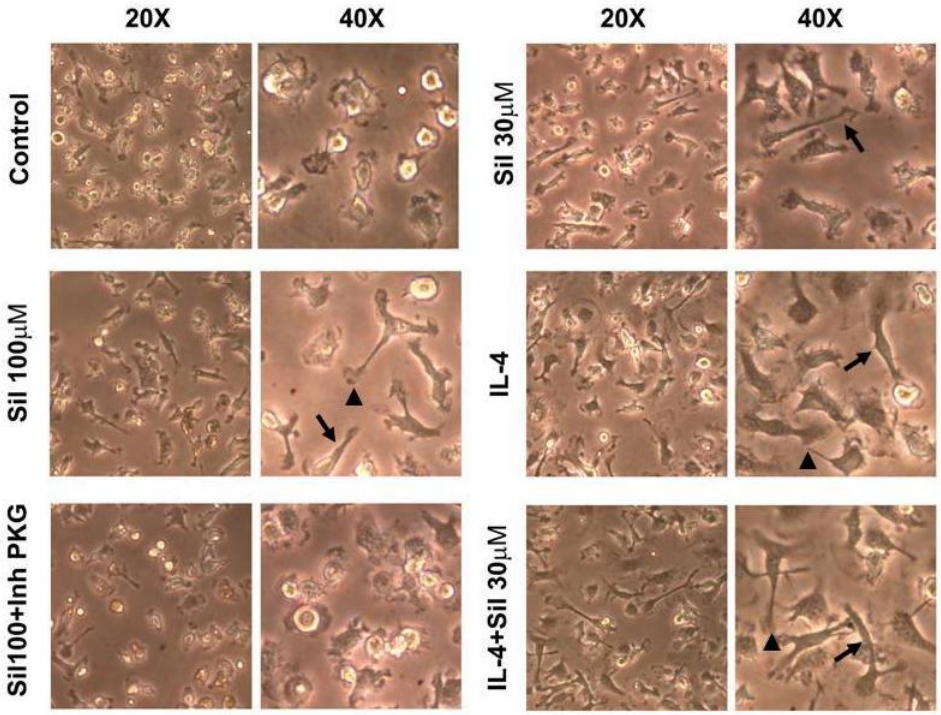

Fig 8: Sildenafil induces a morphological change in BMDM from C57Bl/6 mice. Treatment of BMDM primary cultures with sildenafil for 24 h induced process elongation (arrow) and lamellipodia (arrow head) formation in a concentration-dependent manner (30-100μM). This effect was prevented by the specific protein kinase G (PKG) inhibitor Rp-8pCPTcGMP (1μM) added 30 min before (lower panel, left). The morphological change induced by sildenafil was similar but not identical to that produced by IL-4 (20 ng/ml), an inducer of the M2 phenotype (upper and middle panels). Moreover, sildenafil potentiated the IL-4 effect (lower panel, right).

As shown in Fig 8, when BMDM were treated with sildenafil (30-100 μM) for 24h the morphology of cells changed from a more rounded/amoeboid type to an elongated shape similar to that acquired when BMDM are treated with IL-4 (Durafourt et al. 2012, McWhorter et al. 2013, Vereyken et al. 2011). Moreover, the combination of IL-4 (20 ng/ml) with sildenafil (30 μM) produced

an even more elongated shape (Fig 8). When sildenafil (100 µM) was combined with the PKG inhibitor Rp-8pCPTcGMP (1 µM) cell elongation was prevented.

Since sildenafil treatment induced a morphological change similar to that induced by IL-4 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 that is increased by M2 stimuli (Classen et al. 2009). Cultures were treated with sildenafil alone (1-100 µM) or in combination (added 60 min before) with to LPS+IFNγ or IL-4. After 24h, nitrite concentration in the media and arginase activity in cell extracts were measured. No changes were observed in the concentration of nitrites in the media of cells treated with sildenafil or IL-4 alone or in combination (Fig 9A). As expected, LPS+IFNγ induced a large increase in nitrite concentration that was not significantly affected by the presence of sildenafil, although at the highest concentration (100 µM) used there was a tendency to decrease the LPS effect (Fig 9A). In contrast, arginase activity was increased not only by the M2 stimulus IL-4, but also by LPS+IFNγ (Fig 9B). Sildenafil at the highest concentrations used (30-100 µM) also induced a small non-significant increase in arginase activity in control cells and a small potentiation of the effect of LPS+IFNγ. However, when combined with IL-4 sildenafil produced a large potentiation of the cytokine effect (Fig 9B).

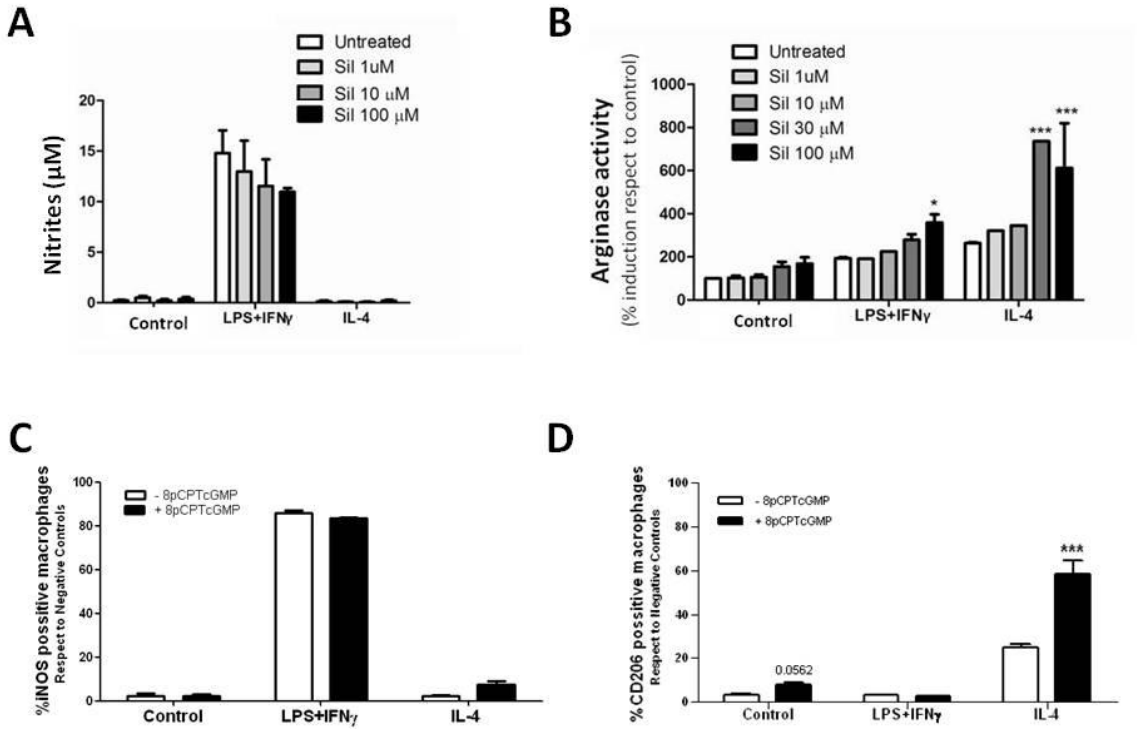

Figure 9: Sildenafil alters the inflammatory phenotype of mouse BMDM. (A-B) Primary cultures of BMDM were incubated for 24h with LPS (100 ng/ml)+INFγ (20 ng/ml) to induce a M1 phenotype, with IL-4 (20 ng/ml) to induce a M2 phenotype, or with sildenafil (1-100 μM) alone or in combination with the inflammatory stimuli (added 30 min before); **(A)** Nitrite accumulation in the media was only induced by LPS+INFγ and was not significantly affected by combination with sildenafil; **(B)** Arginase activity was slightly increased in cells exposed to LPS+INFγ and IL-4. Sildenafil (30-100 μM) alone was little effective but potentiated the effect of the inflammatory stimuli, in particular that of IL-4. Values are mean±SEM of three independent experiments. Two-way ANOVA followed by Bonferroni's post-hoc analysis revealed statistically significant differences respect to untreated cells (*p<0.05;***p<0.001); **(C-D)** FACS analysis of the percentage of BMDM expressing the M1 phenotype marker NOS-2 (n=2) or the M2 phenotype marker CD206 (n=3). BMDM were left untreated (control) or treated for 48h with LPS (10 ng/ml)+IFNγ (20 ng/ml), IL-4 (10 ng/ml) or the cGMP analogue 8pCPTcGMP (50 μM) alone or in combination with the inflammatory stimuli, added 30 min before; **(C)** The % of cells expressing NOS-2 was notably increased in LPS+IFNγ-treated cells and this was not affected by the cGMP analogue. **(D)** The % of cells expressing CD206 was increased by IL-4. The cGMP analogue had a small effect and notably potentiated the effect of IL-4. Values are means±SEM analyzed by two-way ANOVA followed by Bonferroni's post-hoc analysis revealed statistically significant differences (***p<0.001) respect to cells not exposed to the cGMP analogue.

These results indicate that sildenafil can promote a M2 phenotype but not properly induce a shift M1 to M2. In order to confirm these results 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 (added 30 min before) with of LPS (10 ng/ml)+IFNγ (20 ng/ml) or IL-4 (10 ng/ml) for 48h. As shown in Fig 9C, 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 the cGMP analogue or IL-4, alone or in combination. In agreement with the nitrite data, 8pCPTcGMP did not affect the percentage of cells expressing NOS-2 expression in LPS+IFNγ-treated BMDM (Fig 9C). As expected, the number of cells expressing the M2 marker CD206 was increased in BMDM treated with IL-4. The cGMP analogue alone also increased the number of CD206-expressing cells to a small extend and significantly potentiated the effect of IL-4, doubling the number of CD206+ cells, in agreement with the sildenafil effect on arginase activity (Fig 9D). In contrast, no change in the number of CD206+ cells was observed in LPS+IFNγ-treated BMDM (Fig 9D). Furthermore, we analyzed the effect of the cGMP analogue on levels of the pro-inflammatory cytokine (IL-1β) and the anti-inflammatory cytokine (IL-10) in the media of BMDM 18h after treatment with the same stimuli using Luminex technology. Surprisingly, results showed a large increase in both cytokines in the media of LPS+IFNγ-treated but not in IL-4-treated BMDM (Fig 10). The cGMP analogue alone induced a small increase in IL-10 release in control cells and a larger increase in IL-4-treated cells and significantly inhibited the increase in both cytokines induced by LPS+INFγ but that of IL-1β to a much larger extend (Fig 10). These results support the contention that in BMDM cGMP promotes an anti-inflammatory phenotype.

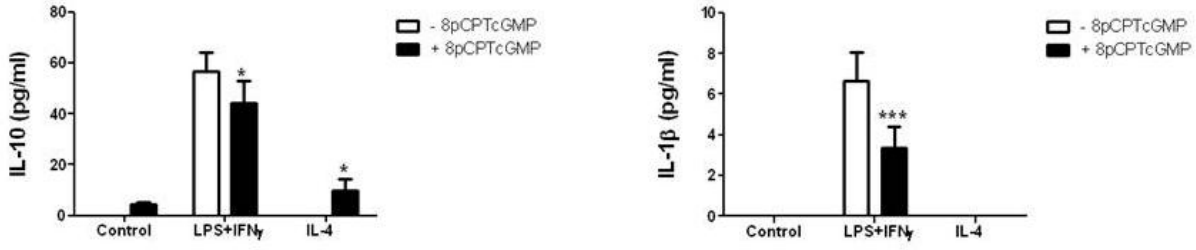

Figure 10: Increasing cGMP induces changes in pro-and anti-inflammatory cytokine release from BMDM. Cytokine was determined in BMDM media by Luminex technology. Cells were left untreated (control) or treated for 18h with LPS (10 ng/ml)+IFNγ (20 ng/ml), IL-4 (10 ng/ml) or 8pCPTcGMP (50 µM) alone or in combination with the inflammatory stimuli, added 30min before. Levels of both the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokine IL-1β were increased by LPS+INFγ but were not affected by IL-4. The cGMP analogue induced a small but significant decrease in IL-10 levels and a larger decrease in IL-1β in LPS+INFγ-treated cells. However, in cells treated with IL-4 the analogue significantly increased IL-10 release. Results are representative of two independent experiments; values are means±SD, of two independent experiments. Two-way ANOVA followed by Bonferroni's post-hoc analysis revealed statistically significant differences (*p<0.05, ***p<0.001) respect to cells not exposed to the cGMP analogue.

3.6. Sildenafil increases myelin phagocytosis in microglia/macrophages in spinal cord of EAE animals and in BMDM

It has been reported that in demyelinating disorders, such as MS, myelin debris interferes with the remyelination process (Kotter et al. 2006). Myelin clearance correlates with increased remyelination and differentiation of OPCs (Kotter et al. 2006, Miron and Franklin 2014). Furthermore, recent reports indicate that M2 cells have higher myelin phagocytic activity than M1 cells (Durafourt et al. 2012) and that M2 cell polarization is essential for efficient remyelination (Miron et al. 2013). To investigate if sildenafil treatment affects myelin debris phagocytosis in EAE mice we performed Oil-red O (ORO) staining in longitudinal SC sections. Results showed that 15 days of sildenafil (10mg/kg)

treatment starting after disease onset increased the amount of ORO-loaded vesicles in infiltrates (Fig 11 A-D). Quantification of the amount of ORO-loaded vesicles relative to infiltrated area in infiltrates of different sizes showed a significant increase in sildenafil-treated mice compared to vehicle-treated controls (Fig 11E). To investigate if this lipid-loaded vesicles stained by ORO were inside M2 cells, SC sections were double-stained for the M2-phenotype marker YM-1 and with ORO and were visualized by confocal microscopy. Confocal image analysis followed by 3D-reconstruction of images showed ORO in vesicles inside Ym-1 possitive macrophage/microgial cells (Fig 11F).

To investigate if sildenafil has a direct effect on the myelin phagocytic capacity of macrophages, a myelin phagocytosis assay was performed on mouse BMDM. Cells were treated with the cGMP analogue 8Br-cGMP (500 µM) or, sildenafil (30-100 µM) alone or in combination with IL-4 (20 ng/ml). After 24h, cells were incubated with Dil-labelled myelin (25 µg/ml) for 90 min and myelin uptake was analyzed by flow cytometry. IL-4, the cGMP analogue and sildenafil significantly alone increased myelin phagocytosis (Fig 11G). The effect of cGMP-increasing stimuli alone was of higher magnitude than that induced by IL-4 and when added together their effects were a little less than additive.

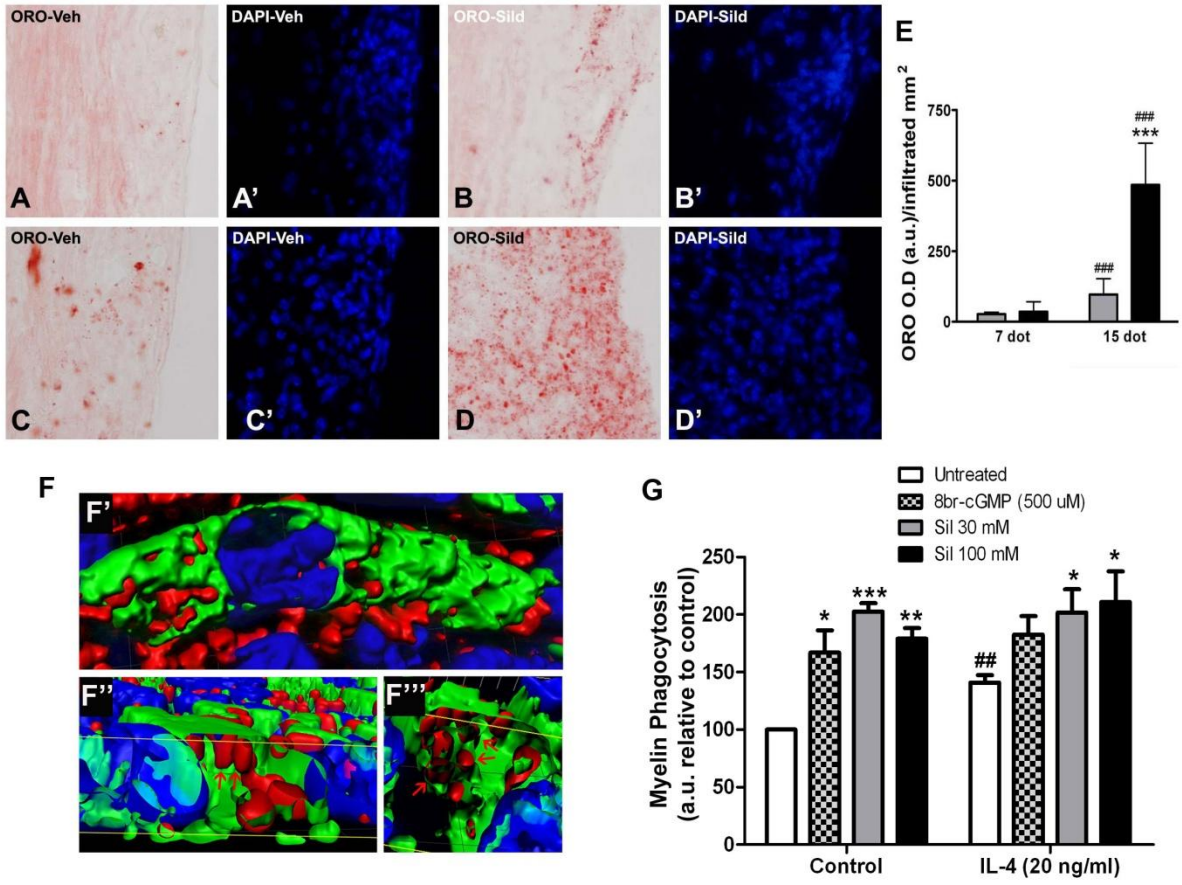

Fig 11. Sildenafil treatment enhances myelin phagocytosis in macrophages/microglia in the spinal cord of EAE animals and in mouse BMDM cultures. (A-D) Phagocytosis of myelin in the spinal cord of EAE animals evidenced by vesicular Oil-red O (ORO) staining. Representative images of ORO staining in infiltrates (cell nuclei stained with DAPI) of different sizes in animals treated with vehicle (A,C) or sildenafil (B, D) for 7 or 15 days (dot) starting at onset of disease; **(E)** Quantification reveals that sildenafil significantly enhances ORO staining after 15 dot. Values are means±SEM (n=4-6). Two-way ANOVA followed by Bonferroni's post-hoc analysis reveals statistically significant differences respect to EAE vehicle-treated animals after 15 dot (*p<0.05). **(F)** 3D rendering of macrophage/microgial cells positive for the M2-phenotype marker YM-1 (green) containing ORO in vesicles (red) in the spinal cord of sildenafil-treated EAE animals; Cell nuclei are shown in blue. **(G)** BMDM were treated for 24h with the cGMP analogue 8Br-cGMP (500 µM) or sildenafil (30 µM-100 µM) alone or in combination with IL-4 (20 ng/ml) and were exposed to Dil-labeled myelin for 90min. Myelin phagocytosis was analyzed by flow-cytometry. Values are means±SEM of three independent experiments. Two-way ANOVA followed by Bonferroni's post-hoc analysis reveals statistically significant differences respect to untreated (*p<0.05, **p<0.01, ***p<0.001) and respect to untreated control ($^{\#\#}$ p<0.01).

DISCUSSION

DISCUSSION

In MS, inflammation, demyelination and axonal damage are important hallmarks of the pathology (Henderson et al. 2009, Lassmann 2014, Peterson and Fujinami 2007). This inflammatory and neurodegenerative nature of MS generates the necessity of the development of treatments that combine immune-modulatory and neuroprotective effects (Aharoni 2010).

In MS models, it has been observed that sildenafil improve clinical symptoms and disease progression. In EAE mice treated for 8 days with sildenafil starting at the acute phase (peak) of the disease, showed significantly amelioration of clinical symptoms associated with an increase in myelin staining and decreased of axonal loss, suggesting that sildenafil may be promoting remyelination and axonal protection; moreover, it was shown that 3 days after sildenafil treatment reduced the amount of infiltrating cells, increased the number of Foxp3+ lymphocyte T regs and decreased microglia/macrophage activation (Pifarre et al. 2011). Sildenafil effects on remyelination were further studied by curpizone demyelination model, showing as well a significant amelioration of clinical symptoms associated with a protective effect on myelin structure, reduction of reactive gliosis (microglia and astrocytes) and decreased expression of pro-inflammatory cytokines (Nunes et al. 2012). Furthermore, treatment with sildenafil starting at the onset of EAE has shown to prevent the disease progression, to maintain myelin and axon structure, decrease microglial activation, down-regulate the innate and adaptative responses decreasing pro-inflammatory cytokine production and proliferation in splenocyte, and has shown to upregulate the alternative

microglia/macrophage activation marker Ym-1 associated with neuroregenerative effects (Pifarre et al. 2014).

The results obtained in this thesis showed that sildenafil treatment in de acute phase indeed ameliorates clinical symptoms decreasing clinical score close to 1, and that extending the treatment from 8 to 15 days does not show further improvement. After 15 days of treatment increase MBP staining was observed, suggesting remyelination enhancement as seen previously (Pifarre et al. 2011). The results obtained also showed that when sildenafil treatment starts at the chronic phase of EAE stills promotes significant amelioration of the symptoms. Further analysis demonstrated that sildenafil promotes remyelination increasing MBP levels after 15 dot starting at chronic phase. This result has clinical significance since it shows that sildenafil exerts remyelinating and neuroprotective effects once EAE becomes chronic, when more infiltrating cells, more lesions and an increased grade of demyelination can be found (Berard et al. 2010).

Taking in account these results and with the aim to study mechanisms involved in sildenafil effects on CNS cells, mouse cerebellar organotypic cultures were established. EAE is induced by an auto-immune reaction against myelin peptide that promotes an acute attack by peripheral immune cells that infiltrate the CNS which is followed by demyelination (Constantinescu et al. 2011, McCarthy et al. 2012). On the other hand, cerebellar organotypic cultures demyelinated with lysophosphatidylcholine (LPC) have proven to be an excellent model to study demyelination-remyelination process without axonal death and excluding the influence of the immune system. This model is accessible to experimental perturbation, which allows testing the effect of different stimuli on the mechanisms that might be implied in the processes that

wants to be studied (Birgbauer et al. 2004, Zhang H. et al. 2011). After LPC demyelination, microglia activation can be observed, myelin debris is generated as a consequence of demyelination and naked axons sometimes suffer damage which is evident by the presence of axonal bulbs, Caspr proteins are no longer visible, OPCs are present in the slices and after demyelination OPCs proliferation is induced (Zhang H. et al. 2011).

The results obtained in this work showed almost complete demyelination of axons after LPC treatment; however after 10 days of treatment with sildenafil myelin sheath reappeared increasing the remyelination index, along with Caspr clusters. Combination of sildenafil and BAY41, a direct activator of NO-GC, revealed a significant increase in myelin protein levels; however no significantly effects where observed in MBP mRNA expression; however, no further increase in remyelination index were observed compared with sildenafil alone. Similar effects on the remyelination index were observed after NMDA treatment in combination with sildenafil. NMDA is a glutamate analogue that activates this neurotransmitter receptors (NMDA-R), in cerebellum present primarily in granular neurons and can be also found in astrocytes and oligodendrocytes. Activation of NMDA-R allows calcium to entry into the cell and the activation of the constitutive form of NOS (NOS-1) and further NO production (Maarsingh et al. 2009, Manucha 2016, Verkhratsky and Kirchhoff 2007). This NO can diffuse across the plasmatic membrane generating cGMP not only in NMDA-R positive cells but also in neighbor cells.

One possible molecular-signaling pathway involved in the increase in remyelination by sildenafil is the NO-cGMP-PKG pathway. In the CNS, NO has been associated with the modulation of synaptic plasticity, brain development, visual and sensory processing, neuro-endocrine secretion and cerebral blood

flow (Garthwaite J. 2000, Guix et al. 2005). Constitutive NOS (NOS-1 and NOS-3) produce small amounts of NO under physiological conditions (Maarsingh et al. 2009, Manucha 2016). On the other hand, NO has been also described as a molecule important in the inflammation modulation, considered as a neuropathological agent responsible for excitotoxic cell death and neuroinflammatory cell damage in many neurological disorders (Duncan and Heales 2005, Murphy 2000). Moreover, one of NO targets is the NO-GC, its binding induces NO-GC activation and further cGMP production. NO-cGMP pathway impairment may be implied in neurological dysfunctions, decreased astroglial expression of the NO-GC β subunit was observed in astrocytes of post-mortem brains of different neurodegenerative diseases' patients (Baltrons et al. 2004). We have observed that effect of sildenafil on remyelination index was inhibited by NO-GC inhibitor (ODQ) and NOS inhibitor (L-NNA) treatments. Similar effects were found when PKG was inhibited with Rp-8pCPTcGMP. On the other hand, immune stainings revealed an increase in NOS-2 after 24h of LPC treatment in organotypic cultures. As said before NOS-2 is induced under pro-inflammatory conditions, LPC demyelination in organotypic cultures leave axons exposed, myelin debris and microglia activation, characteristics of neuroinflammation (Zhang H. et al. 2011). NOS-2 produces higher and longer-lasting amounts of NO after induction and can be expressed primarily in microglia and astrocytes cells (Brown and Neher 2010, García 2004, Maarsingh et al. 2009, Murphy 2000) suggesting that LPC treatment may be responsible of cGMP and NO increase, which can be in part promoting sildenafil effects. Furthermore, PKG targets important pathways involved in remyelination and neuroprotection, such as MEK/ERK and Akt/MTOR pathways (Dai et al. 2014, Guardiola-Diaz et al. 2012). Taking together, these results demonstrate that sildenafil treatment increases production of myelin by actions exerted directly

on CNS cells, restoring myelin sheath and mature internodes which is associated with functional remyelination (Meffre et al. 2015, Zhang H. et al. 2011) suggesting that NO-cGMP-PKG pathway activation is required to promote sildenafil effects.

In relapsing-remiting MS demyelinating episodes are characterized by the death of OLs followed by new OLs generation and migration, but with the recurrence of this episodes decreases their capacity of generation, which in consequence affects remyelination (Copray et al. 2006, Lassmann 2014); however, previous results have shown that sildenafil protects OLs from apoptotic death in EAE model (Pifarre et al. 2014). Taking previous and new results into account, in this work we study the effect of sildenafil in OLs maturation in organotypic cerebellar cultures. OLs express a series of specific molecules, which allows determining their differentiation or maturation stage. Neuron-Glial precursor cells (NG2+) are observed in cerebellar organotypic cultures (Zhang H. et al. 2011). OPCs can also be found in this model, expressing molecules such as: transcriptional factor Nkx2.2 and Platlet Derived Growth Factor α Receptor (PDGFαR) among others. Transcriptional factors Olig1 and Olig2 are express during early to late differentiation stages of immature OLs. More mature OLs express Myelin Basic Protein (MBP), Proteolipid Protein (PLP), Myelin Oligodendrocyte Glycoprotein (MOG), Myelin Associated Glycoprotein (MAG) and Cyclic Nucleotide Phosphodiesterase (CNPase) among others (Fig 12) (Copray et al. 2006, Liu et al. 2007, Meffre et al. 2015, Miron et al. 2010, Nishiyama et al. 2009, Zhang H. et al. 2011).

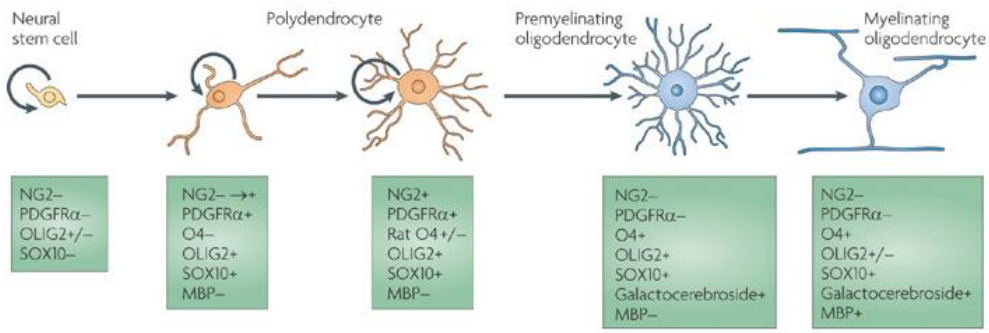

Figure 12: Olygodendrocyte maturation markers expression. Figure modified from Akiko Nishiyama, Mila Komitova, Ryusuke Suzuki & Xiaoqin Zhu; Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. Nature Reviews Neuroscience 10, 9-22 (January 2009). Molecules and protein expression of oligodendroglial cells throughout development (Nishiyama et al. 2009).

Our results showed that sildenafil treatment significantly increased the expression of OPCs transcription factor involved in early stage of OL differentiation (Nkx2.2+) and showed a tendency increased the number of mature OLs (Olig2+MBP+) as well as the number late stage mature OLs (Olig2-MBP+) in SC of EAE mice. Moreover, when ventral and dorsal thoracic sections of EAE mice SC where analyzed, an increase number of late stage mature OLs (Olig2-MBP+) relative to the total MBP+ mature OLs was found in sildenafil treated group. This result was further confirmed in organotypic cultures, where increased number of late stage mature oligodendrocytes relative to the total of mature oligodendrocytes was found with sildenafil treatment combined with BAY41 and with NMDA. Taking together this results demonstrate that cGMP increased by sildenafil is promoting maturation of OLs. In agreement with these results, other studies have demonstrated that an increase in intracellular cGMP derived from NO-GC in OLs promotes their maturation and arborization, extending the reach of each cell in cerebellar slices, the source of NO in this model was partially attributed to an increase production of NO in neighbor granular neurons via NMDA-R activation (Garthwaite G. et al. 2015); however

recent studies have shown that OLs can also express NMDA receptors specially in their processes (Salter and Fern 2005); therefore cGMP levels in OLs could be increased by actions of NMDA-R agonists. Similar to these results, in an ischemic mouse model sildenafil has proven to increase nestin lineage OPCs and mature OLs (CNPAse+ and CC1+) in corpus callosum and striatum of treated mice (Zhang R. L. et al. 2012). Transduction signals of cGMP increase primarily occur via PKG, in OLs this kinase may be targeting different pathways implicated in OLs maturation and protection. Some of these pathways as above mention could be MEK/ERK and Aky/mTOR, different studies *in vitro* have involved them with growth and differentiation of oligodendrocytes (Bibollet-Bahena and Almazan 2009, Kumar et al. 2013). Furthermore, recent *in vitro* studies have shown that ERK I/II activation is required for the progression of oligodendrocyte early progenitors to the development of immature oligodendrocytes, but does not affect the transition from immature to mature oligodendrocytes; however, mTOR activation is not required for the development of immature oligodendrocytes, but it shows to be essential in the transition from immature to mature myelinating oligodendrocytes (Guardiola-Diaz et al. 2012). Additionally, important cross-talk between Akt/mTOR and MEK/ERK pathway has been studied, demonstrating that both pathways regulate MBP mRNA and protein expression and that Akt/mTOR inhibition increase ERK I/II phosphorylation *in vitro* (Dai et al. 2014)

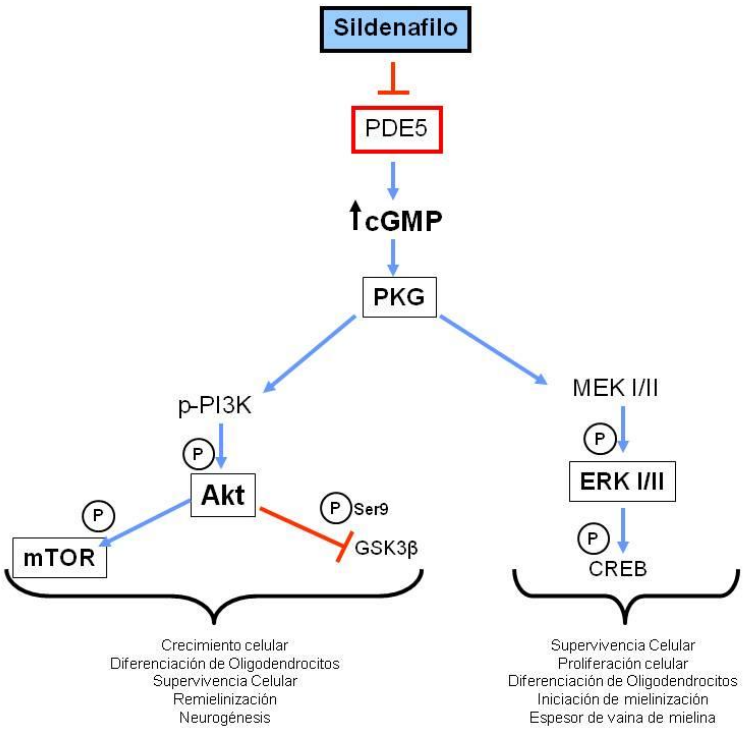

Figure 13: Sildenafil possible mechanism pathway

Therefore, sildenafil not only protects OLs from apoptotic death, but this work shows that it promotes OLs maturation into mature myelinating OLs.

Other mechanism possibly implicated in sildenafil ameliorating and remyelinating effects was the generation of trophic factors. Previous studies have demonstrated that sildenafil increases BDNF expression in splenocytes of EAE mice after sildenafil treatment and in SC sections, denoting that the increased BDNF expression in SC was mainly generated by infiltrating leukocytes and in axons (Pifarre et al. 2014). Another important neurotrophic factor associated with remyelination and regeneration process is CNTF. The results of this work showed that sildenafil treatment to EAE-mice increased CNTF expression in the SC, and was observed primarily by astrocytes. Furthermore cGMP increase also induced CNTF gene expression in cerebellar organotypic cultures. The CNTF produced in our model could be enhancing sildenafil effects on OLs, it has been recognized influence of CTNF on OL

differentiation (Stankoff et al. 2002) and it has been recently shown that this growth factor acts as a quemoatractant controlling OL progenitor migration during remyelination (Vernerey et al. 2013). In agreement with the results shown in this work, it has been recently demonstrated *in vitro* that the NO-cGMP-protein kinase G pathway up-regulates CNTF expression in astrocytes and that astrocyte-derived CNTF enhances OLs differentiation (Paintlia et al. 2013).. Moreover, studies have demonstrated that CNTF plays an important role in myelin protein expression and in oligodendrocyte protection against apoptotic death (Modi et al. 2013); therefore sildenafil effect on oligodendrocyte protection against apoptotic death observed in previously the EAE model (Pifarre et al. 2014) may be partially exerted through sildenafil increase expression of CNTF. Moreover, treatment with exogenous CNTF injection (s.c.) in the cuprizone-demyelinating mouse model, increased the myelin protein MOG expression after demyelination, thus suggesting that CNTF may be potentiating remyelination (Salehi et al. 2013). Treating EAE mice with mesenchymal cells overexpressing CNTF induced functional recovery of the disease, reduced demyelination, increased NG2+ cells (OLs precursors), decreased pro-inflammatory cytokines (TNFα and IFNγ) and increased anti-inflammatory cytokine IL-10 (Lu et al. 2009). This evidence suggests that sildenafil enhances the production not only of BDNF but also CNTF, and this increased expression of neurotrophic factors may develop important mechanisms and actions in the CNS after demyelinating and inflammatory lesions, promoting oligodedroglial protection and differentiation.

In EAE model it was previously described that sildenafil treatment significantly decreased microglial/macrophage activation and showed down regulation of adaptative immune response by decreasing pro-inflammatory cytokines (IL-2, INFγ and TNFα) release by splenocytes re-stimulated with MOG,

it was also described that sildenafil increased alternative activation microglia/macrophage marker Ym-1 (Pifarre et al. 2014); these results suggest that sildenafil besides having remyelinating and neuroprotective effects, it can also be promoting anti-inflammatory effects in the IS and CNS and may be promoting M2 microglia/macrophage phenotype. In order to study sildenafil effects on CNS inflammatory microglial phenotype, cytokines and microglia/macrophage phenotypic markers were determined.

The M1 phenotype has been related to release of pro-inflammatory cytokines, cytotoxicity, radical oxygen species (ROS) release, auto antigen presentation to T cells and therefore contributing with disease progression as has been observed Alzehimer's disease, MS and stroke studies (Chhor et al. 2013, Rawji et al. 2016). The M2 anti-inflammatory phenotype has been related with the production of anti-inflammatory cytokines and is associated with regeneration properties and wound healing (Gensel and Zhang 2015, Ransohoff and Perry 2009). Furthermore, the M2 phenotype resolution and regenerative properties have been related with inducing OPCs differentiation, remyelination and promoting myelin clearance (Durafourt et al. 2012, Kotter et al. 2006, Miron et al. 2013, Wang et al. 2015, Yu et al. 2015).

Our results showed that BAY41+Sil treatment in LPC demyelinated cerebellar cultures induced tendency to an early increased in Arg-1 (M2 phenotype marker) and COX-2 (M1 phenotype marker), no changes were found in CD206 (M2 phenotype marker) expression and a late decrease tendency in NOS-2 (M1 phenotype marker) expression was observed.

Increased arginase activity has been associated with alternatively activated microglia/macrophages (M2) (Gensel and Zhang 2015, Ransohoff and Perry 2009) which converts L-arginine into ornithine, a precursor of proline and

polyamines. Polyamines have been related with oxidative stress amelioration and cellular proliferation (Guasco Herrera et al. 2014). Curiously, in our results the treatment induced a tendency to increase M1 marker, COX-2 simultaneously with the increase Arg-1; COX-2 increase has usually been associated to pro-inflammatory process (Deininger and Schluesener 1999, Minghetti 2004). However recent studies propose anti-inflammatory properties of COX-2, demonstrating the presence of a group of lipids in postmortem brain samples of Alzehimer's disease patients, with pro-resolving and anti-inflammatory properties called pro-resolving lipid mediators (SPMs) synthesized by 5-lipoxygenase (LOX) and COX; this SPMs have shown to improve neuronal survival and $A\beta_2$ phagocitosys *in vitro* (Zhu et al. 2016). Therefore, the early increase in COX-2 observed in the cerebellar organotypic cultures after sildenafil treatment could be promoting the production of SPMs. Moreover, COX-2 increase has been associated with M2b/c (immune-regulatory/immune-suppressor) phenotype associated with the production of anti-inflammatory prostaglandins (Chhor et al. 2013, Ransohoff and Perry 2009, Wu et al. 2013), and wound healing after spinal cord injury (Gensel and Zhang 2015). On the other hand, within sildenafil treatment doses tendency to decrease M1 phenotype was observed, suggesting that PDE5 inhibitor may be promoting healing and regeneration of tissue by enhancing a M2-like phenotype and diminishing the pro-inflammatory phenotype.

In order to confirm the inflammatory effect of sildenafil in CNS, cytokine expression was determined in organotypic cerebellar cultures. Results showed that BAY41+Sil treatment significantly increase IL-10 and IL-6 24h after first dose, no changes were found in IL-1β and TNFα, pro-inflammatory cytokines, expression.

IL-10 is a known anti-inglammatory cytokine, its functions are exerted primarly on dendirtic cells and macrophages, IL-10 inhibits major histocompatibility complex class II expression and inhibits the production of proinflammatory cytokines and mediators (IL-1, IL-6, IL-12, TNFα) (Mosser and Zhang 2008). In agreement with previous results in EAE model (Pifarre et al. 2014), recent studies have shown that the increased expression of IL-10 and promotion of M2 microglia phenopype after estrogen treatment promotes neuroprotection preventing EAE progression (Benedek et al. 2016). Moreover, it has been shown that IL-10 increase expression resulted in attenuated inflammatory response, decreased microglia activation and less neurodegeneration in EAE model (Mayo et al. 2016). On the other hand, IL-6 was classically conceived as a pro-inflammatory cytokine; recently, more studies come to light showing its anti-inflammatory properties. In agreement with our results, studies performed by using interleukin-6 (IL-6) fused to its soluble receptor (sIL-6R) protein have shown that IL-6 increase *in vivo* has effects on myelination, inducing OPCs to differentiate into mature oligodendrocytes and promoting their survival (Haggiag et al. 2001, Valerio et al. 2002). Moreover, increase immune-regulatory response, by increasing IL-6 and IL-10 expression, has been associated with myelin repair in chronic phase of EAE in IL-4Rα$^{-/-}$ mice (Gaupp et al. 2008). Even though previous studies have shown that sildenafil treatment reduced pro-inflammatory cytokines expression in mouse cerebella of cuprizone-demyelinated model (Nunes et al. 2012) and in splenocytes of EAE mice (Pifarre et al. 2014), in our results BAY41+Sil treatment in cerebellar slices did not show significant changes in pro-inflammatory cytokines expression. In the case of TNFα excessive generation in brain has been involved in the pathogenesis of stroke, traumatic brain injury and Alzehimer's disease (Clark and Vissel 2016) and IL-1β is a

known pro-inflammatory cytokine, the release of this cytokine has been associated with cytotoxicity (Rawji et al. 2016).

Taking these results together suggests that sildenafil is promoting a mixed microglia phenotype, since an early increase in the expression of Arg-1, COX-2 was observed accompanied by an increase expression of IL-10 and IL-6; these features are recently being associated with M2b/c microglia/macrophage phenotype which shows immune-regulatory and immune-suppressor properties by promoting inflammatory resolution, tissue repair, production of anti-inflammatory and prostaglandins (Colton 2009, Chhor et al. 2013, Gensel and Zhang 2015, Ransohoff and Perry 2009, Wu et al. 2013) and even more, this phenotype has been associated with OPCs differentiation (Miron et al. 2013). Recent studies have indentified another mixed microglia/macrophage phenotype not classically M1 nor alternatively activated M2 cells; they were called “resolution-phase” macrophages (rM), since this particular phenotype has been observed in macrophages isolated from the resolving phase of acute inflammation. rM showed to increase M2 marker CD206 and anti-inflammatory molecules (IL-10 and PGD_2) expression, along with M1 markers including NOS-2, COX-2 (Bystrom et al. 2008). Moreover, new evidence has emerge showing that after spinal cord injury, exogenous IL-4 treatment increase the population of M2 phenotype, but it also appeared another population of cells suggesting to be infiltrating macrophages with a mixed phenotype co-expressing M1 markers (NOS-2, COX-2, LOX, CD16/32) and M2 markers (Arg-1, CD206) as well as anti-inflammatory cytokines IL-10 and TGFβ1 and were associated with rM, known to be important in the resolving of inflammation (Francos-Quijorna et al. 2016). Even though the phenotype markers and cytokines expressed after BAY41+Sil treatment in our cerebellar slices is very similar to those observed in the rM, our treatment in this model despite increasing Arg-1 failed in

promoting CD206; moreover no expression of NOS-2 or any other pro-inflammatory molecule besides COX-2 was observed. Therefore it is not clear yet if sildenafil is promoting rM unique phenotype in CNS.

Mouse cerebellar organotypic cultures are complex models to study inflammatory phenotype changes; this can result as a consequence of the presence of the other cells that are part of the CNS, such as astrocytes that could also be contributing in the production of cytokines and other inflammatory molecules (like NOS-2). In order to study more specifically sildenafil effects on microglia/macrophage phenotype BMDM cultures were established. The study of these cells grants clinical significance since EAE model as well as in MS peripheral immune cell infiltrates can be found (Berard et al. 2010, Henderson et al. 2009).

BMDM cultures were established as previously described (Classen et al. 2009) and were polarized into M1 phenotype with LPS+INFγ or into M2 phenotype with IL-4; a un-polarized BMDM population was used as control. Results showed nitrite accumulation only in M1 BMDM and that sildenafil treatment showed a tendency to decrease this accumulation. Additionally NOS-2 positive cell number significantly increased in LPS+INFγ treated BMDM; however treatment with cGMP analogue did not decreased the number NOS-2 positive cell population. On the other hand, the three populations of BMDM showed arginase activity; nevertheless, sildenafil treatment showed a tendency to increase arginase activity in control BMDM, being significant in M1 BMDM at the highest concentration of sildenafil and significantly doubled arginase activity in M2 BMDM. Moreover, CD206 positive cell number increased after IL-4 treatment; cGMP analogue induced an increase in CD206 positive population

in control BMDM and further potentiated the number of CD206 positive cells in IL-4 treated BMDM.

In order confirm the effect of cGMP increase on inflammatory phenotypes; IL-10 and IL-1β release were determined in the culture media of BMDM 18h after treatments. Results showed that LPS+INFγ treatment significantly increased IL-10 release in BMDM, these results agree with those of Howes, *et al.* that demonstrated that C57BL/6 BMDM produce higher levels of IL-10 in response to bacterial products such as LPS (Howes et al. 2016), similar IL-10 increase has been observed in primary microglia after LPS treatment (Chhor et al. 2013); nevertheless, cGMP analogue treatment significantly reduced IL-10 production in LPS+INFγ treated BMDM. Interestingly, IL-10 production was not observed in control or IL-4 stimulated BMDM; however, cGMP analogue increased IL-10 production in both populations, being significant in IL-4 BMDM. On the other hand, there was only IL-1β release on behave of LPS+INFγ BMDM and treatment with cGMP analogue significantly decreased the production of IL-1β in this M1 population. In agreement with this results, inhibiting PDE activity in human monocytes decreased the release of IL-1β and this effect was mimic by cGMP analogues and NO donor suggesting NO-GC activation is implied in the effect (von Bulow et al. 2005). Taking these results together suggest that increase cGMP levels promoted by sildenafil affect inflammatory outcomes, do not significantly reverse M1 phenotype but it does promote M2 phenotype and anti-inflammatory mediators.

One of the important actions of microglia/macrophage in neurodegenerative diseases is their phagocytic activity of cellular and myelin debris produced in the lesions (Ransohoff and Perry 2009). In neurodegenerative diseases, such as MS, olygodendrocyte differentiation is not

observed in demyelinated lesions, and it has been proved that the presence of myelin debris can impair remyelination by inhibition of OPCs differentiation (Kotter et al. 2006). For this reason myelin clearance by phagocytic cells has gained relevance. Studies have shown that M2 macrophages phagocyte greater amount of myelin than M1 (Durafourt et al. 2012). Myelin phagocytosis was assessed in EAE model through ORO staining, making evident that the lipid vesicles around infiltrates increase in EAE mice treated with sildenafil at the onset of the disease; furthermore, it was observed that most of the vesicles where inside Ym-1 stained M2 microglia/macrophages. To confirm that sildenafil was promoting myelin phagocytosis in M2 cells, control and M2 BMDM where treated with sildenafil and a cGMP analogue for 24h before being exposed to purified myelin. Flow cytometry analysis revealed that sildenafil treatment increased myelin phagocytosis in IL-4 stimulated BMDM compared with controls and LPS treated BMDM (data not shown); moreover sildenafil significantly increases myelin pahocytosis in control and M2 BMDM, this effect was mimicked by cGMP analogue. This increase in myelin clearance is another mechanism promoted by sildenafil essential for remyelination to take place.

The results observed in organotypic cerebellar and BMDM cultures did not showed a clear pro or anti-inflammatory phenotype induction. These results showed that sildenafil can promote inflammatory changes through cytokine expression and could be generating a mixed microglia/macrophage phenotype. Nowadays increasing evidence of mixed or overlapping microglia/macrophage phenotype can be found, and has been associated with the recovery after damage. IL-4 treatment of macrophages *in vitro* produced IL-6 and co-expressed CD206 (M2 marker) without affecting immunosuppressive properties of M2 phenotype macrophages (Casella et al. 2016). Moreover, the

expression of Arg-1 as well as IL-1β was associated with neurite growth promotion and spinal cord injury recovery (Fenn et al. 2014). IL-4 gene therapy to EAE mice decreased demyelination and axonal loss showing an increase in the expression of CD206 in microglia/macrophages, but also an increase IL-6 (Casella et al. 2016). Even more, it has comes to light evidence that a pro-inflammatory environment at the beginning of the lesion followed by anti-inflammatory environment with increase of pro-resolving and tissue regenerating molecules is required for an effective resolution and repair after CNS injury (Rawji et al. 2016) and that the whole spectrum of microglia/macrophage phenotypes are required in the resolution of CNS injury (Gensel and Zhang 2015, Rawji et al. 2016).

Indeed classifying microglia/macrophages as classically (M1) or alternatively-activated (M2) in *ex-vivo* (such as organotypic cerebellar cultures) or *in vivo* models is a complex matter. Some authors are starting to consider as either of both phenotypes, since this nomenclature was generated from monocyte-derived macrophages treated *in vitro* using defined cytokine stimuli and controlled environment, but after inflammatory stimuli *in vivo* microglia/macrophage plasticity promotes the generation of a broad spectrum of slightly different phenotypes that can be required for the resolution and healing process to take place (Gensel and Zhang 2015, Stables et al. 2011). Therefore, it cannot be said that sildenafil treatment is inducing a shift into M1 or M2 phenotype, but it is affecting immune response in CNS and in IS, generating anti-inflammatory mediators that promotes resolution of inflammatory process. More importantly sildenafil treatment increased myelin phagocytosis in non-polarized BMDM suggesting that this restoring and essential process can be promoted despite microglia/macrophage phenotype.

CONCLUSIONS

CONCLUSIONS

1. Sildenafil treatment ameliorates clinical symptoms in mouse chronic EAE model when administrated in the acute and in chronic phase.
2. Sindenafil treatment promotes remyelination of axons and mature-functional internodes increasing myelin protein levels.
3. The NO-cGMP-PKG pathway is required in sildenafil-induced remyelination.
4. Some of the mechanisms underliying sildenafil-induced remyelination and neuroprotection are associated with:
    - Maturation of oligodendocytes
    - Increased CNTF expression in CNS
    - Modulation of inflammatory process
    - Increased myelin phagocytic capacity

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