Regulation of neuroinflammation by cGMP-mediated pathways.

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ABSTRACT

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In the present work we investigated the involvement of intracellular cGMP levels in regulating different aspects of neuroinflammation. In the first chapter we observed that cultured rat astrocytes and microglial cells express the necessary machinery to synthesize cGMP in response NPs and to degrade the nucleotide. We also investigated the effects of NP stimulation in the expression of the pro-inflammatory protein iNOS. We observed that pretreatment with ANP down-regulates iNOS protein levels induced by the pro-inflammatory agent LPS by an NPR-A-PKG dependent mechanism in rat cultured microglial cells. In addition we found that this down-regulation seems to be done at translational level, without affecting transcription or protein degradation rate.

Based on evidence obtained in our laboratory in cultured glial cells indicates that cGMP-mediated pathways regulate cytoskeleton dynamics, GFAP expression and motility in astrocytes, as well as inflammatory gene expression in microglia found in the first chapter, suggesting a role in the regulation of the glial reactive phenotype. In the second chapter we wanted to examine if cGMP regulates the glial inflammatory response in vivo following CNS damage caused by a focal cryolesion onto the cortex in rats and mice. We investigated the effect of 3 doses of treatment with two different cGMP-phosphodiesterase (PDE) inhibitors, zaprinast and sildenafil. We observed that the non-selective GMP-PDE inhibitor zaprinast enhances astrogliosis around the lesion while decreasing macrophage/microglial activation, oxidative stress and neuronal death in rat. We observed also that treatment with the selective PDE5 inhibitor sildenafil reproduces in mice the changes in glial reactivity and the antioxidant and antiapoptotic effects previously observed with zaprinast in rats indicating that inhibition of PDE5 is responsible for these neuroprotective actions. However, sildenafil effects were not observed in mice deficient in MT-I/II. We further show that sildenafil significantly increases MT-I/II protein levels in lesioned cortical homogenates and MT-I/II immunostaining in glial cells around the lesion, and decreases activation of the transcriptor factor STAT3, supporting the involvement of these proteins in the neuroprotective effects of sildenafil in focal brain lesion. As a result of the anti-inflammatory and neuroprotective effects observed by PDE5 inhibitors in the cryolesion model, in the third chapter we investigate if treatment with sildenafil could have beneficial effects in a MOG_{35-55}-induced EAE model, an animal model of MS, a disease

where an altered inflammatory response occurs. We show that treatment with sildenafil after disease onset markedly reduces the clinical signs of EAE by preventing axonal loss and promoting remyelination. Furthermore, sildenafil decreases CD3+-leukocyte infiltration and microglial/macrophage activation in the spinal cord, while increasing Foxp3-Tregs. In addition, sildenafil treatment decreased ICAM-1 in spinal cord infiltrated cells. The presence of reactive astrocytes forming scar-like structures around infiltrates was enhanced by sildenafil suggesting a possible mechanism for restriction of leukocyte spread into healthy parenchyma. We show also that treatment with sildenafil at the onset of clinical symptoms, when the inflammatory process is stronger, prevent disease advance and regulates peripheral adaptative immune response and PDE5 levels.

Taking in account all the results obtained we evidenced that modulation of intracellular cGMP levels have beneficial effects in neuroinflammatory processes and that this benefits are related to regulation of reactive gliosis, oxidative stress, antioxidant factors, adaptative immune response and infiltration of immune cells into CNS leading to decrease neuronal damage.

RESUMEN

En el trabajo aquí presentado se investigó la participación de los niveles intracelulares de GMP-cíclico (CMPC) en la regulación de diferentes aspectos de la neuroinflamación. En el primer capítulo hemos observado que los cultivos de astrocitos y de celulas microgliales aisladas de rata expresan la maquinaria necesaria para sintetizar GMPc en repuesta a Péptidos Natriuréticos (PN) y también los enzimas necesarios para degradar el nucleótido. También se investigaron los efectos de la estimulación por PN en la expresión de la proteína pro-inflamatoria iNOS. Hemos observado que el pretratamiento con ANP hace disminuir los niveles de la proteína iNOS inducida por el agente pro-inflamatorio LPS a través de un mecanismo dependiente de NPR-A-PKG en células microgliales cultivadas de rata. Además hemos observado que esta regulación a la baja parece estar hecha a nivel de traducción, sin afectar a la transcripción o la tasa de degradación de la proteína.

Sobre la base de datos obtenidos en nuestro laboratorio en cultivos de células gliales, se ha demostrado que las vías mediadas por GMPc regulan la dinámica del citoesqueleto, la expresión de GFAP en los astrocitos y la motilidad, así como la expresión de genes inflamatorios en la microglía que se demuestra en el primer capítulo, lo que sugiere un papel en la regulación del fenotipo reactivo glial. En el segundo capítulo, hemos querido verificar si el GMPc regula la respuesta inflamatoria glial in vivo después de un daño producido en el SNC causado por una criolesión focal en la corteza cerebral en ratas y ratones. Se investigó el efecto de 3 dosis de tratamiento con dos inhibidores de diferentes fosfodiesterasas de GMPc (PDE), zaprinast y sildenafilo. Hemos observado que el inhibidor no selectivo de PDE-GMPc zaprinast potencia la astrogliosis alrededor de la lesión, mientras que produce una disminución de la activación de macrófagos/microglía, el estrés oxidativo y muerte neuronal en la rata. Se observó también que el tratamiento con el inhibidor selectivo de la PDE5 sildenafilo reproduce en los ratones los cambios en la reactividad glial y los efectos antioxidantes y antiapoptóticos observados anteriormente con el zaprinast en ratas, indicando que la inhibición de la PDE5 es responsable de estas acciones neuroprotectoras. Sin embargo, los efectos de sildenafilo no se observaron en los ratones deficientes en MT-I/II. Asimismo, se muestra que el sildenafilo aumenta significativamente los niveles de las proteínas MT-I/II en homogenados corticales de ratones lesionados así como la inmunotinción de MT-I/II en las células gliales alrededor de la lesión, y disminuye la

activación del factor transcriptor de STAT3, apoyando la participación de estas proteínas en los efectos neuroprotectores del sildenafilo en la lesión cerebral focal.

Como resultado de los efectos antiinflamatorios y neuroprotectores observados por los inhibidores de la PDE5 en el modelo criolesión, en el tercer capítulo se investigó si el tratamiento con sildenafilo podría tener efectos beneficiosos en un modelo de EAE inducido por MOG_{35-55}, un modelo animal de esclerosis múltiple, una enfermedad en la que existe una respuesta inflamatoria alterada. Se demuestra que el tratamiento con sildenafilo después del inicio de la enfermedad reduce notablemente los síntomas clínicos de la EAE mediante la prevención de la pérdida axonal y la promoción de la remielinización. Por otra parte, el sildenafilo disminuye la infiltración de leucocitos CD3+ así como la activación de microglia/macrófagos en la médula espinal, al tiempo que aumenta la presencia de Foxp3-Tregs. Además, el tratamiento con sildenafilo disminuye la expresión de ICAM-1 en las células infiltradas de la médula espinal. La presencia de astrocitos reactivos que forman estructuras similares a cicatrices alrededor de los infiltrados se potenció por el tratamiento con sildenafilo, sugiriendo un posible mecanismo para restringir la propagación de leucocitos en el parénquima sano. Se demuestra también que el tratamiento con sildenafilo al inicio de los síntomas clínicos, cuando el proceso inflamatorio es más fuerte, impide el avance de la enfermedad y regula la respuesta adaptativa inmune periférica y los niveles de la PDE5.

Teniendo en cuenta todos los resultados obtenidos se demuestra que la modulación de los niveles intracelulares de GMPc tiene efectos beneficiosos en los procesos neuroinflamatorios y que estos beneficios están relacionados con la regulación de la gliosis reactiva, el estrés oxidativo, factores antioxidantes, la respuesta inmune adaptativa y la infiltración de células inmunes en el SNC que conducen a una disminuición en el daño neuronal.

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ABBREVIATIONS

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AD	Alzheimer's disease	**IS**	Immune System
cAMP	Cyclic adenosine monophosphate	**LFB**	Luxol fast blue
ANP	atrial natriuretic peptide	**LPS**	lipopolysaccharide
AP-1	Activator protein 1	**MAPKs**	mitogen-activated protein kinases
APC	Antigen presenting cell	**MHC**	Major histocompatibility complex
Aβs	Amyloide beta peptides	**MOG**	Myelin oligodendrocyte glycoprotein
BBB	Blood-Brain Barrier	**MS**	Multiple Sclerosis
BNP	brain natriuretic peptide	**MT**	Metallothionein
BSA	Bovine serum albumin	**NF-kB**	Nuclear transcription factor kappa beta
cGMP	Cyclic guanosine monophosphate	**NMDA**	N-methyl-D-aspartate
8br-cGMP	8-bromo-cGMP	**NMDA-R**	N-methyl-D-aspartate type glutamate receptor
CBF	cerebral blood flow	**NMMA**	N^G-monomethyl-L-arginine
CD	Cluster of differentiation	**NO**	Nitric oxide
CFA	Complete Freund's adjuvant	**NOS**	nitric oxide synthase
CNP	C-type natriuretic peptide	**NP**	natriuretic peptide
CNS	Central Nervous System	**NPR**	natriuretic peptide receptor
COX-2	Ciclooxygenase-2	**ODQ**	1H-[1,2,4]oxodiazolo-[4,3-a]quinoxalin-1-one
dbcAMP	dibutyril-cAMP	**PDE**	phosphodiesterase
dbcGMP	dibutyril-cGMP	**PFA**	paraformaldehyde
EAE	Experimental autoimmune encephalomyelitis	**PKA**	cyclic AMP-protein kinase
GCNO	soluble guanylyl cyclase	**PKG**	cyclic GMP-protein kinase
GFAP	Glial fibrillar acidic protein	**ROS**	Reactive oxygen species
HIV	Human immunodeficiency virus	**SNP**	sodium nitroprusside
IBMX	3-Isobutyl-1-methylxanthine	**STATs**	Signal transducers and activators of transcription
IFN-γ	Interferon-gamma	**TGF-β**	Tumor growth factor beta
IL	interleukine	**TNF-α**	tumour necrosis factor alpha
iNOS	Inducible nitric oxide synthase	**VEGF**	Vascular endothelial growth factor

INTRODUCTION

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1. NEUROINFLAMMATION

1.1. Immunity in the Central Nervous System

The Central Nervous System (CNS) presents many distinctive characteristics such as high energy supply, activity-dependent modulation of local blood flow, strict control of permeability and composition of the narrow extracellular space, as well as complex neurotrophic interactions to maintain neuronal survival and modulate synaptic plasticity. 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, Kawamata et al. 2003).

1.1.1. 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 (Fig. 1), 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 and promote recovery, but uncontrolled neuroinflammation can induce secondary injury (Abbott, Patabendige et al. 2010).

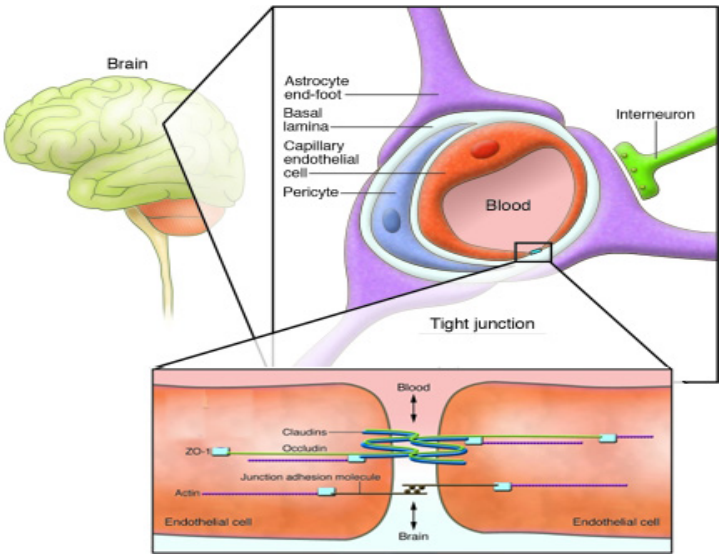

Figure 1. Structure of the BBB and tight junctions. Scheme of the cell components of the BBB and detail of a tight junction established by the interaction between the transmembrane proteins on adjacent endothelial cells. Adapted from Chou and Messing (Chou and Messing 2008).

In addition to the BBB, several factors contribute to restricting the immune response in the CNS. One is the absence of a conventional lymphatic system since there are no defined lymphatic vessels draining the brain. However, involvement of cervical lymph nodes in CNS immune reactions, and different routes of lymphatic drainage through interstitial fluid and cerebrospinal fluid have been demonstrated. Another feature contributing to the immunological privilege of the brain is 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, Gasque et al. 2009; Weller, Galea 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) and experimental autoimmune encephalomyelitis (EAE).

1.1.2. Resident immune effector cells in the CNS

Besides neurons, the other major cell types in the CNS are glial cells: astrocytes, microglia and oligodendrocytes, which have vital importance in the maintenance of homeostasis, formation of myelin and providing support and protection to neurons. 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, Nielsen 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 (Streit, Conde et al. 2005; Ghafouri, Amini et al. 2006; Kim and Joh 2006; Wyss-Coray 2006; Sofroniew and Vinters 2010)

MICROGLIA

The presence of microglial cells in the CNS represents the most obvious necessity of presence of the immune system and its surveillance even at an immune-privileged site. They are the primary sensors of brain pathology and are rapidly recruited to sites of infection, trauma or inflammation. This population represents 10-20 % of total glial cells and was first described by Del Rio-Hortega (1932), who used silver impregnation techniques to visualize non-neuronal cells.

Developmentally, microglial cells are considered to be the resident macrophages of the CNS, descendants of the monocytic lineage that invade the central nervous system early during ontogenesis (Fedoroff and Hao 1991; Hailer 2008). They express monocytic cellular markers such as the αMβ2 integrin (also known as CD11b/CD18 and MAC-1), IgG receptors (CD16/CD32), ionized calcium-binding adaptor protein-1 (Iba-1) and the major histocompatibility complex (MHC) (Raivich and Banati 2004; 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 with each other, expanding throughout the CNS (Raivich,

Jones 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, Kirchhoff et al. 2005). CNS injury provokes immediate and focal activation of microglia, which is thought to occur before astrocyte activation, switching their behaviour from resting to defending cells in the injured site, with a different response depending on the stimulation provided (Town, Nikolic et al. 2005). Reactive microglial cells proliferate, retract its ramifications and extend new pseudopodia that enable active migration, and acquire amoeboid morphology and phagocytic capacity for removing dead cells and cell debris. These characteristics together with their antigen presenting capacity in MHC-II, make them the APCs of the CNS initiating the adaptive immune response (Fig.2) (Aloisi 2001; Kim and de Vellis 2005; Hailer 2008).

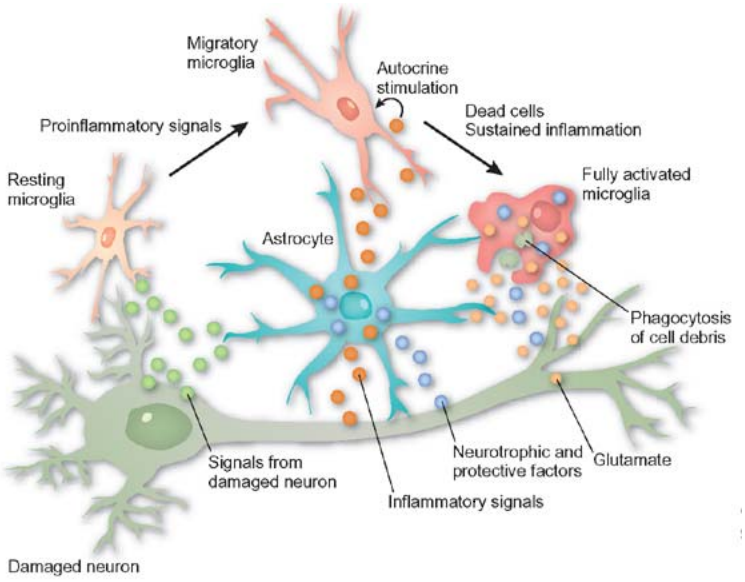

Figure 2. Microglial activation sequence. Different stages of microglial activation in response to damaging signals and segregation of protective and pro-inflammatory factors from CNS cells. Adapted from Monk and Shaw (Monk and Shaw 2006).

Activation of microglia in response to brain injury involves a continuum spectrum of phenotypes rather than one simple stage. The reactive microglia phenotype oscillates between two end-points depending on the stimulatory environment, one corresponds to the innate activation with a phagocytic phenotype and anti-inflammatory cytokine secretion, and the other is the adaptive activation with release of pro-

inflammatory cytokines that is associated with capacity for stimulation of Th1 and Th2 subpopulations (Aloisi 2001; Town, Nikolic et al. 2005).

Concomitant with cell shape changes, reactive microglia can release pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) and express enzymes such as nitric oxide synthase-2 (NOS-2), ciclooxygenase-2 (COX-2), and NADPH oxidase that produce inflammatory mediators (NO, arachidonic acid metabolites, reactive oxygen species (ROS)) that can inhibit neurite outgrowth and affect cell survival (Kim and de Vellis 2005; Brown and Neher 2010). Expression of these inflammatory genes can be up- or down-regulated in microglial cells by activation of several transcription factors such as NF-κB, C/EBPs, AP-1 or STATs, by pro- or anti-inflammatory cytokines, neurotransmitters, contact with components of apoptotic cells or receptor activation, among others (Herdegen and Waetzig 2001; Satriotomo, Bowen et al. 2006; Ejarque-Ortiz, Medina et al. 2007; Kaltschmidt and Kaltschmidt 2009).

ASTROCYTES

Astrocytes are the most abundant cellular type in the CNS, greatly outnumbering neurons. These cells play important roles in the function of the healthy CNS, including regulation of blood flow, provision of energy metabolites to neurons, participation in brain development, synaptic function and plasticity, and maintenance of the extracellular balance of ions, fluid, pH and transmitters. Astrocytes are also primary responding cells to injury and disease and can acquire characteristics of effector immune cells. Pro- or anti-inflammatory effects have been attributed to astrocytes depending on the nature and spatio-temporal location of the stimuli by secreting different molecules that will affect the response of other cells such as microglia and neurons, having the potential to contribute to CNS diseases (Sofroniew and Vinters 2010). Reactive astrogliosis includes a wide range of phenotypes depending on the inducing stimulus. After mild trauma, at distant sites from a severe injuries, or after moderate metabolic or molecular insults or infections, astrocytes become hypertrophic, show increased expression of intermediate filament proteins such as vimentin, nestin and in particular glial fibrillary acidic protein (GFAP), present little anatomical overlap of the processes with neighboring astrocytes (isomorphic gliosis), and exhibit the potential for resolution if the triggering insult is removed or resolves (Fig. 3A). When more severe insults occur astrocytes proliferate and present a pronounced hypertrophy

of cell bodies potently up-regulating GFAP and other genes, and no longer occupy discrete domains and instead have overlapping processes (anisomorphic astrogliosis). They ultimately form a compact glial scar along the borders of areas of tissue damage and inflammation that also incorporates fibromeningeal and other glial cells with deposition of a dense collagenous extracellular matrix (Fig. 3B) (Sofroniew 2009). Mature glial scars tend to persist for long periods and act as barriers not only to axon regeneration but also to inflammatory cells in a manner that protects healthy tissue from nearby areas of intense inflammation (Liberto, Albrecht et al. 2004; Silver and Miller 2004; Voskuhl, Peterson et al. 2009; Toft-Hansen, Fuchtbauer et al. 2011).

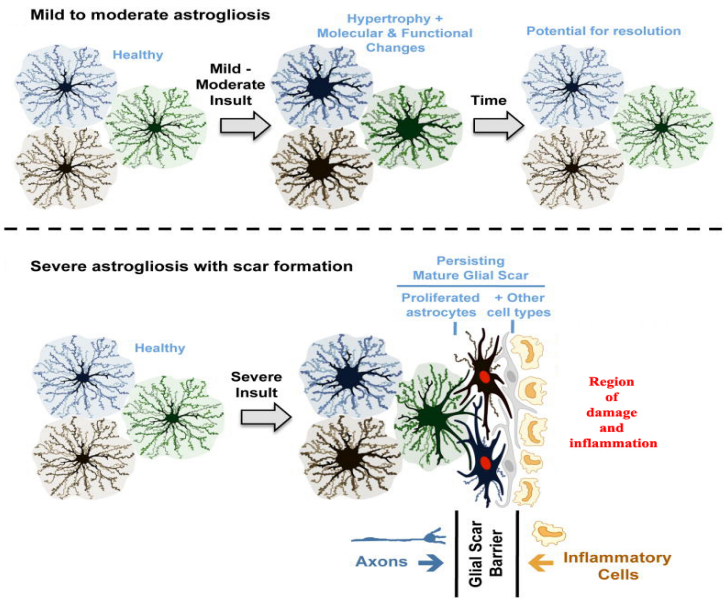

Figure 3. Schematic representations of different gradations of reactive astrogliosis that vary with insult severity. Adapted from Sofroniew (Sofroniew 2009).

The signaling molecules and regulators of astrogliosis that can be released by neurons, microglia, oligodendrocytes, pericytes, endothelial cells or other astrocytes include ROS, growth factors such as fibroblast growth factor-2 (FGF2), cytokines such as IL-6, TNF-α or IFN-γ, neurotransmitters such as glutamate and noradrenalin and purines such as ATP. Other external factors or insults that can produce astrocyte activation are inducers of innate immunity such as lipopolysaccharide (LPS), hypoxia and glucose deprivation, neurodegeneration associated products such as β-amyloide peptide (Aβ) or systemic metabolic toxicity-associated molecules such as NH_4^+ (Hamby, Hewett et al. 2006; Farina, Aloisi et al. 2007; Sofroniew and Vinters 2010).

When activated, astrocytes can play detrimental roles by loosing physiological functions and by participating in neuroinflammatory processes by secretion of the above mentioned factors leading to activation of other astrocytes or microglial cells. In contrast, increasing evidence indicates that cytokine-stimulated astrocytes can also have beneficial roles and can promote recovery of CNS function by increasing production of energy substrates and neurotrophic factors, by protecting from oxidative stress by production of glutathione, by uptake of potentially excitotoxic glutamate, by protection against NH_4^+ toxicity, degradation of Aβ, repair of the BBB or by reducing vasogenic edema after insults (Bush, Puvanachandra et al. 1999; Chen, Vartiainen et al. 2001; Swanson, Ying et al. 2004; Sofroniew 2009).

2. cGMP-GENERATING PATHWAYS IN CNS CELLS

In the SNC as well as in peripheral tissues cGMP is generated by guanylyl cyclases (GCs) that catalyze the conversion of GTP into cGMP. There are two main classes: NO-sensitive guanylyl cyclases (GC_{NO}) and particulate guanylyl cyclases (pGCs) that in the CNS belong to the natriuretic peptide (NP) receptor (NPR) group (Fig.4).

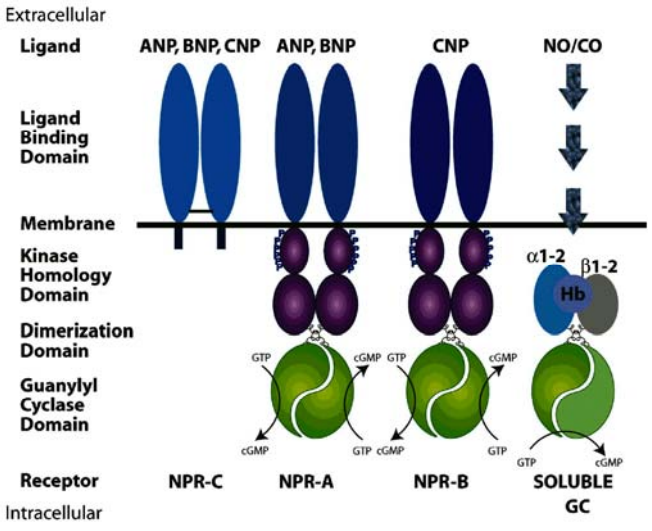

Figure 4. Schematic representation of enzymes related to cyclic GMP formation and their ligands. Adapted from Potter (Potter 2011).

2.1. NO-cGMP pathway

Nitric oxide (NO) is a potent physiological mediator and its synthesis is ubiquitous in mammalian tissues playing many important functions. In macrophages NO acts as antimicrobial agent, in vascular smooth muscle cells as a vasodilator and in the CNS and Peripheral Nervous System (PNS) is recognized to be a neurotransmitter. In the CNS, NO was first found to mediate stimulation of cGMP formation by the excitatory amino acid glutamate (Garthwaite, Charles et al. 1988) and has been implicated in the modulation of synaptic plasticity, brain development, visual and sensory processing, neuroendocrine secretion and cerebral blood flow (Garthwaite 2000; Guix, Uribesalgo et al. 2005). Furthermore, NO has been also described as a neuropathological agent responsible for excitotoxic cell death and neuroinflammatory cell damage in many neurological disorders (Murphy 2000; Duncan and Heales 2005).

2.1.1. Nitric oxide synthases

NO is synthesised by a family of nitric oxide synthases by oxidation of one of the guanidine nitrogens of L-arginine, using O_2 and NADPH as co-substrates and forming L-citrulline as by-product. NOS activity requires flavin adenine dinucleotide, flavin mononucleotide, heme, tetrahydrobiopterin (BH_4) and calmodulin. NO is a small, highly diffusible, and reactive molecule with a short half-life ($t_{1/2}$=seconds) and is rapidly oxidized to the stable, inactive end-products, nitrite and nitrate (Zhang and Snyder 1995). Three distinct isoforms of NOS encoded by different genes and several splice variants have been identified. nNOS (or NOS-1) is the isoform first purified and cloned and is predominantly expressed in neurons (Bredt and Snyder 1990); iNOS (NOS-2) that was first found in immunologically activated macrophages (Hevel, White et al. 1991; Stuehr, Cho et al. 1991), and eNOS (NOS-3) that is expressed in vascular endothelial cells (Pollock, Nakane et al. 1993). The three forms are only active as homodimers. Both, nNOS and eNOS, are constitutive isoforms with calcium-calmodulin-dependent activity 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 (NMDA-R) stimulated calcium entry that leads to activation of nNOS. However, excess NO production by overstimulation of NMDA-R has been implicated in excitotoxicity (Dawson, Dawson et al. 1991). In contrast, iNOS is inducible at the transcriptional level and its activity is calcium-independent since it has tightly-bound calmodulin. iNOS

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), Aβ peptides or HIV coat proteins, that can cause neurotoxicity (Murphy 2000; García 2004; Brown and Neher 2010). In the CNS, microglia and to a minor extend astroglia, are the main cellular site for iNOS expression in rodent brain and its generation of NO has been implicated not only in the response to infectious organisms but also in the pathogenesis of various insults as well as of multiple neurological disorders (Murphy 2000; Brown and Neher 2010; Steinert, Robinson et al. 2011).

The stimuli and conditions that determine iNOS expression are species and cell-specific. Transcriptional induction of iNOS, as well as that of other inflammatory genes such as TNF-α in glial cells involves activation of the transcription factors NF-kB, CREB, C/EBP, STAT1 and AP-1 among others, that bind to promoter response elements of the gene (Pautz, Art et al. 2010). Several factors, such as NO, heat shock proteins, cAMP, TGF-β and glucocorticoids, can affect iNOS induction, potentiating or decreasing iNOS depending on the cell type, by interfering with transcription factor activation or binding to the gene promoter (Saha and Pahan 2006). Regulation of iNOS mRNA stability by a complex network of RNA binding proteins such as HuR or AUF-1, of mRNA translation and of protein stability have been also described in different cell types including macrophages (Vodovotz, Bogdan et al. 1993; Pautz, Art et al. 2010).

2.1.2. NO-sensitive guanylyl cyclases

GC_{NO} is recognised as the major receptor for NO, and thus mediates numerous of its physiological functions. GC_{NO} was initially thought to be entirely cytosolic and was thus named “soluble GC”, however it has also been found associated to membranes (Zabel, Kleinschnitz et al. 2002). GC_{NO} is composed of an α and a β subunit and both subunits are required for catalytic activity (Koesling, Russwurm 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, Wedel et al. 1999) (Fig. 4).

In contrast to the ubiquitous NO formation in CNS parenchymal cells, NO-dependent cGMP synthesis by activation of GC_{NO} 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, Pifarre 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 GC_{NO} at the protein and mRNA level in rat brain astroglial cell cultures and after intracerebral administration in adult rat brain (Baltrons and Garcia 1999; Baltrons, Pedraza et al. 2002; Pedraza, Baltrons et al. 2003). In addition, decreased astroglial expression of the GC_{NO} β subunit was observed in post-mortem brains of Alzheimer's disease (AD), Multiple Sclerosis and Creutzfeld-Jacob disease patients, suggesting that an impairment of the astroglial NO/cGMP system may bear some relation to neuronal dysfunction (Baltrons, Pifarre et al. 2004).

2.2. Natriuretic peptide-cGMP pathway

Natriuretic peptides (NPs) form a family of structurally related peptides derived from separate genes. The three major members of this family in mammals are atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Both ANP and BNP elicit vascular, renal and endocrine effects directed towards regulation of vascular tone, sodium and water balance and other cardiovascular functions. On the other hand, CNP does not seem to function as a cardiac hormone (Cao and Yang 2008). Not much is known about the role of NPs in brain tissue but accumulating evidence indicates possible regulatory roles of NPs in neural development (Waschek 2004), synaptic transmission (Decker, Wojtowicz et al. 2008) and processing of information (Decker, Wojtowicz et al. 2009), and neuroprotection (Nogami, Shiga et al. 2001; Fiscus 2002; Wiggins, Shen et al. 2003).

2.2.1. Particulate guanylyl cyclases

NPs exert their actions through binding to three types of membrane receptors, namely NP receptor A (NPR-A), NP receptor B (NPR-B) and NP receptor C (NPR-C). NPR-A, that is sensitive to both ANP and BNP, and NPR-B that is highly specific for CNP, have guanylyl cyclase (GC) activity and are also named GC-A and GC-B (Cao and Yang 2008). Both isoforms have a similar structure including an extracellular ligand binding domain, a single transmembrane domain, a kinase homology domain and a GC catalytic domain in the intracellular portion of the protein (Fig. 4) (Potter 2011; Potter 2011). Their stimulation increases intracellular cGMP that mediates most of the known cardiovascular effects of NPs. NPR-C lacks GC activity and acts as a clearance

receptor for all three NPs, controlling their local concentration by receptor-mediated internalization and degradation, although a signalling function for this receptor has been also demonstrated, playing an important role in bone growth. All three receptor types are widely distributed in a variety of tissues, including the CNS (Potthast and Potter 2005; Potter, Yoder et al. 2009).

Although NPs have been localized predominantly in neuronal perikarya and fibers throughout the brain, NPRs expression has been reported to predominate in astroglia. Using cGMP immunocytochemistry in rat brain slices it has been well documented that the majority of the ANP-responding cells in the rat brain are astroglial cells supporting a predominant localization of NPR-A receptors in these cells (de Vente, Bol et al. 1989; De Vente, Bol et al. 1990; Markerink-Van Ittersum, Steinbusch et al. 1997; Vles, de Louw et al. 2000). However, studies in rat cultured astrocytes have also shown NPR-B expression (Sumners and Tang 1992). Using spheroid cultures from rat brain, Teunissen et al. (Teunissen, Steinbusch et al. 2000) observed that, in addition to astrocytes, cells labelled with a microglial marker presented cGMP immunoreactivity after stimulation with ANP but not with a NO donor suggesting that microglia can express NPRs but no GC_{NO}. Additionally, when this work was underway, expression of mRNAs for NPR-A and NPR-B as well as ANP was found in embryonic rat microglial cells (Moriyama, Taniguchi et al. 2006).

2.3. cGMP inactivation

2.3.1. Cyclic GMP phosphodiesterases

Phosphodiesterases (PDEs) are the enzymes responsible for the hydrolysis of cGMP and cAMP, restricting both the amplitude and duration of the cyclic nucleotide signal. In mammals, PDEs are encoded by 21genes, producing 11 distinct PDE families (PDE1 to PDE11) that are expressed in multiple tissues, including CNS and PNS. According to the specificity for the nucleotide, PDEs are divided in three groups: PDEs hydrolysing cAMP and cGMP (PDE1, PDE2, PDE3, PDE10 and PDE11), PDEs hydrolysing cAMP (PDE4, PDE7 and PDE8) and PDEs highly specific for cGMP (PDE5, PDE6 and PDE9) (Conti and Beavo 2007; Omori and Kotera 2007). These three cGMP specific PDEs are expressed in the CNS cells, PDE6 is unique to the retina, PDE5 is prominently expressed in brain vessels and cerebellar Purkinje cells and PDE9 is ubiquitously distributed in brain (Andreeva, Dikkes et al. 2001; Cote 2004). In

addition, dual-substrate isoforms are also found in multiple brain areas, with exception of PDE11 that is unique to the pituitary (Reneerkens, Rutten et al. 2009). Increases in cGMP immunorreactivity after stimulation with NO or ANP in slices from different brain regions have been shown to be potentiated in the presence of non-specific as well as specific PDE inhibitors in both neuronal populations and astrocytes (De Vente 2000).

Typically, mammalian PDEs are found as homodimers with the exception of PDE1 and PDE6, which are heterotetramers. Monomers contain a catalytic domain and a regulatory domain. Different mechanisms of regulation of PDE activities that can induce stimulation or inhibition have been described, including phosphorylation by cGMP- or cAMP-protein kinases (PKG and PKA), binding of cyclic nucleotides to the regulatory domains, and autoinhibition provided by physical constraints imposed by dimer contacts (Omori and Kotera 2007; Francis, Blount et al. 2011).

PDE inhibitors were initially developed as pharmacological tools due to their capacity for regulation of the cardiovascular system function. The first inhibitors used were methylxanthines (caffeine, theophylline, 3-isobutyl-1-methylxanthine (IBMX)) that inhibited practically all PDE activities and were later shown to also interact with adenosine receptors. A second generation of more selective inhibitors for each of the PDEs families was later developed. PDE5 inhibitors were soon developed because of the important role of this PDE in controlling cGMP levels in vascular smooth muscle and its effects on vascular tone. Zaprinast was the first PDE5 inhibitor developed as a vasodilator but was an unsuccessful clinical drug that was later found to inhibit other PDEs (PDE1, PDE6, PDE9, PDE11). However, it was a precursor to the chemically-related PDE5 inhibitors sildenafil (Viagra), vardenafil (Levitra), and tadalafil (Cialis) which successfully reached the market as vasodilators for the treatment of erectile dysfunction and pulmonary arterial hypertension (Bell and Palmer 2011).

Numerous studies have shown beneficial effects of PDE5 inhibition in the CNS. Prickaerts et al (Prickaerts, Steinbusch et al. 1997) were the first to describe memory-enhancing effects of zaprinast administration in rats. Later on, more selective PDE5 inhibitors were shown to have positive effects in cognitive function in impaired and unimpaired rodents (Reneerkens, Rutten et al. 2009). Studies in different models of Alzheimer's disease and accelerated senescence have shown that treatment with sildenafil improves synaptic function and cognitive deficits (Puzzo, Staniszewski et al. 2009; Cuadrado-Tejedor, Hervias et al. 2011; Orejana, Barros-Minones et al. 2012). Amelioration of memory deficits caused by other diseases such as diabetes or

hyperammonemia has been also demonstrated (Patil, Singh et al. 2004; Erceg, Monfort et al. 2005; Patil, Singh et al. 2006). In addition, after experimental stroke treatment with selective PDE5 inhibitors has been shown to increase neurogenesis and improve neurological functional recovery (Zhang, Zhang et al. 2005; Zhang, Zhang et al. 2006; Menniti, Ren et al. 2009). PDE5 inhibitors have been also demonstrated to be neuroprotective against 3,4-methylenedioxy-methamphetamine (MDMA, "ecstasy") toxicity in rats, an effect involving a long lasting increase in MnSOD expression (Puerta, Hervias et al. 2009; Puerta, Barros-Minones et al. 2012), and against 3-nitropropionic acid-induced striatal lesions that mimic neuropathological features of Huntington's disease (Puerta, Hervias et al. 2010). Protective effects after spinal cord injury in rats (Serarslan, Yonden et al. 2010) and amelioration of peripheral neuropathy in a mouse model of diabetes (Wang, Chopp et al. 2011) have also been described.

2.4. cGMP targets

Among the downstream intracellular targets for cGMP are cyclic nucleotide regulated ion channels, PKGs (PKGIα, PKGIβ and PKGII) and cGMP-regulated phosphodiesterases (PDE2, PDE3 and PDE5) (Fig. 5). Cyclic nucleotide-gated channels are all non-selective cation channels that have calcium permeability under physiological conditions. They are directly activated by cGMP and/or cAMP binding and have been shown to play important roles in signal transduction in retinal photoreceptors and in olfactory neurons, and have been also implicated in regulation of synaptic plasticity (Bradley, Reisert et al. 2005).

PKGs are serine/threonine-kinases that become activated by cGMP binding and mediate most of the nucleotide effects. PKGI isozymes are soluble proteins while PKGII is membrane-bound. All three are functional as homodimers and regulate multiple signaling pathways by phosphorylation of ion channels, G proteins and associated regulators and cytoskeleton-associated proteins, among others. Nearly all cells contain at least one of the three isoforms, but the PKGI subtypes have been found to predominate in the CNS, being widely distributed in different regions, with higher concentrations found in cerebellum and hippocampal structures (Hofmann, Bernhard et al. 2009).

cGMP regulates the activity of target PDEs by binding to their regulatory domains. In PDE5, cGMP binding into its regulatory domain is stabilized by a phosphorylation that is preferentially catalyzed by PKG, and is facilitated by allosteric

cGMP occupation of the catalytic site. PDE2 undergoes a conformational change upon binding of cGMP the regulatory domain that increases its enzymatic activity towards cAMP. In the case of PDE3 that has affinity for both, cAMP and cGMP, the rate of cGMP hydrolysis is around 10% that of cAMP but cGMP can compete with cAMP for binding to the catalytic site and inhibit its breakdown. Thus changes in cGMP levels can affect cAMP hydrolysis and thereby regulate cAMP signaling (Fig. 5) (Tsai and Kass 2009; Francis, Blount et al. 2011).

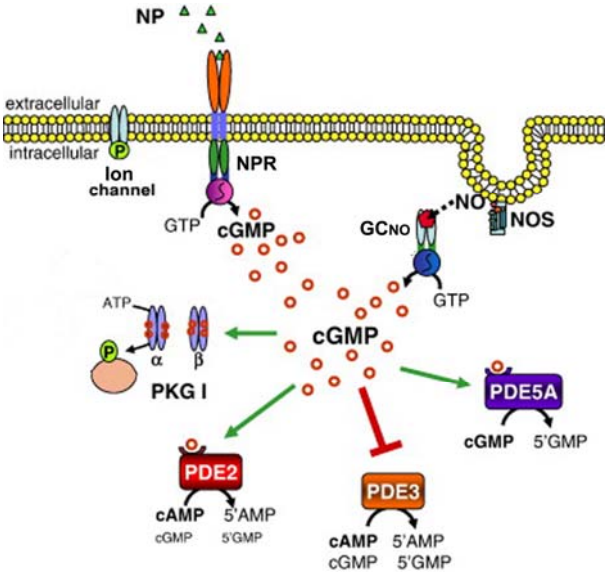

Figure 5. cGMP signaling cascade. Intracellular cGMP produced in response to NPs or NO regulate different intracellular targets. Adapted from Tsai and Kass (Tsai and Kass 2009).

3. cGMP IN NEUROINFLAMMATION

Both NO- and NP-dependent cGMP formation has been implicated in the regulation of expression of more than 60 different genes, many of them inflammatory (Pilz and Broderick 2005; Vollmar 2005; Vellaichamy, Kaur et al. 2007). In most cases cGMP regulation of gene expression occurs at the transcriptional level, but post-transcriptional and post-translational mechanisms have also been described (Wang, Bruderer et al. 1999; Begum, Sandu et al. 2002) and most effects involve regulation via PKG of specific transcription factors such as CREB, NF-κB and c-Fos (Vollmar 2005).

In microglial cells the available data about cGMP regulation of inflammatory gene expression is scarce and contradictory. Studies in a murine microglial cell line

(N9) suggested an anti-inflammatory role for cGMP since its analogue 8-bromo-cGMP (8brcGMP) was able to inhibit Aβ-induced release of leukotriene B4 (Paris, Town et al. 1999) and to reduce LPS-induced secretion of TNF-α (Paris, Town et al. 2000). In the same cell line, the PDE5 inhibitor sildenafil has been recently shown to reduce LPS-induced iNOS expression as well as release of the pro-inflammatory cytokines TNF-α and IL-1β (Zhao, Zhang et al. 2011). However, another study in a different murine microglial cell line (BV2) showed that the NO-cGMP pathway mediated LPS-induced expression of the inflammatory gene CD11b (Roy, Fung et al. 2006). Contradictory results have been also reported using rat brain microglial cultures. Whereas Choi et al. (Choi, Choi et al. 2002) observed that the cGMP- PDE inhibitor zaprinast and the cGMP-analogue dibutyryl-cGMP (dbcGMP) enhanced TNF-α and IL-1β secretion as well as iNOS and MHC-II expression, more recently Moriyama et al. (Moriyama, Taniguchi et al. 2006) described ANP inhibition of NF-κB and AP-1 activation and decreases in LPS-induced nitrite and IL-1β production. In agreement with an anti-inflammatory effect of cGMP are studies in macrophages, cells of the same lineage as microglia. It has been shown that the ANP-cGMP-PKG pathway reduces LPS-induced transcription of iNOS and TNF-α by reducing activation of NF-κB (Vollmar 2005). Other aspects of microglial activation have also been shown to be affected by cGMP pathway. Studies in leech subjected to nerve cord injury have shown that cGMP formed in response to the NO generated in the lesioned area controls microglial migratory direction towards the lesion (Bicker 2005; Duan, Sahley et al. 2009).

In astrocytes, cGMP regulates different aspects of their inflammatory response. Brahmachari et al. (Brahmachari, Fung et al. 2006) reported that cGMP mediates NO-induced increases in GFAP expression in cultured astrocytes. Furthermore, studies in our laboratory have shown that cGMP formed in response to ANP or NO induces via PKG a rapid and reversible reorganization of glial fibrillary acidic protein (GFAP) and actin filaments that results in a dramatic change in cell morphology and increased migration in a scratch wound assay in vitro (Boran and Garcia 2007). Additionally, it has been shown that the NO-cGMP-PKG pathway inhibits LPS-induced matrix metalloproteinase-9 expression in rat astrocytes (Shin, Lee et al. 2007).

4. METALLOTHIONEINS

Metallothioneins I and II (MT-I/II) belong to a family of low molecular weight, heavy metal-binding, cystein-rich proteins with antioxidant and neuroprotective properties (Hidalgo, Aschner et al. 2001; Chung and West 2004). The Mt1 and Mt2 genes are expressed coordinately in most tissues including the CNS. It is well accepted that MT-I/II proteins are highly inducible by a range of stimuli that include metals, hormones, cytokines, oxidative agents, inflammation and stress (Yagle and Palmiter 1985; Sato and Bremner 1993; West, Hidalgo et al. 2008). The main cell expressing these MT isoforms in the CNS is the astrocyte, in particular the reactive astrocyte. Neurons express MT-I/II to a much lower extent while in the normal brain oligodendrocytes and microglia are essentially devoid, but the later cells up-regulate MT-I/II expression in response to injury (West, Hidalgo et al. 2008; West, Leung et al. 2011). In vitro studies have shown that reactive astrocytes secrete MT-I/II that can be internalized by neurons and promote regenerative growth after injury (Chung, Penkowa et al. 2008). Moreover, exogenous MT-I/II induces a reactive phenotype in cultured astrocytes that is permissive to neurite outgrowth (Leung, Pankhurst et al. 2010). Using a model of focal cortical injury by cryolesion in mice it has have shown that MT-I/II over-expression is neuroprotective by decreasing microglia/macrophage activation, oxidative stress and apoptotic cell death (Giralt, Penkowa et al. 2002). Additionally, MT-I/II over-expression causes increased astroglial reactivity around the lesion accelerating formation of the glial scar and wound healing (Giralt, Penkowa et al. 2002). Conversely, genetically modified mice with null MT-I/II expression exhibit a worse outcome following a range of CNS injuries (Penkowa, Carrasco et al. 1999; Carrasco, Penkowa et al. 2000; Asanuma, Miyazaki et al. 2002; Trendelenburg, Prass et al. 2002).

5. 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, Montalban 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 (Friese, Montalban et al. 2006; Bynoe, Bonorino et al. 2007). 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, Peterson et al. 2009). Recent evidence aditionally indicates that demyelination and oligodendrocyte degeneration follows inflammation-induced astrocyte dysfunction (Sharma, Fischer et al. 2010). High levels of inflammatory mediators are secreted by infiltrating immune cells and resident CNS cells and characterize the inflammatory environment during disease (Raivich and Banati 2004). 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, Staykova et al. 2007), suggesting that NO may be neuroprotective. As mentioned above, the major target for NO is GC_{NO} but there are not reports on the implication of cGMP in the neuropathology of EAE.

AIMS

AIMS

In cells from peripheral tissues the cGMP-mediated pathways are able to regulate inflammatory gene expression. In macrophages, the ANP-cGMP pathway down-regulates iNOS and TNF-α expression. In the inflamed CNS, glial cells and in particular microglia, show increased expression of iNOS and the NO produced has been implicated in neurotoxicity. However, when this work was started there were no reports about the regulation of iNOS expression by cGMP-mediated pathways in glial cells. While it was well established that astrocytes could generate cGMP in NO- and NP-dependent manners, there was no information about the capacity of microglia to synthesize and degrade the nucleotide. **The first aim of this thesis was to examine if microglial cells could generate cGMP and if cGMP mediated-pathways could regulate iNOS expression.**

Our results and those of other authors using cultures of glial cells have shown that cGMP-mediated pathways down-regulate inflammatory responses in microglia and promote a reactive phenotype in astrocytes. **The second aim of this thesis was to investigate if increasing cGMP by treatment with cGMP-PDE inhibitors had the same effects in the glial inflammatory response in vivo in a model of focal cortical injury.**

Results in the cortical injury model indicated that treatment with PDE5 inhibitors exerted anti-inflammatory and neuroprotective effects. **The third aim of this thesis was to investigate if treatment with a PDE5 inhibitor could have beneficial effects in an animal model of multiple sclerosis, a disease where an altered inflammatory response is the leading cause of neurodegeneration and functional disability.**

MATERIALS AND METHODS

MATERIALS AND METHODS

1. MATERIALS

1.1. Reagents

Culture material

Dulbecco's modified Eagle's medium (DMEM), Red blood cell lysing buffer (RBC), RPMI medium, cytosine arabinoside (Cyt-ara), Trypan Blue, penicillin, streptomycin, L-Glutamine, albumin from bovine serum, β-mercaptoethanol, trypsin from bovine pancreas, Soybean Trypsin inhibitor Type I-S and DNase I were obtained from Sigma-Aldrich Química. Foetal-calf serum (FCS) was from Ingelheim Diagnostica. L-methionin and L-cystein free DMEM (Gibco), trypsin and Hanks' solutions were from Invitrogen.

General reagents

Lipopolysaccharide (LPS, from *Salmonella typhimurium*), 1H-[1,2,4]oxodiazolo-[4,3-a]quinoxalin-1-one (ODQ), dibutyril-cGMP (dbcGMP), dibutyril-cAMP (dbcAMP), 8-bromoguanosine 5'-monophosphate (8br-cGMP), sodium nitroprusiate (SNP), cicloheximida (CHX), HEPES, chloroform, isopropanol, m-xilene, DPX mountant, paraformaldehyde (PFA), methanol, Triton X-100, bovine serum albumin (BSA), BrdU, β-2-mercaptoethanol, 4´,6-diamidino-2-phenylindole (DAPI), eosin, Harris Hematoxilin solution, luxol fast blue solution, cresylEcht Violet, lithium carbonate, phosphatase inhibitors, protease type XIV and 3-Isobutyl-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich Química. TRIzol reagent, dNTPs, RNAse inhibitor, Superscript reverse transcriptase, random primers and Taq polymerase were from Invitrogen. Ethylendiaminetetraacetic acid (EDTA), Sodium dodecyl sulfate (SDS), glicerol, ammonium persulfate, sodium nitrite, sucrose, glucose, NaCl, KCl, KH_2PO_4, Na_2HPO_4, $CaCl_2$, NaOH, glicine and sulfanilamide by Merck; iQ Sybr Green Supermix, acrilamide/bisacrilamide, TEMED and agarose by BioRad. Rhodamine-phalloidin, BODIPY FL-phallacidin, red fluorescent inert particles (excitation/emission maxima = 580/608), acetylated low-density lipoprotein (Ac-LDL) labelled with 1,1´ dioctadecyl-3,3,3´,3´ tetramethylindocarbocyanine perchlorate (Dil), (Dil-Ac-LDL) or with Alexa 488 (Alexa 488-Ac-LDL) were from Molecular Probes; polyvinyl difluoride (PDVF) membranes, Catch and Release kit and chemiluminiscence detection kit from Millipore Iberica; OxiBlot TM detection kit was from Chemicon; ethanol and methanol from Alcoholes Gual, 8-bromo-

β-phenyl-1,N²-ethenoguanosine-3',5'-cyclic monophosphorothioate (Rp-8Br-PET-cGMPS) and adenosine-3',5'-cyclic monophosphorothioate (Rp-cAMPS), protein kinase G and A (PKG and PKA) inhibitors were from Biolog Life. Atrial natriuretic peptide (ANP, 3-28 aminoacids), C-type natriuretic peptide (CNP), cANP (4-23 aminoacids, amide) were from American Peptide. In Situ Cell Death Detection Kit, LightCycler FastStart DNA Masterplus SYBR green I system, LightCycler capillary tubes, specific primers and protease inhibitor cocktail were from Roche Molecular Biochemicals. Peroxidase substrate Kit DAB was from Vector Laboratories. CBA mouse Th1/Th2/Th17 cytokine kit was from BD Bioscience. RNeasy Plus Mini Kit was from QIAGEN.

1.2. Antibodies

Primary antibodies

Mouse anti-actin, mouse anti-GFAP antibodies and lectin from *Lycopersicon esculentum* conjugated with biotin were obtained from Sigma-Aldrich Química. Rabbit anti-GFAP and rabbit purified anti-CD3 were from DAKO. Mouse anti-iNOS was from BD Transduction Laboratories. Mouse anti-ICAM-1 was from Pharmingen. Mouse anti-SMI-32 was from Covance. Goat anti-malondialdehide (MDA), mouse anti-CD68 and anti-CD11b antibodies were from Serotec. Rabbit anti-Iba-1 was from WAKO (Osaka, Japan). Mouse anti-NeuN was from Chemicon. Rabbit anti-VEGF was from Abcam. Sheep anti-cGMP antibody developed against PFA-fixed cGMP linked to thyroglobulin was kindly donated by J. de Vente. Mouse anti-Foxp3 was a gift kindly donated by Dr. G. Roncador (Monoclonal Antibodies Core Unit, Spanish National Cancer Research Center (CNIO), Madrid.

Secondary antibodies

Antibodies used for immunostainings: Goat anti-mouse antibody conjugated to alkaline phosphatase was obtained from Sigma-Aldrich Química. Alexa 488, 555 or 568 coupled to anti-IgG secondary antibodies were from Molecular Probes. Secondary biotin-conjugated anti-rabbit, anti-goat and anti-mouse and streptavidin-horseradish peroxidase (HRP) were from Vector. Antibodies used for Western Blots: HRP-conjugated anti-rabbit and anti-mouse IgG was from Amersham Life Science.

1.3. Instruments and Software

Images were acquired using Nikon Digital camera DXM 1200F and Nikon Act-1 software or confocal (model TSC SP5, Leica) microscopes, and then analysed by using AnalySIS (version 3.2, Soft Image Analysis System). Absorbance and fluorescent measurements were obtained with a multilabel plate reader VICTOR 3 (PerkinElmer). Western blot membranes were developed by Versa Doc Imaging System (Bio-Rad) and then individual bands were quantified by Quantity One Analysis Software (Bio-Rad). Agarose gels were visualized by GelDoc Imaging system (Bio-Rad). The flow cytometer (model FACSCanto) was from BD Biosciences and the data obtained was analysed by FCAP Array™ Software (BD Biosciences). Data analyses were carried out using Graph Pad Prism Software (version 4). For Real time PCR LyghtCycler 2.0 carrousel-based termocycler (Roche) or the 96 wells-plate termocycler Bio-Rad CFX96 (Bio-rad) were used. For quantification of protein, RNA or DNA concentrations QUBIT nanofluorimeter system was used (Invitrogen). The analysis software Scion Image (NIH) was used to quantify histological staining and the Image J program to manually count stained positive cells.

2. METHODS

2.1. Primary glial cell cultures

To prepare glial cell cultures Sprague-Dawley rats were used. Cortical cultures were prepared from 1-3 day-old pups and cerebellar cultures from 7-8 day-old pups. These animals were breed at the Universidad Autónoma de Barcelona and kept with their mothers until sacrifice.

2.1.1. Astrocyte-enriched cultures

Astrocyte-enriched cultures were prepared as described previously with little modifications (Agullo, Baltrons et al. 1995). Briefly, rats were decapitated and cerebellum or cortex immediately dissected out and maintained in D1 media (NaCl 137 mM; KCl 5,5 mM; KH_2HPO_4 2,22 mM; Na_2HPO_4 0,17 mM; glucose 5 mM and sucrose 58,5 mM; pH 7,4). After meninges and blood vessels were removed, the tissue was minced and incubated in Ca^{2+}-free Krebs-Ringer buffer containing 0.025% trypsin. Cells were then mechanically triturated through a glass pipette and filtered through a 40 μm nylon mesh in the presence of 0.52 mg/ml soybean trypsin inhibitor and 170 IU/ml DNase I. After centrifugation, cells were stained by Trypan Blue and viable cells counted in a Neubauer chamber. Cells resuspended in

90% DMEM, 10% FCS, 20 U/ml penicillin (P), 20 μg/ml streptomycin (S) and L-glutamine 2 mM were seeded on plastic Petri dishes at $1.25x10^5$ cells/cm^2. Cells were maintained in a humidified atmosphere of 90% air-10% CO_2. The medium was changed every 3-4 days and cells were used after 14-15 days *in vitro* (DIV). These cultures that are enriched in astrocytes also contain microglial cells that show a drastic increase in number when the astrocyte monolayer reaches confluency (10 DIV) representing up to 20-30 % of the total amount of cells at 14 DIV (Agullo, Baltrons et al. 1995). In some experiments, confluent cultures (10 DIV) were treated with 10 μM cytosine arabinoside (Cyt-ara) until the day of use (14 DIV) in order to decrease microglial proliferation (Agullo, Baltrons et al. 1995).

2.1.2. Microglial isolated cultures

Microglial cultures were prepared by mild trypsinization of confluent astrocyte-enriched cultures as described by Saura et al. (Saura, Tusell et al. 2003). Once these cultures reached confluency, monolayers were washed twice with DMEM and were incubated at 37ºC with a trypsin solution (0.25% trypsin, 1 mM EDTA in Hank's buffered salt solution (HBSS)) diluted 1:4 in DMEM until the astrocyte monolayer detached (about 1 h), then the medium was aspirated, cells washed once and DMEM with serum was added. Cells were maintained in this medium for at least 2 days. In these cultures more than 98% of the cells were microglia as estimated by Dil-Ac-LDL and CD68 staining. Cells were treated with the different compounds in complete DMEM for the indicated times and concentrations.

2.2. Splenocyte cultures

Spleens from EAE animals (see 2.4) were mechanically dissociated and cells were flushed out from the spleens gently using a syringe plunger with phosphate-buffered saline (PBS: 2.6 mM KCl, 1.4 mM KH_2PO_4, 136 mM NaCl, 8mM Na_2HPO_4) and centrifuged at 4ºC. The pellet was resuspended and left in RBC lysis buffer (17 mM Tris, 140 mM NH_4Cl) to remove erythrocytes. After further centrifugation, cells were washed with PBS, centrifuged and resuspended in complete medium (RPMI with L-glutamine supplemented with 15% FCS, P/S antibiotics, 1 mM sodium piruvate and 50 μM β-mercaptoethanol). Cells were seeded in 24-well plates at a density of 2–4 x 10^6 cells/ ml and exposed or not to MOG35–55 (10 μg/ml, 72 h). Cytokine release was determined in the media of 24-well plates with BD CBA mouse Th1/Th2/Th17 cytokine kit following the manufacturer instructions. Cells were stored for mRNA analysis (see below).

2.3. Cryolesion procedure

For the cryolesion procedure we used two-month-old male Sprague-Dawley rats or five to ten-week-old 129S1/SvImJ wild-type or homozygous MT-I/II deficient mice, with 129S7/SvEvBrd genetic background, (Masters et al. 1994) initially obtained from Jackson Laboratories (Bar Harbor, MA) and colonies maintained at the UAB. Animals were separated in four groups: two groups of unlesioned animals and two groups of cryolesioned animals that were treated with vehicle or zaprinast (rats) and sildenafil (mice). The number of animals per treatment are summarized in Table 1.

Table 1: Animals and treatments used in the cryolesion experiments

	Experiment	Unlesion		Cryolesion	
	Exp.1	Vehicle	Zaprinast	Vehicle	Zaprinast
Rat	Sprague-Dawley	n=4	n=4	n=6	n=6
	Exp.2	Vehicle	Sildenafil	Vehicle	Sildenafil
Mouse	129S1/SvImJ MTI/II KO	n=4	n=4	n=5 n=4	n=7 n=6

Animals were cryolesioned under tribromethanol anaesthesia. The skull over the right frontoparietal cortex was exposed, and a focal cryoinjury was carried out on the surface of the brain by applying a 0.4 mm diameter dry ice pellet (at -78ºC) for 30 or 60 seconds (for mice and rats, respectively) as previously described (Giralt, Penkowa et al. 2002). Animals were allowed to recover and returned to the animal room until they were sacrificed 3 days post lesion (dpl). Rats were injected intraperitoneally (i.p.) with zaprinast (10 mg/kg, Sigma), or vehicle (0.05 M NaOH) and mice were injected subcutaneously (s.c.) with Sildenafil (10 mg/ kg, Pfizer) or vehicle (water), 2 h before and 24 and 48 h after cryolesion. Unlesioned animals were used as controls. After decapitation, the entire ipsilateral cortex was dissected out and divided into two parts cutting in the middle of the lesion. Half of the lesioned cortex was fixed in 4% PFA for immunohistochemistry and the lesioned area of the other half was frozen in liquid nitrogen and stored at -80ºC until use for biochemical studies. All experiments were carried out in a human manner and were approved by the proper ethical committees.

2.4. EAE

2.4.1. EAE Induction

Two-month-old female C57BL/6 mice (Charles River) were housed in the animal care facility of the UAB under constant temperature and provided food and water ad libitum. EAE was induced by immunization with MOG35–55 peptide (Scientific Technical Service, Universitat Pompeu Fabra, Barcelona, Spain). On day 0, mice were injected subcutaneously (s.c.) into the hind flanks with an emulsion of 100 µl MOG35–55 (3 mg/ml) and 100 µl of 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 which was repeated 2 days after immunization. As controls, a group of mice were immunized with BSA instead of MOG or with CFA supplemented with mycobacterium.

2.4.2. Clinical score evaluation in EAE model

Mice were clinically scored for EAE daily according to the following criteria: 0 no signs of disease; 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.

2.4.3. Treatments and sacrifices

Animals were clinically scored for EAE daily and treated with sildenafil (10 mg/kg, s.c.; Pfizer) or vehicle (water) once daily. Two protocols of treatment were used in different experiments. In Experiment 1, treatment started 18 days post-immunization (dpi), at the peak of the disease, when more than 90 % of animals were sick and the group mean score was approximately 2. To study inflammatory cell infiltration and neuropathology animals were sacrificed after 3 or 8 days of treatment (21 and 26 dpi). In Experiment 2, animals were treated at the onset of clinical symptoms (clinical score around 1, 10-16 dpi) and sacrificed after receiving sildenafil treatment for 7 or 15 days. Mice were sacrificed under isofluorane anaesthesia. Blood, spinal cords, brains and spleens were collected. The number of animals and the treatments are summarized in Table 2.

Table 2: Animals and treatments used in EAE experiments

Experiment	CFA/BSA		EAE	
	Vehicle	Sildenafil	Vehicle	Sildenafil
Exp.1(peak)	n=6	n=6	n=17	n=17
Exp.2(onset)	n=5	n=2	n=22	n=15

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

3. STAININGS

3.1. Immunocytochemistry (ICC)

Cultured cells were fixed with 4% PFA in PBS at room temperature (RT). After several washes, monolayers were permeabilized by incubation with PBS-0.1% Triton X-100. After blocking non-specific binding with 1% BSA in PBS, cells were incubated overnight (ON) at 4ºC with primary antibody in 0.1% BSA-PBS, and 1h at room temperature with the appropriated secondary antibody conyugated with Alexa (555, 568 or 488) for fluorescence studies (dilution 1:1,000). The primary antibody was omitted in controls.

For F-actin staining, cells were incubated overnight at 4ºC with BODIPY FL-phallacidin or rhodamine-phalloidin (diluted 1:200 in 1% BSA-PBS). When double stainings were performed, the corresponding F-actin marker was added during the secondary antibody incubation.

In some experiments, microglia were labelled by incubating live cells for 4 h at 37ºC with the specific marker Dil- or Alexa 488-Ac-LDL (10 µg/ml) before fixation.

For nuclei labelling, DAPI (0.25 µg/ml) was added together with secondary antibodies or actin markers.

3.2. Immunohistochemistry (IHC)

Dissected fresh brains or the lumbar-thoracic region of the spinal cords were fixed in 4 % PFA in PBS and embedded in paraffin according to standard procedures. Brains of cryolesioned animals were cut in serial coronal sections and spinal cords of EAE animals were cut in longitudinal sections (8 µm). Sections were deparaffinised by serial immersion from xilene to ethanol 50%. Antigen retrieval was performed by one of three standard protocols. Heat-induced by boiling in citrate buffer (pH 6.0) in a microwave oven, heating to

37ºC protease type XIV solution (0,1 % in TBS) or heating samples at 70ºC in Tris-EDTA. After temper the samples at room temperature (RT), they were washed in TBS and incubated in 1.5% H_2O_2, 70 % methanol in 0.05 M Tris to quench endogenous peroxidase. If necessary, permeabilization was done by incubation in TBS+0.5% triton X-100 (TBS-T). To block non-specific binding, samples were incubated in blocking buffer (10% FCS in TBS-T). Then, sections were incubated overnight at 4ºC with primary antibodies in a wet-chamber. The slices were tempered for one h at RT and after washing, sections were incubated at RT with the appropriate secondary antibody conjugated to biotin (1:200) that was detected using streptavidin-HRP (1:300) developed using the peroxidase substrate Kit DAB following manufacturer instructions. Finally, samples were dehydrated by several immersions in serial concentrations of ethanol-xilol and mounted with DPX. Control sections were incubated in the absence of primary antibodies.

3.3. Histological methods

Longitudinal sections of the lumbar-thoracic region of the spinal cords of EAE animals were stained with haematoxylin and eosin (HE), Luxol Fast Blue (LFB) or Bielschowsky silver staining for evidence of infiltrates, demyelination and axonal integrity, respectively, following standard procedures (Giuliani, Metz et al. 2005).

3.3.1. Haematoxylin-Eosin (H&E)

The histological score for infiltration was evaluated on HE-stained sections blindly according to the following criteria: 0 no inflammation; 1-cellular infiltrates only around blood vessels and meninges; 2-moderate cellular infiltrates in parenchyma, less than 50% of the white matter (WM); 3-severe infiltrates in parenchyma, deep and/or more than 50% of the WM.

3.3.2. Luxol Fast Blue (LFB)

The demyelination score was evaluated on LFB-stained sections blindly according to the following criteria: 0 no demyelination; 0.5 little demyelination only around infiltrates; 1-superficial demyelination, which involves less than 25% of the white matter (WM); 1.5-superficial demyelination which involves more than 25% but less than 50% of the WM; 2-deep demyelination and/or demyelination that involves more than 50% of the WM; 3-diffuse and widespread demyelination.

3.3.3. Bielschowsky silver technique

Axonal density was evaluated blindly by Bielschowsky's silver impregnation. Slides were incubated with fresh 20% silver nitrate solution previously preheated in a microwave. After washing thrice in distilled water slides were placed in ammoniacal silver solution. Then samples were incubated in working developer solution containing formaldehyde, citric acid, nitric acid and ammonium hydroxide. Slices were checked under microscope before washing in fresh ammonia water and then washed in distilled water. Slides were then placed in 5% Hypo (Sodium thiosulfate) and washed with distilled water, dehydrated and mounted with DPX.

3.4. Other staining methods.

3.4.1. Counterstaining

In some cases sections were counterstained with 0.25%cresylEcht Violet, rinsed with water and posterior differentiation in 95% ethanol or hematoxylin before mounting.

3.4.2. Lectin staining

Cells of myelo-monocytic lineage and blood vessels were also stained with lectin from Lycopersicon esculentum conjugated with biotin as previously described (Penkowa, Carrasco et al. 1999) and lectin were detected using streptavidin-HRP. Control sections were incubated in the absence of primary antibodies to evaluate the extent of non-specific binding of the secondary antibody.

3.4.3. In situ detection of DNA fragmentation

For detection and quantification of apoptotic cell death at a single cell level, terminal deoxynucleotide transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-fluoresceine nick end labelling (TUNEL) was performed using the In Situ Cell Death Detection Kit on paraffin brain sections, following the protocol for difficult tissues according to the manufacturer's recommendations. This kit is based on labelling of DNA strand breaks (TUNEL reaction). Positive (DNAase I-treated) and negative (without terminal transferase) controls were included in each experiment.

3.5. Quantification and statistical analysis

3.5.1. Cryolesion

Quantification of GFAP, lectin and Iba-1 staining in cryolesioned animals was carried out at the border of the cortical lesion where inflammation is prominent using the Scion Image program. For each parameter analyzed, brain sections from four to five animals per group were used and a mean value of 4–6 different 0.5 mm^2 areas per brain section was calculated. Quantification areas were from the central part of the lesion border where staining is more homogeneous than at the edges. Quantification of VEGF staining was performed in 5 different 0.5 mm2 areas of the lesioned cortex at least 50 μm away from the lesion border using the Scion Image program. TUNEL- and NeuN-positive cells as well as lectin-stained or hematoxylin–eosin-stained blood vessels were counted in 5–7 different 0.5 mm^2 random areas of the lesioned cortex at least 50 μm from the lesion border. For the predominantly apoptotic cell death marker TUNEL, positively stained cells were defined as cells with nuclear staining. Criteria for identifying degenerating neurons using the neuronal nuclei marker NeuN were the presence of condensed or dystrophic cell bodies. Quantification of TUNEL, NeuN and vessels was carried out by two independent investigators, who were blinded to animal identity and treatment.

3.5.2. EAE

Four to eight areas (1 mm^2) randomly chosen along the length of each of 3–4 different longitudinal spinal cord sections separated at least 300 μm were analyzed by two independent investigators blinded to treatment and clinical score. The analysis software Scion Image (NIH) was used to quantify LFB, Bielschowsky, SMI-32, ICAM-1, Iba1 and GFAP staining. Quantification of CD3+ and FoxP3+ cells was performed manually in infiltrates (4–8 infiltrates per section).

4. DETERMINATION OF mRNA LEVELS BY RT-PCR

4.1. RNA extraction

RNA from cultured astrocytes and from splenocytes from EAE mice was extracted using TRIzol reagent following manufacturer's instructions. Briefly, the cellular lisate was incubated for complete dissociation of nucleo-protein complexes. Chloroform was added and after centrifugation the upper aqueous phase was collected and RNA was precipitated by addition of isopropanol. After centrifugation the pellet was washed with cold 75% ethanol and dried at RT. Once ethanol was completely evaporated RNA was resuspended in DEPC treated water (see 7.3).

RNA from isolated microglial cultures was extracted using RNeasy Plus Mini Kit (QIAGEN) following manufacturers' instructions.

Final concentration of RNA was measured with QUBIT nanofluorimeter system and RNA integrity was evaluated in 1% agarose gels.

4.2. RT-PCR.

4.2.1. RNA Reverse Transcription

RNA was transcribed to complementary DNA (cDNA) by using Random Primers and Superscript II or III reverse transcriptase (RTase). In a final volume of 20 µl, 12 µl of mix reaction (4 µl 5X reaction buffer, 4µl of dNTPs mixture (dATP/dCTP/dTTP/dGTP; 2,5mM each), 2 µl of DTT (100mM), 10 units of RNA inhibitor and 200 units of RTase) were added to 8 µl of mix of random primers and samples, containing the same amount of RNA each. The reaction was done at 42ºC during 1 h and finally samples were heated at 95ºC.

4.2.2. Amplification by PCR and Real time PCR

Semi quantitative PCR was done in a final volume of 50 µl containing 40 µl of reaction mix (5 µl of 10X reaction buffer, 1.5 µl $MgCl_2$ (50 mM), 4µl of dNTPs mixture (2.5 mM), 0.5 µl of forward (F) primer, 0.5 µl of reverse (R) primer, both at 50 µM) and 5 µl of c-DNA and 5 µl of Taq polymerase (5 Units/µl).

For real time PCR in LightCycler Carousel-Based System, the LightCycler FastStart DNA Masterplus SYBR green I system (Roche) was added to 5 µl of first strand cDNA that was used as template.

For real time PCR in 96-well plate termocycler, iQ Sybr Green Supermix (Bio-Rad) was applied and 2 µl of first strand cDNA was used as a template.

For standardization in both systems 5 or 2 µl, respectively, of serial dilutions of the gel purified and sequence verified PCR products were used as standards in separate reactions. All samples and standards were analyzed in duplicates.

PCR products were subjected to agarose gels (1-2% agarose/ 0.5 mg/ml ethidium bromide) and were visualized by UV light. Each amplicon was analyzed between the cell cycles where cDNA amplification was lineal. The intensity of bands was quantified by using Quantity One software (Bio-Rad) and values were normalized for GAPDH levels.

In real-time experiments SYBR green fluorophore was employed. This fluorophore is incorporated into double strand DNA and is quantified in each cycle of the reaction obtaining a curve in which we can extrapolate the initial value of each specific cDNA concentration. Values were accepted if error was not higher than 5 % and the difference in duplicates was lower than half cycle.

The primers used to amplify sequences of interest were validated by BLAST software and are listed in table 3.

Table 3: List of specific primers used for each of the gens analysed

	Sequence 5'-3'	Amplicon size
GAPDH-F GAPDH-R	GCC AAG TAT GAT GAC ATC AAG AAG TCC AGG GGT TTC TTA CTC CTT GGA	262 base pairs (bp)
iNOS-F iNOS-R	CTG CAT GGA ACA GTA TAA GGC AAA C CAG ACA GTT TCT GGT CGA TGT CAT GA	228 bp
NPRA-F NPRA-R	ATC ACA GTG AAT CAC CAG GAG TTC AGA TGT AGA TAA CTC TGC CCT TTC	97 bp
NPRB-F NPRB-R	ATG GTC AGA GGC CGT ATT TCC CTT GTT AAA CCG GCG AAT GA	149 bp
NPRC-F NPRC-R	CCTACAATTTCGACGAGACCAAA TCGCTCACTGCCCTGGAT	71 bp
PDE1A-F PDE1A-R	GAT CGC TCT GTC CTT GAG AAT CTC TGT TGA GTC TGT CAG AAG	462 bp
PDE1B-F PDE1B-R	GAG GAC CAT TGT TTT TGA GTT GCT GCC TCC AGA AAA CTC ATC AGA AA	90 bp
PDE2A-F PDE2A-R	CCC AAA GTG GAG ACT GTC TAC ACC TAC CTG GCC ACA GTG CAC CAA GAT GA	279 bp
PDE5A-F PDE5A-R	CAG GAA ATG GTG GGA CCT TC AAG GCT TCC AGG AAC TGC TC	619 bp
PDE9A-F PDE9A-R	CAC TTG GCT GTC CTA GAG AAA CGC CTC CAG CGG TGA GAT GTC ATT GTA	606 bp
CD19-F CD19-R	CCT CTC CCT GTC TCC TTC CT TGG TCT GAG ACA TTG ACA ATC A	259 bp
FoxP3-F FoxP3-R	CAG CTG CCT ACA GTG CCC CTA G CAT TTG CCA GCA GTG GGT AG	362 bp
RORγt-F RORγt-R	TCA CCT GAC CTA CCC GAG G TCC AAG AGT AAG TTG GCC GTC	194 bp
TNF-α-F TNF-α-R	GGA GGA GAA GTT CCC AAA TG CGG ACT CCG TGA TGT CTA AG	497 bp

5. DETECTION OF PROTEIN LEVELS

5.1. Preparation of homogenates

Treated cell cultures were scraped out of the plates in PBS and collected by brief centrifugation and resuspended in ice-cold homogenization buffer (50 mM Tris-HCl, 1 mM EDTA buffer, pH 7.4 at 37ºC) containing anti-proteases and anti-phosphatases cocktail. In the case of fresh frozen brain tissue, it was homogenized in 10 % weight/volume of the same buffer. Cutured astrocytes and tissue were homogenized with a glass-Teflon Potter-Elvehjeim homogenizer (20 strokes, mechanically driven at 800 rpm). For microglial cultures, which contained very low quantity of cells in a little volume of homogenization buffer, cells were lysed with addition of 1% NP40 detergent and vortexed for 1 minute.

In some experiments, cytosolic and membrane fractions were obtained by centrifugation of the cell lysate at 100.000 x g . The membrane fraction was washed once by resuspension and centrifugation.

5.2. Measurement of protein levels

Protein concentration was determined in some cases by the method of Lowry (Lowry, Rosebrough et al. 1951) using BSA as standard or by QUBIT nanofluorimeter system following manufacturer's instructions.

5.3. Western Blot (WB)

Samples from total homogenate, cytosolic o membrane fractions containing equal amounts of protein (15-40 μg) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The percentage of acrylamide/bis-acrylamide was 10 or 12 depending on the molecular weight of the protein. After running the samples, proteins were transferred to PDVF membranes, and probed with rabbit anti-GFAP (1:500), mouse anti-iNOS (1:1000), purified monoclonal IgG anti-rat CD11b (1:500), polyclonal rabbit anti-Iba1 (1:3000) or mouse anti-actin (1:100.000) primary antibodies. This was followed by incubation with anti-IgG-HRP-labelled secondary antibodies (1:3000) and subsequent detection with an enhanced chemiluminescence detection kit (Millipore).

5.4. Detection of protein oxidation

Protein oxidation was detected in immunoblots of cortex homogenates (20 μg protein) by detection of carbonyl groups using the OxiBlot TM detection kit (Chemicon) following the manufacturer's recommendations.

5.5. ELISA for MT I-II

Concentrations of MT I+II were measured using a competitive double-antibody ELISA assay as described previously (Gasull, Rebollo et al. 1993; Gasull, Giralt et al. 1994). Briefly, 96 well plates were coated ON at 25ºC with 12 ng of MT-II. After blocking with 1% BSA, 100 μl of solution containing the unknown sample (200 μg of total protein) or standard (0-400 ng MT) and 100 μl of anti-MT-I/II antibody were added (final dilution of antibody 1:2000) and incubated at 37ºC. The plates were then incubated with anti-rabbit IgG-HRP conjugate (1:3000) before adding substrate solution. After stopping the reaction with sulphuric acid, the absorbance was read at 595 nm.

5.6. Metabolic labelling with ^{35}S-methionin/cystein and immunoprecipitation

Microglial cultures were treated with ANP for 1 h before addition or not of LPS. After 12 h incubation, medium was replaced by L-methionin and L-cystein free DMEM for 45 min. Afterwards, medium was aspirated and new media containing L-[^{35}S]- Met/Cys (24 μCi/ml) and incubated for additional 5 h. After washes with cold PBS, cells were scrapped and centrifuged. Cells were resuspended in homogenization buffer containing 1% triton X-100 and vortexed. Samples were centrifuged at 100,000xg and supernatants collected and protein concentration quantified.

Immunoprecipitation with anti-iNOS antibody was performed using Catch and Release kit (Millipore) following manufacturers' instructions starting with 500 μg of total protein for each treatment. As control an equal amount of protein was incubated with mouse immunoglobulin G.

Immunoprecipitated samples were subjected to SDS-PAGE at 8 % of acrylamide/bis-acrylamide. After completion of the run, the gels were dried with heat in vacuum, and visualized by autoradiography.

6. STATISTICAL ANALYSIS

Experiments were reproduced the indicated number of times. Quantifiable determinations were expressed as means ± SEM or SD of the indicated number of experiments. Significance of differences was evaluated by one-way ANOVA followed by Turkey's post-hoc test or two-way ANOVA followed by Bonferroni's post-hoc test when there were more than two groups. For comparison of two groups the Student's t-test was used. For comparison of some experiments which were normalized as percent of one treatment, One sample t-test was used. Data analyses were carried out using Graph Pad Prism Software (version 4).

7. OTHER METHODS

7.1. Measurement of nitrites

Nitrite concentration in the culture medium was assayed by the Greiss reaction. In brief, an equal volume of Griess reagent (0.5% sulfanilamide, 0.05% N-(1-naphthyl) ethylenediamide dihydrochloride, and 2.5% phosphoric acid) was added to the medium sample and incubated at RT. Absorbance was measured at 550 nm.

7.2. cGMP stimulation.

Cultures were washed and pre-incubated at 37ºC in PBS before stimulation with SNP (100 μM), ANP (1 μM) or CNP (1 μM) for 3 min or the indicated times in the presence or absence of PDE inhibitors at the indicated concetrations. Immediately after the incubation, cells were fixed with 4% PFA and stained for cGMP by the standard procedure (see 3.1).

7.3. Cytokine analysis by Flow Cytometry

Media from cultured splenocytes that had been exposed or not to MOG35–55 (10 μg/ml, 72 h) were collected and cytokine levels determined with BD CBA mouse Th1/Th2/Th17 cytokine kit following manufacturers' instructions.

7.4. DEPC treatment of water

For inhibition of RNAses filtered water was treated with 0.1% of diethylpyrocarbonate (DEPC) mixing ON and then autoclaved to inactivate traces of DEPC.

CHAPTER 1

CHAPTER 1: REGULATION OF iNOS EXPRESSION BY cGMP IN MICROGLIAL CELLS

1.1. cGMP metabolism in microglial cells

We have investigated if microglial cells are able to accumulate cGMP by stimulating rat cerebellar mixed glial cultures or purified cortical microglial cultures with the NO donor SNP (100 μM) or with ANP (1 μM) in the presence or absence of PDE inhibitors. cGMP formation was examined by immunostaining of fixed cultures with an antibody against PFA-fixed cGMP linked to thyroglobulin. As shown in figure 1A, in mixed glial cultures both SNP and ANP stimulated cGMP formation in astrocytes in agreement with previous reports (Teunissen, Steinbusch et al. 2001; Baltrons, Pifarre et al. 2004). However, only ANP was able to increase cGMP in microglia as shown by colocalization with the microglial marker Ac-LDL (Fig. 1A). Similar results were obtained in purified cortical microglial cultures (Fig. 1B). Intracellular accumulation of the nucleotide was increased in microglia in the presence of the nonspecific PDE inhibitor IBMX as well as the more selective cGMP-PDE inhibitors zaprinast (PDE5, PDE9 and PDE1) and sildenafil (PDE5), indicating that these cells express cGMP-degrading PDEs. In view of these results, we examined the expression of NP receptors and of cGMP-PDE isoforms in purified cortical and cerebellar microglial cultures in comparison with astrocytes. As shown in Fig. 1C, expression of mRNAs for NPR-A and NPR-B was observed in rat microglia from both regions but NPR-C was only detected in microglia from cerebellum. Notably, in cortical microglia NPR-A appears to be the predominant isoform, in contrast to astrocytes where NPR-A is barely detectable while NPR-B is highly expressed. However, in cerebellum both NP receptor types are abundantly expressed in astrocytes and much less expressed in microglia. We also detected expression of cGMP-specific PDE isoform mRNAs in cultures of cortical microglia and astrocytes (Fig. 1D). Both cell types expressed mRNA for the cGMP-specific PDE5A but not for PDE9A which is ubiquitously expressed in neuronal populations (van Staveren, Glick et al. 2002). Microglia also expressed dual-substrate PDE1A and PDE1B, whereas astrocytes did not express PDE1A but expressed PDE1B at higher levels than microglia, and also PDE2A that appeared to be absent in microglia. Thus, in addition to astrocytes, microglia has the enzymatic machinery to synthesize and degrade cGMP.

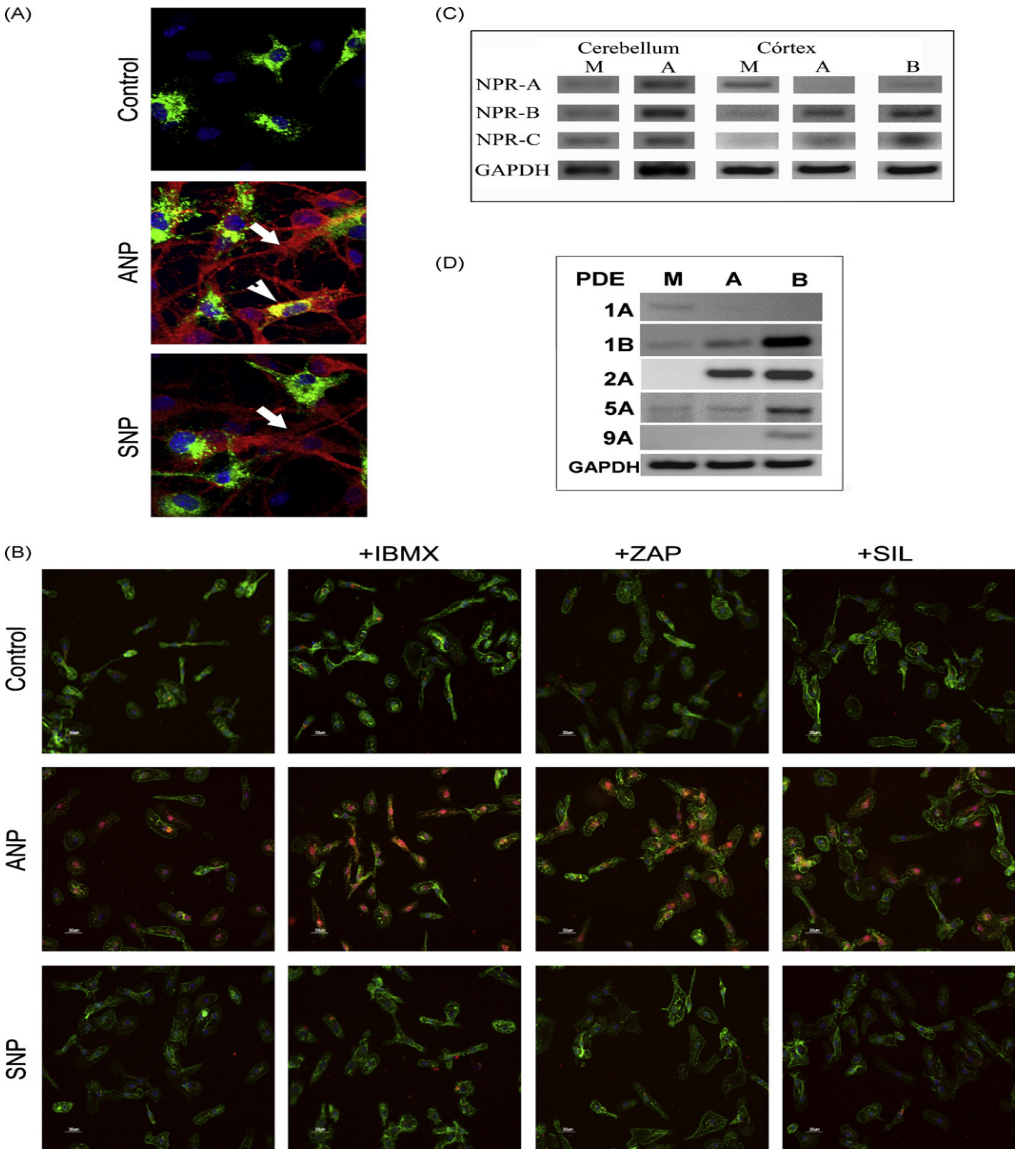

Figure 1. Cyclic GMP formation and expression of NPRs and PDEs in cultured glial cells. (A) Mixed astroglia-microglia primary cultures from rat cerebellum were treated for 3 min with vehicle (control), SNP (100 µM) or ANP (1 µM) in the presence of 1 µM IBMX. Cells were immunostained for cGMP (red), the microglial marker Alexa 488-Ac-LDL (green) and nuclei (DAPI, blue) and observed by confocal microscopy. Note that cGMP immunostaining is increased in astrocytes (arrows) in response to both SNP and ANP, but only in response to ANP in microglia (arrowhead). (B) Primary cultures of rat cortical microglia were treated with SNP (100 µM, 10 min) or ANP (1 µM, 30 min) in the absence or presence of IBMX (1 µM), zaprinast (ZAP; 100 µM) or sildenafil (SIL; 100 nM). Cells were immunostained for cGMP (red), F-actin (BODIPY FL-phallacidin, green) and nuclei (DAPI, blue). As in cerebellar microglia ANP but no SNP stimulated cGMP formation in cortical microglia and the effect was enhanced by cGMP-PDE inhibitors. (C) Expression of NPR mRNAs in cortical and cerebellar cells and (D) PDE mRNAs in cortical cells was analysed by RT-PCR. Microglia (M), astrocytes (A) and 1-day-old rat brain (B). GAPDH was used as control for differences in cDNA synthesis efficiency.

1.2. ANP down-regulates iNOS protein expression in microglia

We first analysed the effect of ANP on iNOS protein expression in mixed astroglial-microglial cultures exposed to LPS (10 ng/ml). iNOS protein can be detected by western blotting after 6 h of LPS treatment and increases until at least 24 h, the last time studied (Fig. 2). When cultures were stimulated with LPS for different times in the presence or absence of ANP (1 μM) added one hour before LPS, we observed a significant reduction on iNOS inducion of around 40 % after 24 h (Fig. 2). This effect was not observed when ANP was added at the same time as LPS. These results indicate that ANP down-regulates iNOS protein expression in glial cells and that the effect requires that ANP acts previous to the iNOS inducing stimulus.

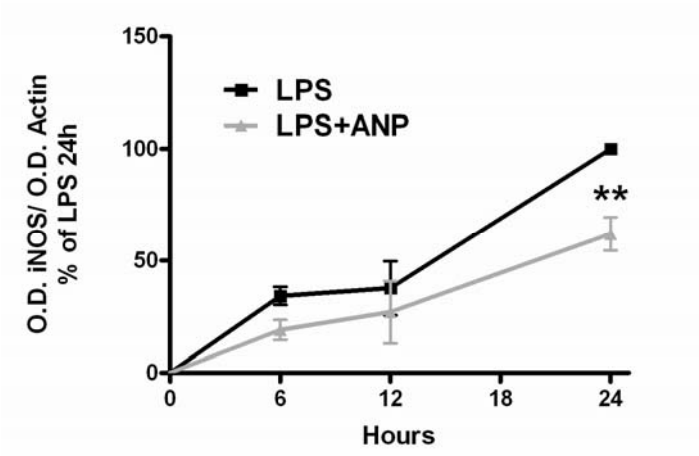

Figure 2. Effect of ANP on iNOS protein levels in homogenates of mixed glial cultures. ANP (1 μM) was added to microglial cultures 1 h before LPS (10 ng/ml). After the indicated times, cell were harvested and homogenates (40-60 μg protein) were subjected to WB. Immunoreactive iNOS bands were analyzed by densitometry and normalized to actin levels. Results, represented as % of LPS values at 24 h, are means ± SEM of 2 to 5 independent experiments. Significantly different (**$p<0.01$) from LPS value at 24 h analyzed by one-sample Student's t-test.

Microglia is known to be the major source of NO produced after iNOS induction by LPS in glial cells (Kim & de Vellis 2005). In agreement with this, when we examined LPS-induced nitrite accumulation in the media of mixed glial cultures treated with the cytostatic agent Cyt-Ara (10 μM) to reduce microglial proliferation (Fig. 3A), we observed a drastic reduction in nitrite levels (Fig. 3B). Thus, further studies on the effect of ANP on LPS-induced iNOS were performed in cultures of purified microglia.

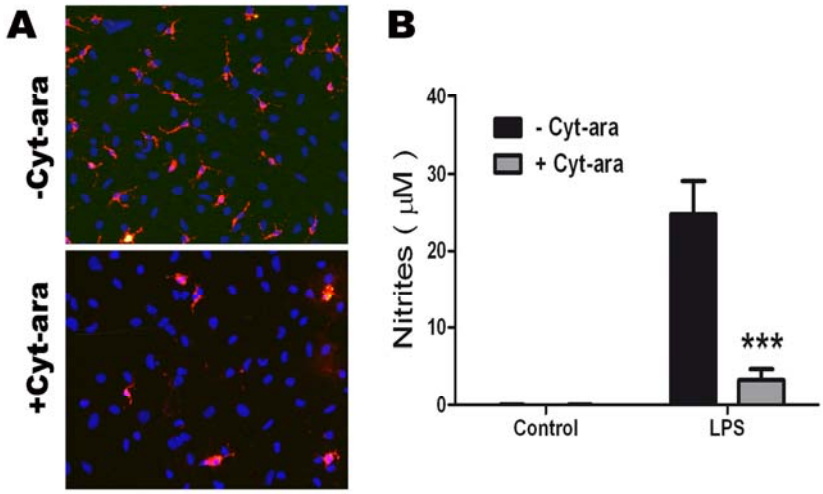

Figure 3. LPS-induced nitrite accumulation is drastically reduced in mixed glial cultures treated with cytosine arabinoside (Cyt-ara) to decrease microglial proliferation. Cultures were treated or not with cyt-ara (10 μM) at almost confluence (see methods) and after 4-5 days cells were treated with LPS (10 ng/ml) for 24 h (A) Representative images of mixed glial cultures where microglia was stained with Dil-Ac-LDL (red) and nuclei with DAPI (blue) showing that microglial contamination is drastically reduced by treatment with Cyt-ara. (B) Quantification of nitrite levels in the culture media. Results are means ± SEM of 4 independent experiments. Significant difference vs Cyt-ara untreated cultures by Student's t-test (***$p<0.001$).

As we previously observed in mixed glial cultures, iNOS protein expression in isolated microglial cultures from rat cerebellum was increased by treatment with LPS for 24 hours and ANP pretreatment for 1 hour significantly reduced the LPS effect (Fig. 4).

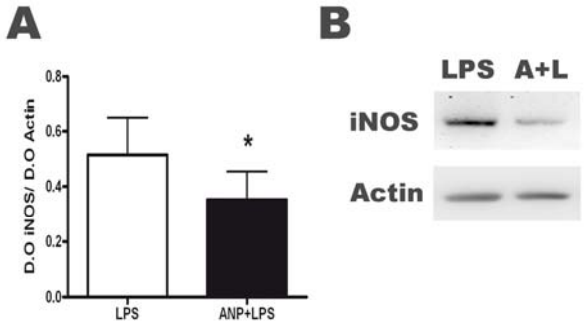

Figure 4. Effect of ANP on iNOS protein levels induced by LPS in homogenates of microglial cultures. Homogenates (40-60 μg protein) were subjected to WB. Immunoreactive bands were analyzed by densitometry and normalized to actin levels. (A) Quantification of 10 independent experiments (means ± SD). Statistically significant difference by paired Student's t-test (*$p<0.05$); (B) Representative WB showing the effect of ANP (1 μM) pretreatment (A+L) in LPS-induced iNOS.

ANP can bind with similar affinity to NPR-A and NPR-C, and with much lower affinity to NPR-B. Since as shown in figure 1C, the three receptor types are expressed in cerebellar microglial cells, we investigated if treatment with the NPR-C selective agonist cANP (1 μM) or with CNP (1 μM) that binds preferentially to NPR-B, mimicked the effect of ANP. As shown in Fig. 5, none of these agonists were able to decrease LPS-induced iNOS. Interestingly, cANP tended to increase iNOS protein although the effect did not reach significance. Thus, the effect of ANP appears to be mediated by NPR-A receptors.

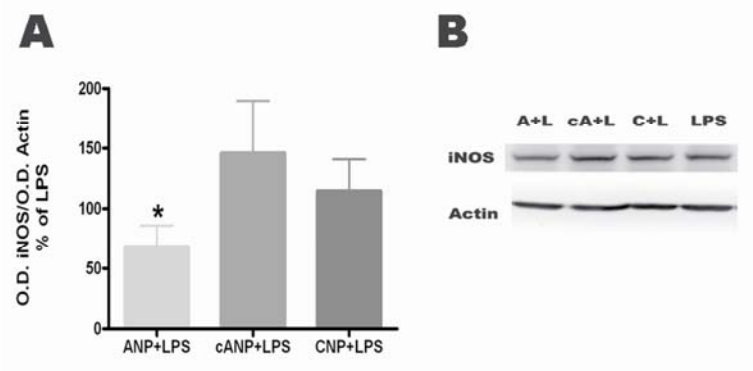

Figure 5. Specific agonists of NPR-B and NPR-C do not reproduce the ANP down-regulation of LPS-induced iNOS. ANP (1 μM), cANP (1 μM) or CNP (1 μM) were added to pure microglial cultures 1 h before LPS and iNOS protein levels were analyzed by WB after 24 h (A) Quantified data are expressed as % LPS effect and are means ± SEM of ANP (n=6), cANP (n=6) and CNP (n=3). One sample Student's t-test shows statistically significant differences (*$p<0.05$) compared to LPS. (B) Representative WB showing the effect of the different NPRs agonists in LPS-induced iNOS.

To evaluate if PKG was implicated in the ANP effect, cerebellar microglial cultures were pretreated with the PKG inhibitors Rp-8-Br-PET-cGMPS (0.5 μM) and KT-5823 (1 μM) for 30 minutes before addition of ANP. As shown in Fig. 6, both PKG inhibitors prevented the effect of ANP on LPS-induced iNOS. PKG-inhibitors did not affect iNOS induction by LPS (not shown). These results indicate that cGMP activation of PKG is involved in ANP down-regulation of iNOS expression.

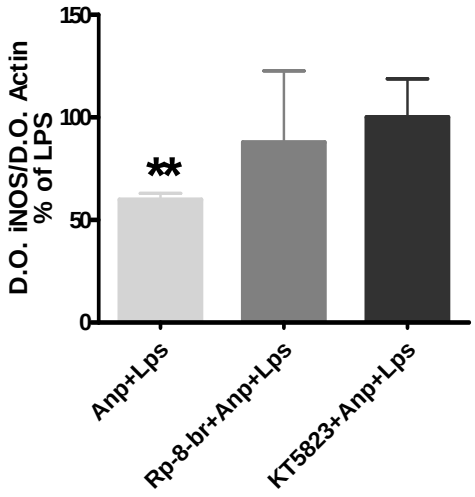

Figure 6. PKG inhibitors prevent ANP down-regulation of iNOS induction. Microglial cultures were treated with the PKG inhibitors Rp-8br-PET-cGMP (0.5 μM) or KT-5823 (1 μM) added 30 min before ANP. Results, expressed as % of LPS effect, are means ± SEM of n=2-3 independent experiments. One sample t-test shows statistically significant differences (*$p<0.05$) compared to LPS alone.

Increased cGMP levels can inhibit cAMP specific PDE-3 and lead to accumulation of this nucleotide and activation of cAMP-dependent protein kinase (PKA) (Francis, Blount et al. 2011). Furthermore, the cAMP-PKA pathway has been reported to down-regulate iNOS induction in glial cells (Galea and Feinstein 1999). To rule out the possibility that this pathway was involved in the reduction in iNOS induction by ANP we treated microglial cultures with the PKA inhibitor KT-5720 (1 μM), added 30 min before ANP. As shown in Fig. 7, KT-5720 was not able to prevent the effect of ANP. On the contrary, KT-5720 tended to decreased LPS-induced iNOS expression both in the absence or presence of ANP. These results indicate that the ANP effect is not mediated by the cAMP-PKA pathway and furthermore suggest that this pathway up-regulates LPS-induced iNOS expression in microglia, as reported before in macrophages (Won, Im et al. 2004; Saha and Pahan 2006). This was confirmed by the observation that treatment of microglial cultures with the permeable cAMP analogue dibutiryl-cAMP (dbcAMP) drastically increased LPS-induced iNOS protein (Fig. 8). Interestingly, co-treatment with ANP was able to significantly reduce by 50% the combined effect of LPS and dbcAMP.

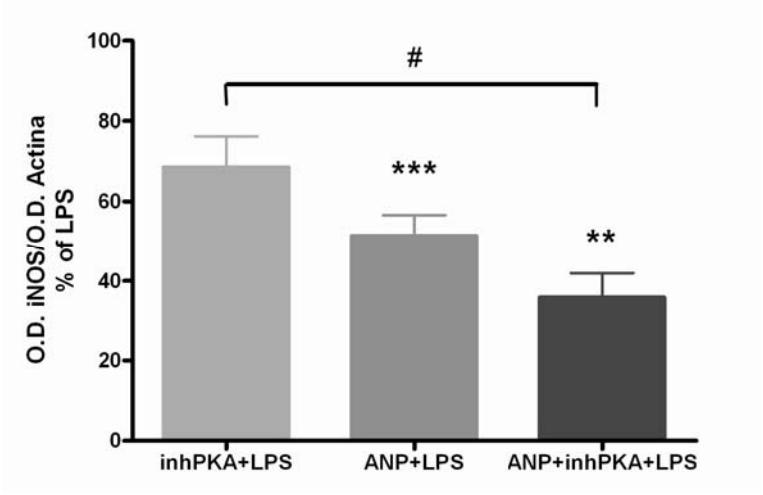

Figure 7. The PKA inhibitor KT-5720 does not prevent ANP down-regulation of LPS-induced iNOS. Microglial cultures were preincubated with KT-5720 (1 μM) for 1 h before adding LPS or 30 min before ANP when used in combination. Results, expressed as % of LPS effect, are means ± SEM of 3 independent experiments One sample t-test shows statistically significant differences from LPS value (***p<0.001, **p<0.01). One-way ANOVA shows statistically significant differences between inhPKA+LPS and inhPKA+ANP+LPS treatments (#p<0.05).

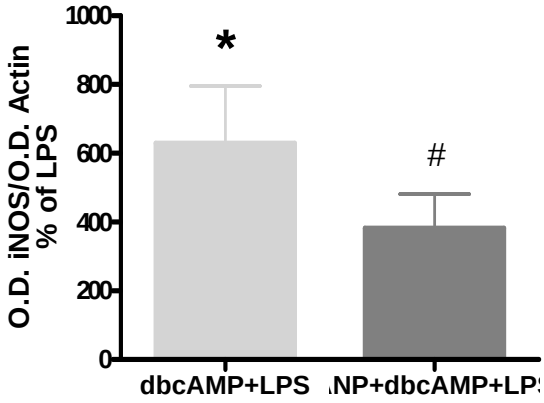

Figure 8. dbcAMP potentiates LPS-induced iNOS expression and ANP down-regulates this effect. Microglial cultures were treated with dbcAMP (10 μM) added 1h before LPS in the absence or presence of ANP. Results are represented as % of LPS and are means ± SEM of 4 diferent experiments. Significantly different from LPS by one sample t-test (*p<0.05). Significantly different from dbcAMP+LPS by paired t-test (#p<0.05).

1.3. Mechanism of the down-regulation of LPS-induced iNOS expression by ANP

Regulation of iNOS transcription is the major mechanism to control iNOS expression (for review see (Pautz, Art et al. 2010). In order to elucidate if this mechanism was responsible for ANP reduction of LPS-induced iNOS protein expression, mRNA from microglial cultures treated or not with LPS, in the presence or absence of ANP, were analyzed by real-time PCR at different times after addition of LPS up to 8 h. As shown in Fig. 9, the

maximum levels of iNOS mRNA in cells exposed to LPS are reached by 4 h and no further increases are observed by 8 h. ANP did not affect mRNA levels at any of the times studied indicating that it does not affect iNOS transcription. The lack of effect of ANP on iNOS mRNA levels even after reaching maximal induction by LPS also suggests that ANP does not affect mRNA stability. Similar results were obtained in mixed glial cultures (results not shown).

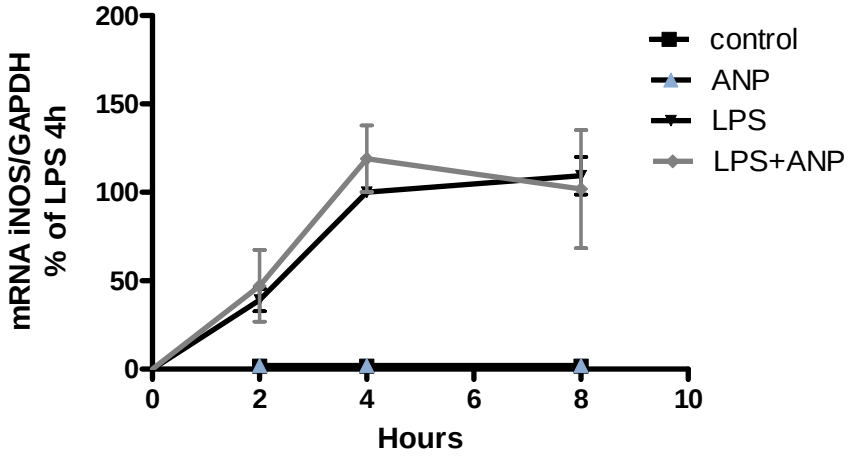

Figure 9. ANP has no effect on iNOS mRNA levels in microglial cultures exposed to LPS. The time-course of iNOS mRNA levels was determined by real-time PCR in microglial cultures treated or not with LPS in the absence or presence of ANP. Results are presented as % of LPS levels after 4 h of treatment and are means ± SEM of 3-4 independent experiments. Two-way ANOVA show no significant differences between LPS and ANP+LPS at any time studied.

Another mechanism that has been shown to regulate iNOS protein levels in cells closely related to microglia such as macrophages is the decrease in protein stability (Vodovotz, Bogdan et al. 1993). To investigate if this mechanism was involved in the ANP effect glial cultures were stimulated for 18 h with LPS in the presence or absence of ANP and after that time the supernatant was replaced by fresh media without LPS or ANP. As shown in Fig. 10, once LPS is eliminated the levels of iNOS protein begin to decrease reaching almost undetectable levels 12 h later. As expected, treatment with ANP added 30 min before LPS diminished iNOS protein levels at 18 h, and after replacing the media iNOS again decreased to undetectable levels 12 h later. To compare the degradation rate of the protein we referred protein levels at each time point to the level at 18 h. As shown in the insert of Fig. 10, the slope of the degradation curve is not modified by pretreatment with ANP, thus ruling out a decrease in the half-life of iNOS as the mechanism for the ANP-induced down-regulation of the protein.

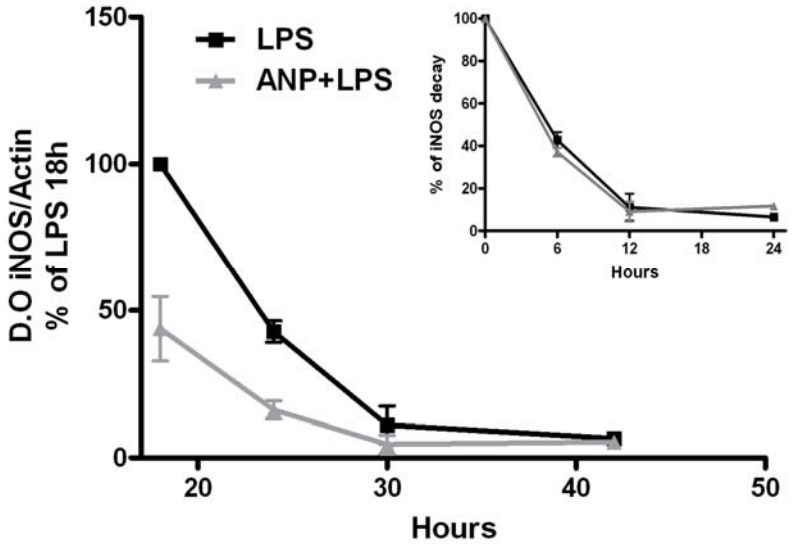

Figure 10. ANP does not affect the rate of iNOS protein degradation. Mixed glial cultures were stimulated with LPS in the absence or presence of ANP for 18 h and then the media was replaced by fresh media. iNOS protein levels were analyzed by WB at different time points. Results, represented as % of LPS levels at 18 h, are mean±SD of 2 different experiments. In the insert, the same results are represented as % of levels at 18 h for each treatment. No significant differences were observed at any time point analyzed by Student's t-test.

Regulation of iNOS protein expression at the translational level has also been described in macrophages stimulated with the pro-inflammatory cytokine IFN-γ (Vodovotz, Bogdan et al. 1993). To investigate if ANP was affecting iNOS protein synthesis we performed an experiment in which cells were maintained in a medium without methionine (Met) and cystein (Cys) and afterwards ^{35}S-labelled Met and Cys were added to label new translated protein. After stimulation with LPS in the presence or absence of ANP, protein extracts were immunoprecipited with anti-iNOS antibody and were subjected to electrophoresis and autoradiography. As shown in Fig.11, LPS treatment induces a small but significant increase in total protein synthesis that is not affected by ANP co-treatment. However, the increase in ^{35}S incorporation to immunoprecipited iNOS protein was reduced in the presence of ANP. These results suggest that the ANP down-regulation of iNOS protein may result from an effect in the process of mRNA translation to protein.

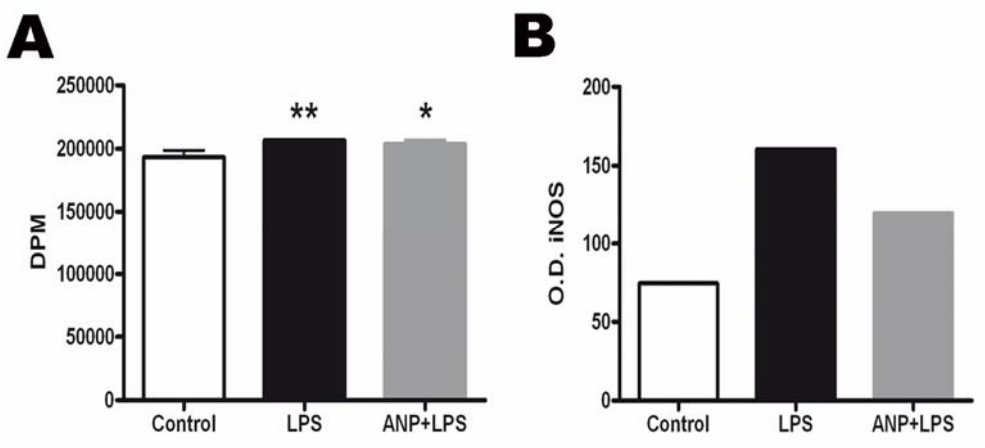

Figure 11. ANP appears to reduce iNOS translation without affecting total protein synthesis. (A) Total incorporation of ^{35}S-Met and ^{35}S-Cys in mixed glial cultures treated or not with LPS in the absence or presence of ANP. Results are means ± SD of five culture dishes. One-way ANOVA shows statistically significant differences compared to control cultures (**p< 0.01, *p<0.05). (B) Quantification of autoradiographic bands after SDS-PAGE of anti-iNOS immunoprecipited ^{35}S-labeled protein from homogenates of mixed glial cultures treated or not with LPS with or without ANP. Equal amount (500 μg) of protein extracted from a pull of 5 different dishes for each treatment was immunoprecipited and subjected to SDS-PAGE before autoradiographic exposure. Quantification was done by Quantity-One software.

DISCUSSION

Neuroinflammation is characterized by activation of innate immunity resident cells in CNS. Previous studies have implicated cGMP-mediated pathways in the regulation of pro-inflammatory gen expression in a variety of cells types including macrophages (Vollmar 2005). However, when this Thesis was started very little was known about expression of components of cGMP-mediated pathways in microglia and their possible implication in the regulation of inflammatory responses. In this work we demonstrate that there is a differential expression of components of the cGMP pathway in cultured microglia and astroglia from rat cortex and cerebellum. In agreement with previous reports (de Vente, Bol et al. 1989; De Vente, Bol et al. 1990; Markerink-Van Ittersum, Steinbusch et al. 1997; Baltrons, Boran et al. 2008), we observed that astrocytes are able to produce cGMP in response to NO donors as well as in response to ANP. However microglial cells produce cGMP only in response to ANP. This agrees with the work of Teunissen et al. (Teunissen, Steinbusch et al. 2000) that showed using spheroid cultures from rat brain that a few cells labelled with a microglial marker presented cGMP immunoreactivity after stimulation with ANP but not with a NO donor. Also in agreement with previous reports (Cao and Yang 2008), we showed that astrocytes express mRNA for NPRs with GC activity, and we found that their expression differs in cortical and cerebellar cells. Both NPR-A and NPR-B are highly expressed in cerebellar astrocytes but only NPR-B is expressed in cortical astrocytes. These receptors were also detected in microglia from cerebellum but to a much lower extend than in astrocytes. Interestingly, in clear contrast to astrocytes, NPR-A is highly expressed in cortical microglia but NPR-B is barely detected. When our work was underway, Moriyama et al. (Moriyama, Taniguchi et al. 2006) reported expression of mRNAs for NPR-A and NPR-B in cultured microglia from whole embryonic rat brain. These authors also found mRNA expression for ANP in the same cultures and suggested that NP may be an autocrine regulator in microglia.

Accumulation of cGMP in response to ANP in cortical microglial cultures was potentiated in the presence of the cGMP-PDE5 inhibitors zaprinast and sildenafil. Accordingly, we found mRNA expression of PDE5 in these cells. This data is consistent with the reported PDE5 immunoreactivity in microglia-like cells in the penumbra of an ischemic lesion in rat cortex (Menniti, Ren et al. 2009). Whereas sildenafil is a selective inhibitor of PDE5, zaprinast inhibits PDE5 with relatively high potency (IC_{50}=0,13 μM) but is also effective inhibiting PDE9 (IC_{50}=29 μM), the isoform with highest affinity for cGMP that is widely expressed in brain, as well as dual substrate PDE1 variants (IC_{50}=6 μM) ((Bender and

Beavo 2006), and references therein). We did not find mRNA for PDE9 in microglia or astrocytes, thus ruling out the contribution of this isoform to the regulation of cGMP formation in these glial cells. However, we found expression for both PDE1A and PDE1B in microglia and of PDE1B in astrocytes, suggesting that in addition to PDE5, PDE1 isoforms can play a role in controlling cGMP levels. In agreement with this, we previously showed increases in cGMP accumulation in the presence of vinpocetine, a selective PDE1 inhibitor, in cortical astrocytes (Agullo and Garcia 1997). Interestingly, expression of PDE1B has been shown to increase during monocyte to macrophage differentiation and to play a major role in the regulation of cGMP levels in these cells (Bender and Beavo 2006). Additionally astrocytes, but not microglia, showed high expression of mRNA for PDE2, another dual substrate PDE whose inhibition was previously shown to increase cGMP in astrocytes in rat hippocampal slices (van Staveren, Markerink-van Ittersum et al. 2001).

Knowing that microglia can synthesize cGMP in response to ANP, we investigated if this second messenger was regulating the expression of iNOS as has been demonstrated in macrophages (Vollmar 2005). Here we show that ANP also reduces LPS induction of iNOS in microglia when it is added to cells before LPS. As in macrophages the ANP effect seems to be mediated by NPR-A receptors, since it was not mimicked by NPR-B (CNP) or NPR-C (cANP) agonists, and involves down-stream stimulation of PKG as demonstrated by inhivbition of the ANP effect by pre-treatment with two different PKG inhibitors. Since microglial cells were shown to express both NPR-A and NPR-B it was surprising that the peptide CNP, that binds with high affinity to NPR-B and is expected to stimulate cGMP formation, did not have the same effect as ANP on iNOS induction. A possible explanation for the lack of effect of CNP is the reported compartmentalization of ANP-NPR-A-PKG components in discrete membrane domains. It has been described by Airhart et al. (Airhart, Yang et al. 2003) in yeast and cardiac rat cells that ANP treatment is able to recruit PKG to the plasma membrane establishing an association with NPR-A. This leads to phosphorylation of the receptor and a gain of function of its cyclase activity that results in a 2-fold increase in the ANP effect (Airhart, Yang et al. 2003). Similar mechanism has not been shown for CNP acting through NPR-B.

Regulation of cAMP-specific PDE3 by cGMP leads to its inhibition and can thus increase intracellular cAMP levels (Francis, Blount et al. 2011). Opposite effects of stimulation of the cAMP-PKA pathway on iNOS expression have been described in different cell types. Whereas down-regulation has been observed in rat primary astrocytes cultures, C6

glial cells and hepatocyte, increased expression was shown in mesangial cells, cardiomyocites, murine fibroblasts and rat peritoneal macrophages (Galea and Feinstein 1999; Won, Im et al. 2004; Saha and Pahan 2006). In the present work, implication of PKA in ANP effects has been ruled out by the observation that pre-treatment with a PKA inhibitor did not affect ANP effects on iNOS induction. Furthermore, in accordance with previous results in macrophages (Won, Im et al. 2004), the cAMP analogue db-cAMP was able to potently potentiate LPS induction of iNOS expression in microglia, and pre-treatment with ANP was able to reduce iNOS levels to the same extend (by 40%) as in cells exposed only to LPS.

Regulation of transcription is the most common mechanism by which iNOS levels are controlled but regulation at the translational and protein degradation levels has been also described in murine macrophages (Vodovotz, Bogdan et al. 1993; Won, Im et al. 2004). We have observed that in microglia exposure to ANP before LPS does not affect the time-course of iNOS mRNA expression neither during the rising phase, nor 4 h after having reached maximal levels, indicating the ANP does not affect transcription or mRNA stability. An effect of ANP on the degradation of the iNOS protein was also ruled out by the observation that co-incubation with ANP did not affect the degradation rate of induced iNOS protein after removing the LPS stimulus. Thus, we examine the possibility that ANP was affecting iNOS mRNA translation to protein. Our preliminary results analysing the incorporation of ^{35}S-labelled aminoacids into iNOS protein, suggest that ANP reduces iNOS protein synthesis. Further experiments are needed to confirm this hypothesis.

Previous works demonstrated that poorly hydrolysable cGMP analogues reduced the release of anti-inflammatory compounds leukotriene B4 and TNF-α in a murine microglial cell line (N9) (Paris, Town et al. 1999; Paris, Town et al. 2000). More recently, using the same microglial cell line, Zhao et al., (Zhao, Zhang et al. 2011) have shown that the PDE5 inhibitor sildenafil reduces LPS-induced iNOS induction and release of IL-1β and TNF-α by blocking nuclear factor-κB (NF-κB) and MAPKs activation. Additionally, while our work was underway and in agreement with our results, Moriyama et al. (Moriyama, Taniguchi et al. 2006) reported that ANP decreased LPS-induced nitrite accumulation in rat embrionic brain microglial cultures. In contrast, Choi et al. (Choi, Choi et al. 2002) had reported that a high concentration of the cGMP analogue dibutyril-cGMP and of zaprinast potentiated iNOS expression in rat brain microglial cultures. However, these conditions could lead to cross-activation of the cAMP-PKA pathway that, as previously reported (Feinstein, Heneka et al. 2002; Dello Russo, Boullerne et al. 2004; Liu, Zhao et al. 2011; Savchenko 2012) and also

shown here, increases expression of iNOS. Also in contrast with an anti-inflammatory effect of cGMP in microglia, it has been reported that NO via cGMP up-regulates LPS-induced expression of the inflammatory gene cd11b in the murine microglial cell line BV2 (Roy, Fung et al. 2006). However, our results in vivo in cortically cryolesioned rats and mice ((Pifarre, Prado et al. 2010); Chapter II) as well as in EAE mice treated with PDE5 inhibitors ((Pifarre, Prado et al. 2011); Chapter III) clearly show a decreased microglial activation thus supporting an anti-inflammatory role of cGMP-mediated pathways in microglia. Moreover, a recent study has shown that administration of brain natriuretic peptide down-regulates microglial activation in murine models of traumatic brain injury and intracerebral hemorrhage (James, Wang et al. 2010).

CHAPTER 2

CHAPTER 2: EFFECT OF cGMP-PDE INHIBITORS ON GLIAL INFLAMMATORY RESPONSE AND NEURODEGENERATION ELICITED BY FOCAL BRAIN INJURY IN RODENTS

2.1. Effect of treatment with the non-selective cGMP-PDE inhibitor zaprinast in cortically cryolesioned rats

As shown in chapter 1, rat cultured astroglial and microglial cells express cGMP specific PDE5 and dual substrate PDE1 and in both cell types in vitro studies have shown that cGMP-mediated pathways regulate the inflammatory response. With the aim of studying if cGMP regulates the glial inflammatory response *in vivo* we used an animal model of focal cortical lesion by cryoinjury. In a first experiment we treated rats with the cGMP-PDE inhibitor zaprinast that inhibits cGMP-specific PDE5 with relatively high potency (IC50 = 0,13 μM) and is also effective inhibiting PDE1 (IC50 = 6 μM) and PDE9 (IC50 = 29 μM), the isoform with highest affinity for cGMP that is widely expressed in neuronal populations in brain (Bender and Beavo 2006).

2.1.1. Effect on the inflammatory response

Cryoinjury to the cortex produces a profound inflammatory response with activation of resident microglia, recruitment of macrophages and reactive astrogliosis that occurs in a highly temporal-specific manner reaching maximal levels at 3 days post lesion (dpl) (Penkowa, Carrasco et al. 1999). In this study, rats were treated with zaprinast (10 mg/kg) 2 h before and 24 and 48 h after cryolesion and were sacrificed 3 dpl. Reactive astrogliosis manifested by a significant increase in GFAP immunoreactivity was clearly visible around the lesioned area in vehicle treated animals (Fig. 1c and e). In agreement with this, GFAP-protein levels were significantly increased in homogenates of the lesioned cortex (Fig. 2a). Treatment with zaprinast enhanced GFAP immunostaining around the lesion by five- to six-fold (Fig. 1d, f and g) and GFAP protein levels in homogenates by two- to three-fold (Fig. 2a) respect to lesioned vehicle-treated animals. Interaction between factors was significant for both GFAP immunostaining and protein levels ($p<0.001$), indicating that zaprinast potentiates the effect of the cryolesion. These results suggest that zaprinast may accelerate the formation of the glial scar around the injured tissue. In unlesioned rat cortex, zaprinast induced a small but significant increase in GFAP immunoreactivity (Fig. 1a, b and g), indicating that cGMP can also regulate astroglial physiology under normal conditions.

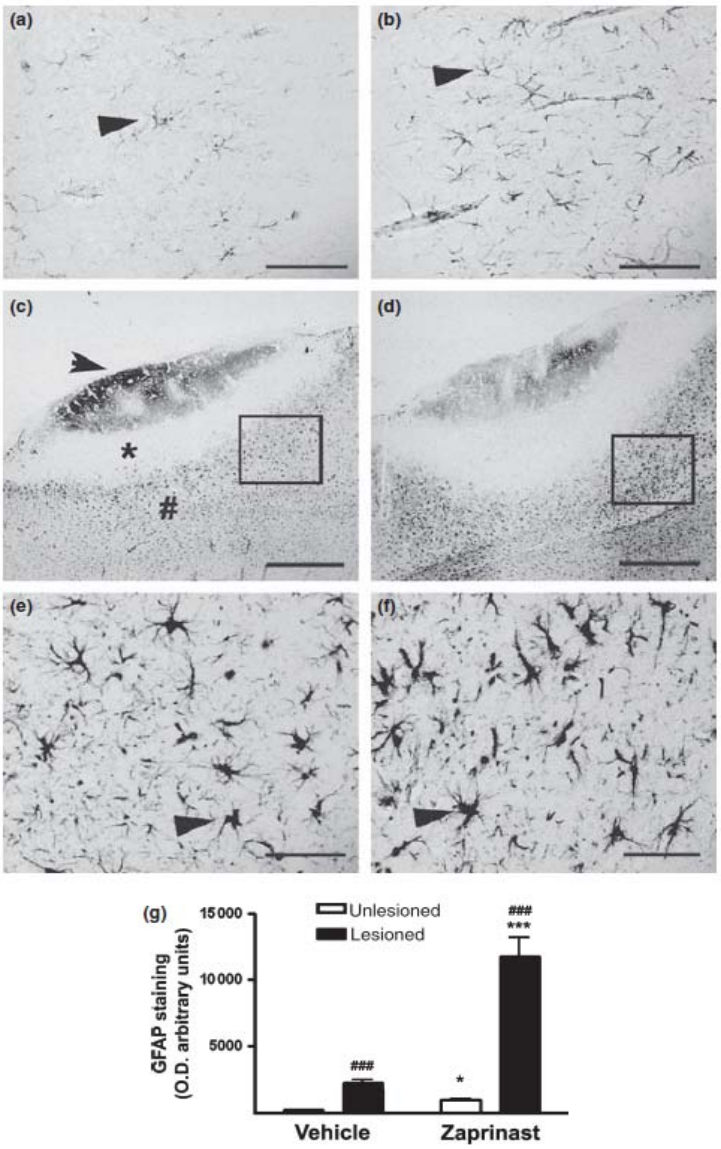

Figure 1. Effect of zaprinast on GFAP immunostaining in cortex slices of unlesioned and cryolesioned rats. GFAP staining in unlesioned animals was weak (a) and increased slightly but significantly after treatment with zaprinast (b); GFAP staining in cryolesioned animals 3 dpl was prominent around the lesion (c; arrow points to the necrotic area; *area of microglial activation, #area of astroglial activation), and treatment with zaprinast greatly increased the number and intensity of GFAP-stained cells (d); (e) and (f) are higher magnification of squares in (c) and (d) showing reactive astrocytes with swollen cell bodies and enhanced immunostaining; (g) GFAP staining intensity was quantified with the Scion Image program in 4–6 areas of 0.5 mm^2 per brain chosen at random in unlesioned animals (n=4) or from the border of the lesion (excluding the edges) in cryolesioned animals (n=5). Statistically significant differences vs. unlesioned animals (###$p<0.001$) and vs. vehicle-treated animals (*$p<0.05$ and ***$p<0.001$). Two-way ANOVA shows significant interaction between lesion and treatment ($p<0.001$). Bar: (a, b, e, f) 30 µm; (c, d) 400 µm.

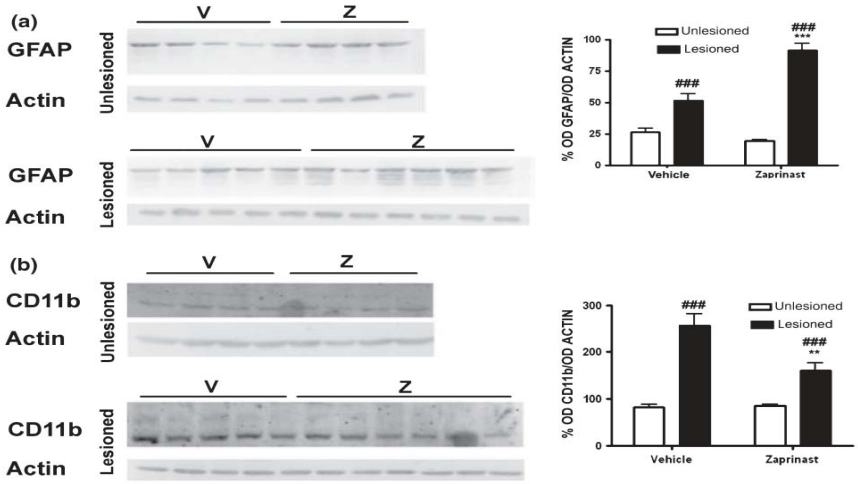

Figure 2. Effect of zaprinast on GFAP and CD11b protein levels in cortex homogenates of unlesioned and cryolesioned rats. Homogenates (40 µg protein) from unlesioned (n=4) or cryolesioned rat cortex (n=6), treated with vehicle (V) or zaprinast (Z) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunobloted. GFAP (a) and CD11b (b) immunoreactive bands were analyzed by densitometry and following normalization to actin levels, values were expressed relative to one cryolesioned zaprinast-treated animal that was included in all blots. Statistically significant differences vs. unlesioned animals (###$p<0.001$) and vehicle-treated lesioned animals (***$p<0.001$ and **$p<0.01$). Two-way ANOVA shows significant interaction between lesion and treatment (GFAP: $p<0.001$; CD11b: $p<0.05$).

Using lectin staining that reveals resting (ramified) and activated (round) microglia and infiltrating macrophages, as well as blood vessels, a prominent recruitment and activation of microglia/macrophages (round/ameboid lectin positive cells) was observed at the border of the lesion in brain parenchyma of vehicle-treated rats, (Fig. 3c and g). In agreement with this, staining for the specific microglia/macrophage cytosolic protein Iba-1 (Fig. 3e and h) and levels of the microglia/macrophage surface marker CD11b analyzed by WB in homogenates (Fig. 2b) were significantly increased in the lesioned-cortex. In contrast to astrogliosis, a notable decrease in lectin-positive cells and in the intensity of lectin staining was observed in zaprinast-treated animals (Fig. 3d and g). Furthermore, staining for Iba-1 revealed less activated (more ramified) microglia/macrophage morphologies in addition to decreased staining intensity (Fig. 3f and h). A significant decrease in CD11b levels in homogenates (Fig. 2b) confirmed the decreased activation of these cells in zaprinast treated animals. Interaction between factors was significant for lectin and Iba-1 staining and CD11b protein ($p<0.05$, <0.001 and <0.05, respectively), which indicates that zaprinast reduces the effect of cryolesion. Zaprinast did not affect lectin (Fig. 3b and g) and Iba-1 staining (Fig 3h) or CD11b levels (Fig. 2b) in unlesioned animals. These results are indicative of an anti-inflammatory action of the cGMP-PDE inhibitor.

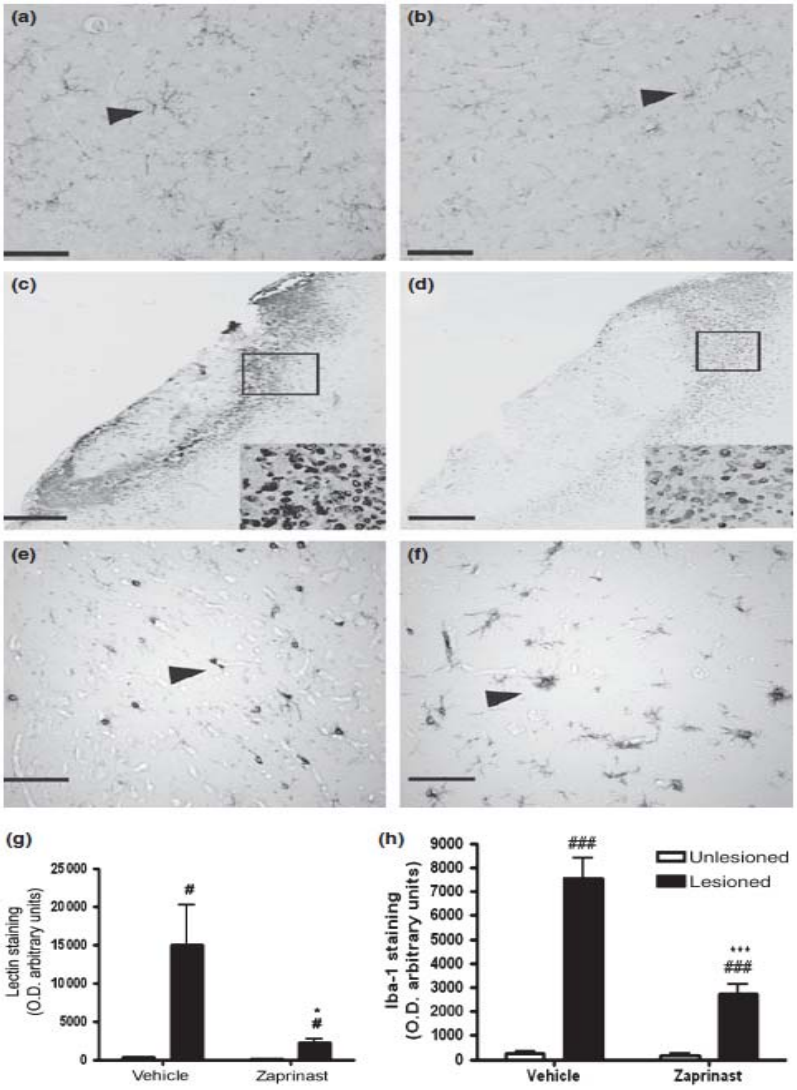

Figure 3. Effect of zaprinast on lectin and Iba-1 staining in cortex slices of unlesioned and cryolesioned rats. Lectin stained ramified microglia similarly in vehicle-treated (a) and zaprinast-treated unlesioned animals (b, arrowheads); At 3 dpl, numerous lectin-positive microglia/macrophages were present around and inside the lesion (c) and treatment with zaprinast greatly decreased the intensity of lectin staining around the lesioned area (d); inserts in (c) and (d) show higher magnification of the squares showing rounded activated microglia/macrophages at the lesion area. (e) Staining for the specific microglia/macrophage marker Iba-1 in vehicle-treated and (f) zaprinast-treated cryolesioned cortex. Zaprinast promoted a less activated cell morphology (arrowheads in e, f) and a significant decrease in staining intensities (h). Lectin and Iba-1 staining intensities were quantified with the Scion Image program in 4–6 areas of 0.5 mm^2 per brain chosen at random in unlesioned animals (n=4) or from the border of the lesion (excluding the edges) in cryolesioned animals (n=4–5) (g, h). Statistically significant differences vs. unlesioned animals (#$p<0.05$, ###$p<0.001$) and vs. vehicle-treated lesioned animals (*$p<0.05$, ***$p< 0.001$). Two-way ANOVA shows significant interaction between lesion and treatment (lectin: $p< 0.05$; Iba-1: $p< 0.001$). Bars: (a, b, e, f) 30 µm; (c, d) 400 µm.

Activated microglia/macrophages are known to produce reactive nitrogen and oxygen species that can induce oxidative stress and neural cell death. We investigated if zaprinast regulated this inflammatory response by measuring levels of proteins with carbonyl groups, a sign of oxidative stress, using a commercial detection kit (OxyBlot TM). As shown in Fig. 4, at 3 dpl saline-treated rats showed increased levels of proteins with carbonyl groups. This effect was significantly decreased by zaprinast treatment in agreement with an anti-inflammatory action of the cGMP-PDE inhibitor.

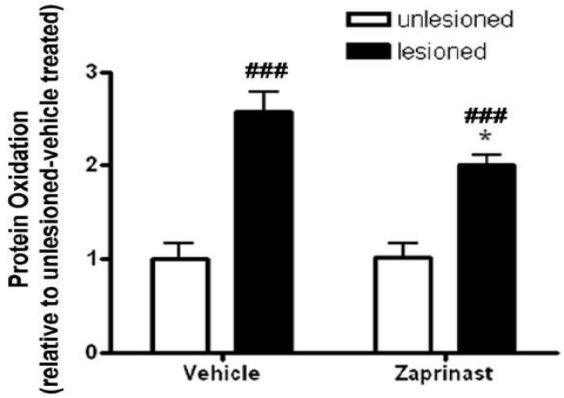

Figure 4. Effect of zaprinast on protein oxidative stress in unlesioned and cryolesioned animals. Carbonyl groups in proteins were detected on WBs of cortex homogenates (20 µg protein) with the OxyBlot TM kit (Chemicon). Unlesioned animals (n=4) showed very low levels of oxidated proteins whereas lesioned rats (n=6) presented two- to threefold higher levels. Zaprinast treatment significantly reduced protein oxidation. Results are presented relative to vehicle-treated unlesioned animals and are means ± SEM of three replicate experiments. Statistically significant differences vs. unlesioned animals (###$p< 0.001$) and vs. vehicle-treated lesioned animals (*$p<0.05$). Two-way ANOVA shows significant interaction between lesion and treatment ($p<0.05$).

2.1.2. Effect on neurodegeneration

As previously shown by Penkowa et al. (Penkowa, Carrasco et al. 1999) and Giralt et al. (Giralt, Penkowa et al. 2002), a significant increase in the number of apoptotic cells is observed in the deepest border of the cryolesion reaching a peak at 3 dpl and decreasing thereafter. We have evaluated the effect of zaprinast on apoptotic cell death by counting TUNEL-positive cells in an area distant at least 50 µm from the border of the lesion. Unlesioned animals showed very few TUNEL-positive cells whether treated or not with zaprinast (not shown). However, as shown en Fig. 5, left panels, numerous TUNEL-positive cells were observed in lesioned vehicle-treated animals and treatment with zaprinast notably decreased these numbers. We also examined if zaprinast was decreasing neuronal injury by analyzing the morphology of NeuN-stained neurons. In the cryolesioned cortex, numerous

NeuN-labeled cells presented an abnormal morphology with shrunken and dystrophic cell bodies (Fig. 5, right panels). In contrast, the majority of neurons in the zaprinast-treated animals appeared normal. This result is consistent with the reduction in oxidative stress produced by the treatment with zaprinast and evidences a neuroprotective effect of the cGMP-PDE inhibitor in the brain cryoinjury model.

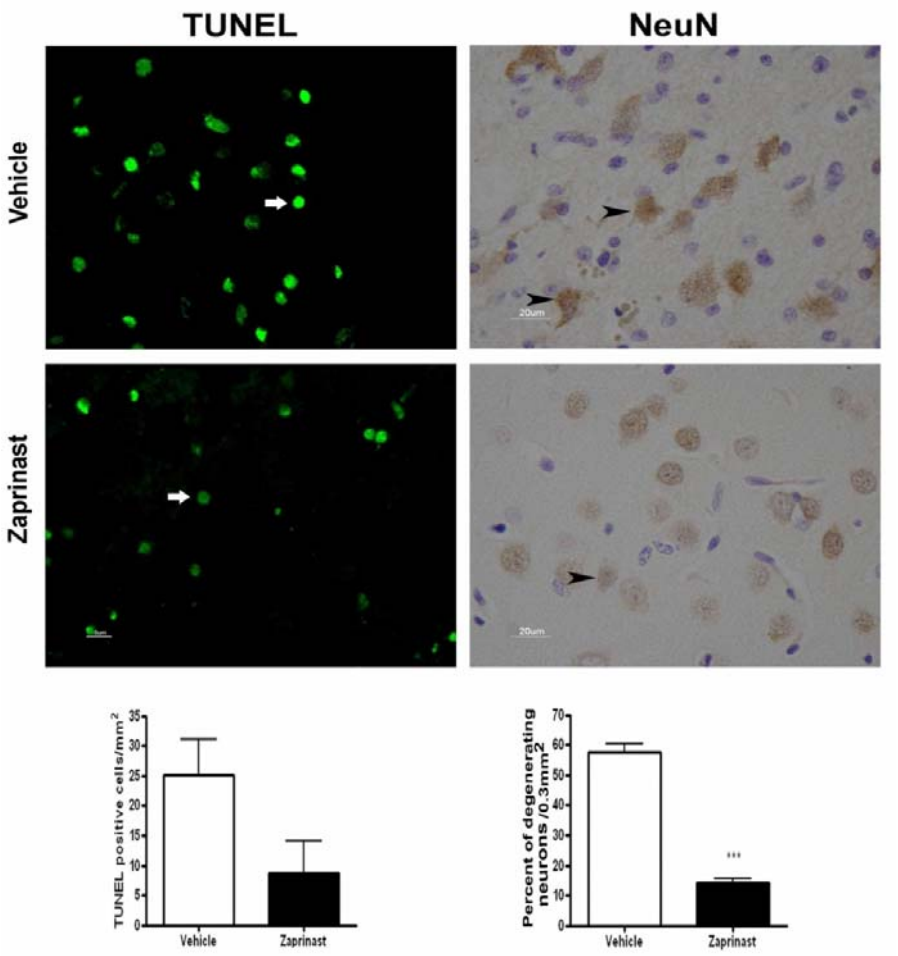

Figure 5. Zaprinast decreases TUNEL-positive cells and attenuates neuronal injury in the cryolesioned cortex. (Left) TUNEL-positive cells labeled using the In Situ Cell Death Detection Kit (Roche); (Right) Neurons stained with the neuronal nuclei marker NeuN. TUNEL-positive cells (cells with nuclear staining, arrows) and injured neurons (condensed or dystrophic cell bodies, arrowheads) were quantified in 5–7 areas/ brain section at least 50 µm away from the necrotic area (n=5). Zaprinast treatment reduced the number of TUNEL-positive cells ($p=0.058$) and of NeuN-labeled degenerating neurons (**$p<0.01$) in the injured cortex. Bars in TUNEL 5 µm, in NeuN 20 µm.

2.1.3. Effect on angiogenesis

Observation of lectin-stained sections revealed increased blood vessel-staining in zaprinast-treated animals. Quantification of clearly visible lectin-stained vessels in random areas at least 50 μm away from the lesion confirmed that zaprinast significantly increased their numbers by around two-fold in the lesioned cortex (Fig. 6, left panels). A similar increase was observed when vessels were counted in hematoxylin–eosin-stained sections (Fig. 6, center panels). This finding is in agreement with the well documented angiogenic action of cGMP-enhancing compounds in peripheral tissues in vitro and in vivo (Ziche and Morbidelli 2000; Pyriochou, Beis et al. 2006), and also demonstrated in rat CNS after stroke (Zhang et al. 2003). Findings in different models of CNS pathologies show that VEGF plays a key role in the angiogenic response to injury and exerts neuroprotective and neurotrophic effects (Zachary 2005). The NO-cGMP pathway has been implicated in VEGF increased expression and angiogenesis induced by cerebral ischemia (Zhang, Wang et al. 2003). To investigate if zaprinast treatment increased VEGF levels in the cryolesioned cortex, we examined VEGF immunostaining. No significant staining was observed in unlesioned cortex (not shown). However, VEGF staining was intense in reactive astrocyte-like cells in the parenchyma as well as surrounding blood vessels in the cryolesioned cortex and staining levels were significantly increased by the zaprinast treatment (Fig 6, right panels). These results suggest that increased angiogenesis in zaprinast-treated animals may be mediated by cGMP-induced VEGF expression in astrocytes.

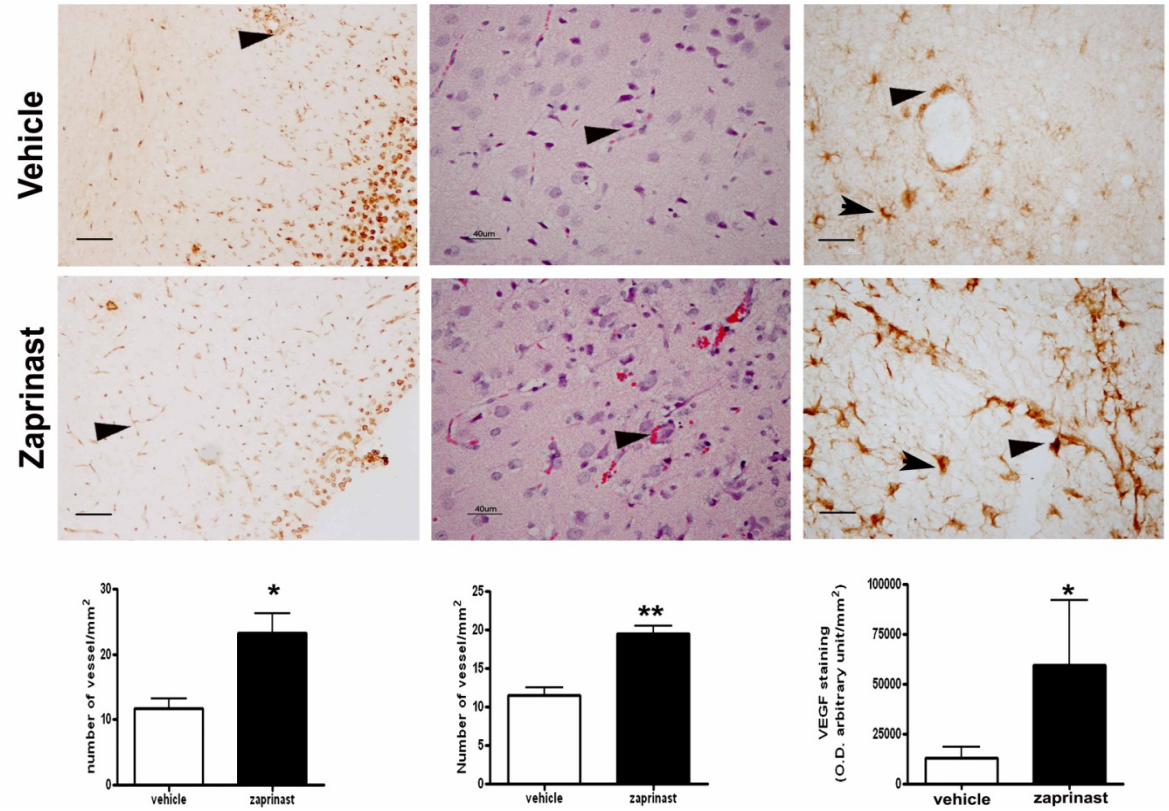

Figure 6. Zaprinast increases vascularisation and vascular endothelial growth factor (VEGF) staining in the cryolesioned cortex. Vessels labeled with lectin (left) or H&E (center) were quantified in 5–7 areas per brain section at least 50 µm away from the necrotic area (n=5). (Right) Immunoreactivity for VEGF is observed in reactive astrocyte-like cells in the parenchyma (arrow) and vessels (arrowhead); VEGF staining was quantified with the Scion Image program in five 0.5 mm^2 areas per brain at least 50 µm from the necrotic area (n=4). Zaprinast treatment significantly increased the number of blood vessels and the expression of VEGF. Statistically significant differences vs. vehicle-treated lesioned animals (**p< 0.01; *p<0.05). Bar: 40 µm.

2.2. MTs-I/II are involved in the anti-inflammatory and neuroprotective effects of the selective PDE5 inhibitor sildenafil in cortically cryolesioned mice

In a second experiment we investigated if the selective PDE5 inhibitor sildenafil reproduces in mice the anti-inflammatory and neuroprotective effects of zaprinast in cryolesioned rats. We also investigated if the antioxidant and neuroprotective proteins MT-I/II are involved in the effects of sildenafil.

2.2.1. Effect on the inflammatory response

In this study, we treated five to ten week-old 129S1/SvImJ wild-type mice with sildenafil (10 mg/kg, s.c.) 2 h before and 24 and 48 h after cryolesion. As shown in Fig 7A, B, sildenafil-treatment did not affect GFAP immunostaining in unlesioned animals but significantly enhanced the increased GFAP immunoreactivity caused by the lesion when compared to vehicle-treated controls. In agreement with this, GFAP protein levels analysed by western blot in cortical homogenates were significantly increased by sildenafil compared to vehicle in lesioned but not in unlesioned animals (Fig. 7C). Two-way ANOVA showed significant interaction between lesion and treatment for GFAP staining ($p<0.001$) indicating that sildenafil potentiates the effect of the cryolesion.

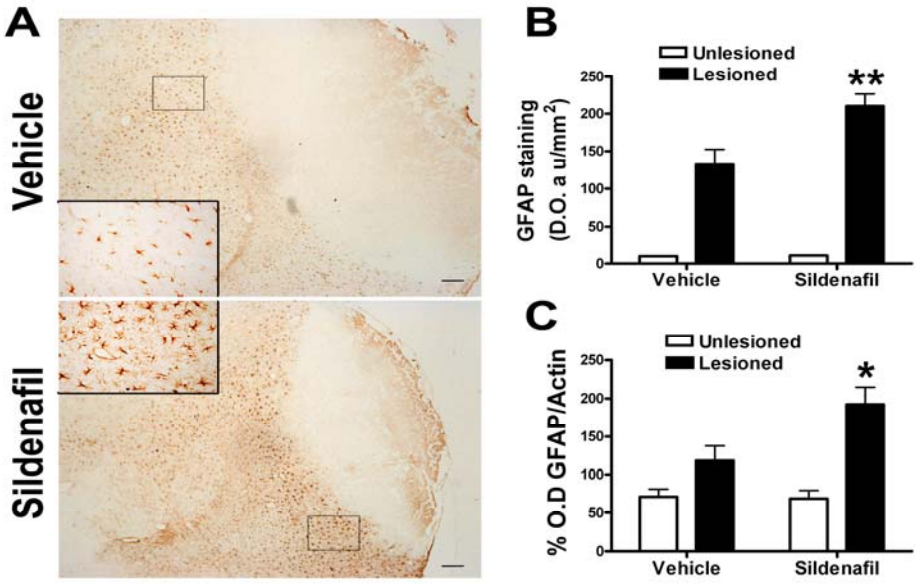

Figure 7. Treatment with sildenafil increases astroglial reactivity in mice cryolesioned cortex. (A) GFAP immunohistostaining in coronal sections of cortex at 3 dpl was prominent around the lesion and increased significantly after treatment with sildenafil. Inserts: higher magnification of squared areas showing reactive astrocytes with swollen cell bodies and enhanced immunostaining. (B) GFAP immunostaining was quantified with the Scion Image software in 4–6 areas of 0.5 mm^2 chosen at random in unlesioned cortex or from the border of the lesion (excluding the edges) in cryolesioned animals. (C) GFAP protein levels analyzed by WB in brain cortical homogenates (40 μg of total protein). Results in (B) and (C) are means±SEM of 3-4 vehicle- and 5-7 sildenafil-treated mice. Two-way ANOVA with lesion and treatment as main factors revealed significant differences between unlesioned and lesioned animals ($p<0.001$) in both GFAP IHC and WB analysis, whereas for sildenafil the effect was significant in the former ($p<0.025$) and marginally significant ($p=0.07$) in the latter analysis. The interaction between both factors was significant in the IHC study ($p<0.025$) and marginally significant ($p=0.07$) in the WB study. Post-hoc analysis revealed that sildenafil increased GFAP levels in lesioned animals in both GFAP IHC and WB analysis (*$p<0.05$; **$p<0.01$). Bar: 200 μm (A).

As expected, a prominent recruitment and activation of microglia/macrophages (round/amoeboid lectin-positive cells) was observed at the border of the lesion in vehicle-treated mice (Fig. 8A). Treatment with sildenafil caused a reduction in lectin-positive cells and in the intensity of lectin staining (Fig. 8A, B). Accordingly, levels of the specific microglia/macrophage cytosolic protein Iba-1 were significantly decreased by sildenafil treatment in homogenates of the lesioned cortex (Fig. 8C).

Staining for the toxic byproduct of lipid peroxidation MDA showed numerous positive cells around the lesion in control animals at 3 dpl (Fig. 8D, E). The number of MDA-positive cells as well as the MDA-intensity of staining in cryolesioned mice was significantly decreased by sildenafil treatment, in agreement with an anti-inflammatory and neuroprotective action of this PDE5 inhibitor.

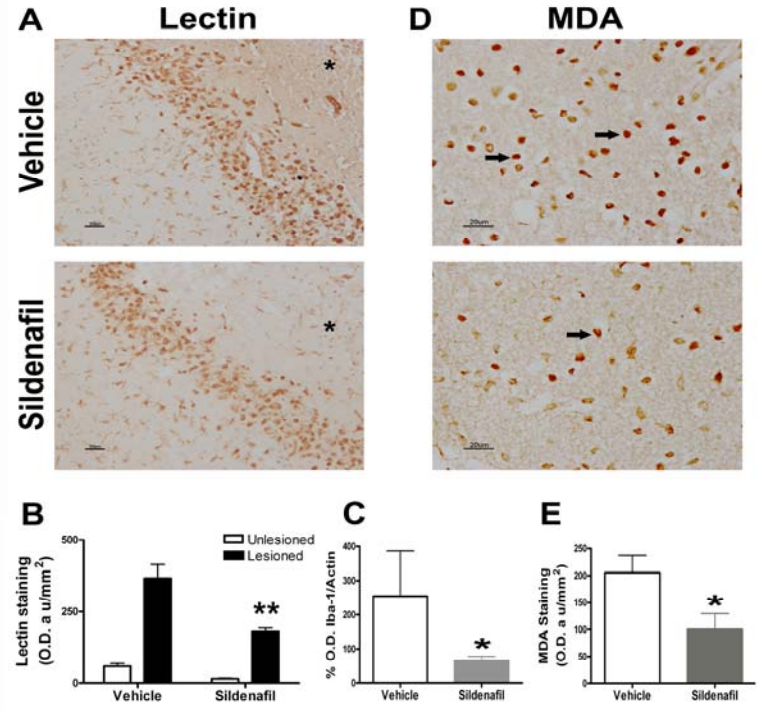

Figure 8. Treatment with sildenafil decreases microglia/macrophage activation and lipid peroxidation in mice cryolesioned cortex. (A) Lectin histochemistry (HC) revealed numerous activated microglia/macrophages around the lesion at 3 dpl. Treatment with sildenafil decreased the intensity of lectin staining and the density of positive cells. Asterisk: necrotic area. (B) Quantification of lectin HC was carried out as for GFAP IHC (n=3-6) and analyzed by two-way ANOVA, which revealed a significant effect of the lesion ($p<0.001$) and of sildenafil ($p<0.025$); the interaction between lesion and treatment was only marginally significant ($p=0.09$). Nevertheless, post-hoc analysis revealed that sildenafil treatment significantly reduces lectin levels in lesioned animals (**$p<0.01$). (C) Protein levels of the specific microglia/macrophage marker Iba-1 analyzed by WB in lesioned cortex homogenates (40 µg) are significantly decreased in sildenafil-treated animals (*$p<0.05$; Student T-test). (D) Staining for the toxic byproduct of lipid peroxidation MDA was strong in numerous cells around the lesion at 3 dpl (arrows) and was reduced by treatment with sildenafil. (E) Quantification of MDA staining (*$p < 0.05$; Student T-test). Results are mean±SEM. Bar: 50 µm (A); 20 µm (D).

2.2.2. Involvement of MT-I/II in the neuroprotective effect of sildenafil in cryolesioned mice

Alterations in the inflammatory response to the cryolesion induced by zaprinast in rats and by sildenafil in mice are similar to those previously observed in mice overexpressing MT-I/II (Giralt, Penkowa et al. 2002), namely increased astrogliosis, decreased recruitment and activation of microglia/macrophages and decreased oxidative stress. Additionally, both zaprinast treatment and MT-I/II overexpression decreased apoptotic cell death, whereas MT-I/II deficiency potentiated the neurodegeneration caused by cryolesion (Penkowa, Carrasco et al. 1999). Thus we set to investigate if MT-I/II could be involved in the neuroprotective effect of PDE5 inhibition. We first examined if treatment with sildenafil was affecting MT-I/II expression in the cryolesioned cortex. As shown in Fig. 9A, we found an increase in MT-I/II immunoreactive cells in the border of the lesion in sildenafil-treated animals compared to vehicle-treated controls. Quantification of MT-I/II immunostaining around the lesion (Fig. 9B) and ELISA analysis in cryolesioned cortex homogenates (Fig. 9C) showed significantly higher levels of MT-I/II in animals treated with sildenafil. Closer examination of consecutive sections of the lesioned cortex stained for MT-I/II, GFAP or lectin (Fig. 9D), showed that cells presenting high immunoreactivity for MT-I/II in sildenafil-treated animals were more abundant in areas rich in GFAP-stained cells than in areas of round heavily lectin-stained amoeboid microglia/macrophages, suggesting a preferential induction of MT-I/II in astrocytes.

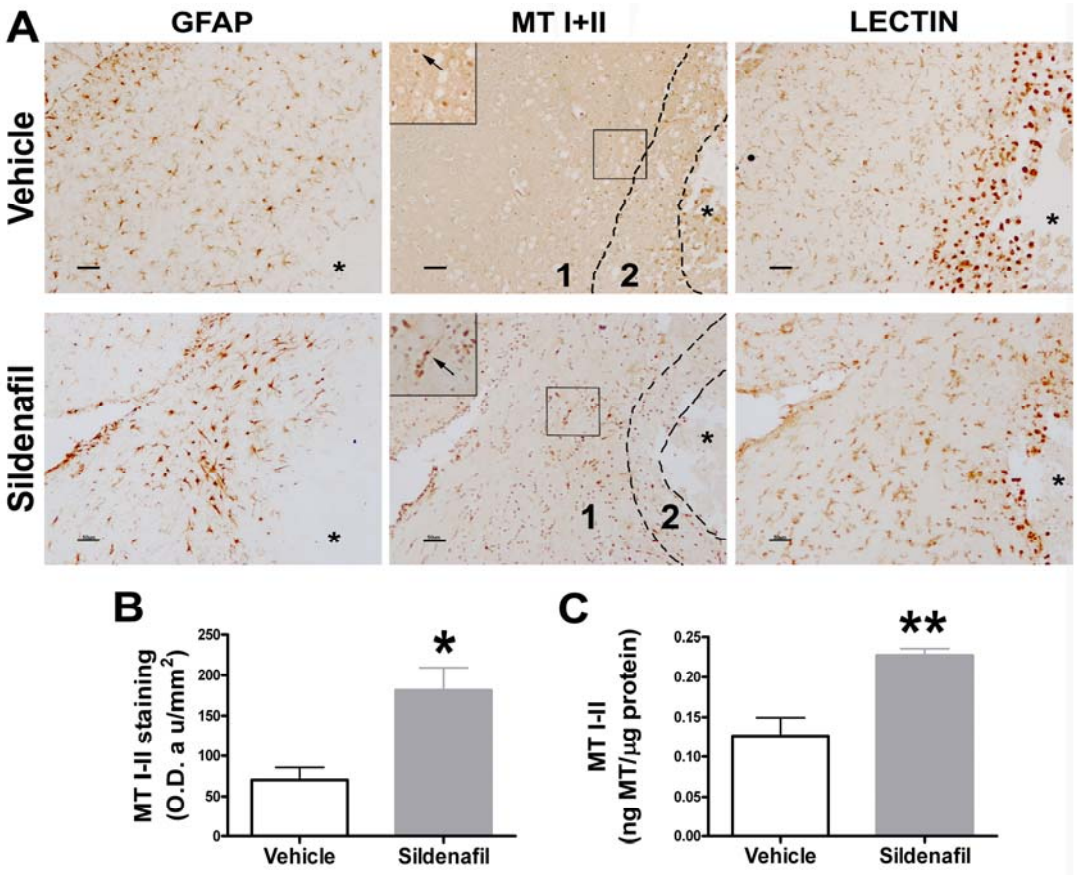

Figure 9. Sildenafil treatment up-regulates MT-I/II expression in the cryolesioned cortex. (A) GFAP (left), MT-I/II (middle) and lectin (right) staining in consecutive serial sections of cryolesioned cortex shows an increase of MT-I/II positive cells in sildenafil-treated animals predominantly in areas of intense astrogliosis (1). (B) Quantification of MT-I/II immunostaining around the lesion was increased by sildenafil. (C) Measurement of MT-I/II protein levels by competitive ELISA in cortex homogenates reveals a significant increase in sildenafil-treated animals. Results are mean±SEM of 4 vehicle- and 7 sildenafil-treated mice. Significant differences: *$p<0.05$, **$p<0.01$, (Student's t-test). Bar: 50 µm. Asterisk: necrotic area. Insert: higher magnification of square areas. Arrows points MT-I/II positive cells.

We next investigated the effect of sildenafil treatment on apoptotic cell death in cryolesioned cortex from wild-type and or homozygous MT-I/II deficient mice. Apoptotic cell death was evaluated by counting TUNEL-positive cells in an area distant at least 50 µm from the border of the lesion. As shown en Fig. 10 A,B, numerous TUNEL-positive cells were observed in vehicle-treated wild-type animals and numbers were even higher in MT-I/II deficient mice in agreement with previous reports (Penkowa, Espejo et al. 2001) In contrast, a drastic reduction in TUNEL-positive cells was observed in sildenafil-treated wild-type mice. However, the drug was not able to reduce apoptotic cell death in MT-I/II deficient mice. Two-way ANOVA showed high interaction ($p<0.001$) between strain and treatment indicating that the strain is critical for the effect of sildenafil.

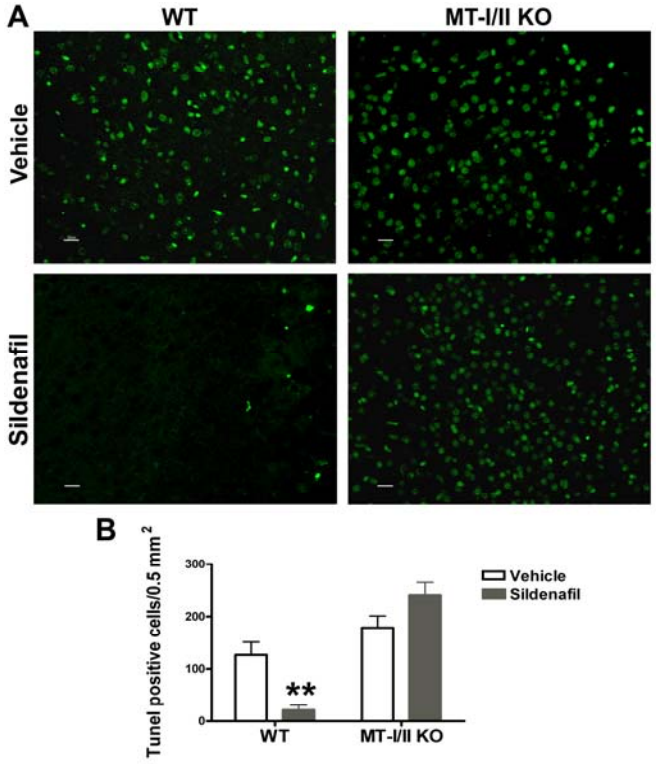

Figure 10. Sildenafil treatment drastically reduces apoptotic cell death in the cryolesioned cortex of wild-type mice but not of MT-I/II-deficient mice. (A) Many TUNEL-positive cells were present surrounding the lesioned cortex, which were dramatically decreased by sildenafil in a strain-dependent manner. (B). TUNEL-positive cells were counted in 5x0.5 mm^2 areas/brain section; results are mean±SEM of 4 vehicle- and 5-7 sildenafil-treated mice. Two-way ANOVA revealed that the strain ($p<0.001$) but not the treatment with sildenafil ($p=0.028$) affected significantly the number of apoptotic cells. The interaction between treatment and strain was highly significant ($p<0.001$), and accordingly post-hoc analysis revealed that sildenafil treatment significantly reduced cell death in wild-type mice but not in MT-I/II-deficient mice (**$p<0.01$).

Staining for GFAP and lectin also showed a lack of effect of sildenafil in the glial response in cryolesioned MT-I/II deficient mice (Table 1). Taken together, these results indicate that induction of MT-I/II is involved in the neuroprotective effect of PDE5 inhibition in the cortical cryoinjury model.

Table 1. Effect of sildenafil on glial activation in cryolesioned cortex of MT-I/II KO mice

Treatment	*GFAP*	*Lectin*
Vehicle	100 ± 14.84	100 ± 14.98
Sildenafil	83.95 ± 16.2	128.34 ± 28.56

Treatment of MT-I/II deficient mice with sildenafil does not affect GFAP or lectin staining intensity around the cortical cryolesion 3dpl. Results, expressed as percent over vehicle-treated animals, are means±SEM of 4-6 animals.

2.2.3. Effects of sildenafil on phosphorylated Signal Transducer and Activator of Transcription 3 (STAT3)

One of the transcription factors involved in the induction of MT-I/II expression under inflammatory conditions is STAT3 (Pedersen, Jensen et al. 2009). Furthermore, exogenous MT has been shown to induce astrogliosis in cultured astrocytes and increased phosphorylation, and consequent activation, of JAK2 and STAT3 (tyr 705) appear to be involved in this effect (Leung, Pankhurst et al. 2010). To investigate if treatment with sildenafil induces activation of STAT3 in parallel with up-regulation of MT-I/II and astroglial reactivity, we measured the levels of pSTAT3 (tyr 705) by WB in homogenates of cryolesioned cortex. Contrary to expectations, sildenafil treatment significantly reduced pSTAT3 (Fig 11) indicating that this transcription factor is not involved in sildenafil induction of MTs expression or increased astrogliosis around the lesion.

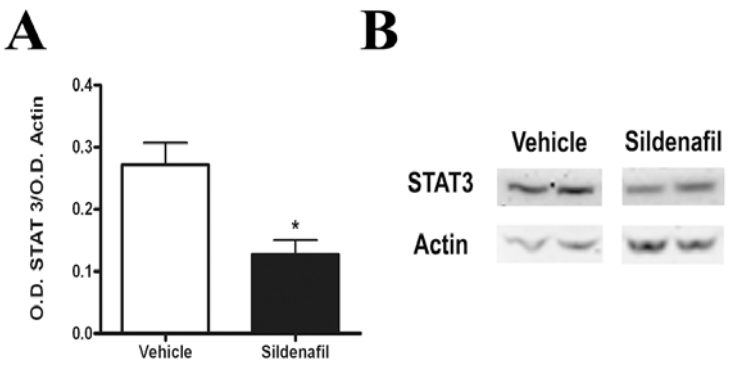

Figure 11. Sildenafil treatment decreases activation of STAT3 in cryolesioned mice cortex. (A) Quantification of pSTAT3 (tyr 705) by WB in cortex homogenates reveals a significant decrease in sildenafil-treated animals. Immunoreactive bands were analyzed by densitometry and normalized to actin levels Results are mean±SEM of 3 vehicle- and 3 sildenafil-treated mice. Significant differences were analyzed by Student's t-test (*$p<0.05$). (B) Representative WB showing the effect of sildenafil treatment in cryolesioned animals.

DISCUSSION

Accumulating evidence demonstrates that treatment with selective PDE5 inhibitors, such as sildenafil and analogues, is neuroprotective in various animal models of CNS injury through different mechanisms. Activation of antiapoptotic pathways, angiogenesis and neurogenesis appear to be responsible for PDE5 inhibitor efficacy in promoting functional recovery after stroke in rats (Zhang, Zhang et al. 2005; Zhang, Zhang et al. 2006; Menniti, Ren et al. 2009), and increases in pCREB and BDNF have been implicated in protection against 3-nitropropionic acid toxicity (Puerta, Hervias et al. 2010) and in improving synaptic function and cognitive deficits in AD models (Puzzo, Staniszewski et al. 2009; Cuadrado-Tejedor, Hervias et al. 2011), as well as in a senescence-accelerated mouse model (Orejana, Barros-Minones et al. 2012). Results shown here after treatment with zaprinast in rats and with sildenafil in mice suggest a further beneficial effect of PDE5 inhibition in brain injury through regulation of glial inflammatory responses.

The experimental model of cryolesion to the parietal cortex used in these studies has been successfully used for several years to examine the role of inflammatory cytokines and of MTs in the CNS response to traumatic brain injury (for a review see (Chung, Hidalgo et al. 2008)). In this model, activation and recruitment of microglia/macrophages and reactive astrogliosis occurs in a highly temporal-specific manner. Round or amoeboid microglia/macrophages appear in the injured area 1 dpl and reach a maximum by 3 dpl making a line of demarcation around the lesion. This response precedes that of astrocytes but numerous reactive astrocytes are also present around the lesion at 3 dpl. Gliosis progressively returns to normal levels and by 20 dpl few signs of glial reactivity are obvious (Penkowa, Carrasco et al. 1999; Giralt, Penkowa et al. 2002).

In this work, we show that treatment with zaprinast in rats or with sildenafil in mice greatly potentiates reactive astrogliosis as evidenced by increased immunoreactivity for GFAP around the lesion and increased GFAP protein levels in homogenates of the lesioned cortex. After brain injury, NO-dependent cGMP formation can occur in astrocytes as a result of iNOS induction in activated glial cells and recruited macrophages (Murphy 2000). In addition, we and others have shown that cGMP can be formed in astrocytes by activation of NPRs ((Deschepper and Picard 1994; Baltrons, Saadoun et al. 1997; De Vente 2000); Chapter 1 in this Thesis). Furthermore, in cultured astrocytes stimulation of the cGMP-PKG pathway by NO and/or ANP has been shown to increase GFAP expression (Brahmachari, Fung et al. 2006) and to induce rearrangements of the GFAP and actin cytoskeleton and to accelerate

astrocyte migration in a scratch-wound assay (Boran and Garcia 2007). Thus, increases in cGMP in astrocytes after PDE5 inhibitor treatment are most probably involved in the observed enhancement of astrogliosis around the lesion that may accelerate glial scar formation. Following CNS injury, astrogliosis and glial scar formation has generally been considered an impediment for axon generation. However, increasing evidence correlates enhanced astrogliosis with positive outcomes in focal injury models (Giralt, Penkowa et al. 2002; Molinero, Penkowa et al. 2003; Klementiev, Novikova et al. 2008). Furthermore, ablating proliferation of astrocytes after forebrain stab injury has been demonstrated to increase lesion size and the spread and persistence of inflammatory cells and to exacerbate neuronal loss, providing evidence that scar-forming astrocytes play essential roles in neural protection and repair (Bush, Puvanachandra et al. 1999; Sofroniew and Vinters 2010).

In addition to increased astrogliosis, we also show here that treatment with both PDE5 inhibitors produces a decrease in microglial/macrophage activation on the lesion border, as demonstrated by the decrease in lectin staining around the necrotic area and of cd11b and Iba-1 protein levels in homogenates from cryolesioned cortex. In agreement with an anti-inflammatory effect of cGMP in microglia, it has been reported that non-hydrolizable cGMP analogues inhibit Aβ-induced release of leukotriene B4 (Paris, Town et al. 1999) and LPS-induced secretion of TNF-α (Paris, Town et al. 2000) in a murine microglial cell line (N9). More recently, Zhao et al., (Zhao, Zhang et al. 2011) have shown that sildenafil attenuates LPS-induced iNOS expression and pro-inflammatory cytokine release in the same microglial cell line. Furthermore, anti-inflammatory actions of cGMP formed in response to ANP has been well documented in macrophages (Vollmar 2005), and also shown in primary rat microglial cultures by us (Chapter 1) and others (Moriyama, Taniguchi et al. 2006). Interestingly, the content of ANP has been reported to increase in reactive glial cells surrounding experimental brain infarction (Nogami, Shiga et al. 2001) and up-regulation of the ANP-cGMP pathway has been suggested to contribute to cortical spreading depression-induced protection against ischemic insult (Wiggins, Shen et al. 2003).

PDE5 is most highly expressed in smooth muscle, notably in the vasculature, where the enzyme regulates NO/cGMP-mediated vascular relaxation (Kass, Champion et al. 2007). In the CNS, in addition to smooth muscle of meningeal arteries and a few smaller blood vessels (Menniti, Ren et al. 2009), immunoreactivity for PDE5 has been shown in cerebellar Purkinje cells, motor neurons, mesencephalic neurons and scattered cortical neurons (Nakamizo, Kawamata et al. 2003; Bender and Beavo 2004; Menniti, Ren et al. 2009). PDE5

immunoreactivity was also reported in glial cells in cortex (Menniti, Ren et al. 2009) and spinal cord white matter (Nakamizo, Kawamata et al. 2003), but the type of glial cell was not clearly identified in those studies. Our results in rat brain primary glial cultures show that both astrocytes and microglia express mRNA for PDE5A (Chapter I; (Prado, Baltrons et al. 2010). Interestingly, Menniti et al. observed that 48 h after middle cerebral artery occlusion in rats increased PDE5 immunoreactivity was evident in blood vessels and in microglia-like cells in the areas of ischemic damage, suggesting that the enhanced functional recovery produced by treatment with PDE5 inhibitors in this model of stroke may involve regulation of cGMP levels in vascular and microglial cell function (Menniti, Ren et al. 2009).

Overproduction of nitrogen and oxygen free radicals by activated microglia is thought to be responsible for oxidative stress and to greatly contribute to cell death, in particular of neurons, in acute tissue damage as well as in chronic neurodegenerative diseases (Block, Zecca et al. 2007). Thus, the reduction in protein and lipid oxidation observed in cryolesioned animals treated with cGMP-PDE inhibitors (Fig. 4 and 8) most probably results from decreased activation of microglia/macrophages and greatly contributes to decreased neuronal degeneration. Additionally, a large body of evidence indicates that cGMP activates anti-apoptotic pathways in neural cells (Kang, Kim et al. 2004). Also increased vascularisation induced by PDE inhibitors will contribute to neuronal survival. Numerous studies have demonstrated that endogenous NO and NO-releasing activators of GC_{NO} enhance endothelial cell migration, growth and organization into capillary-like structures in vitro, as well as angiogenesis in vivo (Ziche and Morbidelli 2000; Zhang, Wang et al. 2003; Pyriochou, Beis et al. 2006). In the rat cryoinjured cortex, zaprinast enhancement of angiogenesis can be exerted by a direct effect on endothelial cell cGMP levels and also by modulating the behavior of supporting cells such as astrocytes. The angiogenic cytokine VEGF is up-regulated in reactive astrocytes following CNS injury (Zachary 2005) and, as shown here, zaprinast treatment further increases VEGF expression in reactive astrocytes in the brain parenchyma and surrounding blood vessels.

An important finding of this work is the identification of MT-I/II induction as a mechanism involved in the neuroprotection afforded by sildenafil. This conclusion is based on the complementary observations that expression of MT-I/II is increased in the cryolesioned cortex of sildenafil-treated animals, and that antiapoptotic and glial effects of sildenafil are not observed in MT-I/II knockout mice. MT-I/II proteins are expressed throughout the CNS

and are highly inducible by a wide range of stimuli, including inflammatory and oxidative agents (Yagle and Palmiter 1985; Sato and Bremner 1993; West, Hidalgo et al. 2008).

Previous studies showed that over-expression of MT-I/II and exogenous administration of these proteins are neuroprotective in cryoinjured mice and other neurotrauma models by similar mechanisms as those shown here for PDE5 inhibitor treatment (van Lookeren Campagne, Thibodeaux et al. 1999; Giralt, Penkowa et al. 2002; Chung, Vickers et al. 2003). In the normal brain, MT-I/II isoforms are expressed in astrocytes and to a lower extend in neurons, but not in microglia and oligodendrocytes. However, in response to injury MT-I/II are up-regulated in reactive astrocytes and also in microglia (Hidalgo, Aschner et al. 2001). In this work, comparison of immunostaining for MT-I/II with that of GFAP and lectin in consecutive sections of the lesioned cortex indicated that sildenafil treatment enhanced MTs expression in areas of reactive gliosis and immunoreactivy appeared stronger in areas with abundant hypertrophied reactive astrocytes (strong GFAP-stained cells) than in areas with highly reactive microglia/macrophages (lectin-positive amoeboid cells) closer to the wound, suggesting a preferential induction of MT-I/II in astrocytes. Several in vivo and in vitro reports support a neuroprotective and neuroregenerative action of astrocytic MT-I/II (Hidalgo, Aschner et al. 2001; West, Leung et al. 2011). Within the astrocyte, MTs may be part of the mechanisms by which these cells are able to handle reactive oxygen species. Additionally, recent evidence indicates that reactive astrocytes secrete MT-I/II that can be internalized by neurons and promote regenerative growth after injury (Chung, Penkowa et al. 2008). Interestingly, exogenous MT-I/II induced a reactive phenotype in cultured astrocytes that was found to be permissive to neurite outgrowth (Leung, Pankhurst et al. 2010).

STAT3 phosphorylation and consequent activation has been implicated in the regulation of glial scar formation after CNS injury (Sofroniew and Vinters 2010). Experiments in conditional knockout mice of STAT3 in astrocytes exhibit failure in the activation of these cells and disruption of glial scar formation that results in increased spread of inflammation, infiltration of immune cells and lesion volume after spinal cord injury (Okada, Nakamura et al. 2006; Herrmann, Imura et al. 2008). On the other hand, STAT 3 has been implicated in the induction of MT-I/II expression by inflammatory stimuli (Pedersen, Jensen et al. 2009), and exogenous MTs have been shown to induce astrogliosis in cultured astrocytes through a JAK/STAT dependent pathway (Leung, Pankhurst et al. 2010). Although sildenafil treatment of cryolesioned mice induces MT- I/II expression and enhances

astrogliosis, we did not observe up-regulation of STAT3 expression; in contrast we showed a significant decrease in pSTAT3 levels in homogenates of the lesioned cortex (Fig. 11). pSTAT3 has been found up-regulated in microglial cells after an ischemic insult and its silencing in these cells has been shown to prevent inflammation as well as neuronal loss (Satriotomo, Bowen et al. 2006). Thus, it would be interesting to investigate if sildenafil treatment increases STAT3 activation in microglia and if this effect is related to decreased microglia/macrophage activation and neuronal death in the cryolesioned cortex.

In summary, our results show that cGMP-elevation in CNS cells after PDE5 inhibitor treatment in cortically cryolesioned animals enhances astrogliosis around the lesion while decreasing macrophage/microglial activation and increasing angiogenesis. These effects result in decreased oxidative stress and neuronal cell death. The observation that PDE5 inhibition enhances MT-I/II expression in the cryolesioned cortex and that its beneficial effects are not observed in MT-I/II knockout mice strongly support MT-I/II induction as part of the mechanisms underlying the neuroprotective effects of PDE5 inhibitors in CNS injury and disease.

CHAPTER 3

CHAPTER 3: EFFECT OF TREATMENT WITH SILDENAFIL ON DISEASE SYMPTOMS AND NEUROPATHOLOGY IN EAE

3.1. Effect of sildenafil administration at the acute phase of the disease

3.1.1. Effects on clinical symptoms

C57BL/6 mice immunized with $MOG_{35–55}$ developed EAE clinical symptoms after 7 days and at 18 dpi, when the incidence of clinical EAE was 90–91% and the average score around 2, animals (16–17 per group) were injected s.c. with sildenafil (10 mg/kg) or vehicle (water) daily and were sacrificed 3 (21 dpi) or 8 (26 dpi) days later for neuropathology and inflammatory cell infiltration analysis in the spinal cord. As shown in Fig. 1 and Table 1, disability in mice treated with vehicle continued to increase between 18 dpi and 26 dpi although at a slower rate, whereas mice treated with sildenafil showed a significantly lower disease score after 4 days of treatment and between 5–8 days of treatment, the clinical score stabilized around 1. After 8 days of sildenafil treatment, the cumulative clinical score (sum of scores from disease onset) and the grade of remission (difference between score at 18 and 26 dpi) were significantly different from vehicle-treated animals (Table 1). Interestingly, more than half of the animals presented virtually full recovery (score 0, n=2; score 0.5, n=7). In a different experiment, animals given 10 doses of 5 mg/kg sildenafil every other day starting at 20 dpi showed a smaller but significant improvement in clinical score by 43 dpi (controls: 2.2 ± 0.2, n=10; sildenafil-treated: 1.6 ± 0.2, n = 7), indicating that the effect depends on the dose. A group of mice (n=6) immunized in parallel with BSA did not present clinical symptoms of EAE (not shown).

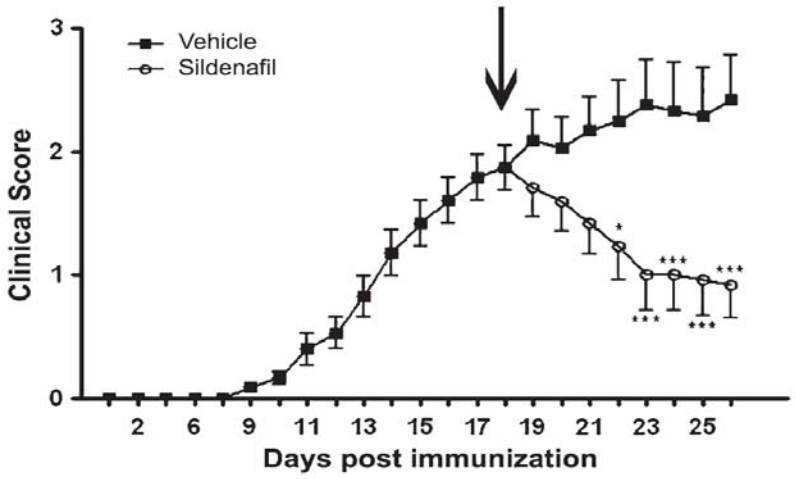

Figure 1. Sildenafil ameliorates clinical symptoms associated with EAE. C57BL/6 mice immunized with MOG35–55 developed clinical symptoms of EAE after 1 week. Sildenafil administration (10 mg/kg, s.c.) daily starting 18 dpi significantly decreased disease severity after 4 days when compared to vehicle-treated animals. Values are mean ± SEM (n = 12–13). Statistical significance: *p<0.05; ***p<0.001.

Table 1. Clinical scores of EAE mice treated or not with sildenafil.

Treatment	EAE clinical score before treatment (18 dpi, n=17 per group)			EAE clinical score after treatment (26 dpi, n=12-13 per group)			Mortality	Grade of remission
	Incidence	Average Score	Cumulative score	Incidence	Average Score	Cumulative score		
Vehicle	90.9	2.42±0.92	9.28±1.55	100	2.84±0.24	29.45±2.9	1	0.42±0.24
Sildenafil	90.3	2.47±0.19	10.97±1.53	84.61	1.00±0.25	18.81±3.16*	0	-1.47±0.23***

EAE incidence (% diseased animals) and clinical scores of MOG-immunized mice before (18 dpi) and after 8 days of sildenafil treatment (26 dpi). Cumulative score (sum of scores from disease onset) and grade of remission (difference between score at 18 and 26 dpi) were significantly different in sildenafil-treated animals. Results are mean ± SEM of the indicated number of animals at each time point. Statistical significance: *p<0.05; ***p<0.001.

3.1.2. Effects on neuropathology

To investigate if clinical improvement was accompanied by decreased neuropathology, we examined demyelination and axonal loss in longitudinal sections of the lumbar-thoracic region of spinal cords by LFB and Bielschowsky's silver staining in comparison to BSA-immunized mice that presented no pathology (Fig.2). As shown in Fig. 3a, the decrease in LFB staining intensity and the corresponding demyelination score were similar in vehicle-treated MOG-immunized mice at both 21 and 26 dpi. However, in 21 dpi animals treated with sildenafil for the last 3 days, demyelination was already less evident and by 26 dpi, after 8 days of sildenafil treatment, there was a significant increase in LFB staining and a decrease in demyelination score suggesting that sildenafil promotes remyelination. In contrast, quantification of Bielschowsky's staining showed that axonal loss increased from 21 to 26 dpi in vehicle-treated animals but not in sildenafil-treated animals (Fig. 3b), indicating that sildenafil prevents further axon degeneration. In agreement with this, staining of non-phosphorylated neurofilaments with anti-SMI-32, a marker of axonal damage, was significantly lower in 8-day sildenafil-treated animals compared to vehicle-treated controls (Fig. 3c). Taken together, these results indicate that the functional recovery produced by sildenafil treatment in MOG-immunized animals results from axonal protection and remyelination.

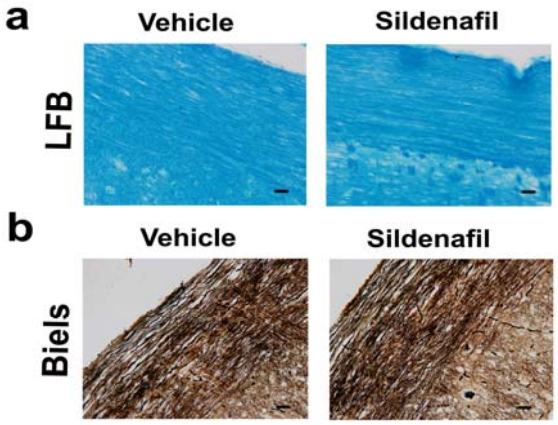

Fig. 2. BSA-immunized mice do not present demyelination or axonal loss. Spinal cord sections from BSA-immunized mice 26 dpi (n = 4), treated or not with sildenafil for 8 days, stained with LFB (a) or Bielschowsky (b).

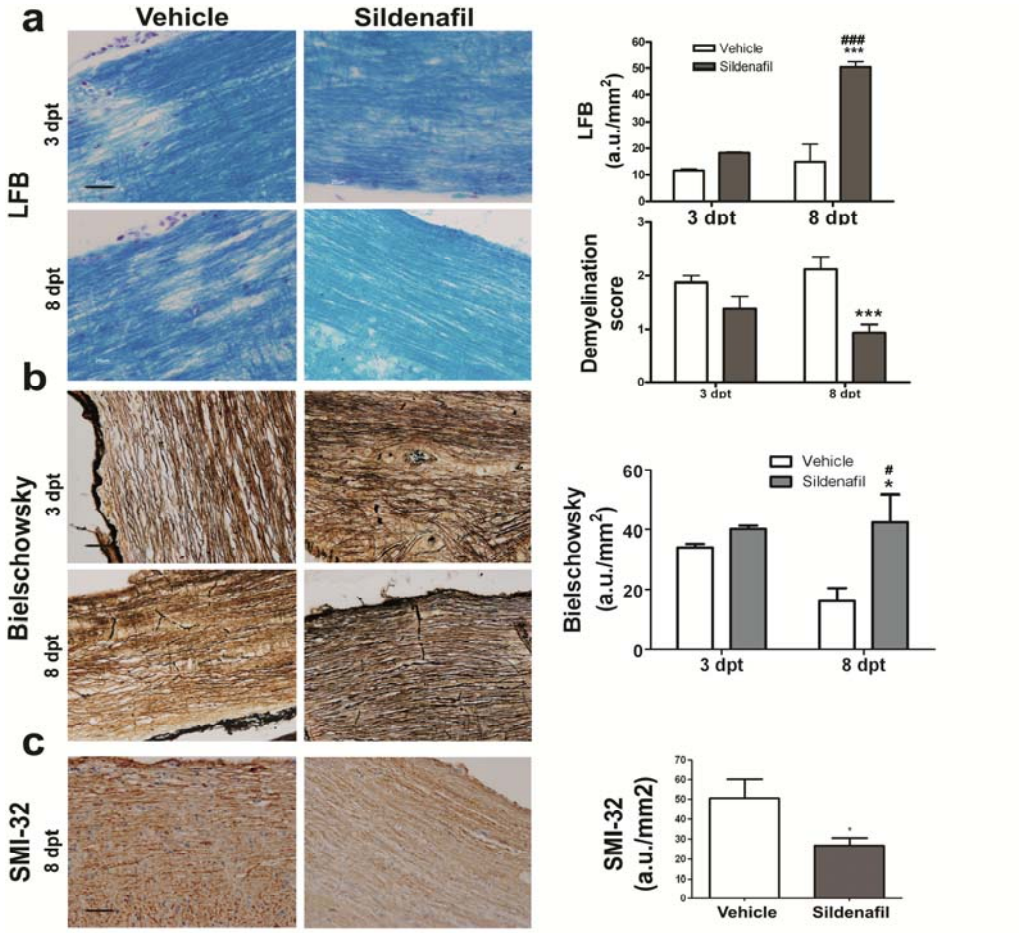

Figure 3. Sildenafil ameliorates neuropathology associated with EAE. Longitudinal lumbar-thoracic spinal cord sections from MOG-immunized mice treated or not with sildenafil for 3 (n= 4) or 8 days (n=12–13) starting at 18 dpi were stained to evaluate (a) demyelination (LFB), (b) axonal loss (Bielschowsky), and (c) axonal damage (SMI-32) (bars 50 μm in a, b; 20 μm in c). Demyelination scores and staining intensities [arbitrary units (a.u.)/mm2] were quantified as described in Materials and Methods. Note that immunization with MOG causes severe demyelination and axonal damage at 26 dpi that is significantly reduced by 8 day treatment with sildenafil. Values are means ± SEM. Statistically significant difference versus vehicle-treated animals: *p<0.05; ***p<0.001; or versus 3 dpt (21 dpi): # p<0.05; ###p<0.001.

3.1.3. Effects on inflammatory cell infiltration and adhesion molecule expression in the spinal cord

Autoreactive T cells infiltrating the CNS are the initiator and early effector cells in EAE development, but infiltrated macrophages, dendritic cells, and resident microglia constitute the ultimate effector cells that amplify neuroinflammation and tissue damage. Thus, we next examined the effect of sildenafil on cellular infiltration in the spinal cord. As estimated by H&E staining at 21 dpi, administration of sildenafil for the previous 3 days dramatically decreased the severity of cell infiltration, being the infiltrates smaller and largely confined to the submeningeal area (Fig. 4a). Quantification of CD3+ T cells in infiltrates showed that sildenafil significantly reduced the proportion of this cell population at 3 dpt (Fig. 4b). At 26 dpi, vehicle-treated animals presented significantly lower infiltration score (H&E) and number of CD3+ T cells per infiltrate respect to 21 dpi, and levels were no different to those determined in animals treated with sildenafil for 3 or 8 days (Fig. 4a,b).

The role of Foxp3+ Tregs in suppressing autoreactive T cells is well established. Additionally, Foxp3+ Tregs have been shown to play a critical role in protection and recovery from EAE [Paust 2005]. Since sildenafil afforded both protection and recovery, we investigated if it affected the population of Foxp3+ cells. As shown in Fig. 4c, at 21 dpi, Foxp3+ cells in spinal cord infiltrates of sildenafil-treated mice were notably increased compared to vehicle-treated controls. Thus, up-regulation of Tregs may be another factor contributing to the beneficial effects of sildenafil in recovery from EAE.

ICAM-1, a type-1 membrane-bound glycoprotein expressed in most leukocyte subtypes, endothelial cells, and CNS glial cells, is involved in leukocyte entry, lymphocyte activation, and other immune responses and plays a central role in the development of MS and EAE (Lebedeva, Dustin et al. 2005; Bullard, Hu et al. 2007). Thus, we examined if sildenafil was affecting ICAM-1 expression in spinal cords of EAE mice at 21 dpi when significant reduction in cell infiltration was observed (Fig. 4b). In control mice, ICAM-1-immunoreactivity was observed in blood vessels and in numerous cells within and around infiltrates (Fig. 4d). In contrast, in sildenafil-treated animals, it was almost absent in infiltrates but was still observed in blood vessels.

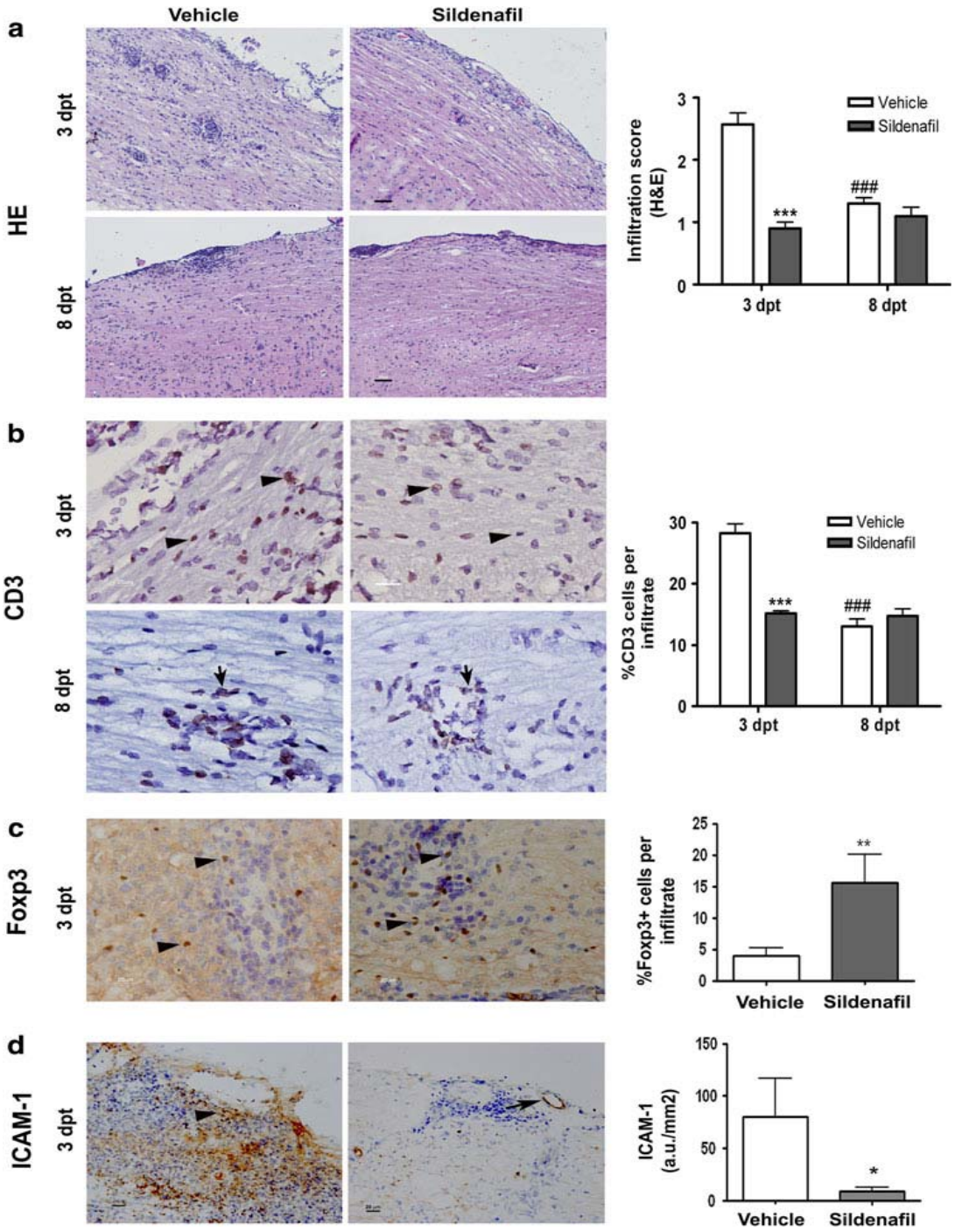

Figure 4. Sildenafil decreases inflammatory cell infiltration and ICAM-1 expression and increases Foxp3+ cells in spinal cords of EAE mice. Spinal cord sections from MOG-immunized mice treated or not with sildenafil for 3 (n=4) or 8 days (n=12–13) starting at 18 dpi were stained to evaluate (a) general cell infiltration (H&E), (b) T lymphocytes (anti-CD3), (c) Tregs (anti-Foxp3), and (d) ICAM-1. Arrowheads point to infiltrated cells and arrow point to blood vessels (bars 50 µm in a; 20 µm in b–d). Sildenafil significantly decreased inflammatory cell infiltration and ICAM-1 in infiltrates and increased Foxp3+ cells after 3 days of treatment. Inflammatory cell infiltration decreased significantly from 21 to 26 dpi in vehicle-treated animals, and 8-day sildenafil treatment did not reduce it further. Values are means ± SEM. Statistically significant difference versus vehicle-treated animals: *p<0.05; **p<0.01; ***p<0.001; or versus 3 dpt (21 dpi): ###p<0.001.

3.1.4. Effects on reactive gliosis

MOG-immunized mice presented heavily activated macrophages/microglia (strongly Iba1+ globoid cells) within and around infiltrates in the spinal cord WM that were significantly reduced by sildenafil at both 3 and 8 dpt (Fig. 5a, b). In addition, EAE animals presented activated ramified microglia (strongly Iba1-stained) throughout the spinal cord, more evident at 21 than at 26 dpi. In contrast, animals treated with sildenafil for 3 or 8 days showed a decreased intensity of Iba-1 staining in activated microglia and increased numbers of cells with long and thin ramifications typical of resting microglia (Fig. 5a, inserts), suggesting that decreased microglial activation may also contribute to the neuroprotective effect of sildenafil.

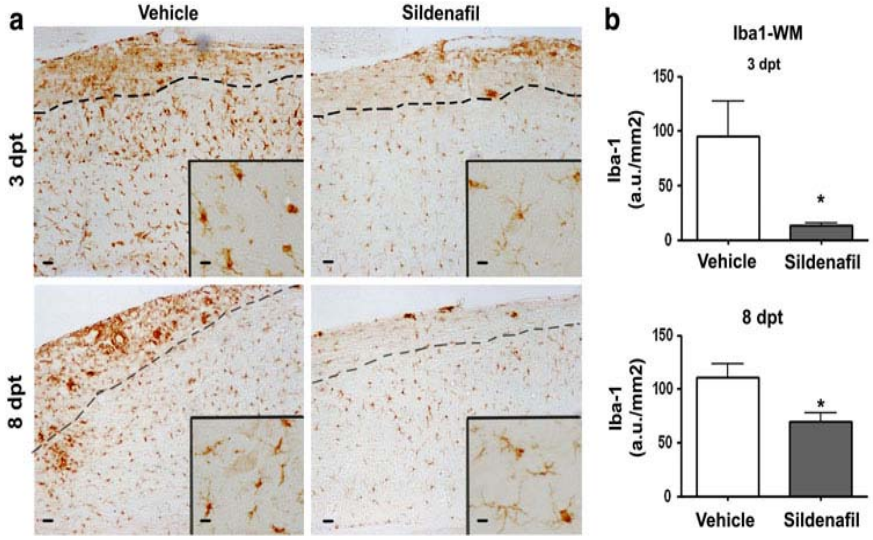

Figure 5. Sildenafil decreases macrophage/microglia activation in the spinal cord of EAE mice. (a) Spinal cord sections from MOG-immunized mice treated or not with sildenafil for 3 (n=4) or 8 days (n=12–13) were immunostained for Iba1 to evaluate macrophage/microglia activation (bar 50 μm); (b) quantification of Iba1 staining intensity in WM. Treatment with sildenafil significant reduced macrophage/microglia activation at both treatment times. Inserts magnification of GM areas showing that sildenafil promotes a resting morphology in microglia (bar 20 μm). Values are mean ± SEM. Statistical significance: *p<0.05.

Increasing evidence indicates that astrogliosis can play a crucial role in the pathogenesis and resolution of demyelinating disease. Thus, we examined the effect of sildenafil on GFAP immunoreactivity in the spinal cord of EAE mice. Different effects were observed in WM and gray matter (GM). Severely reactive astrocytes with long overlapping processes (anisomorphic gliosis) were observed throughout the WM but particularly in areas of heavy inflammatory cell infiltration (Fig. 6a). Although no significant differences in GFAP

overall staining intensity were observed between vehicle- and sildenafil-treated animals, a stronger tendency to form scar-like structures around confined infiltrates could be generally observed in the latter (Fig. 6a), suggesting a role in controlling the spread of infiltration. In the GM moderate astrogliosis was widespread in vehicle-treated mice, and in contrast to WM, sildenafil had a biphasic effect, significantly decreasing GFAP immunoreactivity after 3 days but increasing it after 8 days of treatment (Fig. 6b). At this treatment time, activated astrocytes were evenly distributed in GM and presented a more stellate shape, a feature typical of isomorphic gliosis.

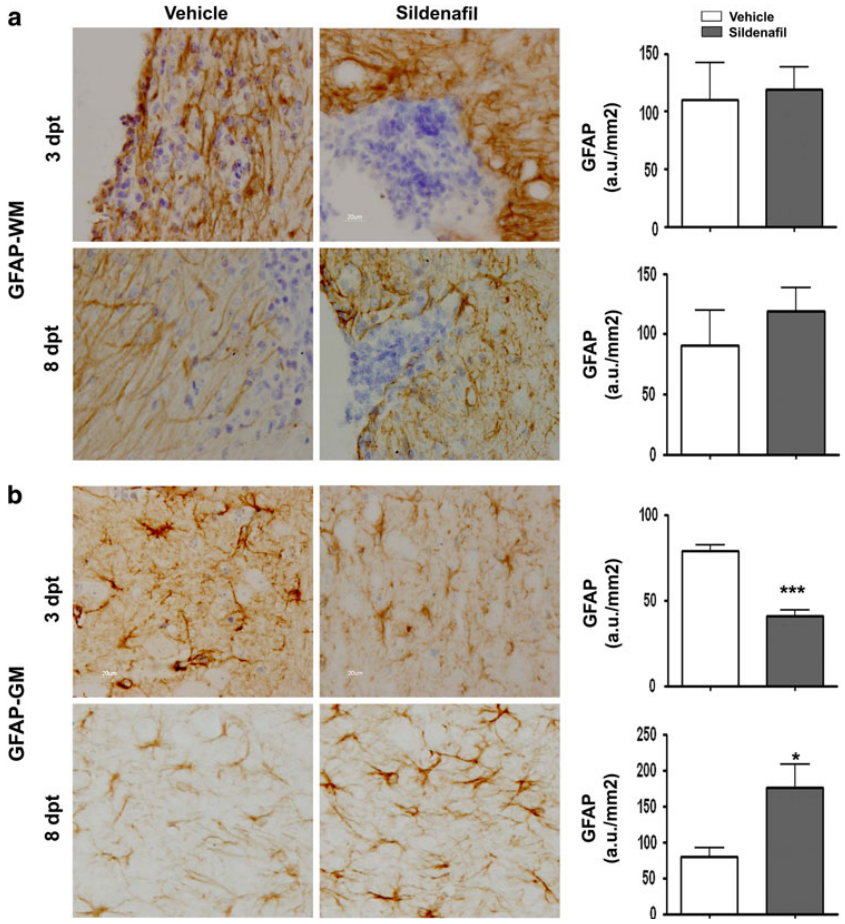

Figure 6. Sildenafil modifies astrogliosis in the spinal cord of EAE mice. Spinal cord sections from MOG-immunized mice treated or not with sildenafil for 3 (n=4) or 8 days (n=12–13) were immunostained for GFAP to evaluate astroglial activation (bar 20 μm). Quantification of GFAP staining in the WM (a) showed no significant differences between treated and untreated animals, but scar-like structures around infiltrates were more prominent in sildenafil-treated animals. In the GM (b), sildenafil had a biphasic effect on astroglial activation, decreasing GFAP staining intensity at 3 dpt but increasing it at 8 dpt. Values are mean ± SEM. Statistical significance: *p<0.05; ***p<0.001.

3.2. Effect of sildenafil administration at the onset of clinical symptoms

3.2.1. Effect on disease development

We have investigated the effect of early administration of sildenafil at the onset of clinical symptoms of MOG35-55-induced EAE when the infiltration of inflammatory cells in the spinal cord is beginning. Animals (15-22 per group) were treated with sildenafil (10 mg/kg, s.c. daily) or vehicle (water) starting when the clinical score was around 1 (10-16 dpi) and were sacrificed 7 or 15 days later. Spinal cords and spleens were removed for central and peripheral immune response analysis. As shown in Fig. 7 and Table 2, disability in vehicle-treated mice continued to increase for the following 4 days after starting the treatment, reaching a plateau with an average score of 2.4 until the end of the experiment. In contrast, the clinical score of mice treated with sildenafil stabilized after 3 days of treatment at a score around of 1.5, indicating that early administration of the drug prevents disease progression. Both the average score and the cumulative score of sildenafil-treated mice after 15 doses of the drug were significantly lower than those of vehicle-treated animals (Table 2). Note that the grade of remission was much lower when sildenafil was administered starting at the onset of the disease (-0.04, Table 2) than when it was administered starting at peak disease (-1.17, Table 1), in accordance with a preventive effect of early administration of the drug in disease development and an ameliorating effect when administered at the acute phase of the disease.

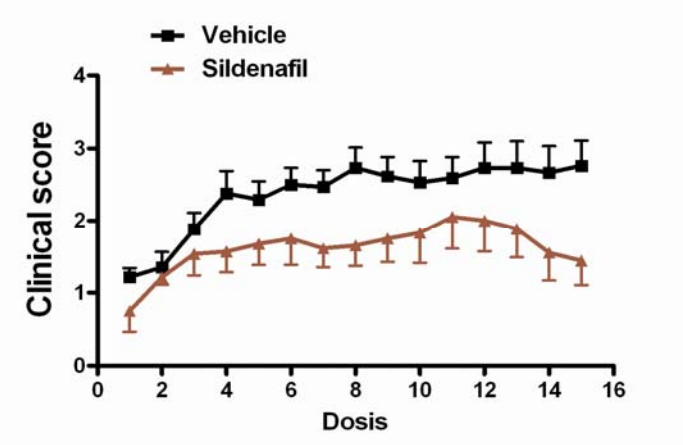

Figure 7. Treatment with sildenafil at the onset of clinical EAE symptoms reduces disease severity. C57BL/6 mice immunized with MOG35–55 developed clinical symptoms of EAE after 1 week. Sildenafil administration (10 mg/kg, s.c. daily) started when animals reached a score around 1 (10-16 dpi) and continued for 15 days. Two–way ANOVA shows significant differences between treatments (***$p<0.001$). Values are mean ± SEM (n = 9–22).

Table 2. Clinical scores of EAE mice treated or not with sildenafil starting at the onset of disease.

Treatment	EAE clinical score before treatment (n=15-22 per group)			EAE clinical score after 15 days of treatment (n=9-15 per group)			Grade of remission
	Incidence	Average Score	Cumulative score	Incidence	Average Score	Cumulative score	
Vehicle	100	1.21±0.12	1,20±0.20	100	2.76±0.36	44,33±3.84	0,47±0.46
Sildenafil	100	0.75±0.28	1.53±0.24	87.5	1.44±0.33**	27.11±4.89***	-0.04±0.21

EAE incidence (% diseased animals) and clinical scores of MOG-immunized mice before and after 15 days of sildenafil treatment. Results are mean ± SEM. Two way ANOVA followed by Bonferroni's post hoc test reveals statistically significant differences in average score and cumulative score (sum of scores from disease onset) but not in the grade of remission (difference between peak score and final score) after 15 days of sildenafil treatment, ** $p<0.01$ *** $p<0.001$.

3.2.2. Effect on the peripheral immune response

To investigate if regulation of the peripheral immune response might be involved in sildenafil reduction of disease severity when administered at the onset of clinical symptoms, we examined the immune response of splenocytes isolated from CFA or MOG35–55 immunized mice treated or not with sildenafil for 7 days and re-exposed or not to MOG35–55. Results were expressed as % of CFA-treated animals. We analyzed splenocyte release of pro-inflammatory (IL-2, IL-4, IL-17, TNF-α and IFN-γ) and anti-inflammatory (IL-10) cytokines by Flow Cytometry. As shown in figure 8A, re-exposure to MOG increased release of all these cytokines. Sildenafil treatment significantly reduced the release of IL-2 and IFN-γ as well as IL-4 indicating that the drug decreases Th1 and Th2 responses in splenocytes from MOG-immunized mice. A marked tendency to decrease the release of TNF-α and IL-17 was also observed indicating that sildenafil also reduces the Th17 response. No effect was observed in the levels of the anti-inflammatory cytokine IL-10.

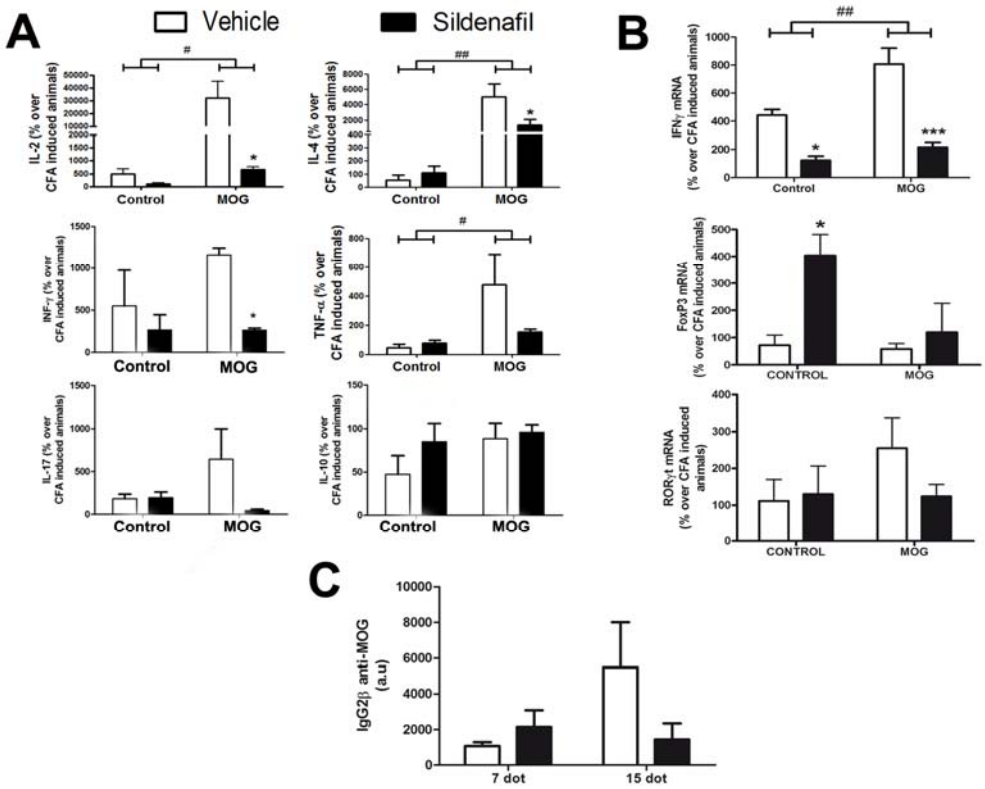

Figure 8. Treatment with sildenafil significantly affects the adaptative immune response in EAE mice. Splenocytes were isolated from MOG35–55 (n=4) or CFA- (n=2) immunized mice treated daily with sildenafil or vehicle for 7 days starting at the onset of disease. Cultured splenocytes were incubated without (control) or with MOG35–55 (10 μg/ml) for 72 h. (A) Release of cytokines measured in the media by Flow Cytometry; (B) mRNA expression of IFN-γ, FoxP3 and ROR-γτ was measured by RT-PCR in the splenocytes. Quantification of the primer specific mRNA was related to the total amount of the housekeeping gene GAPDH. Results are represented as % over CFA induced animal values. (C) Plasma from MOG35–55 immunized animals (n=4-5) treated for 7 or 15 days with vehicle or sildenafil was subjected to ELISA to detect specific IgG2β anti-MOG production by B lymphocytes. Values are means ± SEM and statistical analysis was performed by two-way ANOVA comparing splenocytes treatment (#p<0.05, ## p<0.01) and Bonferroni's post-hoc test comparing with the respective vehicle-treated animals. (*p<0.05, **p<0.01; ***p<0.001)

We also analyzed in splenocytes the mRNA expression of IFN-γ, as well as that of the Th17 and Treg specific transcription factors RORγt and FoxP3, respectively. In agreement with the cytokine results, we observed a marked decrease in the mRNA expression of IFN-γ in splenocytes of sildenafil-treated animals re-exposed to MOG (Fig. 8B). However, we also observed a significant decrease in IFN-γ mRNA in splenocytes not re-exposed to MOG. The level of mRNA for the transcription factor FoxP3 (Tregs) was not affected in splenocytes from vehicle-treated animals re-exposed to MOG, however in those from sildenafil-treated animals a marked increase (4-fold) was observed only in splenocytes not re-exposed to MOG, suggesting that sildenafil has an effect on Treg differentiation. In contrast, mRNA levels of the transcription factor RORγt (Th17) tend to increase after re-exposure to MOG in

splenocytes from vehicle-treated animals but not from sildenafil-treated animals. This is in accordance with the effect observed in IL-17 release (Fig. 8A) further supporting the contention that sildenafil treatment attenuates the Th17 MOG-specific response.

It is well known that B cells contribute to the pathogenesis of EAE by production of anti-myelin antibodies that contribute to demyelination (Mann, Ray et al. 2012). Thus, we analyzed if the B lymphocyte response was affected by sildenafil treatment. We measured by sandwich-ELISA the levels of specific anti-MOG IgG2β in plasma from MOG-immunized mice treated with vehicle or sildenafil for 7 or 15 days (Fig. 8C). As expected, the level of anti-MOG IgG2β increased in plasma of EAE vehicle-treated animals between 7 and 15 days of treatment, however this increase was prevented by treatment with sildenafil (Fig 8C) indicating a significant effect on humoral immune response.

3.2.3. Effects of sildenafil treatment on PDE5 expression in splenocytes

Expression of PDE5 has been reported to increase in blood vessels and in putative microglia in the penumbra of an ischemic lesion in rats (Menniti, Ren et al. 2009) and also in sciatic nerve tissue in mice with diabetic peripheral neuropathy (Wang, Chopp et al. 2011). Furthermore, in this last case, treatment with sildenafil normalized PDE5 levels. Expression of PDE5 in splenocytes has not been reported to date but our observation that sildenafil has effects on the different T cell populations present prompted us to analyzed mRNA expression of PDE5 in splenocytes of non-immunized animals and of CFA- and MOG-immunized mice treated with vehicle or sildenafil for 7 days. We observed detectable levels of PDE5 mRNA in splenocytes from non-immunized mice and, as shown in Fig. 9, immunization with CFA or MOG, produced a notable increase in PDE5 mRNA in vehicle-treated mice (250 % and 375 % of non-immunized mice, respectively) that was not observed in sildenafil-treated animals. These results suggest that decreased intracellular cGMP levels resulting from increased activity of PDE5 could have a permissive effect on T cell responses after MOG immunization, and that inhibition and down-regulation of PDE5 by sildenafil in these cells may be part of the mechanisms implicated in the preventive effect of sildenafil on EAE development.

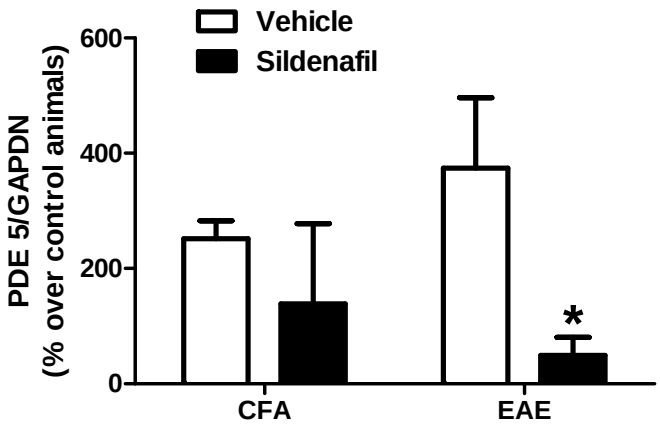

Figure 9. **Sildenafil down-regulates PDE 5 mRNA expression enhanced by MOG-immunization in mice splenocytes**. mRNA expression of PDE5A was measured by RT-PCR in splenocytes isolated from non-immunized or MOG- or CFA-immunized mice at the onset of EAE clinical symptoms and treated with vehicle or sildenafil for 7 days. Results, presented as % over non-immunized animal values, are mean ± SEM (n=2-4). Statistically significant difference was found by two-way ANOVA between CFA and EAE animals ($p<0.05$) and by Bonferroni's post-hoc test between sildenafil- and vehicle-treated animals (*$p<0.05$).

DISCUSSION

The EAE model of MS induced in C57BL6 mice by immunization with MOG_{35-55} peptide has been extensively used to study the development of neuroinflammation and the resulting demyelination occurring in the CNS. MOG_{35-55} immunization induces an acute attack of immune cells to the CNS, starting on the spinal cord around days 7-10 after immunization, followed by a progressive deterioration of myelin sheaths and axons that is accompanied by increased functional disability. Around three weeks after immunization the disease becomes chronic and symptoms progress slowly.

Results of our studies treating MOG-immunized animals with the PDE5 selective inhibitor sildenafil (10 mg/kg, sc) show that daily administration of the drug for up to 15 days (last time studied) starting at the onset of clinical symptoms (10-16 dpi) prevents disease progression as shown by the stabilization of the clinical score (Fig. 7 and Table 2). Furthermore, administration of sildenafil starting at the acute phase of the disease (18 dpi) ameliorates disease symptoms (Fig. 1 and Table 1). Importantly, the beneficial effect of sildenafil is significant after only 4 days of treatment and after 8 days the grade of remission is significantly increased presenting virtually full recovery more than half of the animals. In agreement with the decrease in EAE clinical symptoms observed with this treatment protocol, analysis of the neuropathology in the lumbar-thoracic spinal cord showed that axonal loss was prevented while remyelination was increased. These neuroprotective effects may result at least in part from the reduction in encephalitogenic T cell infiltration induced by sildenafil in the spinal cord as shown by the decrease in the number of CD3+-Tcells per infiltrate occurring at 21 dpi after only 3 days of sildenafil treatment. This effect of sildenafil was not longer observed at 26 dpi after 8 doses of the drug. At this time-point vehicle-treated animals presented significantly lower infiltration score (H&E) and number of CD3+ T cells per infiltrate respect to 21 dpi, in agreement with other reports showing that inflammatory cell infiltration in MOG-immunized mice decreases after peak disease despite generalized axonal loss and demyelination and persistent clinical disability (Herrero-Herranz, Pardo et al. 2008).

On the other hand, activated microglia/macrophages in WM (strongly Iba1+ globoid cells) were significantly reduced by sildenafil at both 3 (21 dpi) and 8 dpt (28 dpi). These results suggest that decreased macrophage/microglial activation may bear a more direct relation to the axon protective effect of sildenafil than decreased T cell infiltration. However, a contribution of a direct effect of sildenafil in axons and oligodendrocytes potentiating cGMP-mediated neuroprotective pathways cannot be ruled out (Garthwaite, Goodwin et al.

1999; Nakamizo, Kawamata et al. 2003; Benjamins and Nedelkoska 2007; Wang, Chopp et al. 2011).

In this work we have observed that the adhesion molecule ICAM-1 that is involved in leukocyte entry, lymphocyte activation and other immune responses, and plays a central role in the development of MS and EAE (Lebedeva, Dustin et al. 2005; Bullard, Hu et al. 2007), is highly expressed in inflammatory cells within and around infiltrates in the spinal cord of EAE mice, and that treatment with sildenafil drastically reduces this expression while that of blood vessel endothelial cells was still observed. Studies on ICAM-1 null mice have evidenced the critical role of ICAM-1 expression in T cells for the modulation of effector T cell responses (Bullard, Hu et al. 2007). Interestingly, NO via cGMP was reported to reduce T cell adhesion to human brain microvessels in vitro, but NO did not modulate adhesion molecule expression in the endothelial cells, suggesting a direct action on the T cells (Wong, Prameya et al. 2005). Thus, down-regulation by sildenafil of ICAM-1 expression in T cells could be involved in decreasing T cell infiltration and/or activation in the spinal cord of EAE mice. Additionally, by increasing cGMP in macrophages sildenafil could attenuate TNF-α release and TNF-α-induced expression of chemokines and adhesion molecules in endothelial cells (Vollmar 2005).

Another mechanism by which sildenafil may be reducing the proportion of autoreactive T cells (Th1 and Th17) in the infiltrates may be related to the observation that sildenafil treatment increases the population of FoxP3 Tregs (Fig 4c), cells that have been extensively implicated in suppressing homing of damaging T cells to the nervous system producing protection and recovery from EAE (Paust and Cantor 2005).

Using Iba-1 immunostaining we have shown that in addition to reducing heavily Iba1-stained globoid macrophages/microglia within and around infiltrates, treatment with sildenafil for 3 or 8 days decreased microglia activation throughout the spinal cord suggesting a further mechanism that may contribute to the neuroprotective effect of the drug. This agrees with results in Chapter II showing that treatment with PDE5 inhibitors decreases recruitment and activation of macrophages/microglia around a cortical cryoinjury in rodents, and also with results from in vitro studies showing that cGMP-mediated pathways reduce expression and release of inflammatory mediators in both macrophages and microglia (Paris, Town et al. 1999; Vollmar 2005; Moriyama, Taniguchi et al. 2006), Chapter 1 in this Thesis). Since microglia does not seem to express a functional GC_{NO} activity but can accumulate cGMP by stimulation with ANP in the presence of cGMP-PDE inhibitors (Chapter 1, (Prado, Baltrons

et al. 2010)), it is tempting to speculate that increases in cGMP in microglia/macrophages in the spinal cord of EAE animals occurs in response to NPs. Interestingly, a recent study showed that administration of brain natriuretic peptide down regulates microglial activation in murine models of traumatic brain injury and intracerebral hemorrhage (James, Wang et al. 2010).

The role of astrogliosis in the pathogenesis and resolution of demyelinating disease is not clear. Astrocytes can promote and perpetuate immune-mediated demyelination by priming autoreactive T cells and expressing cytokines, chemokines and co-stimulatory and adhesion molecules (Dong and Benveniste 2001). However, astrocytes can also promote anti-inflammatory responses and form perivascular barriers that restrict the influx of leukocytes into CNS parenchyma (Liberto, Albrecht et al. 2004; Sofroniew and Vinters 2010). We have previously shown that increasing cGMP in astrocytes regulates cytoskeleton dynamics and accelerates migration in a scratch wound assay in vitro (Boran and Garcia 2007). In addition, we have shown that treatment with PDE5 inhibitors enhances astrogliosis around a cortical cryolesion suggesting that cGMP-mediated pathways can accelerate glial scar formation [Chapter 2, (Pifarre, Prado et al. 2010)]. Here we show that in the spinal cord WM of sildenafil-treated EAE animals heavily reactive astrocytes with long overlapping processes (anisomorphic gliosis) present a marked tendency to form scar-like structures around confined infiltrates (Fig. 7a), suggesting a role in controlling the spreading of infiltration in the healthy parenchyma. In addition, in the GM a short administration (3 days) of sildenafil reduces astrogliosis, whereas a longer treatment (8 days) increases reactivity of astrocytes that present typical features of isomorphic gliosis. Numerous evidences indicate that these activated astrocytes exert suppressive effects on inflammatory cells and release antioxidants and growth factors that protect neurons and oligodendrocytes (Liberto, Albrecht et al. 2004). Thus, it will be of interest to investigate how the different effects of sildenafil on astroglial reactivity in WM and GM observed at the different treatment times relate to the reduced inflammation, axon protection and remyelination the drug produces in EAE mice.

Since sildenafil administration in the chronic phase of the disease decreased the infiltration and activation of inflammatory cells in the CNS parenchyma, we investigated if the drug could affect the peripheral immune response in EAE animals. We analyzed the release of cytokines and the expression of mRNAs specific of different lymphocyte populations in splenocytes isolated from EAE mice treated with sildenafil for 7 days from the onset of clinical symptoms when the inflammatory process and the inflammatory cell

infiltration into the CNS are booming. As expected, the MOG recall experiments showed increased Th1/Th2/Th17 responses as indicated by increased release of the Th1 cytokines IL-2 and IFN-γ, the Th2 cytokine IL-4 and the Th17 cytokines IL-17 and TNF-α and by up-regulation of the mRNA for IFN-γ and the Th17-specific transcription factor RORγt. However, these responses were reduced in splenocytes from animals treated with sildenafil supporting an effect of the drug on the adaptative-immune response. Furthermore, we observed increased mRNA expression of the Treg transcription factor Foxp3 but only in splenocytes from sildenafil-treated animals not re-exposed to MOG. The lack of effect of sildenafil treatment on Foxp3+ cells expression during the recall response may be related to the observation that Th1/Th2-polarizing cytokines potently inhibit Foxp3-Treg differentiation reported Wei et al (Wei, Duramad et al. 2007). These authors showed that IL-4 was the most potent cytokine inhibiting Foxp3 mRNA induction, and as can be observed in Fig. 8A, even though sildenafil significantly reduces IL-4 production, the levels remaining are more than 12-fold higher than in splenocytes not re-exposed to MOG. In contrast to our results Brahmachari and Pahan (Brahmachari and Pahan 2010) reported that in splenocytes from SJL/J mice immunized with myelin basic protein and in B6.129 mice immunized with MOG, re-exposure to the antigens down-regulates FoxP3 mRNA expression in a NO- and cGMP-dependent manner. As previously mentioned, in our work re-exposure to MOG of splenocytes from MOG-immunized mice had no effect on FoxP3 mRNA expression (Fig. 8B). Differences in mice strains and/or immunization protocols and time at which the memory response was examined may explain these conflicting resuslts. In our study, the up-regulation in FoxP3+ mRNA observed in non-primed splenocytes from EAE animals (17-23 dpi) that had been treated with sildenafil for 7 days at the onset od disease, is in accordance with the observed increased in the number of FoxP3+ cells in spinal cords of animals treated with sildenafil for 3 days at peak disease (21 dpi).

Studies in EAE have identified the Th1 subset population as responsible for initiating the inflammatory response in the CNS, whilst Th2 cells were regarded as responsible of stimulating B cells to produce specific antibodies (Gold, Linington et al. 2006; Ozdemir, Akdis et al. 2010). The reduction in Th1 response in splenocytes could be a reflection of a decreased inflammatory response that can contribute to prevent advancement of disease when sildenafil is administered at the onset of clinical symptoms. The balance between Treg and autoreactive Th17 cells has been extensively implicated in EAE progression. While the ratio between Th17 and Treg cells is decanted into Th17 effector cells at the peak of the disease, it

changes during remission, where there is a massive decrease of effector T cells while maintaining Tregs (Korn, Oukka et al. 2007). These cells have been demonstrated to recognize self-reactive T cells and suppress them, thus reducing CNS damage and leading to protection from EAE (Zhou and Littman 2009). The observation in our study of a down-regulation of Th17 lymphocyte markers IL-17 and RORγt concomitant with an up-regulation FoxP3-Tregs suggest another mechanism for the reduced severity of the disease in sildenafil-treated EAE mice. Down-regulation of the adaptive immune response has been generally observed for drugs that ameliorate or prevent EAE such as lithium (De Sarno, Axtell et al. 2008), glatiramer acetate (Aharoni, Eilam et al. 2010) and fingolimod (Chiba, Kataoka et al. 2011).

The role of B cells in the pathogenesis of EAE producing anti-myelin antibodies that contribute to demyelination is well established (Mann, Ray et al. 2012). Since B cell differentiation requires stimulation by Th2 (Ozdemir, Akdis et al. 2010) and sildenafil reduced the peripheral Th2 response in EAE mice, we examined if treatment with the drug affected the accumulation of specific IgG2β anti-MOG in the plasma of EAE mice. We found that time-dependent accumulation of the specific-IgG was prevented after 15 days of sildenafil treatment suggesting that the drug affects B cell differentiation. This effect will be critical for the reduction in the generation of demyelinating lesions and of functional disability in EAE animals (Gold, Linington et al. 2006; Mann, Ray et al. 2012).

Finally, another new finding in this thesis is the increased expression of PDE5 mRNA in splenocytes from CFA- and MOG-immunized mice and the normalization of PDE5 levels in sildenafil-treated animals. An up-regulation of PDE5 has been also described in other pathologies affecting the nervous system such as stroke (Menniti, Ren et al. 2009) and diabetic neuropathy (Wang, Chopp et al. 2011) in which treatment with sildenafil has beneficial effects.

In conclusion, the beneficial effects of sildenafil treatment on the progression of EAE and resolution of EAE seem to be mediated by effects in multiple cells types, which is not surprisingly because cGMP-mediated pathways are ubiquitous. To our knowledge, this is the first report demonstrating efficacy in a mouse model of MS of a PDE5 inhibitor that is well-tolerated drug used in humans for treatment of erectile dysfunction and arterial pulmonary hypertension.

GENERAL DISCUSSION

GENERAL DISCUSSION

Neuroinflammation elicited by infection, trauma, ischemic injury or neurodegenerative disease is characterized by glial activation (reactive gliosis), a phenomenon that involves changes in morphology, proliferation and motility in microglia and astroglia, the cells responsible for the innate immune response in the CNS. Additionally, activated glial cells express a wide range of immunoregulatory molecules including pro- and anti-inflammatory cytokines and their receptors, as well as enzymes that generate reactive nitrogen and oxygen species, such as iNOS and NADPH oxidase, that have been implicated in the pathogenesis of different CNS diseases (Kim and de Vellis 2005; Hailer 2008; Sofroniew and Vinters 2010). In this study we demonstrate that intracellular cGMP regulates glial inflammatory responses *in vitro* and *in vivo*. We have shown in cultured cells that the ANP-cGMP-PKG pathway reduces LPS-induced iNOS protein expression in microglia, in agreement with other reports showing reduced expression and release of pro-inflammatory mediators in microglia and macrophages by ANP-cGMP-mediated pathways (Vollmar 2005; Moriyama, Taniguchi et al. 2006). More importantly, we have demonstrated *in vivo* using two animal models of CNS injury that increasing cGMP by administration of cGMP-PDE inhibitors reduces microglia/macrophage activation. In a model of focal cortical injury by cryolesion in rodents we have shown that treatment with PDE5 inhibitors (zaprinast, sildenafil) decreases recruitment and activation of microglia/macrophages at the lesion border and reduce protein and lipid oxidative stress, an effect most probably contributing to the observed decrease in neuronal cell death. Similarly, in the EAE model of Multiple Sclerosis, in which treatment with sildenafil at peak disease notably ameliorates clinical symptoms, we have observed a reduction in heavily activated amoeboid microglia/macrophages at infiltration sites, as well as in microglia at intermediate stages of activation all along the spinal cord, which may be one of the factors involved in preventing axonal damage and degeneration.

Previous studies in cultured astrocytes had also shown that cGMP can regulate astrogliosis. Data from our laboratory demonstrated that increasing cGMP levels leads to dramatic changes in the GFAP and actin cytoskeleton that result in accelerated closure of a scratch wound (Boran and Garcia 2007) and other authors showed increased expression of GFAP (Brahmachari, Fung et al. 2006). In accordance with this, we have shown here that treatment with PDE5 inhibitors enhances astrogliosis around the lesioned area in the cortical cryolesion model. Thus, as previously demonstrated for other conditions that increase astrogliosis around the lesion (Giralt, Penkowa et al. 2002; Molinero, Penkowa et al. 2003;

Klementiev, Novikova et al. 2008), PDE5 inhibitors are expected to accelerate the formation of the glial scar and the regeneration of lesioned tissue. In addition, we show here that sildenafil treatment up-regulates the expression in astrocytes of the antioxidant MT-I/II proteins that have been shown to be neuroprotective and neuroregenerative in the cryolesion model (Hidalgo, Aschner et al. 2001; Leung, Pankhurst et al. 2010; West, Leung et al. 2011), and of VEGF that may be directly implicated in PDE inhibitor increased angiogenesis, an effect that will also contribute to increase neuronal survival.

In the EAE model, we have also found that treatment with sildenafil increases anisomorphic astrogliosis and formation of scar-like structures around infiltrates in the spinal cord. This effect may contribute to reducing inflammatory cell infiltration in treated animals, since as demonstrated in transgenic mice with compromised proliferation of astrocytes, deficient formation of these scar-like structures leads to uncontrolled spreading of infiltration of immune cells into the spinal cord parenchyma (Sofroniew 2009). Other effects of sildenafil observed in our study that will also contribute to decrease leukocyte entry and lymphocyte activation in the spinal cord of EAE mice are the down-regulation of ICAM-1 in infiltrated cells, since studies on ICAM-1 null mice have evidenced the critical role of this adhesion molecule in the modulation of effector T cell responses (Bullard, Hu et al. 2007), and the up-regulation of Foxp3-Tregs known to suppress autoreactive T cells and to play a critical role in protection and recovery from EAE (Paust and Cantor 2005).

Another important finding in this Thesis is that sildenafil administration to EAE animals at the onset of clinical symptoms prevents disease progression, an effect that may be related the ability of the drug to regulate the adaptive immune response, as observed for other drugs with beneficial effects in EAE (De Sarno, Axtell et al. 2008; Aharoni, Eilam et al. 2010; Chiba, Kataoka et al. 2011). We have found that the Th1/Th2/Th17 recall response is decreased in splenocytes from sildenafil-treated animals while FoxP3-Tregs are upregualted, as also observed in the spinal cord. Likewise, sildenafil treatment has been shown to decrease plasmatic accumulation of specific anti-MOG IgG2β, an effect that will contribute to prevention of demyelination.

Accumulating evidence demonstrates that treatment with selective PDE5 inhibitors is neuroprotective in various animal models of CNS injury through different mechanisms. Activation of antiapoptotic pathways, angiogenesis and neurogenesis appears to be responsible for PDE5 inhibitor efficacy in promoting functional recovery after stroke in rats (Zhang, Zhang et al. 2005; Zhang, Zhang et al. 2006; Menniti, Ren et al. 2009), and increases

in pCREB and BDNF have been implicated in protection against 3-nitropropionic acid toxicity (Puerta, Hervias et al. 2010) and in improving synaptic function and cognitive deficits in Alzheimer's disease models (Puzzo, Staniszewski et al. 2009; Cuadrado-Tejedor, Hervias et al. 2011). Our results add other important mechanisms by which PDE inhibitors can be efficacious as neuroprotectants after neural tissue damage which are the anti-inflammatory effects at the level of the innate immune response, as well as the adaptive immune response, observed in the animal models of focal brain lesion and of EAE. The variety of effects of PDE5 inhibitors are in accord with the ubiquitous expression of cGMP-mediated pathways in different tissues in mammals where different molecular targets of the nucleotides may be expressed. All these studies highlight the relevance of the degradation pathway via PDE5 for the control of cGMP levels. Interestingly, up-regulation of expression of PDE5 has been reported in microglia in the penumbra of ischemic brain lesions in rats (Menniti, Ren et al. 2009), in sciatic nerve of diabetic mice with peripheral neuropathy (Wang, Chopp et al. 2011) and in this work in splenocytes from EAE mice, but whether these changes in PDE5 expression are cause or consequence of the disease, will need to be investigated. Whatever the case, PDE5 inhibitors will restore normal levels of cGMP not only by inhibiting the enzyme activity but also by down-regulating PDE5 expression as shown in splenocytes from sildenafil-treated EAE mice.

CONCLUSIONS

CONCLUSIONS

- Rat brain microglia in primary culture synthesizes cGMP in response to ANP but not to NO and express natriuretic peptide receptors with guanylyl-cyclase activity (NPR-A and NPR-B), as well as cGMP-degrading enzymes (PDE5, PDE1).

- Pretreatment of rat cerebellar microglial cultures with ANP down-regulates LPS-induced iNOS protein through interaction with NPR-A. This effect is mediated by PKG but not by PKA.

- The ANP down-regulation of iNOS protein expression does not result from inhibition of transcription or increased degradation rate of the protein, but appears to involve a decrease in iNOS protein translation.

- In cortically cryolesioned rats, treatment with the non-selective cGMP-PDE inhibitor zaprinast enhances astrogliosis around the lesioned area while decreasing macrophage/microglial activation, oxidative stress and neuronal death.

- In cortically cryolesioned mice, treatment with the selective PDE5 inhibitor sildenafil reproduces the changes in glial reactivity and the antioxidant and antiapoptotic effects previously observed with zaprinast in rats, indicating that inhibition of PDE5 is responsible for these neuroprotective actions.

- Treatment with sildenafil increases expression of the antioxidant and neuroprotective protein MT-I/II in activated glial cells around the cryolesion in wild type mice. Additionally, the neuroprotective effects of sildenafil are not observed in cryolesioned MT-I/II-deficient mice. These observations support the involvement of glial MT-I/II up-regulation in the neuroprotective effects of sildenafil in the criolesioned cortex.

- Sildenafil administration to MOG35-55-immunized mice at the peak of EAE disease symptoms rapidly and markedly ameliorates disease severity by decreasing axonal damage and increasing remyelination in the spinal cord.

The neuroprotective effects of sildenafil in EAE probably relate to the reduction in autoreactive T cell infiltration, microglia/macrophage activation and ICAM-1 expression and to the increase in Foxp3-Tregs induced by the treatment in the spinal cord.

- Sildenafil treatment enhanced the presence of reactive astrocytes forming scar-like structures around infiltrates suggesting a possible mechanism for restricting leukocyte spread into healthy parenchyma.

- Sildenafil administration to MOG35-55-immunized mice at the onset of EAE symptoms prevents disease progression. This effect may result from a direct regulation of the

peripheral adaptative immune response as indicated by the decreased Th1/Th2/Th17 response and increased FoxP3 expression in splenocytes and by the reduction of anti-MOG specific antibodies in the plasma of sildenafil-treated animals.

-PDE5 expression is up-regulated in splenocytes from untreated EAE mice but not in sildenafil-treated animals suggesting that diminished cGMP intracellular levels by increased degradation, may have a permissive effect in immune cell activation.

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Cyclic GMP phosphodiesterase inhibition alters the glial inflammatory response, reduces oxidative stress and cell death and increases angiogenesis following focal brain injury

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Abstract

Recent evidence obtained in cultured glial cells indicates that cGMP-mediated pathways regulate cytoskeleton dynamics, glial fibrillary acidic protein expression and motility in astrocytes, as well as inflammatory gene expression in microglia, suggesting a role in the regulation of the glial reactive phenotype. The aim of this work was to examine if cGMP regulates the glial inflammatory response *in vivo* following CNS damage caused by a focal cryolesion onto the cortex in rats. Results show that treatment with the cGMP phosphodiesterase inhibitor zaprinast (10 mg/kg i.p.) 2 h before and 24 and 48 h after the lesion results 3 days post-lesion in notably enhanced astrogliosis manifested by increased glial fibrillary acidic protein immunoreactivity and protein levels around the lesion. In contrast, zaprinast decreased the number of round/ameboid lectin-positive cells and the expression of the activated microglia/macrophage markers Iba-1 and CD11b indicating decreased recruitment and activation of these cells. This altered inflammatory response is accompanied by a decrease in protein oxidative stress, apoptotic cell death and neuronal degeneration. In addition, zaprinast enhanced angiogenesis in the lesioned cortex probably as a result of vascular endothelial growth factor expression in reactive astrocytes. These results suggest that regulation of the glial inflammatory response may contribute to the reported neuroprotective effects of cGMP-phosphodiesterase inhibitors in brain injury.

Keywords: angiogenesis, cyclic GMP, glial reactivity, neuroprotection, oxidative stress, phosphodiesterase inhibitors.

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Brain inflammation is rapidly induced by acute neuropathologic insults such as infection, trauma or ischemia and also occurs in chronic neurodegenerative disorders. Microglial and astroglial cells are responsible for the innate immune response in the CNS and their activation constitutes one of the most notable characteristics of neuroinflammation. Reactive astrocytes proliferate, become hypertrophic and show increased expression of intermediate filament proteins, in particular glial fibrillary acidic protein (GFAP). They migrate to the site of injury and form a glial barrier that isolates the still intact tissue from secondary lesions (Silver and Miller 2004; Pekny and Nilsson 2005). Increasing evidence indicates that immune-activated astrocytes can promote recovery of CNS function by increased production of energy substrates and neurotrophic factors and enhanced expression of proteins with antioxidant and glutamate inactivating functions (Liberto *et al.* 2004). Focal activation of microglia after CNS injury occurs before astrocyte activation and implies a switch in behavior from resting to defending cells. Normally ramified resting microglia acquires a macrophage-like phenotype presenting increased motility and phagocytic activity (Kim and de Vellis 2005; Town *et al.* 2005). Concomitant with cell shape changes, reactive glial cells release pro-inflammatory cyto-

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Abbreviations used: ANP, atrial natriuretic peptide; GC, guanylyl cyclase; GFAP, glial fibrillary acidic protein; LPS, bacterial lipopolysaccharide; NOS2, NO synthase 2; NPs, natriuretic peptides; PDE, phosphodiesterase; VEGF, vascular endothelial growth factor.

kines and express enzymes such as NO synthase 2 (NOS2) and NADPH oxidase able to generate inflammatory mediators (NO, reactive oxygen species) that can inhibit neurite outgrowth and affect cell survival (Block *et al.* 2007).

The major target for NO in the CNS is the predominantly soluble guanylyl cyclase (GC) and its product cyclic GMP (cGMP), the recognized mediator of important regulatory actions of NO in synaptic plasticity, brain development and cerebral blood flow (Feil and Kleppisch 2008; Garthwaite 2008). cGMP has been also implicated in neuroprotective actions of NO (Kang *et al.* 2004). In mammalian CNS, immunocytochemical and biochemical studies have shown a widespread expression of NO-stimulated GC (NO-GC) in neurons and to a lesser extent in astrocytes but not in microglia (Teunissen *et al.* 2001; Baltrons *et al.* 2004; Ding *et al.* 2004). Astrocytes can also generate cGMP upon binding of natriuretic peptides (NPs) to transmembrane GC receptors NPR-A and NPR-B (de Vente and Steinbusch 2000; Cao and Yang 2008). NPs have been localized at the mRNA and protein levels in neuronal structures and astrocytes in the rat brain. The function of the NP-cGMP pathway in the CNS is largely unknown but available data suggest that it may play a role in brain development and neuroprotection (Wiggins *et al.* 2003; Cao and Yang 2008).

Little is known about the regulation and function of cGMP formation in glial cells during neuroinflammation. We have shown that in response to different neuroinflammatory compounds formation of cGMP in cultured astrocytes initially increases in parallel with NOS2 induction but later decreases because of down-regulation of NO-GC (Baltrons and Garcia 1999; Pedraza *et al.* 2003). Decreased NO-GC expression is observed in adult rat brain after administration of bacterial lipopolysaccharide (LPS), IL-1β or β-amyloid peptides (Baltrons *et al.* 2002; Pedraza *et al.* 2003; Duport and Garthwaite 2005) and in reactive astroglia in post-mortem brain tissue from patients with neurodegenerative diseases (Baltrons *et al.* 2004). The pathophysiological relevance of inflammation-related regulation of astroglial cGMP formation is unknown at present but recent evidences suggest a role for cGMP-mediated pathways in the regulation of the astroglial reactive phenotype. We have shown that cGMP dramatically affects the organization of the actin and GFAP filaments in cultured astrocytes and enhances their migration in a scracht-wound assay (Boran and Garcia 2007). Furthermore, cGMP has been reported to mediate NO-induced increases in GFAP expression (Brahmachari *et al.* 2006). In addition, cGMP-mediated pathways have been implicated in the regulation of inflammatory gene expression in different cell types including microglia in culture (Paris *et al.* 1999, 2000; Choi *et al.* 2002; Pilz and Broderick 2005; Vollmar 2005; Moriyama *et al.* 2006; Roy *et al.* 2006).

The aim of the present work was to investigate if cGMP regulates glial inflammatory responses *in vivo*. For this purpose, we have used a well-characterized experimental model of cryolesion to the parietal cortex in rodents. To increase cGMP levels, animals were treated with the cGMP phosphodiesterase (PDE) inhibitor zaprinast at a dose (10 mg/kg i.p.) that was previously reported to increase cGMP in rat brain and facilitate object recognition memory while having a minimal effects on blood pressure (Prickaerts *et al.* 1997; Domek-Lopacinska and Strosznajder 2008). Results show that administration of zaprinast 2 h before and 24 and 48 h after cryolesion significantly enhances astrogliosis while decreasing microglia/macrophage recruitment and activation at 3 days post-lesion (dpl). This is accompanied by decreased protein oxidative stress, apoptotic cell death and neuronal degeneration and increased angiogenesis and vascular endothelial growth factor (VEGF) expression supporting the therapeutic potential of cGMP-PDE inhibitors as anti-inflammatory and neuroprotective in the CNS.

Materials and methods

Animals and cryolesion procedure

Two-month-old male Sprague-Dawley rats were kept under constant temperature and had free access to food and water. Animals were separated in four groups: two groups of unlesioned animals (four per group) and two groups of cryolesioned animals (six per group) that were vehicle-treated or zaprinast-treated. Animals were cryolesioned under tribromethanol anesthesia. The skull over the right frontoparietal cortex was exposed, and a focal cryoinjury was carried out on the surface of the brain by applying a dry ice pellet for 60 s as previously described (Giralt *et al.* 2002). Animals were allowed to recover and returned to the animal room until they were killed 3 dpl. Animals were injected i.p. with zaprinast (10 mg/kg, Sigma-Aldrich Química S.A., Madrid, Spain) or vehicle (0.05 M NaOH), 2 h before and 24 and 48 h after cryolesion. Unlesioned animals were used as controls. After decapitation, the entire ipsilateral cortex was dissected out and divided into two parts cutting in the middle of the lesion. Half of the lesioned cortex was fixed in 4% paraformaldehyde for immunohistochemistry and the lesioned area of the other half was frozen in liquid nitrogen and stored at −80°C until use for biochemical studies. All experiments were carried out in a human manner and were approved by the proper ethical committees.

Immunohistochemistry

Paraformaldehyde-fixed cortical tissue was dehydrated and embedded in paraffin according to standard procedures and cut in serial coronal 5 μm sections. Sections were rehydrated and underwent heat-induced antigen retrieval by boiling in citrate buffer, pH 6.0, in a microwave oven for 5 min. Afterwards, sections were incubated in 1.5% H_2O_2 in 0.05 M Tris pH 7.4/Triton X-100 (Sigma) to quench endogenous peroxidase, followed by 10% fetal bovine serum to block non-specific binding. Then, sections were incubated overnight at 4°C with polyclonal rabbit anti-GFAP antibody (Dako Diagnostico S.A., Barcelona, Spain, 1 : 900) for astrocyte staining, rabbit polyclonal anti-Iba-1 antibody (Wako Pure Chemical Industries, Ltd., Osaka, Japan, 1 : 200) for microglia/macrophage staining, mouse monoclonal antibody anti-NeuN (Chemicon, Millipore Iberica, Madrid, Spain, 1 : 100) for neuronal nuclei

staining or polyclonal rabbit anti-VEGF (Abcam, Cambridge, UK, 1 : 100). Afterwards, sections were incubated for 1 h at 25°C with anti-rabbit or anti-mouse purified antibody conjugated to biotin (Vector Laboratories, Burlingame, CA, USA, 1 : 200). Cells of myelo-monocytic lineage and blood vessels were also stained with lectin from *Lycopersicon esculentum* conjugated with biotin (Sigma, 1:45), as previously described (Penkowa *et al.* 1999). Biotin-conjugated secondary antibody and lectin were detected using streptavidin/horseradish peroxidase (Vector, 1 : 300) and visualized using the peroxidase substrate Kit DAB (Vector). Control sections were incubated in the absence of primary antibodies to evaluate the extent of non-specific binding of the secondary antibody.

For detection and quantification of apoptotic cell death at a single cell level, we used the *In Situ Cell Death Detection Kit* from Roche Molecular Biochemicals (Barcelona, Spain) on paraffin brain sections (5–8 μm) following the protocol for difficult tissues according to the manufacturer's recommendations. This kit is based on labeling of DNA strand breaks (TUNEL reaction). Positive (DNAase I-treated) and negative (without terminal transferase) controls were included in each experiment.

Western blots

Brain tissue was homogenized in 10 volumes of ice-cold 50 mM Tris–HCl and 1 mM EDTA, pH 7.4, containing anti-protease (Roche) and anti-phosphatase (Sigma) cocktails, with a glass-Teflon Potter-Elvehjeim homogenizer mechanically driven at 800 rpm. Equal amounts of protein (40 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel (10%) electrophoresis followed by transfer to polyvinylidene difluoride membranes (Immobilon polyvinylidene difluoride; Millipore Iberica) at 100 V for 1.5 h at 4°C. Membranes were blocked at 4°C overnight in phosphate-buffered saline (pH 7.4) containing 5% non-fat dry milk and then incubated at 25°C for 2 h with mouse anti-GFAP (Sigma, 1 : 500), purified monoclonal IgG anti-rat CD11b (AbD Serotec, Oxford, UK, 1 : 500) or monoclonal anti-actin (Sigma, 1 : 100 000) primary antibodies. Afterwards, membranes were washed twice with phosphate-buffered saline–Tween 20 (Sigma), incubated with the secondary anti-mouse IgG–horseradish peroxidase-labeled (Amersham Life Science, Barcelona, Spain, 1 : 4000) and subsequently detected with a chemiluminescence detection kit (Immobilon; Millipore).

Protein oxidation was detected in immunoblots of cortex homogenates (20 μg protein) by detection of carbonyl groups using the OxiBlot TM detection kit (Chemicon) following the manufacturer's recommendations.

The intensity of bands was quantified by using Quantity One software (Bio-Rad Laboratories, Barcelona, Spain) and values were normalized for actin levels and expressed relative to the same animal that was included in all blots.

Quantification and statistical analysis

Quantification of GFAP, lectin and Iba-1 staining in cryolesioned animals was carried out at the border of the cortical lesion where inflammation is prominent using the Scion Image program. For each parameter analyzed, brain sections from four to five animals per group were used and a mean value of 4–6 different 0.5 mm^2 areas per brain section was calculated. Quantification areas were from the central part of the lesion border where staining is more homogeneous than at the edges. Quantification of VEGF staining was performed in 5×0.5 mm^2 areas of the lesioned cortex at least 50 μm away from the lesion border using the Scion Image program. TUNEL- and NeuN-positive cells as well as lectin-stained or hematoxylin–eosin-stained blood vessels were counted in 5–7×0.5 mm^2 random areas of the lesioned cortex at least 50 μm from the lesion border. For the predominantly apoptotic cell death marker TUNEL, positively stained cells were defined as cells with nuclear staining. Criteria for identifying degenerating neurons using the neuronal nuclei marker NeuN were the presence of condensed or dystrophic cell bodies. Quantification of TUNEL, NeuN and vessels was carried out by two independent investigators, who were blinded to animal identity and treatment. Data are expressed as mean ± standard error (SEM) of the indicated number of animals. When comparing more than two groups, significance of differences was evaluated by a two-way ANOVA followed by Bonferroni's test, using cryolesion and treatment as main factors. For comparison of two groups the Student's *t*-test was used.

Results

Treatment with zaprinast alters the inflammatory response elicited by cryolesion

We previously showed that cryoinjury to the cortex produces a profound inflammatory response with activation of resident microglia, recruitment of macrophages and reactive astrogliosis that occurs in a highly temporal-specific manner reaching maximal levels at 3 dpl (Penkowa *et al.* 1999). In this study, we demonstrate that treatment with a cGMP-PDE inhibitor leads to a significantly different glial response. Because of the limited information available about cGMP-PDE isoform expression in rat glial cells *in situ*, we have used the PDE-inhibitor zaprinast that inhibits cGMP-specific PDE5 with relatively high potency (IC_{50} = 0,13 μM) and is also effective inhibiting PDE9 (IC_{50} = 29 μM), the isoform with highest affinity for cGMP that is widely expressed in brain, as well as dual substrate PDE1 variants (IC_{50} = 6 μM) (Bender and Beavo 2006a; and references therein). Peripheral administration of zaprinast to rats at the dose used in this study (10 mg/kg) was previously reported to enhance memory performance while having minimal effects on blood pressure (Prickaerts *et al.* 1997). To insure high blood levels at the time of lesion, zaprinast was injected i.p. 2 h before and then treatment was administered 24 and 48 h after the lesion.

Astrocytes

Reactive astrogliosis manifested by a significant increase in GFAP immunoreactivity (two-way ANOVA; $p < 0.001$) was clearly visible around the lesioned area (Fig. 1c and e). In agreement with this, GFAP protein was significantly increased ($p < 0.001$) in homogenates of the lesioned cortex (Fig. 2a). Treatment with zaprinast enhanced GFAP immunostaining around the lesion by five- to sixfold (Fig. 1d, f and g) and GFAP protein levels in homogenates by two- to threefold (Fig. 2a) respect to lesioned vehicle-treated ani-

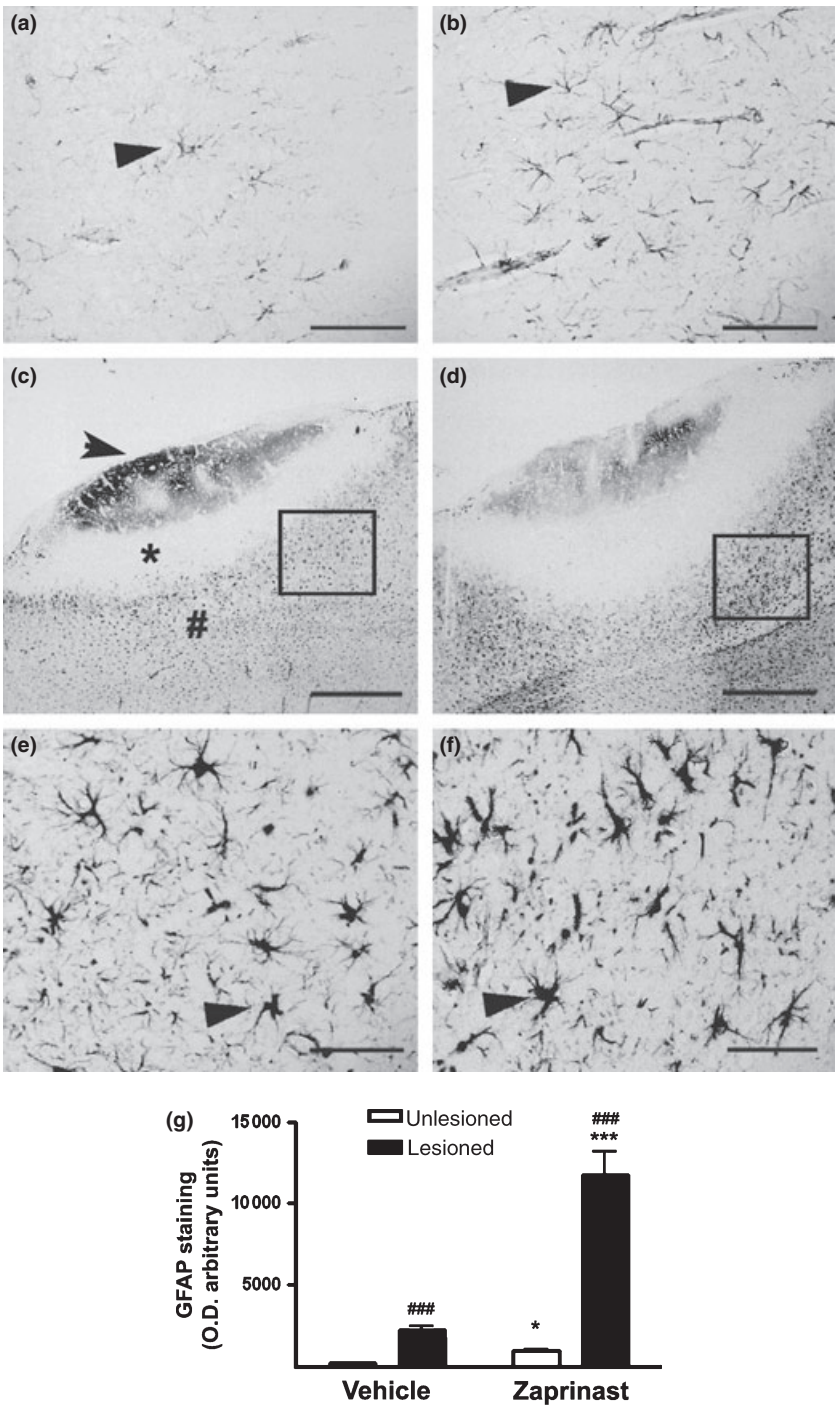

Fig. 1 Effect of zaprinast on glial fibrillary acidic protein (GFAP) immunostaining in cortex slices of unlesioned and cryolesioned rats. GFAP staining in unlesioned animals was weak (a) and increased slightly but significantly after treatment with zaprinast (b); GFAP staining in cryolesioned animals 3 dpl was prominent around the lesion (c; arrow points to the necrotic area; *area of microglial activation, #area of astroglial activation), and treatment with zaprinast greatly increased the number and intensity of GFAP-stained cells (d); (e) and (f) are higher magnification of squares in (c) and (d) showing reactive astrocytes with swollen cell bodies and enhanced immunostaining; (g) GFAP staining intensity was quantified with the Scion Image program in 4–6 areas of 0.5 mm^2 per brain chosen at random in unlesioned animals ($n = 4$) or from the border of the lesion (excluding the edges) in cryolesioned animals ($n = 5$). Statistically significant differences vs. unlesioned animals ($^{###}p < 0.001$) and vs. vehicle-treated animals ($^{*}p < 0.05$ and $^{***}p < 0.001$). Two-way ANOVA shows significant interaction between lesion and treatment ($p < 0.001$). Bar: (a, b, e, f) 30 μm; (c, d) 400 μm.

mals. Interaction between factors was significant for both GFAP immunostaining and protein levels ($p < 0.001$), indicating that zaprinast potentiates the effect of the cryolesion. These results suggest that zaprinast may accelerate the formation of the glial scar around the injured tissue. In unlesioned rat cortex, zaprinast induced a small but significant increase ($p < 0.05$) in GFAP immunoreactivity (Fig. 1a, b and g), indicating that cGMP can also regulate astroglial physiology under normal conditions.

Microglia/macrophages

Lectin staining reveals resting (ramified) and activated (round) microglia and infiltrating macrophages, as well as blood vessels. In vehicle-treated rats, a prominent recruitment and activation of microglia/macrophages (round/ameboid lectin-positive cells) was observed at the border of the lesion (Fig. 3c and g). In agreement with this, staining for the specific microglia/macrophage cytosolic protein Iba-1 (Fig. 3e and h) and levels of the microglia/macrophage surface marker CD11b

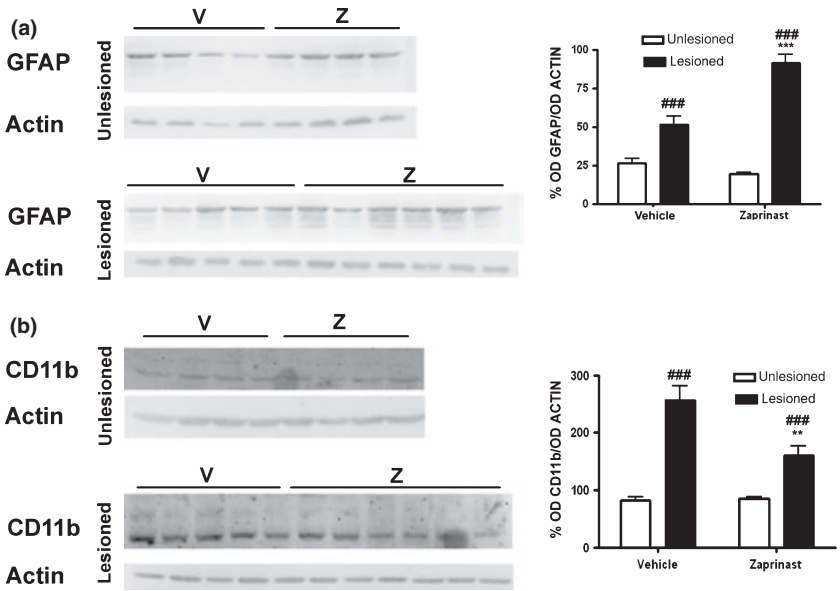

Fig. 2 Effect of zaprinast on glial fibrillary acidic protein (GFAP) and CD11b protein levels in cortex homogenates of unlesioned and cryolesioned rats. Homogenates (40 μg protein) from unlesioned ($n = 4$) or cryolesioned rat cortex ($n = 6$), treated with vehicle (V) or zaprinast (Z) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunobloted. GFAP (a) and CD11b (b) immunoreactive bands were analyzed by densitometry and following normalization to actin levels, values were expressed relative to one cryolesioned zaprinast-treated animal that was included in all blots. Statistically significant differences vs. unlesioned animals ($^{###}p < 0.001$) and vehicle-treated lesioned animals ($^{***}p < 0.001$ and $^{**}p < 0.01$). Two-way ANOVA shows significant interaction between lesion and treatment (GFAP: $p < 0.001$; CD11b: $p < 0.05$).

analyzed by western blot in homogenates (Fig. 2b) were significantly increased in the lesioned-cortex. In contrast to astrogliosis, a notable decrease in lectin-positive cells and in the intensity of lectin staining was observed in zaprinast-treated animals (Fig. 3d and g; $p < 0.05$). Furthermore, staining for Iba-1 revealed less activated (more ramified) microglia/macrophage morphologies in addition to decreased staining intensity (Fig 3f and h; $p < 0.01$). A significant decrease in CD11b levels in homogenates (Fig. 2b; $p < 0.01$) confirmed the decreased activation of these cells in zaprinast-treated animals. Interaction between factors was significant for lectin and Iba-1 staining and CD11b protein ($p < 0.05, < 0.001$ and < 0.05, respectively), which indicates that zaprinast reduces the effect of cryolesion. Zaprinast did not affect lectin (Fig. 3b and g) and Iba-1 staining (H) or CD11b levels (Fig. 2b) in unlesioned animals. These results are indicative of an anti-inflammatory action of the cGMP-PDE inhibitor.

Treatment with zaprinast decreases cryolesion-induced oxidative stress, apoptotic cell death and neuronal degeneration

Activated microglia/macrophages are known to produce reactive nitrogen and oxygen species that can induce oxidative stress and neural cell death. We investigated if zaprinast regulated this inflammatory response by measuring levels of proteins with carbonyl groups, a sign of oxidative stress, using a commercial detection kit (OxyBlot TM, Chemicon, Millipore Iberica). As shown in Fig. 4, at 3 dpl saline-treated rats showed increased levels of proteins with carbonyl groups. This effect was significantly decreased by zaprinast treatment ($p < 0.05$), in agreement with an anti-inflammatory action of the cGMP-PDE inhibitor.

We previously reported that a significant increase in the number of apoptotic cells is observed in the deepest border of the cryolesion reaching a peak at 3 dpl and decreasing thereafter (Penkowa *et al.* 1999; Giralt *et al.* 2002). We have evaluated the effect of zaprinast on apoptotic cell death by counting TUNEL-positive cells in an area distant at least 50 μm from the border of the lesion. Unlesioned animals showed very few TUNEL-positive cells whether treated or not with zaprinast (not shown). However, as shown en Fig. 5, left panels, numerous TUNEL-positive cells were observed in lesioned vehicle-treated animals and treatment with zaprinast notably decreased these numbers. We also examined if zaprinast was decreasing neuronal injury by analyzing the morphology of NeuN-stained neurons. In the cryolesioned cortex, numerous NeuN-labeled cells presented an abnormal morphology with shrunken and dystrophic cell bodies (Fig. 5, right panels). In contrast, the majority of neurons in the zaprinast-treated animals appeared normal. This result is consistent with the reduction in oxidative stress produced by the treatment with zaprinast and evidences a neuroprotective effect of the cGMP-PDE inhibitor in the brain cryoinjury model.

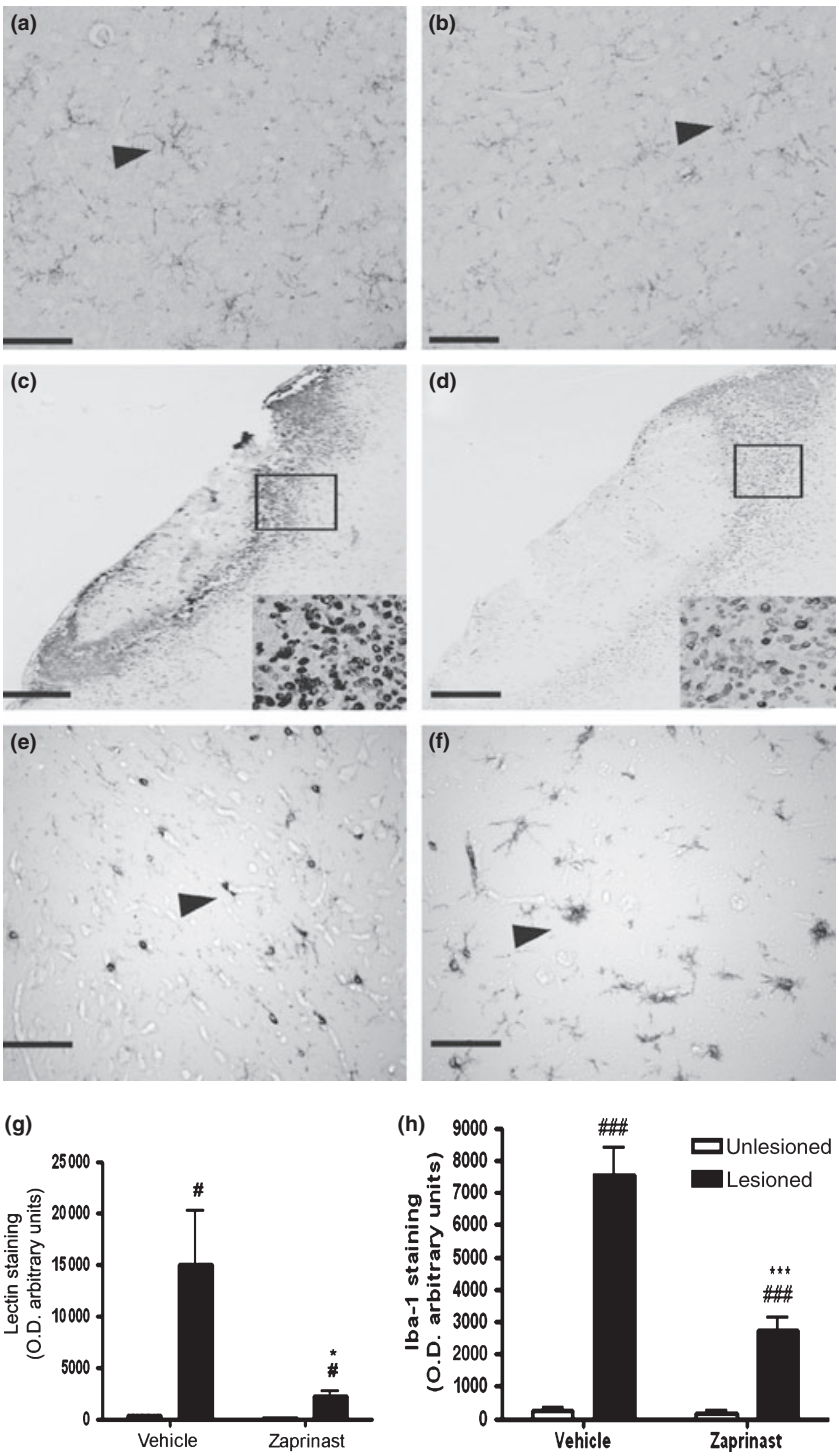

Fig. 3 Effect of zaprinast on lectin and Iba-1 staining in cortex slices of unlesioned and cryolesioned rats. Lectin stained ramified microglia similarly in vehicle-treated (a) and zaprinast-treated unlesioned animals (b, arrowheads); At 3 dpl, numerous lectin-positive microglia/macrophages were present around and inside the lesion (c) and treatment with zaprinast greatly decreased the intensity of lectin staining around the lesioned area (d); inserts in (c) and (d) show higher magnification of the squares showing rounded activated microglia/macrophages at the lesion area. (e) Staining for the specific microglia/macrophage marker Iba-1 in vehicle-treated and (f) zaprinast-treated cryolesioned cortex. Zaprinast promoted a less activated cell morphology (arrowheads in e, f) and a significant decrease in staining intensities (h). Lectin and Iba-1 staining intensities were quantified with the Scion Image program in 4–6 areas of 0.5 mm^2 per brain chosen at random in unlesioned animals ($n = 4$) or from the border of the lesion (excluding the edges) in cryolesioned animals ($n = 4$–5) (g, h). Statistically significant differences vs. unlesioned animals ($^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$) and vs. vehicle-treated lesioned animals ($^{*}p < 0.05$, $^{***}p < 0.001$). Two-way ANOVA shows significant interaction between lesion and treatment (lectin: $p < 0.05$; Iba-1: $p < 0.001$). Bars: (a, b, e, f) 30 μm; (c, d) 400 μm.

Zaprinast significantly increases the number of blood vessels and enhances VEGF expression in the lesioned cortex

Observation of lectin-stained sections revealed increased blood vessel-staining in zaprinast-treated animals. Quantification of clearly visible lectin-stained vessels in random areas at least 50 μm away from the lesion confirmed that zaprinast significantly increased their numbers by around twofold in the lesioned cortex (Fig. 6, left panels). A similar increase was observed when vessels were counted in hematoxylin–eosin-stained sections (Fig. 6, center panels). This finding is in agreement with the well-

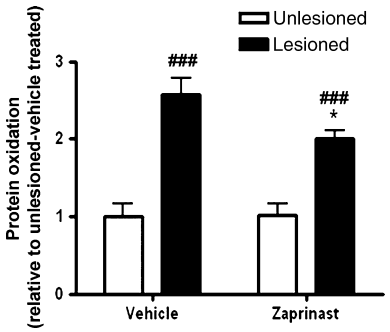

Fig. 4 Effect of zaprinast on protein oxidative stress in unlesioned and cryolesioned animals. Carbonyl groups in proteins were detected on western blots of cortex homogenates (20 μg protein) with the OxyBlot TM kit (Chemicon). Unlesioned animals ($n = 4$) showed very low levels of oxidated proteins whereas lesioned rats ($n = 6$) presented two- to threefold higher levels. Zaprinast treatment significantly reduced protein oxidation. Results are presented relative to vehicle-treated unlesioned animals and are means ± SEM of three replicate experiments. Statistically significant differences vs. unlesioned animals ($^{###}p < 0.001$) and vs. vehicle-treated lesioned animals ($^{*}p < 0.05$). Two-way ANOVA shows significant interaction between lesion and treatment ($p < 0.05$).

documented angiogenic action of cGMP-enhancing compounds in peripheral tissues *in vitro* and *in vivo* (Ziche and Morbidelli 2000; Pyriochou *et al.* 2006), and also demonstrated in rat CNS after stroke (Zhang *et al.* 2003).

Findings in different models of CNS pathologies show that VEGF plays a key role in the angiogenic response to injury and exerts neuroprotective and neurotrophic effects (Zachary 2005). The NO-cGMP pathway has been implicated in VEGF increased expression and angiogenesis induced by cerebral ischemia (Zhang *et al.* 2003). To investigate if zaprinast treatment increased VEGF levels in the cryolesioned cortex, we examined VEGF immunostaining. No significant staining was observed in unlesioned cortex (not shown). However, VEGF staining was intense in reactive astrocyte-like cells in the parenchyma as well as surrounding blood vessels in the cryolesioned cortex and staining levels were significantly increased by the zaprinast treatment (Fig 6, right panels). These results suggest that increased angiogenesis in zaprinast-treated animals may be mediated by cGMP-induced VEGF expression in astrocytes.

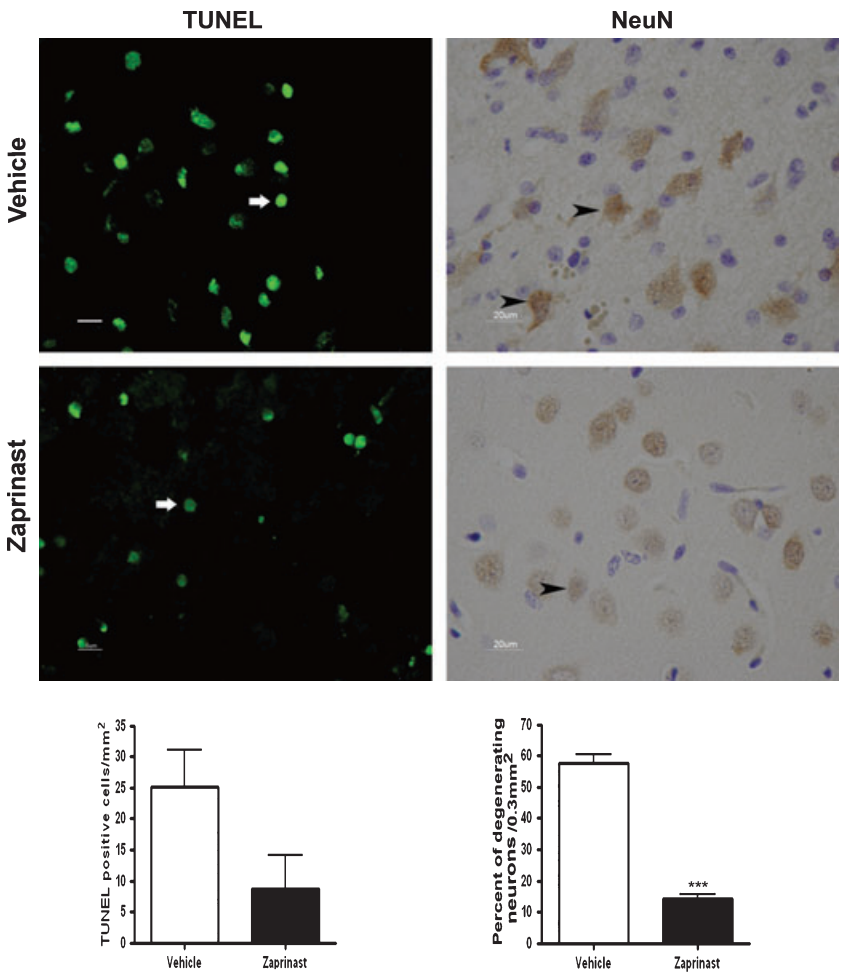

Fig. 5 Zaprinast decreases TUNEL-positive cells and attenuates neuronal injury in the cryolesioned cortex. (Left) TUNEL-positive cells labeled using the *In Situ Cell Death Detection Kit* (Roche); (Right) Neurons stained with the neuronal nuclei marker NeuN. TUNEL-positive cells (cells with nuclear staining, arrows) and injured neurons (condensed or dystrophic cell bodies, arrowheads) were quantified in 5–7 areas/brain section at least 50 μm away from the necrotic area ($n = 5$). Zaprinast treatment reduced the number of TUNEL-positive cells ($p = 0.058$) and of NeuN-labeled degenerating neurons ($^{**}p < 0.01$) in the injured cortex. Bars in TUNEL 5 μm, in NeuN 20 μm.

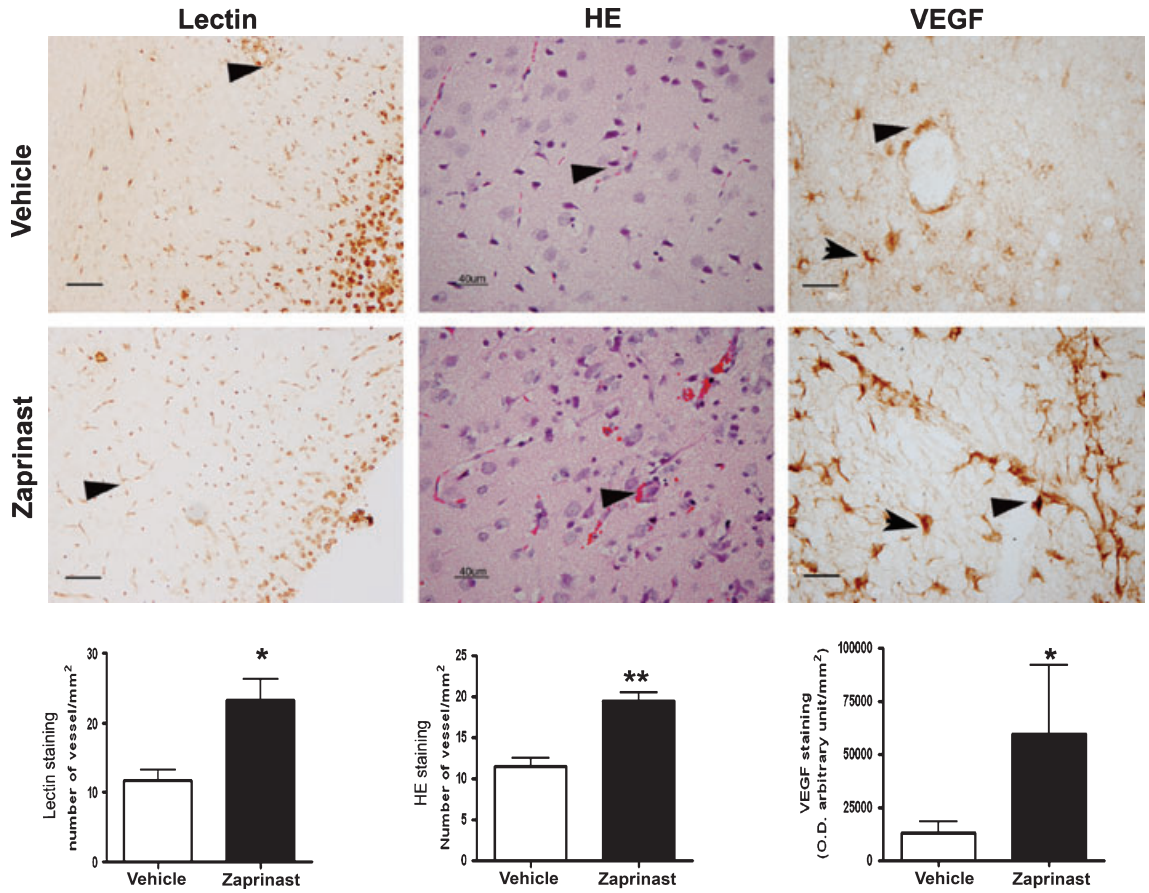

Fig. 6 Zaprinast increases vascularisation and vascular endothelial growth factor (VEGF) staining in the cryolesioned cortex. Vessels labeled with lectin (left) or hematoxylin–eosin (HE) (center) were quantified in 5–7 areas per brain section at least 50 μm away from the necrotic area ($n = 5$). (Right) Immunoreactivity for VEGF is observed in reactive astrocyte-like cells in the parenchyma (arrow) and vessels (arrowhead); VEGF staining was quantified with the Scion Image program in five 0.5 mm^2 areas per brain at least 50 μm from the necrotic area ($n = 4$). Zaprinast treatment significantly increased the number of blood vessels and the expression of VEGF. Statistically significant difference vs. vehicle-treated lesioned animals (**$p < 0.01$; *$p < 0.05$). Bar: 40 μm.

Discussion

In this work, we demonstrate for the first time that peripheral administration of the cGMP-PDE inhibitor zaprinast dramatically alters the inflammatory response of astrocytes and microglia/macrophages to focal brain injury, decreases oxidative stress and neurodegeneration and increases angiogenesis and VEGF expression. Because information about cGMP-PDE isoform expression in glial cells *in situ* is lacking, we used the PDE-inhibitor zaprinast that inhibits the specific cGMP-PDEs PDE5 and PDE9, as well as dual substrate PDE1 variants (Bender and Beavo 2006a; and references therein). PDE1 activity has been shown to regulate cGMP in astrocytes and macrophages in culture (Agullo and Garcia 1997; Bender and Beavo 2006b). A number of studies have shown positive effects of peripherally administered zaprinast and other more specific PDE5 inhibitors in different tests of cognition in unimpaired and impaired rodents (Puzzo *et al.* 2009; Reneerkens *et al.* 2009). Additionally, PDE5 inhibitors have been reported to increase functional recovery after stroke in rats (Zhang *et al.* 2005, 2006). Results shown here suggest a further beneficial effect of cGMP-PDE inhibition in brain injury through regulation of glial inflammatory responses.

The experimental model of cryolesion to the parietal cortex used in this study has been successfully used by us for several years to examine the role of metallothioneins and inflammatory cytokines in the CNS response to traumatic brain injury (Chung *et al.* 2008). In this model, activation and recruitment of microglia/macrophages and reactive astrogliosis occurs in a highly temporal-specific manner. Round or ameboid microglia/macrophages appear in the injured area 1 dpl and reach a maximum by 3 dpl making a line of demarcation around the lesion. This response precedes that of astrocytes but numerous reactive astrocytes are also present around the lesion at 3 dpl. Gliosis progressively

returns to normal levels and by 20 dpl few signs of glial reactivity are obvious (Penkowa *et al.* 1999; Giralt *et al.* 2002). In this work, we show that treatment with zaprinast greatly potentiates reactive astrogliosis as evidenced by increased immunoreactivity for GFAP around the lesion and increased GFAP protein levels in homogenates of the lesioned cortex. Thus, as previously demonstrated by us and others for other conditions that increase astrogliosis around the lesion (Giralt *et al.* 2002; Molinero *et al.* 2003; Klementiev *et al.* 2008), zaprinast is expected to accelerate the formation of the glial scar and the regeneration of the lesioned tissue. After brain injury, NO-dependent cGMP formation can occur in astrocytes as a result of NOS2 induction in activated glial cells and recruited macrophages (Murphy 2000). In addition, cGMP can be formed in astrocytes by activation of NPRs (Deschepper and Picard 1994; de Vente and Steinbusch 2000). As reported in rat brain slices, zaprinast can increase astroglial cGMP independent of the stimulus for its formation (van Staveren *et al.* 2001). Interestingly, the content of atrial natriuretic peptide (ANP) has been reported to increase in reactive glial cells surrounding experimental brain infarction (Nogami *et al.* 2001) and up-regulation of the ANP-cGMP pathway has been suggested to contribute to cortical spreading depression-induced protection against ischemic insult (Wiggins *et al.* 2003). We have recently shown that increasing cGMP levels in cultured rat astrocytes by stimulation with NO or ANP leads to dramatic changes in the GFAP and actin cytoskeleton that result in accelerated closure of a scratch wound (Boran and Garcia 2007). In addition, the cGMP-protein kinase G pathway has been implicated in NO-induced increase in GFAP expression in mouse astrocytes (Brahmachari *et al.* 2006). Thus, potenciation of cGMP effects by zaprinast inhibiton of cGMP-PDE in astrocytes is most probably responsible for the increased astrogliosis observed around the cryolesion in zaprinast-treated animals.

Another important finding of this study is that zaprinast treatment notably reduces the recruitment and activation of microglia/macrophages caused by the lesion as manifested by the significant decrease in lectin-stained round/ameboid cells and of specific markers for microglia/macrophage activation Iba-1 and CD11b in the lesioned cortex. Overproduction of nitrogen and oxygen free radicals by activated microglia is thought to be responsible for oxidative stress and to greatly contribute to cell death, in particular of neurons, in acute tissue damage as well as in chronic neurodegenerative diseases (Block *et al.* 2007). Thus, the reduction in protein oxidative stress, apoptotic cell death and neuronal degeneration observed in zaprinast-treated animals most probably results from decreased activation of microglia/macrophages. Available data indicate that rat brain microglia do not express functional NO-GC and are thus unable to form cGMP in response to NO (Teunissen *et al.* 2000; van Staveren *et al.* 2005; Pifarre *et al.* 2009). However, rat brain microglia in culture has been reported to express mRNA for NPR-A and NPR-B (Moriyama *et al.* 2006) and expression of PDE1B has been shown to increase during monocyte to macrophage differentiation and to play a major role in regulating cGMP levels in these cells (Bender and Beavo 2006b). Thus, it is possible that potentiation by zaprinast of NP-induced cGMP formation in microglial/macrophages is involved in decreased cell activation. An anti-inflammatory role of cGMP formed in response to ANP has been well documented in macrophages (Vollmar 2005), and ANP inhibition of LPS-induced NO secretion and IL-1β expression has been reported in rat microglial cultures (Moriyama *et al.* 2006). Also in agreement with an anti-inflammatory role for cGMP in microglia, cell-permeable cGMP analogs were reported to inhibit β-amyloid-induced release of leukotriene B4 (Paris *et al.* 1999) and LPS-induced secretion of tumour necrosis factor-α (Paris *et al.* 2000) in a murine microglial cell line (N9). However, other works implicated cGMP in NO-induced pro-inflammatory responses in cultured rodent microglia (Choi *et al.* 2002; Roy *et al.* 2006). The reason for this discrepancy is difficult to ascertain. In any case, we cannot rule out with the present results that the anti-inflammatory effect of zaprinast in the cryoinjured cortex is secondary to the neuroprotective effect. A large body of evidence indicates that cGMP activates anti-apoptotic pathways in neural cells (Kang *et al.* 2004). Additionally, the zaprinast-increased vascularisation will also contribute to neuronal survival. Numerous studies have demonstrated that endogenous NO and NO-releasing activators of NO-GC enhance endothelial cell migration, growth and organization into capillary-like structures *in vitro*, as well as angiogenesis *in vivo* (Ziche and Morbidelli 2000; Zhang *et al.* 2003; Pyriochou *et al.* 2006). In the cryoinjured cortex, zaprinast could enhance angiogenesis through a direct effect on endothelial cell cGMP levels and also by modulating the behavior of supporting cells such as astrocytes. The angiogenic cytokine VEGF is up-regulated in reactive astrocytes following CNS injury (Zachary 2005) and, as shown here, zaprinast treatment further increases VEGF expression in reactive astrocytes in the brain parenchyma and surrounding blood vessels.

In conclusion, our results support the notion that regulation of glial inflammatory responses by cGMP may be part of the mechanisms underlying the neuroprotective effects of cGMP-PDE inhibitors reported in animal models of acute and chronic CNS disease.

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Glial cells as sources and targets of natriuretic peptides

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ABSTRACT

Natriuretic peptides and their receptors are widely expressed in mammalian CNS and increasing evidence implicates them in the regulation of neural development, synaptic transmission and processing of information, and neuroprotection. Although the peptides have been mainly localized in neuronal populations they are also produced in glial cells. Astroglia and microglia also express functional natriuretic peptide receptors that can regulate important physiological responses. In this article we review evidence on the localization of natriuretic peptides and their receptors in astroglial and microglial cells and summarize data supporting the participation of this signalling system in neuron-glia and glia-brain blood vessel communication relevant to CNS function.

1. Introduction

Natriuretic peptides (NPs) form a family of structurally related peptides derived from separate genes. The three major members of this family in mammals are atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). ANP was the first NP to be identified in rat atrial extracts as a potent blood pressure lowering component (de Bold et al., 1981). BNP and CNP were first isolated from porcine brain (Sudoh et al., 1988, 1990) but BNP was later found at much higher concentrations in cardiac tissues (Mukoyama et al., 1991). Both ANP and BNP elicit vascular, renal and endocrine effects directed towards regulation of vascular tone, sodium and water balance and other cardiovascular functions. On the other hand, CNP is largely expressed in the CNS, vascular endothelial cells, and chondrocytes and does not seem to function as a cardiac hormone. However, disruption of the murine CNP gene results in severe dwarfism. NPs exert their actions through binding to three types of membrane receptors, namely NP receptor A (NPR-A), NP receptor B (NPR-B) and NP receptor C (NPR-C). NPR-A, that binds both ANP and BNP, and NPR-B that is preferentially stimulated by CNP, have guanylyl cyclase (GC) activity and are also named GC-A and GC-B. Their stimulation increases intracellular cGMP that mediates most of the known effects of NPs. NPR-C lacks GC activity and acts as a clearance receptor for all three NPs controlling their local concentration by receptor-mediated internalization and degradation, although a signalling function for this receptor has been also demonstrated. All three receptor types are widely distributed in a variety of tissues, including the CNS. The reader is referred to excellent recent reviews (Cao and Yang, 2008; Kuhn, 2009; Pandey, 2008; Potter et al., 2006, 2009).

2. Natriuretic peptides and natriuretic peptide receptors: structure and signalling

ANP, BNP and CNP are synthesized as preprohormones that after elimination of a signal sequence and further proteolytic cleavage give rise to the biologically active peptides. The 28 amino acid sequence of mature ANP is well conserved across mammalian species with rodents and humans differing in only one amino. PreproBNP sequences in mammals are more variable across species and proteolytic cleavage gives rise to circulating BNP molecules of 32 amino acids in length in humans and pigs and 45 amino acids in rodents. Two mature CNP molecules of 53 and 22 amino acids have been also identified. The longer peptide is the predominant form in the CNS. All three mature NPs have a 17-amino-acid conserved sequence flanked by two cysteines that form a disulfide-linked ring essential for biological activity (Potter et al., 2006; Rosenzweig and Seidman, 1991).

The three NP receptors (NPRs) contain a relatively large (about 450 amino acids in length) extracellular ligand binding domain and a single membrane-spanning region. NPR-A and NPR-B contain three intramolecular disulfide bonds and five N-linked glycosylation sites in their extracellular domain, an equally large and highly conserved intracellular domain consisting of a kinase-homology domain (KHD) that resides close to the transmembrane region, an amphipathic α-helical hinge region and a guanylyl cyclase catalytic domain located in a 250-amino-acid region of the

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carboxyl-terminus portion (Misono et al., 2005; Potter et al., 2006). NPR-A exists as a homodimer or homotetramer in its native state and oligomerization is ligand-independent. However, ligand binding with a stoichiometry of 2:1, appears to induce a rotation of the juxtamembrane domains that brings the single GC domains of each monomer to an optimal proximity and orientation for GC catalytic activity (Misono et al., 2005). The function of the KHD is not well known. Although it binds ATP, no kinase activity has been detected but it appears to modulate the activity of the GC domain. Under basal conditions NPR-A is phosphorylated in four serine and two threonine residues within the N-terminal portion of the KHD. Studies on NPR-A overexpressing cells showed that phosphorylation is essential for its activation and that dephosphorylation is involved in homologous desensitization by prolonged exposure to NPs, as well as in heterologous desensitization by exposure to angiotensin II, endothelin and growth factors. However, the kinase(s) and phosphatase(s) involved in phosphorylation and dephosphorylation of NPR-A are largely unknown (Potter et al., 2006). Signalling mediated by NPR-A and NPR-B involves synthesis of cGMP as an intracellular signaling molecule which, in the same way as cGMP generated by the NO/soluble GC system, will modulate the activity of specific target proteins, such as phosphodiesterases (PDEs 2, 3 and 5), cyclic nucleotide regulated ion channels, and cGMP-dependent protein kinases (cGK I and II) (Lucas et al., 2000). However, recent evidences in cells expressing both NO- and NP-sensitive GCs show significant differences in the spatio-temporal distribution and signalling of the cGMP produced by these GCs (Fischmeister et al., 2006)

In contrast to NPR-A and NPR-B, NPR-C is a disulfide-linked dimer, has a short cytoplasmic tail of 37 amino acids and lacks a GC domain. It binds ANP, BNP and CNP with an affinity similar to NPR-A and NPR-B and with a stoichiometry of one NP molecule per dimer (Potter et al., 2006; Rose and Giles, 2008). NPR-C is widely distributed in tissues and cell types and its main function appears to be removal of NPs from the circulation modulating their availability at target organs. The increased half-life of ANP observed in the circulation of transgenic mice lacking NPR-C gave strong support for this "clearance" function (Matsukawa et al., 1999). In addition, there is solid evidence that NPs via NPR-C can inhibit adenylyl cyclase and its down-stream signalling molecules in different tissues through a pertussis toxin-sensitive inhibitory guanine nucleotide regulatory protein (Gi) (Anand-Srivastava, 2005). Functional Gi-activator sequences similar to those identified in the insulin-like growth factor receptor were found in the 37 amino acid cytoplasmic domain of NPR-C (Murthy and Makhlouf, 1999; Pagano and Anand-Srivastava, 2001). The Gi proteins activated by NPR-C can also activate the phospholipase C β isoform (PLCβ). Whereas the inhibition of adenylyl cyclase is mediated by the Gi α subunit, the activation of PLCβ is exerted by the βγ subunits (Murthy et al., 2000; Pagano and Anand-Srivastava, 2001). Both signalling pathways have been shown to regulate ionic currents in heart and vascular cells. In cardiac myocytes, reduction in intracellular cAMP seems to be implicated in NPR-C mediated selective inhibition of L-type Ca^{2+} currents, while in cardiac fibroblasts NPR-C agonists activated a non-selective cation current mediated by transient receptor potential (TRP) channels through PLC activation. In addition, in vascular smooth muscle cells binding of CNP to NPR-C can cause hyperpolarization and elicit a relaxation through activation by Gi βγ subunits of an inwardly rectifying K^+ channel of the GIRK type (Rose and Giles, 2008).

3. Expression and function of natriuretic peptides and their receptors in the CNS

ANP binding sites were discovered in the brain nearly 25 years ago (Quirion et al., 1984), soon after the peptide was identified in rat atrial myocardial extracts. Since the discovery, widespread expression of all three types of NPs and their receptors has been shown by a variety of methods in discrete loci in rodent and human CNS. NPs present a predominant neuronal localization and their highest concentrations are found in the hypothalamus. Significant concentrations are also found in various telencephalic areas, cerebellar cortex, spinal cord and retina. In general, CNP is more highly expressed, and both BNP and CNP are more widely distributed than ANP, in CNS structures (reviewed in Cao and Yang, 2008). In contrast to NPs, different lines of evidence show that NPRs are mainly localized in astroglial cells in several brain regions (see below) indicating the involvement of these cells in the physiological actions of NPs in the CNS. Not much is known about the role of NPs in brain tissue but accumulating evidence indicates possible regulatory roles of NPs in neural development, synaptic transmission and processing of information, and neuroprotection.

Various studies reveal widespread but discrete patterns of NP and NPR gene expression in the early embryonic rat CNS, suggesting region- and stage-specific roles for NPs during CNS development (reviewed in Cao and Yang, 2008; Waschek, 2004). Functionally, studies on embryonic mice with an inactive NPR-B or deficient for PKG I have provided evidence that NP-GC-cGMP signalling controls axonal bifurcation and pathfinding at the dorsal root entry zone (Schmidt et al., 2007). Furthermore, addition of CNP to neurotrophin-treated olfactory neuronal precursor cells results in inhibition of proliferation and promotion of maturation (Simpson et al., 2002). During postnatal development transient expression of ANP and CNP has been observed in various regions of the rat brain further supporting NP functions in CNS developmental processes. NP precursor mRNA levels increased markedly in most brain regions during the 2 first postnatal weeks and decreased to adult levels by postnatal day 28 suggesting that NPs may regulate synaptogenesis or gliogenesis (Ryan and Gundlach, 1998). A recent study using receptor affinity labelling and GC assays to characterize comparatively NPR-A and NPR-B expression during development showed that whereas NPR-A levels increase between embryonal day 18 and adult, NPR-B expression in brain is highest and widely distributed around postnatal day 1. Interestingly, the perinatal NPR-B peak coincides with elevated expression of nestin, a neural stem/progenitor cell marker (Muller et al., 2009) supporting the implication of the CNP–NPR-B–cGMP pathway in the regulation of neural progenitor cell proliferation and/or maturation.

Evidence for a modulatory role of NPs in synaptic transmission and processing of information comes from a series of reports demonstrating that NPs regulate the release and uptake of noradrenaline and dopamine in different brain preparations, and the electrical properties of neurons in the hypothalamus, spinal cord and retina (reviewed in Cao and Yang, 2008). Furthermore, recent reports demonstrate that application of CNP to acute hippocampal slices decreases the population spike amplitude after high frequency stimulation, thus affecting long term potentiation (LTP), presumably by modifying the distribution of pre- and postsynaptic proteins (Decker et al., 2008). The same group has shown that CNP through NPR-B significantly decreases hippocampal network oscillations that are related to short- and long-term memory (Decker et al., 2009). These observations are in line with previous reports showing that in vivo CNP influences emotional behavior, such as anxiety and arousal (Wiedemann et al., 2000) as well as learning and memory processes (Telegdy et al., 1999). As previously described for the NO–GC–cGMP system, recent evidence indicates that NPs via cGMP–cGK are also involved in nociceptive processing. NPR-B and cGKI have been shown to colocalize in dorsal root ganglion cells and a cGKI inhibitor to antagonize the pronociceptive effects of the NPR-B ligand CNP (Schmidtko et al., 2008).

Evidence for neuroprotective actions of ANP and BNP mediated by cGMP and cGK have been obtained in different neuronal cell lines (Fiscus et al., 2002) and in cultured retinal neurons (Kuribayashi et al., 2006). In vivo, a prolonged increase in cortical ANP expression has been reported to occur after an acute episode of cortical spreading depression (CSD) (Wiggins et al., 2003). Increased cortical levels of cGMP have been also reported after CSD (Read et al., 2001) suggesting that ANP via cGMP may contribute to the neuroprotection afforded by CSD preconditioning against a subsequent ischaemic insult. A role for ANP in regulation of cerebral blood flow (CBF) in the infarcted area is supported by its increased content in reactive glial cells surrounding experimental brain infarction (Nogami et al., 2001). Increased cGMP immunoreactivity after stimulation with ANP was found in brain blood vessels in acute rat cortical slices (de Vente et al., 1989) and increased permeability of brain capillaries in response to ANP via cGMP has been observed in vitro (Grammas et al., 1991; Sarker and Fraser, 2002). Paradoxically, ANP attenuated sodium and water accumulation, as well as brain edema in rat models of cerebral ischemia (Naruse et al., 1991) and intracerebral hemorrhage (Rosenberg and Estrada, 1995). In addition to effects on CBF, the beneficial effects of ANP in ischemic injury may result from its ability to inhibit activation of infiltrated inflammatory cells (Vollmar, 2005) as well as resident microglia (see below). Recently, BNP administered by tail vein injection was reported to improve neurological function after traumatic brain injury in mice and this effect was associated to increased cerebral blood flow and decreased microglial activation (James et al., 2010).

4. Expression of natriuretic peptides and their receptors in glial cells

Although NPs have been localized predominantly in neuronal perikarya and fibers throughout the brain, NP expression has been also detected in astroglial cells. McKenzie (1992) first documented the presence of ANP-immunoreactive astrocytes throughout the canine brain. The same group later described the distribution of ANP-immunoreactivity in astroglial cells in human cerebral and cerebellar cortices. Strong immunostaining was observed in protoplasmic astrocytes in all layers of the cingulate and striate cerebral cortex gray matter, in astrocytes within and immediately subjacent to the glia limitans and in fibrous astrocytes in the white matter (McKenzie et al., 1994). In the cerebellar cortices, immunoreactive astrocytes with many short, thick processes were distributed sparsely throughout the granular layer with processes surrounding granule cells and parenchimal blood vessels. Fibrous astrocytes in the white matter were also stained but the most numerous subpopulation of ANP-immunoreactive astrocytes in human cerebellum was identified as Bergmann glia (McKenzie et al., 2001). Immunocytochemical techniques also allowed detection of ANP, BNP and CNP in human and rat retina astroglial cells (Cao et al., 2004; Rollin et al., 2004). In the rat retina, immunoreactivity for BNP was strong in the main trunks and major processes of Müller cells but hardly detectable in the endfeet. The expression profile for ANP was similar, but with a much lower level. On the contrary, the endfeet and major processes in the inner retina were strongly CNP-positive suggesting that this peptide may be involved in glia-ganglion cell communication. Moreover, CNP-positive Müller cell endfeet were observed closely enwrapping blood vessels suggesting a participation in the regulation of blood flow and intraocular pressure (Fernandez-Durango et al., 1999).

Release of ANP from cultured rat brain astrocytes has been described to occur by calcium-dependent exocytosis (Krzan et al., 2003), suggesting that calcium increases induced in astrocytes as a result of neuronal activity could regulate NP release. Vesicles containing ANP also appear to contain ATP (Pangrsic et al., 2007). Expression of CNP was also demonstrated in cultured mouse astrocytes (Yeung et al., 1996) and the neurotrophic peptide pituitary adenylate cyclase-activating polypeptide (PACAP) was reported to increase its expression (Fujikawa et al., 2006).

The first evidence of expression of functional NPRs in astrocytes was obtained by Friedl et al. (1989, 1985) using primary cultures from rat forebrain. These authors reported that ANP increased cGMP levels in a time- and concentration-dependent manner in astrocyte-enriched cultures but not in neuronal cultures. Some years later, BNP and CNP were shown to stimulate cGMP formation in rodent brain astrocytes, and CNP appeared to be the most potent NP (Kobayashi et al., 1993; Yeung et al., 1992, 1991). This was confirmed by Deschepper and Picard (1994) who additionally showed that astrocytes from diencephalon accumulated more cGMP in response to NPs than astrocytes from cortex in agreement with regional differences described in brain (Quirion, 1989). Differential expression of NPR-A and NPR-B mRNA was observed in astrocytes and neurons from rat hypothalamus and brain stem, with astrocytes expressing predominantly the NPR-A subtype and neurons the NPR-B subtype (Sumners and Tang, 1992). However, information on the distribution of the different NPRs in different astrocyte populations in mammalian brain in situ is scarce.

Using cGMP immunocytochemistry in rat brain slices it has been well documented that the majority of the ANP-responding cells in the rat brain are astroglial cells supporting a predominant localization of NPR-A receptors in these cells (de Vente et al., 1990, 1989; Markerink-Van Ittersum et al., 1997). In agreement with this, Goncalves et al. (1995) demonstrated that ANP-induced cGMP accumulation in slices from rat brain diencephalon was totally blocked by the gliotoxic substance fluorocitrate whereas the response to a NO donor and to CNP were only partially affected. ANP-responding astrocytes have been observed in discrete regions of the brain including the median preoptic area, the olfactory bulb, the hippocampus, the lateral septum, the medial amygdala and the spinal cord. Not all astrocytes in these areas were responsive to ANP demonstrating cell heterogeneity (de Vente et al., 1990; Markerink-Van Ittersum et al., 1997). In some cases it was clearly demonstrated that the same asctrocytes express both NO- and ANP-sensitive GCs (Teunissen et al., 2001). In the immature cerebelum intense cGMP immunostaining was reported in Bergmann cell fibres in the molecular layer and Bergmann cell bodies in the Purkinge cell layer, and astrocytes in the inner granule cell layer and in the white matter (de Vente et al., 1990).

Independent of cGMP formation, ANP and BNP exert antiproliferative effects in astrocytes by binding to NPR-C (Levin and Frank, 1991; Sumners et al., 1994). Using diencephalic astrocyte cultures Levin's group demonstrated that ANP antagonizes positive effects of the glial mitogen endothelin-3 on the transcription of the immediate-early genes egr-1 and Tis 8 and transactivation of basic fibroblast growth factor (bFGF) (Biesiada et al., 1996; Hu and Levin, 1994). ANP inhibition of mitogen-activated protein kinase appears to be involved in these effects (Prins et al., 1996).

Using spheroid cultures from rat brain, Teunissen et al. (2000) observed that, in addition to astrocytes, cells labelled with a microglial marker presented cGMP immunoreactivity after stimulation with ANP but not with a NO donor. In agreement with this, we have observed that ANP but not sodium nitroprusside (SNP), stimulates cGMP formation in microglia co-cultured with astrocytes, as well as in isolated microglia from rat cortex and cerebellum (Fig. 1A and B). Intracellular accumulation of the nucleotide was increased in microglia in the presence of the non-specific PDE inhibitor IBMX, as well as the more selective cGMP-PDE5 inhibitors zaprinast and sildenafil, indicating that these cells express cGMP-degrading PDEs. Expression of mRNAs for NPR-A and NPR-B as well as ANP was reported in rat brain microglia in culture (Moriyama et al., 2006) suggesting that ANP may be an

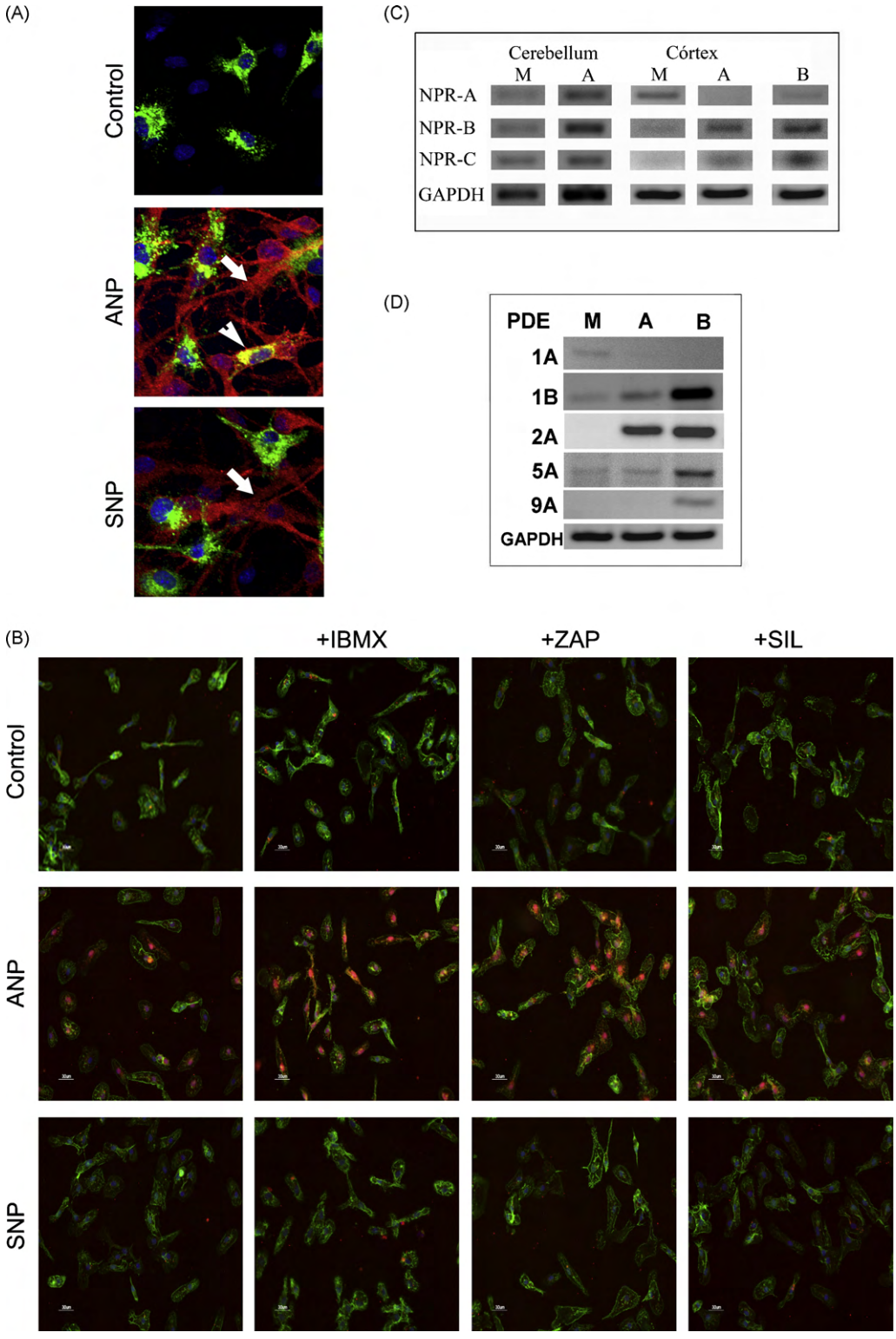

Fig. 1. Cyclic GMP formation and expression of NPRs and PDEs in cultured glial cells. (A) Mixed astroglia-microglia primary cultures from rat cerebellum were treated for 3 min with vehicle (control), the NO donor SNP (100 μM) or with ANP (1 μM) in the presence of 1 mM IBMX. Cells were immunostained for cGMP (red), the microglial marker Alexa 488-Ac-LDL (green) and nuclei (DAPI, blue) and observed by confocal microscopy. Note that cGMP immunostaining is increased in astrocytes (arrows) in response to both SNP and ANP, but only in response to ANP in microglia (arrowheads). (B) Primary cultures of rat cortical microglia were treated with SNP (100 μM, 10 min) or with ANP (1 μM, 30 min) in the absence or presence of the non-specific PDE inhibitor IBMX (1 mM) or the cGMP-PDE inhibitors zaprinast (ZAP; 100 μM) or sildenafil (SIL; 100 nM). Cells were immunostained for cGMP (red), F-actin (BODIPY FL-phallacidin, green) and nuclei (DAPI, blue). As in cerebellar microglia, ANP but no SNP stimulated cGMP formation in cortical microglia and the effect was enhanced by cGMP-PDE inhibitors. (C) Expression of NPR mRNAs in cortical and cerebellar cells and (B) PDE mRNAs in cortical cells was analysed by RT-PCR. Microglia (M), astrocytes (A) and 1-day-old rat brain (B). GAPDH was used as control for differences in cDNA synthesis efficiency. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

autocrine regulator in these cells. We have confirmed expression of mRNAs for NPR-A and NPR-B in rat microglia from cortex and cerebellum and also detected NPR-C in microglia from cerebellum (Fig. 1C). Notably, in cortical microglia NPR-A appears to be the predominant isoform, in contrast to astrocytes where NPR-B is more highly expressed. However, in cerebellum both receptors types are more abundantly expressed in astrocytes. We have also detected expression of cGMP-specific PDE isoform mRNAs in cortical cultures of microglia and astrocytes (Fig. 1D). Both cell types express mRNA for the cGMP-specific PDE5A but not for PDE9A which is ubiquitously expressed in neuronal populations (van Staveren et al., 2002). Microglia also expresses dual-substrate PDE1A and PDE1B, isoforms that present higher affinity for cGMP than for cAMP (Bender and Beavo, 2006a). Interestingly, expression of PDE1B has been shown to increase during monocyte to macrophage differentiation and to play a major in the regulation of cGMP levels in these cells (Bender and Beavo, 2006b). PDE1B was also detected in astrocytes that in addition presented strong expression of PDE2A another dual-substrate PDE whose inhibition was previously shown to increase cGMP in astrocytes in rat hippocampal slices (van Staveren et al., 2001). Thus, in addition to astrocytes, microglia has the enzymatic machinery to synthesize and degrade cGMP.

5. NPs in glial cell physiology

Despite the numerous data accumulated during the last 20 years on the expression and release of NPs in glial cells and the glial localization of functional NP receptors, solid evidence about specific roles of the glial NP–NPR system in the regulation of specific CNS functions is lacking. However, circumstantial evidence underlines particular areas of CNS physiology where autocrine or paracrine actions of NPs in glial cells could be relevant, namely, neuron-glia communication, regulation of cerebral blood flow and water balance and neuroinflamation.

Using cultured astrocytes we showed that ANP via cGMP and cGK induces a rapid and reversible reorganization of glial fibrillary acidic protein (GFAP) and actin filaments that results in a dramatic change in cell morphology involving soma retraction and process elongation and branching (Boran and Garcia, 2007). Since astrocyte processes contact neuronal membranes as well as blood vessels, enhancement of astrocyte process formation may have consequences for neuronal and vascular function in the CNS. At the synaptic level astrocyte processes contact presynaptic and postsynaptic elements in a structure defined as the tripartite synapse (Araque et al., 2001). One of the important functions of this astrocyte–synaptic relation is the clearance of transmitters, in particular glutamate. Astrocytes express high densities of plasma-membrane transporters for glutamate that once taken up is converted into glutamine that is shuttled back to the neuron to be reused as a source of the transmitter. Additionally, astrocytes can release a variety of gliotransmitters into the extracellular space by different mechanisms. Calcium-dependent exocytotic mechanisms have been described for release of glutamate, D-serine, ATP and peptides such as brain-derived neurotrophic factor and ANP (Parpura and Zorec, 2009). Processes of the same astrocytes that contact synaptic membranes can form endfeet around blood vessels. Thus, calcium increases triggered by neuronal activity in astrocytes followed by exocytotic release of ANP can increase the permeability of brain capillaries by acting on NPRs in endothelial cells (Grammas et al., 1991; Sarker and Fraser, 2002). Through this action astroglial-derived ANP can regulate blood–brain fluid exchange and provide metabolic support for neuronal activity.

In cultured astrocytes, in addition to regulation of astrocyte stellation via cGMP, ANP, by binding to NPR-C, inhibits cell proliferation induced by growth factors (Levin and Frank, 1991; Sumners et al., 1994). These two actions of ANP may be relevant to astrocyte differentiation and brain development. Interestingly, NPR-A levels increase postnatally during a period of active gliogenesis (Muller et al., 2009). Alternatively, ANP may be involved in the phenotypic changes occurring in astrocytes after brain injury. Associated to cell shape changes, we have observed that ANP increases migration in a scratch wound assay in vitro (Boran and Garcia, 2007). Moreover, cGMP via cGK was reported to increase the expression of the intermediate filament protein GFAP (Brahmachari et al., 2006). Using a cortical cryolesion model of acute brain injury in rats we have recently shown that treatment with a cGMP phophodiesterase inhibitor increases astrogliosis around the lesion suggesting the participation of enhanced astroglial cGMP in this potential beneficial effect that will accelerate wound healing (Pifarre et al., 2010). Whether NO- and/or NP-dependent mechanisms are involved remains to be determined. The increased immunoreactivity for ANP reported in reactive astroglial cells surrounding experimental brain infarction (Nogami et al., 2001) suggest that an autocrine action of the peptide is possible. In this experimental paradigm, a further paracrine action of astroglial ANP in capillary endothelial cells is suggested by the increased angiogenesis reported in the ischemic boundary after systemic administration of the selective PDE5 inhibitor sildenafil (Zhang et al., 2005, 2006).

In response to certain insults, i.e., ischemia and traumatic brain injury, astrocyte swelling may occur even in the absence of GFAP accumulation. Changes in pH, elevated levels of glutamate and of extracellular K^+ are recognized causes of swelling. Although osmotic changes are part of a normal physiological response to maintain brain homeostasis, exaggerated swelling can lead to pathological conditions, for example triggering neuronal damage via the release of glutamate to the extracellular environment. Interestingly, NPs have been reported to inhibit osmotic swelling in cultured astrocytes (Latzkovits et al., 1993) and retina Müller cells (Kalisch et al., 2006). Moreover, CNP and cGMP analogues have been shown to decrease intracellular pH in astrocytes by inhibiting a Na^+/H^+ exchanger (Touyz et al., 1997). The most abundant brain water-channel protein, aquaporin 4 (AQP4), is normally found at the border between brain parenchyma and major fluid compartments in astrocyte foot processes (brain–blood) and glia limitants (brain-subarachnoid CSF) as well as ependymal cells and subependymal astrocytes (brain-ventricular CSF), suggesting that it controls water flow in and out of the brain (reviewed in Tait et al., 2008). AQP4 is up-regulated in astrocytes associated to brain edema and in reactive astrocytes. Furthermore, AQP4 involvement in astrocyte migration has been demonstrated in culture and impaired glial scarring has been shown in APQ4-null mice in vivo (Tait et al., 2008). Increases in AQP4 mRNA and protein have been reported in cultured astrocytes in response to CNP suggesting an additional role for this NP in water flux control in the CNS (Miyajima et al., 2004) and also a possible mechanism for NP enhanced astrocyte migration.

Alterations in NPR astroglial levels may contribute to neural damage in pathophysiological conditions. Tang et al. (1993) reported lower number of ANP receptors and ANP stimulated cGMP levels in astrocyte cultures from spontaneously hypertensive rats compared with their respective normotensive rat cultures. Recently, a decrease in CNP stimulated cGMP synthesis was demonstrated in the astrocyte compartment of rat cerebral cortical slices from hyperammonemic rats and it was suggested that down-regulation of NPR-B function in astrocytes may contribute to the neurophysiological symptoms of hyperammonemia (Zielinska et al., 2007).

Numerous evidences implicate the ANP–NPR-A system in the regulation of inflammatory processes (Vellaichamy et al., 2007; Vollmar, 2005). In bacterial toxin (LPS)-activated macrophages, the

ANP-cGMP pathway reduces NO synthase-2 expression by transcriptional and post-transcriptional processes. It also reduces pro-inflammatory cytokine expression and release. Inhibition of NF-κB activation is largely responsible for the transcriptional effects (Vollmar, 2005). Microglia, the resident macrophages in the CNS, are activated during CNS injury and switch in behavior from apparently resting but vigilant cells to defending cells. A role for ANP as regulator of microglial inflammatory responses is supported by the demonstration that ANP inhibits NF-κB and AP-1 activation, and decreases LPS-induced nitrite and IL-1β production in rat brain microglial cultures (Moriyama et al., 2006). In the same line, studies in a murine cell line (N9) showed that a cGMP analogue was able to inhibit Aβ-induced release of leukotriene B4 (Paris et al., 1999) and to reduce LPS-induced secretion of TNF-α (Paris et al., 2000). In agreement with an anti-inflammatory effect of cGMP in microglia, we have recently reported that in rats with a cortical cryolesion, treatment with a cGMP-PDE inhibitor decreases the number of round/amoeboid lectin-positive cells and the expression of the activated microglia/macrophage markers Iba-1 and CD11b indicating decreased recruitment and activation of these cells. Furthermore, this altered inflammatory response is accompanied by a decrease in protein oxidative stress and neuronal degeneration (Pifarre et al., 2010). Since rat microglia does not seem to express the NO-sensitive GC (de Vente et al., 1990, 1989; Fig. 1) it is tempting to speculate that increases in cGMP occur in response to NPs in this setting. This hypothesis is supported by a recent in vivo study showing that administration of BNP in murine models of traumatic brain injury and intracerebral hemorrhage downregulates microglial activation (James et al., 2010).

6. Concluding remarks

Numerous evidences indicate that NPs participate in the regulation of important CNS functions. In addition to neurons, astroglial cells have been demonstrated by in vitro and in situ data to express NPs and functional NPRs. A few in vitro studies also point to microglia as potential sources and targets of NPs but in situ confirmatory data are lacking. Circumstantial evidence suggests that the NP–NPR system of glial cells participates in neuron-glia and glia-blood vessel communication and could play important roles in the regulation of blood–brain barrier function, neuroinflammation and neuroprotection. Further investigation is needed on the mechanisms regulating synthesis and release of the peptides and expression and activity of their receptors in glial cells in normal and pathological states in order to understand the neurophysiological relevance of this signalling system. Application of gene targeting technology in mice has provided invaluable information about the molecular physiology and biological functions of the NP–NPR system in cardiovascular function. However, this experimental approach has not been exploited to study the biological functions of this signalling system in the CNS. Given the cellular complexity of this tissue and the expression of components of the NP–NPR system in neuronal as well as glial and blood vessel endothelial cells, selective deletion of NPRs in particular cell types will be the approach of choice to unequivocally implicate a cellular action of the peptides in a CNS physiological or pathological response.

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ORIGINAL PAPER

Sildenafil (Viagra) ameliorates clinical symptoms and neuropathology in a mouse model of multiple sclerosis

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Abstract Cyclic GMP (cGMP)-mediated pathways regulate inflammatory responses in immune and CNS cells. Recently, cGMP phosphodiesterase inhibitors such as sildenafil, commonly used to treat sexual dysfunction in humans including multiple sclerosis (MS) patients, have been reported to be neuroprotective in animal models of stroke, Alzheimer's disease, and focal brain lesion. In this work, we have examined if sildenafil ameliorates myelin oligodendrocyte glycoprotein peptide ($MOG_{35–55}$)-induced experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. We show for the first time that treatment with sildenafil after disease onset markedly reduces the clinical signs of EAE by preventing axonal loss and promoting remyelination. Furthermore, sildenafil decreases CD3+ leukocyte infiltration and microglial/macrophage activation in the spinal cord, while increasing forkhead box transcription factor 3-expressing T regulatory cells (Foxp3 Tregs). However, sildenafil treatment did not significantly affect $MOG_{35–55}$-stimulated proliferation or release of Th1/Th2 cytokines in splenocytes but decreased ICAM-1 in spinal cord infiltrated cells. The presence of reactive astrocytes forming scar-like structures around infiltrates was enhanced by sildenafil suggesting a possible mechanism for restriction of leukocyte spread into healthy parenchyma. These results highlight novel actions of sildenafil that may contribute to its beneficial effects in EAE and suggest that treatment with this widely used and well-tolerated drug may be a useful therapeutic intervention to ameliorate MS neuropathology.

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Introduction

Experimental autoimmune encephalomyelitis (EAE) has been extensively used as an animal model of multiple sclerosis (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 [13]. 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 [7, 13]. In contrast, accumulating evidence indicates that local astroglial activation is neuroprotective. As in locally triggered innate immune responses caused by trauma or stroke, reactive astrocytes in EAE have been reported to form scar-like barriers that restrict leukocyte infiltration into the CNS parenchyma [33, 35].

Additionally, astrocytes are known to release anti-inflammatory cytokines, ROS scavengers, and growth factors that could restrict local inflammation and promote nerve recovery [18]. Interestingly, recent evidence indicates that demyelination and oligodendrocyte degeneration follows inflammation-induced astrocyte dysfunction [30]. High levels of inflammatory mediators (cytokines, chemokines, and NO) are secreted by infiltrating immune cells and resident CNS cells and characterize the inflammatory environment during disease [28]. Up-regulation of NO synthase type 2 (NOS2) has been reported in MS and EAE, and NO and peroxynitrite, the highly reactive product of NO reaction with superoxide, have been implicated in tissue damage [28]. However, NOS2-deficient mice develop more severe EAE [38], suggesting that NO may be neuroprotective. A major target for NO in many cell types including CNS cells and lymphocytes is NO-sensitive guanylyl cyclase (GC) [12]. It is well established that NO via cGMP regulates important neuronal functions such as synaptic plasticity processes involved in memory formation; however, very little is known about the function of this pathway in glial cells or the contribution of cGMP to NO effects during neuroinflammation. In both neurons and astrocytes, the NO-cGMP pathway has been reported to activate antiapoptotic mechanisms [32]. Furthermore, we have recently shown that in astrocytes this signaling pathway is involved in the rearrangement of actin and GFAP filaments that results in astrocyte stellation and enhanced motility suggesting a role in the regulation of the reactive phenotype [3]. In agreement with this, other authors have demonstrated that the same pathway is involved in up-regulation of GFAP, a marker of astrogliosis [4]. Immune and glial cells also produce cGMP by stimulation of natriuretic peptide (NP) receptors [26]. Atrial natriuretic peptide (ANP) has been shown to affect innate and adaptive immune responses and to reduce production of proinflammatory mediators by macrophages and microglia [21, 34]. Neuroprotective actions of NPs have been reported in animal models of cerebral ischemia [37] and traumatic brain injury [16].

The cGMP signal can be terminated by the action of several members of the large family of cyclic nucleotide phosphodiesterase (PDE) [8]. cGMP-selective PDE5, well known for its important actions in the cardiovascular system, is also expressed in neural and immune cells [8]. PDE5 inhibitors, such as sildenafil, which are widely used for treatment of erectile dysfunction in humans, have been shown to improve cognition in unimpaired and impaired rodents [27, 29], to increase neurogenesis and enhance functional recovery after stroke [40] and to regulate glial inflammatory responses and decrease neuronal cell death after cortical cryoinjury [25]. In this work, we show that sildenafil administration after disease onset rapidly ameliorates $MOG_{35–55}$-induced EAE in mice by preventing axonal loss and enhancing remyelination. Sildenafil regulation of inflammatory cell infiltration and glial reactivity may underlay these beneficial effects. Results suggest that this drug may be beneficial in MS.

Materials and methods

Animals and treatments

Two-month-old female C57BL/6 mice (Charles River) were housed in the animal care facility of the Universitat Autonoma de Barcelona (UAB) under constant temperature and provided food and water ad libitum. EAE was induced by immunization with $MOG_{35–55}$ peptide (Scientific Technical Service, Universitat Pompeu Fabra, Barcelona, Spain). On day 0, mice ($n = 34$) were injected subcutaneously (s.c.) into the hind flanks with an emulsion of 100 µl $MOG_{35–55}$ (3 mg/ml) and 100 µl CFA (Sigma) supplemented with 4 mg/ml *Mycobacterium tuberculosis* H37RA (Difco). Additionally, animals received an intraperitoneal injection of 500 ng *pertussis toxin* (Sigma), which was repeated 2 days after immunization. As controls, a group of mice were immunized with BSA ($n = 6$). Animals were treated with sildenafil (10 mg/kg, s.c.; extracted from Pfizer Viagra tablets according to [11]) or vehicle (water) once a day starting at 18 days post-immunization (dpi). Mice were clinically scored for EAE daily according to the following criteria: 0 no signs of disease; 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. Mice were sacrificed under isofluorane anesthesia at 21 of 26 dpi (3 or 8 days post-initiation of treatment (dpt) respectively), and spinal cords and spleens were removed. Experiments were approved by the UAB Animal and Human Experimentation Ethics Committee.

Histological methods

The lumbar-thoracic region of the spinal cords was fixed in paraformaldehyde and embedded in paraffin for histological analysis. Longitudinal sections (8 µm) were stained with hematoxylin and eosin (H&E) or Luxol Fast Blue (LFB) for evidence of infiltrates, and demyelination and histological scores were evaluated blindly according to the following criteria. For cell infiltration (H&E): 0 no inflammation; 1 cellular infiltrates only around blood vessels and meninges; 2 moderate cellular infiltrates in parenchyma, less than 50% of the white matter (WM); 3 severe infiltrates in parenchyma, deep and/or more than 50% of the WM. For demyelination (LFB): 0 no demyelination; 0.5 little demyelination only around infiltrates; 1 superficial demyelination, which involves

less than 25% of the WM; 1.5 superficial demyelination which involves more than 25% but less than 50% of the WM; 2 deep demyelination and/or demyelination that involves more than 50% of the WM; 3 diffuse and widespread demyelination. Axonal density was evaluated by Bielschowsky's silver impregnation.

Immunostaining of longitudinal lumbar-thoracic sections was performed as previously described [25]. Antigen retrieval was done by the appropriate standard protocol (citrate, EDTA or protease type XIV; Sigma). Sections were incubated with the primary antibodies: polyclonal rabbit purified anti-glial fibrillary acidic protein (GFAP; Dako, Cat. no. Z0334; 1:900) for astrocytes; polyclonal rabbit purified anti-Iba1 (WAKO, Cat. no. 19-19741; 1:250) for macrophages/microglia; polyclonal rabbit purified anti-CD3 (Dako, Cat. no. A0452; 1:100) for T lymphocytes; monoclonal anti-Foxp3 (a gift from Dr. G. Roncador, Monoclonal Antibodies Core Unit, Spanish National Cancer Research Center (CNIO), Madrid [1]) for Tregs; monoclonal purified anti-ICAM-1 (Pharmingen, Cat. no. 554967; 1:100); monoclonal purified anti-SMI-32 (Covance, Cat. no. SMI-32R; 1:300) for axonal damage. Appropriate secondary antibodies conjugated to biotin (Vector, 1:200) were used and detected using streptavidin/horseradish peroxidase (Vector, 1:300) and the peroxidase substrate DAB Kit (Vector). Control sections were incubated in the absence of primary antibodies. Sections were counterstained with 0.25% cresyl Echt Violet (Sigma) or hematoxylin before mounting. Images were acquired using Nikon Digital camera DXM 1200F and Nikon Act-1 software.

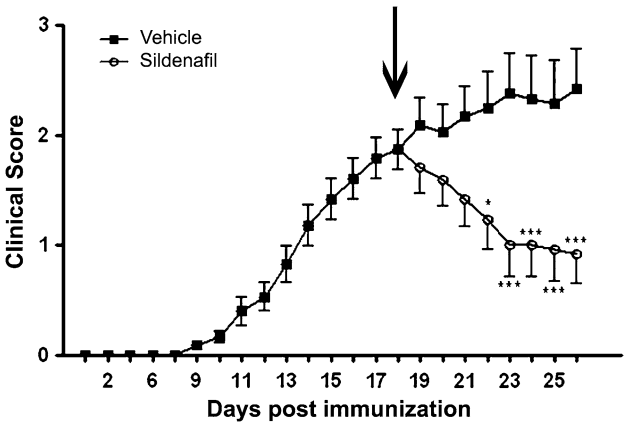

Fig. 1 Sildenafil ameliorates clinical symptoms associated with EAE. C57BL/6 mice immunized with $MOG_{35–55}$ developed clinical symptoms of EAE after 1 week. Sildenafil administration (10 mg/kg, s.c.) daily starting 18 dpi significantly decreased disease severity after 4 days when compared to vehicle-treated animals. Values are mean ± SEM (n = 12–13). Statistical significance: $*p < 0.05$; $***p < 0.001$

Staining quantifications

Four to eight areas (1 mm^2) randomly chosen along the length of each of 3–4 different longitudinal spinal cord sections separated at least 300 μm were analyzed by two independent investigators blinded to treatment and clinical score. The analysis software Scion Image (NIH) was used to quantify LFB, Bielschowsky, SMI-32, ICAM-1, Iba1 and GFAP staining. Quantification of CD3- and Foxp3-positive cells was performed manually in infiltrates (4–8 infiltrates per section).

Splenocyte responses

Splenocytes were isolated from the spleens of animals killed at 21 dpi. Cells were flushed out from the spleens gently using a syringe plunger with PBS and centrifuged (220×g, 4°C). The pellet was resuspended and left 5 min at 37°C in 5 ml of RBC lysis buffer (Sigma, 17 mM Tris, 140 mM NH_4Cl) to remove erythrocytes. After further centrifugation, cells were washed once again with PBS, centrifuged and resuspended in 10 ml of complete medium (RPMI + L-glutamine supplemented with 15% FCS (Ingelheim diagnostic, Barcelona, Spain), pen/strep antibiotics, 1 mM sodium piruvate and 50 μM β-mercaptoethanol). Cells were seeded at a density of 2–4 × 10^6 cells/ml in 96- or 24-well plates and exposed to $MOG_{35–55}$ (10 μg/ml, 72 h). Proliferation was measured in 96-well plates using EZ4U kit (Biomedica Gruppe, Vienne, France). Cytokine release was determined in the media of 24-well plates with BD CBA mouse Th1/Th2 cytokine kit.

Table 1 Clinical scores of EAE mice treated or not with sildenafil

Treatment	EAE clinical score before treatment (18 dpi, n = 17 per group)			EAE clinical score after treatment (26 dpi, n = 12–13 per group)			Mortality	Grade of remission
	Incidence	Average score	Cumulative score	Incidence	Average score	Cumulative score		
Vehicle	90.9	1.75 ± 0.27	9.28 ± 1.55	100	2.41 ± 0.36	29.45 ± 2.9	1	0.66 ± 0.24
Sildenafil	90.3	2.09 ± 0.22	10.97 ± 1.53	84.61	0.92 ± 0.27	18.81 ± 3.16*	0	−1.17 ± 0.23***

EAE incidence (% diseased animals) and clinical scores of MOG-immunized mice before (18 dpi) and after 8 days of sildenafil treatment (26 dpi). Cumulative score (sum of scores from disease onset) and grade of remission (difference between score at 18 and 26 dpi) were significantly different in sildenafil-treated animals. Results are mean ± SEM of the indicated number of animals at each time point. Statistical significance: * $p < 0.05$, *** $p < 0.001$

Statistical analysis

Differences in clinical scores, LFB, Bielschowsky, histological scores, CD3, and cytokines were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. Student's t test was used for two group comparisons (ICAM, Foxp3, Iba-1, GFAP, and SMI32). Results shown are mean ± SEM of the indicated number of animals.

Results and discussion

Sildenafil administration after disease onset ameliorates clinical symptoms and neuropathology in a chronic model of EAE

C57BL/6 mice immunized with $MOG_{35–55}$ developed EAE clinical symptoms after 7 days and at 18 dpi, when the incidence of clinical EAE was 90–91% and the average score around 2; animals (16–17 per group) were injected s.c. with sildenafil (10 mg/kg) or vehicle (water) daily and were killed 3 (21 dpi) or 8 (26 dpi) days later for central and peripheral immune response analysis. As shown in Fig. 1 and Table 1, disability in vehicle-treated mice continued to increase, whereas mice treated with sildenafil showed a lower cumulative score and a rapid recovery that was already significant after 4 days of treatment. During 5–8 days of treatment, the clinical score stabilized around 1, and interestingly more than half of the animals presented virtually full recovery (score 0, $n = 2$; score 0.5, $n = 7$). In a different experiment, animals given 10 doses of 5 mg/kg sildenafil every other day starting at 20 dpi showed a smaller but significant improvement in clinical score by 43 dpi (controls: 2.2 ± 0.2, $n = 10$; sildenafil-treated: 1.6 ± 0.2, $n = 7$), indicating that the effect depends on the dose. A group of mice ($n = 6$) immunized in parallel with BSA did not present clinical symptoms of EAE (not shown).

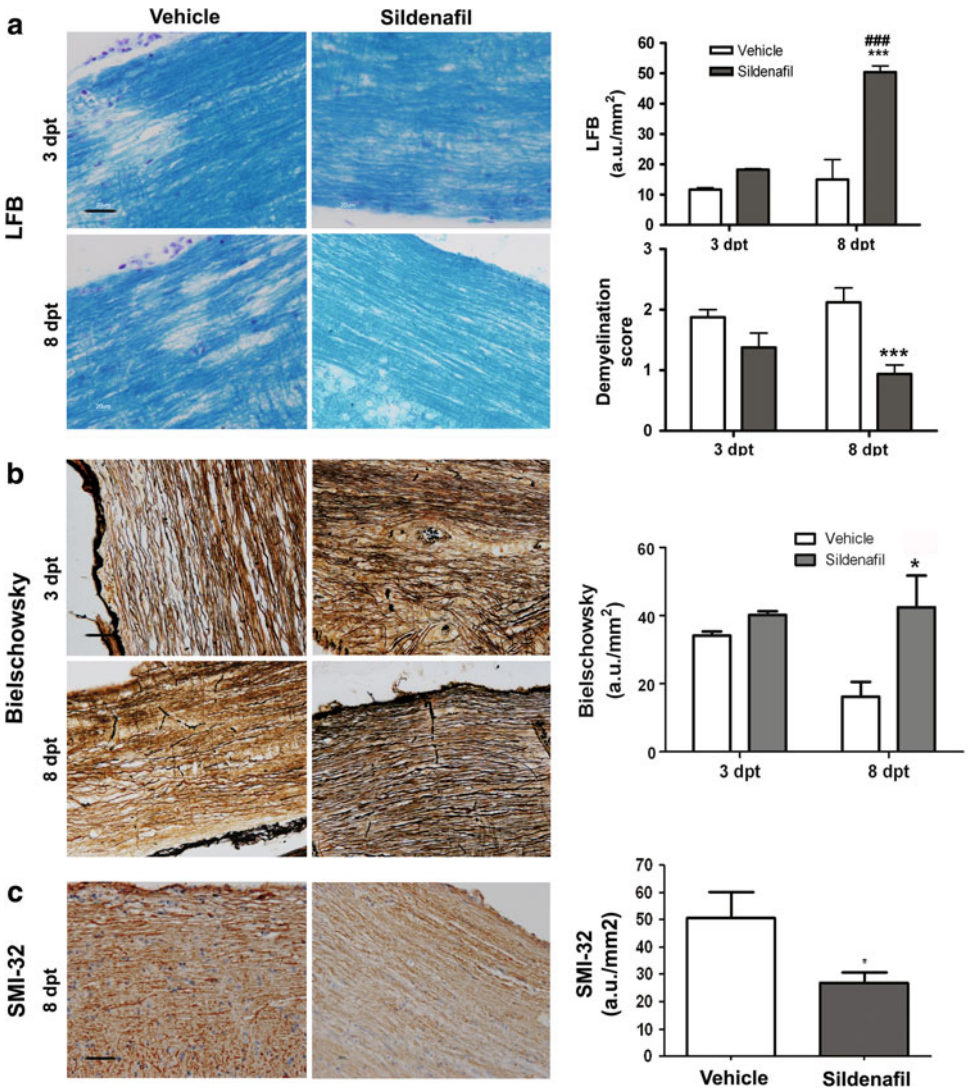

Fig. 2 Sildenafil ameliorates neuropathology associated with EAE. Longitudinal lumbar-thoracic spinal cord sections from MOG-immunized mice treated or not with sildenafil for 3 ($n = 4$) or 8 days ($n = 12–13$) starting at 18 dpi were stained to evaluate **a** demyelination (LFB), **b** axonal loss (Bielschowsky), and **c** axonal damage (SMI-32) (*bars* 50 μm in **a**, **b**; 20 μm in **c**). Demyelination scores and staining intensities [arbitrary units (a.u.)/mm^2] were quantified as described in "Materials and methods". Note that immunization with MOG causes severe demyelination and axonal damage at 26 dpi that is significantly reduced by 8 day treatment with sildenafil. Values are mean ± SEM. Statistically significant difference versus vehicle-treated animals: $^{*}p < 0.05$; $^{***}p < 0.001$; or versus 3 dpt (21 dpi): $^{\#\#\#}p < 0.001$

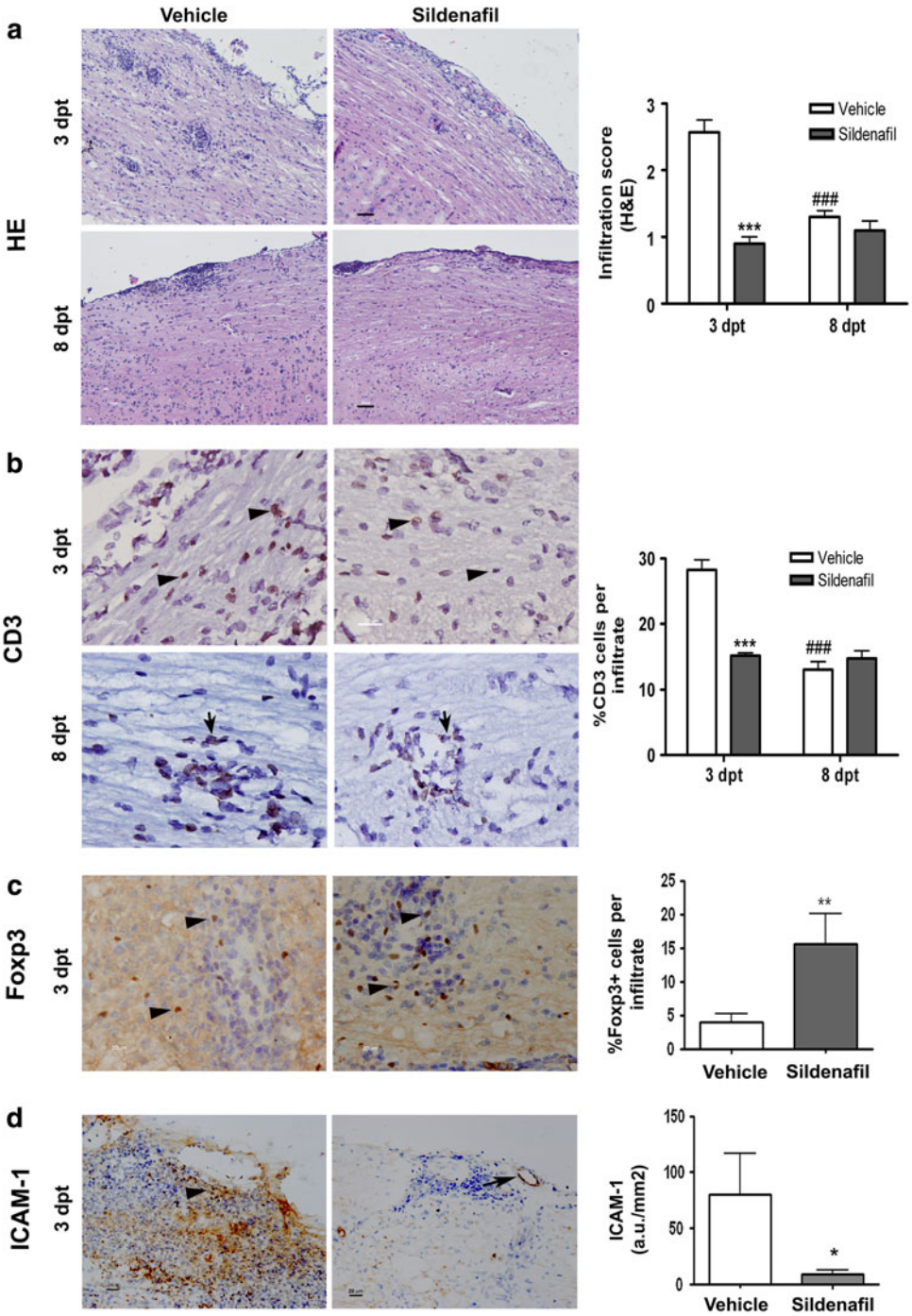

Fig. 3 Sildenafil decreases inflammatory cell infiltration and ICAM-1 expression and increases Foxp3+ cells in spinal cords of EAE mice. Spinal cord sections from MOG-immunized mice treated or not with sildenafil for 3 ($n = 4$) or 8 days ($n = 12$–13) starting at 18 dpi were stained to evaluate **a** general cell infiltration (H&E), **b** T lymphocytes (anti-CD3), **c** Tregs (anti-Foxp3), and **d** ICAM-1. *Arrowheads* points to infiltrated cells and *arrow* points to blood vessel (*bars* 50 μm in **a**; 20 μm in **b**–**d**). Cell infiltration score, percentage of CD3+ and Foxp3+ cells in infiltrates and ICAM-1 staining were quantified as indicated in "Materials and methods". Sildenafil significantly decreased inflammatory cell infiltration and ICAM-1 in infiltrates and increased Foxp3+ cells after 3 days of treatment. Inflammatory cell infiltration decreased significantly from 21 to 26 dpi in vehicle-treated animals, and 8-day sildenafil treatment did not reduce it further. Values are mean ± SEM. Statistically significant difference versus vehicle-treated animals: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; or versus 3 dpt (21 dpi): $^{\#\#\#}p < 0.001$

To investigate if clinical improvement was accompanied by decreased neuropathology, we examined demyelination and axonal loss in longitudinal sections of the lumbar-thoracic region of spinal cords by LFB and Bielschowsky's silver staining in comparison to BSA-immunized mice that presented no pathology (Supplemental Fig. 1). As shown in Fig. 2a, the decrease in LFB staining intensity and the corresponding demyelination score were similar in vehicle-treated MOG-immunized mice at both 21 and 26 dpi. However, in 21 dpi animals treated with sildenafil for the last 3 days, demyelination was already less evident and by 26 dpi, after 8 days of sildenafil treatment, there was a significant increase in LFB staining and a decrease in demyelination score suggesting that sildenafil promotes remyelination. In contrast, quantification of Bielschowsky's staining showed that axonal loss increased from 21 to 26 dpi in vehicle-treated animals but not in sildenafil-treated animals (Fig. 2b), indicating that sildenafil prevents further axon degeneration. In agreement with this, staining of non-phosphorylated neurofilaments with anti-SMI-32, a

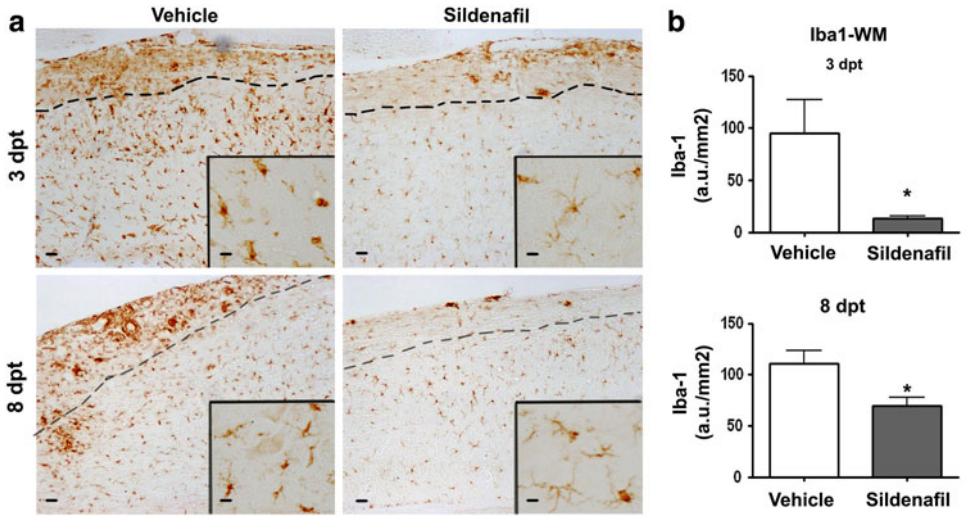

Fig. 4 Sildenafil decreases macrophage/microglia activation in the spinal cord of EAE mice. **a** Spinal cord sections from MOG-immunized mice treated or not with sildenafil for 3 ($n = 4$) or 8 days ($n = 12$–13) were immunostained for Iba1 to evaluate macrophage/microglia activation (*bar* 50 μm); **b** quantification of Iba1 staining intensity in WM. Treatment with sildenafil significant reduced macrophage/microglia activation at both treatment times. *Inserts* magnification of GM areas showing that sildenafil promotes a resting morphology in microglia (*bar* 20 μm). Values are mean ± SEM. Statistical significance: $*p < 0.05$

marker of axonal damage, was significantly lower in 8-day sildenafil-treated animals compared to vehicle-treated controls (Fig. 2c). Taken together, these results indicate that the functional recovery produced by sildenafil treatment in MOG-immunized animals results from axonal protection and remyelination.

Sildenafil treatment decreases inflammatory cell infiltration and ICAM-1 expression while increasing the proportion of Foxp3+ Tregs in spinal cord of EAE mice

Autoreactive T cells infiltrating the CNS are the initiator and early effector cells in EAE development, but infiltrated macrophages, dendritic cells, and resident microglia constitute the ultimate effector cells that amplify neuroinflammation and tissue damage. Thus, we next examined the effect of sildenafil on cellular infiltration in the spinal cord. As estimated by H&E staining at 21 dpi, administration of sildenafil for the previous 3 days dramatically decreased the severity of cell infiltration, being the infiltrates smaller and largely confined to the submeningeal area (Fig. 3a). Quantification of CD3+ T cells in infiltrates showed that sildenafil significantly reduced the proportion of this cell population at 3 dpt (Fig. 3b). At 26 dpi, vehicle-treated animals presented significantly lower infiltration score (H&E) and number of CD3+ T cells per infiltrate respect to 21 dpi, and levels were no different to those determined in animals treated with sildenafil for 3 or 8 days (Fig. 3a, b). However, activated macrophages/microglia in WM (strongly Iba1+ globoid cells) were significantly reduced by sildenafil at both 3 and 8 dpt (Fig. 4a, b). These results agree with a recent report showing that inflammatory cell infiltration in MOG-immunized mice decreases after peak disease despite generalized axonal loss and demyelination and persistent clinical disability [15] and suggest that decreased macrophage/microglial activation may bear a more direct relation to the axon protective effect of sildenafil than decreased T cell infiltration. Additionally, a contribution of a direct effect of sildenafil in axons and oligodendrocytes potentiating cGMP-mediated neuroprotective pathways cannot be ruled out [2, 14, 22].

To investigate if regulation of the peripheral immune response might be involved in sildenafil reduction of inflammation in the spinal cord, we examined the immune response of splenocytes upon ex vivo stimulation with $MOG_{35–55}$. No significant differences were found in the proliferative response or the release of INF-γ, TNF-α, IL-2 or IL-6 in splenocytes from vehicle- and sildenafil-treated animals 3 dpt (Fig. 5), thus ruling out that sildenafil compromises the ability of T cells to be activated.

ICAM-1, a type-1 membrane-bound glycoprotein expressed in most leukocyte subtypes, endothelial cells, and CNS glial cells, is involved in leukocyte entry, lymphocyte activation, and other immune responses and plays a central role in the development of MS and EAE [6, 17]. Thus, we examined if sildenafil was affecting ICAM-1 expression in spinal cords of EAE mice at 21 dpi when significant reduction in cell infiltration was observed (Fig. 3b). In control mice, ICAM-1-immunoreactivity was observed in blood vessels and in numerous cells within and

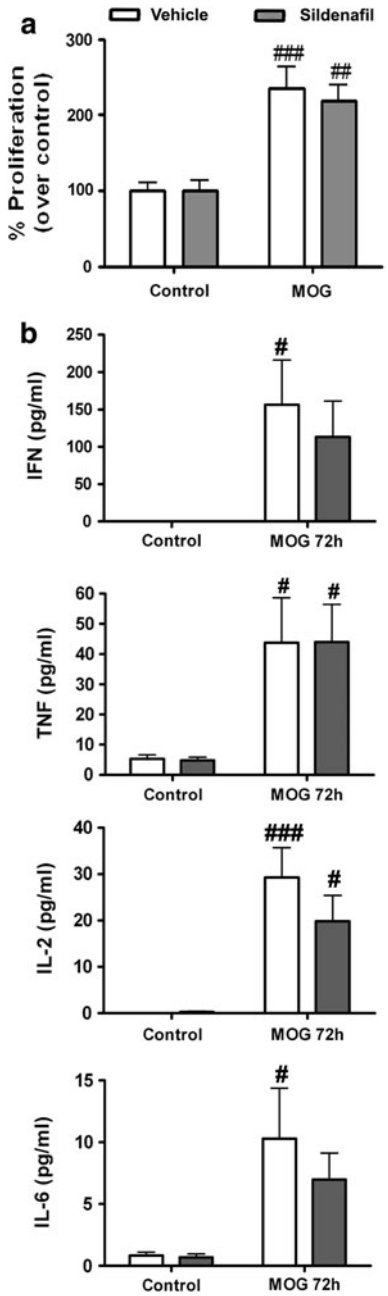

Fig. 5 Sildenafil treatment does not affect in vitro responses to $MOG_{35–55}$ in splenocytes from EAE mice. Splenocytes isolated from vehicle- or sildenafil-treated MOG-immunized mice (21 dpi) were incubated with or without $MOG_{35–55}$ (10 μg/ml) for 72 h, and the proliferative response (**a**) and the release of cytokines (**b**) were measured. Values are mean ± SEM ($n = 4$). Statistically significant differences versus control splenocytes: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$

around infiltrates (Fig. 3d). In contrast, in sildenafil-treated animals, it was almost absent in infiltrates but was still observed in blood vessels. Studies on ICAM-1 null mice have evidenced the critical role of ICAM-1 expression in T cells for the modulation of effector T cell responses [6]. Interestingly, NO via cGMP was reported to reduce T cell adhesion to human brain microvessels in vitro, but NO did not modulate adhesion molecule expression in the endothelial cells, suggesting a direct action on the T cells [39]. Thus, down-regulation by sildenafil of ICAM-1 expression in T cells could be involved in decreasing T cell infiltration and/or activation in the spinal cord of EAE mice. Additionally, by increasing cGMP in macrophages sildenafil could attenuate TNF-α release and TNF-α-induced expression of chemokines and adhesion molecules in endothelial cells [34].

The role of Foxp3+ Tregs in suppressing autoreactive T cells is well established. Additionally, Foxp3+ Tregs have been shown to play a critical role in protection and recovery from EAE [24]. Since sildenafil afforded both protection and recovery, we investigated if it affected the population of Foxp3+ cells. As shown in Fig. 3c, at 21 dpi, Foxp3+ cells in spinal cord infiltrates of sildenafil-treated mice were notably increased compared to vehicle-treated controls. Thus, up-regulation of Tregs may be another factor contributing to the beneficial effects of sildenafil in recovery from EAE. This result is in clear contrast with a recent report on splenocytes showing that NO inhibits expression of Foxp3 in myelin basic protein-primed T cells via cGMP [5]. This discrepancy may be reconciled in light of the observation that development of Foxp3+ Tregs in response to antigen stimulation is antagonized by Th1/Th2 lineage differentiation activities [36]. This may occur in splenocytes from immunized mice but not in the spinal cord of animals treated with sildenafil where T cell infiltration is greatly reduced.

Sildenafil treatment regulates reactive gliosis

In addition to heavily Iba1-stained globoid macrophages/microglia within and around infiltrates, MOG-immunized mice presented activated ramified microglia (strongly Iba1-stained) throughout the spinal cord, more evident at 21 than at 26 dpi. In contrast, animals treated with sildenafil for 3 or 8 days showed a decreased intensity of Iba1 staining in activated microglia and increased numbers of cells with long and thin ramifications typical of resting microglia (Fig. 4a, inserts), suggesting that decreased microglial activation may also contribute to the neuroprotective effect of sildenafil. In vitro studies have shown that cGMP-mediated pathways reduce expression and release of inflammatory mediators from both macrophages and microglia [21, 23, 34]. Furthermore, we have recently shown that treatment with cGMP-PDE inhibitors decreases recruitment and activation of macrophages/microglia around a cortical cryoinjury in rodents [25]. Since microglia does not seem to express NO-sensitive GC [26], it is tempting to speculate that increases in cGMP occur in response to NPs. Interestingly, a recent study showed that

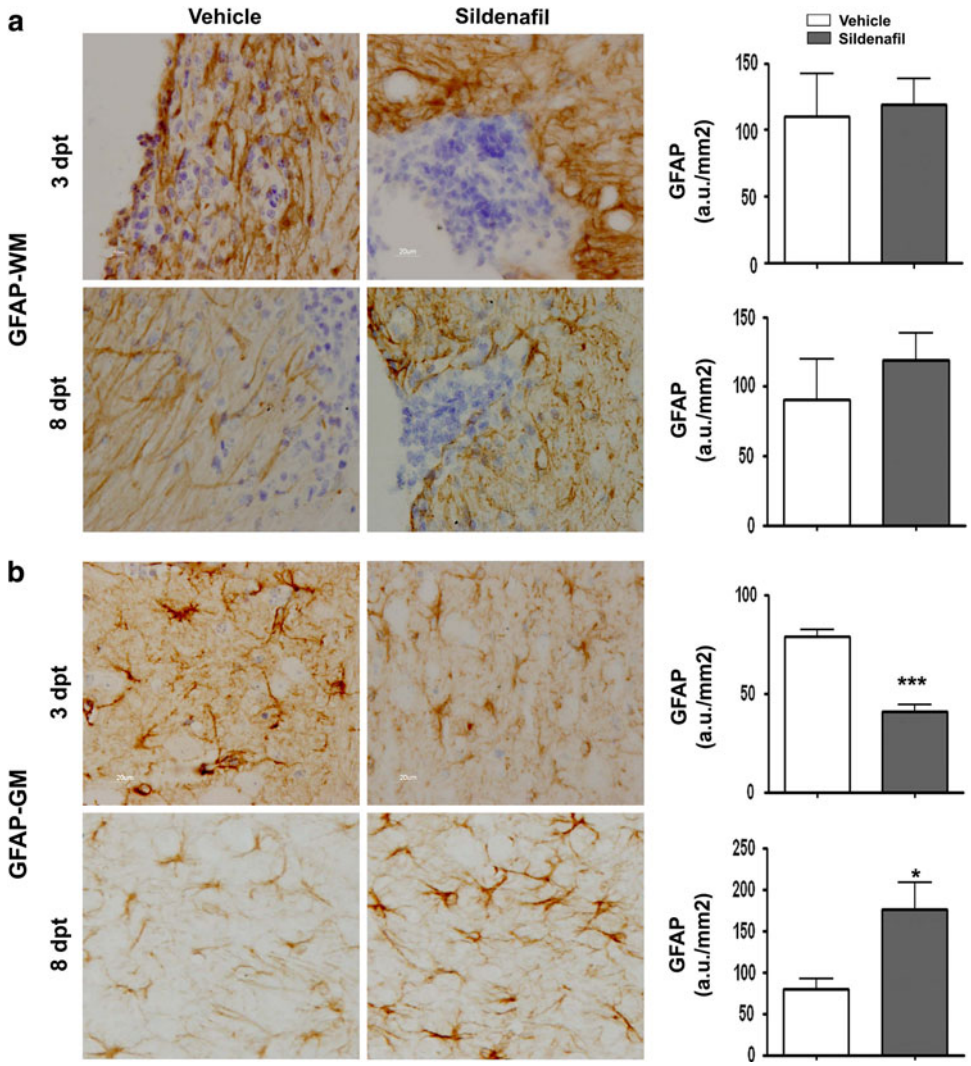

Fig. 6 Sildenafil modifies astrogliosis in the spinal cord of EAE mice. Spinal cord sections from MOG-immunized mice treated or not with sildenafil for 3 ($n = 4$) or 8 days ($n = 12$–13) were immunostained for GFAP to evaluate astroglial activation (*bar* 20 μm). Quantification of GFAP staining in the WM (**a**) showed no significant differences between treated and untreated animals, but scar-like structures around infiltrates were more prominent in sildenafil-treated animals. In the GM (**b**), sildenafil had a biphasic effect on astroglial activation, decreasing GFAP staining intensity at 3 dpt but increasing it at 8 dpt. Values are mean ± SEM. Statistical significance: $*p < 0.05$; $***p < 0.001$

administration of brain natriuretic peptide downregulates microglial activation in murine models of traumatic brain injury and intracerebral hemorrhage [16].

Increasing evidence indicates that astrogliosis can play a crucial role in the pathogenesis and resolution of demyelinating disease. Astrocytes can promote and perpetuate immune-mediated demyelination by priming autoreactive T cells and expressing cytokines, chemokines, and costimulatory and adhesion molecules [9]. However, astrocytes also promote anti-inflammatory responses and form perivascular barriers that restrict the influx of leukocytes into CNS parenchyma [18, 31]. We have previously shown that increasing cGMP in astrocytes regulates cytoskeleton dynamics and accelerates migration in a scratch wound assay in vitro [3]. In addition, we have shown that treatment with PDE5 inhibitors enhances astrogliosis around a cortical cryolesion suggesting that cGMP-mediated pathways can accelerate glial scar formation [25]. Thus, we examined the effect of sildenafil on GFAP immunoreactivity in the spinal cord of EAE mice. Different effects were observed in WM and gray matter (GM). Severely reactive astrocytes with long overlapping processes (anisomorphic gliosis) were observed throughout WM but particularly in areas of heavy inflammatory cell infiltration (Fig. 6a). Although no significant differences in GFAP overall staining intensity were observed between vehicle- and sildenafil-treated animals, a stronger tendency to form scar-like structures around confined infiltrates could be generally observed in the latter (Fig. 6a), suggesting a role in controlling the spreading of infiltration. In the GM moderate astrogliosis was widespread in vehicle-treated mice, and in contrast to WM, sildenafil had a biphasic effect, significantly decreasing GFAP immunoreactivity after 3 days but increasing it after 8 days of treatment (Fig. 6b). At this treatment time, activated astrocytes were evenly distributed in GM and presented a more stellate shape, features typical of isomorphic gliosis. Numerous evidences indicate that these activated astrocytes exert suppressive effects on

inflammatory cells and release antioxidants and growth factors that protect neurons and oligodendrocytes [18]. Thus, it will be of interest to investigate how the different effects of sildenafil on astroglial reactivity in WM and GM observed at the different treatment times relate to the reduced inflammation, axon protection, and remyelination the drug produces in EAE mice.

To our knowledge, this is the first report demonstrating efficacy of a PDE5 inhibitor in a mouse model of MS. Sexual dysfunction is a common symptom in MS patients, and treatment with PDE5 inhibitors including sildenafil [10] and tadalafil [19] are commonly used to treat these symptoms. However, to our knowledge, there have not been any published studies to determine if these treatments modify MS pathology although a presentation at the 2005 ECTRIMS meeting suggested radiological benefit in a small cohort of MS patients [20].

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*Title page

METALLOTHIONEINS I/II ARE INVOLVED IN THE NEUROPROTECTIVE EFFECT OF SILDENAFIL IN FOCAL BRAIN INJURY

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Keywords: cGMP-phosphodiesterase, sildenafil, metallothionein, brain injury, neuroprotection

Running title: Metallothioneins in sildenafil neuroprotection.

ABSTRACT

Increasing evidence indicates that treatment with the cGMP-specific phosphodiesterase type 5 (PDE5) inhibitor sildenafil has beneficial effects in animal models of acute and chronic CNS injury. We recently reported a neuroprotective effect of the non-selective cGMP-PDE inhibitor zaprinast in cortically cryoinjured rats that was accompanied by enhanced astrogliosis around the lesion and decreased macrophage/microglial activation and oxidative stress. These effects are similar to those observed in cryolesioned mice overexpressing metallothioneins I/II (MT-I/II), metal-binding cysteine-rich proteins that are up-regulated in response to injury. Here we show that treatment with the selective PDE5 inhibitor sildenafil (10 mg/kg, sc) 2h before and 24h and 48h after induction of cortical cryolesion produces in wild-type mice the changes in glial reactivity and the antioxidant and antiapoptotic effects previously observed with zaprinast in rats, indicating that inhibition of PDE5 is involved in these neuroprotective actions. However, these effects of sildenafil were not observed in mice with null MT-I/II expression. We also show that sildenafil significantly increases MT-I/II protein levels in the lesioned mice cortex and MT-I/II immunostaining in glial cells around the lesion. These results indicate that cGMP-mediated pathways regulate expression of MT-I/II and support the involvement of these proteins in the neuroprotective effects of sildenafil in focal brain lesion.

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1. INTRODUCTION

In the CNS, the intracellular messenger cGMP can be formed by NO-sensitive guanylyl cyclase (NO-GC) and by natriuretic peptide receptor-GC in both neuronal and glial cells and it is now well established that cGMP-mediated pathways regulate synaptic plasticity, brain development and cerebral blood flow (Feil and Kleppisch, 2008; Garthwaite, 2008). Additionally, in vitro studies have shown that cGMP-via protein kinase G (PKG) activates antiapoptotic mechanisms in neurons, astrocytes and oligodendrocytes (Benjamins and Nedelkoska, 2007; Fiscus, 2002; Takuma et al., 2001a; Takuma et al., 2001b) and regulates astroglial and microglial inflammatory responses (Boran and Garcia, 2007; Brahmachari et al., 2006; Moriyama et al., 2006; Zhao et al., 2011).

The cGMP signal is terminated by the action of several members of the large family of cyclic nucleotide phosphodiesterases (PDEs) (Conti and Beavo, 2007). The cGMP-specific PDE5, well known for its implication in cardiovascular system function, is expressed in different brain regions and has been detected in neuronal populations as well as in glial cells (Bender and Beavo, 2004; Menniti et al., 2009; Prado et al., 2010). In agreement with this, PDE5 inhibitors, such as sildenafil, have been shown to increase cGMP in cortex, hippocampus and striatum (Marte et al., 2008; Puerta et al., 2009; Zhang et al., 2006). Several studies have demonstrated that treatment with PDE5 inhibitors improves cognition in rats and mice and restores memory in impaired rodents, including Alzheimer's disease models (Cuadrado-Tejedor et al., 2011; Fiscus,

2002; Orejana et al., 2011; Puzzo et al., 2009; Reneerkens et al., 2009). Moreover, accumulating evidence supports a neuroprotective action of sildenafil and analogues in animal models of chemical (Puerta et al., 2010; Puerta et al., 2009) and ischemic brain injury (Ko et al., 2009; Menniti et al., 2009; Zhang et al., 2006) by promoting survival pathways, angiogenesis and neurogenesis. In addition, we have shown that treatment with the non-selective cGMP-PDE inhibitor zaprinast in cortically cryolesioned rats increases astrogliosis and decreases microglia/macrophage activation around the lesion, effects that are associated with decreased oxidative stress and apoptotic cell death (Pifarre et al., 2010). We have also shown anti-inflammatory and axon and myelin protective effects of sildenafil treatment in the spinal cord of mice with experimental autoimmune encephalomyelitis (EAE) that result in a significant amelioration of clinical symptoms (Pifarre et al., 2011). Thus, in addition to their well known vasodilator and angiogenic actions, cGMP-PDE inhibitors, can affect a wide range of cellular processes in neural and inflammatory cells that make them promising drugs for the treatment of CNS injury and neurodegenerative disease.

Metallothioneins (MTs) are a family of low molecular weight, cystein-rich proteins, with metal-binding and free radical scavenging properties. The isoforms MT-I and II (MT-I/II), which are expressed coordinately in most tissues, occur throughout the brain and spinal cord and the main cell expressing these MT isoforms is the astrocyte. In response to CNS injury, MT-I/II expression is rapidly induced in reactive astrocytes and appears to play important neuroprotective and neuroregenerative roles (Hidalgo et al., 2001; West et al.,

2008). We and others have shown that genetically modified mice with null MT-I/II expression exhibit worse outcomes following a range of CNS injuries (Asanuma et al., 2002; Carrasco et al., 2000; Giralt et al., 2002a; Penkowa et al., 1999; Trendelenburg et al., 2002). Conversely, mice overexpressing MT-I/II show improved recovery (Giralt et al., 2002b; Molinero et al., 2003; van Lookeren Campagne et al., 1999). In cortically cryolesioned MT-I/II overexpressing mice we have observed decreased oxidative stress, inflammation and apoptosis (Giralt et al., 2002b). Additionally, while cryolesioned MT-I/II knockout mice exhibited decreased astroglial reactivity at early times after injury, MT-I/II overexpressing animals showed increased astrogliosis around the lesion (Giralt et al., 2002a; Giralt et al., 2002b). These effects are similar to those induced by treatment with zaprinast in cryolesioned rats (Pifarre et al., 2010). Zaprinast, initially considered a selective PDE5 inhibitor, is now known to also inhibit with high potency PDE9, the isoform with the highest affinity for cGMP that is widely expressed in CNS structures, as well as PDE1, PDE 6 and PDE 11 isoforms (Bender and Beavo, 2006), and references therein). In this work, we show that treatment with the selective PDE5 inhibitor sildenafil reproduces in cryolesioned mice the neuroprotective effects previously observed with zaprinast in rats indicating that inhibition of PDE5 is involved. Furthermore, we show that sildenafil treatment up-regulates MT-I/II in glial cells around the cryolesion in wild-type mice and that the neuroprotective effect of sildenafil is absent in MT-I/II knockout mice. Taken together these results indicate that up-regulation of MT-I/II is involved in the neuroprotective effects of sildenafil in focal brain lesion.

2. MATERIALS AND METHODS

2.1. Animals and cryolesion procedure

Five to ten weeks old 129S1/SvImJ wild-type or homozygous MT-I/II deficient mice, with 129S7/SvEvBrd genetic background, were produced by suppression of their Mt1 and Mt2 genes by homologue recombination (Masters et al., 1994). Mice were initially obtained from Jackson Laboratories (Bar Harbor, MA), and colonies were maintained at the Autonomous University of Barcelona. All mice were kept under constant temperature, with a standard 12 h light/darkness cycle and with free access to water and food. Wild-type mice were separated in 4 groups: 2 groups of unlesioned animals (n=4) and 2 groups of cryolesioned animals (n=5-7), that were treated with vehicle or with sildenafil. Cryolesioned MT-I/II null mice were separated in 2 groups (n=4-6), that were treated with vehicle or with sildenafil. Animals were cryolesioned under tribromethanol anesthesia. The skull over the right frontoparietal cortex was exposed, and a focal cryoinjury was carried out on the surface of the brain by applying a dry ice pellet for 30s as previously described (Giralt et al., 2002b). Animals were allowed to recover and returned to the animal room until they were sacrificed 3 days post-lesion (dpl). Sildenafil (10 mg/kg, Pfizer) or vehicle (0.05 M NaOH) was administered subcutaneously 2 h before and 24 and 48 h after cryolesion. The dose of sildenafil is equivalent to 0.81 mg/kg in humans (Reagan-Shaw et al., 2008) and is in the range of those previously used by us and others in rodents (Pifarre et al., 2011; Puerta et al., 2009; Puzzo et al., 2009; Reneerkens et al., 2009; Zhang et al., 2005).

After decapitation, the entire ipsilateral cortex was dissected out and divided into two parts by cutting in the middle of the lesion. Half of the lesioned cortex was frozen in liquid nitrogen and stored at -80ºC until use for biochemical analysis and the other half was fixed in 4% paraformaldehyde for immunohistochemistry. All experiments were carried out in a human manner and were approved by the proper ethical committees.

2.2. Histological stainings

Paraformaldehyde-fixed cortical tissue was dehydrated and embedded in paraffin according to standard procedures and cut in serial coronal 5 µm sections. Immunostaining of the sections was performed as previously described (Pifarre et al., 2010). Antigen retrieval was done by the appropriate standard protocol (citrate or protease type XIV; Sigma). Afterwards, sections were incubated overnight at 4ºC with primary antibodies; rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; Dako, Cat. no. Z0334; 1:900), rabbit polyclonal anti-MT-I/II (obtained as described in Gasull et al., 1993) or goat polyclonal anti-malondialdehide (MDA; AbD Serotec, Cat. no. AHP797; 1/75). Afterwards, sections were incubated for 1 h at 25ºC with anti-rabbit or anti-goat purified antibodies conjugated to biotin (Vector Laboratories, Burlingame, CA, USA, 1: 300). Cells of myelo-monocytic lineage were stained with lectin from Lycopersicon esculentum conjugated with biotin (Sigma, 1:45), as previously described (Pifarre et al., 2010). Biotin conjugated secondary antibodies and lectin were detected using streptavidin/horseradish peroxidase (Vector, 1:300) and visualized using the peroxidase substrate Kit DAB (Vector). Control sections were incubated in the absence of primary antibodies to evaluate the

extent of non-specific binding of the secondary antibody. For detection and quantification of apoptotic cell death at a single cell level, we used the *In Situ Cell Death Detection Kit* from Roche Molecular Biochemicals (Barcelona, Spain) on paraffin brain sections (5–8 μm) following the protocol for difficult tissues according to the manufacturer's recommendations. This kit is based on labeling of DNA strand breaks (TUNEL reaction). Positive (DNAase I-treated) and negative (without terminal transferase) controls were included in each experiment.

2.3. Western blots

Brain tissue was homogenized in 10 volumes of ice-cold 50 mM Tris–HCl and 1 mM EDTA, pH 7.4, containing anti-protease (Roche) and anti-phosphatase (Sigma) cocktails, with a glass-Teflon Potter-Elvehjeim homogenizer mechanically driven at 800 rpm. Equal amounts of protein (40 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel (10%) electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene difluoride membranes (Immobilon PDVF; Millipore Iberica) at 100 V for 1.5 h at 4ºC. Membranes were blocked at 4ºC overnight in phosphate-buffered saline (pH 7.4) containing 5% non-fat dry milk and then incubated at 25ºC for 2 h with rabbit anti-GFAP (Sigma, 1:500), rabbit polyclonal anti-Iba1 (Wako, 1:3000) or monoclonal anti-actin (Sigma, 1:100 000) primary antibodies. Afterwards, membranes were washed twice with 0.05% Tween 20 (Sigma) in phosphate-buffered saline (PBS), incubated with the secondary antibody IgG–horseradish peroxidase-labeled (HRP; Amersham Life Science, Barcelona, Spain, 1:4000) and subsequently detected with a chemiluminescence detection kit (Immobilon;

Millipore). The intensity of bands was quantified by using Quantity One software (Bio-Rad Laboratories, Barcelona, Spain) and values were normalized for actin levels and expressed relative to the same animal that was included in all blots.

2.4. ELISA

Levels of MT-I/II were measured in homogenates of cryolesioned cortex using a competitive double-antibody ELISA assay as previously described (Gasull et al., 1994; Gasull et al., 1993). Briefly, 96-well plates were coated overnight at 25ºC with 12 ng of MT-II. After blocking for 2 h with 1% bovine serum albumin, 100 µl of solution containing the unknown sample (200 µg of total protein) or standard (0-400 ng MT-I/II) (all in triplicates) and 100 µl of antibody were added (final dilution of antibody 1:2000) and incubated for 2h at 37ºC. The plates were then incubated for 2h at 37ºC with anti-rabbit IgG-HRP conjugate (1:3000) before adding substrate solution. Three washes were done between each step with 0.05% Tween 20 in PBS. After stopping the reaction with sulphuric acid, the absorbance was read at 595 nm.

2.5. Quantification and statistical analysis

Quantification of GFAP, lectin, MDA and MT-I/II staining in cryolesioned animals was carried out at the border of the cortical lesion where inflammation is prominent using the Scion Image program. For each parameter analyzed, brain sections from four to seven animals per group were used and a mean value of 4–6 different 0.5 mm^2 areas per brain section was calculated. Quantification areas were from the central part of the lesion border where staining is more homogeneous than at the edges. Quantification of MDA staining was performed

in 5 x 0.5 mm^2 areas of the lesioned cortex at least 50 µm away from the lesion border using the Scion Image program. TUNEL-positive cells were counted by two independent investigators, who were blinded to animal identity and treatment in 5 x 0.5 mm^2 areas/brain section at least 50 µm from the lesion border. Positively stained cells were defined as cells with nuclear staining. Data are expressed as mean ± standard error (SEM) of the indicated number of animals. When comparing more than two groups, significance of differences was evaluated by a two-way ANOVA followed by Bonferroni's test, using strain and treatment as main factors. For comparison of two groups the Student's t-test was used.

3. RESULTS

3.1. Treatment with sildenafil alters the inflammatory response elicited by cryolesion in mice cortex

Cryoinjury to the cortex produces a profound inflammatory response with activation of resident microglia, recruitment of monocytes and reactive astrogliosis that occurs in a highly temporal-specific manner reaching maximal levels at 3 dpl (Penkowa et al., 1999). As shown in Fig 1A and B, treatment with the selective PDE5 inhibitor sildenafil (10 mg/kg, sc) 2 h before and 24 and 48 h after the lesion, significantly increased astroglial reactivity as judged by the enhancement of GFAP immunoreactivity caused by the lesion when compared to vehicle-treated controls but did not affect GFAP immunostaining in unlesioned animal cortex. In agreement with this, GFAP protein levels analyzed

by western blot in cortical homogenates were significantly increased by sildenafil in lesioned but not unlesioned animals (Fig. 2C). Two-way ANOVA showed significant interaction between lesion and treatment for GFAP staining ($p < 0.025$) indicating that sildenafil potentiates the effect of the cryolesion. This suggests that the treatment may accelerate the formation of the glial scar and the resolution of the injury.

Lectin staining reveals resting (ramified) and activated (round) microglia and infiltrating macrophages, as well as blood vessels. As expected, prominent recruitment and activation of microglia/macrophages (round/amoeboid lectin-positive cells) was observed at the border of the lesion in vehicle-treated mice (Fig. 2A). Treatment with sildenafil caused a reduction in lectin-positive cells and in the intensity of lectin staining (Fig. 2A, B). Accordingly, levels of the microglia/macrophage cytosolic protein Iba1 in homogenates of the lesioned cortex were significantly decreased in sildenafil-treated animals (Fig. 2C). These results indicate that the treatment decreases microglia/macrophage activation and support the anti-inflammatory potential of the drug.

Activated microglia/macrophages are known to produce reactive nitrogen and oxygen species that can induce oxidative stress. We investigated if sildenafil regulated this inflammatory response by staining for the toxic byproduct of lipid peroxidation MDA. As expected, numerous MDA-positive cells were observed around the lesion in control animals at 3 dpl. Sildenafil-treatment significantly decreased the number of MDA-positive cells as well as the intensity of staining

(Fig. 2 D, E), in agreement with an anti-inflammatory and neuroprotective action of this PDE5 inhibitor.

3.2. Treatment with sildenafil increases MT-I/II expression in the cryolesioned cortex and reduces apoptotic cell death in wild-type but not in MT-I/II knockout mice

The alterations in the inflammatory response to the cryolesion induced by sildenafil are similar to those we previously observed in mice overexpressing MT-I/II (Giralt et al., 2002b). Additionally, MT-I/II overexpression decreased apoptotic cell death, whereas MT-I/II deficiency potentiated the neurodegeneration caused by cryolesion (Penkowa et al., 1999). Thus we set to investigate if MT-I/II could be involved in the neuroprotective effect of PDE5 inhibition. We first examined if treatment with sildenafil was affecting MT-I/II expression in the cryolesioned cortex. As shown in Fig. 3A, we found an increase in MT-I/II immunoreactive cells in the border of the lesion in sildenafil-treated animals compared to vehicle-treated controls. Quantification of MT-I/II immunostaining around the lesion showed significantly higher levels in animals treated with sildenafil (Fig. 3B). Competitive ELISA analysis of the lesioned cortex homogenates confirmed that treatment with sildenafil significantly increased MT-I/II protein levels (Fig. 3C). Closer examination of consecutive sections of the lesioned cortex stained for MT-I/II, GFAP or lectin (Fig. 3A), showed that cells presenting high immunoreactivity for MT-I/II in sildenafil-treated animals were more abundant in areas rich in GFAP-stained cells than in areas of round heavily lectin-stained amoeboid microglia/macrophages, suggesting a preferential induction of MT-I/II in astrocytes.

We next investigated the effect of sildenafil treatment on apoptotic cell death in cryolesioned cortex from wild-type and MT-I/II knockout mice. Apoptotic cell death was evaluated by counting TUNEL-positive cells in an area distant at least 50 μm from the border of the lesion. As shown en Fig. 4A and B, numerous TUNEL-positive cells were observed in vehicle-treated wild-type animals and numbers were even higher in MT-I/II deficient mice in agreement with previous results (Penkowa et al., 2001). In contrast, a drastic reduction in TUNEL-positive cells was observed in sildenafil-treated wild-type mice; however the drug was not able to reduce apoptotic cell death in MT-I/II deficient mice. Two-way ANOVA shows high interaction ($p<0.001$) between strain and treatment indicating that the strain is critical for the effect of sildenafil. Staining for GFAP and lectin also showed a lack of effect of sildenafil in the glial response in cryolesioned MT-I/II deficient mice (not shown). Taken together, these results indicate that induction of MT-I/II is involved in the neuroprotective effect of PDE5 inhibition in the brain cryoinjury model.

4. DISCUSION

Accumulating evidence demonstrates that treatment with selective PDE5 inhibitors, such as sildenafil and analogues, is neuroprotective in various animal models of CNS injury through different mechanisms. Activation of antiapoptotic pathways, angiogenesis and neurogenesis appears to be responsible for PDE5 inhibitor efficacy in promoting functional recovery after stroke in rats (Menniti et al., 2009; Zhang et al., 2005; Zhang et al., 2006), and increases in pCREB and BDNF have been implicated in protection against 3-nitropropionic acid toxicity

(Puerta et al., 2010) and in improving synaptic function and cognitive deficits in Alzheimer's disease models (Cuadrado-Tejedor et al., 2011; Puzzo et al., 2009). Additionally, we have recently reported that sildenafil ameliorates clinical symptoms of EAE protecting myelin and axons and these beneficial effects are associated with a potent anti-inflammatory action (Pifarre et al., 2011). Results from our previous work with zaprinast in rats (Pifarre et al., 2010) and from the present work with sildenafil in mice show decreased microglia/macrophage activation and decreased oxidative stress associated with decreased apoptotic cell death supporting the involvement of an anti-inflammatory action in the neuroprotective effect of PDE5 inhibition in brain injury. Furthermore, in both these studies, we observed enhanced astrogliosis around the lesioned area which according to previous studies for other conditions having the same effect in cryolesion models is expected to accelerate the formation of the glial scar and the regeneration of the lesioned tissue (Giralt et al., 2002b; Klementiev et al., 2008; Molinero et al., 2003). These results suggest that inhibition of PDE5 in different CNS cell types can contribute to neuroprotection.

PDE5 is most highly expressed in smooth muscle, notably in the vasculature, where the enzyme regulates NO/cGMP-mediated vascular relaxation (Kass et al., 2007). In the CNS, in addition to smooth muscle of meningeal arteries and a few smaller blood vessels (Menniti et al., 2009), immunoreactivy for PDE5 has been shown in cerebellar Purkinje cells, motor neurons, messencephalic neurons and scattered cortical neurons (Bender and Beavo, 2004; Menniti et al., 2009; Nakamizo et al., 2003). PDE5 immunoreactivity has been also reported in glial cells in cortex (Menniti et al., 2009) and spinal cord white matter

(Nakamizo et al., 2003) but the type of glial cell was not clearly identified in those studies. Interestingly, 48h after middle cerebral artery occlusion in rats increased PDE5 immunoreactivity was reported both in blood vessels and in microglia-like cells in the areas of ischemic damage, suggesting that PDE5 inhibitors enhance functional recovery in this model of stroke through effects on vascular and microglial cell function (Menniti et al., 2009). Expression of PDE5 in glial cells is also supported by data obtained in vitro. We have shown that sildenafil and zaprinast increase cGMP accumulation in primary cultures of astroglia and microglia and that mRNA for PDE5 can be detected in these cell types (Prado et al., 2010). Furthermore, Zhao et al., (Zhao et al., 2011) recently reported that sildenafil attenuates LPS-induced pro-inflammatory responses in a murine microglial cell line (N9). An anti-inflammatory role of cGMP formed in response to atrial natriuretic peptide has been well documented in macrophages (Vollmar, 2005), and also shown in primary rat microglial cultures (Moriyama et al., 2006). Anti-inflammatory effects of non-hydrolizable cGMP analogues were also reported in N9 microglia (Paris et al., 2000). On the other hand, in cultured astrocytes stimulation of the cGMP-PKG pathway has been reported to increase GFAP expression (Brahmachari et al., 2006) and to induce rearrangements of the GFAP and actin cytoskeleton and accelerated astrocyte migration in a scratch-wound assay (Boran and Garcia, 2007). Thus, PDE5 inhibitor induced increases in cGMP in astrocytes are possibly involved in the observed enhancement of astrogliosis around the cryolesion. Following CNS injury, astrogliosis and glial scar formation has generally been considered an impediment for axon generation. However, increasing evidence correlates

enhanced astrogliosis with positive outcomes in different CNS injury models (Giralt et al., 2002b; Klementiev et al., 2008; Molinero et al., 2003).

An important new finding of this work is the identification of MT-I/II induction as a mechanism involved in the neuroprotection afforded by sildenafil. This conclusion is based on the complementary observations that expression of MT-I/II is increased in the cryolesioned cortex of sildenafil-treated animals, and that antiapoptotic and glial effects of sildenafil are not observed in MT-I/II knockout mice. MT-I/II proteins are expressed throughout the CNS and are highly inducible by a wide range of stimuli, including inflammatory and oxidative agents (Sato and Bremner, 1993; West et al., 2008; Yagle and Palmiter, 1985). We and others have previously shown that overexpression of MT-I/II and exogenous administration of these proteins is neuroprotective in cryoinjured mice and other neurotrauma models by similar mechanisms as sildenafil (Chung et al., 2003; Giralt et al., 2002b; van Lookeren Campagne et al., 1999). In the normal brain, MT-I/II isoforms are expressed in astrocytes and to a lower extend in neurons, but not in microglia and oligodendrocytes. However, in response to injury MT-I/II are up-regulated in reactive astrocytes and also in microglía (Hidalgo et al., 2001). In this work comparison of immunostaining for MT-I/II with that of GFAP and lectin in consecutive sections of the lesioned cortex indicated that sildenafil treatment enhanced MTs expression in areas of reactive gliosis and immunoreactivity appeared stronger in areas with abundant hypertrophied reactive astrocytes (strong GFAP-stained cells) than in areas with highly reactive microglia/macrophages (lectin-positive amoeboid cells) closer to the wound (Fig. 4), suggesting a preferential induction of MT-I/II in astrocytes. Several in vivo and in vitro reports support a neuroprotective and

neuroregenerative action of astrocyte MT-I/II. Within the astrocyte, MTs may be part of the mechanisms by which these cells are able to handle reactive oxygen species. Additionally, recent evidence indicates that MT-I/II secreted by reactive astrocytes can be internalized by neurons and promote regenerative growth after injury (Chung et al., 2008). Furthermore, it has been demonstrated that exogenous MTs induce a reactive morphology and elevated GFAP expression in cultured astrocytes. Interestingly, the MT-induced astrogliosis was permissive to neurite outgrowth (Leung et al., 2010).

In summary, the parallelism in the anti-inflammatory, antioxidant, astrogliosis-inducing and neuroprotective effects of sildenafil and MT-I/II overexpression in the rodent cortical cryolesion model, together with the absence of a sildenafil effect in MT-I/II null mice and the up-regulation of MTs in glial cells in the lesioned cortex of sildenafil-treated animal, strongly support that MT-I/II induction is involved in the beneficial effects of PDE5 inhibitors in acute CNS injury.

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FIGURE LEGENDS

Fig. 1. Treatment with sildenafil increases astroglial reactivity in mice cryolesioned cortex.

(A) GFAP immunohistochemistry (IHC) in coronal sections of cortex at 3 dpl was prominent around the lesion and increased significantly after treatment with sildenafil. Inserts: higher magnification of squared areas showing reactive astrocytes with swollen cell bodies and enhanced immunostaining. (B) GFAP immunostaining was quantified with the Scion Image software in 4–6 areas of 0.5 mm^2 chosen at random in unlesioned cortex or from the border of the lesion (excluding the edges) in cryolesioned animals. (C) GFAP protein levels analyzed by western blot (WB) in brain cortical homogenates (40 µg protein). Results in (B) and (C) are means±SEM of 3-4 vehicle- and 5-7 sildenafil-treated mice. Two-way ANOVA with lesion and treatment as main factors revealed significant differences between unlesioned and lesioned animals ($p<0.001$) in

both GFAP IHC and WB analysis, whereas for sildenafil the effect was significant in the former ($p<0.025$) and marginally significant ($p=0.07$) in the latter analysis. The interaction between both factors was significant in the IHC study ($p<0.025$) and marginally significant ($p=0.07$) in the WB study. Post-hoc analysis revealed that sildenafil increased GFAP levels in lesioned animals in both GFAP IHC and WB analysis (*$p<0.05$; **$p<0.01$). Bar: 200 µm.

Fig. 2. Treatment with sildenafil decreases microglia/macrophage activation and lipid peroxidation in mice cryolesioned cortex.

(A) Lectin histochemistry (HC) revealed numerous activated microglia/macrophages around the lesion at 3 dpl. Treatment with sildenafil decreased the intensity of lectin staining and the density of positive cells. Asterisk: necrotic area. (B) Quantification of lectin HC was carried out as for GFAP IHC (n=3-6) and analyzed by two-way ANOVA, which revealed a significant effect of the lesion ($p<0.001$) and of sildenafil ($p<0.025$); the interaction between lesion and treatment was only marginally significant ($p=0.09$). Nevertheless, post-hoc analysis revealed that sildenafil treatment significantly reduces lectin levels in lesioned animals (**$p<0.01$). (C) Protein levels of the specific microglia/macrophage marker Iba-1 analyzed by western blot in lesioned cortex homogenates (40 µg protein) are significantly decreased in sildenafil-treated animals (*$p<0.05$; Student's t-test). (D) Staining for the toxic byproduct of lipid peroxidation MDA was strong in numerous cells around the lesion at 3 dpl (arrows) and was reduced by treatment with sildenafil. (E) Quantification of MDA staining (*$p<0.05$; Student's t-test). Results are means±SEM. Bar: 50 µm (A); 20 µm (D).

Fig. 3. Sildenafil treatment up-regulates MT-I/II expression in the cryolesioned cortex. (A) GFAP (left), MT-I/II (middle) and lectin (right) staining in consecutive serial sections of cryolesioned cortex shows an increase of MT-I/II positive cells in sildenafil-treated animals more prominent in areas of intense astrogliosis (1) than in areas of microglia/macrophage activation (2). Quantification of MT-I/II immunostaining (B) and measurement of MT-I/II protein levels by competitive ELISA in cortex homogenates (C), revealed significant increases in sildenafil-treated animals. Results are means±SEM of 4 vehicle- and 7 sildenafil-treated mice. Significant differences: *p<0.05, **p<0.01, (Student's t-test). Bar: 50 µm. Asterisk: necrotic area. Inserts: higher magnification of squared areas. Arrows point to MT-I/II positive cells.

Fig. 4. Sildenafil treatment drastically reduces apoptotic cell death in the cryolesioned cortex of wild-type mice but not of MT-I/II-deficient mice. (A) Numerous TUNEL-positive cells were present in the lesioned cortex in wild-type and MT-I/II-deficient mice and sildenafil dramatically decreased their numbers in wild-type but not in MT-I/II-deficient mice. (B) TUNEL-positive cells were counted in 5x0.5 mm^2 areas/brain section; results are means±SEM of 4 vehicle- and 5-7 sildenafil-treated mice. Two-way ANOVA revealed that the strain ($p<0.001$) significantly affected the number of apoptotic cells. The interaction between treatment and strain was highly significant ($p<0.001$). Post-hoc analysis revealed that sildenafil treatment significantly reduces cell death in wild-type mice but not in MT-I/II-deficient mice (**$p < 0.01$).

Figure 1

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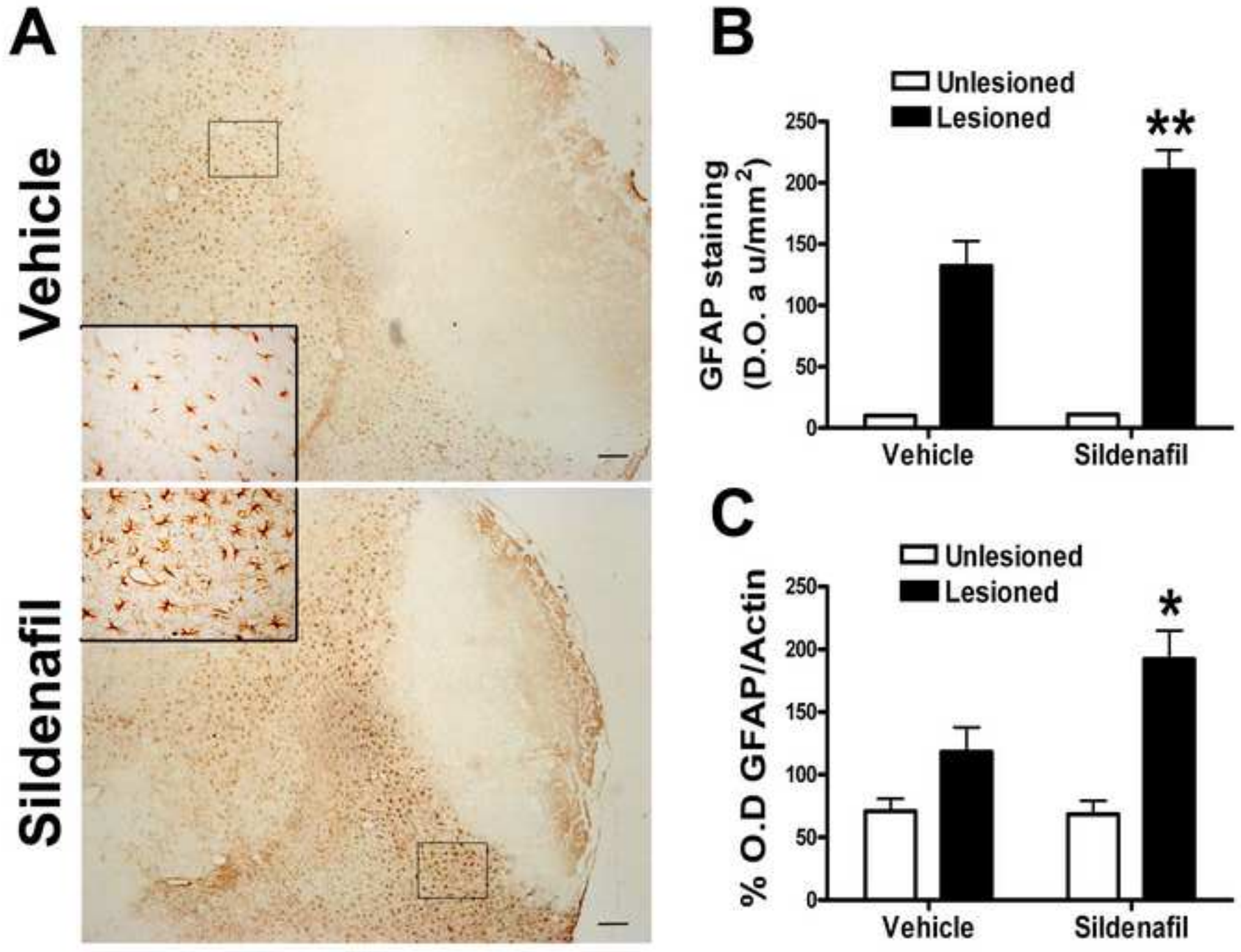

Figure 2

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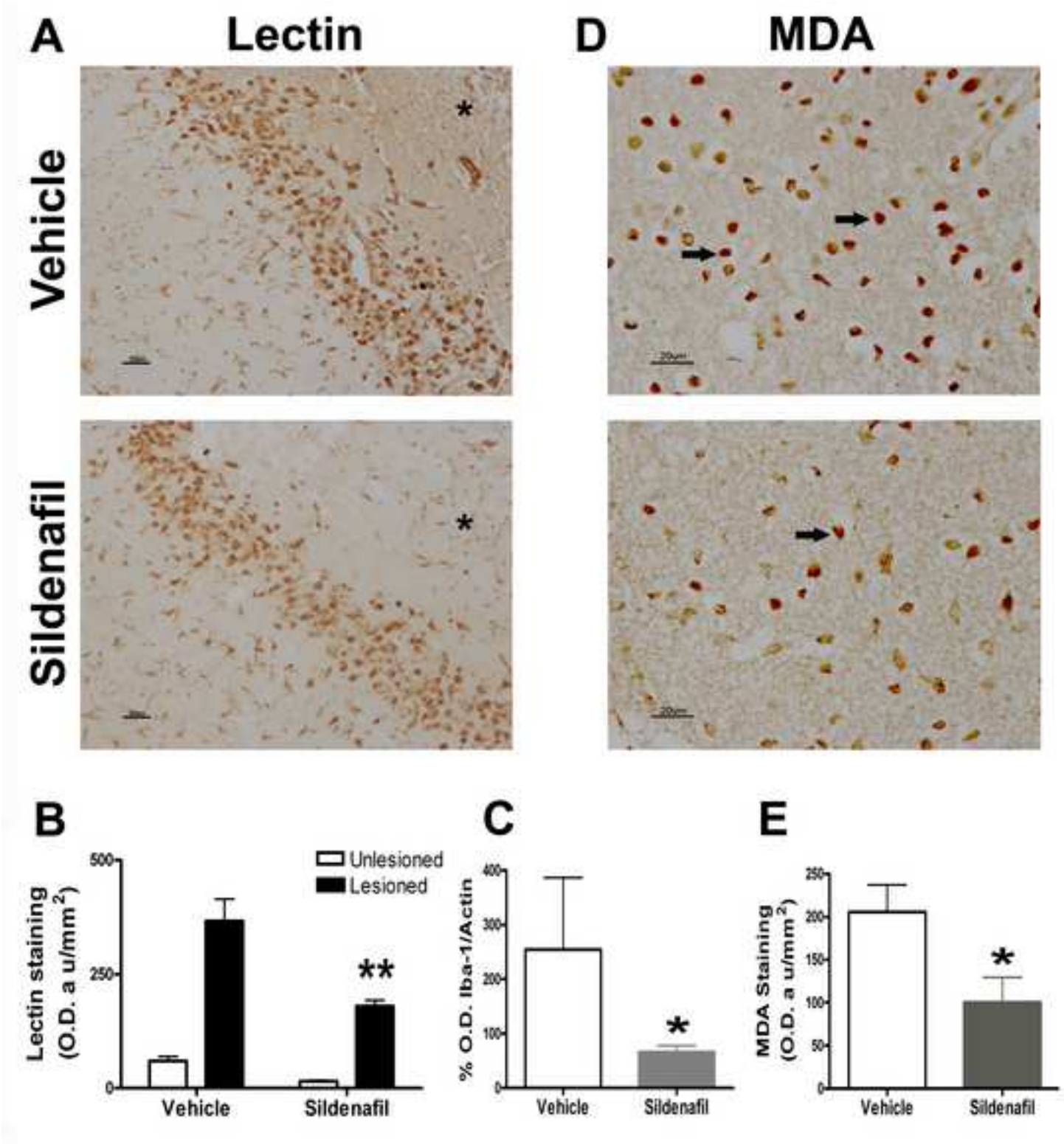

Figure 3

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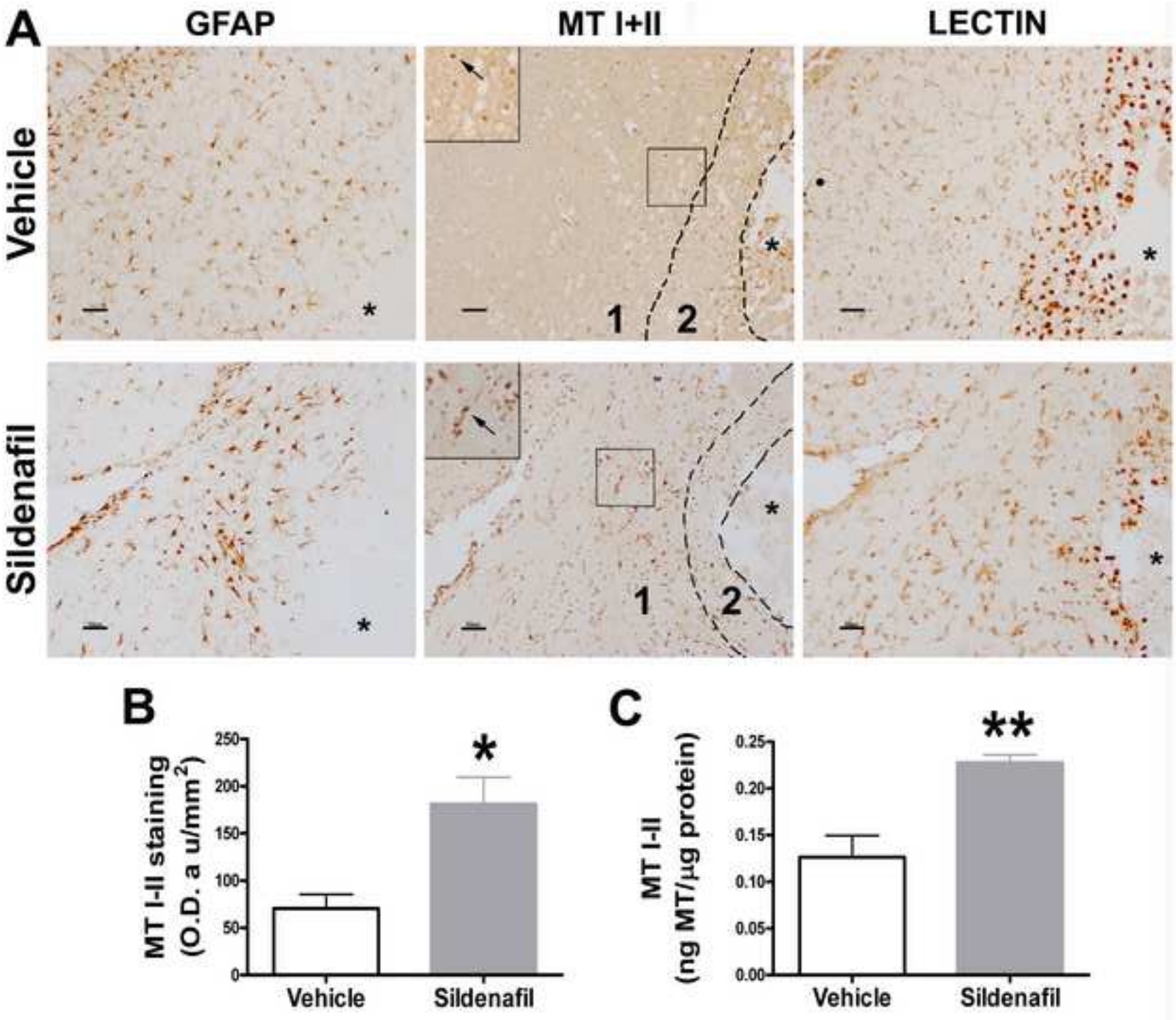

Figure 4

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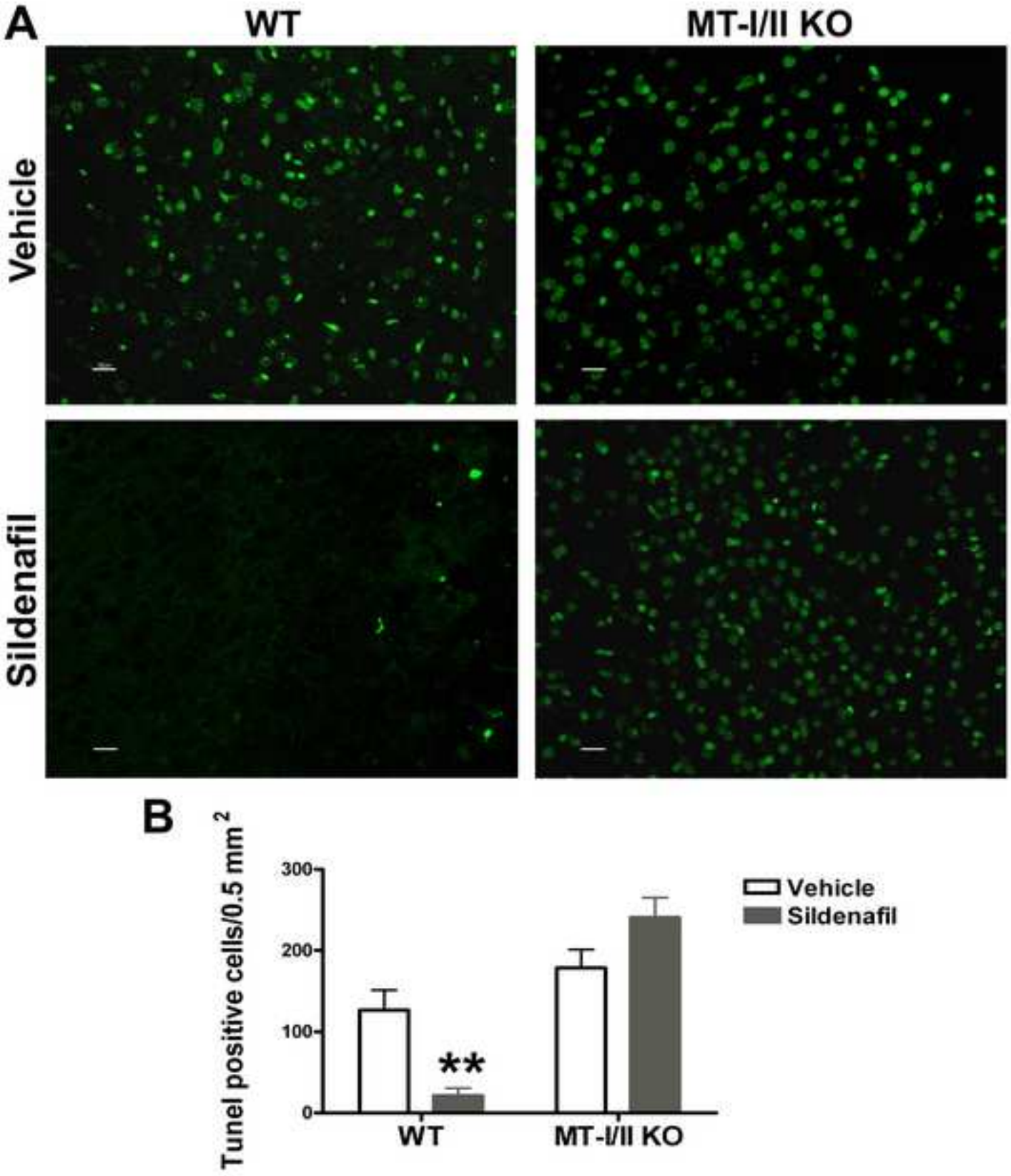

HIGHLIGHTS

-Sildenafil alters the inflammatory response elicited by cryolesion in mice cortex.

- Sildenafil increases MT-I/II expression in mice cryolesioned cortex.

- Sildenafil reduces apoptotic cell death in WT but not in MT-I/II KO mice.