Tesis Doctoral

Neuroprotective and immunomodulatory roles of anti-inflammatory neuropeptides on central nervous system disorders

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Granada, Septiembre 2015

Editor: Universidad de Granada.Tesis Doctorales
Autora: Marta Pedreño Molina
ISBN: 978-84-9125-291-7
URI: http://hdl.handle.net/10481/40979

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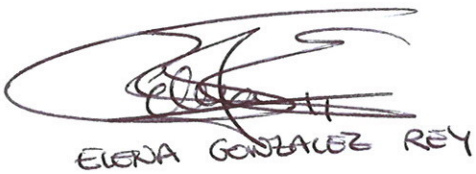

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ABBREVIATIONS

AD	Alzheimer's disease
ADNP	Activity-dependent neuroprotector homeobox
AM	Adrenomedullin
APC	Antigen presenting cell
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CCL2	(C-C motif) ligand 2
CCL5	(C-C motif) ligand 5
ConA	Concanavalin A
CNS	Central nervous system
CSF	Cerebrospinal fluid
CRLR	Calcitonin receptor-like receptor
CST	Cortistatin
CXCL10	C-X-C motif chemokine 10
DAT	Dopamine active transporter
DC	Dendritic cell
DLN	Draining lymph nodes
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
GHSR	ghrelin receptor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPCR	G-protein-coupled receptor
Iba1	ionized calcium-binding adapter molecule

IFNγ	Interferon gamma
iNOS	inducible nitric oxid synthase
LB	Lewy Bodies
LPS	Lipopolysaccharide
MAO B	Monoamine oxidase B
MBP	Myelin basic protein
MHC	Major Histocompatibility Complex
MPP	1-Methyl-4-phenylpyridinium
MOG	Myelin oligodendrocyte glycoprotein
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple Sclerosis
NO	Nitric oxide
Olig-2	oligodendrocyte lineage transcription factor 2
OPC	Oligodendrocyte precursor cell
OPN	Osteopontin
PD	Parkinson's disease
PDGF	platelet-derived growth factor
PLP	Proteolipid protein
RAMP	Receptor-activity-modifyins protein
ROS	Reactive oxygen species
Syn	Synuclein
SSTR	Somatostatin receptor
Th	T helper
TH	Tyrosine hydroxylase
TNFα	Tumor necrosis factor alpha

Treg	Regulatory T
VIP	Vasoactive intestinal peptide

SUMMARY

Nervous system disorders affect to more than one billion people worldwide and constitute around 35% of the total pool of all diseases in Europe. Due to their high severity and incidence, Multiple Sclerosis (MS) and Parkinson's disease (PD) are two important diseases of central nervous system (CNS). MS is a neurodegenerative and autoimmune disease characterized by a chronic inflammatory demyelination and axonal damage of the CNS, being the main cause of non-traumatic disability in young adults. MS pathology is mediated by a Th1/Th17 autoreactive response against the myelin sheath. On the other hand, PD is the second most common neurodegenerative disorder in adults over the age of 65. It is characterized by a progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNpc) and their projections to the striatum. In the last years it has been accepted that the immune system also has a role in PD, where neuroinflammation seems to be a hallmark of its pathogenesis. Due to their complex pathophysiology, there is no effective cure for both disorders, being necessary looking for new treatments that act through a multitarget approach. In the last years, our lab has identified several neuropeptides that show anti-inflammatory and immunomodulatory profiles. Among them, adrenomedullin and cortistatin seem also to have neuroprotective roles. The aim of the present study is to investigate the role of both neuropeptides in diseases of the CNS characterized by a disrupted immune response leading to neuroinflammation and neurodegeneration.

We used a well-established pre-clinic model of MS, the experimental autoimmune encephalomyelitis (EAE) to assay the therapeutic potential of adrenomedullin. We demonstrated that adrenomedullin is able to reduce the incidence and severity of the disease, even when the disease was fully established. Moreover, the treatment of mice suffering EAE with adrenomedullin reduced the inflammatory infiltration, the number of demyelinating plaques, and the production of pro-inflammatory mediators in spinal cord and/or brain. Furthermore, adrenomedullin was able to modulate the Th1/Th17 autoreactive response in the lymphoid organs and in the CNS. Interestingly, this effect was specific of the encephalitogenic antigent, and

it seems to be related with the fact that the treatment with adrenomedullin generated immune tolerance by inducing regulatory T (Treg) cells and tolerogenic dendritic cells that were able to control the progression of the disease. Additionally, adrenomedullin promoted neuroprotective responses by up-regulating the expression of neurotrophic factors. Accordingly, using a focal model of demyelination induced by the injection of the toxin lysolecithin, we demonstrated that adrenomedullin decreased the demyelinating area and increased the number of oligodendrocytes, suggesting a role on remyelination. Otherwise, adrenomedullin inactivates resident cells of CNS in presence of pro-inflammatory stimulus.

On the other hand, we previously described the beneficial effect of cortistatin in EAE. Therefore, we decided to analyze the potential therapeutic effect of this neuropeptide in a preclinical mouse model of PD induced by acute exposure to the neurotoxin MPTP. We observed that treatment with cortistatin reduced the loss of dopaminergic neurons in the SNpc and their projections to the striatum caused by MPTP injection. This effect correlated with an improvement in the locomotor activity. Furthermore, in an in vitro model of PD, cortistatin decreased cell death of dopaminergic neurons caused by MPP+. Moreover, cortistatin diminished the presence and activation of microglia cells and astrocytes in the SNpc of MPTP-mice, suggesting a role of this neuropeptide in the regulation of neuroinflammation associated to this disorder. Furthermore, cortistatin induced the expression of neurotrophic factors. We also observed that cortistatin modulated the immune response of glial cells in the presence of α-synuclein, as extracellular stimuli. Finally, animals deficient in cortistatin also revealed the relevance of this neuropeptide in the development and progression of this neurodegenerative disorder.

Together, our results suggest that adrenomedullin and cortistatin emerge as potential new therapeutic agents that combine their anti-inflammatory, immunomodulatory and neuroprotective properties to regulate at multiple levels the progression of CNS disorders.

RESUMEN

Las enfermedades del sistema nervioso central (SNC) afectan a más de mil millones de personas en todo el mundo, y constituyen alrededor del 35% del total de enfermedades en Europa. Entre ellas, destacan la esclerosis múltiple (EM) y la enfermedad de Parkinson (EP) debido a su alta severidad e incidencia. La EM es una enfermedad neurodegenerativa y autoinmune caracterizada por una desmielinización e inflamación crónica, que cursa con daño axonal en el SNC, siendo la principal causa de discapacidad no traumática en jóvenes adultos. La patología de la EM está mediada por una respuesta autoreactiva tipo Th1/Th17 contra la vaina de la mielina. Por otra parte, la EP es el segundo desorden neurodegenerativo más común, por detrás de la enfermedad de Alzheimer, que afecta a personas mayores de 65 años. Se caracteriza por una degeneración progresiva de neuronas dopaminérgicas de la sustancia negra pars compacta (SNpc) y sus proyecciones en el estriado. En los últimos años se ha demostrado que el sistema inmunitario juega un papel importante en el desarrollo de la EP, siendo la neuroinflammación un proceso importante en su patogénesis. Debido a sus complejas patofisiologías no existe una cura eficaz para estas enfermedades. Por este motivo se hace necesaria la búsqueda de nuevos tratamientos que tengan varias dianas simultáneas con un papel relevante en la progresión y desarrollo de estos desórdenes. En los últimos años, nuestro laboratorio ha identificado distintos neuropéptidos con un perfil anti-inflamatorio e inmunomodulador. Entre ellos se encuentran adrenomedulina y cortistatina, de los que además se ha demostrado un potencial papel neuroprotector. En esta tesis, hemos trabajado con ambos neuropéptidos caracterizando su potencial terapéutico en EM y EP.

Para demostrar el papel terapéutico de adrenomedulina en EM usamos un modelo pre-clínico de esta enfermedad, la encefalomielitis autoinmune experimental (EAE). Demostramos que adrenomedulina es capaz de reducir la incidencia y severidad de la enfermedad, incluso cuando ésta se encuentra plenamente establecida. Además, el tratamiento redujo la infiltración de células inflamatorias, el número de placas de desmielinización y la producción de mediadores pro-inflamatorios en cerebro y médula espinal. Por otra parte, adrenomedulina fue capaz de modular la respuesta

autoreactiva tipo Th1/Th17 en órganos linfoides y en el SNC. Interesantemente, el efecto de adrenomedulina fue específico de antígeno y parece estar relacionado con su capacidad de generar tolerancia inmunológica mediante la inducción de células T reguladoras y células dendríticas tolerogénicas que son capaces de controlar la progresión de la enfermedad. Además, adrenomedulina promovió respuestas neuroprotectoras induciendo la expresión de factores neurotróficos. Corroborando estos resultados, mediante el uso de un modelo focal de desmielinización inducido por la inyección de la toxina lisolecitina en el cuerpo calloso, demostramos que el tratamiento con adrenomedulina redujo el área de desmielinización e incrementó el número de oligodendrocitos en la zona lesionada, sugiriendo un papel de este neuropéptido en promover remielinización. Asimismo, adrenomedulina fue capaz de inactivar células residentes del SNC en presencia de un estímulo pro-inflamatorio.

Por otra parte, basándonos en un estudio previo en el que demostrábamos el efecto beneficioso de cortistatina en EAE, decidimos estudiar el efecto terapéutico de este neuropéptido en un modelo pre-clínico de EP, inducido por la intoxicación aguda con la neurotoxina MPTP. Observamos que el tratamiento sistémico con cortistatina reducía la pérdida de neuronas dopaminérgicas en la SNpc y sus proyecciones en el estriado. Este efecto se correlacionó con una mejora de la actividad locomotora de los ratones. Además, mediante un modelo in vitro de EP, cortistatina fue capaz de proteger de la muerte neuronal causada por MPP+, la forma tóxica de MPTP. Igualmente, cortistatina disminuyó la presencia y activación de microglía y astrocitos en la SNpc de ratones inyectados con MPTP, sugiriendo un papel de este neuropéptido en la regulación de la neuroinflamación asociada a esta enfermedad. Por otra parte, el tratamiento con cortistatina aumentó la producción de factores neurotróficos. Observamos también que cortistatina moduló la respuesta inmunitaria de células gliales en presencia de α-sinucleína. Finalmente, la inducción del modelo de EP en ratones deficientes en cortistatina resultó en una mayor sensibilidad a la neurotoxina, sugiriendo la participación endógena de este neuropéptido en el desarrollo y progresión de esta enfermedad.

En conjunto, estos resultados sugieren que adrenomedulina y cortistatina emergen como nuevos agentes terapéuticos, con capacidad de regular la progresión de enfermedades del SNC actuando a diferentes niveles y combinando propiedades anti-inflamatorias, inmunomoduladoras y neuroprotectoras.

1. INTRODUCTION

The fact that the immune and nervous systems interact with each other was suspected and postulated more than a century ago. More recently it has been clearly established that the immune system communicates in a bidirectional way with the nervous system. This dialog implies that the immune system acts as a sixth sense informing the nervous system that a systemic immune/ inflammatory response to infection or tissue injury is occurring. The nervous system responds to this call by modifying behavioral responses (fever, reduction of locomotor activity, appetite and reproduction) and by triggering molecular pathways, in general anti-inflammatory and immunosuppressive in nature, which restrain and limit the immune response. This implies that both systems do not function as independent systems, being closely interconnected, functionally and anatomically in order to maintain the organism in homeostasis. In fact, they share a common pool of molecules that are conserved in invertebrates and vertebrates, supporting the hypothesis that both these systems could have a common evolutionary origin and that could coevolved together (1, 2). Because of the presence of the blood-brain barrier and the lack of a lymphatic system, the central nervous system (CNS) has been traditionally considered as an immune-privileged site. However, evidence now demonstrates that, under some pathological conditions, such as a trauma, infection or stroke (3), inflammatory and immune responses in the CNS can occur and uncontrolled immune responses are in the base of the most prevalent neurodegenerative disorders, including multiple sclerosis (MS), Parkinson's disease (PD), Alzheimer Disease (AD) or amyotrophic lateral sclerosis (ALS) (4). Thus, new concerns about neurodegenerative diseases involve taking into account the connection between the neuroendocrine and the immune systems and to consider it as a relevant context for understanding the aetiology, development and progression of these disorders as well as for developing new therapeutic strategies. Therefore, let us to introduce some general concepts about both systems and the actors involved in their communication.

1.1. Immune system: the daunting job of defending against pathogens while maintaining tolerance against self

The immune system is a whole of biological structure that protects us against disease. To perform its function, the immune system has developed two mechanisms, the innate and adaptive immunity.

The innate response is mediated by pattern recognition receptors (PRRs) that are expressed by immune cells. These receptors recognize pathogen-associated molecular patterns (PAMPs) belonging to pathogens. In this way, white blood cells such as neutrophils and macrophages recognize pathogens and initiate an inflammatory response characterized by phagocytosis and the release of inflammatory mediators such as cytokines, chemokines, reactive oxygen species… (5). A successful inflammatory response results in the elimination of the pathogen, and is followed by a resolution and repair phase (6). However, although the inflammatory response is necessary in order to remove the dangerous insult, the inflammation needs to be regulated, because a breakdown in this regulation can result in the emergence of inflammatory chronic diseases (7).

On the other hand, the adaptive immune recognition is mediated by antigen receptors that are generated by somatic recombination leading to the generation of a diverse repertoire of receptors with a wide rank of specificities and the potential to recognize almost any antigenic determinant in a specific manner. These receptors are found in T and B lymphocytes. In this way, after antigen presentation by antigen-presenting cells (APCs) such as dendritic cells and macrophages, B and T cells suffer activation and initiate an immune response against pathogens. B cells perform an antibody-mediated immunity and T cells a cell-mediated immunity (8).

According to function and surface markers, T cells can be classified in T helper ($CD4^+$ T cell) cells that assist other immune cells in the immune response, and cytotoxic T cells ($CD8^+$ T cell) that kill tumour cells or cells infected by virus. Following activation, $CD4^+$ T cells can differentiate towards different subsets (see Fig. 1.1), including Th1, Th2, Th17 or regulatory T cells (Treg), depending on microenvironmental factors such as cytokine milieu. The differentiation towards each lineage depends on the up-

regulation of specific transcription factors, as Tbet for Th1, GATA-3 for Th2, RORγT for Th17 and Foxp3 for Tregs. It is generally accepted that Th1 subset drives the immune response against intracellular pathogens, Th17 against extracellular pathogens, Th2

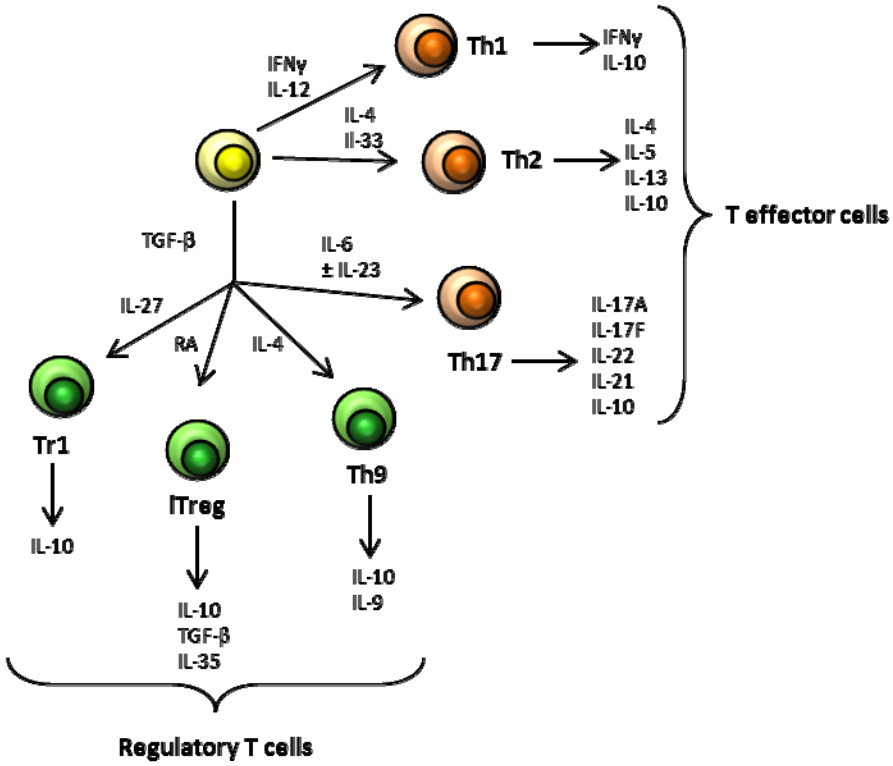


Figure 1.1: Primary polarization of diverse CD4+ T cell subsets. Depending on cytokine milieu, T naïve cells (yellow) will polarize toward different subsets of T effector cells and regulatory T cells. Modified from (9).

promotes humoral responses, and Treg cells prevent the development of autoimmunity suppressing activated T cell expansion (10). T cell differentiation has been traditionally considered linear and irreversible, but recent findings show that this process is flexible depending on cytokine milieu (11), and Th cells can change their profile of cytokine production (12).

Therefore, in the presence of pathogens, the innate and adaptive immunity are combined in order to control the infection. However, there must be a homeostasis in this process, since a wrong or exacerbated response can drive to chronic inflammation that is related to autoimmune diseases, disorders in which our own proteins are attacked like foreign antigens by autoreactive T and B cells that have lost tolerance

capability (13). For this reason, the regulation of the immune response and the mechanisms of tolerance are crucial for the correct function of the body.

The immune system has to be able to respond against foreign antigens, nevertheless it has to be tolerant to self-antigens from our body. Therefore, the immune system has developed different mechanisms to distinguish between self and foreign antigens, called immune tolerance that is necessary to maintain homeostatic balance. The immune tolerance is initially exerted in the thymus and in bone marrow (called central tolerance) for educating T cells and B cells, respectively. In the case of the thymus, it is mounted a first positive selection of T cells with low avidity to recognize peptides bound to MHC molecules, and then a second negative selection of self-reactive T cells (14). However some autoreactive T cells can escape to this control and they must be inactivated in the periphery (out of thymus) in order to prevent autoreactive reactions and subsequent autoimmune diseases. Autoreactive T cells are controlled in the periphery by different mechanisms, including the induction of T cell ignorance, anergy and deletion in the absence of costimulatory stimulation and an inflammatory milieu, and the suppressive action of two critical immune cell populations, such as tolerogenic dendritic cells and Treg cells (15).

The activation of T cells occurs through antigen presentation by APCs, such as dendritic cells (DCs). DCs can present antigens to CD8+ T cells or CD4+ T cells through Major Histocompatibility Complex type I or II (MHC class I or MHC class II), respectively (16). To perform the antigen presentation, antigens are captured, internalized and processed by DCs, inducing DC maturation (by up-regulation of MHCII and co-stimulatory molecules such as CD80 and CD86) while they migrate to the lymph nodes, where they present the antigen to T cells. There are evidences that suggest that depending on cytokine milieu DCs perform distinct maturation processes toward tolerogenic DCs or immunogenic DCs (see Fig. 1.2). Contrary to immunogenic DCs, tolerogenic DCs have low expression of surface MHC class II and co-stimulatory molecules and low production of inflammatory cytokines upon stimulation (17). In this way, when a tolerogenic DC tries to present the antigen to T cells it induces anergy or promotes apoptosis in T effector cells and alternatively, they generate Treg cells (17).

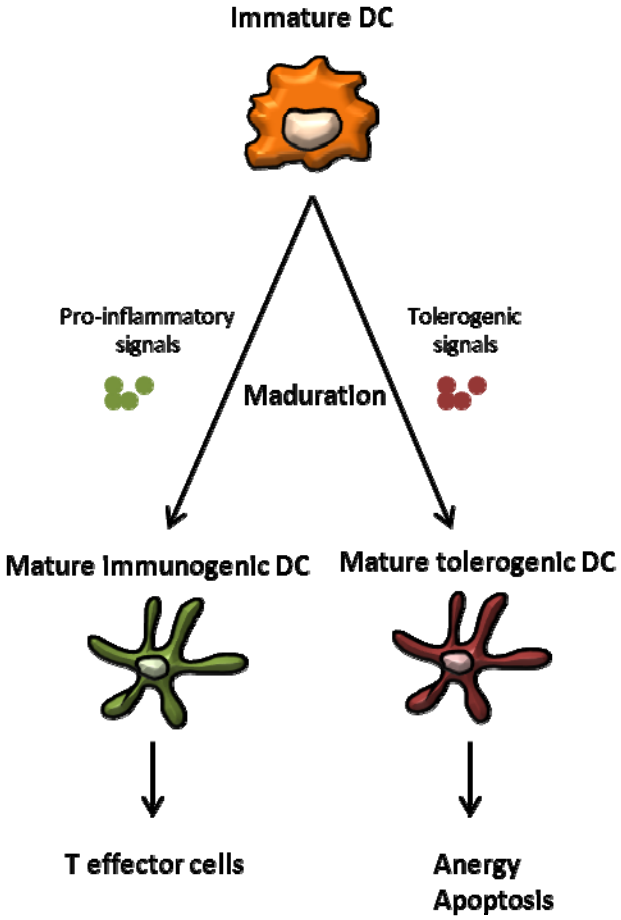


Figure 1.2: Maturation of dendritic cells. Depending on the presence of pro-inflammatory or tolerogenic signals, immature dendritic cells will mature toward immunogenic dendritic cells or tolerogenic dendritic cells. Thus, proinflammatory signals such as IL-6, TNFα or LPS produce dendritic cell maturation to an immunogenic phenotype, whereas, tolerogenic signals such as IL-10 or TGFβ favour a differentiation toward tolerogenic dendritic cells. DC: dendritic cell. Modified from (18).

Treg cells play an important role in both central and peripheral immune tolerance. In fact, the development of autoimmune diseases has been related to the malfunction of Treg cells (18). According to their origin, two populations of Treg cells can be found (see Fig. 1.3): Natural Treg cells, which express the cell-surface marker CD25 and the transcriptional factor FOXP3, constitute 5-10% of peripheral T cells and are developed in the thymus and later colonize peripheral tissues where they suppress the activation of self-reactive T cells (19). Inducible Treg cells (iTreg), which are generated from naïve $CD4^+$ $CD25^-$ T cells and $CD8^+$ $CD25^-$ T cells in the periphery under different conditions, including presence of tolerogenic DCs, IL-10 and TGF-β. There are different

subsets of iTreg cells, such as $CD8^+$ regulatory T cells, T regulatory 1 (Tr1) cells and T helper 3 (Th3) cells (19).

Treg cells can exert their function by different mechanisms such as cell to cell-contact by reverse signalling via CTLA-4 engagement of B7 on DCs, secretion of cytokines as TGF-B and IL-10, and cytotoxicity by releasing perforin and granzyme A (20). Independently of the mechanism used to induce tolerance, Treg cells inhibit the proliferation of effector T cells and the production of cytokines by these cells (19). In the same way that DCs can generate Treg cells, Treg cells can modulate the function of DCs leading to the inhibition of T cell activation by DCs (21).

As mentioned above, the differentiation of the different subsets of T cells is not irreversible, changing to another subset under the influence of the microenvironment. Similarly, Foxp3+ Treg cells can loss Foxp3 expression and develop features characteristics of effector T cells (22).

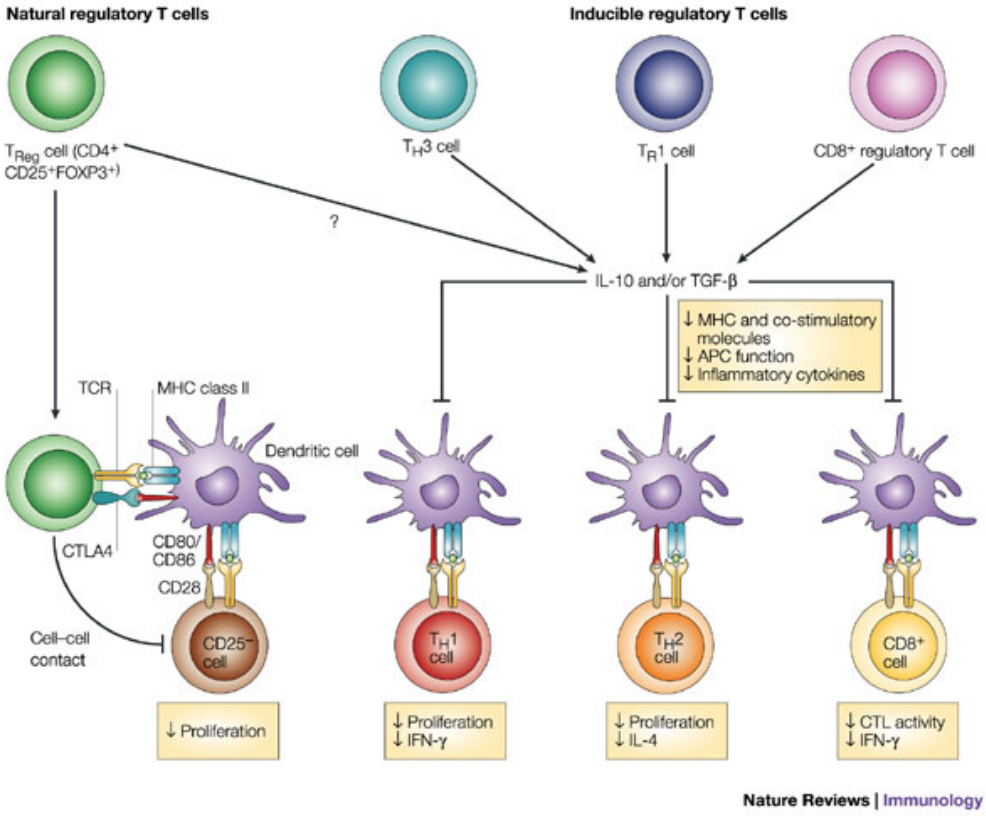


Figure 1.3: Different subsets of Treg cells that are involved on immune tolerance maintenance. $CD4^+CD25^+FOXP3^+$ natural regulatory T cells (Treg cells) inhibit the proliferation of $CD25^-$ T cells, by cell-cell contact, and/or the expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4), which interacts with CD80/CD86 of APCs resulting in a negative signal for T cell activation. On the other hand, inducible regulatory T cells (Th3, Tr1 and $CD8^+$ Tregs) secrete IL-10 and/or transforming growth factor beta (TGF-β), which inhibit the proliferation of effector T cells (Th1, Th2 and $CD8^+$ cytotoxic T cells) by decreasing the expression of MHC-II and coestimulatory molecules, the function of APCs, and down-regulating the production of pro-inflammatory cytokines. From (19).

1.2. Nervous System: healthy brain vs neuroinflammation

The nervous system is the part of animal's body that coordinates voluntary and involuntary actions, transmitting signals between different body systems. It is formed by two main parts, the CNS and the peripheral nervous system (PNS). The CNS is constituted by brain and spinal cord and is responsible for integrating sensory information and responding accordingly. On the other hand, the PNS is constituted by

cranial, spinal and peripheral nerves, and their motor and sensory endings, being its function related to transmitting information to and from the CNS.

The nervous system is composed by two main cell types, neurons and glial cells. Neurons are the cells that transmit information through electrical and chemical signals, and they are composed by soma (cell body), dendrites and the axon (see Fig. 1.4).

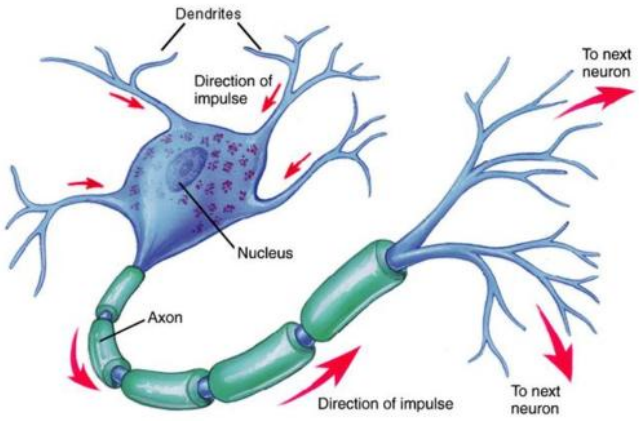


Figure 1.4: Diagram of neuron. From (23).

Otherwise, the 90% of human brain consists on glial cells (microglia, astrocytes, oligodendrocytes and Schwann cells) which traditional functions include supporting neural survival, neurotransmission, and insulating axons to speed up electrical communication. Microglia are resident immune cells implicated in the phagocytosis of cellular debris, oligodendrocytes (in the CNS) and Schwann cells (in the PNS) generate myelin that wraps axons, and astrocytes provided physical support to neurons, as well as energy and substrates for neurotransmission. In addition, different studies have shown that glial cells have functions that are crucial in the formation, operation and adaptation of the neural circuitry (24).

Far of its traditional vision as an an immune-privileged site where immune system did not play any role, numerous evidences indicate that CNS may be affected by some conditions such as trauma, stroke, or invading pathogens that can trigger an immune response within CNS, leading to a neuroinflammatory environment. Thus, there

is an activation of innate immunity carried out by resident immune cells that release inflammatory mediators such as chemokines and cytokines, and also trigger oxidative stress (25, 26). While acute inflammation is generally beneficial, since it decreases further injury, repairs the damage tissue and provides neurotrophic responses in CNS (27), long-term inflammation can activate adaptive immunity and disrupt blood-brain barrier leading to the consequent infiltration of peripheral immune cells (28). This chronic neuroinflammation (see Fig. 1.5) is associated with harmful consequences for the CNS and linked to neurodegenerative disorders such as MS, PD, AD or ALS (29, 30).

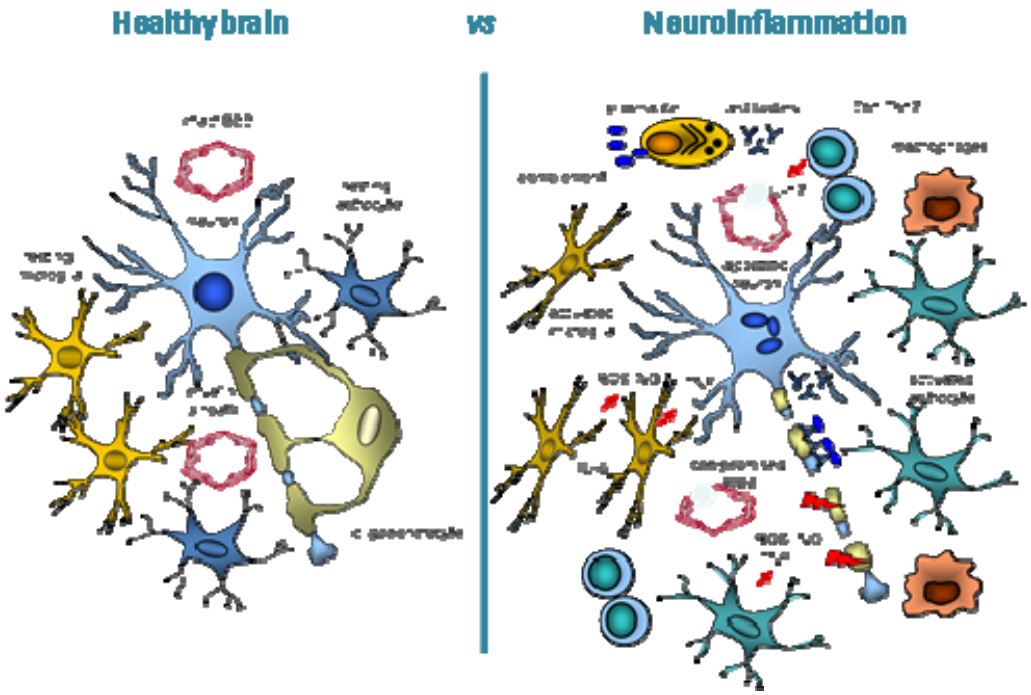


Figure 1.5: Healthy brain *vs* neuroinflammation. In a healthy brain state, immune system can produce an acute inflammation in response to infection or injury in order to resolve the dangerous stimulus. However long term inflammation can activate adaptive immunity, disrupt the blood-brain barrier (BBB), activate glial cells (astrocytes and microglia) and generate a chronic neuroinflammation, which is associated with harmful consequences for the CNS.

Besides the cellular components of the immune system, resident glial cells, mainly microglia and astrocytes, are critical players in neuroinflammation.

Microglia are resident myeloid-derived immune cells in the CNS that act as the first immune defence in nervous system. Microglia constitute about 10% of total cells in the adult CNS and its density is different depending on brain regions, predominating in hippocampus, olfactory telencephalon, basal ganglia and substantia nigra (31). Microglia are known as the resident macrophages of the CNS, and like them, microglia are activated in response to a variety of stimuli, releasing immune mediators to control damage and promote tissue repair. Similar to the M1-M2 polarization of macrophages, microglial cells exposed to LPS and IFNγ undergo M1-like phenotypic changes characterized by amoeboid shape, production of pro-inflammatory cytokines, high phagocytic capacity, up-regulation of iNOS (inducible Nitric Oxide Synthase) and costimulatory molecules. On the other hand, in response to IL-4 and IL-10, microglia acquire an M2-like phenotype characterized by thin cell bodies, up-regulated Arginase I and secretion of neurotrophic factors and anti-inflammatory mediators. M2-like microglia attenuate inflammation induced by M1-like microglia and promote tissue reparation (32). Despite these similarities with macrophages, microglia have other important functions for CNS maintenance, are implicated on neuronal firing activity and synaptic function, and are needed for maturation of excitatory synaptic transmission. Besides their phagocytic function removing the corpses of neurons after programmed cell death, microglia are also able to induce apoptosis in neurons. In addition, microglial cells produce neurotrophic factors such as BDNF (brain-derived neurotrophic factor), a neurotrophin that regulates synaptic plasticity and that its lack produces deficits in contextual fear conditioning and motor learning and in motor-learning-associated spine formation (33). Beyond beneficial roles of microglia, these cells can become overactivated, producing an excess of cytotoxic factors such as nitric oxide, superoxide anion, TNFα and glutamate (34, 35, 36), leading to a progressive neurotoxicity (37).

Astrocytes are star-shaped glial cells with a neural origin, which function has been classicaly described as a brain support. However, different studies demonstrated that they also have an active role in the normal function of the CNS. On a healthy brain, they support physiological processes, such as control and formation of neuronal synapses, removal of the excess of neurotransmitters from extracellular space, or regulation of blood-brain barrier permeability in order to increase the delivery of oxygen and glucose when neuronal activity is enhanced (24). Moreover, they have a role clearing ROS, and it has been shown that regions with low density of activated astrocytes are more susceptible to oxidative damage (38, 39). On the other hand, when CNS is damaged, astrocytes become reactive, migrate to the injury site and form a glial scar, a response called reactive astrogliosis. Reactive astrocytes may exacerbate tissue damage and release proinflammatory cytokines that can inhibit neurite outgrowth and kill oligodendrocytes. In addition, reactivated astrocytes share the neurotoxic behaviour of microglia during chronic inflammation. In this way, reactivated astrocytes release inflammatory mediators that activate microglial cells, exacerbating chronic microglial activation, and, by the way, activated microglia release inflammatory mediators that activate astrocytes (32). Conversely, there is an astrocyte activation that is less aggressive, produces growth factors that enhance the survival of neurons and glia and is associated with improved recovery from tissue damaging insults (40)

In summary, microglia and astrocyte participate in the dual role of neuroinflammation by releasing proinflammatory mediators and increasing neurotoxicity, but also producing growth factors and isolating the damage in order to improve neuronal survival and tissue recovery (see Fig. 1.6). The identification of factors that balance these functions is critical to restore homeostasis in neurodegenerative disorders.

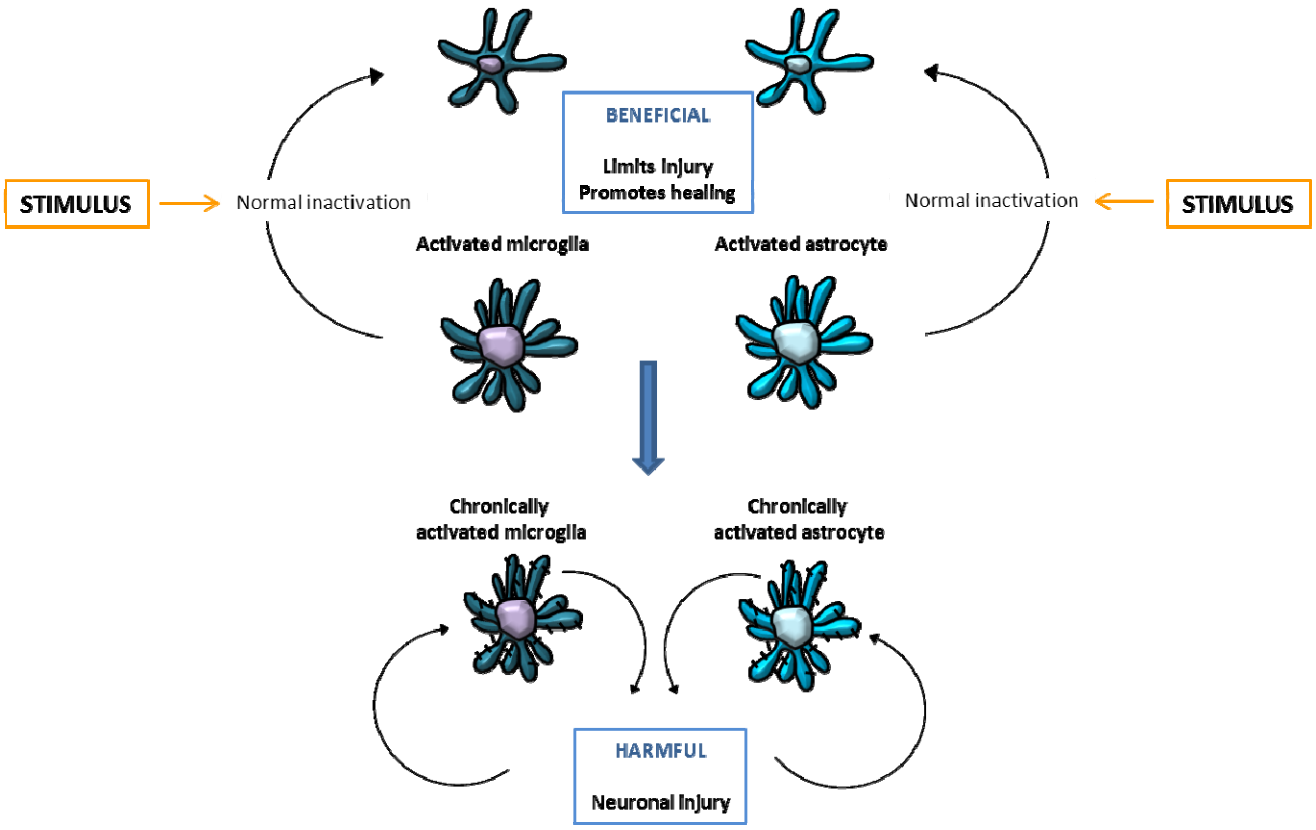


Figure 1.6: Dual role of microglia and astrocytes. Some stimulus, such as hypoxia, trauma, infection or stroke can activate microglia and astrocytes in order to control the damage. In this state, they release growth factors and anti-inflammatory mediators, which limit injury and promote healing. Following this, there is a normal inactivation of these cells. However a chronic uncontrolled astrocytic and microglia activation have detrimental consequences such as neuronal injury. Modified from (41).

1.3. Multiple Sclerosis (MS)

Multiple sclerosis is a neurodegenerative disease characterized by a chronic inflammatory demyelination and axonal damage of the CNS, being the main cause of non-traumatic disability in young adults (42). This disorder was initially described by Robert Carswell in 1838 as “A remarkable lesion of the spinal cord accompanied with atrophy” (43), and now around 2.5 millions of people are affected worldwide by MS, with a higher incidence in North America and the north of Europe. The disease normally begins between the ages of 20 and 40 years and women are affected

approximately twice as often as men (42). Life expectancy is reduced in 7-10 years and normally the cause of death is medical complications (44).

1.3.1. Clinical Signs and Diagnosis

MS patients show a wide variety of clinical signs, being common paresthesias or numbness, monocular visual disturbances, motor weakness, diplopia, incoordination, vertigo and dizziness. Moreover, there are other symptoms that can be presented, such as fatigue, ataxia, spasticity, neuropathic pain, depression or other emotional changes (45).

There are four MS clinical courses (see Fig. 1.7) (46):

- Relapsing remitting (RRMS): Characterized by episodes of new or worsening symptoms followed by partial or complete recovery periods. This is the most common course and affect to 85 % of the patients.

- Secondary progressive (SPMS): After RR course, the disease will begin to progress more steadily with or without any relapses. Most of patients with RRMS evolve to SPMS.

- Primary progressive (PPMS): Characterized by steady worsening of neurologic functioning without any relapse. The progression is continuous.

- Progressive relapsing (PRMS): Patients with this course experience steadily worsening neurologic function from the beginning, but they have occasional relapses. It is the least common course, affecting to 5 % of patients with MS.

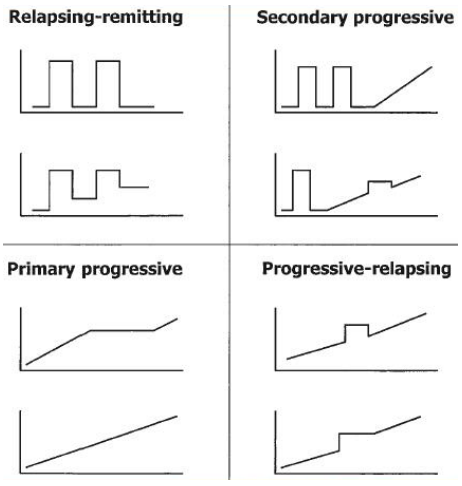


Figure 1.7: Clinical courses of multiple sclerosis.
Y axis: clinical signs; X axis: time
From (47)

For the diagnosis of MS, clinical criteria are been mainly used, based on the demonstrations of symptoms and signs attributable to white matter lesions that are disseminated in time and space. To support clinical diagnosis, different tests are performed, such as determination of immunoglobulin concentration on cerebrospinal fluid (an increase is observed in >90% of patients). Moreover, imaging techniques (mainly magnetic resonance) are used to detect MS lesions (44).

Although autoantibodies play an important role in MS pathogenesis, as indicate the increase in immunoglobulins (Igs) in cerebrospinal fluid in MS patients, the specific autoantibody responsible of the self-reactive response still remains unknown (48).

1.3.2. Etiology

Despite the etiology of MS is mostly unknown, there are diverse studies that indicate a central role of immune system that categorize MS as an autoimmune disease, in which autoreactive cells attack myelin sheath. Moreover, the disease pathogenesis is influenced by genetic and environmental factors.

Different genetic studies show familial aggregation in MS, where the risk of having MS increases among relatives of MS patients, and there is a concordance rate of 30% among monozygotic twins (44). As in other autoimmune diseases, the human leukocyte antigen (HLA) locus, specifically the haplotype DRB1*15:01-DQA1*01:02-DQB1*06:02, is strongly associated with MS. Furthermore, several genes with a function in the immune system have been associated with MS, such as interleukin 12A, interleukin 12B, interleukin 7, interferon regulatory factor 8 or the costimulatory molecule CD86 (49). However, although the genetic variants are a risk factor to develop MS, they are not a determinant factor to suffer the disease.

On the other hand, environmental factors are related to MS triggering, such as vitamin D deficiency, infections or cigarette smoking (50). There are three hypotheses that relate pathogen infection and MS (51):

- Hygiene hypothesis: proposed more than 30 years ago, it suggests that exposure to several infectious agents early in life is protective against MS.

- Poliomyelitis hypothesis: The infection with an unknown virus increases the risk for MS when is acquired in late childhood or adulthood, but it confers protection if is acquired in infancy.

- Prevalence hypothesis: MS is caused by a pathogen that is more common in regions of high MS prevalence.

Although several viruses have been proposed as triggers for MS, Epstein-Barr virus, the causative agent of mononucleosis, has become the leading candidate. There are different hypothesis for the mechanism of action of this virus in the pathogenesis of MS, being one of them the idea that, in genetically susceptible individuals, the antigens from virus can cross-reacts with myelin antigens or there is an activation of superantigens (51).

1.3.3. Pathogenesis

MS is a chronic inflammatory demyelinating disease of the CNS that, typically, affects the brain, spinal cord and optical nerves. Besides activation of glial cells, peripheral immune cells enter to CNS due to disturbances of the blood-brain barrier (BBB) and contribute to the inflammatory environment and disease progression (52). Moreover, the course of disease is accompanied by axonal damage and demyelination, which occurs in all phases, but is more pronounced early during the disease course, and this correlates with cellular infiltration (53).

There are two hypotheses about the formation of focal areas or “plaques” of demyelination: 1) inflammation induces demyelination, which leads to secondary axon degeneration; 2) MS is a neurodegenerative disease with secondary inflammation and demyelination. These hypotheses try to explain the different lesion phenotypes in RRMS, where the pathology is initially more inflammatory and demyelinating; and in the progressive MS, which is more neurodegenerative (54).

Complete demyelination is accompanied by a variable degree of acute axonal injury and axonal loss which in part is counteracted by remyelination. This process occurs at the peripheral margins of the plaques or within the whole white matter lesion. Complete remyelinated plaques are known as “shadow plaques” where myelin

density is reduced and thinner. Remyelination is found during early stages, but usually fails in patients with progressive MS (55).

Although MS is a disease of unknown etiology, it is clear that the immune system plays a central role in the disease pathogenesis, where inflammation is dominated by T cells and activated macrophages and microglia. Indeed, characterization of MS lesions indicates that they contain CD8+ T cells and, in a lesser degree, CD4+ T cells, monocytes, and B cells (56, 57, 58, 59). It has been postulated that early RRMS is mainly mediated by the adaptive immune system and SPMS by the innate system, where activated microglia interacts with astrocytes, leading oligodendrocyte destruction (59). Moreover, the presence of IgG on cerebrospinal fluid isolated from MS patients and the effectiveness of anti-CD20 antibodies-based treatments in MS (60) support that B cells and plasma cells have an important role on the pathogenesis of MS.

On the other hand, evidence based in several studies in animal models of MS indicates that CD4+ T cells seem to have an important role in the disease initiation in humans (61). In fact, although Th1 cells were previously thought to drive MS inflammation, now it is widely recognized that Th17 cells are more involved in the initiation of pathogenesis (59). Moreover, the frequency of Th17 cells is increased in the cerebrospinal fluid of patients with MS, compared to that of individuals with non-inflammatory neurologic diseases, and dramatically increases during relapses. In addition, Th17 cell clones from the cerebrospial fluid and peripheral blood of patients with MS expressed higher basal levels of activation markers, costimulatory molecules and adhesion molecules than Th1 cell clones (62), suggesting a major role of Th17 cells.

Due to the low availability of human samples, numerous murine demyelinating models (virus-induced, autoimmune or toxic-induced models), that reflect the clinical and pathological variability observed in human disease, have been developed in order to study the MS pathogenesis and assay new therapies.

1.3.4. Animal models of MS

1.3.4.1. Experimental autoimmune encephalomyelitis (EAE)

EAE is one of the most intensively studied animal models of immune disease and MS. Although it has greatly contributed to understand what is happening in MS and has helped to find novel treatments for MS, there are several limitations and differences in the pathogenesis of EAE compared to MS (63), including: it is difficult to study remyelination in EAE; EAE affects the spinal cord white matter whereas MS is mainly a brain disease with prominent demyelination of the cerebral and cerebellar cortex; EAE is useful to study the role of CD4+ T cells but not for CD8+ T cells; EAE is primarily a T cell-mediated disease, making difficult to study the role of glial cells. Other distinct features in EAE are the destruction of the myelin sheaths of the nerve fibres, the presence of distributed plaques, the temporal maturation of lesions from inflammation through demyelination, the gliosis and the presence of Igs in the cerebrospinal fluid (64).

EAE can be induced in a wide range of species (guinea pigs, rabbits, goats, rats, mice…). In this study, we will refer to the EAE-induction in mice. EAE is induced by immunization with myelin proteins, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP), supplemented with adjuvants into susceptible mouse strains. Depending on myelin protein fragment and mouse strains used, the clinical course of the disease resembles to relapsing-remitting (using PLP protein and SJL mice) or chronic progressive (using MOG protein and C57CL/6 mice) clinical courses (65). Moreover, besides the immunization with myelin proteins (that produces an active EAE), the disease can be induced passively by inoculating into naïve mice myelin-specific T cells isolated from active EAE mice.

Beyond the possibility of studying the pathogenesis of MS, the use of EAE model has allowed the development of different treatments for MS, such as glatiramer acetate, mitoxantrone or natalizumab (66).

EAE is a disease mediated by CD4+ T cells, specifically by Th1 and Th17 cells. During last years, there has been a controversy about the subset of Th cells that trigger the development of EAE. EAE was initially considered a Th1 (IFNγ-secreting cells)

mediated autoimmune disease; however, some studies have shown not only that IFNγ is not indispensable for the development of EAE (67), but even the lack of IFNγ can increase the severity of the disease (68). On the other hand, recent findings have suggested a primary role for Th17 cells (IL-17-secreting cells). In this way, it has been seen that the lack of IL-17, although does not affect disease incidence, decreases the severity of the disease and delays the onset (69). Moreover, cytokines implicated on the expansion of IL-17 secreting cells, such as IL-23 (70), also appear to be important on EAE pathogenesis, since it has been reported that this cytokine is essential for the induction of EAE (71). Otherwise, it has been showed that Th17 cell population can promote BBB disruption, favouring the entry of immune cells to the CNS and neuroinflammation (72). Interestingly, O'Connor and coworkers reported that Th1 cells infiltrate the CNS, orchestrate inflammation and cellular recruitment, and then facilitate the entry of Th17 cells (73). While this remains the subject of some debate, evidence now indicates that IL-17-producing T cells are critical for EAE (69, 74, 75, 76), and that T cell infiltration and inflammation in the CNS in EAE occur only when Th17 cells outnumber Th1 cells. Nevertheless, other authors have showed that there is not just one Th subset responsible of the development of EAE, being different Th subsets able to induce EAE with different pathological phenotypes. In addition, the capability of different Th subsets of induce EAE could explain the heterogeneity in MS lesions (77).

In any case, Th1 and Th17, reactive to components of the myelin sheath, cross the BBB and trigger a CNS inflammation. The entry of T cells disrupts the BBB facilitating the entry of peripheral mononuclear phagocytes into CNS parenchyma. Moreover, there is an activation of peripheral macrophages and resident cells such as microglia and astrocytes. Activated resident and infiltrating immune cells release inflammatory mediators and free radicals that enhance the inflammation, leading to myelin sheath destruction and oligodendrocyte loss (see Fig. 1.8) (65).

Diverse studies indicate that a deregulation in the mechanisms involved in the maintenance of immune tolerance contribute to the establishment and progression of the autoimmune response. It has been reported that there is a decrease in the effector

function of a Treg population in MS patients compared to healthy donors (78). In fact, CD4+ CD25+ Treg cells confer protection to EAE induction (79).

Besides the importance of T cell populations in the development of EAE, glial niche also has an important role in the pathogenesis of EAE. In this way, microglial activation may be related to BBB disruption by producing matrix metalloproteinases (28). Furthermore, the use of inhibitors of macrophages/microglia attenuates EAE severity (80, 81). However, besides detrimental role of microglia and macrophages, they also have a beneficial role when they are polarized to M2 phenotype (82). Astrocytes are also another important cell population on EAE progression, providing a suitable environment for T cell activation, secreting cytokines and chemokines that potentiate inflammation and recruitment of immune cells. Moreover, although astrocytes are fully capable of priming T cells during EAE, they are not necessary to initiate disease, being mostly involved in the potentiation and exacerbation of ongoing disease (83). Otherwise, astrocytes have a dual role on remyelination and repair, with detrimental and beneficial functions (83).

Another main cell target in MS is the oligodendrocyte, a cell that produces myelin and involved on remyelination (process in which myelin sheaths are restored from demyelinated axons). This event can occur spontaneously in MS, but it has been seen that it fails or is inadequate in diverse MS patients, being unclear why some patients suffer remyelination and others do not. Remyelination involves the generation of new mature oligodendrocytes derived from a population of oligodendrocyte precursor cells (OPCs), which have to be recruited and differentiated to myelin expressing oligodendrocytes. The efficiency of remyelination is affected by age, sex and genetic background. Aging usually correlates with a decrease of both OPC recruitment and differentiation (84). Moreover, remyelination depends on the presence of positive factors that enhance it, being microglia and astrocytes the major source of these factors, such as IL-1β and TNFα. However other pro-inflammatory cytokines, such as IFNγ, can act negatively in remyelination (85). Therefore, it is crucial for the treatment of demyelinating disease, searching for therapies able to modulate the negative and positive factors involved on remyelination.

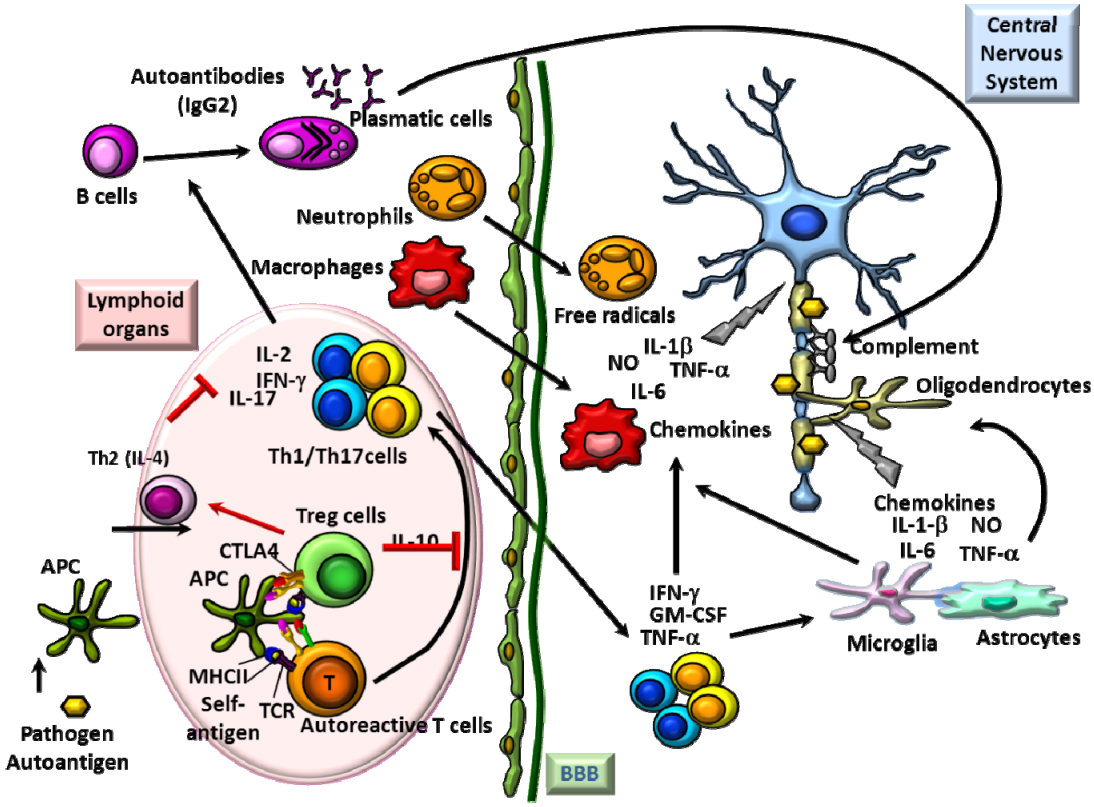


Figure 1.8: Immunopathogenesis on EAE: In this autoimmune disease, antigen presenting cells (APC) present myelin autoantigens to autoreactive T CD4 cells (Th1 and Th17). These cells migrate to CNS, release pro-inflammatory cytokines (TNF-α and IFN-γ) and chemokines, and produce a subsequent recruitment and activation of inflammatory cells (macrophages and neutrophils), which produce cytotoxic factors such as cytokines, nitric oxide (NO) and free radicals. These events lead to oligodendrocyte and a myelin sheath loss. Moreover, in this process there is IgG2a autoantibodies production by plasmatic cells. Normally, Treg cells control the action of autoreactive T cells, having a balance between Tregs and Th1/th17 cells, however in autoimmune diseases there is an unbalance between both T cell populations.

1.3.4.2. Demyelination models induced by toxins

Understanding why remyelination process fails in EAE is difficult, mainly due to the presence of the immune response associated to the disease. Thus, in order to characterize this process, other MS animal models have been developed lacking the

influence of the peripheral immunity (85). The experimental models of demyelination based on the use of toxins, mainly lysolecithin or cuprizone, do not exactly mimic MS pathogenesis, but they are extremely useful for studying the biology of remyelination and have a great value to find therapies designed to repress demyelination or accelerate remyelination. Although toxin-induced demyelination models are characterized by a main role of oligodendrocytes, astrocytes and microglia, it has been recently seen that lymphocytes also infiltrate CNS in such models (86).

- Lysolecithin model: Lysolecithin is a chemical compound derived from phosphatidylcholines that stimulates phagocyte recruitment and phagocytosis of the myelin sheath. It thought that demyelination occurs by activation of recruited macrophages and microglia (87). The injection of lysolecithin into the white matter of brain or spinal cord of rats or mice produces an ellipsoid-shaped area of demyelination that extends over 1-2 mm. Within this lesion, remyelination occurs spontaneously ending at 1 month post injection.

- Cuprizone model: Cuprizone is a copper chelator that inhibits the copper-dependent mitochondrial enzymes cytochrome oxidase and monoamine oxidase, leading a disturbance in energy metabolism and apoptosis of oligodendrocytes, and subsequent demyelination (88). Cuprizone intoxication in mice produces demyelination on the corpus callosum, white matter in thalamus or internal capsule, and remyelination occurs by a recruitment of OPCs and later differentiation, which can be blocked by continued toxin exposure (89).

Because none of the MS models exactly mimics the complexity of this disease in humans, it is necessary combining “MS models based on adaptative immunity” and “demyelination models of MS with a less immune influence” to study the pathogenesis and to find an effective therapy for MS.

1.3.4.3. Theiler’s Virus Infection

Although the role of virus infection has not been found as an exclusive agent that causes MS, some viruses have been related to MS pathogenesis (90). For this reason viral infection has been used as a model for MS.

The Theiler's virus infection is the most common animal model used for demyelinating autoimmune inflammatory disease induced by virus (91). This model is induced by intracranial injection of the virus to susceptible breed mice (SJL). After injection there is an acute phase that is followed by a chronic phase. The acute phase is characterized by infiltrates on CD4 T cells, CD8 T cells, macrophages, and few B cells, and mice show few clinical signs (92). On the other hand, in the chronic phase there are demyelinating lesions in the white matter with infiltrates of CD4 and CD8 T cells. It can be observed neurologic impairment and axonal loss which lead to the appearance of clinical signs (93).

1.3.5. Multiple sclerosis therapies

Current drugs in MS are directed to target inflammation being effective against RRMS. However, at the present, there is no treatment for PPMS, characterized by a nerve degeneration rather than inflammation. Thus, recent research is also focused on therapies affecting to neuroprotection and/or remyelination. A list with the main current therapies approved is shown:

- Interferon β (IFNβ): It was approved by the US Food and Drug Administration (FDA) 20 years ago and is effective for RRMS and SPMS. The interferon group exerts distinct biological activities, including immunomodulation, anti-inflammatory and antiviral effects. Although the mechanism of action by which IFNβ produces beneficial effects in MS patients has not been fully clarified yet, it appears to interfere with T-cell activation, reduce the production of proinflammatory cytokines, downregulate the antigen presentation and inhibit T cell penetration into CNS. IFNβ reduces relapses by about 1/3 and the appearance of new MRI lesions by about 2/3. In some patients, IFNβ is not effective, due probably to the presence of neutralizing antibodies to the IFNβ. The most common adverse events of IFNβ treatment are flu-like symptoms (affecting to 43-61% of patients), injection-site reactions (affecting to 52-85% of patients), lymphopenia in the majority of patients, and is associated with depression and suicidal ideation (94).

- Glatiramer acetate (GLAT): It was approved by the US FDA in 1996 as a treatment for RRMS. Treatment with GLAT produces a reduction in the relapse rate of around 30%. It has been proposed that GLAT promotes differentiation of CD4+ T cells into Th2, decreases the release of proinflammatory cytokines, enhances production of IL-10 by monocytes, increases the frequency of CD25+ FoxP3+ Treg cells and has immunomodulatory effects on B cells. Beyond the effect in both innate and adaptive immune system, it has been suggested that GLAT has neuroprotective and/or remyelinating properties. Like IFNβ, also it has been observed flu-like symptoms and injection-site reactions (95).

- Mitoxantrone: It was the first cytotoxic drug used for the treatment of SPMS. Mitroxantrone is an anthracenedione that produces DNA strand topoisomerase II, interfering with DNA repair. This treatment is injected intravenously and causes the reduction of lymphocytes. It has several adverse events such as cardiotoxicity and leukemia. Nevertheless it remains as a therapeutic option, especially for SPMS (96).

- Natalizumab: It is a monoclonal antibody against the α4 subunit of the very late antigen (VLA-4), preventing the entry of leukocytes through BBB. Despite their beneficial effects, natalizumab has serious adverse effects such as the association with progressive multifocal leukoencephalopathy in 1% of patients, and it is used when others therapies (IFN β and GLAT) have failed (96).

- Fingolimod: It was the first oral drug approved for RRMS. It is a synthetic modulator of the S1P receptor, promoting that T and B cells are unable to leave the secondary lymphoid tissues, thereby impairing their recruitment into the CNS. However, the treatment with fingolimod induces lymphopenia. Despite these effects, this therapy does not trigger a general immunosupression since it has not affect the egress of memory T cells from secondary lymph organs and has not effect of inflammatory cytokines production. Furthermore, new experimental evidences suggest that fingolimod may contribute to neuroprotection and/or remyelination, and it is currently being investigated in PPMS. Despite its beneficial effects, the European Medicines Agencia (EMA) has approved fingolimod only as second-line escalation therapy in RRMS (97, 98).

In summary, due to the critical role of inflammation and autoimmunity in MS, current therapies are focused on immunomodulation but they are no curative and they have minor effects on neuroprotection and/or myelin repair, being inefficients against progressive MS. For this reason, it is crucial that clinical research focuses on finding therapies with neuroprotective and/or myelin repair properties complementary to immune modulatory actions. Some of these new neuroprotective therapies based on channel blocking agents, the induction of growth factors, the administration of cannabinoids or the development of cell therapy, are currently assayed in clinical trials (99).

1.4. Parkinson's disease (PD)

Parkinson's disease is the second most common neurodegenerative disorder in adults over the age of 65. PD is characterized by progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNpc) and their projections to the striatum. Discovered in 1817 by James Parkinson, PD appears at mean age of 55 and the incidence increase with age from 20/100.000 to 120/100.000 at age 70 (100).

1.4.1. Clinical signs and diagnosis

PD is characterized by changes in the motor function, such as bradykinesia (slowness of movement), rigidity, postural instability and rest tremor (101). The disease is diagnosed based on clinical criteria, although there is no a definitive test. The observation of Lewy bodies is the main feature for diagnosis; however this is a post-mortem analysis. In clinical practice, diagnosis criteria are based on the presence of a pool of motor dysfunctions and response to levodopa. On the other hand, PD inherited can be diagnosed by gene testing, but this method is effective in a reduced number of cases.

Although in the last two decades, it has made a remarkable progress in genetics and clinical imaging for the diagnosis, it is necessary to improve it to avoid confusion with other parkinsonism disorders such as multiple-system atrophy, dementia with Lewy Bodies or atypical parkinsonism (102, 103).

1.4.2. Etiology

PD can be divided according to its etiology in familial, sporadic and youth PD. The most of cases of PD are sporadic with an unknown origin, and it is generally assumed that the etiology include both genetic and environmental components. Around 10% of cases show a clear genetic-link and diverse studies in familial PD have showed that PD is associated with a mutation of distinct genes, including α-synuclein, parkin, PTEN-induced putative kinase I, DJ-1 and Leucine-rich repeat kinase 2 (104).

Typical late onset PD is mostly idiopathic and usually is determinated by environmental factors. The finding that people intoxicated with 1-methil-4-phenyl1-1,2,3,6-tetrahydropyridine (MPTP) developed a PD-like syndrome (parkinsonism), supported the hypothesis that the neurodegeneration caused in PD patients may be produced by exposure to dopaminergic neurotoxins. Besides MPTP, other neurotoxins have been proposed to have an important role on PD pathogenesis, such as the pesticides paraquat, rotenone and others. However, there is no data that can associate any specific toxin to sporadic PD. On the other hand, there are environmental factors that are inversely associated to PD, such as smoking or coffee drinking, supporting the idea that environmental factors can modify PD susceptibility (100).

1.4.3. Pathogenesis of PD

Regarding the pathogenesis of the disease, there are different non exclusive pathogenic factors involved on PD (see Fig. 1.9):

- Misfolding and aggregation of proteins:

Protein aggregates are associated with several neurodegenerative disorders such as AD, Huntington's disease and ALS. Some evidences suggest that inclusion formation may be a defensive mechanism since misfolded proteins can be neurotoxic (105). However, other studies have demonstrated the toxicity of the protein aggregates themselves (106, 107). PD is characterized by the presence of deposits of aggregated α-synuclein (α-Syn) in intracellular inclusions, known as Lewy bodies, in the SNpc of the brain. α-Syn is a 140-amino acid protein that is highly enriched in presynaptic neuronal terminals, in particular in the neocortex, hippocampus and SNpc,

as well as within astrocytes and oligodendroglia. The physiological role of α-Syn is still being established, but its interaction with pre-synaptic membranes suggests that one function may be the regulation of synaptic vesicle pools, including control of dopamine levels (108). In PD, it is thought that α-Syn protofibrils are toxic (109). Accordingly, pathogenic mutations showed in patients with inherited PD may produce toxic protein conformation and cause the disease. Moreover, it has been seen that some pesticides induce misfolding or aggregation of α-Syn (110), suggesting this as one of their neurotoxic mechanism. In fact, one of the hallmarks of PD is the formation of Lewy bodies, which were described 90 years ago by Friederich H. Lewy and are intraneuronal eosinophilic cytoplasmic inclusions composed primary by α-Syn. Lewy bodies have a widespread distribution and it may correspond to a variety of motor and non-motors symptoms of PD (111). Finally, PD is an aging-related disease and it has been showed that aging also increases the accumulation of misfolding proteins by an impaired activity of proteasome (100).

- Mitochondrial dysfunction and oxidative stress:

There are some evidences that suggest that nigral cells from PD patients are under oxidative stress state, since an elevation of products from lipid, protein and DNA oxidation was observed (109). Moreover, different studies show a disrupted mitochondrial metabolism (i.e., decreased activity of complex I of the electron transport chain) in PD patients. Mitochondrial dysfunction also can contribute to increase oxidative stress, and both mitochondrial dysfunction and oxidative stress contribute to accumulation of α-syn, because they induce α-syn aggregation and impair proteasomal ubiquitination and degradation of proteins (112). On the other hand, mutations that promote α-syn protofibrils increase the levels of cytoplasmic dopamine, which increase oxidative stress, fueling a destructive cycle (109). According to the role of oxidative stress on PD pathogenesis, it has been shown that some pesticides inhibit the function of complex I of electron transport chain and various genetic mutations are associated with mitochondrial dysfunction (112).

- Neuroinflammation:

As it was explained above, CNS has been traditionally considered as an immune-privileged site, but now it has been demonstrated that neuroinflammation occurs and that when it becomes chronic it could be associated with the development and progression of neurodegenerative disorders such as PD. According to this hypothesis, post mortem studies have reported the presence of activated microglia within the SNpc of patients with PD (39). Activated microglia can produce different inflammatory toxic mediators and also contribute to oxidative stress. In this sense, activated microglia up-regulate nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) and iNOS, two enzymes that produce superoxide anion and nitric oxide (NO), respectively (39, 113). Moreover, heterogeneous distribution of astrocytes has been also observed, with low density of astrocytes in SNpc compared to other brain areas. The limited astroglial environment has been associated with PD since astrocytes might contribute to neuroprotective mechanism by detoxifying oxygen free radicals or secreting neurotrophic factor such as GDNF (40). So, astrocytes can have a dual role, contributing to a neuroinflammatory state (producing proinflammatory mediators) and releasing neuroprotective factors.

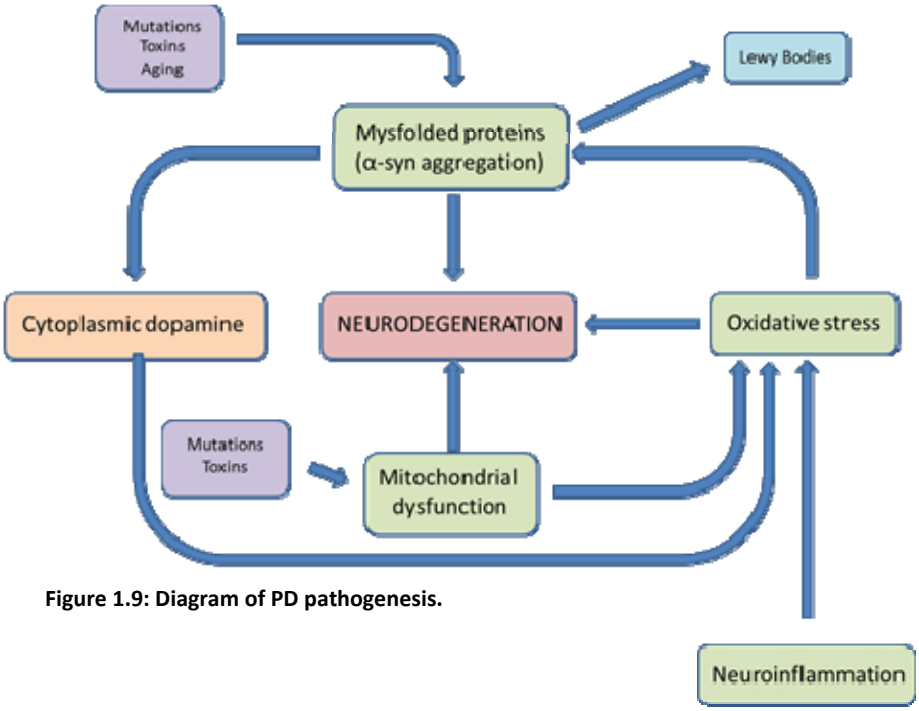


Figure 1.9: Diagram of PD pathogenesis.

Moreover, the influence of neuroinflammation on PD pathogenesis has been supported by different studies with animal models of PD. Indeed, microglial activation

has been seen in brain of mice and monkeys after MPTP injection (114, 115) and the blockade of microglial activation was reported as neuroprotective in a MPTP mouse model (116). On the other hand, astroglial activation has been reported in the SNpc and the striatum of mice exposed to MPTP and rats exposed to 6-hydroxydopamine (6-OHDA) (117, 118).

Besides the role that microglia and astrocytes play on PD, it has been reported that peripheral immune system also plays a main role. Epidemiological studies reported that the use of nonsteroidal anti-inflammatory drugs decreases the risk of developing PD (119). A higher production of proinflammatory cytokines such as TNFα, IL-1β, IFNγ in the SNpc and striatum and of IL-2, TNFα, IL-6 and RANTES in the serum of PD patients have been found compared to healthy controls (120, 121). Moreover, it has been reported serum antibodies that recognize components of dopaminergic neurons (39), and CD8+ and CD4+ T cells on post mortem brain of PD patients (122), suggesting that adaptive immune system have an important role on PD pathogenesis. Using a mouse model for PD, it has been described that MPTP-induced dopaminergic cell death is attenuated in the absence of T lymphocytes and that dopaminergic toxicity is mediated by CD4+ T cells (122).

1.4.4. Animal models of PD

Understanding PD makes necessary the development of preclinical models. However, none of them show the complexity of the human disease. This is mainly due to the lack of the chronic progressive neurodegenerative component. However, all the animal models recapitulate certain features of human aging or disease, and their availavility allows the opportunity to assess the efficacies of new pharmacotherapies.

- Genetic models

The discovery of mutations associated to PD has promoted the development of genetic animal models. α-synuclein transgenic mice reproduce the motor and non-motor dysfunctions observed at early stages of PD but they do not show nigrostriatal degeneration; mice overexpressing LRRK2 mutation form show progressive motor-activity deficits, but do not course with neuronal death; transgenic mice for others

proteins involved on inherited PD do not show neuronal abnormalities (123). Because genetic models do not show a loss of nigral dopaminergic neurons, which is the main characteristic of PD, and PD patients showing a genetic link represent a small number (approximately 10%), toxin-based models remain being the most used experimental models for PD.

- Toxin-based models

Several neurotoxins, including 6-OHDA, MPTP, paraquat and rotenone, have been used to induce dopaminergic neurodegeneration in vivo.

6-OHDA: Was introduced by Ungerstedt in 1968. The stereotaxic injection into the SNpc produces dopaminergic degeneration starting at 24h post injection. However, the clinical signs do not mimic what is hapening with PD patients and the formation of Lewy bodies is lost. Moreover, although 6-OHDA induces dopaminergic degeneration, remains unclear if the mechanism is the same than in PD patients (100).

Paraquat: It has been reported that exposure to this herbicide may increase the risk for PD and mice intoxicated with paraquat show inclusions of α-syn (100). Paraquat induces dopaminergic degeneration, although it may affect to other cell types.

Rotenone: Rotenone induces dopaminergic degeneration by inhibiting the complex I of electron transport chain and generating reactive oxygen species (ROS). Intravenous injection of rotenone induces clinical signs in rats resembling to PD signs, as ataxia, hypokinesia, rigidity or resting tremor. Moreover, animals develop intraneuronal cytoplasmic inclusions of α-syn (124).

MPTP: (See figure 1.10). The neurotoxic potential of MPTP was discover in 1983 when drug users developed a progressive parkinsonian syndrome by accidental injection of MPPP, an analog of the narcotic meperidine, which was contaminated with MPTP, responsible of parkinsonian symptoms (125). MPTP produces a parkinsonism disorder in humans, monkeys and mice, being irreversible and severe in primates. Clinical symptoms remember to those human PD, including resting tremor, rigidity slowness of movement, postural instability and freezing. Moreover, like in PD, damage induced by

MPTP intoxication increases with age. After systemic administration, MPTP cross the BBB and is then converted to MPP+ by monoamine oxidase B (MAO B) in glial cells. MPP+ enters into dopaminergic neurons using plasma membrane dopamine transporter (DAT) (126). DAT is necessary for MPTP damage since it was reported that mice lacking dopamine transporter are protected from MPTP-induced neuronal death (127). Once inside neurons, MPP+ can follow at least two routes: 1) it can bind to the vesicular monoamine transporter-2 (VMAT2), which translocates MPP+ into synaptosomal vesicles (128), which appears to protect cells from MPTP –induced neurodegeneration; 2) it can be concentrated within the mitochondria where inhibit the complex I of electron transport chain leading to a reduction of ATP and increase of ROS levels (100). Moreover, ROS production appears to increase α-syn aggregation, and viceversa, overexpression of α-syn appears to increase ROS production (124). However, acute MPTP intoxication is not able to form Lewy bodies (129). The magnitude of the lesion induced by MPTP depends on the toxin dose and the route of administration; it has been reported that chronic administration of MPTP induces Lewy bodies-like inclusions (124). Systemic injection of MPTP in mouse is the classic and established model used to study the molecular pathways involved in PD neuronal cell death and to test the effectiveness of neuroprotective drugs (129).

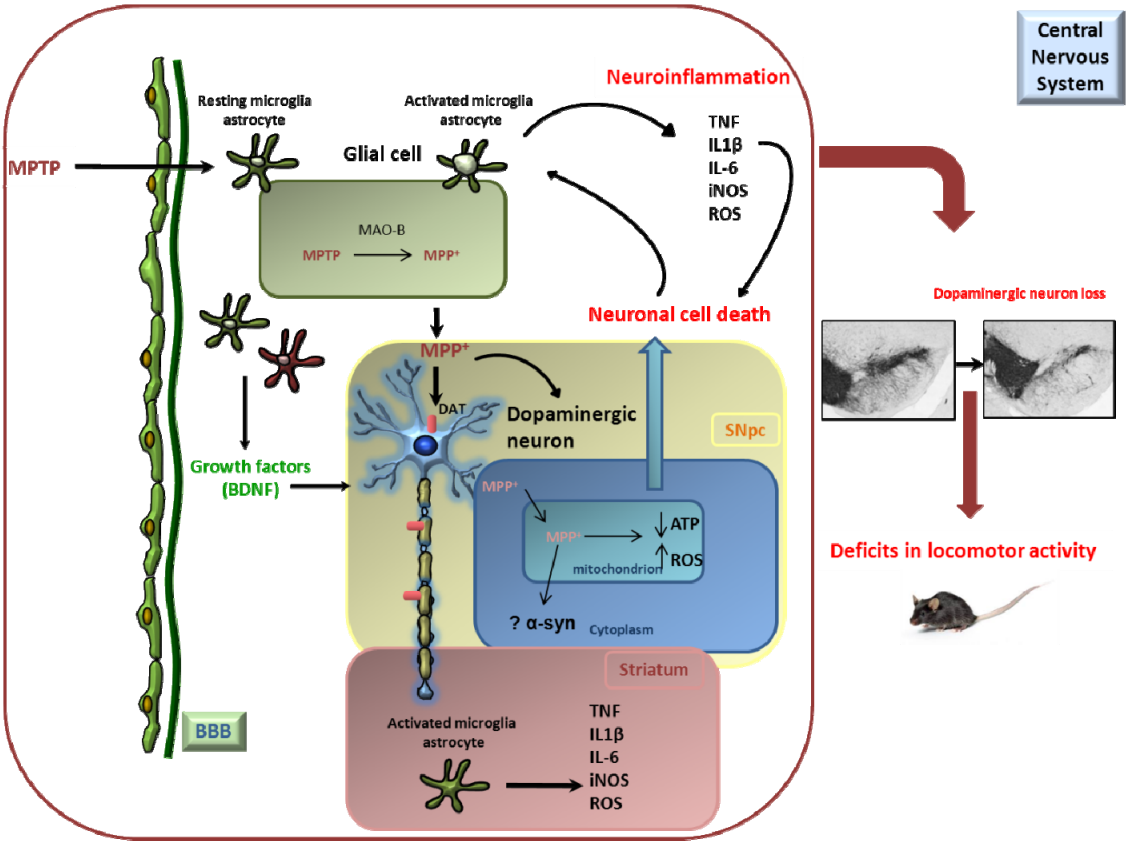


Figure 1.10: Mechanism of action of MPTP: MPTP crosses the BBB and is converted to MPP^+ by MAO-B in glial cells (astrocytes and microglia). While glial cells are produinge growth factors and mediators that protect neurons, after MPP^+ activation, microglia and astrocytes can iniciate a neuroinflammatory process by releasing pro-inflammatory mediators and lead to neuronal cell death, mainly in SNpc and striatum. On the other hand, MPP^+ can enter into dopaminergic neurons through the transporter DAT (found in the soma and projections of dopaminergic neurons). Inside of neurons MPP^+ produces the reduction of ATP and increases of ROS, leading to neuronal cell death. Moreover, neuronal cell death produces glial actiation, becoming a destructive cycle. Together, the toxic effects of MPTP trigger a loss of dopaminergic neurons in the nigra-striatal dopaminergic pathway that results in deficits of locomotor activity in mice.

1.4.5. PD therapies

Despite 50 years of research, no efficient long-term treatment has been developed for PD and current therapies are able to alleviate the symptoms but they fail to stop disease progression. Nowadays, there are different therapeutic strategies to treat PD: symptomatic treatment, neuroprotective therapy and cell replacement therapy.

a) Symptomatic treatment is mainly based on using the dopamine-precursor L-DOPA. This is the most commonly used treatment for PD and it has been used for approximately 40 years. However, although it successfully improves motor symptoms at the beginning, half of patients show adverse events related to motor complications whithin the first 5 years of use. Other symptomatic treatments commonly used are based on the administration of MAO-B inhibitors. Nevertheless none of these treatments efficiently stop disease progression (130).

b) Due to the inefficiency of L-DOPA and MAO-B inhibitors to stop disease progression, the goal of current research is to find treatment with a neuroprotective and anti-oxidant potential. At present time there are some drugs on clinical trials able to act at neuroprotective level and decreasing PD symptoms. Platelet-derived growth factor (PDGF), granulocyte-colony stimulating factor (G-CSF) and Coenzyme 10 are in phase I, phase II and phase II clicinal trial, respectively (131). Also, a specific aim is to activate the endogenous production of neurotrophic factors as they can not be supplemented easily due to their problems of innaccessibility to the brain from the periphery.

c) Because in PD there is a selective loss of dopaminergic neurons, a novel therapy strategy is based on replacing these cells by transplantation of fetal neurons derived from stem cells. However, all of these therapeutic options are still in an experimental phase (130).

Finally, because the immune system and neuroinflammation have important roles on the pathogenesis of PD, new treatments targeting components of the neuroinflammatory response are studied lately. In base of these findings, we speculate that an ideal treatment should be a drug with anti-oxidant effects, neuroprotective and able to modulate the immune response produced in PD.

1.5. Focusing on neuroimmunology as a endogenous therapy for MS and PD

As it was mentioned above, an effective therapy in CNS diseases characterized by neurodegeneration and/or neuroinflammation and autoimmunity, such as MS and PD,

should be a treatment with multiple targets, which includes the regulation of the autoreactive process, the inflammatory response and the immune tolerance together with the induction of neuroprotection and trophic support. From our point of view, endogenous factors shared by the immune and neuro-endocrine systems could be the key in modulating the physiology and pathology of CNS.

1.5.1. Bidirectional communication between neuroendocrine and immune systems

It has become lately evident that the body is in homeostatic equilibrium when there is a controlled balance between physiological systems modulating the internal environment of the organism. The relationship between the neuro-endocrine and immune systems, mediated by a complex networks of primary and accessory cells and mediators, allows to modulate a coordinated response against different stimulus (see Fig. 1.11) (132). In this sense, the immune system receives stimuli and alerts to the neuro-endocrine system of a dangerous situation. The CNS responds by different ways in order to control the dangerous signal. Inversely, the immune system is controlled by the CNS, in response to environmental and physical stress (133). Nervous system can control the immune system at both local and systemic levels. PNS, in response to inflammation, releases some neuropeptides, such as substance P and corticotrophin-releasing hormone (CRH), from sensory peripheral nerves, which enhance the inflammatory response (vasodilatation, vascular leakiness, edema and pain) in order to favor the pathogen clearance (133). However, neuro-endocrine system also can limit the inflammation by releasing other molecules with anti-inflammatory effects in order to avoid a chronic response. This function is carried out by the sympathetic and parasympathetic nervous system, in combination with the hypothalamic-pituitary-adrenal (HPA) axis. This axis is the main anti-inflammatory pathway, and corticoids are the classic agents released in response to systemic inflammation and stress. Other agents with this function and released in the same conditions are opioids, hormones, and neuropeptides have recently been added to the list (134).

The interaction between neuro-endocrine and immune system is based on sharing the same biochemical language. Cytokines produced by cells from immune

system act on cells of the neuro-endocrine system, and viceversa, molecules such as neurotransmitters, neuropeptides and hormones produced by the neuro-endocrine system can act on cells of the immune system. Moreover, some neuropeptides can be released by both systems in response to inflammatory signals, and can act in autocrine and paracrine ways by binding to specific receptors expressed in immune cells (135).

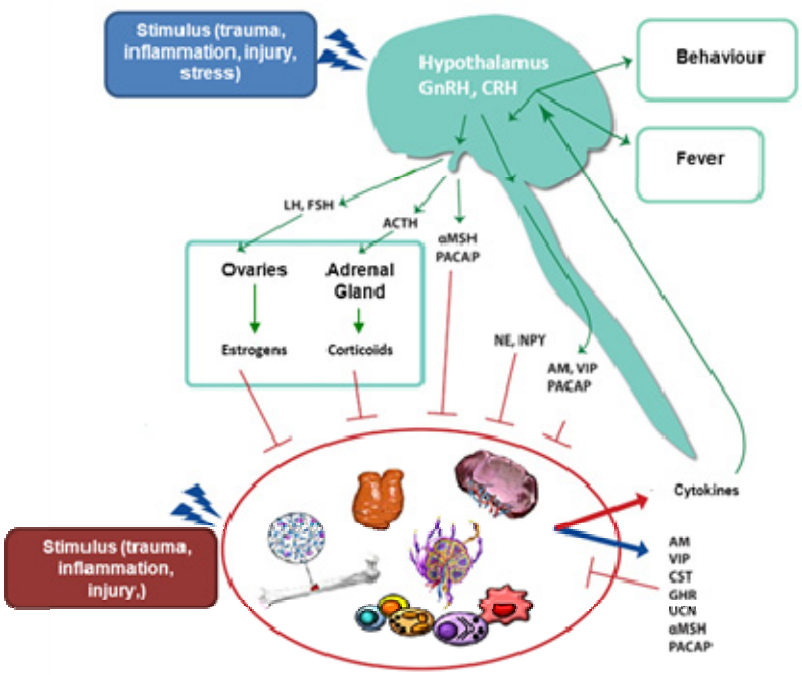


Figure 1.11: Interaction between neuro-endocrine and immune systems: Immune cells communicate to nervous system through the release of cytokines when there is a dangerous stimulus. Nervous system responds releasing CRH that leads to the production of several hormones, such as corticoids, generating an anti-inflammatory response to restore the immune homeostasis. CRH also stimulates the release of cathecolamines such as NE and NPY with an anti-inflammatory effect. In this response also contribute the LF and FSH. In addition, some neuropeptides with an anti-inflammatory role (AM, VIP, CST, GHR,) can be produce and exerted their function in both, the immune and the neuro-endocrine system. Abbreviations: GnRH, gonadotropin-releasing hormone; CRH, corticotropin releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; ACTH, adrenocorticotropic hormone; NE, norepinephrine; NPY, neuropeptide Y; αMSH, melanocyte stimulating hormone; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating peptide; AM, adrenomedullin; CST, cortistatin; GHR, ghrelin; UCN, urocortin.

1.5.2. Neuropeptides: key mediators in immune-nervous communication

Neuropeptides are molecules released at the peripheral peptidergic endings of sensory and efferent nerves in close proximity to immune cells in response to various stimuli (invasive and inflammatory). Moreover, they also can be released by immune cells as lymphocytes, macrophages, mast cells and neutrophils (15), being part of the crosstalk between neuroendocrine and immune systems.

During the last two decades, distinct neuropeptides with immunomodulatory properties and therapeutic potential in some animal models of autoimmune and inflammatory diseases have been described. Among them, vasoactive intestinal peptide (VIP), α-melanocyte stimulating hormone (αMSH), urocortin, adrenomedullin, cortistatin or ghrelin highlight by their pleiotropic immunomodulatory actions. They belong to different protein families but, however, they share some characteristics that makes them very attractive to be considered as immunomodulatory agents: they are produced by immune cells under inflammatory conditions, their actions are mediated through G-protein-coupled receptors (GPCRs) and the signaling is through activation of cyclic adenosine monophosphate (cAMP)/ protein kinase A, downregulating the expression of proinflammatory mediators (see Fig. 1.12) (15).

Different studies have showed the anti-inflammatory effects of these neuropeptides. For example, VIP is able to modulate innate immune response inhibiting the phagocytic function and free radical production, reducing the production of pro-inflammatory cytokines and chemokines by macrophages and microglia, down-regulating the expression of iNOS and COX-2, increasing the production of anti-inflammatory cytokines and reducing degranulation of mast cells (136).

Besides their function modulating the innate immune response, some of them also are also able to modulate the adaptive immune response. In *in vitro* studies, it has been reported that VIP inhibits the expression of costimulatory molecules in activated macrophages, microglia and dendritic cells (137, 138, 139). Moreover, VIP inhibits IL-2 production and T cell proliferation, and is able to induce the differentiation of Th naïve

cells towards Th2 cells by inducing the production of Th2-cytokines by macrophages. Furthermore, these findings have been supported by *in vivo* studios (140).

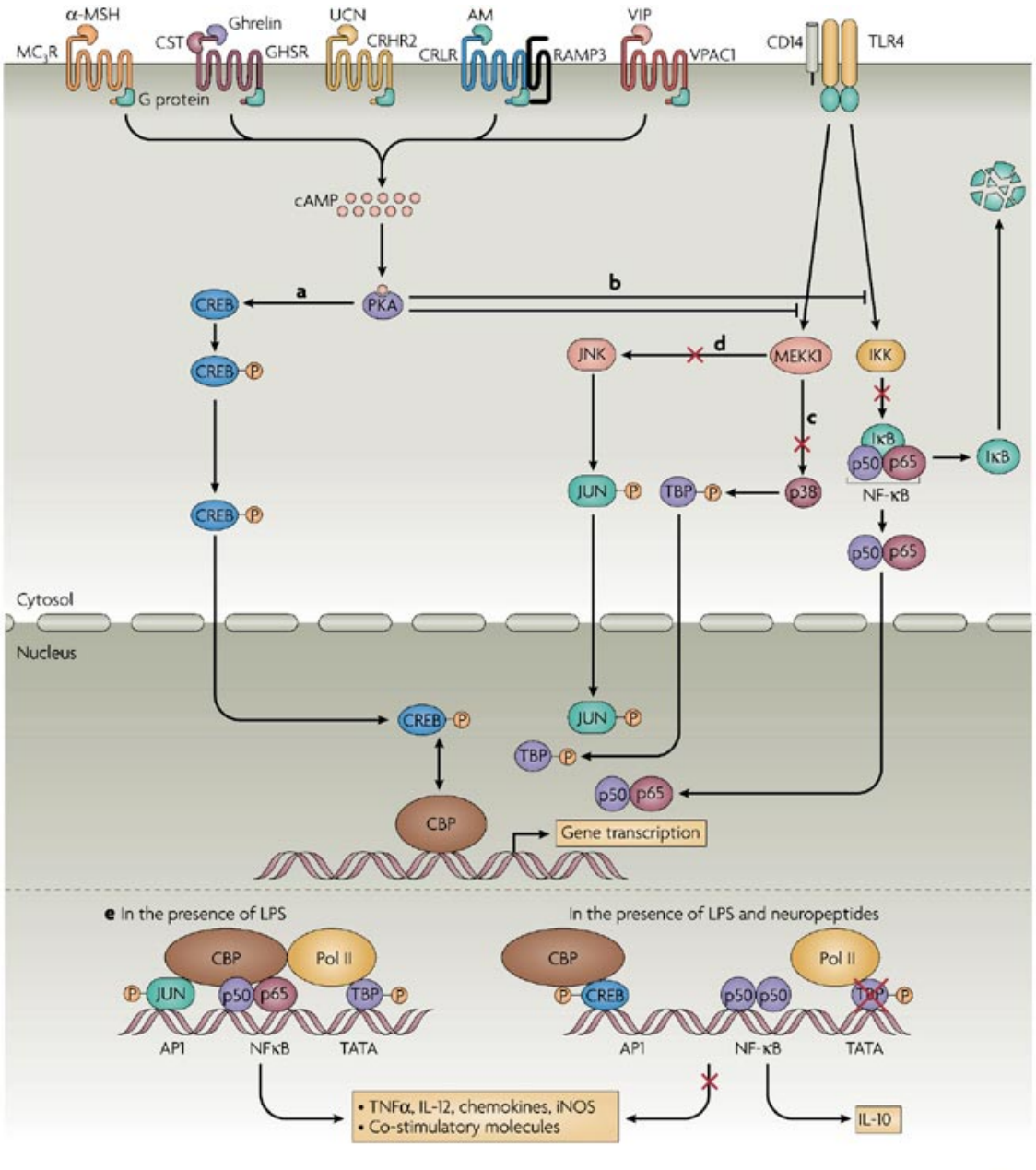


Figure 1.12: Next page.

Figure 1.12: Molecular mechanisms and transcription factors involved in the anti-inflammatory effect of neuropeptides. The binding of each neuropeptide with its corresponding receptor increases cyclic AMP (cAMP) and activates protein kinase A (PKA), which has four main effects. a) First, PKA activation induces the phosphorylation of the cAMP responsive-element-binding protein (CREB), which, owing to its high affinity for the co-activator CREB-binding protein (CBP), prevents the association of CBP with p65 (which is a key component of nuclear factor-κB (NF-κB)). b) Second, activated PKA inhibits IκB kinase (IKK), which stabilizes the IκB inhibitor and prevents nuclear translocation of p65. c) Third, PKA activation inhibits MAPK/ERK kinase kinase 1 (MEKK1) phosphorylation and activation, and the subsequent activation of p38 and TATA-binding protein (TBP). Non phosphorylated TBP lacks the ability to bind to the TATA box and to form an active transactivating complex with CBP and NF-κB. A reduction in the amounts of nuclear p65, CBP and phosphorylated TBP inhibits the formation of the conformationally active transactivating complex that is required for the transcription of most cytokine and chemokine genes. d) Fourth, inhibition of MEKK1 by PKA subsequently deactivates JUN kinase (JNK) and cJUN phosphorylation. The composition of the activator protein 1 (AP1) complex changes from the transcriptionally active cJUN–cJUN, to the transcriptionally inactive JUNB–cFOS or CREB. e) The final consequence is that the transcriptional machinery, which is perfectly assembled to the gene promoters of several inflammatory mediators (tumour-necrosis factor-α (TNFα) is shown as an example) after the signalling of lipopolysaccharide (LPS) through Toll-like receptor 4 (TLR4), is significantly disrupted by treatment with these neuropeptides. AM, adrenomedullin; CRHR2, corticotropin releasing hormone receptor type 2; CRLR, calcitonin-related-like receptor; CST, cortistatin; GHSR, growth hormone secretagogue receptor; IL, interleukin; iNOS, inducible nitric oxide synthase; MC3 R, a melanocortin receptor; α-MSH, α-melanocyte-stimulating hormone; Pol II, RNA polymerase II; RAMP3, receptor-activity-modifying protein 3; UCN, urocortin; VIP, vasoactive intestinal peptide; VPAC1, a VIP receptor. From (15).

Neuropeptides are also able to induce peripheral expansion of new antigen-specific CD4+ CD25+ FoxP3+ Treg cells which suppress the activity of self-reactive T cells (136).

Due to their inmmunomodulatory properties, neuropeptides are attractive therapeutic candidates for the treatment of inflammatory disorders and autoimmune diseases. In fact, several pre-clinical studies have showed the therapeutic potential of these peptides in experimental models of sepsis, inflammatory bowel disease, collagen-induced arthritis or multiple sclerosis (15). Moreover, some of them are in clinical phase, for example the use of VIP for the treatment of sarcoidosis (141).

Besides their immunomodulatory properties, some neuropeptides show neuroprotective features. For example, ghrelin displays neuroprotective role after brain injury in rats, and treatment with ghrelin of neurons exposed to oxygen-glucose deprivation inhibits cell death and apoptosis (142). Furthermore, VIP shows a neuroprotective role increasing neuronal survival, inducing neuronal differentiation of murine embryonic stem cells, preventing astrocytic cell death and increasing BDNF and

ADNP production (143). On the other hand, it has been reported the therapeutic effect of ghrelin and VIP in a mouse model of PD (144, 145).

In this study, we have focused on characterizing the role of the neuropeptides adrenomedullin and cortistatin in neurodegenerative disorders that course with a neuroinflammatory component.

1.6. Adrenomedullin

Adrenomedullin is a neuropeptide discovered in 1993 by Kitamura, 'a novel hypotensive peptide discovered in human pheochromocytoma by monitoring the elevating activity of platelet cAMP' (146). It was designated as "adrenomedullin" because its aboundance in adrenal gland. This neuropeptide has a wide body distribution, including its presence in cardiovascular, respiratory, reproductive, neurological, gastrointestinal, renal, endocrine and immune tissues. Moreover, adrenomedullin is also found in plasma, urine cerebrospinal and amniotic fluid. However, its half-life is short, about 22 minutes in plasma (147).

Adrenomedullin has numerous biological actions which are of potential importance for cardiovascular homeostasis, growth and development of cardiovascular tissues. Systemic adrenomedullin administration reduces arterial pressure, decreases peripheral vascular resistance and increases heart rate and cardial outpus. Moreover this peptide exerts a renal function controlling the body fluid volume, affecting to diuresis. It has been reported that intracerebral infusion of adrenomedullin inhibits salt intake and thirst. On the other hand, adrenomedullin also may have reproductive functions, since it has been detected in the uterus and plancenta, being its expression higher during pregnancy (148).

Human adrenomedullin consists in 52 amino acids and it has a ring structure formed by a disulfide bond and an amidated carboxyl terminus. Adrenomedullin shows 27% homology with calcitonin gene related peptide (CGRP), suggesting that AM belongs to the CGRP superfamily (149). The adrenomedullin gene is highly conserved among different species, including humans, mice and rats. It is synthesized as part of a larger precursor molecule, termed preproadrenomedullin. This molecule contains a 21-

amino acid N-terminal signal peptide that immediately precedes a 20-amino acid amidated peptide. The human gene encoding preproadrenomedullin is localized in a single locus of chromosome 11 and it contains 4 exons and 3 introns (150). Murine adrenomedullin gene is organized into four exons and three introns and is localized on a distal region of mouse chromosome 7. In humans preproadrenomedullin is formed by 185 amino acids, while murine preproadrenomedullin peptide contains 184 amino acids (151). Adrenomedullin synthesis can be influenced by physical factors such as stress, and by cytokines (TNFα, IL-1B), hormones and lipopolysaccharide (147).

Due to the homology that adrenomedullin and CGRP share, adrenomedullin binds to the calcitonin receptor-like receptor (CRLR). The receptor of adrenomedullin and CGRP is a heterodimer formed by CRLR and a receptor-activity-modifying protein (RAMP), which exists as different subtypes, RAMP1, RAMP2 and RAMP3. It has been reported that while CGRP binds to the heterodimer CRLR-RAMP1, adrenomedullin binds to CRLR-RAMP2 or CRLR-RAMP3 (see Fig. 1.13)(147).

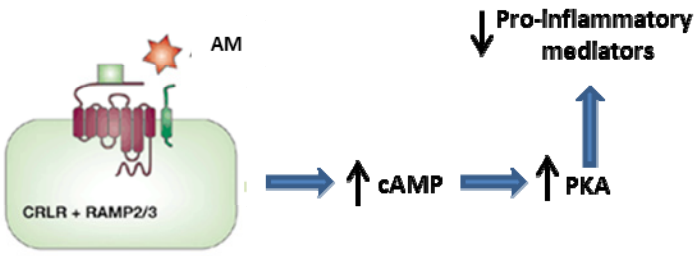


Figure 1.13: Adrenomedullin receptor: Through binding to CRLR-RAMP2/3 adrenomedullin (AM) exerts different functions, by increasing cAMP and activating protein Kinase A, leading to the decrease of pro-inflammatory mediators. Modified from (152).

- **Immunoregulatory properties of adrenomedullin**

The neuropeptide adrenomedullin is expressed by both innate and adaptive immune cells, such as macrophages and T cells (153, 154) On the other hand, adrenomedullin receptor is also expressed by immune cells (15), and it is up-regulated under inflammatory conditions (155), suggesting an important role of adrenomedullin in inflammation. See table 1.1.

Adrenomedullin exerts its function by binding to adrenomedullin receptor, CRLR-RAMP2/3. After binding, there is an activation of cAMP/protein kinase A, downregulating the expression of proinflammatory mediators (see Fig. 1.12), such as cytokines (TNFα, IL-6, IL-12, IL-18, IL-1β, MIF), chemokines (CCL5, IP-10, MIP-1α, MIP2, MCP-1) and nitric oxide (15).

Beside the anti-inflammatory role of adrenomedullin, it also shows immunoregulatory properties, such as the induction of IL-10 production and the generation of Treg cells (156, 157).

Due to its anti-inflammatory and immunoregulatory features, adrenomedullin has been tested as a therapy in different animal models of inflammatory and autoimmune diseases. Thus, adrenomedullin prevents lethal endotoxemia by decreasing the local and systemic levels of a wide spectrum of inflammatory mediators, including cytokines, chemokines, and the acute phase protein serum amyloid A (158). Other studies have shown the therapeutic effects of adrenomedullin in autoimmune diseases as Crohn's disease (156) and rheumatoid arthritis (157), where the therapeutic effect of adrenomedullin was associated with the downregulation of both inflammatory and Th1 driven autoimmune responses, including regulation of a wide spectrum of inflammatory mediators. Moreover the effect was related tothe involvement of IL-10 secreting-Tregs in Crohn's disease (156), and the generation or activation of efficient $CD4^+CD25^+$ regulatory T cells in arthritis (157). Furthermore, the infusion of adrenomedullin improves acute myocarditis by reducing the infiltration of inflammatory cells and decreasing the expression of the chemokine MCP-1 (159).

- **Adrenomedullin on the nervous system**

Adrenomedullin is expressed in cerebral vascular endothelial cells and all the major cell types within the brain, including neurons, astrocytes, microglia and oligodendrocytes. Because most of these cells express adrenomedullin receptor (160), it has been thought that this neuropeptide can act in an autocrine and paracrine

manner. On the other hand, adrenomedullin is able to cross the blood-brain barrier (161) and seems to play an important role in the regulation of BBB (162, 163).

In pathological conditions, adrenomedullin seems to have an important role too, since it has been seen that this neuropeptide is up-regulated after ischemia process in rats (164). Moreover, adrenomedullin down-regulation increases ROS production after focal ischemia in mice (165). In addition, AM-KO mice are less resistant to hypobaric hypoxia than wild-type mice (166). On the other hand, adrenomedullin promotes differentiation of OPCs under pathological conditions *in vitro* (167). Due to the role of adrenomedullin on nervous system, this neuropeptide has been tested in animal models of stroke and traumatic brain injury (168, 169), where it showed a therapeutic role.

Based on the anti-inflammatory and neuroprotective properties of adrenomedullin, this neuropeptide emerges as an interesting new therapeutic approach to be characterized in autoimmune and neuroinflammatory disorders, such as multiple sclerosis. There is no data about the potential therapeutic role of this neuropeptide in such disease and if it could affect both, peripheral and local components of this disorder. Specially, there is no data about the ability, if any, of adrenomedullin in regulating *in vivo* a disturbed glial niche and the associated mechanisms.

1.7. Cortistatin

Cortistatin is a neuropeptide belonging to the somatostatin family. It was discover in the cortex of rat brain in 1996 by L. de Lecea (170). Cortistatin is encoded by the CORT gene and is synthetized as preprocortistatin that contains 114 amino acids in mouse and 105 amino acids in humans. Precortistatin is modified to procortistatin after proteolytic process leading different peptides: CST-17 and CST-29 in humans, and CST-14 and CST-29 in rodents (171).

Cortistatin shows high structural homology to somatostatin; thus, CST-14 shares 11 of its 14 residues with somatostatin-14 and displays an amino acid sequence (FWKT) identical to somatostatin in the binding site to receptors. In fact, cortistatin

binds to the five receptors of somatostatin (SST1-5) performing similar functions to somatostatin, such as depression of neuronal activity, decrease of cAMP and cellular proliferation (172). However, cortistatin shows distinct physiological functions such as induction of slow-wave sleep and reduction of locomotor activity (173).

Besides its binding to SSTR1-5, cortistatin is also able to bind to ghrelin receptor (GHSR), leading to activation of cAMP/PKA pathway, which decreases the production of proinflammatory mediators (174). Furthermore, cortistatin binds to truncated variants of SSTR-5 (171). Moreover, in humans has been described other specific receptor for cortistatin, MrgX2, for which somatostatin shows low affinity. However, in mice this receptor has not been found yet. So, the differential effects that cortistatin and somatostatin show could be explained by the existence of different receptors for cortistatin (Fig. 1.14).

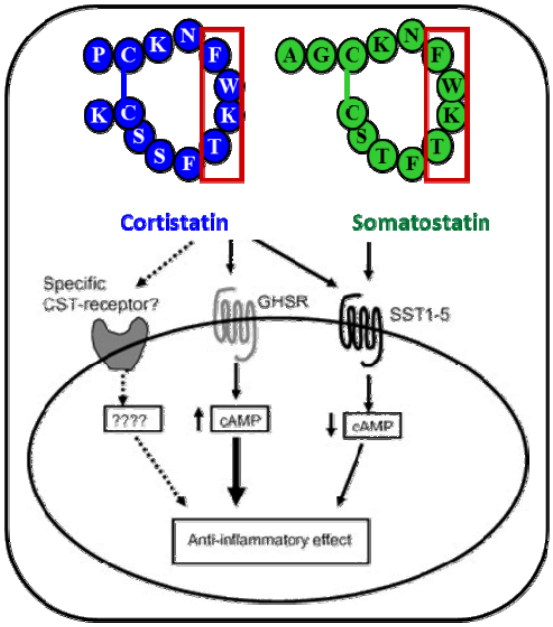


Figure 1.14: Cortistatin receptors. Although, cortistatin and somatostatin share a common binding site (red rectangle), cortistatin is able to bind to different receptors, having unique functions that do not share with somatostatin. Binding to GHSR produce an increase of cAMP; whereas binding to SST1-5 the effect is through reduction of cAMP, producing both anti-inflammatory effects. Modified from (175)

Moreover, certain receptor-activity-modifyng proteins (RAMPs) could act with SSTR to form a receptor that preferentially bidns to cortistatin, where the RAMP associated determines the binding specificity for the ligand and the activity of the receptor (175).

The use of mice deficient in cortistatin has been useful to characterize unique functions of this peptide: stimulatory role on prolactin secretion, inhibition of growth hormone and adrenocorticotropin hormone axes, as well as the gender-dependent role of cortistatin in the regulation of glucose-insulin homeostasis (176). Moreover, mice lacking cortistatin show deficiencies in the function of the hypothalamus-pituitary-adrenal (HPA) axis, leading to elevated levels of corticosterone (176) and a chronic state of anxiety (177)

- **Immunoregulatory properties of cortistatin**

The neuropeptide cortistatin, but not somatostatin, and its receptors, are expressed by both innate and adaptive immune cells, such as macrophages, dendritic cells and lymphocytes. Moreover the levels of cortistatin correlate with the state of activation of these cells, suggesting that cortistatin may have a role on the regulation of the immune system. In fact, the anti-inflammatory role of cortistatin has been reported both *in vitro* and *in vivo* (171). Cortistatin has a role modulating the innate immune response. For example, cortistatin is able to decrease cytokines such as TNFα, IL-6, IL-1β, IL-12, chemokines and nitric oxide, and to induce the production of IL-10 (a regulatory cytokine) by macrophages activated by LPS. See table 1.1.

Beside its function as an anti-inflammatory agent, it has been also showed that cortistatin has an immunoregulatory role on adaptive immune response. In fact, cortistatin is able to impair the activation of T cells toward Th1 or Th17, and favors a Th2 response (178, 179, 177). Moreover, cortistatin is able to induce Treg cells *in vivo* (15, 177, 180).

Due to its anti-inflammatory and immunoregulatory properties, cortistatin has been tested as a therapy in different animal models of inflammatory and autoimmune diseases. Cortistatin showed a therapeutic effect in sepsis (181) by decreasing at local

and systemic levels a wide spectrum of inflammatory mediators, including cytokines, chemokines and acute phase proteins; Moreover, cortistatin has a therapeutic effect in inflammatory bowel disease (178) by downregulating the inflammatory and Th1-driven autoimmune response, including the involvement of IL-10 secreting-Tregs. Cortistatin also shows a therapeutic effect in rheumatoid arthritis (179), where its role is associated with a striking reduction in the two deleterious components of the disease-that is, the Th1-driven autoimmune and inflammatory responses (downregulating the production of pro-inflammatory cytokines and chemokines. In addition, cortistatin has a role as therapy in multiple sclerosis, an autoimmune and neuroinflammatory disorder, by decreasing the encephalitogenic action of Th1 and Th17 cells in the periphery and nervous system, being able to downregulate pro-inflammatory mediators and to ncrease the number of Tregs, and favouring neuroprotective/regenerative responses (177).

Mice deficient in cortistatin show exacerbated responses to inflammation and T cell stimulation, since macrophages and T cells increases the production of pro-inflammatory cytokines induced by LPS and the proliferation induced by stimulation with anti-CD3, respectively. However, the induction of EAE model to cortistatin deficient mice results in a delayed disease onset and reduction of clinical signs (177). This paradoxical effect of cortistatin could be explained by the fact that mice lacking cortistatin have elevated levels of glucocorticoids, which compensate the lack of cortistatin (176).

- **Cortistatin on nervous system**

Preprocortistatin is expressed in cortical and hippocampal neurons, and also in olfactory bulb, striatum and in GABAergic neurons. However, cortistatin is not expressed in thalamus, or cerebellum (157, 151).

Cortistatin has diverse functions on nervous system. For example, it is able to induce a slow-wave sleep (182). Moreover, intracerebroventricular injection of cortistatin in rats showed that it can decrease locomotor activity (183), and impairs long-term memory in passive avoidance test (184, 185). According to this, cortistatin

could have an important role in memory, since cortistatin expression is affected in the cortex of a mouse model of Alzheimer's disease (186), and the levels of cortistatin are reduced in Alzheimer's disease patients (187). Furthermore, cortistatin has shown neuroprotective effects in cell-based systems and pre-clinical models of ischemia (188) and bacterial meningoencephalitis (189). We have recently reported that cortistatin provides a highly effective therapy in mice with EAE, in which it impaired the autoimmune and inflammatory responses, modulated glial activity and mounted an active program of neuroprotection. Cortistatin did not merely act as an immunosuppressant, but it deactivated the inflammatory response of resident glial cells, and at the same time it induced their CNS supporting roles keeping intact the phagocytosis and trophic functions of these cells (177). Of note, cortistatin levels are decreased during progression of EAE, in the temporal lobe of Alzheimer´s patients, and in the retina of diabetic patients, in which a correlation exists between increased retinal neurodegeneration and glial activation (190).

On base of these previous results that demonstrate the involvement of cortistatin on neuroinflammation and neuroprotection, we wondered about the potential beneficial effect of cortistatin as a multitarget therapeutic approach against a complex neurodegenerative disorder such as PD.

	Family	Main source	Immune source	Receptor	Receptor in immune cells	Main actions	Immune functions	Pre-clinical models
AM	Calcitonin CGRP amylin	Adrenal CNS Peripheral tissues (except thyroid)	T, Mϕ, Mo DC	CRLR-RAMP2 CRLR-RAMP3	CRLR RAMP2/3 in T, Mo, Mϕ, DC	Vasodilatation, bronchodilatation, ↑ cardiac output, smooth muscle relaxation	↓Inflammatory factors (TNFα, IL12, IL6, IL18, MIF, NO, IL1β) ↓Chemokines (rantes, IP10, MIP1α, MIP2, MCP1) ↑IL10 production ↓T cell proliferation ↓Th1 response (IL2 and IFNγ production) Induces regulatory T cells	IBD RA Sepsis Myocarditis Pancreatitis Stroke TBI
CST	Somatostatin	CNS, kidney, stomach	T, Mϕ, Mo	SSTR1-5 MRGX2 GHSR	SSTR1-5 in T, Mo, Mϕ, DC GHSR in T, Mϕ, Mo	↓ locomotor activity Sleep induction ↓ cellular proliferation	↓ Pro-inflammatory cytokines and chemokines ↑IL10 production ↓T cell proliferation ↓Th1 response Induces regulatory T cells	Sepsis RA IBD MS Ischemia Bacterial meningoencephalitis

Table 1.1: Properties of the neuropeptides adrenomedullin and cortistatin: Abbreviations: AM, adrenomedullin; CST, cortistatin; CGRP, calcitonin gene-related peptide; CNS, central nervous system; T, T cells; Mϕ, macrophages; Mo, monocytes; DC, dendritic cells; CRLR, calcitonin receptor-like receptor; RAMP, receptor activity-modifying proteins; SSTR, somatostatin receptors; MRGX2, mas-related gene X2; GHSR, growth hormone secretagogue receptor; IBD, inflammatory bowel disease; RA, rheumatoid arthritis; TBI, traumatic brain injury; MS, multiple sclerosis.

2. HYPOTHESIS AND OBJECTIVES

Neurodegenerative diseases, such as Parkinson's disease (PD), Multiple Sclerosis (MS), Alzheimer's disease or Amyotrophic Lateral Sclerosis, affect to more than one billion of people worldwide and constitute about 35% of the total pool of all diseases in Europe.

MS is a disabling inflammatory autoimmune disease of the central nervous system (CNS), characterized by inflammation and Th1/Th17-mediated autoimmune attack against myelin components that causes important neurological disorders. This pathological loss of myelin is followed by a phenomenon of remyelination, in which oligodendrocytes synthesize new myelin sheaths to cover the naked axons in CNS. Remyelination occurs in early lesions but fails with disease progression. Current treatments are not curative as are mainly focused on modulating the deregulated immunological response with minor attention to neuroprotective approaches.

On the other hand, PD is the second most common neurodegenerative disorder, characterized by a complex pathophysiology that involves neuroinflammation and loss of nigral dopamine neurons and neurotrophic factors. This complexity is the main cause of therapeutic failure for this disorder. Current treatments alleviate the symptoms but effective cure is not available.

Due to the intrinsic multifactorial components and diversity of targets of these disorders of the CNS (neuroinflammation, neurodegeneration, chronicity, limited access of drugs through the blood-brain-barrier, lack of biomarkers of susceptibility and/or progression,...), approaches for developing new therapeutic strategies are highly desirable. From our point of view, the best therapeutic agent for these diseases should combine immunomodulatory and neuroprotective effects.

Adrenomedullin (AM) and Cortistatin (CST) are two neuropeptides produced by the CNS and immune system that have different physiological effects throughout the

body. AM has cardiovascular and anti-inflammatory effects and recently has also shown neuroprotection in experimental brain disease models as ischemic stroke and traumatic brain injury. CST has also shown immunomodulatory and neuroprotective effects in cell-based systems and pre-clinical models of ischaemia, excitotoxicity, and bacterial meningoencephalitis. Recently, we have identified that CST provides a highly effective therapy for the pre-clinical model of MS by regulating the autoimmune and inflammatory responses and mounting an active program of neuroprotection by modulating glial activity.

Based on their immunomodulatory and neuroprotective actions, we hypothesize that addition of exogenous CST and AM may have the potential to be a disease modifying therapy for CNS disorders acting through a multitarget approach.

The general aim of this project is developing a novel strategy to treat MS and PD in which downregulation of neuroinflammation is combined with endogenous trophic support in order to orchestrate the reversal of neurodegeneration. We propose to examine pre-clinical models that recapitulate human pathology in MS and PD to further explore whether AM and CST provides benefit in these models.

Key objectives of this work are:

1. To study the therapeutic effect of adrenomedullin in a pre-clinical model of multiple sclerosis, the experimental autoimmune encephalomyelitis (EAE):
    a. To examine the effect of the systemic injection of adrenomedullin in the clinical and histopathological parameters characteristics of EAE.
    b. To analyze the role of adrenomedullin on the production of inflammatory mediators in the periphery and the CNS of mice with EAE.
    c. To investigate the effect of adrenomedullin in the encephalitogenic Th1 and Th17 response.
    d. To determine the role of adrenomedullin in the activity of the glial niche under neuroinflammatory states.

2. To investigate the ability of adrenomedullin to generate immune tolerance in EAE:
    a. To characterize the presence and function of regulatory T cells induced by adrenomedullin during the progression of EAE.
    b. To determine the capacity of adrenomedullin to generate dendritic cells with tolerogenic activity in EAE.
3. To characterize the neuroprotective and neuroregenerative effect of adrenomedullin in a focal model of demyelination induced by lysolecithin.
4. To investigate the therapeutic role of cortistatin in a preclinical model of PD caused by acute exposure to MPTP.
    a. To study the effect of cortistatin on dopaminergic nigro-striatal pathway, neuroinflammation, brain-derived growth factor depletion and motor deficits induced by MPTP.
    b. To characterize the mechanisms mediating the neuroprotective effect of cortistatin in cultures of MPP+-treated dopaminergic neurons and alpha-synuclein-treated glial cells.

5. To analyze the endogenous role of cortistatin by determining the progression of MPTP-induced PD in cortistatin-deficient mice.

3. MATERIALS AND METHODS

3.1. Peptides

MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) was acquired from GeneScript, dissolved in PBS without Ca^{++} and Mg^{++} and stored at -80ºC until use. Mouse Adrenomedullin and Cortistatin-29 were acquired from American Peptides, dissolved in phosphate buffer 20mM pH7.0 and stored at -80ºC until use.

3.2. Culture media

Complete DMEM: DMEM medium (Dulbecco-modified Eagle Medium, PAA) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/ml, Gibco), streptomycin (100 µg/ml, Gibco) and L-glutamine (2 mM, Gibco).

DMEM 10:10:1: DMEM medium supplemented with 10% FBS, 10% horse serum (Gibco), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM).

B + S medium: FBS free DMEM/F12 medium (DMEM/ Nutrient Mixture F-12, Gibco) supplemented with apo-transferrin (25 µg/ml, Sigma), biotin (10 nM, Sigma), sodium selenite (30 nM, Sigma), putrescine (1 µg/ml, Sigma), insulin (5 µg/ml, Sigma), hydrocortisone (20 nM, Sigma), progesterone (20 nM, Sigma), penicillin (100 U/ml), streptomycin (100 mg/ml), bFGF (fibroblast growth factor-basic) (5 ng/ml, Peprotech), PDGF (Platelet-derived growth factor) (5 ng/ml, Peprotech) and 0.1% BSA (bovine serum albumin, fraction V, Gibco). For oligodendrocyte differentiation this media was supplemented with Thyroid hormone T3 (30 nM, Sigma).

Complete RPMI medium: RPMI medium (Roswell Park Memorial Institute medium-1640, PAA) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml, Gibco), L-glutamine (2 mM), and B-mercaptoethanol (50 µM, Sigma).

Complete neurobasal medium: Neurobasal medium (Gibco) supplemented with serum free supplement B27 (Gibco), 15% FBS, L-glutamine (2 mM), ascorbic acid (0.2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml).

SH-SY5Y culture medium: DMEM/F12 medium, 10% FBS, L-glutamine (2 mM), HEPES (15 mM) (Sigma) and penicillin (100 U/ml) and streptomycin (100 µg/ml).

3.3. Buffers and solutions

Dissecting buffer for mesencephalic neurons: HBSS (without Ca^{++} and Mg^{++}), D-glucose, ascorbic acid (0.2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml)

Lysis buffer for erythrocytes (ACK): 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA

Phosphate-buffered saline (PBS): 130 mM NaCl, 70 mM Na_2HPO_4, 3 mM $NaHPO_4$, pH 7.2

Borate buffer (0.1 M): 1.24 g boric acid, 1.9 g borax, 500 ml ddH_20, pH8.5

Sodium citrate buffer: 10 mM sodium citrate, 0.05% Tween 20, pH 6.0.

Lysis buffer for tissue protein extractions: 50 mM Tris-HCl pH 7.4, 0.5 mM dithiothreitol, 10 µg/ml protease inhibitor mixture (phenylmethylsulfonyl fluoride, pepstatin and leupeptin)

Washing buffer for flow cytometry: 10% FBS, 1% sodium azide, in PBS

Washing buffer for ELISA: 0.1% Tween-20 in PBS

Blocking buffer for ELISA: 10% FBS in PBS

Solution A for ELISA: 0.2 M NaH_2PO_4

Solution B for ELISA: 0.2 M Na_2HPO_4

Binding buffer pH 9 0.1M: 10 ml solution B + 9 ml ddH_2O

Binding buffer 0.1M, pH 6.0: 8.77 ml solution A + 1.23 ml solution B + 10 ml ddH_2O

PB 0.2 mM: 50 ml solution 1 (24 g sodium phosphate monobasic in 1L ddH_2O), 50 ml solution B (28.4 g sodium phosphate dibasic in 1 L ddH_2O)

Stock solution for storage of free-floating sections: 30% glycerol, 30% etylen glycol, 10% PB 0.2 mM and 30% ddH_2O

3.4. Animal models

C57BL/6 mice were obtained from Charles River. Mice lacking the gene for cortistatin (cortistatin-deficient mice, KOCST) were generated in a C57BL/6 background (166), bred in-house, and matched by sex and weight with wild-type (WT) C57BL/6 mice. All experiments with animals were performed in accordance with the European ethical guidelines and approved by the Animal Care Unit Committee from the Institute of Parasitology and Biomedicine Lopez-Neyra-Consejo Superior Investigaciones Científicas and by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine. All animals were housed in a 12-h dark/light cycle in a climate-controlled room at 28-30ºC and 30-40% relative humidity. They were maintained with water *ad libitum* and standard diet.

3.4.1. EAE model

Induction of EAE and treatment with adrenomedullin:

Previous to induction, female C57BL/6 mice (8 week-old) were anesthetized intramuscularly with a mixture of ketamine (0.8 mg/kg, Imalgene 1000, Merial) and acepromazine (2 mg/kg, CalmoNeosan, Pfizer). To induce chronic EAE, mice were immunized subcutaneously (s.c.) with 200 µg of MOG_{35-55} emulsified in complete Freund's adjuvant (CFA) supplemented with 400 µg of *Mycobacterium tuberculosis* H37RA (Difco). MOG was administrated on the back between the shoulders and on both sides of the midline on the lower back. Mice also received intraperitoneal (i.p.) injections of 200 ng of pertussis toxin (Sigma) dissolved in PBS on days 0 and 2 (see Fig. 3.1). Mice were observed and scored daily for signs of EAE according to the following clinical scoring system (65):

Score	Clinical signs
0	No clinical signs
0.5-1	Partial loss of tail tonicity
1	Complete loss of tail tonicity
2	Limp tail and hind limb weakness
3	Hind leg paralysis
4	Hind leg paralysis with body paresis
5	Hind and fore leg paralysis
6	Dead

The treatment with adrenomedullin consisted in the i.p. injection of the neuropeptide at 1 nmol/day dissolved in PBS for 5 consecutive days and was initiated in different phases of the disease:

- After disease onset: The treatment started when mice showed a clinical score of 0.5-1.
- During acute phase: The treatment was initiated at two points of this phase, when mice showed a clinical score between 1 and 1.5 or with a score >2.

The control animals were administrated only with PBS instead neuropeptide.

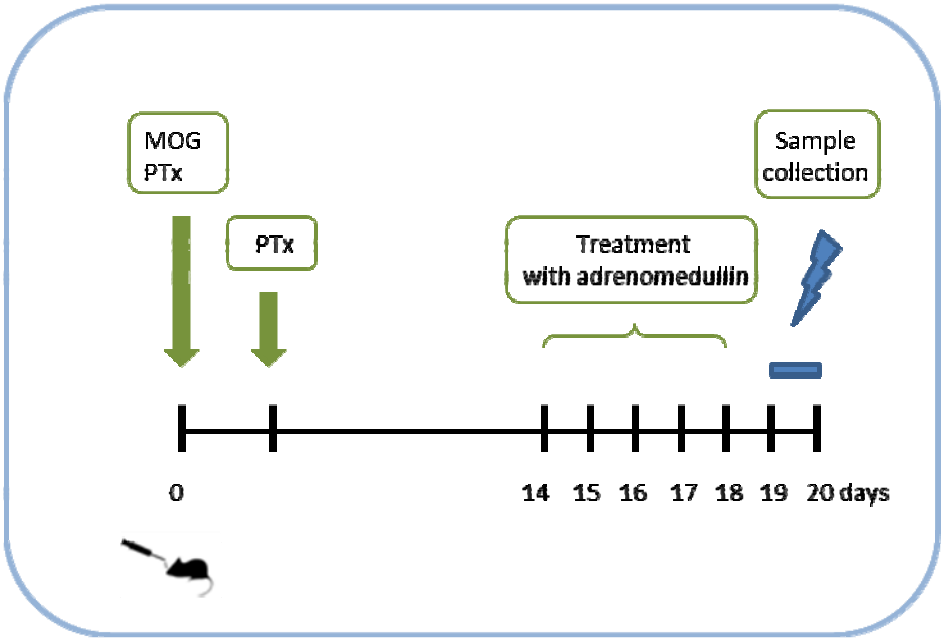


Figure 3.1: Induction of EAE and treatment with Adrenomedullin

Tissue collection:

Untreated and adrenomedullin-treated mice with EAE were sacrificed by CO_2 at various time points after inmunization and spinal cord, brain, spleen, draining lymph nodes (DLNs: inguinal and cervical lymph nodes) and serum were isoated. Brain and spinal cord segments of the cervical and lumbar regions were prepared separately and used for histopathological analysis and for RNA isolation and protein extraction as described below. Single-cell suspensions were obtained from spleen or pooled DLNs

and used for flow cytometry analysis, determination of autoreactive responses and adoptive transfer of EAE as described below. Brain and spinal cord mononuclear cells were isolated by enzymatic tissue digestion and gradient centrifugation and used for flow cytometry analysis and determination of autoreactive responses (see below).

Histopathological analysis:

- Kluver-Barrera staining:

For light microscopy, cervical and lumbar spinal cord segments were fixed with buffered 10% formalin for 48 h and processed for paraffin inclusion and sectioning. 4-µm thickness transversal sections were deparaffinized and hydrated with 95% alcohol. The samples were stained with Luxol Fast Blue Solution at 56ºC overnight. The excess of alcohol was eliminated rising with ddH_20. In order to differentiate the gray and white matter, the samples were incubated with 0.05% lithium carbonate for 30 seconds and 70% alcohol, rinsed with ddH_20 and incubated with 0.1% cresyl violet acetate for 6 minutes and with hematoxylin for 5 minutes (to counterstain nuclei). After various washes, the samples were dehydrated and mounted with Eukitt. Samples were analyzed for the presence of areas of demyelination and cell infiltration using a light microscope.

- Myelin basic protein (MBP) detection:

Spinal cord sections were obtained as described for paraffin processing followed by incubation steps with peroxidase blocking reagents, heat-treated in 1mM EDTA pH 8.0 at 95ºC during 20 minutes for antigenic unmasking, and then incubated for 30 minutes at room temperature with polyclonal anti-MBP antibody. The immunohistochemical processing was performed on an Autostainer480 (Thermo Fisher Scientific Inc) using the polymer-peroxidase-based method and developed with diaminobenzidine (DAB) substrate. We used a non-specific peroxidase-conjugated IgG anti-rabbit serum as a negative control of isotype. Finally, nuclei were hematoxylin-counterstained and the samples were mounted with Eukitt.

- Immunofluorescence detection of cell infiltration:

For immunofluorescence staining, cervical and lumbar spinal cord segments were fixed in 4% paraformaldehyde (PFA) for 4-8 h at 4ºC, equilibrated in 30% sucrose for 24 h, and embebbed in OCT. Transversal cryosections (10-µm thickness) were blocked with 10% goat serum in PBS-T (PBS + 0.2% Triton X-100) for 30 min at room temperature and incubated with FITC-labelled anti-CD4, PE-labelled anti-CD45 or anti-Iba1 antibodies (see table 3.1 for dilutions) for 18 h at 4ºC. The slides were incubated with secondary antibodies when required (see table 3.1) for 1 h at room temperature. Hoechst (Sigma) was used for nuclear staining and sections were mounted using Prolong (Invitrogen). Between steps, samples were extensively washed with PBS-T, and finally were observed in a fluorescence microscope (Olympus IX81).

Protein extraction and cytokine determination in CNS:

Cytokine and chemokine contents were obtained from culture supernatants and from tissue extracts. In the later, proteins were extracted from cervical and lumbar segments of spinal cord and brain by homogenization (50 mg tissue/ml) in lysis buffer for protein extraction. Samples were centrifuged (20,000 g, 15 minutes, 4ºC) and the supernatants were assayed by sandwich ELISA using capture and biotin-conjugated detection antibodies (BD Pharmingen and Peprotech), following the manufacturer's instructions for each one. Briefly, 96-well plates (Nunc. Maxisorb) were incubated with capture antibody dissolved in specific buffer (see table 3.4) for 18 h at 4ºC, then washed twice with washing buffer and blocked with blocking buffer for 2 h at room temperature. After that, samples and standard diluted in blocking buffer were added to the plates for 18 h at 4ºC (see table 3.3). Plates were then washed four times and incubated with biotinylated antibodies (see table 3.5) for 1 h at room temperature. After six washes, Streptavidin-Horseradish Peroxidase (HRP) was added to the each well and incubated for 45 minutes. Finally, plates were washed 8 times and incubated with the chromogenic substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Fluka, 0.1 M) and 0.03% H_2O_2 (Sigma) and developed at room temperature in the dark. After few minutes, absorbance of each well was read at 405 nm using VersaMax spectrophotometer and SoftMax Pro software (Molecular Devices).

Adrenomedullin levels were determined using a competitive ELISA kit (Phoenix Pharmaceutical) following manufacturer's instructions.

Determination of autoreactive response:

Spleen and DLNs were removed from mice at peak of clinical EAE and mechanically homogenized in complete RPMI medium. Cell suspension was centrifugated at 300 g for 5 minutes and resuspended in ACK lysis buffer for 5 minutes at room temperature to eliminate erythrocytes. Finally, spleen and DLN cells were resuspended in complete RPMI medium and cultured at 10^6 cells/ml until stimulation.

Brain and spinal cord mononuclear cells recovered from mice at peak of clinical EAE and isolated after tissue digestion with 10 ml HBSS containing DNAse I (0.1 mg/ml for brain and 0.05 mg/ml for spinal cord) and Liberase (0.05 mg/ml for brain and 0.025 mg/ml for spinal cord) for 45 minutes at 37ºC with shaking. Digested tissue was incubated with 10% FBS, 10mM EDTA in HBSS and, after pelleted, it was resuspended in 10 ml of 30% isotonic Percoll and underlaided with 70% isotonic Percoll. Mononuclear cells were isolated from the 30/70 interphase after gradient centrifugation (5000 rmp for 20 minutes at 20ºC). Finally, cells were washed with complete RPMI medium and cultured at 10^6 cells/ml for further stimulation.

Brain/ spinal cord mononuclear cells and spleen/DLN cells were stimulated in complete RPMI medium with 15 µM MOG_{35-55}. Concanavalin A (ConA, 2.5 µg/ml) was employed as control for non-specific polyclonal T cell stimulation. In brain/spinal cord cell cultures, plastic-adherent splenocytes (2 x 10^5/ml) were added to cultures as antigen presenting cells. After 48 h cytokine and chemokine contents in supernatants were determined by sandwich ELISA as described above. Cell proliferation was evaluated after 72 h by adding 2.5 µCi/ml tritiated thymidine ([^{3}H]-TdR, Amersham) during the last 8 h of culture. Cells were collected and immobilized with 70% ethanol into a glass fiber membrane (Wallac), heated in a microwave for 2 minutes and submerged into scintillation fluid. [^{3}H]-TdR incorporation was measured in a Microbeta counter 1450 (Wallac) and expressed as counts per minute (cpm).

To investigate the direct effect of adrenomedullin on autoreactive responses, the neuropeptide (100 nM) was added to brain/spinal cord mononuclear cell and spleen/DLN cell cultures isolated from untreated EAE mice at the peak of disease and stimulated with 15 µM MOG_{35-55}. After 48 h, supernatants were collected and the

production of cytokines was measured by ELISA and cell proliferation was evaluated after 72 h.

Determination of intracellular cytokines by flow cytometry:

For intracellular analysis of cytokines, brain/spinal cord mononuclear cells, spleen and DLN were isolated at the peak of the disease and cells were stimulated at 10^6 cells/ml with 25 ng/ml Phorbol-12-myristate-13-acetate (PMA, Sigma) and 500 ng/ml ionomycin (Sigma) for 8-12 h, in the presence of 3 µM monensin (Sigma) for the last 6 h of culture. After treatment, cells were collected and distributed into polystyrene tubes (BD) for flow cytometry at 0.5 x 10^6 cells/tube and washed twice with washing buffer (300 g for 5 minutes at 4ºC). Before specific staining, cells were blocked with mouse BD Fc Block anti-2.4G2 (BD Bioscience) to avoid nonspecific binding of antibodies to Fc-receptors, and marked with 7-AAD (7-amino-actinomycin D, Calbiochem) to distinguish dead cells. Then, cells were incubated with APC-labelled anti-CD4 antibody (diluted in washing buffer) for 18 h at 4ºC. Cells were then fixed/permeabilized using BD Cytofix/Cytoperm kit. Finally, cells were incubated with FITC-and PE-conjugated anti-cytokines (anti-IFNγ, anti-IL-17, anti-IL-4 or anti-IL10) diluted in Fix/Perm solution, for 30 minutes at 4ºC (for dilutions see table 3.2). In all cases, we used isotype-matched Abs as controls (see table 3.2). For sample acquisition and analysis, a FACScalibur flow cytometer and Cell Quest Pro program (Becton Dickinson) were used.

Serum collection and determination of autoantibodies:

At the peak of the disease, EAE mice were anesthetized and blood (approximately 1 ml) was extracted by cardiac puncture into the left ventricle with 22G needle and 1 ml syringe. Samples were maintained 3 h at room temperature for blood coagulation and then centrifuged at 2000 rpm for 10 minutes at room temperature. Upper layer serum was collected and stored at -20ºC until use.

ELISA was used to determine the specific anti-MOG antibody responses. Maxisorb plates (Millipore) were coated 18 h at 4ºC with MOG_{35-55} (10 µg/ml) in 0.1 M biphosphate buffer pH 9.5, blocked with PBS + 10% FBS and incubated for 2 h at 37ºC with serial dilutions of collected sera. Biotinylated anti-IgG1 or anti-IgG2a antibodies (2.5 µg/ml, Serotec) were added to plates for 1 h at 37ºC. After washing, the plates

were incubated with HRP, developed with ABTS and the absorbance was determined using a spectrophotometer.

Determination of Treg cell content and function:

To determine the content of Treg cells, brain/spinal cord mononuclear cells, spleen and DLN were isolated at the peak of the disease and cells ($0.5x10^6$ cells/tube) were blocked with mouse BD Fc Block anti-2.4G2 and marked with 7-AAD. Cells were incubated with conjugated anti-surface antigens (APC-labelled anti-CD4 and FITC-labelled anti-CD25 antibodies, diluted in washing buffer) for 18 h at 4ºC (for dilutions see table 3.2) and then processed for FoxP3 staining using a specific kit from eBioscence following the manufacturer's recomendations. Isotype-matched antibodies were used as controls (see table 3.2). For sample acquisition and analysis, a FACScalibur flow cytometer and Cell Quest Pro program (Becton Dickinson) were used.

To determine the regulatory activity of T cells generated in vivo by the treatment of adrenomedullin in mice with EAE, cells from spleen and DLNs from untreated and adrenomedullin-treated EAE mice were isolated at the peak of the disease (18 days postimmunization) and were T-cell enriched by plastic adherence during 2 h at 37ºC. Then, non-adherent cells were collected and injected i.p (10^7 cells/mouse) into EAE mice (clinical score 0.5-1) (see Fig. 3.2). When indicated, isolated CD4 cells were processed to deplete the CD25 population (before injection to the mice) by negative immunomagnetic selection using the CD4+ CD25+ Treg cell isolation kit (Miltenyi Biotec) following manufacturer's instructions. CD4+ CD25- T cells were injected i.p. (15×10^6 cells/mouse) into EAE mice at disease onset and clinical signs were scored daily as described above.

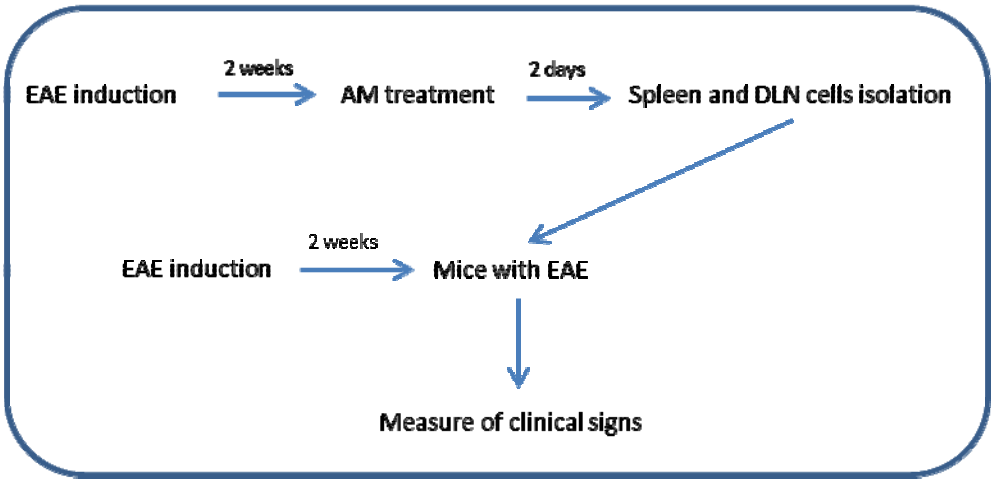


Figure 3.2: Transference of Tregs

DC isolation and treatments:

DCs were differentiated from bone marrow cells obtained from femurs and tibiae of C57Bl/6 mice. Bone marrow cells ($2x10^6$ cells) were incubated in Petri dishes in complete RPMI medium containing mouse GM-CSF (20 ng/ml, Peprotech). At day 6 of culture, non-adherent cells were collected (containing 80-90% $CD11c^+$ cells) and stimulated with 1 µg/ml bacterial lipopolysaccharide (LPS from *Escherichia coli* clone 055:B5, Sigma-Aldrich) and pulsed with $MOG_{35–55}$ (20 µg/ml) in the absence (DCs-control) or presence (DCs-adrenomedullin) of adrenomedullin (100 nM). After 48 h of culture, flow cytometry analysis for CD40, CD80 and CD86 was performed using conjugated anti-surface antigens FITC-labelled anti-CD40, FITC-labelled anti-CD80 or PE-labelled anti-CD86 antibodies (for dilutions see table 3.2) as previously described (169) and the content of cytokines in the culture supernatants was determined by ELISA. After 24 h of culture, MOG-pulsed DCs-control or DCs-adrenomedullin (2×10^5/ml) were added to DLN cells (10^6/ml) isolated from EAE mice at peak of disease and restimulated with $MOG_{35–55}$ (15 µM) or Concanavalin A (2.5 µg/ml) to determine the proliferative response and production of cytokines. Alternatively, MOG-pulsed DCs-control or DCs-adrenomedullin (2×10^6/mouse) were injected i.p. in mice with EAE at disease onset and the progression of the disease was evaluated as described above (see Fig 3.3).

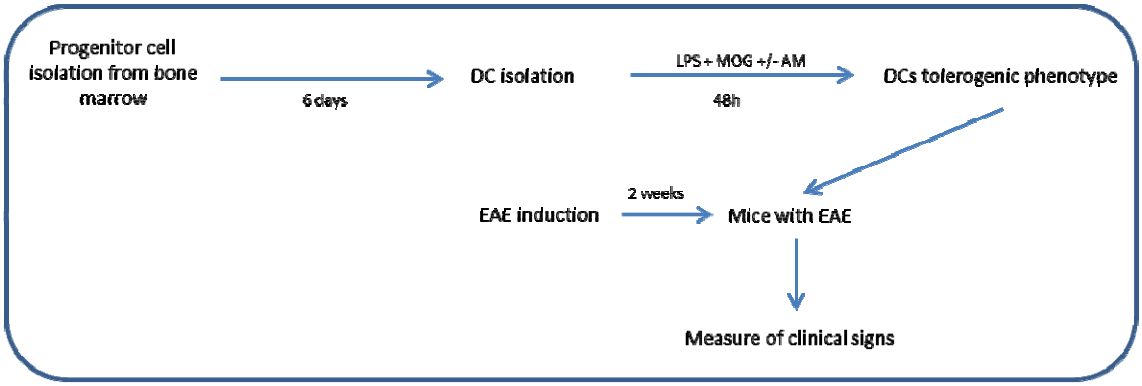


Figure 3.3: Transference of DCs

Neuron and glial cell isolation and culture:

Primary mixed neuron-glia and microglia, astrocytes and oligodendrocytes were obtained from C57Bl/6 newborn mice (postnatal days 1-3). After sacrifice by decapitation, brains were obtained and meninges, cerebellum and olfactory bulb were removed under a dissecting microscope. Next, brains were homogenized in DMEM 10:10:1, and tissue extracts were centrifugated at 1000 rpm for 10 minutes. Finally, pelleted cells were resuspended in DMEM 10:10:1 and plated in $75cm^2$ flasks (2 brains/flask in 10 ml) that were previously coated with Poly-D-lysine (PDL from Sigma, 5µg/ml for 30 minutes at 37ºC and washed with sterile ddH_2O). Culture was maintained at 37ºC with 5% CO^2 for 10-14 days and then used for mixed neuron-gial cultures or isolation of microglia, oligodendrocytes or astrocytes.

i. Isolation of microglia:

After 10-14 days of culture, flasks were shaked at 200 rpm for 3 h at 37ºC. The non-adherent cells (consisting mainly in microglia) were collected and centrifuged at 1000 rpm for 10 minutes. The purity of the population (>97% $CD11b^+$ cells) was determined before culture by immunofluorescence anlaysis. For stimulation, microglial cells were resuspended in DMEM 10:10:1 and plated at $2x10^5$ cells/ml into wells previously coated with PDL (5 µg/ml). After 24h, the medium was changed to DMEM/2% FBS and cells were stimulated with LPS (0.1 µg/ml) in the absence or presence of adrenomedulin (100 nM) for 24 h. Supernatants were stored at -20ºC until

cytokine production was determined by sandwich ELISA. Intracellular ROS levels were also assayed using different stimuli (see below).

ii. Isolation of oligodendrocyte precursor cells (OPCs):

After collecting microglia, DMEM 10:10:1 was added to the flasks and incubated again at 37ºC for 3 h. Flasks were then shaked at 260 rpm at 37ºC overnight. Thereafter, cells in the supernatant (consisting mainly in OPCs, >85% Olig-2^+) were collected and plated ($2x10^5$cells) into PDL-coated 96-well plates in B + S medium. Half medium was changed every 3 days. Mature oligodendrocytes were generated by incubation of precursors with T3 hormone (30 nM) for 3–5 days. Oligodendrocyte cell death was caused by oxidative stress by incubation with 200 µM H_2O_2 in the absence or presence of adrenomedullin (100 nM) for 24 h, and cell survival was assayed by the reduction of MTT (3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide). Briefly, cultures were incubated with 1:10 dilution of MTT solution (5 mg/ml, dissolved in PBS) for 4 h at 37ºC, and the supernatant was then removed and 100 µl of DMSO were added to culture for 1 h at 37ºC. Finally, absorbance was measured at 570 nm with VersaMax spectrophotometer.

iii. Isolation of astrocytes:

Subsequently to microglia and OPCs collection, the cell monolayer remaining in the flask is mainly composed by astrocytes. To obtain them, flasks were incubated with 12 µM cytosine β-D-arabinofuranoside (ARA-C, Sigma) for 3-5 days to avoid glial cell proliferation. Cells were then trypsinized for 5 minutes, collected by centrifugation at 1000 rpm for 10 minutes and resuspended in complete DMEM at 10^5 cells/ml. The isolated cells were 99% GFAP$^+$. Once astrocytes grew until 80% confluence, the medium was changed to DMEM/2%FBS and cells were stimulated with LPS (0.1 µg/ml) in the absence or presence of adrenomedullin (100 nM) for 24 h. Supernatant was stored at -20ºC until cytokine determination by ELISA. Intracellular ROS was also assayed using different stimuli (see below).

iv. Mixed neuron-glia cultures:

Alternatively, we used brain cells isolated from neonates and cultured for 10-14 days as described above as mixed neuron-glia cocultures. Cocultures (5 x 10^5 cells/ml) were incubated in DMEM/10%FBS or stimulated with LPS (0.1 µg/ml) in the absence or

presence of adrenomedullin (100 nM) for 24 h. Supernatant was stored at -20ºC until cytokine determination by ELISA. Our neuron-glia cocultures consisted of 27.28% ± 1.8% neurons, 43.9% ± 2.4% astrocytes, and 8.9% ± microglia. The cellular composition of the neuron and glial cultures were determined by immunofluorescence as follow. Cell cultures were fixed with 2% PFA for 15 minutes at room temperature, blocked with 10% goat serum in PBS + 0.1% Triton X-100 for 1 h, and sequently incubated with primary antibodies (anti-MAP2, anti-GFAP, anti-CD11b) and then with secondary antibodies (Alexa Fluor 594 and 488) at indicated dilutions (table 3.1). For nuclear staining, samples were stained with Hoechst, and mounted with Prolong. Between steps, samples were extensively washed with PBS and finally observed in a fluorescence microscope (Olympus IX81).

Determination of intracellular reactive oxygen species (ROS):

Production of intracellular ROS was measured using 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA), a chemically reduced, acetylated form of fluorescein. This nonfluorescent molecule is readily converted to a green-fluorescent form when the acetate groups are removed by intracellular esterases and oxidation (by the activity of ROS). Murine microglial and astrocyte cultures were seeded at 3 x 10^4/well and 2 x 10^4/well, respectively, in 96-well plates and stimulated with H_2O_2 (300 µM, microglia; 400 µM, astrocytes) in serum-free medium and α-synuclein monomers (1 µg/ml) in DMEM/2% SBFi in the presence or absence of adrenomedullin (100 nM). Carboxy-H_2DCFDA was added (5 µM, prewarmed Hank´s balanced salt solution) together with H_2O_2 or after 4h of α-synuclein stimulation. After incubation with Carboxy-H_2DCFDA (1 h for H_2O_2, 30 minutes for α-synuclein) at 37ºC in the dark, fluorescence was measured in a plate reader at Ex_{485} and Em_{530}. Each sample was run in quatriplicate and means were normalized to their respective controls (% of control).

Determination of gene expression by real-time PCR:

RNA extraction:

Brain or spinal cord were frozen in liquid nitrogen immediately after sacrifice and stored at -80ºC until use. RNA isolation was performed by mechanical homogenization in 1 ml of Tripure (Roche), followed by extraction with 200 µl chloroform and incubation for 15 minutes at room temperature. Samples were centrifuged at 12000 g for 15 minutes at 4ºC and the aqueous phase was collected, incubated with 500 µl of isopropanol for 15 minutes at room temperature and centrifuged at 12000 g for 15 minutes at 4ºC. Precipitated RNA was washed with 70% ethanol and centrifuged at 7500 g for 5 minutes at 4ºC. The RNA samples were dried at room temperature, resuspended in RNAse free water (Invitrogen) and stored at -80ºC until use. RNA was quantified measuring the absorbance in Nanodrop 100 spectrophotometer (Thermo Scientific).

Reverse transcription:

RNA samples (1 µg) were incubated with 2 U of DNAse (Sigma) for 15 minutes at room temperature. To inactivate the reaction, stop solution was added to samples for 10 minutes at 65ºC. After that, RNA was reverse trancribed using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas). Briefly, RNA samples were incubated with 5µM Random Hexamer Primers, 1 nM deoxynucleotide (dNTP) Mix, 5X buffer reaction, 10 U/µl RevertAid RT enzyme and 1 U/µl Ribolock RNAse Inhibitor at 25ºC for 5 minutes, at 42ºC for 60 minutes and at 70ºC for 5 minutes. The final cDNA product was diluted 1/4 with RNAse-free water and stored at -20ºC until amplification.

Real-time polymerase chain reaction (qPCR):

cDNA was used for quantifying gene expression of activity-dependent neuroprotective protein (ADNP) and brain-derived neurotrophic factor (BDNF) by real time quantitative RT-PCR (60ºC as annealing temperature) by using iQ SYBR Green Supermix (BioRad) according to the manufacturer's instructions. β-actin and GAPDH were used for normalization and fold change expression was estimated using the Delta-Delta Ct method. The sequences of primers used for each gene are displayed in table 3.6.

3.4.2. Model of focal demyelination induced by lysolecithin

Induction of focal demyelinization and treatment with adrenomedullin:

Male C57Bl/6 mice (12 week-old) were anesthetized with 2% isofluorane, head shaved and subsected to a skin incision of 1 cm at the cranial middle line. Mice were placed into a stereotactic frame and lysolecithin (2 µl of a stock solution at 1% dissolved in PBS, Sigma) was delivered by microinjection into the left side of corpus callosum (5.5 mm rostral and 1 mm to the left of lambda, 2 mm in depth). Following injection, the wound was sutured with clips. The treatment with adrenomedullin consisted in daily i.p. injection (2 nmol/day), starting 24 h after lysolecithin administration. After 7 and 14 days, brain was collected and used for histopathology analysis as described below (see Fig. 3.4).

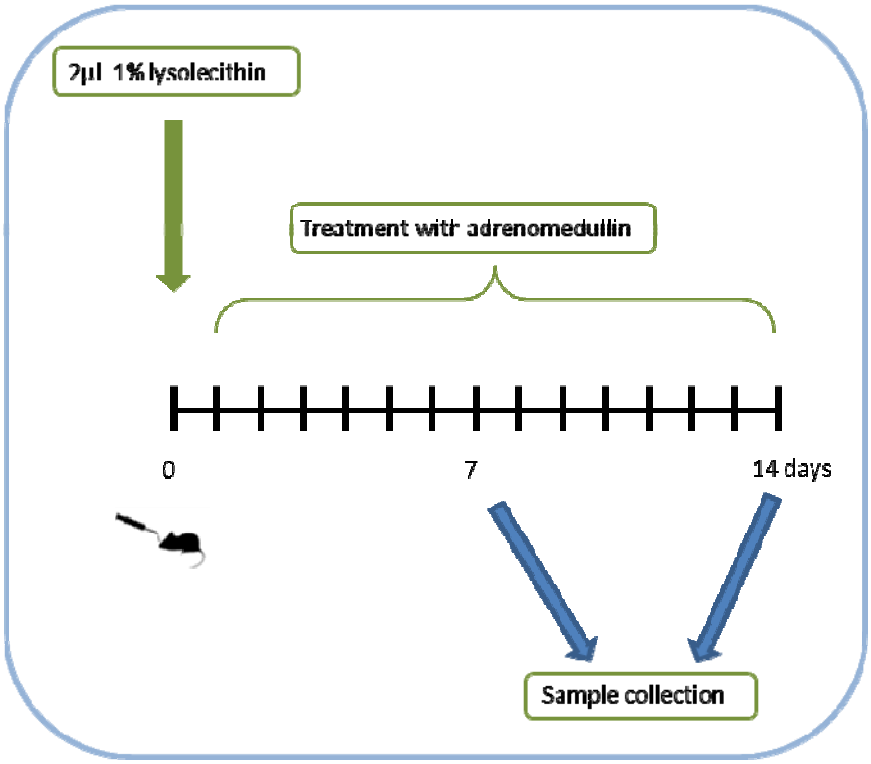


Figure 3.4: Model of focal demyelination induction

Determination of demyelinization and content of oligodendrocytes:

Mice were deeply anesthetized with an i.p injection of chloral hydrate (350 mg/Kg) and perfused through the heart with PBS followed by 4% PFA. Brains were removed, postfixed with the same fixative overnight and left in 30% sucrose in PBS for 24 h. Brains were then embedded in OCT mounting medium (Tissue Tek), cryosectioned and stored at -80ºC. Coronal cryosections (20-µm thickness) were

blocked with 10% goat serum in PBS + 0.3% Triton X-100 for 1 h at room temperature. The immunostainning for myelin was performed with FluoroMyelin™ Red Fluorescent Myelin stain (Molecular Probes) for 1 h at room temperature and nuclei-counterstained with Hoechst. For Olig-2 detection, sections were processed for antigen retrieval (incubated at 100º C with sodium citrate buffer for 20 minutes) and sequentially incubated with anti-Olig2 antibody (for dilutions see table 3.1), and then with secondary antibody (Alexa Fluor 594), nuclei-counterstained with Hoechst and mounted in Prolong. Between steps, samples were extensively washed with PBS-T. Samples were observed in a confocal microscope Leica TCS SP5 and serial stacks were collected using 1 µm on the Z-axis and assembled into projections. To quantify demyelination, the fluoromyelin negative area was measured at 10x magnification using ImageJ software. Olig2-positive cells were quantified within the hypercellular lesion area in at least five fields (at 20x magnification) per animal, four animals per time point.

3.4.3. Model of Parkinson disease induced with MPTP toxin

Induction of PD model and treatment with cortistatin:

To induce PD, male C57BL/6 mice (12 week-old) were i.p injected with MPTP dissolved in PBS (20 mg/kg, 4 times at intervals of 2 h). Naive mice injected with saline were used as sham controls. Treatment consisted in i.p injection of saline or cortistatin (2 nmol of cortistatin-29) at 2 h and 4 h after the last MPTP injection, and then once per day for 6 additional days. Mice were sacrifized 2 and 7 days after the last MPTP injection by deep anesthesia (i.p injection of 350 mg/kg chloral hydrate) and intracardially perfused with PBS and 4% PFA as described above. Brains were isolated and processed for immunohistochemistry and RNA extraction as described below. Mice sacrifized on day 7 were also subjected to tests of lomomotion and coordination (see Fig. 3.5 for experimental schedule).

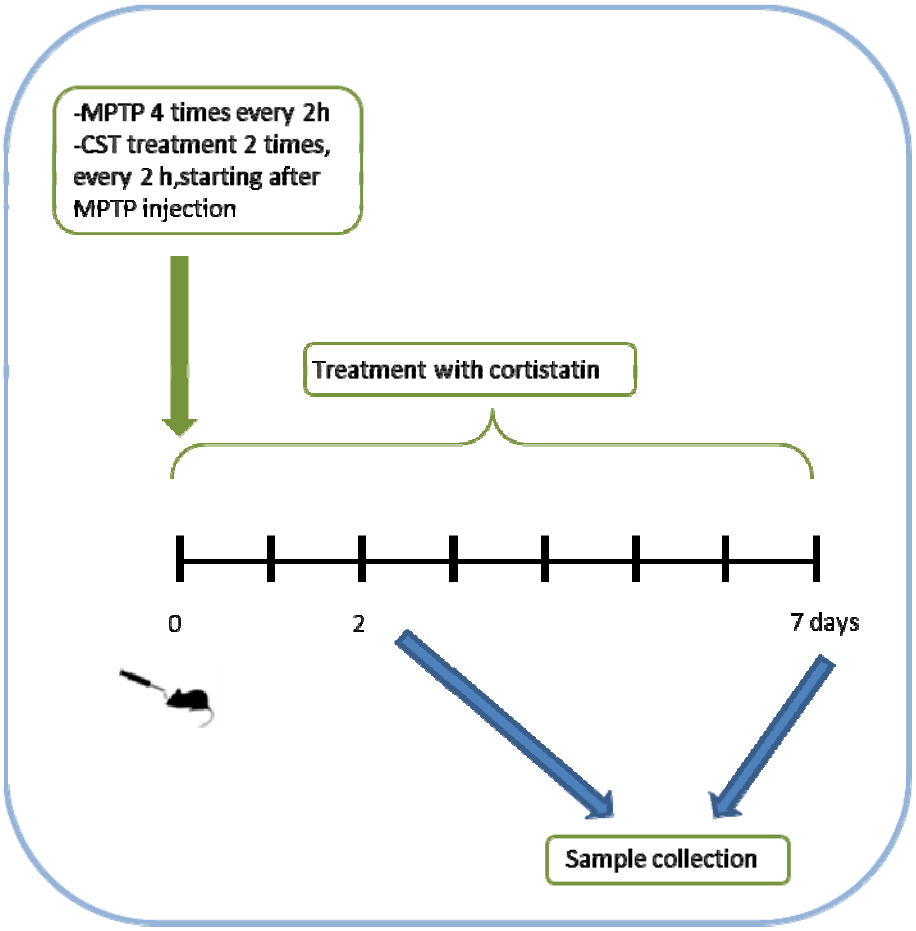


Figure 3.5: Induction of MPTP model of PD

Locomotor and coordination tests:

Before sample collection at day 7, mice were tested for locomotor activity, coordination and neuromuscular strength using the following tests:

- *Rotarod performance test*: test based on a rotating rod with forced motor activity. The test measures parameters such as riding time (seconds) or endurance to evaluate motor coordination. The rotarod consisted on a rotating rod of 3 cm of diameter divided into five 5 cm lanes. On the same day of testing, mice were trained on the apparatus for three consecutive trials in which the rod was kept at constant speed: one trial at 0 rpm and two trials at 4 rpm. When animals were able to stay on the rod rotating at 4 rpm during 60 seconds, the test was initiated. Then, animals were placed individually for four consecutive trials (30 minutes inter-trial intervals) on the rod rotating with an

accelerating speed from 4 to 40 rpm in 300 seconds. The latency to fall off the rod was recorded automatically.

- *Gait test:* This test measures basal ganglia-related movement disorders. Mice were placed on an illuminated runway (4.5 cm wide, 50 cm long) and were allowed to run toward their home cage. Before perform the test, animals were acclimatized over two trials. The stride length was measure for forelimb and hindlimb. In order to differentiate between forelimb and hindlimb strides, front and back paws were painted with blue and black ink, respectively. Mice runaway in a paper, then the paw kept marked on the paper. Stride length was measured as the distance between successive paw prints. The average of three strides was taken for each animal.
- *Hang test:* This test measures neuromuscular strength. Animals were placed on a horizontal grid (total size 12 cm^2 with openings of 0.5 cm^2) and supported until they grabbed the grid. The grid was then inverted (20 cm height) and mice were allowed to hang upside down. Animals were allowed to stay on the grid for 30 seconds and 5 chances were given with 1 minute of interval.

Tyrosine Hydroxylase (TH) detection:

After transcardial perfusion of mice with PBS and 4% PFA, brains were removed, postfixed overnight with 4% PFA at 4ºC and then equilibrated in a 30% sucrose solution for 1-2 days at 4ºC until they sank. Brains were embebbed into OCT, stored at -80ºC and then cryosectioned to obtain sequential coronal sections (30 µm) that include striatum and substantia nigra pars compacta (SNpc). Sections were stored in tissue stock solution (see section 3.3) at 4ºC until required. Stored sections were processed by free floating method for TH staining using the VECTASTAIN Elite ABC system (VECTOR laboratories) following manufacturer's protocol. Briefly, tissue sections were washed 5 times for 5 minutes each with 1 ml of PB WASH (PB 0.1 M + 0.05% triton X-100) and incubated with PB 0.1 M+ 1% H_2O_2 during 15 minutes at room temperature. After washing, sections were blocked for 30 minutes (PB 0.1 M + 0.3% triton X-100 + 2% goat serum) and then incubated with primary anti-TH antibody (see dilution on table 3.1) for 24 h at 4ºC. Sections were washed 5 times for 5 minutes each

and incubated with the secondary antibody (diluted on blocking buffer, see table 3.1) 1.5 h at room temperature. After several washes, sections were incubated with ABC reagent for 45 minutes, washed again and finally revealed with DAB substrate solution until obataining the desired stain intensity. Thereafter, sections were rinsed with cold PB 0.1 M and mounted in 50% glycerol in PBS + 0.03% sodium azide.

The TH-stained SNpc neurons were showed in the right and left SNpc of every six sections throughout the entire extent of the SNpc. The sections used covered the entire rostrocaudal axis of the SNpc [AP, -2.7 to -3.8 mm from bregma according to the atlas of Franklin and Paxinos (191)] that generally yielded 30-36 sections in a serie. To determine striatal density of TH immunoreactivity, five striatal sections between bregma +0.98 and +0.02 mm (according to the atlas of Franklin and Paxinos) of each mouse were stained for TH. Staining intensity of the striatum was determined using a light-transmitted microscope (Olympus BX43) and a digital camera (Olympus DP70). Optical density of striatal TH innervation was measured from digitized pictures using the NIH Image software Image J as previously described (192). The concentration of anti-TH antibody and DAB, and the duration of incubation of striatal sections in DAB were optimized to fall within the linear range of the plot of the immunostaining intensities. A square frame of 900 x 900 µm was placed in the dorsal part of the striatum. A second square frame of 200 x 200 µm was placed in the region of the cortex to measure background value. To control for variations in background illumination, the average of the background density readings from the cortex was subtracted from the average of density readings of the striatum for each section.

Tissue immunodetection of glial cells:

Iba-1 and GFAP expression was analyzed in SNpc and striatum by immunostatining basically following the free floating method described for the detection of TH with some modifications. Briefly, tissue sections (30 µm) were washed 5 times for 5 minutes with PB WASH (PB 0.1 M + 0.05% triton X-100), blocked for 30 minutes with PB 0.1 M + 0.3% triton X-100 + 2% goat serum, incubated with primary antibodies for 24 h at 4ºC (see table 3.1 for dilutions) and with the secondary antibody (Alexa Fluor 594-labelled anti-rabbit) for 1.5 h at room temperature. After several

washes, nuclei were staining with Hoechst and sections were mounted using Prolong and observed in a fluorescence microscope (Olympus IX81).

Determination of gene expression by real-time PCR:

Brain was collected from mouse sacrificed 2 and 7 days after the last MPTP injection. SNpc and striatum were isolated by microdissection, frozen in liquid nitrogen and stored at -80ºC until use. RNA extraction, reverse transcription and qPCR was performed as described for the EAE model. cDNA was used for quantifying gene expression of BDNF, TH, dopamine transporter (DAT) and α-synuclein (SYN) by real time quantitative RT-PCR by using iQ SYBR Green Supermix (BioRad) according to the manufacturer's instructions. GAPDH was used for normalization and we estimated fold change expression with Delta-Delta Ct method. The sequences of primers used for each gene are displayed in table 3.6.

Mesencephalic neuron-glia culture:

The ventral mesencephalon was removed from embryonic tissue (at 12 to 14 days of gestation) in dissection buffer using a dissecting microscope and immediately homogenized mechanically by pipetting several times. The isolated cells were seeded in complete Neurobasal medium at a density of 2 x 10^5 cells/cm^2 in glass coverslides pre-coated with PDL in 0.1 M borate buffer (pH 8.4). After 20 minutes at 37ºC, adhered cells were supplemented with complete neurobasal medium (500 µl/well). 24 h after plating, cells were transferred to serum-free defined medium and cultured in a humidified chamber at 37ºC and 5% CO2 atmosphere. Half medium was replaced by fresh medium after 3 days of culture. After 7-8 days of culture, cells were stimulated with 5 µM 1-methyl-4-phenylpyridinium ion (MPP^+) in presence or absence of cortistatin (100 nM) for 48 h. Cell death was quantified by counting the number of TH positive cells using immunofluorescence analysis as follow. Cell cultures were fixed with 2% PFA for 15 minutes at room temperature, blocked with 10% goat serum in PBS + 0.1% Triton X-100 for 1 h at room temperature, and sequently incubated with anti-TH or anti-MAP2 antibodies (at 4 ºC, overnight), and with the correspondin secondary antibodies (Alexa Fluor 594 and 488) for 1 h at room temperature (see table 3.1 for dilutions). Samples were nuclei-counterstained with Hoechst and mounted with

Prolong. Between steps, samples were extensively washed with PBS and finally observed in a fluorescence microscope (Olympus IX81).

Cell culture, differentiation and treatment of SH-SY5Y cells:

The human neuroblastoma cell line SH-SY5Y (ATCC, Manassas) was cultured and differentiated at 37°C in a humidified atmosphere of 95% air and 5% CO2 in collagen pre-treated plates. Cells were maintained in SH-SY5Y culture medium. At 60–70% of confluence, cells were sub-cultured and differentiated into dopaminergic TH-positive phenotype with 10 µM retinoic acid (RA) for the first 3 days, and then with 80 nM PMA for 3 additional days (193, 194). Upon differentiation, TH immunoreactivity was up-regulated. Differentiation medium contained reduced serum (3–5%). Cells were incubated with MPP^+ (1 mM) in the absence or presence of cortistatin (100 nM) for 48 h and cell viability was determined by MTT assay as described above.

3.5. Statistical analysis

All data are expressed as the mean ±SEM (standard error of the mean). As clinical scores to assess EAE are non-linear, we used non-parametric statistical analysis. These include Mann–Whitney U test or the Kruskal–Wallis test when comparing more than two groups. We used these tests for detecting statistical differences between the individual EAE scores on a specific day for the adrenomedullin-treated and untreated EAE mice. In addition, their scores over each day of the experiment were compared by two way ANOVA or one-way ANOVA (for more than two groups) with appropriate post tests. The rest of experiments were statistically analyzed by using one-way ANOVA, and by unpaired *t*-test for two groups. We considered significance at $p< 0.05$. All statistical analyses were performed using the Graph-Pad Prism software and Sigma-Plot software.

Table 3.1: Immunodetection antibodies for immunofluorescence and immunohistochemistry

Antibodies	Company	Catalog number	Stock dilution	Working dilution
Anti-CD4-FITC	BD bioscience	557807	0.5 mg/ml	2.5 µg/ml
Anti-CD45-PE	BD bioscience	553081	0.2 mg/ml	1 µg/ml
Anti-Iba1	Wako	19-19741	0.5 mg/ml	1 µg/ml; 0.25 µg/ml (floating)
Alexa Fluor 546	Invitrogen	A10040	2 mg/ml	2 µg/ml
Anti-CD11b -FITC	BD bioscience	557396	0.2 mg/ml	0.2 µg/ml
Anti-Olig2	R&D system	AF2418	0.2 mg/ml	0.4 µg/ml
Anti-MBP	Millipore	MAB386	Ascites 1ml	1/1000
Anti-GFAP	DAKO	Z0334	2.9 mg/ml	2.9 µg/ml; 1.4 µg/ml (floating)
Alexa Fluor 594	Invitrogen	A11012	2mg/ml	2 µg/ml
Alexa Fluor 488	Invitrogen	A11017	2mg/ml	2 µg/ml
Anti-TH	Santa Cruz	Sc-14007	200µg/ml	0.5 µg/ml; 0.1 µg/ml (floating)

Table 3.2: Cytometry antibodies

Fluorochrome-conjugated antibodies	Company	Catalog number	Stock dilution	Working dilution
Anti-mouse IgG FOXP-3 – PE	ebioscience	12577380	0.2 mg/ml	4 µg/ml
Anti-mouse IgG CD4 - PERCP	BD bioscience	553052	0.2 mg/ml	4 µg/ml
Anti-mouse IgG CD25 - FITC	BD bioscience	553071	0.5 mg/ml	5 µg/ml
Anti-mouse IgG IFN-γ - FITC	BD bioscience	554411	0.5 mg/ml	5 µg/ml
Anti-mouse IgG IL-17 - PE	BD bioscience	554467	0.2 mg/ml	4 µg/ml
Anti-mouse IgG IL-4 - APC	BD bioscience	554467	0.2 mg/ml	4 µg/ml
Anti-mouse IgG IL-10 - PE	BD bioscience	554467	0.2 mg/ml	4 µg/ml
Anti-mouse IgG CD40-FITC	BD Pharmingen	553723	0.5mg/ml	5 µg/ml
Anti-mouse IgG CD80-FITC	BD Pharmingen	553768	0.5mg/ml	5 µg/ml
Anti-mouse IgG CD86-FITC	BD Pharmingen	553691	0.5mg/ml	5 µg/ml
(Isotype) IgG2a- PE	BD bioscience	553930	0.2 mg/ml	4 µg/ml
(Isotype) IgG1 - FITC	BD bioscience	553953	0.5 mg/ml	5 µg/ml
(Isotype) IgG2a- APC	Biolegend	402012	0.2 mg/ml	4 µg/ml

Table 3.3: Recombinant proteins for ELISA standard curve

Recombinant proteins		
Protein	**Company**	**Catalog number**
IL-2	BD biosciences	550069
OPN	RD System	2799-ML
IL-6	BD biosciences	554582
IL-10	BD biosciences	550070
IL-12	BD biosciences	554592
IL-17	ebiosciences	14-8171
TNFa	Peprotech	315-01A
IFN-γ	BD biosciences	554587
GM-CSF	BD biosciences	554586
CCL-2	Peprotech	250-10
CCL-5	Peprotech	250-07
CXCL10	Peprotech	250-16

Table 3.4: Capture antibodies for ELISA

Capture antibodies	Company	Catalog number	Stock dilution	Working dilution	Buffer
Anti-IL-2	BD biosciences	554424	0.5 mg/ml	2 µg/ml	pH 9
Anti-OPN	RD System	AF808	0.2mg/ml	0.8µg/ml	pH 6
Anti- IL-6	BD biosciences	554400	0.5 mg/ml	2 µg/ml	pH 9
Anti-IL-10	ebiosciences	14-7101	0.5 mg/ml	2 µg/ml	pH 6
Anti-IL-12	BD biosciences	551219	1 mg/ml	4 µg/ml	pH 9
Anti- IL-17	BD biosciences	14-7175	0.5 mg/ml	2 µg/ml	pH 6
Anti-TNFa	BD biosciences	551225	0.5 mg/ml	2 µg/ml	pH 6
Anti- IFN	BD biosciences	551309	0.5 mg/ml	2 µg/ml	pH 9
Anti-GM-CSF	BD biosciences	555403	1mg/ml	4 µg/ml	pH 7
Anti-CCL-2	Peprotech	500-P113	0.5 mg/ml	1 µg/ml	pH 6
Anti-CCL-5	Peprotech	500-P118	0.5 mg/ml	1 µg/ml	pH9
Anti-CXCL10	Peprotech	500-P129	0,5 mg/ml	1 µg/ml	pH9

Table 3.5: Biotinylated antibodies for ELISA

Biotinylated antibodies	Company	Catalog number	Stock dilution	Working dilution
Anti-IL-2	BD biosciences	554426	0.5 mg/ml	2 µg/ml
Anti-OPN	RD System	BAF1834	50µg/ml	0.2 µg/ml
Anti- IL-6	BD biosciences	554402	0.5 mg/ml	2 µg/ml
Anti-IL-10	ebiosciences	13-7102	0.5 mg/ml	2 µg/ml
Anti-IL-12	BD biosciences	554476	0.5 mg/ml	2 µg/ml
Anti- IL-17	ebiosciences	13-7177	0.5 mg/ml	2 µg/ml
Anti-TNF-α	BD biosciences	554415	0.5 mg/ml	2 µg/ml
Anti- IFN-γ	BD biosciences	551506	0.5 mg/ml	2 µg/ml
Anti-GM-CSF	BD biosciences	554407	0.5mg/ml	2 µg/ml
Anti-CCL-2	Peprotech	500-P113 Bt	0.5 mg/ml	2 µg/ml
Anti-CCL-5	Peprotech	500-P118 Bt	0.5 mg/ml	2 µg/ml
Anti-CXCL10	Peprotech	500-P129 Bt	0.5 mg/ml	2 µg/ml

Table 3.6: Primer sequence for real-time PCR

	Primer sequences		
Genes	**sense**	**anti sense**	**T_m**
BDNF	5´- CCC TCC CCC TTT TAA CTG AA - 3´	5´- GCC TTC ATG CAA CCG AAG TA - 3´	60ºC
ADNP	5´- AGA AAA GCC CGG AAA ACT GT - 3´	5´- AAG CAC TGC AGC AAA AAG GT - 3´	60ºC
DAT	5´- AGA TCT GCC CTG TCC TGA AAG-3´	5´- ATC GAT CCA CAC AGA TGC CTC -3´	60ºC
α-Syn	5´- GGA GTG ACA ACA GTG GCT GA- 3´	5´- GCT CCC TCC ACT GTC TTC TG- 3´	60ºC
Actin	5´- TGT TAC CAA CTG GGA CGA CA - 3´	5´- GGG GTG TTG AAG GTC TCA AA - 3´	60ºC
GAPDH	5´- AAC TTT GGC ATT GTG GAA GG- 3´	5´- ACA CAT TGG GGG TAG GAA CA-3´	60ºC

4. RESULTS

4.1. Therapeutic effect of adrenomedullin in preclinical models of MS

We investigated the potential therapeutic effect of adrenomedullin on MS by using a model of chronic progressive EAE induced by MOG_{35-55} and in a focal demyelination model induced by lysolecithin, both models developed in in C57BL/6 mice. The first model helped us to understand the immunoregulatory role of adrenomedullin in MS, and the second one allowed us to study the neuroprotective role of this neuropeptide in demyelinating diseases without the interferences of the peripheral immune response.

4.1.1. Treatment with adrenomedullin reduces severity and incidence of chronic EAE

We observed that EAE mice without treatment developed moderate to severe clinical symptoms without subsequent recovery (Fig. 4.1). Systemic administration of adrenomedullin after the onset (Fig. 4.1A) or during the effector phase of the disease (Fig. 4.1B and 4.1C) greatly reduced disease incidence and severity (Fig. 4.1; Table 1). Noteworthy is the fact that most of the adrenomedullin-treated EAE mice showed mild symptoms and that a significant number of treated mice recovered completely, being entirely asymptomatic 30-40 days after disease onset (Table 4.1). In addition, it is remarkable that a short term treatment with the neuropeptide was enough to generate a long lasting protective effect (Fig. 4.1).

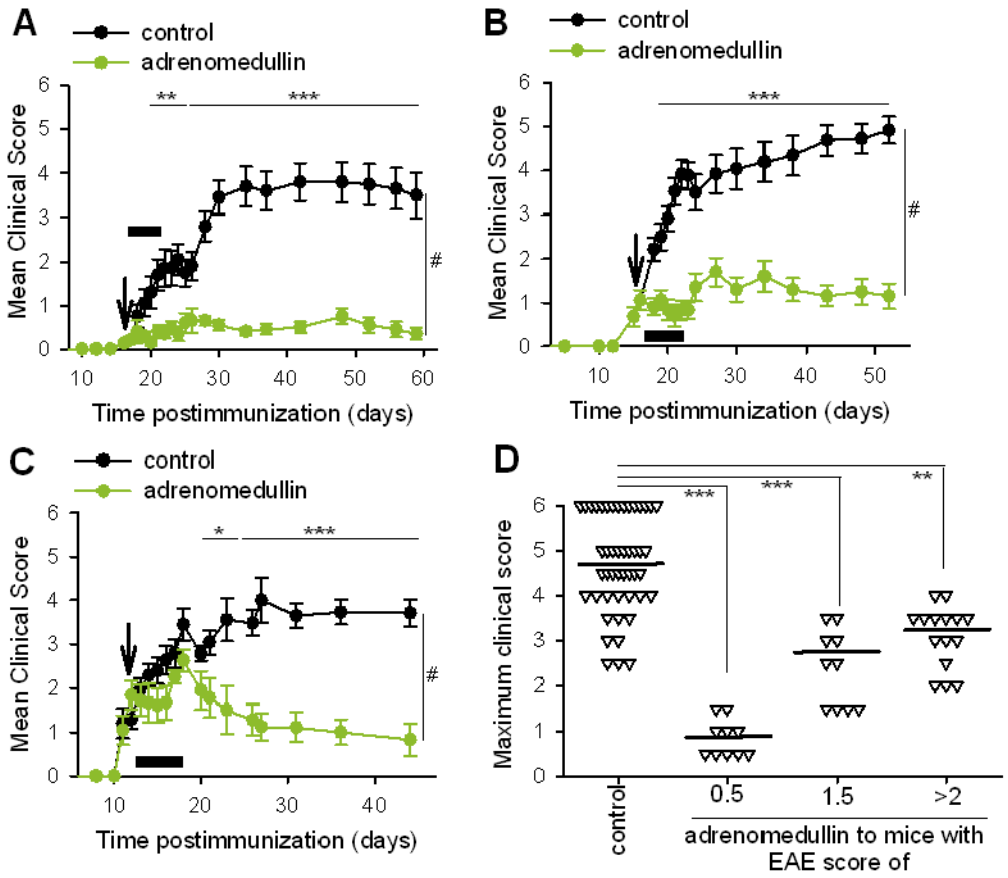


Figure 4.1: **Treatment with adrenomedullin reduces severity of chronic EAE.** Chronic progressive EAE was induced in C57BL/6 mice by immunization with MOG_{35-55}. Mice were treated i.p. with PBS (control) or adrenomedullin (1 nmol/day, black bars) for 5 consecutive days at disease onset (A, in animlas with a clinical score <1) or at two points during during the acute phase (in animals with clinical score 1-2 in B, or with clinical score >2 in C). Arrows point out the day when adrenomedullin treatment started. Data represent the mean clinical score ± SEM. D) Maximum peak of disease adquired by each animal (horizontal line represents the mean; each triangle corresponds to a individual mouse). n = 10 mice/group in A; n = 13 mice/control group and 10 mice/adrenomedullin group in B; n = 24 mice/control group and 15 mice/adrenomedullin group in C; all performed in three independent experiments. *p <0.05; **p <0.01; ***p < 0.001 *vs*. control with Mann-Whitney test (A-C) and Kruskal Wallis Test (D). # p <0.001 with ANOVA t-test.

Timing of Adrenomedullin administration in EAE	Incidence[a]				Mortality	CDI[b]
	None	Mild	Moderate	Severe		
1. Disease Onset						
Control (n=10)	0%	0%	70%	30%	20%	76.2±9.6
adrenomedullin (n=10)	30%	70%	0%	0%	0%	17.6±1.7***
2. Early acute phase						
Control (n=13)	0%	0%	54%	46%	46%	103.6±7.8
adrenomedullin (n=10)	10%	80%	10%	0%	0%	33.1±4.5***
3. Advanced acute phase						
Control (n=24)	0%	0%	54%	46%	29%	82.5±7.1
adrenomedullin (n=15)	13%	86%	0%	0%	0%	41.2±4.1**

Table 4.1: Effect of adrenomedullin in chronic progressive EAE. Chronic progressive EAE was induced in C57BL/6 mice by immunization with MOG_{35-55}. Immunized mice were treated i.p. for 5 days with PBS (control) or with adrenomedullin (1 nmol/day) starting at the onset of clinical signs (with a clinical score of 0.5) or at two points during the acute phase of the disease (early acute phase with a clinical score of 1-2; or advanced acute phase with a clinical score >2). The number of animals per group used in each condition is shown in parenthesis. Data represent results from three independent experiments.

[a]Disease incidence (expressed as percentage) is graded as severe (clinical score: >4), moderate (clinical score: 2-4), mild (clinical score: <2) or none (no clinical signs) at day 36.

[b]Cumulative Disease Index (CDI) is the mean of the sum of the daily disease scores for each animal between days 20 and 44 postimmunization.

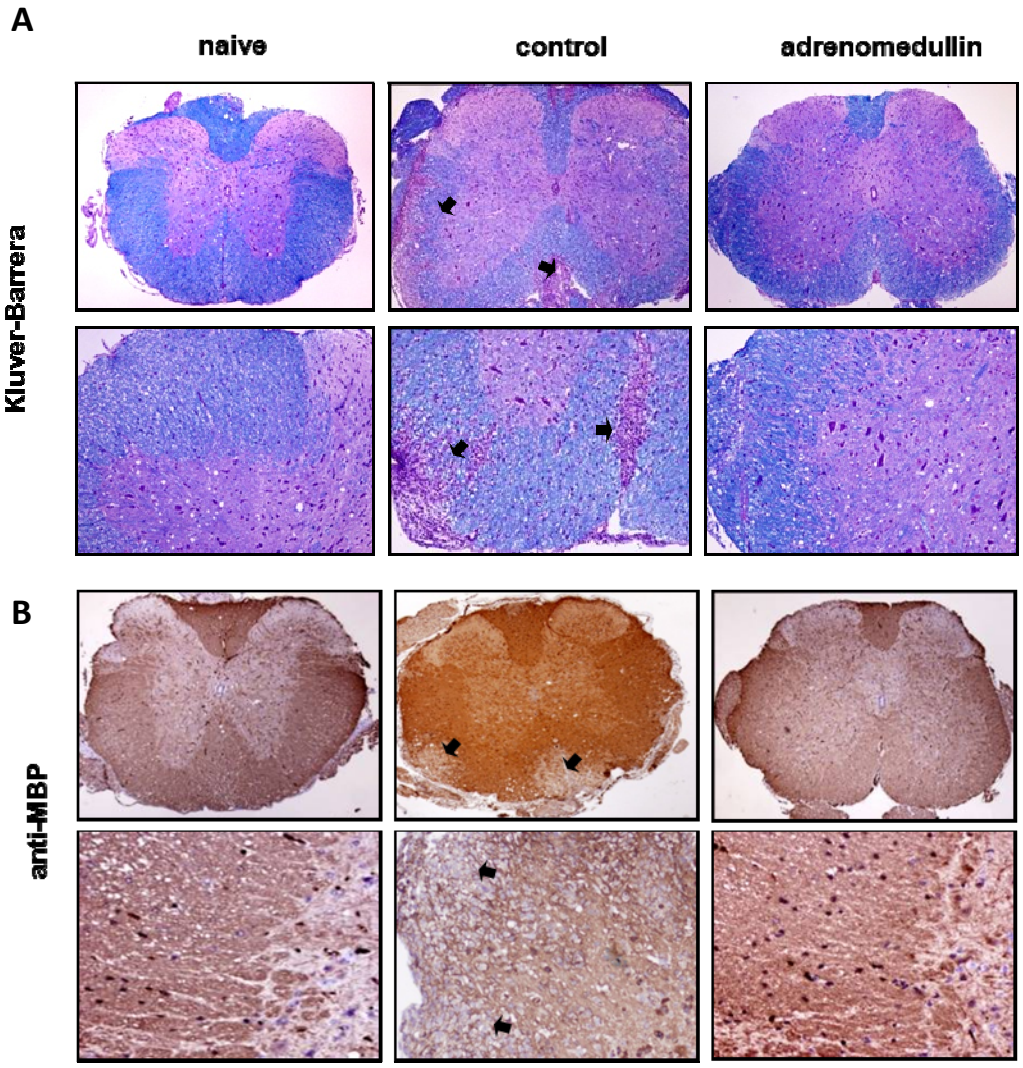
** p<0.005; ***p<0.0001 *vs*. control

4.1.2. Adrenomedullin reduces the inflammatory infiltration and demyelination in the CNS of EAE mice

We next analyzed the mechanisms underlying the beneficial effects of adrenomedullin during the effector phase of EAE in mice with established clinical signs (scores between 1 and 2).

The pathology of MS and EAE is characterized by focal areas of inflammatory infiltration and demyelination, accompanied by oligodendrocyte depletion (55). Histopathologic analysis of spinal cords in both lumbar and cervical regions showed

that treatment with adrenomedullin drastically decreased the number of plaques with inflammatory infiltrates and areas of demyelination (Fig. 4.2), being similar to spinal cords from mice without EAE (naïve). After that, we evaluated the proinflammatory profile of CNS infiltrates of untreated and adrenomedullin-treated EAE mice by immunofluorescence microscopy. This analysis showed that inflammatory cells ($CD45^+$) close to the perivascular area were mostly $CD4^+$ and $Iba1^+$ microglia/macrophages. Interestingly, adrenomedullin was able to reduce the numbers of $CD45^+$, $CD4^+$, and $Iba1^+$ cells (Fig. 4.3). Moreover, the reduction of CNS infiltrating cells in the adrenomedullin-treated EAE mice correlated with the decrease in the amounts of inflammation-related cytokines and chemokines, including IL-6, IL-17, IL-2, IFNγ, CCL5, CCL2 and CXCL10 in spinal cords (Fig. 4.4). Furthermore, treatment with this neuropeptide reduced the contents of GM-CSF and osteopontin in the CNS of EAE mice (Fig. 4.3), two inflammatory markers that play a major role in the induction and progression of EAE and MS (195).



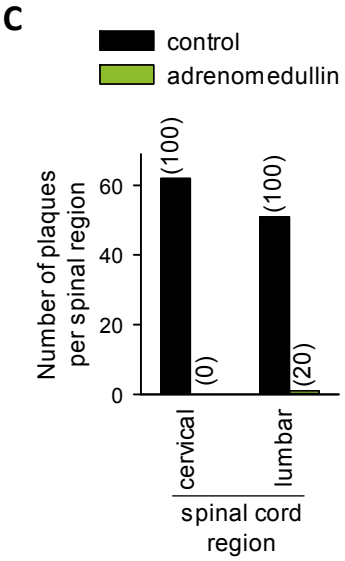
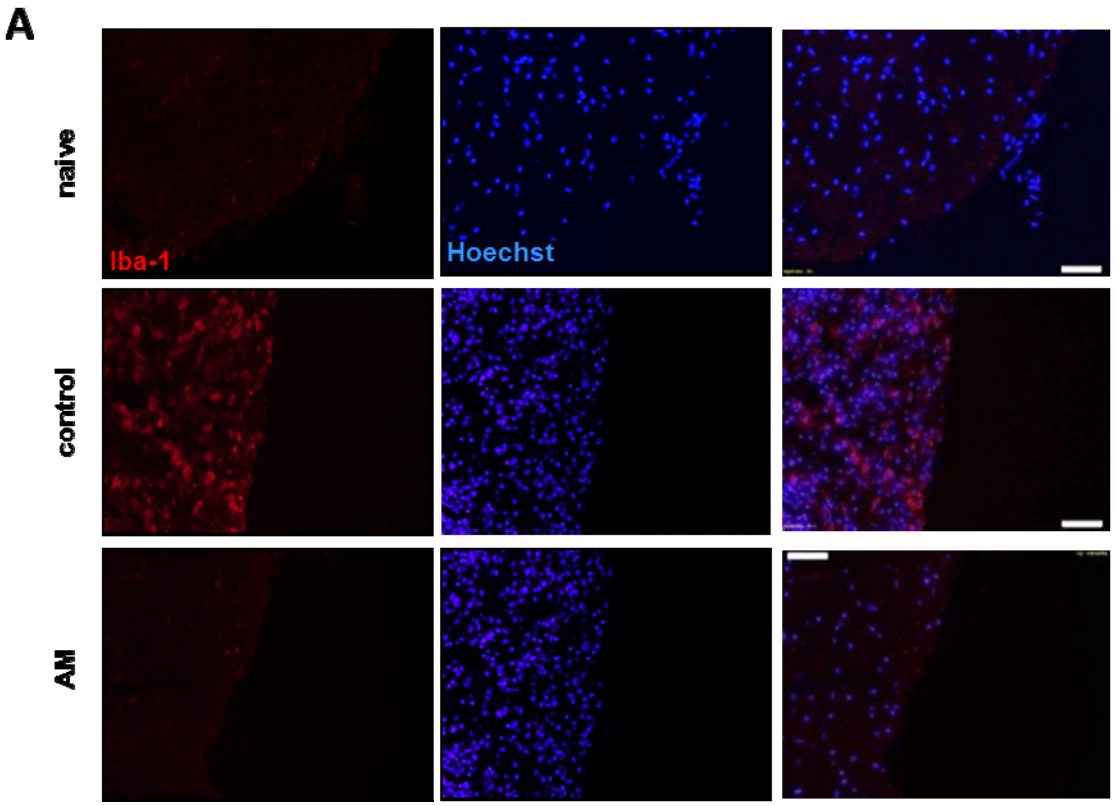
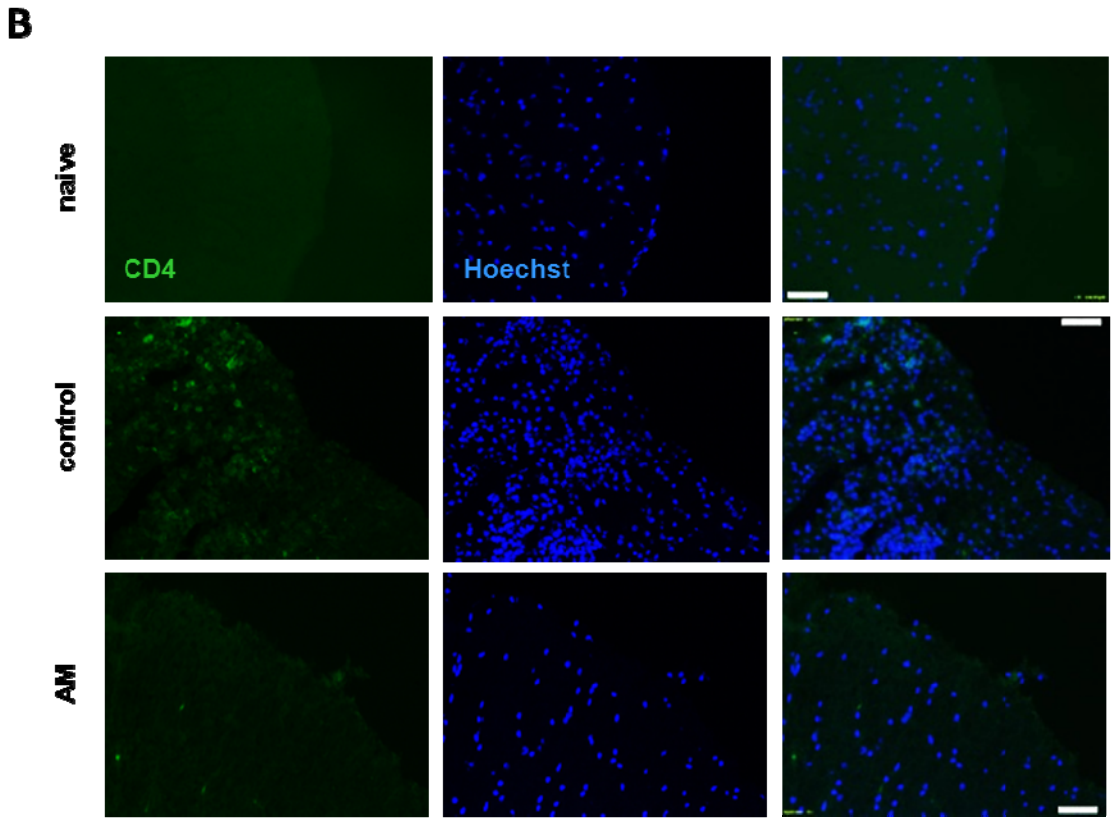


Figure 4.2: Adrenomedullin reduces inflammatory infiltration and demyelination in CNS of mice with EAE. Mice with MOG_{35-55}-induced chronic EAE were treated with PBS (control) or with adrenomedullin for 5 days starting after disease onset (score 1-2) (6/group, two independent experiments). Naïve animals were used as negative controls. Transverse sections of spinal cord randomly selected at the peak of clinical disease were stained with Kluver-Barrera (A) or immunostained for myelin (anti-MBP) (B). Original magnification for upper images, 40x; lower, 100x for Kluver-Barrera and 200x for anti-MBP images. Arrows point to areas of demyelination and inflammatory infiltration. The mean number of plaques of demyelination in the lumbar and cervical regions and the incidence of occurrence of plaques (in parenthesis) was determined (C)


A
naive
control
AM
Iba-1
Hoechst


B
naive
control
AM
CD4
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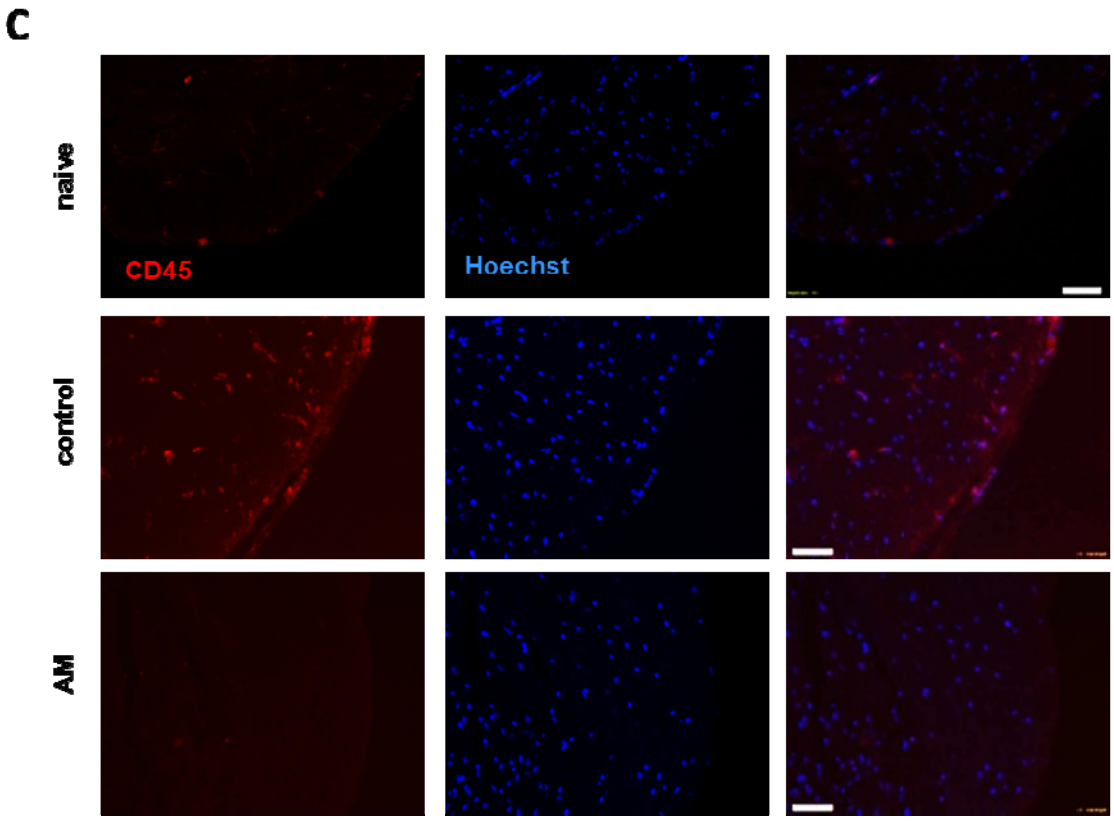

Figure 4.3: Characterization of inflammatory infiltrations in CNS of mice with EAE. Mice with MOG_{35-55}-induced chronic EAE were treated with PBS (control) or with adrenomedullin for 5 days starting after disease onset (score 1-2) (6/group, two independent experiments). Naïve animals were used as negative controls. The phenotype of the infiltrating cells in the lumbar region of spinal cord was assayed by immunofluorescence for Iba-1 (A), CD4 (B) and CD45 (C). AM: adrenomedullin. Scale bars: 50 µm.

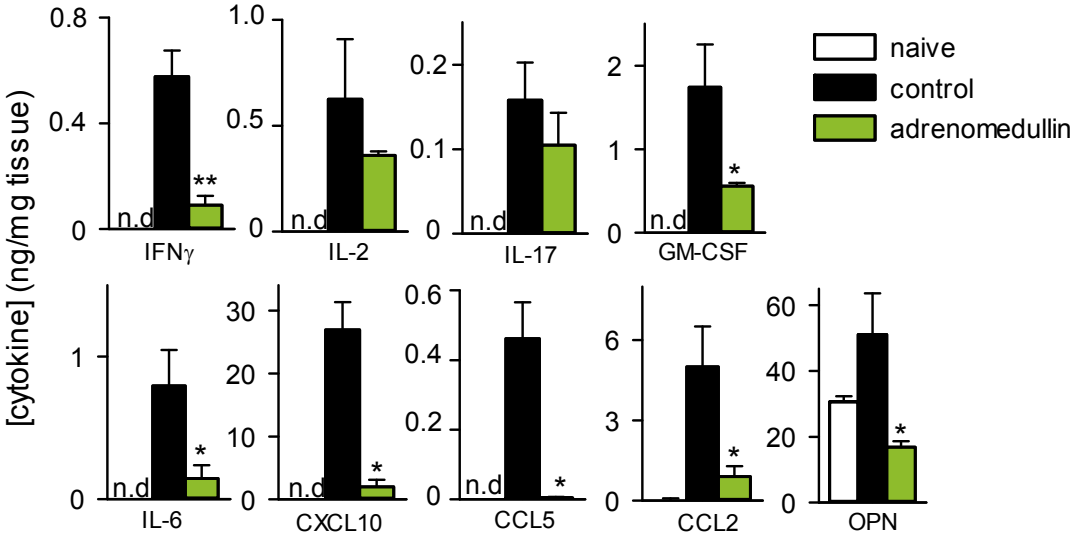

Figure 4.4: Adrenomedullin reduces the production of inflammatory markers in the CNS of EAE mice. Mice with MOG_{35-55}-induced chronic EAE were treated with PBS (control) of adrenomedullin for 5 days starting after disease onset (score 1-2). Levels of inflammatory cytokines, chemokines, GM-CSF, and osteopontin (OPN) were determined by ELISA in protein extracts purified from spinal cord at peak of the disease. Samples from naïve mice were used as reference (n.d.: under detection limit). n = 6 mice/group, two independent experiments. *p < 0.05; **p < 0.01, *vs*. control with Mann-Whitney test.

4.1.3. Adrenomedullin modulates the autoimmune component of EAE in the periphery

In EAE, autoreactive IFNγ-producing Th1 cells and IL-17-secreting Th17 cells infiltrate CNS and promote disease progression (76, 77). Therefore, we investigated whether adrenomedullin could ameliorate EAE by reducing encephalitogenic T-cell responses and/or their migration to CNS. For that, we determined the autoreactive response of T cells, isolated from DLNs and spleen of EAE control mice and adrenomedullin-treated EAE mice, and stimulated *in vitro* with MOG (specific stimulus) or concanavalin A (polyclonal stimulus). As we can observe in figure 4.5, lymphocytes from DLNs and spleen of EAE mice showed marked MOG-dependent proliferation (Fig. 4.5A) and production of IFNγ, IL-2 and IL-17 (Fig. 4.5B). However, T cells isolated from adrenomedullin-treated mice proliferated much less and did not produce Th1 and Th17 cytokines in the MOG-specific recall response (Fig. 4.5). In addition, adrenomedullin treatment reduced the production of GM-CSF and OPN in a MOG-

dependent manner. The effect of adrenomedullin appears to be antigen specific since polyclonal T cell activation with concanavalin A resulted in similar proliferation and cytokine secretion (except for IFNγ) in both groups (Fig. 4.5).

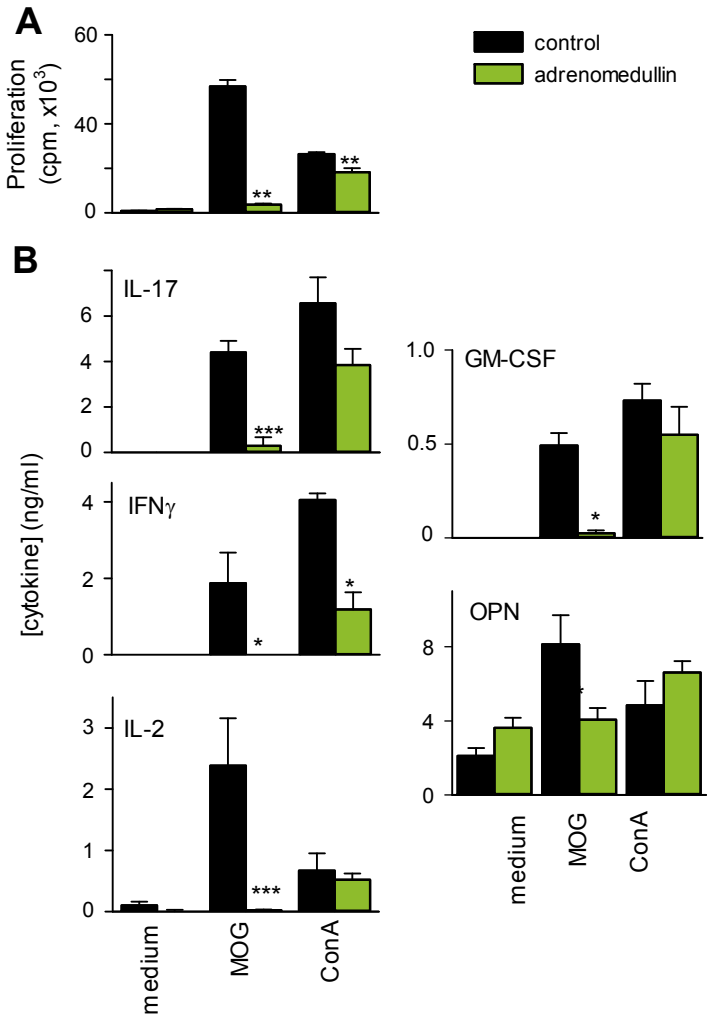

Figure 4.5: Adrenomedullin reduces proliferation and proinflammatory cytokine production by Th1 and Th17 cells from DLNs. Mice with MOG_{35-55}- induced chronic EAE were treated with PBS (control) or adrenomedullin for 5 days after disease onset (clinical score 1-2). Proliferation (A) and cytokine production (B) by DLN cells isolated at peak of the disease and stimulated with medium, the encephalitogenic antigen (MOG_{35-55}) or a polyclonal stimulus (ConA). We obtained similar results with spleen cells (data not shown). N = 6 mice/group, two independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 *vs*. control with Mann-Whitney test.

Moreover, adrenomedullin treatment reduced the number of effector T cells secreting IFNγ, but not IL-17, in spleen and DLNs, while at the same time increased the

percentage of IL-4 and IL-10-expressing CD4 cells (Fig. 4.6). These results indicate that adrenomedullin administration during the effector phase of EAE partially inhibits autoreactive Th1 and Th17 cell activation and clonal expansion in the periphery.

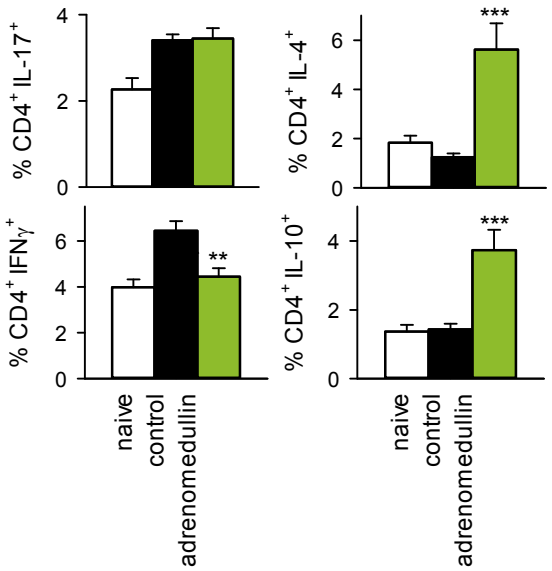

Figure 4.6: Adrenomedullin decreases the number of autoreactive Th1 and Th17 cells and increases the number of Th2 cells. Mice with MOG_{35-55}-induced chronic EAE were treated with PBS (control) or adrenomedullin for 5 days after disease onset (clinical score 1–2). Spleen cells isolated at peak of the disease were assayed for intracellular cytokine expression by flow cytometry in the CD4 gated population. We used naïve mice as basal controls. We obtained similar results with DLNs (data not shown). N = 6–8 mice/group, three independent experiments. **p<0.01; ***p<0.001 *vs* control with t-test.

In order to study if the action of adrenomedullin is exerted directly on lymphoid cells, we assayed the *in vitro* effect of adrenomedullin on MOG-induced proliferation and cytokine production by DLNs isolated from EAE mice. As figure 4.7 shows, adrenomedullin can deactivate MOG-specific recall responses *in vitro*.

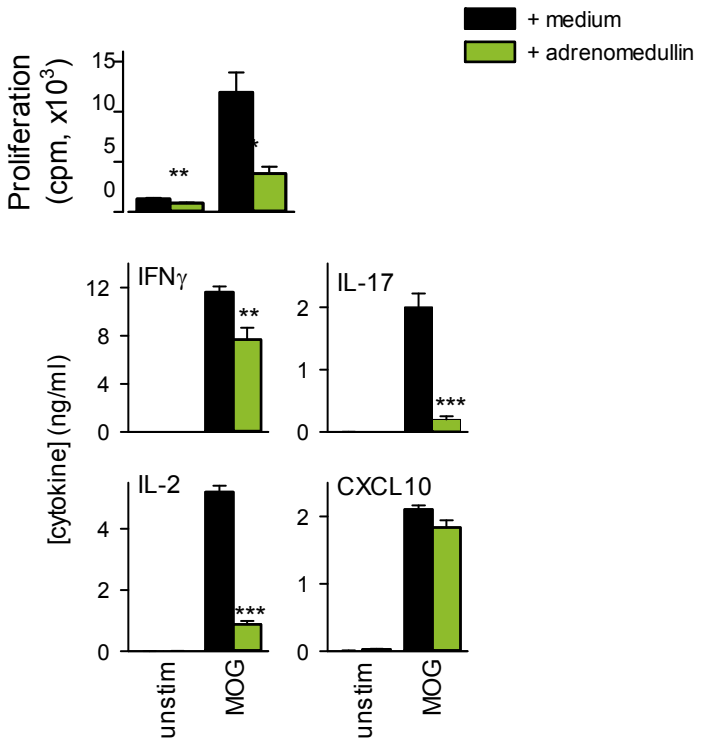

Figure 4.7: Adrenomedullin directly reduces proliferation and proinflammatory cytokine production by Th1 and Th17 *in vitro*. Proliferation and cytokine production by DLN cells isolated from EAE mice at the peak of the disease, and restimulated *ex vivo* with MOG_{35-55} in presence or absence (medium) of adrenomedullin (100 nM). N = 5–6 mice/group, two independent experiments. **p<0.01; ***p<0.001 *vs* control with Mann-Whitney test.

Finally, because high levels of circulating antibodies directed against myelin antigens invariably accompany the development of MS and EAE, and are major factors in determining susceptibility to EAE (196), we next evaluated the effect of adrenomedullin treatment on the serum levels of MOG-specific IgG autoantibodies. We can see in the figure 4.8 that adrenomedullin reduced the ratio between MOG-specific IgG2a and IgG1 isotypes, generally reflective of Th1 and Th2 activities, respectively.

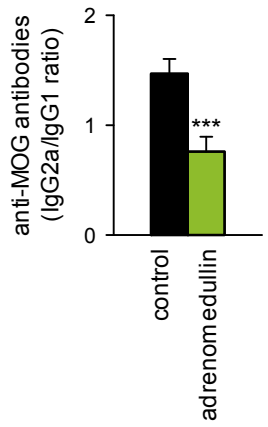

Figure 4.8: Adrenomedullin reduces the production of MOG-specific IgG2a autoantibodies. Mice with MOG_{35-55}-induced chronic EAE were treated with PBS (control) or adrenomedullin for 5 days after disease onset (clinical score 1–2). Sera was isolated at disease peak and assayed for MOG-specificIgG1 and IgG2a levels. N = 14 mice/group, three independent experiments. ***p<0.001 *vs* control with t-test

4.1.4. Adrenomedullin impairs the encephalitogenic response in the CNS

In order to know if, beside its effect in the periphery, adrenomedullin exerts any local effect in the CNS, we characterized brain infiltrating mononuclear cells. In agreement with the results obtained from the histopathological study (Fig. 4.3), flow cytometry analysis of infiltrating mononuclear cells isolated from brains of EAE mice showed that adrenomedullin administration resulted in lower numbers of infiltrating $CD4^+$ T cells, particularly of IFNγ- and IL-17-producing Th1 and Th17 cells (Fig. 4.9).

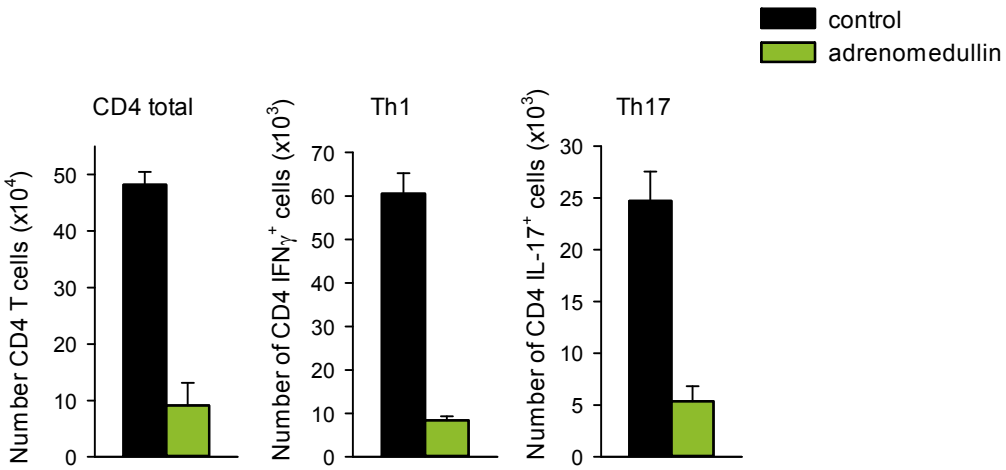

Figure 4.9: Treatment with adrenomedullin decreases Th1 and Th17 infiltrating cells. Phenotypic analysis by flow cytometry of brain infiltrating mononuclear cells isolated at the peak of disease. Results show total numbers of CD4 T cells, $CD4^+IFN\gamma^+$ cells or $CD4^+IL\text{-}17^+$ cells per brain. N = 6 mice/group, two independent experiments. *p<0.05 *vs* control with Mann-Whitney test.

Next, we investigated the encephalitogenic capacity of the few infiltrating T cells found in the CNS parenchyma of adrenomedullin-treated EAE mice. Infiltrating mononuclear cells isolated from brain and spinal cord of untreated EAE mice (control) showed strong proliferation (Fig. 4.10A), revealed by the observation of many clusters of activation, and a Th1/Th17 cytokine profile upon *ex vivo* restimulation with MOG (Fig. 4.10B). In contrast, CNS mononuclear cells from adrenomedullin-treated EAE mice neither proliferated nor produced Th1/Th17 cytokines in the MOG-specific recall response, although they did respond to polyclonal activation (Fig. 4.10).

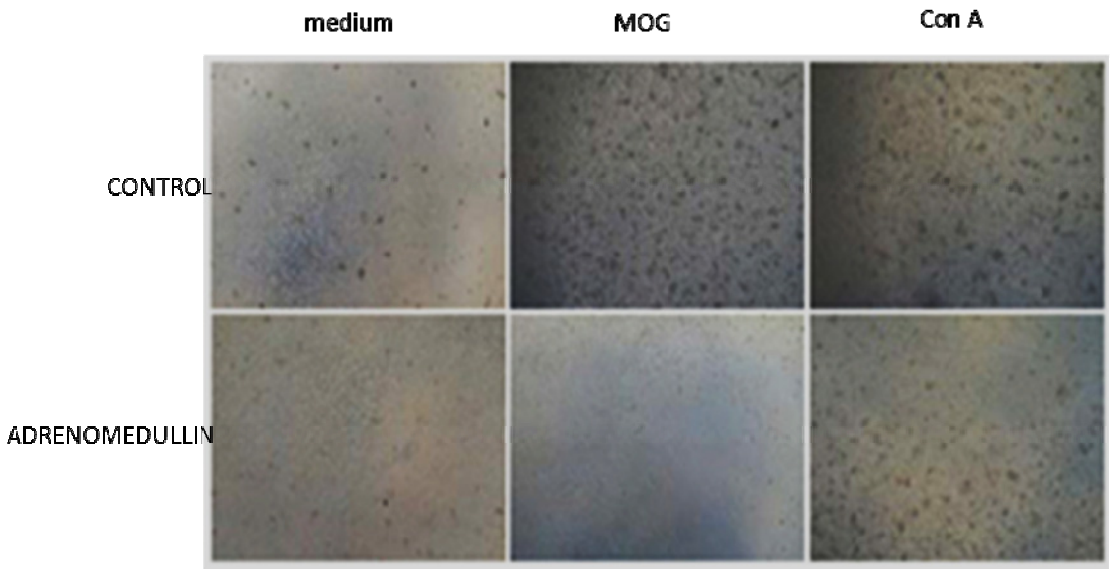

Figure 4.10: see next page

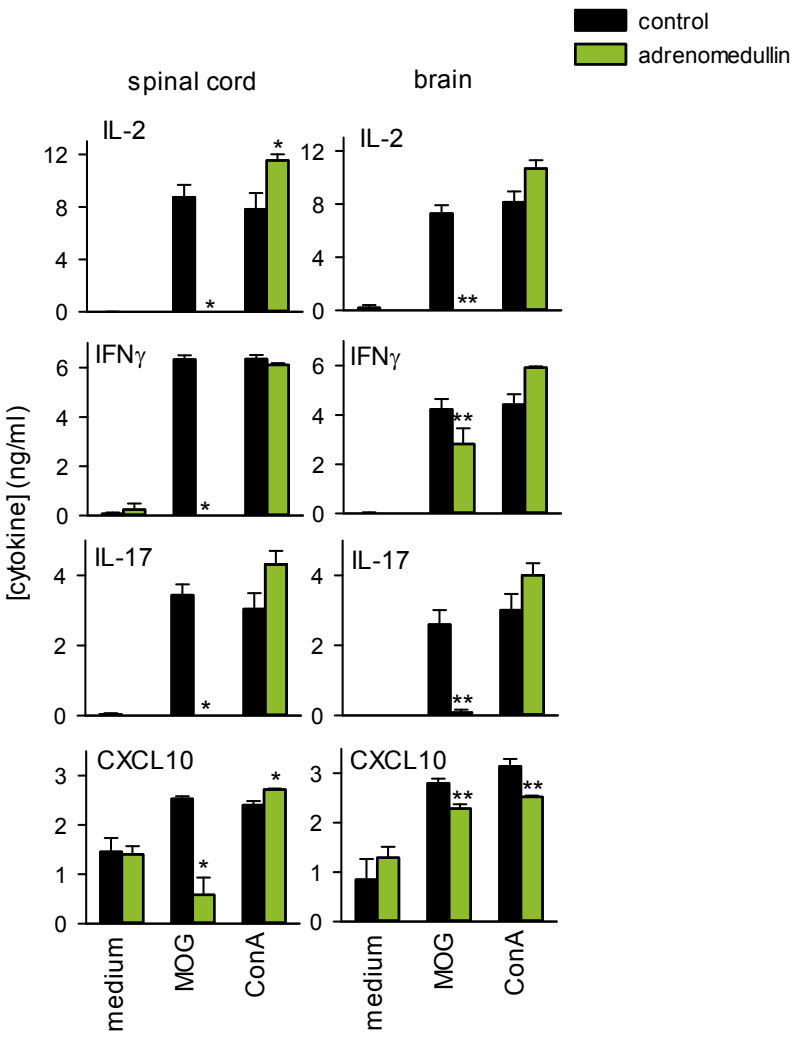

Figure 4.10: Treatment with adrenomedullin impairs the encephalitogenic response in the CNS of mice with EAE. Mice with MOG_{35-55}-induced chronic EAE were treated with PBS (control) or adrenomedullin for 5 days after disease onset (clinical score 1–2). (A) Proliferation (depicted by the formation of cell clusters of activation; original magnification, 100x) and (B) cytokine production by mononuclear cells isolated from brains and spinal cords at peak of the disease and restimulated with MOG_{35-55} or with a polyclonal stimulus (ConA). N = 6–8 mice/group, two independent experiments. AM: adrenomedullin.

To know if the effect of adrenomedullin on T cell population at CNS is exerted directly on T cell population, we added adrenomedullin directly to cultures of MOG-stimulated CNS mononuclear cells isolated from EAE mice. The *ex vivo* treatment with adrenomedullin reduced cell proliferation (data not shown) and the production of IL-2 and IL-17, but not IFNγ (Fig. 4.11). These results support the idea that adrenomedullin could regulate the encephalitogenic response of infiltrating immune cells in the CNS parenchyma during the progression of EAE.

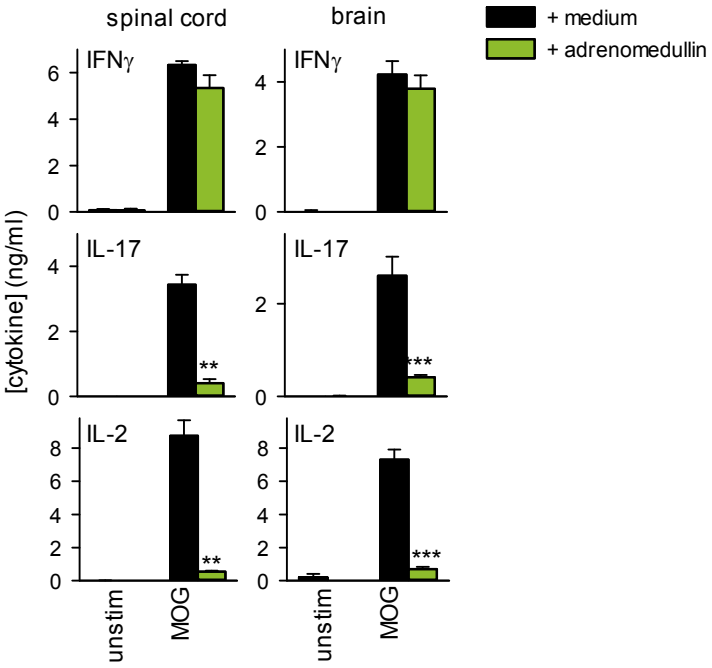

Figure 4.11: Adrenomedullin ameliorates EAE severity by imparing the encephalitogenic response in the CNS *in vitro*. Cytokine production by CNS mononuclear cells isolated from EAE mice at the peak of the disease, and stimulated with medium (unstimulated) or MOG_{35-55} in the absence (medium) or presence of adrenomedullin (100 nM). N = 6–8 mice/group, three independent experiments. **p<0.01; ***p<0.001 *vs* control with Mann-Whitney test.

4.1.5. Adrenomedullin increases the number of functional Treg cells in EAE

Because evidence supports the idea that Treg cells confer significant protection against EAE by deactivating autoreactive T cells and their homing to the CNS (79), we next investigated if adrenomedullin would be able to generate Treg cells during EAE. For that, we isolated $CD4^+$ T cells from spleen and DLNs and we analyzed the number and percentage of IL-10-secreting $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells. As we can see on

figure 4.12A, the percentage and number of these cells were increased in DLNs and spleens of adrenomedullin-treated EAE mice. Moreover, we evaluated the percentage of $CD4^+$ $FoxP3^+$ Treg cells in CNS infiltrating T cells. Despite the low numbers of infiltrating CD4+ T cells, adrenomedullin increased significantly the percentage of $FoxP3^+$ cells in CNS of EAE mice (Fig. 4.12B).

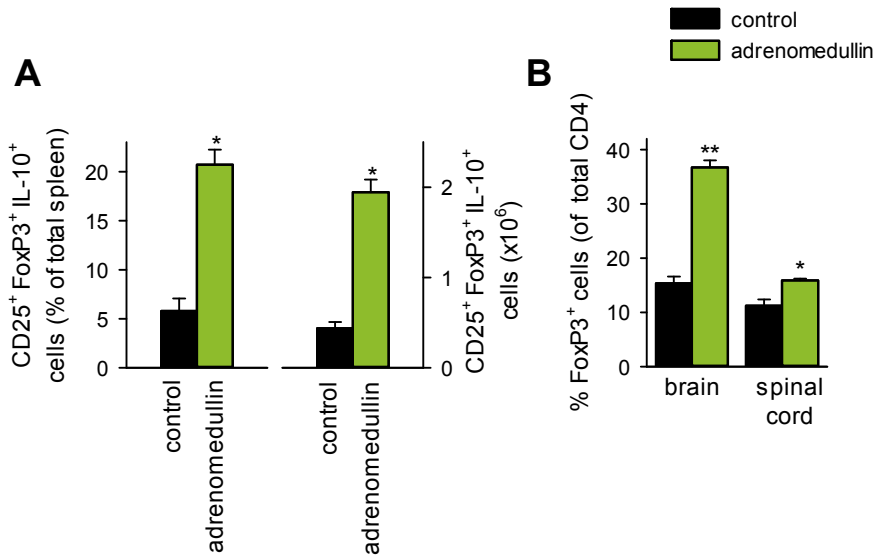

Figure 4.12: Treatment with adrenomedullin increases the number of Treg cells. Mice with MOG_{35-55}-induced chronic EAE were treated with PBS (control) or adrenomedullin for 5 days at disease onset (clinical score 1–2). (A) Adrenomedullin induces the emergence of peripheral Treg cells in EAE mice. Percentage and number of $CD4^+CD25^+FoxP3^+IL\text{-}10^+$ Treg cells in spleens isolated at peak of the disease. n=18 mice/group, three independent experiments. (B) Adrenomedullin increases the percentages of Treg cells in the CNS inflammatory infiltrates. Spinal cord and brain mononuclear cells isolated at peak of the disease were analyzed for the presence of $CD4^+CD25^+FoxP3^+$ Treg cells by flow cytometry and expressed as percentage of $FoxP3^+$ cells in the CD4 population. N = 10 mice/group, two independent experiments *p<0.05; **p<0.01 *vs* control with Mann-Whitney test.

To evaluate the suppressive capacity of Treg cells generated by adrenomedullin treatment *in vivo*, we examined their potential protective effect in the progression of EAE. For this, we isolated T cells from spleen and DLNs from untreated and adrenomedullin-treated mice and we injected them into EAE mice with score between 0.5 and 1. We can observe on figure 4.13 (left panel) that injection of T cells from spleen/DLNs of adrenomedullin-treated EAE mice, but not of untreated EAE mice, into EAE mice ameliorated clinical symptoms. Moreover, in order to know if the protection was mediated by Treg cells, we performed a depletion of $CD25^+$ T cell population prior

to transfer and evaluated the progression of the disease. As we could expect, the transfer of $CD25^-$ T cells abrogated the protective effect *in vivo*, suggesting that the the capacity to generate tolerance resides in the $CD4^+CD25^+$ Treg population (Fig. 4.13, right panel).

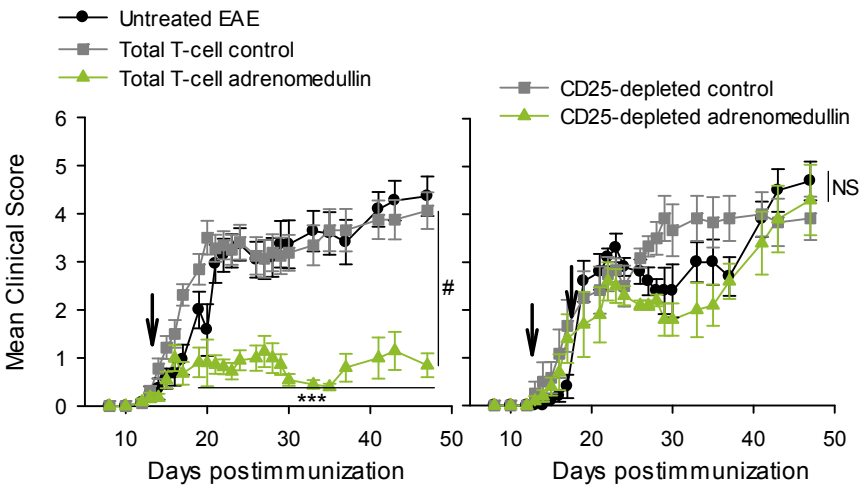

Figure 4.13: Treg cells induced by treatment with adrenomedullin suppress EAE progression. Treatment (arrow) of EAE mice with CD4 T cells or CD25-depleted CD4 T cells isolated from spleen/DLNs of EAE mice that were previously treated with PBS (control) or adrenomedullin. Untreated EAE mice were used as reference. N = 7–9 mice/group, two independent experiments. ***p<0.001; #p<0.01; NS: not significant *vs* control with Kruskal-Wallis test

4.1.6. Adrenomedullin generates dendritic cells (DCs) that suppress the encephalitogenic response in vitro and in vivo

Although the induction of Treg cells by adrenomedullin would definitively contribute to the induction of immune tolerance in EAE mice, we also investigated the effect of this neuropeptide in the generation of DCs with tolerogenic capacity and their potential involvement on the therapeutic effect of adrenomedullin in EAE, due to DCs play a major role in antigen presentation during autoimmune responses. Despite Rullé and coworkers demonstrated previously the capacity of adrenomedullin to regulate DC function by inducing a semi-mature phenotype (197), we analyzed this function in the scenario of EAE. First, we confirmed that addition of adrenomedullin during the process of activation/maturation of DCs with LPS reduced the expression of

coestimulatory molecules CD40 and CD80 and the production of the inflammatory cytokines TNFα, IL-12 and IL-6, while it induced IL-10 secretion (Fig. 4.14).

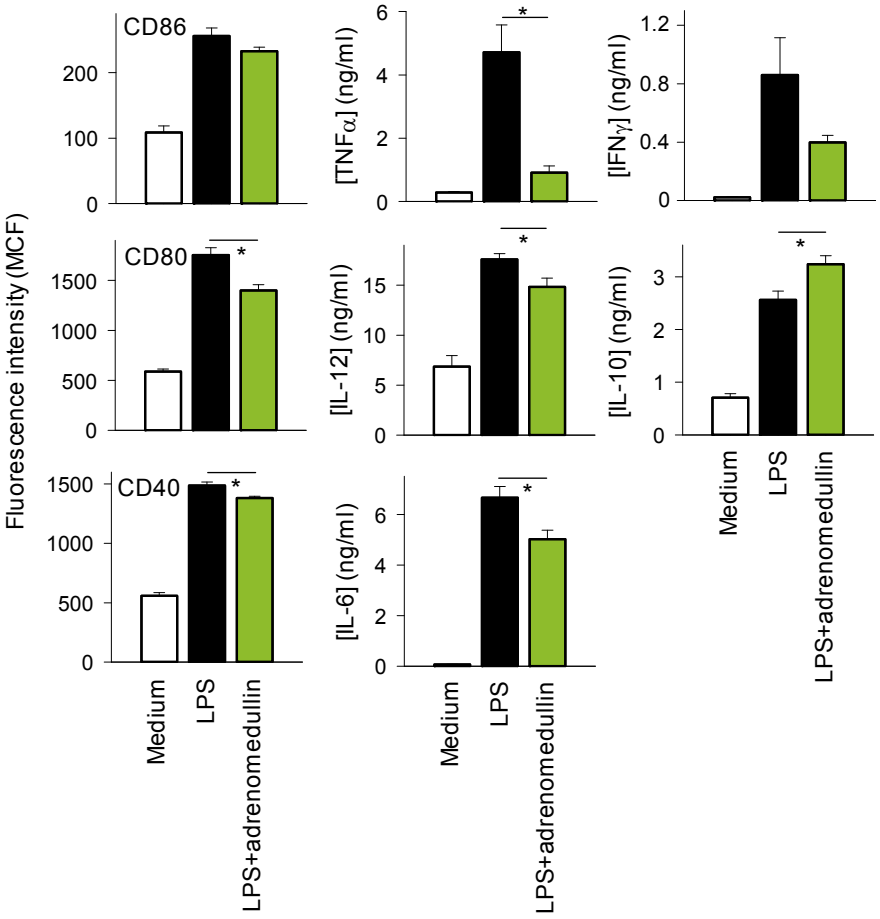

Figure 4.14: Adrenomedullin induces a semi-mature phenotype in DCs Expression of costimulatory molecules and production of cytokines by DCs cultured with medium or matured/activated with LPS in the absence or presence of adrenomedullin (100 nM). MCF: mean channel fluorescence. N = 3, in duplicate. *p<0.05 *vs* control with Mann-Whitney test.

Moreover, we wanted to assay the functional properties of these DCs after their exposure to adrenomedullin. As we can observe in the figure 4.15, MOG-pulsed DCs activated in the presence of adrenomedullin impaired the proliferative response and the production of IL-2 and IL-17, but not of IFNγ, by DLN cells isolated from EAE mice and reactivated with MOG *ex vivo*. Interestingly, DCs stimulated with adrenomedullin did not affect the polyclonal proliferation/production of inflammatory cytokines by T cells.

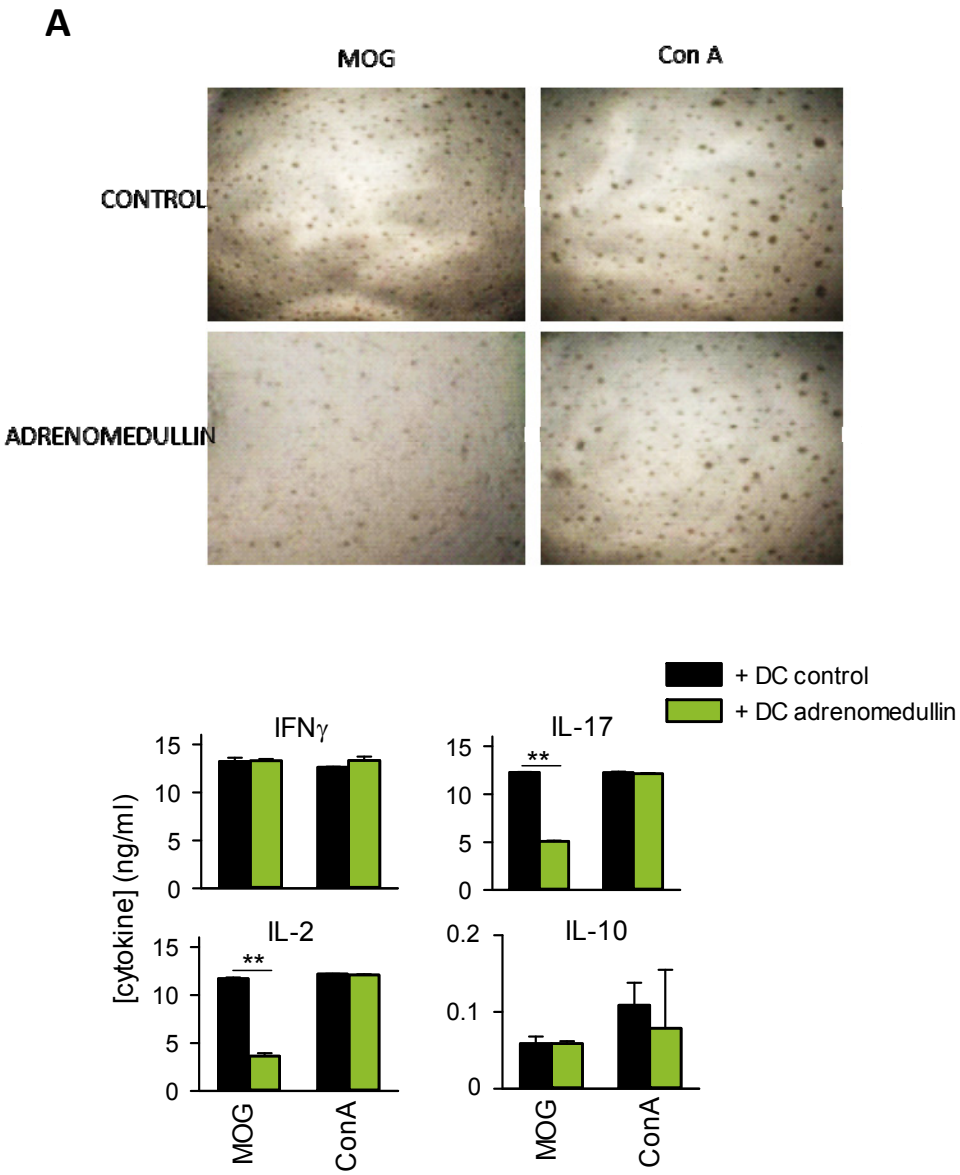

Figure 4.15: Adrenomedullin generates DCs with the capacity to deactivate the encephalitogenic response *in vitro*. DCs matured with LPS and pulsed with MOG_{35-55} in the absence (DC-control) or presence of adrenomedullin (DC-adrenomedullin) were co-incubated with DLN cells isolated from mice suffering EAE and stimulated with MOG_{35-55} or ConA. Cell proliferation (A) (evidenced by the presence of clusters of activation in the culture; original magnification, 100x) and the production (B) of cytokines were determined 48 h later. N = 5, in duplicate. **p<0.01 *vs* control with Mann-Whitney test

Next, once demonstrated that adrenomedullin can generate a semi-mature phenotype in DCs and that these cells impair MOG-induced proliferation and

production of inflammatory mediators *in vitro*, we wanted to know whether these cells could keep their suppressive activity *in vivo.* For that, MOG-pulsed DCs exposed to adrenomedullin were injected into EAE mice and we determined the progression of the disease. We found that administration of adrenomedullin-treated DCs reduced disease severity and incidence of EAE (Fig. 4.16), suggesting that adrenomedullin could generate antigen-specific responses during EAE progression by modulating DC function.

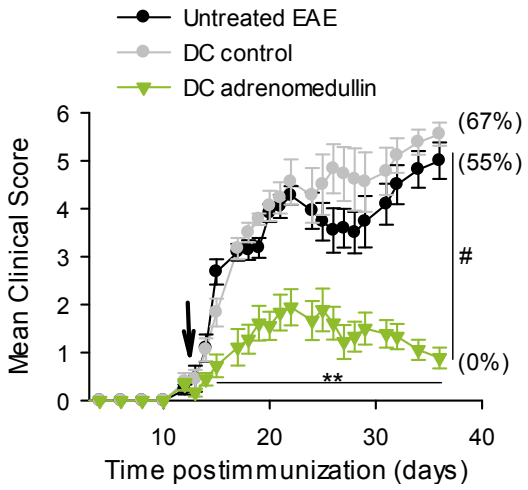

Figure 4.16: Adrenomedullin generates DCs with the capacity to deactivate the encephalitogenic response *in vivo*. DCs matured with LPS and pulsed with MOG_{35-55} in the absence (DC- control) or presence of adrenomedullin (DC-adrenomedullin) were administered i.p. to EAE mice at disease onset (arrow). Untreated EAE mice were used as reference. Numbers in parenthesis represent the mortality rate in each group. N = 6–8 mice/group, two independent experiments. **p<0.01 *vs* control with Mann-Whitney test; #p<0.001 *vs* control with Kruskal-Wallis test.

4.1.7. Adrenomedullin promotes neuroprotective responses

Becasue neurotrophic factors like BDNF and activity-dependent neuroprotective factor (ADNP) play a role during development and differentiation of neuronal and glial cells, promoting cell survival of neurons, axons and oligodendrocytes (198, 199), and they are involved in processes of remyelination, axonal growth and neuroregeneration, we next analyzed the expression of these factors in untreated and adrenomedullin-treated EAE mice. We found that treatment with adrenomedullin significantly increased the expression of these neuroprotective factors in spinal cords of EAE mice (Fig. 4.17). These data suggest that adrenomedullin could promote protective responses in EAE by inducing CNS neurotrophic factors.

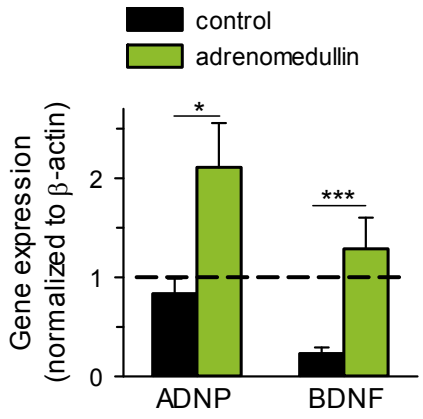

Figure 4.17: Adrenomedullin increases neurotrophic factors in EAE mice. BDNF and ADNP gene expression (relative to β-actin) in spinal cords isolated from EAE mice treated with PBS (control) or adrenomedullin at disease onset. Dashed line corresponds to expression in naïve mice normalized to 1. N = 8 mice/group, three independent experiments. *p<0.05; ***p<0.001 *vs* control with Mann-Whitney test.

4.1.8. EAE mice have reduced levels of adrenomedullin in the CNS

As the exogenous administration of adrenomedullin has a beneficial effect on EAE progression, we wondered whether the endogenous levels of this neuropeptide changed during the disease progression. Interestingly, we observed that the content of adrenomedullin decreased in CNS of EAE mice compared to naïve mice (Fig. 4.18). This result supports the idea that there is an inverse correlation between EAE progression and production of adrenomedullin, and suggests the relevance of endogenous adrenomedullin in the development and progression of EAE.

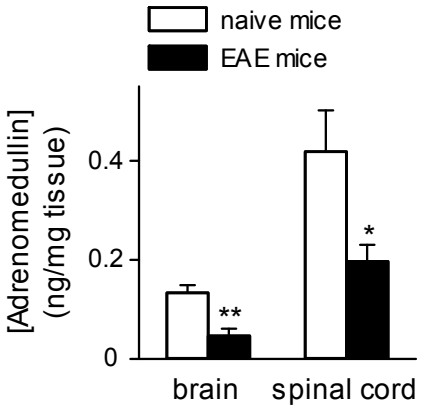

Figure 4.18: EAE mice show decreased adrenomedullin contents in CNS. Levels of adrenomedullin in protein extracts isolated from brain and spinal cords of naïve and EAE mice at the peak of disease. N = 5–8 mice/group, in duplicates). *p<0.05; **p<0.01 *vs* naïve with t-test.

MS is a neurodegenerative disease characterized by a chronic inflammatory demyelination and axonal damage of the CNS. Remyelination is observed during early stages, but usually fails in patients with progressive MS (200). In order to look for an effective therapy for this disorder, the ideal treatment must be able to control the

immune response, while regulating the demyelination/remyelination process at the same time. For this reason, we next investigated whether adrenomedullin could have a beneficial effect on remyelination using the model of lysolecithin-induced demyelination. In contrast to MOG-induced EAE, demyelination and remyelination are temporally distinct in lysolecithin model, with demyelination occurring at 1 day post injection (dpi) after lysolecithin injection, followed by a phase of proliferation/recruitment of OPCs, a differentiation phase beginning at 7 dpi, and a remyelination process starting around 14 dpi and almost completed at 30 dpi (Fig. 4.19A). Therefore, this model provides a significant time frame for analyzing the remyelination process after adrenomedullin treatment.

We used stereotactic microinjection of lysolecithin to induce focal demyelinating lesions in the corpus callosum of 12-week-old mice (Fig. 4.19A). Next, we followed the time course of lesion formation and resolution over a 2 weeks period using fluoromyelin staining and confocal imaging. Administration of adrenomedullin was initiated at 1 dpi and further continued daily for 14 days. Lysolecithin microinjection into the left side of the corpus callosum resulted in demyelination with lesions larger at 7 dpi (corresponding to maximal demyelination) than at 14 dpi (corresponding to onset of repair) (Fig. 4.19A and B). Systemic treatment with adrenomedullin diminished significantly the maximal cross-sectional fluoromyelin negative demyelinated area compared with untreated controls at 7 and 14 dpi (Fig. 4.19B). This suggests a role of adrenomedullin, not only in reducing demyelination, but also inducing active remyelination.

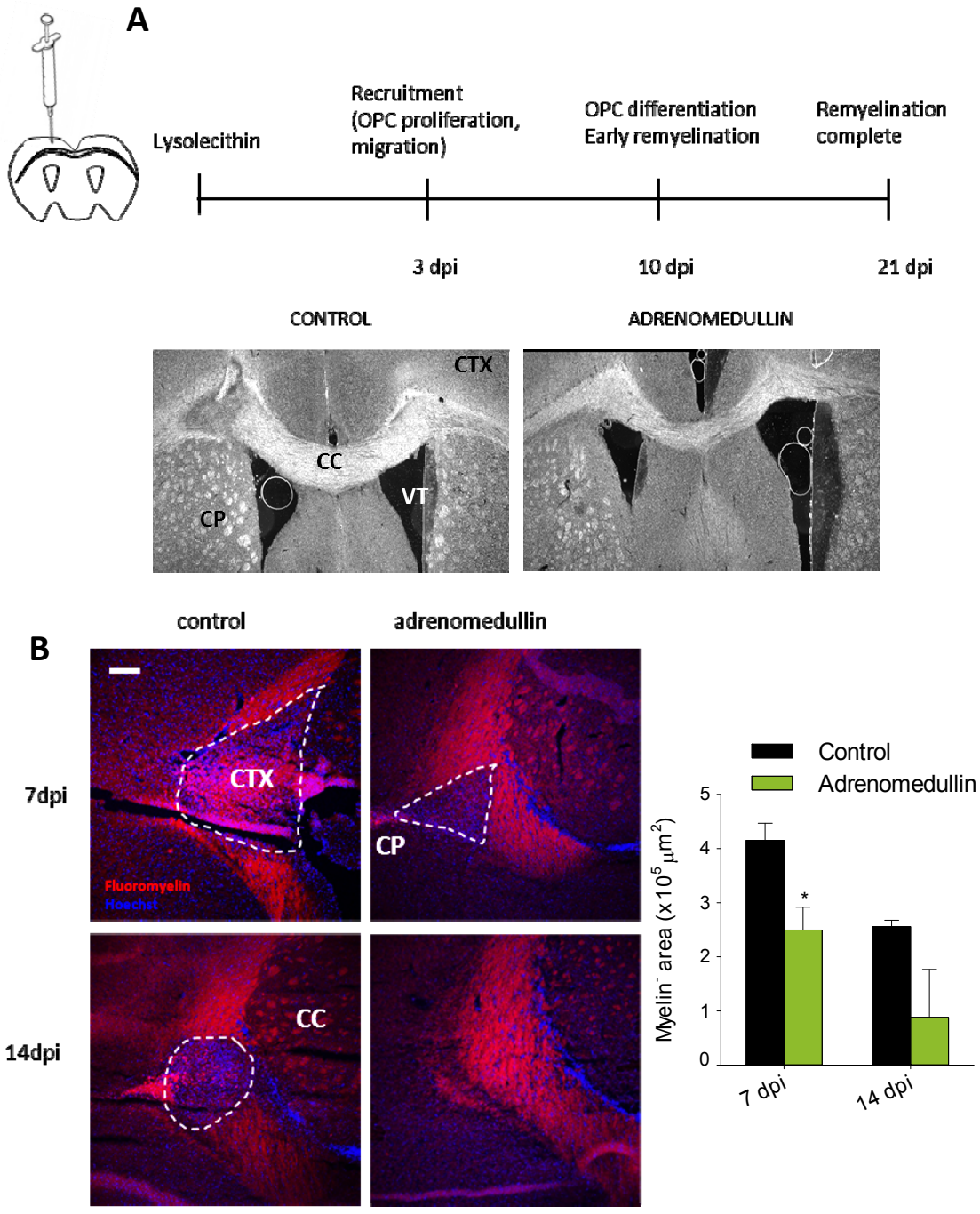

Figure 4.19: Adrenomedullin treatment reduces demyelination in the corpus callosum induced by lysolecithin. (A) C57BL/6 mice were stereotactically microinjected with 2µl 1% lysolecithin into the left corpus callosum at 5.5mm anterior to lambda, 1mm lateral to bregma and 2mm deep, and sacrified at 7-14 dpi. Top, scheme represents the oligodendroglial lineage cell responses in the corpus callosum after model induction. Bottom, representative images of lysolecithin-induced lesion in the corpus callosum of control and adrenomedullin-treated mice after 7dpi (magnification, 40x; CTX: cortex; CC, corpus callosum; CP: caudate putamen; VT, lateral ventricle) (B), Serial 20µm sections were stained for myelin (fluoromyelin) and nuclei (Hoechst). Morphometric data were quantified from z-series projections as described in Materials and Methods. Scale bars: 100µm. Results are from 4 animals per condition. *p<0.05 *vs* control with t-test.

We next investigated the effect of adrenomedullin on oligodendrocyte survival after microinjection of lysolecithin, because oligodendrocytes are the main producers of myelin sheath and are dramatically affected during MS. We identified oligodendrocytes by immundetection of the oligodendrocyte lineage transcription fator 2 (Olig2), which is highly enriched in premyelinating oligodendrocytes, although is also expressed at later stages in the oligodendrocyte lineage. Our results indicated that adrenomedullin treatment increased the percentage of Olig2+ cells in the lesioned area in comparison to untreated mice (Fig. 4.20).

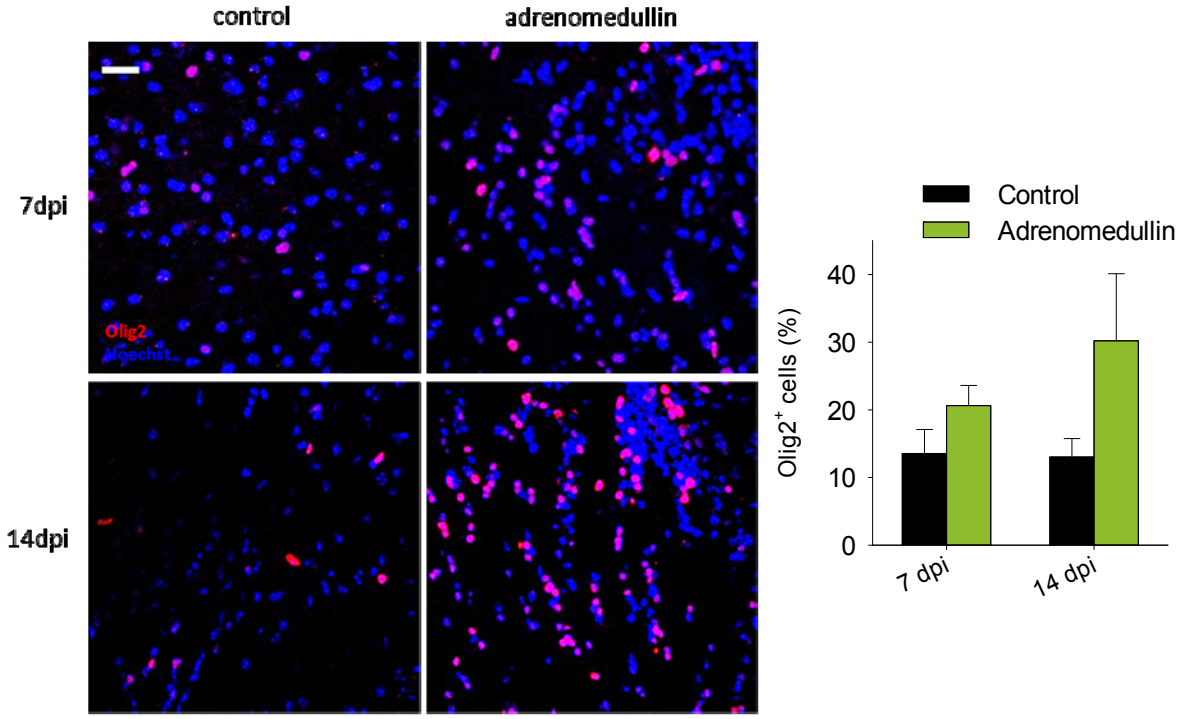

Figure 4.20: Effect of adrenomedullin on oligodendroglial cells in corpus callosum lesions. C57BL/6 mice were stereotactically microinjected with 2µl of 1% lysolecithin into the left corpus callosum and treated i.p. with saline (control) or with adrenomedullin (1 nmol/mouse) daily for a period of 14 days, starting 1 dpi. Mice were sacrificed at 7 and 14 dpi and serial sections of corpus callosum were immunostained for Olig2 and nuclei (Hoechst). Olig2+ cells were quantified from z-series projections and expressed as percentage of total cells in the area. Results are from 4 animals per condition. Scale bars: 50µm.

4.1.9. Effect of adrenomedullin on the glial niche

Resident cells of CNS play an important role on the pathogenesis of MS/EAE and other neuroinflammatory diseases, promoting and enhancing the pro-inflammatory milieu by producing pro-inflammatory mediators and ROS. The local production of these cytotoxic factors contributes to demyelination, oligodendrocyte loss and axonal degeneration (201). Taking this in account, we next investigated whether adrenomedullin exerts any effect in CNS by directly acting on resident cells by regulating the inflammatory milieu generated by activated glial cells. Astrocyte, microglia and neuron-glia cultures generated from newborn mice were stimulated with LPS in presence or absence of adrenomedullin. As we can observe in the figure 4.21A, adrenomedullin downregulated the production of inflammatory cytokines like TNFα, IL-6 and IL-12 by LPS-activated glial cells. Interestingly, adrenomedullin reduced ROS production induced by oxidative stress by microglia, but not by astrocytes (Fig. 4.21B). Notably, adrenomedullin decreased cell death induced by oxidative stress in precursor and mature oligodendrocytes (Fig. 4.21C). These data suggest that adrenomedullin could have a protective effect in EAE and other neuroinflammatory disorders by downregulating the destructive inflammatory response mediated by resident glial cells and by directly preventing oligodendrocyte cell loss.

On the other hand, several studies report that immune cells including lymphocytes, macrophages and DCs secrete adrenomedullin following their activation (202, 203, 204, 205, 197, 206). Thus, we investigated the capacity of glial cells to produce adrenomedullin. We found that, similarly to peripheral immune cells, microglia and astrocytes increase the secretion of adrenomedullin under pro-inflammatory conditions mimicked by LPS stimulation (Fig. 4.22).

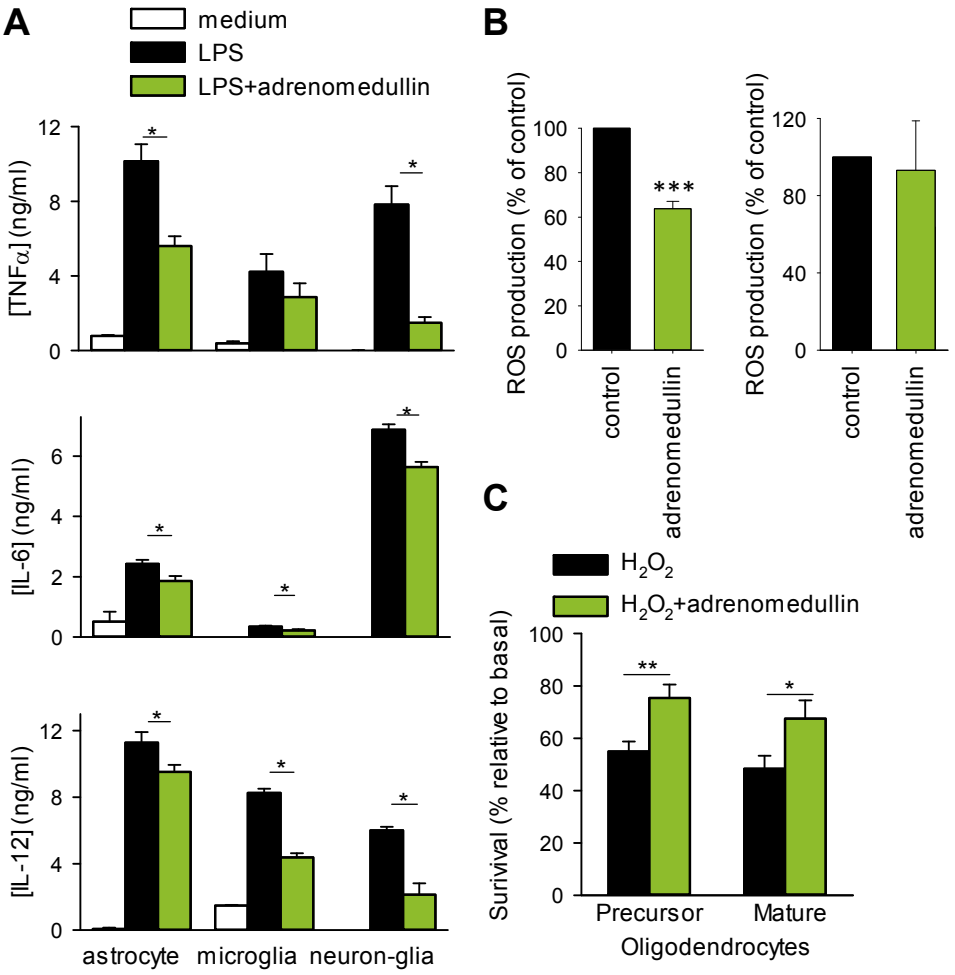

Figure 4.21: Adrenomedullin downregulates inflammatory mediators by glial cells and protects oligodendrocytes from oxidative stress. (A) Adrenomedullin downregulates the inflammatory response of CNS resident cells. Microglia, astrocytes and mixed neuron-glia isolated from newborn mice were cultured in medium or stimulated with LPS in the absence or presence of adrenomedullin (100 nM) and cytokine contents in culture supernatants were determined 24h later. N = 4, in duplicates. (B) Adrenomedullin reduces ROS production by glial cells. Microglia and astrocytes were cultured for 1h with H_2O_2 (microglia, 300µM; astrocytes, 40 µM) in the absence or presence of adrenomedullin (100 nM). Production of ROS was detected by fluorometry using the fluorescence probe carboxy-H_2DCFDA; n = 3, in quatriplicates ***p<0.0001 *vs.* control with t-test. (C) Adrenomedullin protects oligodendrocytes from oxidative-induced cell death. Cell survival of precursor and mature oligodendrocytes cultured for 24h with 200 µM H_2O_2 in the absence or presence of 100 nM adrenomedullin. N = 3, in duplicates. * p<0.05; **p<0.01 *vs*. control with Mann–Whitney test.

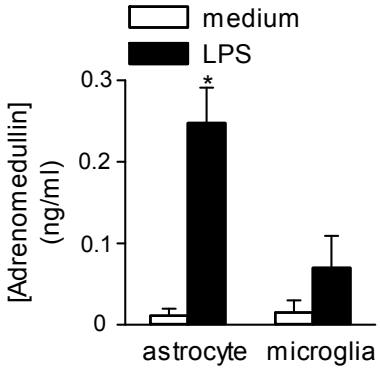

Figure 4.22: Glial cells produced adrenomedullin under pro-inflammatory conditions. Levels of adrenomedullin in culture supernatants of LPS-activated astrocytes and microglia (at 24h). N = 5 in duplicates. *p<0.05 *vs* medium with Mann-Whitney test

4.2. Beneficial effect of cortistatin in the MPTP mouse model of Parkinson´s disease is exerted at multiple levels.

PD is the second most common neurodegenerative disorder, characterized by a complex pathophysiology that involves neuroinflammation and loss of nigral dopamine neurons and neurotrophic factors. This complexity is the main cause of therapeutic failure and approaches for developing new therapeutic strategies are highly desirable. From our point of view, the best therapeutic agent for this disease should combine immunomodulatory and neuroprotective effects. Recently, we have described the therapeutic effect of the neuropeptide cortistatin in both chronic and remitting-relapsing EAE. Cortistatin reduced clinical severity and incidence of EAE, the appearance of inflammatory infiltrates in spinal cord, and the subsequent demyelination and axonal damage, affecting to autoimmune and inflammatory components. Moreover, cortistatin regulated glial activity and favoured an active program of neuroprotection/regeneration (177). These results open the possibility that cortistatin could have an important role as therapy for other neuroinflammatory and neurodegenerative disorders such as PD.

To study the potential neuroprotective effect of this neuropeptide in PD, we used the preclinical model induced by the toxin MPTP. This is the most widely parkinsonism mouse model used to study the idiopathic PD. MPTP model resembles some pathological hallmarks of the disease as it causes a dramatic neurodegeneration

of the nigrostriatal pathway, with the presence of both neuroinflammation and oxidative damage. We analyzed the effect of exogenous administration of cortistatin and also its lack in the development and progression of this disorder. In addition, we performed *in vitro* studies using the human neuroblastoma cell line SH-SY5Y and ventral midbrain cultures from mice.

4.2.1. Treatment with cortistatin ameliorates MPTP-induced locomotor dysfunctions.

PD is characterized by changes in the motor function, such as bradykinesia (slowness of movement), rigidity, postural instability and rest tremor (207). Some of these alterations are mimicked in the MPTP model and can be assayed by using the rotarod, gait and hang task assays. These tests measure rodent locomotion and motor coordination in a standardized manner, so as to evaluate brain functions that are mainly affected by alterations of the dopaminergic systems (208, 209). All of them were performed seven days after the last MPTP injection. Mice injected with MPTP reduced the latency to fall from the rod compared to sham mice in all trials in the rotarod test (Fig. 4.23A). Treatment with cortistatin significantly increased the latency to fall in the MPTP-injected mice (Fig. 4.23A). Moreover, we evaluated the coordination and neuromuscular strength of mice with the gait test and the hang test, respectively. Cortistatin-treated mice increased the stride length of both forelimbs and hindlimbs, showing a similar patter than that naïve mice (Fig. 4.23B). Furthermore, hang test showed that although MPTP-intoxicated mice treated with cortistatin did not stay longer on the grid, they remained less time inactive and moved around more and faster than untreated MPTP-injected mice (Fig. 4.23C).

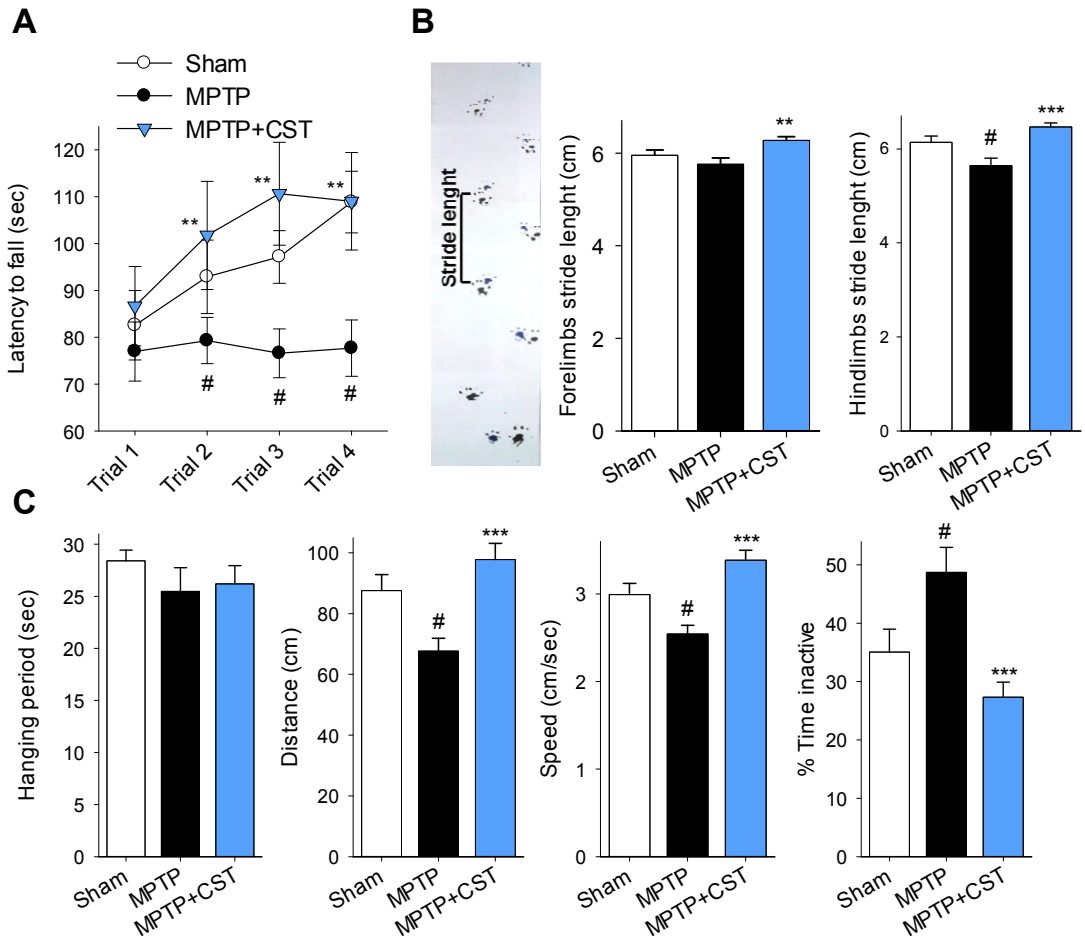

Figure 4.23: MPTP-induced deficits in locomotor activity are avoided after treatment with cortistatin. C57BL/6 mice were administrated with MPTP (20mg/kg; 4 i.p. injections at 2h intervals) and treated with cortistatin during 7 consecutive days. Behavior of mice was analyzed at 7 dpi. (A) Motor coordination on rotarod of sham mice, MPTP mice (MPTP) and cortistatin-treated MPTP mice (MPTP+CST). Graph represents latency to fall off a rotating rod accelerating from 4 to 40 rpm in 300 sec, over 4 consecutive trials. (B) Determination of basal ganglia-related movement disorders with the gait test: forelimb and hindlimb strides were differentiated using blue and black ink for front and back paws, respectively. Stride length was measured as the distance between successive paws prints. The average of three strides was taken for each animal. (C) Hang test for determining neuromuscular strength. Mice were placed on a horizontal grid and once mice grabbed the grid, it was inverted. Mice were allowed to hang upside down for 30 sec. Graphs show the average of 5 trials measuring hanging period, locomotor activity (distance travelled and speed) and resting time. All data are reported as means ± s.e.m. of 20 mice/group (from 4 independent experiments). **p<0.01, ***p<0.001, MPTP+CST-treated mice *vs* MPTP-mice; #p<0.05, MPTP-mice *vs* Sham animals. Group differences were analyzed by One-way ANOVA. Post-hoc comparisons were conducted using the Tukey´s multiple comparison test.

4.2.2. Cortistatin attenuates MPTP-induced dopaminergic neurodegeneration

Once we observed that cortistatin improved the clinical signs of MPTP mice, we investigated its effect on the integrity of the nigrostriatal pathway. We processed the brain of the animals one week after MPTP administration for determining dopaminergic neuronal cell loss in SNpc and the striatum using immunostaining analysis of tyrosine hydroxylase (TH+ neurons). TH is an enzyme found in dopaminergic neurons, responsible for catalyzing the conversion of the amino acid L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA), which is the precursor of dopamine. As observed on figures 4.24 and 4.25, acute systemic administration of MPTP resulted in a dramatic reduction of dopaminergic TH+ neurons in SNpc and in a loss of dopaminergic fibers in the striatum in comparison to sham mice. Systemic administration of cortistatin significantly prevented the MPTP-induced loss of TH+ neurons and reduced the loss of striatal dopaminergic terminals (Fig. 4.24 and 4.25). Thus, cortistatin can attenuate neurotoxic effects of MPTP on the dopaminergic cell bodies in the SNpc and nerve fibers in the striatum.

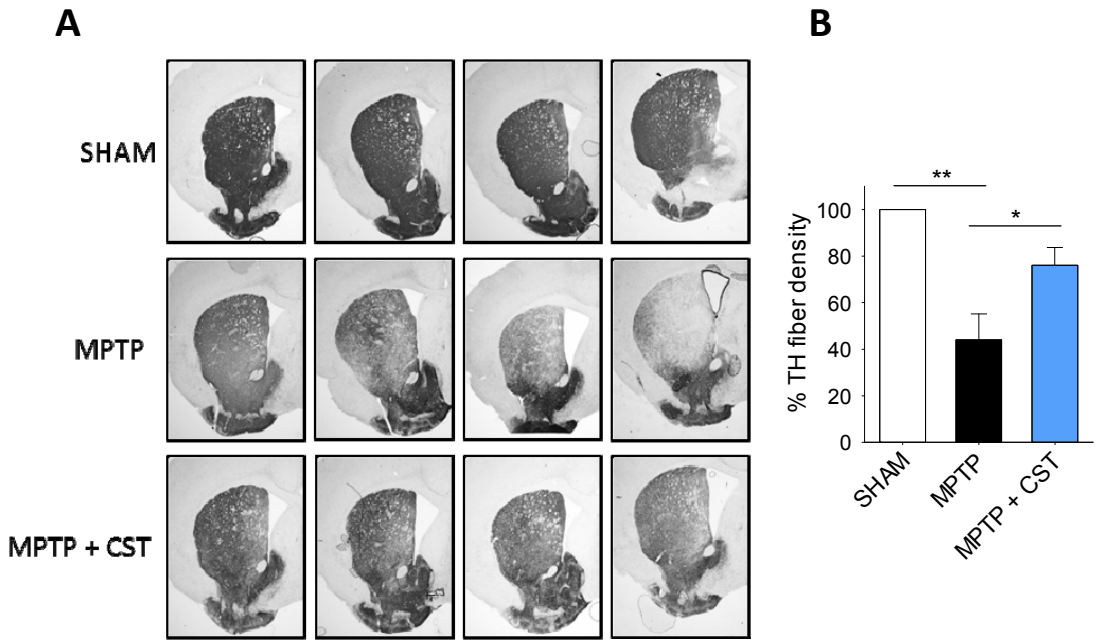

Figure 4.24: Cortistatin protects from dopaminergic-related toxicity induced by MPTP in the striatum. C57BL/6 mice were administrated with MPTP (20mg/kg; 4 i.p. injections at 2h intervals) and treated with cortistatin during 7 consecutive days. Immunoreactivity for TH was analized in midbrain isolated 7 dpi. (A) Coronal sections of striatal fibers immunopositive for TH (stained in black). Five sections/mouse were analyzed; 4 animals/group are showed. Magnifications at 40X. (B) Quantification of density of striatal TH-immunopositive fibers in mice treated with vehicle (sham), MPTP mice (MPTP) and cortistatin-treated MPTP mice (MPTP+CST). N = 5 animals/group. *p<0.05, MPTP+CST vs MPTP-mice; **p<0.01, MPTP-mice *vs* sham animals. Statistical analysis was performed with unpaired Student´s t-test

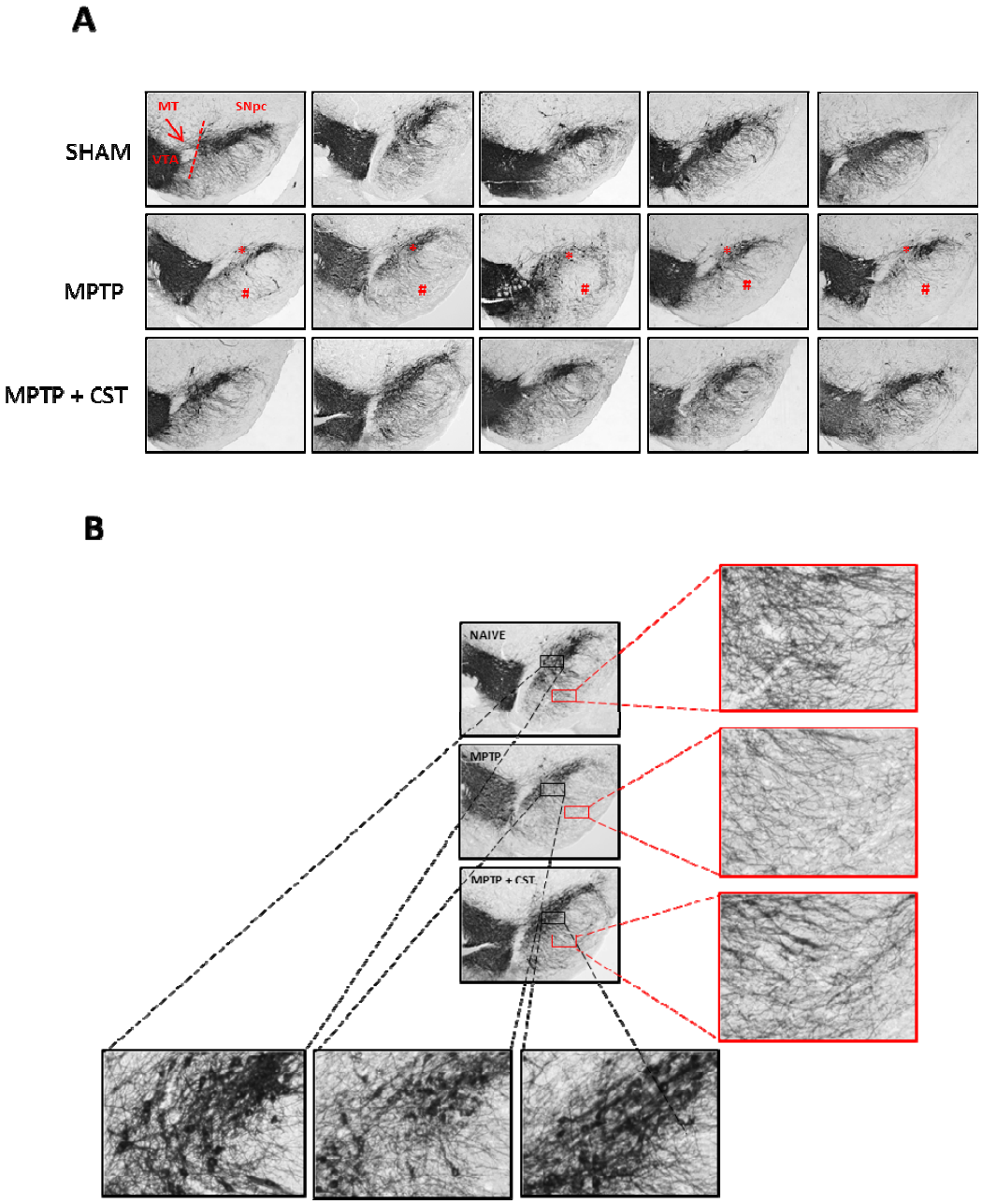

Figure 4.25: **Neuroprotective effect of cortistatin on dopaminergic loss in the SNpc after MPTP intoxication**. C57BL/6 mice were administrated with MPTP (20mg/kg; 4 i.p. injections at 2h intervals) and treated with cortistatin during 7 consecutive days. Immunoreactivity for TH was analized in brains isolated 7 dpi. (A) TH immunostaining of ventral mesencephalon sections showing the loss of dopaminergic nigral neurons in MPTP mice and the protection of these neurons in the cortistatin-treated mice (7 sections per mouse were analyzed; 5 animals are showed). TH: black; cell bodies: red*; cell prolongations: red#. Magnification at 100X. (B) The insets show higher magnifications of the density of neuron bodies (black dashed lines) and cell proyections (red dashed lines). SNpc: substantia nigra pars compacta; VTA: ventral tegmental area; MT: medial terminal nucleus.

4.2.3. Cortistatin modulates the neuroinflammatory response induced by acute MPTP administration

Although the exact molecular mechanisms underlying MPTP-induced dopaminergic neurotoxicity are still unclear, reactive gliosis may play a key role in the pathogenesis of human PD and the MPTP-induced PD model (210, 211, 212, 213). To investigate whether the neuroprotective effect of cortistatin is associated with a reduction of the MPTP-induced glial activation in the SNpc *in vivo* (where viability of dopaminergic neurons is mainly affected by glial responses), we analyzed by immunostaining the expression of Iba1, a specific marker for microglial activation during neurodegenerative inflammation (211). Naïve sham mice only showed a few faintly immunoreactive microglia with resting morphology (ramification with small cell bodies and thin processes) in the SNpc at 7 dpi (Fig. 4.26A). As we expected, MPTP administration resulted in an increase in the number of activated microglia with larger cell bodies (amoeboid-shaped cells) and thick processes, and systemic administration of cortistatin significantly reduced the MPTP-induced microgliosis, as demonstrated by the very few SNpc Iba1^{+} cells observed in these animals (Fig. 4.26A), that showed an intermediate phenotype more similar to the one of sham mice. In contrast to SNpc, striatum of MPTP mice did not show microglial activation at 7 dpi (Fig. 4.26B). On the other hand, activated astrocytes express enhanced levels of GFAP, which is considered as a marker protein for astrogliosis (214). MPTP intoxication led to marked increase in GFAP+ cells in the SNpc and striatum at 7 dpi, which was drastically reduced by cortistatin treatment (Fig. 4.27).

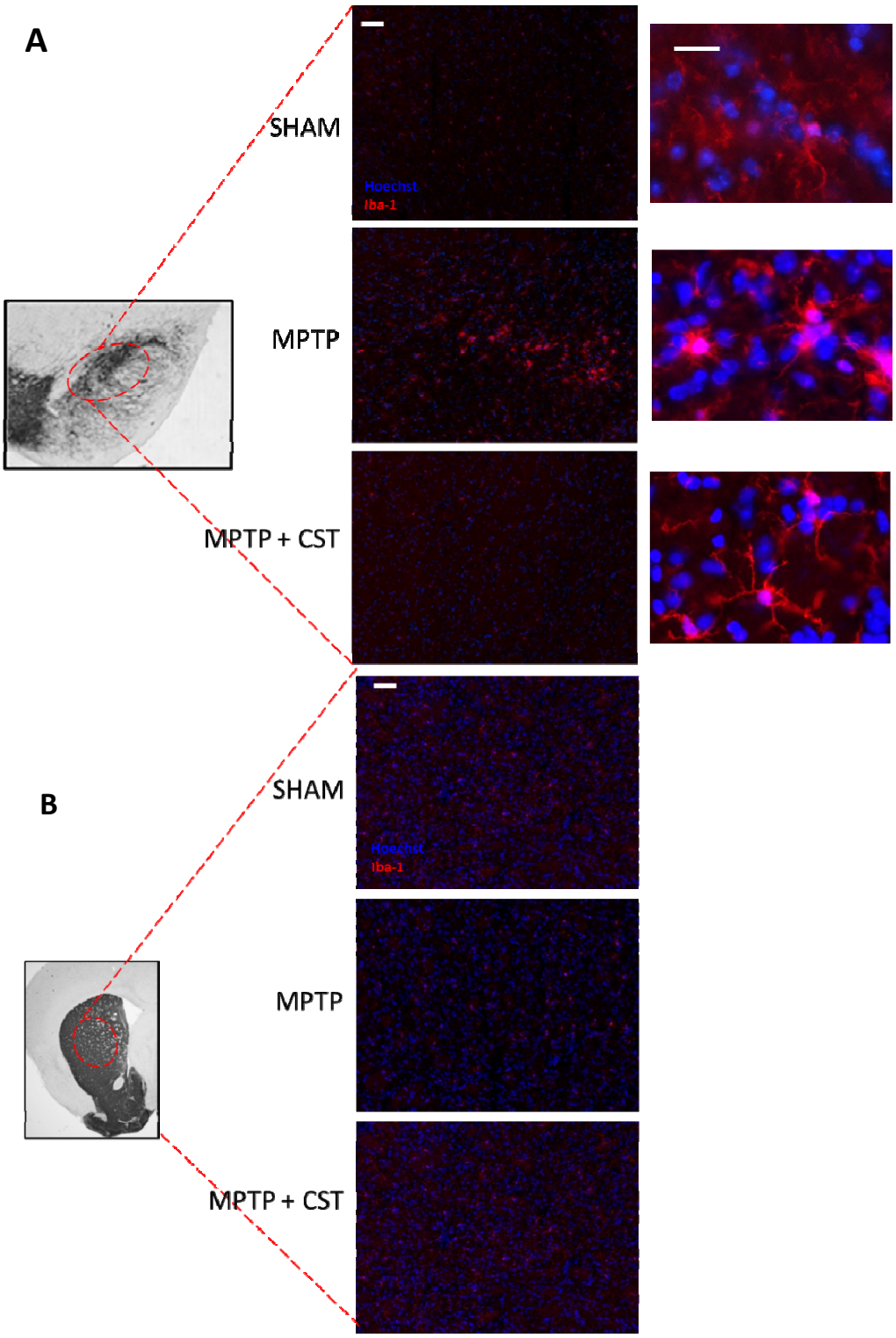

Figure 4.26: Cortistatin reduces MPTP-induced activation of microglia in the SNpc. C57BL/6 mice were administrated with MPTP (20mg/kg; 4 i.p. injections at 2h intervals) and treated with cortistatin for 7 consecutive days. Immunoreactivty for Iba-1 was analized after last injection of cortistatin in coronal sections of the SN region of ventral midbrain (A) or striatal midbrain (B). Nuclei were stained with Hoechst. Sham naïve animals were used as reference controls. Representative images from 5 animals are shown. Scale bar: 50µm. Right pictures in panel (A) show morphological details of microglia phenotype in each group, scale bar: 20µm.

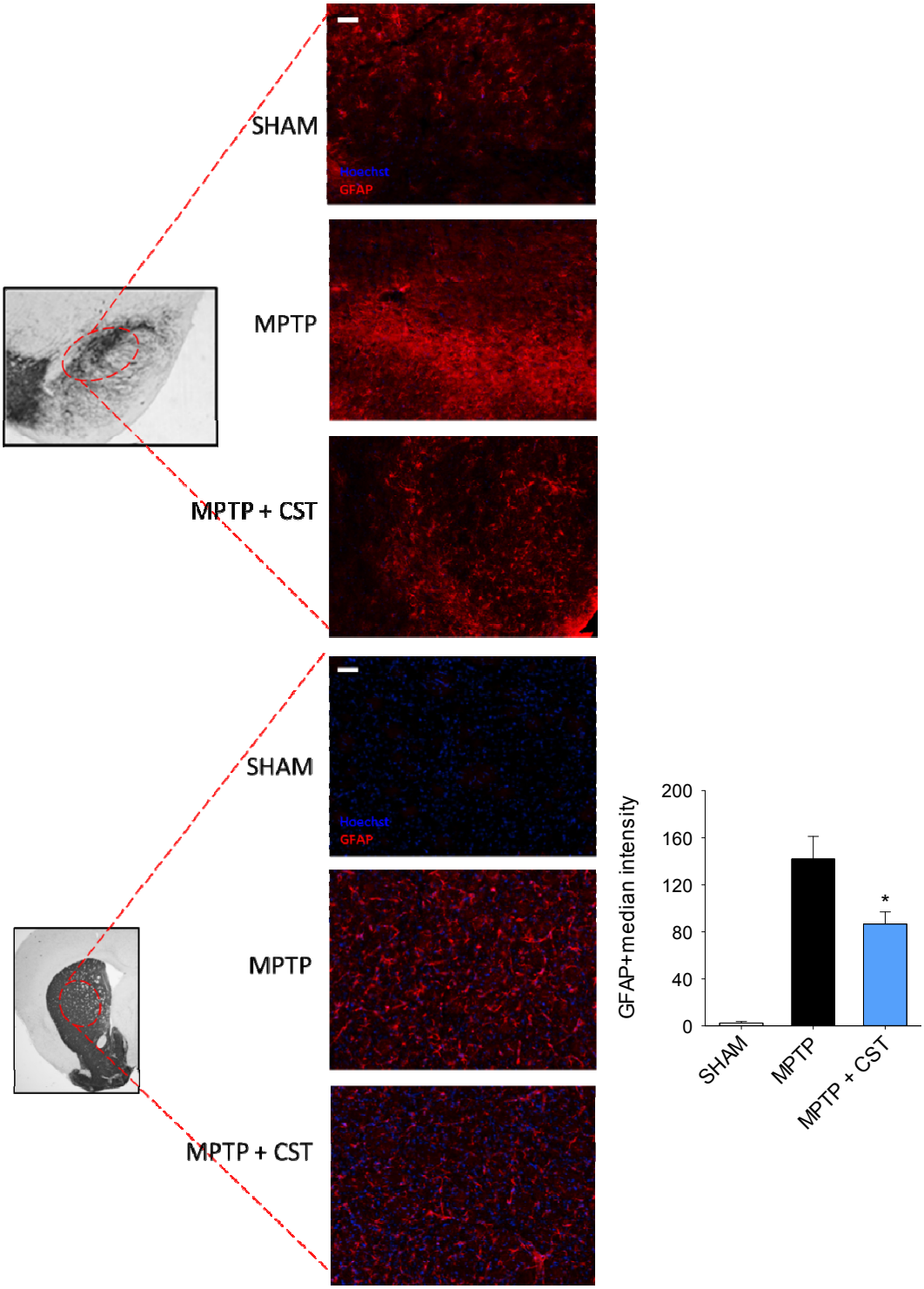

Figure 4.27: Cortistatin diminished astrocytic activation induced by MPTP intoxication. C57BL/6 mice were administrated with MPTP (20mg/kg; 4 i.p. injections at 2h intervals) and treated with cortistatin for 7 consecutive days. Immunoreactivty for GFAP was analized by optical density after last injection of cortistatin in coronal sections of the SN region of ventral midbrain (A) or striatal midbrain (B). Nuclei were stained with Hoechst. Sham mice were used as reference controls. Representative images from 5 animals are shown. Scale bar: 50µm.

4.2.4. Cortistatin regulates the expression of nigral-striatal dopaminergic factors in experimental PD

Analysis of gene expression profiles in MPTP models or PD brains using genechip microarrays suggested that certain genes are related to the neurodegenerative mechanism in PD brains and might be used to develop therapeutic drugs that alleviate the symptoms of PD patients (215). In our study, we decided to analyze temporal changes after cortistatin treatment (at 2 and 7dpi) in the expression through the complete nigra-striatal pathway of selected genes that are related to dopamine synthesis (TH) and uptake (DAT), associated with familial forms of PD and protein folding (α-synuclein), and neurotrophins (BDNF). We found that MPTP injection significantly decreased mRNA expression of TH and DAT and increased gene expression of α-synuclein in SNpc at 2 dpi (Fig. 4.28). Similarly, TH expression in striatum was also reduced in MPTP mice, although no changes occurred in the expression of α-synuclein or DAT in this brain region. Treatment with cortistatin avoided the decrase in expression of TH in both striatum and SNpc, and of DAT in SNpc (Fig. 4.28). Interestingly, cortistatin down-regulated drastically the expression of DAT in striatum. Expression of BDNF did not significantly changed by MPTP or cortistatin treatment at 2 dpi, but it increased significantly in SNpc and striatum at 7 dpi. At this time, it is also significant the diminished expression of DAT (as correlated with dopaminergic cell loss) in SNpc and striatum from MPTP mice in contrast with normal expression found in cortistatin-treated MPTP mice (Fig. 4.28). No changes were observed for TH and α-synuclein expression in the nigral-striatal dopaminergic pathway at 7 dpi.

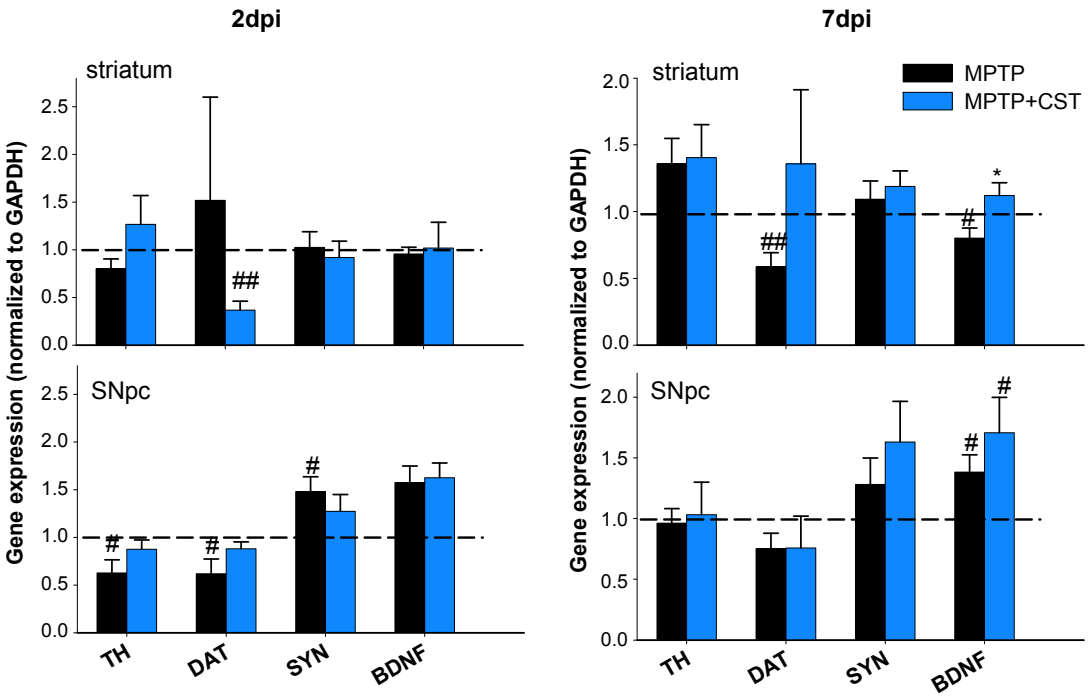

Figure 4.28: **Cortistatin regulates the expression of nigral-striatal dopaminergic factors in the MPTP model**. Midbrain containing striatum and SNpc was isolated and dissected 2 or 7 dpi. Total RNA was isolated and analyzed by Real-time PCR for the expression of tyroxine hydroxylase (TH), dopamine trasporter (DAT), α-synuclein (SYN) and brain-derived neurotrophic factor (BDNF). Graphs represents the expression of each gene using Delta-Delta Ct method normalized with GAPDH (as a housekeeping gene), and are relative to the expression of each factor in sham animals (showed as 1, dashed line). #p<0.05, ##p<0.01, MPTP *vs* Sham mice; *p<0.05, MPTP+CST vs MPTP-mice.

4.2.5. Protective effect of cortistatin in dopaminergic neurons exposed to MPP+

The neurotoxin MPTP is converted in the brain into MPP^+ by the enzyme MAO-B, and this is the functional metabolite that causes toxicity specifically to dopamine neurons. To investigate the effect of cortistatin on MPP^+-induced dopaminergic neurodegeneration, we first used mixed primary cultures of foetal ventral midbrain neuronal-enriched cultures. These cultures have been previously characterized (216) with a cellular composition of 70-80% neurons, of which around 1% are TH^+, and 10-15% glial cells (mostly astrocytes). As shown in the figure 4.29A, the incubation of these mesencephalic cultures with 5µM MPP^+ resulted in a significant decrease in the number of TH-positive neurons (almost 70% of baseline). The addition of cortistatin to cultures significantly reduced the loss of TH^+ neurons and avoided the marked shortening of cell processes in the remainder TH^+ cells induced by MPP^+ (Fig. 4.29A).

Next, we determined the effect of cortistatin on MPP+-induced neurotoxicity in SH-SY5Y human neuroblastoma cells. Although SH-SY5Y is a cell line, these cells were chosen as they are human cells (and it is highly relevant to test the potential neuroprotective effect of cortistatin in human cell cultures), and they can be differentiated into diverse phenotypes as dopaminergic (which allows more homogenous presence of TH+ cells than in mesencephalic mixed cultures described above) (216). We observed that MPP+ exposure caused an important loss in cell viability (80% of control cells without the neurotoxicant) that was significantly reduced by cortistatin (Fig. 4.29B).

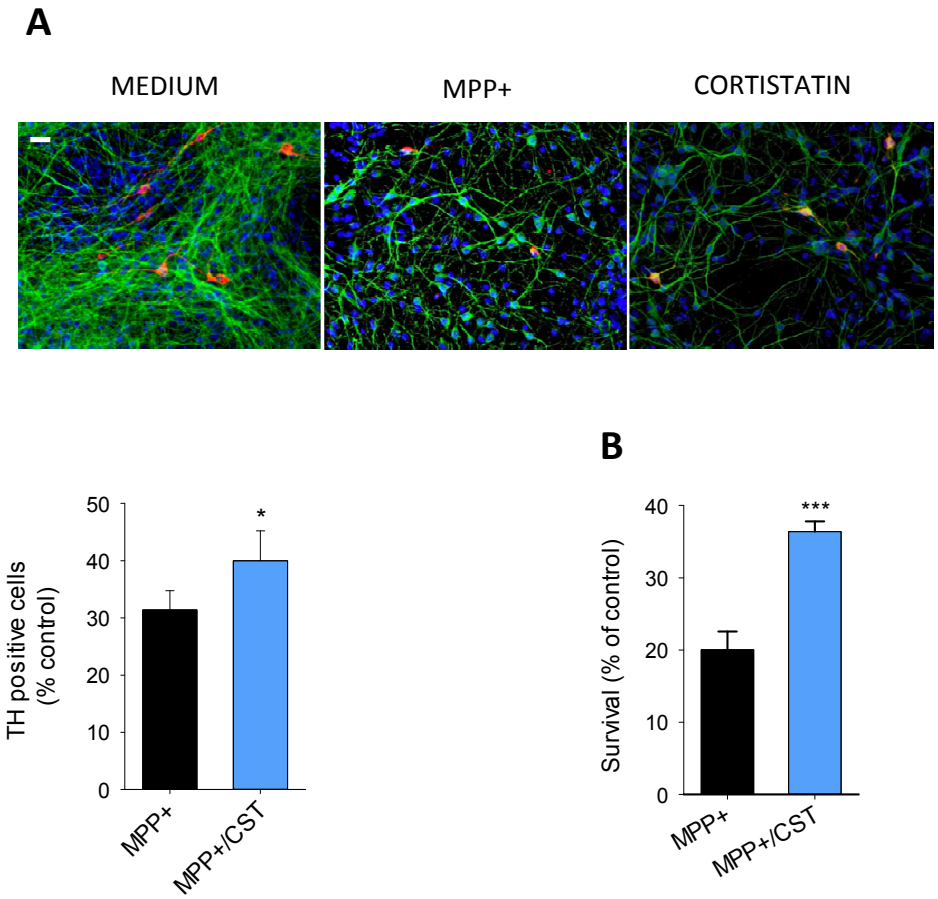

Figure 4.29: Cortistatin protects dopaminergic neurons from MPP^+-associated cell death. (A) Ventral midbrain cultures were prepared from mesencephalon of embryonic mice (E13.5-E14) and treated for 48 h with MPP^+ (5μM) in the absence or presence of cortistatin (CST, 100 nM). The number of dopaminergic neurons in the cultures was determined by counting of TH positive cells and referenced to control cultures treated with medium alone. Magnification 100X. (B) SH-SY5Y cells were differentiated to dopaminergic TH-positive cells by 10 μM retinoic acid during 3 days and then with 80 nM PMA. Seven-day-old cultures were treated with MPP^+ (1 mM) in the presence or absence of cortistatin (100 nM) for 48h. Cell survival was determined by MTT method and expressed relative to culture controls treated with medium alone.*$p<0.05$, ***$p<0.001$, with unpaired t-test.

4.2.6. Cortistatin modulates the inflammatory phenotype of glial cells activated by α-synuclein monomers

As previously described, microglia and astrocytes contribute to neuroinflammation and PD pathogenesis by releasing proinflammatory cytokines and increasing oxidative stress (39, 113). However, the underlying molecules and factors involved in these immune-triggered mechanisms remain largely unexplored. It has been recently described that non-aggregated extracellular wild-type and PD-linked mutant α-synuclein (α-syn) variant A30P, activate glial cultures and induce the release of Th1- and Th2-type cytokines and chemokines (108). α-syn has been found both in its aggregated and monomeric forms in cerebrospinal fluid and blood plasma of PD patients (108), and different studies have demonstrated a role for this protein in the ethiology of PD, forming aggregated amyloid-like forms. These insoluble aggregates are recognized as a key event in PD pathogenesis and other related diseases (217). These results have pointed out the importance of studying the effects of extracellular α-syn on surrounding cells in the brain as a new target for PD therapy. As it was previously demonstrated the capacity of cortistatin to modulate the inflammatory response of glial cells (177), we wondered whether cortistatin could also regulate activation of glial cells by the α-syn variants, which could be related with the role of immune responses on the onset and progression of PD (108). In this thesis, we used the MPTP model, that although replicates many of the pathological signs and motor features of PD in primates and rodents, it does not involve α-syn inclusion body formation. Thus, in order to characterize the role of cortistatin on α-syn-induced glial activation, we performed *in vitro* cultures of microglia and astrocytes stimulated with monomeric preparations of WT and A30P mutant protein in the absence or presence of cortistatin. We observed that both monomers (WT and A30P mutant) induced the production of ROS and TNFα by microglia and astrocytes (Fig. 4.30). Cortistatin was able to reduce ROS production by activated microglia (Fig. 4.30A) and downregulated the inflammatory response of both glial cells (Fig. 4.30B).

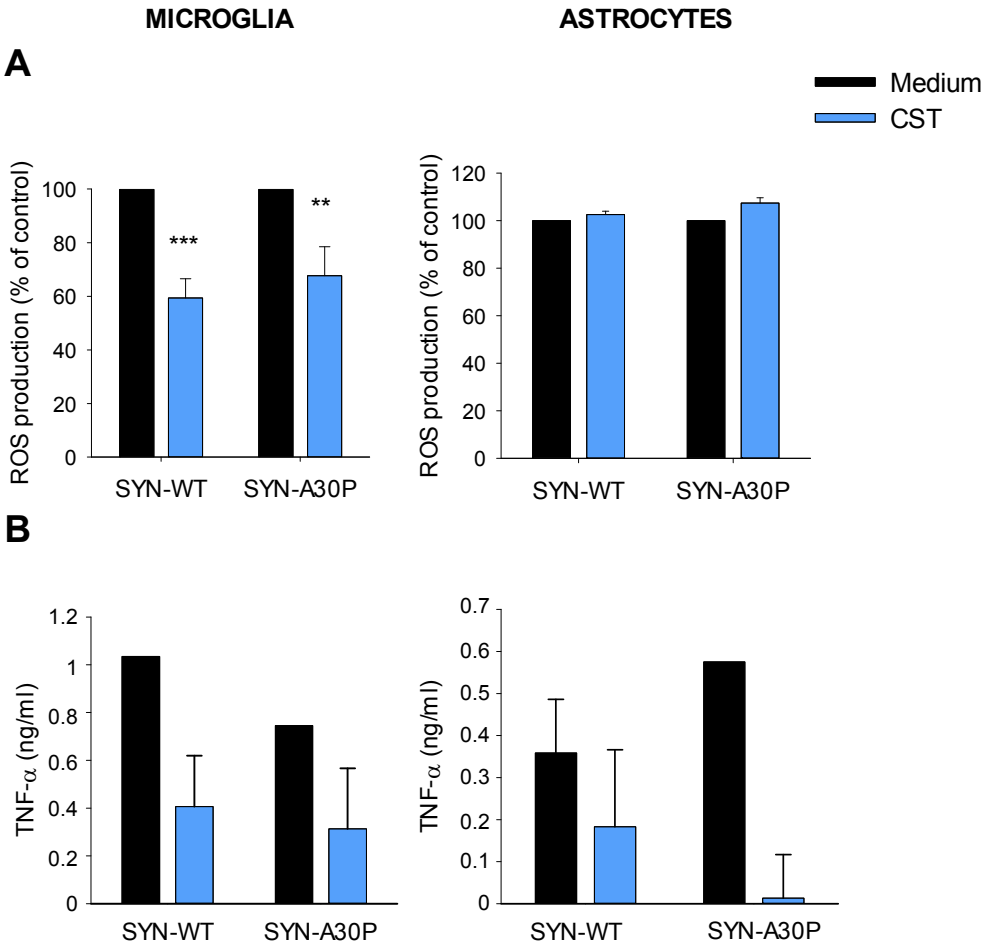

Figure 4.30: Cortistatin modulates the inflammatory phenotype of glial cells activated by α-synuclein monomers. Microglia and astrocytes isolated from newborn mice were cultured with 1 µg/µl α-syn-WT monomers orl a-syn-A30P in the presence or absence of cortistatin (100 nM). After 1 h of culture, production of ROS was detected by fluorometry using the fluorescence probe carboxy-H2DCFDA (A). After 24 h of culture, TNFα levels in supernatants were determined by ELISA (B). n = 4 in duplicates. **p<0.01; ***p<0.0001 *vs* control, with t-test.

4.2.7. Effect of cortistatin deficiency in MPTP-induced PD

Once demonstrated the protective action of cortistatin in the MPTP model of PD, with cortistatin-treated MPTP mice showing less neurodegeneration of the nigrostratial pathway, we asked about the role played by endogenous cortistatin in the development and progression of this disorder. To address this objective, we induced the Parkinsonism model of MPTP in cortistatin-deficient mice (KO-CST). In our hands, less than 20% of animals died by the acute intoxication with MPTP in wild-type mice (as it has been previously described by others, 218). However, around 50% of KO-CST mice died after last MPTP injection. Interestingly, all wild-type mice that were treated with cortistatin survived to MPTP intoxication (Fig. 4.31). Although experiments are in progress in order to evaluate whether the protective effect of endogenous cortistatin affects to the dopaminergic pathway and/or the systemic acute inflammation induced by MPTP, these preliminary results suggest that cortistatin could have a crucial role regulating the neurotoxic effect of MPTP.

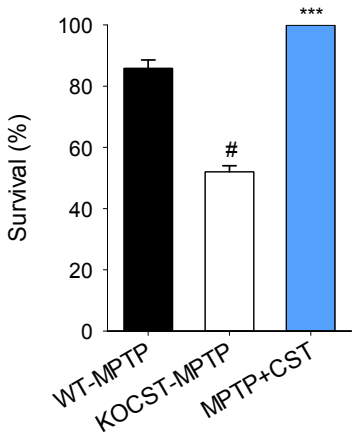

Figure 4.31: Animals deficient in cortistatin showed increased susceptibility to MPTP intoxication. Survival of mice intoxicated with MPTP was monitored during the first 24 hours, in wild-type mice (WT-MPTP), cortistatin deficient mice (KOCST-MPTP) and wild-type mice treated with MPTP and exogenous cortistatin (MPTP+CST). Bars represent means ± SEM of 10 mice/group (three independent experiments). ***p<0.001, MPTP+CST *vs* WT-MPTP and KOCST-MPTP, #p<0.05, KOCST-MPTP *vs* WT-MPTP, using one way ANOVA, post-hoc comparisons with Tukey's multiple comparison test.

5. DISCUSSION

Far to be considered two independent systems, evidences in the last two decades have confirmed that the nervous and immune systems are closely interconnected functionally and anatomically. This implies that both systems regulate their function, homeostasis and pathological states, each other. Thus, the immune response does not solely depend on traditionally considered immune factors, but nervous triggers, such as stress, also orchestrate the final defense response against pathogens or other external and internal insults. Similarly, acting as a sixth sense, the immune system exerts a fine tuning of the neuroendocrine response against a certain stimuli. In addition, many neurodegenerative disorders are caused and/or mainly driven by immune mediators and cells, especially under some conditions such as trauma and infection. Therefore, the understanding of the biochemical mediators involved in the bidirectional communication that exists between nervous and immune systems is critical to maintain the organism in homeostasis, to prevent the development of pathological states, and ultimately, to develop new therapeutic strategies to treat neurodegenerative disorders. In this study, we propose that two endogenous neuropeptides, which are produced by neural and immune cells mainly during the ongoing inflammatory response, can exert a dual role in immune and nervous system and protect against neurodegenerative disorders that course with exacerbated neuroinflammatory responses, such as MS and PD.

5.1. Multiple sclerosis and adrenomedullin

MS is a disabling demyelinating disease of the CNS affecting more frequently young people. Although the mechanisms of disease pathogenesis remain unclear, MS is considered as an archetypal neuroinflammatory and autoimmune disease in which Th1 and Th17 CD4 cells that are reactive to components of the myelin sheath infiltrate the CNS parenchyma, release proinflammatory cytokines and chemokines, and further promote inflammatory cell infiltration and activation (219, 66). The production of inflammatory mediators such as cytokines and free radicals by infiltrating immune and resident glial cells plays a critical destructive role in the process of demyelination and contributes to oligodendrocyte loss and axonal degeneration. Moreover, evidence

indicates that a deregulation in the mechanisms involved in the maintenance of immune tolerance, especially those affecting Treg cells, critically contributes to the establishment and progression of the autoimmune response (78).

Although available therapies based on immunosuppressive agents reduce the relapse rate or delay disease onset, they do not suppress progressive clinical disability. Therefore, we need novel multistep therapeutic approaches that prevent the inflammatory and autoimmune components of the disease, restore immune tolerance and promote and/or allow mechanisms of neuroprotection and neuroregeneration.

In this context, we decided to study the potential therapeutic effect of adrenomedullin, a neuropeptide known for its cardiovascular and anti-inflammatory effects, in a preclinical model of MS, analyzing its effect at both peripheral (in the DLNs and spleen) and local (in the glial niche and CNS infiltrating cells) levels.

Adrenomedullin regulates inflammation and autoimmunity on EAE and exerts neuroprotective properties

Using a chronic model of EAE induced by MOG, we observed that treatment with adrenomedullin reduced the incidence and the severity of this disease (Fig. 4.1; table 4.1), and that this beneficial effect was kept on even during the effector phase, when the disease was fully established. We demonstrate that adrenomedullin reduced the inflammatory infiltration in spinal cord (Fig. 4.2), decreasing the presence of immune cells. Consequently, EAE mice treated with adrenomedullin have reduced number of demyelination plaques in both cervical and lumbar regions of spinal cord. This therapeutic effect is associated with a striking reduction of the two deleterious components of the disease, the autoimmune and neuroinflammatory responses.

Our data indicate that treatment with adrenomedullin decreased the presence of encephalitogenic Th1 and Th17 cells in the periphery and CNS. This effect is mostly exerted by regulating the encephalitogenic sensitization in the peripheral immune compartment (Fig. 4.5). Adrenomedullin impaired the proliferation of T cells and production of Th1/Th17 cytokines, in a MOG-specific manner. While adrenomedullin decreased the percentage of $CD4^+$ IFN^+ T cells, a Th1 cell phenotype (Fig. 4.6), the

treatment did not affect to the percentage of $CD4^+$ $IL\text{-}17^+$ T cells, although it seems that these cells failed to secrete IL-17. At the same time, adrenomedullin treatment increased the percentage of IL-4 and IL-10 expressing $CD4^+$ cells, a Th2 phenotype, being this feature important since Th2 cells are crucial in controlling Th1 and Th17 cell subsets by producing IL-4 and IL-10 (10). The effect of adrenomedullin on the Th2 cells is also reflected by a class switch in autoantibodies (Fig. 4.8). Interestingly, adrenomedullin did not produce a general immunosuppression, since the response to a polyclonal T cell stimulation remained mostly unaffected in the adrenomedullin-treated EAE mice. This property of adrenomedullin makes it an attractive therapy for MS, since current treatments usually generate a generalized immunosuppression (42).

Due to Th1 and Th17 cells infiltrate to CNS parenchyma, we assessed whether the treatment with adrenomedullin could also impair locally the encephalitogenic response by these cells into CNS. Although infiltration of Th1 and Th17 cells was decreased by adrenomedullin, the few cells that infiltrated into parenchyma produced much less levels of pro-inflammatory cytokines such as IL-2, IFNγ and IL-17, and did not proliferate in a MOG-specific recall response (Figs. 4.9 and 4.10), suggesting that adrenomedullin could affect the encephalitogenic response in the CNS.

Moreover, our experiments *in vitro* (Fig. 4.7) indicate that adrenomedullin could regulate the self-reactive T cell responses directly in the peripheral lymphoid organs. However, whether this effect is exerted directly on T cells, affecting their activation, clonal expansion or differentiation, or indirectly through the modulation of APCs, remains to be determined. Nevertheless, we have data that could support either possibility, since we have previously found that adrenomedullin inhibited the activation of T cells in the absence of APCs. Furthermore, here we demonstrate that adrenomedullin regulates DC function by generating a semi-mature phenotype (Fig. 4.14) with the capacity to impair the encephalitogenic response both *in vitro* and *in vivo* (Figs. 4.15 and 4.16). The effect on DCs could partially explain the fact that adrenomedullin inhibited the T cell response in an antigen-specific manner.

Beside its effect in the peripheral immune compartment, our *in vitro* experiments suggest that adrenomedullin could exert direct actions on infiltrating

encephalitogenic T cells in the CNS parenchyma following traffic through the compromised blood-brain-barrier. In this case, whereas adrenomedullin fully suppressed MOG-specific Th17 responses, it failed to inhibit the production of IFNγ (Fig. 4.11). For over a decade, Th1 cells were thought to be the driving force behind MS neuroinflamation (220). However, this traditional view has been challenged by studies describing exacerbated EAE development in animals deficient in IFNγ, which supports a protective role for endogenous IFNγ (221, 67). While this remains the subject of some debate, evidence now indicates that IL-17 producing T cells are critical for EAE (69, 74, 75, 76), and that T cell infiltration and inflammation in the CNS in EAE occur only when Th17 cells outnumber Th1 cells (76). Therefore, the potent suppressive effect of adrenomedullin on the activation of peripheral and central encephalitogenic Th17 cells might be an important component in its protective effect in EAE. Although we previously reported the inhibitory effect of adrenomedullin on Th1 responses in experimental colitis and arthritis (156, 157), the present study is the first demonstrating the impairment of self-reactive Th17 responses by this neuropeptide. Of relevance is also the fact that adrenomedullin downregulated the production of GM-CSF and osteopontin by encephalitogenic T cells, since both cytokines serve a nonredundant function in the initiation of autoimmune inflammation and in the generation of Th1 and Th17 cells in EAE and MS (195, 222, 223, 224, 225). These cytokines present elevated levels in MS patients (226, 227) and it has been demonstrated the therapeutic relevance of decreasing the levels of osteopontin for other conventional MS therapies, such as IFNβ treatment (227).

On the other hand, the treatment with adrenomedullin also increased the number of IL-10-producing $CD4^+CD25^+FoxP3^+$ Treg cells in lymphoid organs and CNS (Fig. 4.12). Our study also demonstrated the involvement of functional Tregs in the therapeutic action of adrenomedullin in autoimmunity (Fig. 4.13). These findings could partially explain the antigen specificity of the long lasting protective response generated by adrenomedullin (Fig. 1.1), and the fact that adrenomedullin administration subsequent to the activation/differentiation of antigen-specific effector Th1/Th17 cells still inhibited the inflammatory phase of EAE. In agreement with the present study, our group previously described the generation of $CD4^+CD25^+$ Treg cells

by adrenomedullin in animal models of rheumatoid arthritis and inflammatory bowel disease (156, 157). Further investigations are necessary to determine whether adrenomedullin affects the expansion of already existing Treg cells or their *de novo* generation. Other neuropeptides, such as VIP and urocortin, increased Treg cells by promoting their generation from the non-Treg compartment through direct actions on T cells and indirectly on tolerogenic DCs (15). A recent study has demonstrated that stimulation of DCs with adrenomedullin induces the expression of indoleamine 2, 3-dioxygenase and the expansion of $FoxP3^+$ Treg cells *in vitro* (197). Here, we found that DCs stimulated and pulsed with MOG in the presence of adrenomedullin are capable to transfer tolerance to mice with EAE (Fig. 4.16), although it remains to be determined whether they induce antigen-specific Treg cells *in vivo*. The IL-10-secreting CD4 Treg cells increased by adrenomedullin in EAE mice could represent a subtype of Tr1-like cells generated by tolerogenic DCs (228).

Regarding the inflammatory response in EAE, it is evident that the regulation of a wide spectrum of inflammatory mediators by adrenomedullin has an advantage over therapies directed against single mediators. The reduction of inflammation in CNS in adrenomedullin-treated EAE mice is associated with a decrease in the levels of chemokines in CNS parenchyma. This is especially relevant for chemokines such as CXCL10 (chemotactic for Th1 cells), CCL5 (for T cells) and CCL2 (for macrophages and T cells) that contribute to MS neuropathology (219, 201). Whether the effect on chemokines is exerted at local level or as a consequence of the peripheral action of adrenomedullin on IL-17 or GM-CSF, which play major roles in the initial recruitment of inflammatory cells to the CNS parenchyma in EAE, remains to be determined. In addition, the effect of adrenomedullin decreasing inflammation on CNS parenchyma could be exerted at local level, since Consonni et al reported the capacity of adrenomedullin to inhibit the secretion of CCL2 and CCL3 by activated microglia *in vitro* (229). Moreover, our study and results from others (229) demonstrate that adrenomedullin might exert its anti-inflammatory action locally by downregulating the production of cytokines and ROS by astrocytes, microglia and neuron-glia cultures (Fig. 4.21). TNFα is involved on MS pathology, as there are elevated levels of this cytokine in active lesions of MS patients and in in serum and cerebrospinal fluid that correlate

with the severity of lesions (230). Furthermore, peripheral blood mononuclear cells from MS patients increase the secretion of TNFα before exacerbation of symptoms (231, 232). The decrease of TNFα production by activated astrocytes in presence of adrenomedullin is very interesting for the treatment of neuroinflammatory and neurodegenerative disorders since TNFα overproduction by astrocytes is enough to triggering the inflammation and degeneration of CNS (233). Moreover, it has been described that astrocytes are efficient presenting MOG peptides and stimulating T cells, but less than DCs, suggesting that astrocytes could act as a secondary line of APCs in the CNS and that adrenomedullin could partially exert its therapeutic effect on the EAE model by reducing the activation of astrocytes (234). IL-6 is reduced by adrenomedullin treatment in activated microglia. It has been reported that IL-6 blockade shows a protective effect in EAE mice (235). Moreover, IL-6 is able to suppress the generation of Treg cells by TGF-B, favouring the induction of Th17 cells (236). Therefore, the inhibition of IL-6 production by adrenomedullin in microglia and in the CNS parenchyma could be apparently an important mechanism for the generation of Treg cells and the suppression of Th17 cells. To note, adrenomedullin did not completely deactivate glial cells, but it modulates their function. This is important because maintaining physiological levels of inflammatory cytokines (IL-6 and TNFα) and a controlled activation of microglia and astrocytes is beneficial for the regulation of the CNS homeostasis (83, 237, 41, 238, 239). Adrenomedullin was also able to reduce the oxidative stress by decreasing ROS production by microglia under oxidative stress conditions (Fig. 20B). ROS play an important role in the pathogenesis of MS, particularly in the loss of myelin/oligodendrocyte complex (240). In fact, some authors suggest that in the search of an effective treatment for MS is necessary to look for antioxidant therapies. However, antioxidant therapies must be taken early in the disease (241). Although it was previously described the antioxidant role of adrenomedullin in the endothelial system (242) we have described it for the first time in the glial niche. Together, these results indicate that the effect on resident inflammatory cells, together with the inhibitory action of adrenomedullin on infiltrating macrophages (158, 156), probably contributes to the protection against oligodendrocyte/neuronal cell loss and axonal damage in this inflammatory milieu.

On the other hand, we observed that adrenomedullin-treated EAE mice showed higher expression levels of two neurotrophic factors, such as ADNP and BDNF, in spinal cord (Fig. 4.17). ADNP is an important neurotrophin involved on the development of the CNS. In fact, ADNP-deficient mice show a cranial neural tube closure failure and die during embryonic development (243), and it is reduced in peripheral blood mononuclear cells of MS patients (199). On the other hand, BDNF is involved in important roles, including axonal growth and modulation of neuronal activity, memory and learning. Furthermore, it is also involved on functions under pathological conditions (198). Thus, BDNF is produced by neurons and by activated astrocytes in MS and EAE, suggesting that BDNF is up-regulated under demyelinating processes (244). However, BDNF levels are reduced in MS patients compared to healthy controls, and are increased significantly after MS relapses, supporting the idea that BDNF is important during the recovery of acute demyelinating inflammatory lesion (245). In fact, BDNF enhances CNS myelination acting directly on oligodendrocytes (246) and mice deficient in BDNF show an impaired differentiation of oligodendrocytes (247). Interestingly, BDNF and ADNP are also produced by immune cells, such as monocytes, T cells and B cells *in vitro* and in MS or EAE lesions (198, 199). Thus, it remains unclear if the effect of adrenomedullin was exerted directly on cells of CNS or on immune cells, since it has demonstrated that both systems express adrenomedullin receptor. Independently of the source of neurotrophic factors, the ability of adrenomedullin for increasing ADNP and BDNF is an important finding in order to look for an effective endogenous therapy, due to the limited access to CNS parenchyma following exogenous administration of these neurotrophic factors, which are not able to cross the BBB. In fact, some of the current treatments approved by FDA for MS treatment, such as glatiramer acetate, increase also the levels of BDNF by T cells (198).

Role of adrenomedullin on demyelination and remyelination

Although MS lesions can be potentially repaired by endogenous OPCs (248), remyelination fails in most cases, resulting in irreversible neurological disability (249). Current treatments mainly target the inflammatory component of the disease, with none or little impact on myelin repair. Therefore, development of therapies promoting

remyelination and neuroprotection would represent an important advance in MS treatment. Interestingly, the delayed treatment with adrenomedullin to EAE mice induced complete recovery in a significant number of animals, suggesting a role of this neuropeptide in repair and/or neuroregeneration. Thus, we decided to characterize a possible role for adrenomedullin on oligodendrocytes survival and function in an aggressive environtment. First, we found that adrenomedullin protected *in vitro* both OPCs and mature oligodendrocytes from cell death caused by an oxidative milieu (Fig. 4.21). Then, we used a distinct *in vivo* model for MS, the lysolecithin-induced demyelination model to analyze its effect in the demyelination and remyelination processes in an immune response-independent manner. Our data indicated that adrenomedullin protected from the demyelination induced by lysolecithin injection and accelerated remyelination (Fig. 4.19). The spontaneous remyelination process starts 10 days after lysolecithin injection, and we observed that adrenomedullin accelerated this process as the adrenomedullin-treated mice showed smaller demyelinating areas in the corpus callosum than untreated control mice at 7 dpi, and the differenceces in size of the demyelinating lesion time between untreated and adrenomedullin-treated mice were kept on at 14 dpi.

Remyelination involves the generation of new mature oligodendrocytes derived from a population of OPCs, which have to be recruited to the site of lesion and then differentiated to myelin expressing oligodendrocytes (84). We observed that adrenomedullin treatment increased over time the number of $Olig2^+$ cells in the lesioned area of corpus callosum of lysolethicin-treated mice (Fig. 4.20). Olig2 is highly enriched in premyelinating oligodendrocytes, although it is not an exclusive marker of OPCs, because the oligodendrocyte lineage expresses it at later stages (250). So, it remains unclear if these $Olig2^+$ cells are OPC or mature oligodendrocytes, although the increased remyelination observed after adrenomedullin treatment supports the latter possibility.

Because several factors modulate oligodendrocyte differentiation, further experiments have to be performed in order to elucidate the role of adrenomedullin in proliferation, recruitment and/or differentiation of OPCs in demyelinating disorders. For example, endogenous myelin repair relies on the delivery of growth factors, such

as BDNF, that regulate survival, proliferation or differentiation of oligodendroglial cells (251, 252). Moreover, it has been recently reported that Treg cells enhance oligodendrocyte differentiation *in vitro* (253). Due to the capacity of adrenomedullin to increase the endogenous expression of BDNF on lesioned spinal cord and the numbers of Treg cells in animals suffering EAE, it is plausible that this neuropeptide regulates proliferation and maturation of oligodendrocytes during remyelination indirectly through both mechanisms. On the other hand, according to our findings describing a role of adrenomedullin on remyelination, it has been described that adrenomedullin promotes OPC differentiation under pathological conditions *in vitro* (167), which supports a direct effect on OPCs. In addition, a recently generated brain conditional adrenomedullin-knockout mouse demonstrated the role of this neuropeptide in the differentiation of adult neural stem/progenitor cells and the development of oligodendrocytes. In this study, authors reported that the lack of adrenomedullin in CNS resulted in reduced proportions of neurons and astrocytes, and in an immature phenotype of oligodendrocytes (254).

Mechanism of action of adrenomedullin

How does adrenomedullin regulate such a wide spectrum of mediators? The answer to this question could lie in the fact that adrenomedullin is the endogenous ligand of the calcitonin-related-like receptor (CRLR), which forms complexes with various receptor activity-modifying proteins (RAMP1, RAMP2 and RAMP3). The binding specificity for the ligand and the activity of the receptor depend on the RAMP subtype associated to CRLR (255). In contrast to CGRP, which specifically binds to the CRLR-RAMP1 complex, adrenomedullin binds preferentially to CRLR associated to RAMP2 and RAMP3 (256). Because CRLR-RAMP2/3 complexes are coupled to Gαs proteins, adrenomedullin signals through the elevation of cAMP and activation of protein kinase A, an intracellular pathway generally involved in the downregulation of inflammatory mediators, inhibition of Th1 responses, generation of Treg cells and induction of tolerogenic DCs (257, 258, 259). Noteworthy, macrophages, lymphocytes and DCs specifically express CRLR-RAMP2/3 complexes, which are differentially up-regulated during inflammatory processes (155), or during the differentiation and maturation of

DCs (197, Appendix I). According to the direct effect exerted by adrenomedullin on glial cells, we also demonstrated that microglia, astrocytes and oligodendrocytes also express adrenomedullin receptors (Appendix I). On the other hand, some controversy exists about the role of CGRP and its receptor in EAE development. Thus, Sardi et al. reported that CGRP deficiency increased severity of EAE and that CGRP administration in cerebrospinal fluid slightly reduced the clinical score (260). In contrast, Mikami et al. observed that CGRP increased Th17 responses, RAMP1-KO mice were completely resistant to EAE, and Th17 cell function and EAE induction were suppressed in T cell specific RAMP1-KO mice (261). Moreover, although CGRP decreased the activation of microglia, it did not alter GFAP expression, lymphocytic infiltration and peripheral production of inflammatory cytokines IFNγ, TNFα, IL-17 and IL-2 in EAE mice (260). These findings could explain the higher efficiency found for adrenomedullin in comparison to CGRP in the treatment of EAE. These evidences suggest that, although adrenomedullin is able to bind to CRLR-RAMP1 with low ability, the main effect of adrenomedullin controlling the encephalitogenic response driven by Th1 and Th17 is probably exerted by CRLR-RAMP2/3 receptors. However, this hypothesis needs to be confirmed by using selective CRLR-antagonists and/or RAMP-KO mice.

Another important issue is that, the local effect of adrenomedullin in CNS on resident and infiltrating cells implies its access to CNS parenchyma through BBB. This could be garantized not only by the existence of specific transporters for neuropeptides in the BBB, but also because a small molecule such adrenomedullin could easily cross the compromised BBB found in EAE mice and MS patients.

Role of endogenous adrenomedullin in EAE

In this study, we described that adrenomedullin is an endogenous immunomodulatory factor produced by astrocytes and microglia under inflammatory stimulation (Fig. 4.22). Other studies support these results. Thus, adrenomedullin is produced in response to inflammatory-related substances, cytokines and oxidative stress by immune and glial cells (202, 203, 204, 205, 197, 206). Additionally, adrenomedullin is increased in several diseases with an inflammatory and autoimmune component, including rheumatoid arthritis, systemic lupus erythematosus and

systemic sclerosis (262, 263, 264, 265). However, in the present study we found that adrenomedullin levels decreased in the CNS of EAE mice (Fig. 4.18), supporting the idea that that endogenous adrenomedullin could have an important role on EAE and MS. Because adrenomedullin deficiency is embryonic lethal due to severe prenatal vascular failures (267), cell- and tissue-conditional knockout mice are required to study the role of endogenous adrenomedullin. To note, a recently generated brain conditional knockout mouse demonstrated that mice lacking adrenomedullin are less resistant to hypoxia (166) and the role of adrenomedullin in the differentiation of adult neural stem/progenitor cells and the development of oligodendrocytes (254). Moreover, mice heterozygous for adrenomedullin exhibit a more extreme inflammatory response to endotoxin-induced septic shock (267). Finally, it was demonstrated that carriers of a single nucleotide polymorphism in the proximity of the adrenomedullin gene have lower levels of circulating peptide (147, 268). Therefore, it should be interesting to investigate whether this single nucleotide polymorphism and low circulating adrenomedullin correlates with the susceptibility to suffer the disease or with its severity in patients with MS or other autoimmune encephalomyelitis.

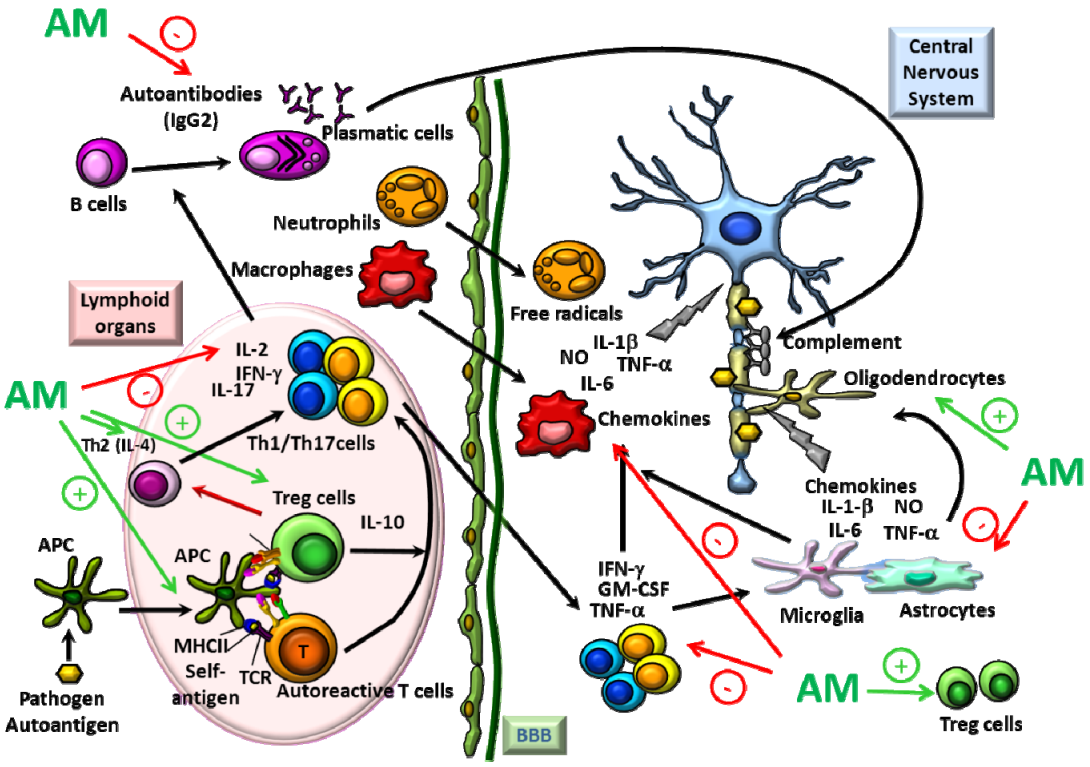

Figure 5.1: Conclusions I. Effect of adrenomedullin in EAE. A short-term systemic treatment with adrenomedullin reduced clinical severity and incidence of EAE, the appearance of inflammatory infiltrates in spinal cord and the subsequent demyelination and axonal damage. This effect was exerted at multiple levels affecting both early and late events of the disease. Adrenomedullin decreased the presence/activation of encephalitogenic Th1 and Th17 cells and down-regulated several inflammatory mediators in peripheral lymphoid organs and CNS. Adrenomedullin also decreased the levels of circulating complement-fixing autoantibodies, an effect probably mediated indirectly through its effect of Th1 cells. Noteworthy, adrenomedullin inhibited the production by encephalitogenic cells of osteopontin and GM-CSF, two critical cytokines in the development of EAE. At the same time, adrenomedullin increased the number of Th2 cells and IL-10-producing Treg cells with suppressive effects on the progression of EAE, and generated dendritic cells with a semi-mature phenotype that impaired encephalitogenic responses. Finally, adrenomedullin regulated inflammatory glial activity and favored an active program of oligodendrocyte protection and remyelinization. Red arrows: inhibitory effects; green arrows: stimulatory effects.

5.2. Parkinson´s disease and cortistatin

PD is one of the most complex disorders of the CNS characterized by the relationship between dopamine depletion due to the loss of dopaminergic (DA) neurons, neuroinflammation and reduction of growth factors. The contribution of neuroinflammation to the progression of PD is supported by evidence of accumulation of inflammatory mediators, activation of inflammatory pathways, and oxidative

damage to proteins in the cerebrospinal fluid and brains of PD patients as well as in experimental PD models (39, 113, 114, 115). Indeed, it has been suggested a mechanistic link between protein aggregation and enhanced neurodegeneration driven by neuroinflammation (105, 106, 107). Epidemiological studies have shown that the use of nonsteroidal anti-inflammatory drugs may reduce the risk of developing PD. Different compounds with anti-inflammatory actions are able to rescue nigral DA neurons *in vitro* and in animal models under a variety of neurotoxic insults (119). However, anti-inflammatory therapy in humans has revealed contradictory results probably due to the complexity of the human disease and the interactions of inflammatory pathways. Regarding the trophic factors, multiple studies *in vitro* and in animal models of PD indicate that most of them protect DA neurons from neurotoxins, although only few of them restore function in the dopaminergic system (269). However, trophic factors neither are given orally nor cross the BBB. Due to this limited delivery, the benefits of therapies including trophic factors in pre-clinical models have not yet replicated in human trials. Together, this multifactorial disorder requires a therapeutic intervention involving protection of the nigrostriatal pathway from death-inducing inflammatory insults and stimulation of endogenous production of neurotrophic factors to restore the function of DA neurons.

We recently characterized the immunomodulatory and neuroprotective effects of cortistatin, a cyclic-neuropeptide produced by brain cortex and immune cells. Cortistatin showed beneficial activities in cell-based systems and pre-clinical models of ischemia, glutamate-induced excitotoxicity, bacterial encephalitis, and MS (188, 270, 271, 189, 190). By using cortistatin-deficient mice, we found that lack of cortistatin caused exacerbated inflammatory responses and overeactivity of glial cells. Together, these results suggest a crucial role for endogenous cortistatin in modulating neurodegeneration. Therefore, we wondered if cortistatin could be a key endogenous factor that may give us the clue in the relationship between neuroinflammation and neurodegeneration in PD. In this study, we used the pre-clinical model of MPTP-induced neurotoxicity in wild-type and cortistatin-deficient mice to evaluate the influence of cortistatin in the susceptibility to develop PD. We evaluated some hallmarks of PD pathology as glial activation, dopaminergic neuron functions, trophic

factors and behavioral tests to detect motor deficits. We also performed cell culture experiments to identify the pathways in which cortistatin may be involved.

Protective effect of cortistatin in the MPTP-induced PD model

In this study, we demonstrated that systemic administration of cortistatin protects the nigrostriatal dopaminergic pathways from the parkinsonian toxicity of MPTP in mice by reducing the dopaminergic neuron death in SNpc and protecting from the loss dopaminergic nerve terminal in striatum (Figs. 4.24 and 4.25). Concurrent with the effect in the nigrostriatal pathway, cortistatin treatment attenuated the decrease in nigrostriatal TH, DAT and BDNF expression (Fig. 4.28) and improved motor disfuctions induced by MPTP.

Regarding the mechanism of action of cortistatin, one possibility is that cortistatin delays rather than prevents dopaminergic neuron death. However, the facts that cortistatin has exhibited neuroprotective effects in other experimental models (188, 189, 190,) and that cortistatin significantly inhibited MPP^+-induced dopaminergic cell loss in mesencephalic cultures and human neuroblastoma cells (Fig. 4.29), suggest that this neuropeptide may function as a direct neuroprotective factor for dopaminergic neurons.

On the other hand, we observed that the effect of cortistatin was accompanied by the inhibition of microgliosis and astrogliosis and concomitant release of glial-derived inflammatory mediators (Figs. 4.26, 4.27 and 4.30). After its systemic administration, MPTP crosses the BBB and is metabolized to MPP^+, the active toxic form of the molecule, by glial cells, and then released into the extracellular space (272). Due to its high-affinity for the DAT transporter, MPP+ is concentrated in dopaminergic neurons, within which it blocks the mitochondrial electron transport chain complex I, resulting in the enhanced production of ROS, decreased synthesis of ATP and subsequent cell death (272). Microglia and astrocytes can be rapidly activated in response to neuronal damage at an early progression stage of the MPTP-induced PD model, reaching a maximum 1 dpi and keeped on about a week (118, 273, 274). Similar to these findings, we found that MPTP increased gliosis in both the SNpc and striatum.

Glial activators, such as α-synuclein, released from dying or stressed dopaminergic neurons activate glial cells to produce pro-inflammatory and neurotoxic factors, which are toxic to surrounding neurons, fuelling a cycle of progressive dopaminergic neurodegeneration (39, 275). Therefore, the neuroprotective agents targeting microglial activation may be able to break this vicious cycle and modulate the course of neurodegenerative processes. In fact, several agents, including other neuropeptides, have been found to be neuroprotective due to their ability to suppress the activation of microglia in various PD models (ref: 276, 277, 278, 279). In this regard, we propose that the ability of cortistatin to prevent microglial activation in both the SNpc and striatum in the *in vivo* model and also after activation *in vitro* with α-synuclein plays an important role in its neuroprotective effect in this model (Figs. 4.26, 4.27, and 4.30). Furthermore, we have previously reported the inactivation of microglial cells by cortistatin in a progressive model of MS (177). Interestingly, while microglial cells found in SNpc of MPTP showed an activated phenotype, the few $Iba1^{+}$ cells found in cortistatin-treated mice showed a more resting phenotype similar to that found in brain parenchyma of healthy animals. The activated phenotype of microglia in MPTP model has been associated with an exacerbated phagocytosis of MPP+-damaged dopaminergic neurons, but also of viable neurons in a neuroinflammatory environment (280, 281), which might be harmful for the surrounding parenchyma. Therefore, modulation of microglia activation by cortistatin might be relevant to prevent glial-mediated inflammation and neuronal elimination. In fact, because microglial activation is aggressively initiated in early PD but remains stable for years (282, 283), it might be essential to consider treatments that block reactive microglia.

Together with neuroinflammation, oxidative stress is well recognized as a key pathophysiological event contributing to the progressive loss of nigral dopaminergic neurons in PD (284). In fact, oxidative stress contributes to accumulation of α-syn that increases the levels of cytoplasmic dopamine, which augments the oxidative stress resulting in a positive feedback loop (109). Here, we reported that cortistatin was able to decrease the production of ROS induced by α-syn monomers (Fig. 4.30) in microglia, suggesting that this neuropeptide could also exert neuroprotection in the MPTP model through its antioxidant properties.

Molecular targets of the neuroprotective effect of cortistatin

It has been reported that MPTP-induced neurodegeneration is correlated with selective alterations of gene expression in mice, mainly by regulating the expression of TH, DAT, α-syn, and neurotrophic factors (285). Our results showed that MPTP decreased TH gene expression, the rate-limiting enzyme for dopamine synthesis, while cortistatin treatment partially avoided this loss at 2 dpi (Fig. 4.28). Interestingly, no changes were found in TH expression at 7 dpi in both untreated and cortistatin-treated MPTP mice. It should be noted that MPTP could target TH at either the gene expression or protein level (285). If MPTP damages TH at the protein level, the gene expression of TH should increase to compensate this loss. However, if MPTP targets TH gene expression, the direction of the alterations of TH gene and protein expression should be the same. Thus, our data indicates that MPTP targets TH protein expression, as we observed a decrease in TH immunodetection in the nigrostriatal pathway of MPTP mice compared with cortistatin-treated mice at 7 dpi. As previously indicated, MPP^+ penetrates in dopaminergic neurons through DAT. In this study, we showed that mRNA expression of DAT was downregulated at 2 dpi in the striatum of cortistatin-treated MPTP mice (Fig. 4.28), suggesting a protective role of this neuropeptide by preventing the MPP^+ input into dopaminergic neurons. However, while DAT expression was greatly reduced at 7 dpi in untreated MPTP mice (as correlated with dopaminergic cell loss), the recovery of DAT levels in cortistatin-treated MPTP mice may represent either an upregulation of DAT in the remaining dopaminergic terminals or a process of reinnervation by the surviving dopaminergic neurons. On the other hand, the upregulation of DAT at 7 dpi in cortistatin-treated mice could be also related to the fact that the molecule MPTP has been excreted in the urine at this time point, being not detected in the mice (286), and the toxic metabolite MPP^+ is not present anymore, which reduces the pressure over the modulation of the DAT expression. Protective effects in animal models of PD have been obtained with various molecules including glial cell line-derived neurotrophic factor, neurturin, BDNF, and basic FGF (287, 288). Regarding BDNF, it is strongly expressed by dopaminergic neurons in the SNpc and is reduced by 70% in PD patients, in part, due to loss of dopaminergic neurons that

express BDNF (289). Thus, clinical application of BDNF would be desirable but it has been limited because of difficulties in delivery and side effects. This peptide does not readily diffuse across the BBB or ventricular lining and has limited or unstable bioavailability and some toxicity (290). According to this, the loss of dopaminergic neurons in the SNpc observed in MPTP mice at 7 dpi correlates with the reduction of BDNF expression in the striatum and with the fact that cortistatin increased its levels (Fig. 4.28). To note, we have previously reported the *in vivo* up-regulation of BDNF by cortistatin in a progressive model of MS (177). Regarding the role of α-syn, although its pathological relevance is not fully clear, aggregates of this protein have been observed in PD patients and mutations in α-syn is linked to familial PD. Thus, it is important to evaluate the expression of this gene following MPTP treatment. In this study, we have only detected slight increases in α-synuclein expression in SNpc of mice intoxicated with MPTP at 2 dpi. There are discrepancies about the induction of α-syn expression by MPTP, with different authors reporting down-regulation (285) and up-regulation (291) of this gene. These differences can be due to difference of the ages of aminals, and doses regimens. Althought PD patients show aggregation of α-syn, they do not show changes at its mRNA level; thus, the mRNA of level α-syn might not be a good marker for MPTP neurotoxicity (285).

Molecular pathways involved in the neuroprotective effect of cortistatin

Cortistatin can exert its effect through somatostatin receptors (SSTR1-5) and ghrelin receptor (GHSR1a). It has been reported the expression of SSTR1-4 in midbrain and striatum of mice (292). Moreover, GHSR1a expression has been described in MES35.5, a cell line of dopaminergic neurons (293), and in dopaminergic neurons in the SNpc (294). Interestingly, the neuropeptide ghrelin, acting through GHSR1a, also showed a therapeutic effect in MPTP mice (294). These evidences suggest that the protective effect of cortistatin observed in our study may be exerted through the same receptor. Anyway, more experiments are needed in order to discard the action of other cortistatin receptors. We have previously observed that astrocytes and microglia express somatostatin receptors but not GHSR1a (anexo), indirectly supporting that the effect of cortistatin on glial cells should be mediated through SSTR.

Role of endogenous cortistatin in PD

Which could be the role of endogenous cortistatin in the context of this complex disorder? Evidence indicates that cortistatin is an endogenous immunomodulator: a) immune cells produce cortistatin in response to inflammatory/immune stimulation (295); b) cortistatin produced by macrophages and lymphocytes plays an autocrine/paracrine regulatory role in the immune response, as lack of cortistatin predisposes to stronger response to immunostimulation (177); c) an inverse correlation exists between EAE severity and the levels of cortistatin in the CNS (177); and a deficiency of cortistatin in the retina of diabetic patients with retinopathy correlated with increased retinal neurodegeneration and glial activation (190). In addition, expression of cortistatin has been described in the basal ganglia (296), where striatum and SNpc are located. Moreover, we have observed that cortistatin is expressed in the mesencephalon of embryonic mice and that this expression is upregulated after MPP+ incubation (Appendix II). These findings suggest that endogenous cortistatin might normally provide protection against neurodegenerative and neuroinflammatory pathologies. In this study, we report that mice deficient in cortistatin showed a higher and faster mortality than wild-type mice exposed to MPTP (Fig. 4.31), suggesting a main role of this neuropeptide in the initiation of the disease. Previously, we demonstrated that the lack of cortistatin delayed the onset and progression of the disease in the EAE model (177). This unexpected phenotype was associated with elevated circulating glucocorticoids that are well-known immunosuppressive factors with widespread actions in different components of the immune system (176). However, cortistatin deficient mice were more susceptible to suffer diseases characterized by a local inflammatory response such as cutaneous leishmaniasis or monoarticular inflammation. The differential effect exerted by cortistatin observed in the EAE and MPTP models can be due to the fact that progression of EAE depends on a coordinated systemic immune response, while the neurotoxin produces a more localized neuroinflammation. In this sense, the absence of

cortistatin could influence to exacerbate this inflammatory response leading to a drastic damage in the target tissue.

Finally, we are currently focused on investigating the endogenous role of cortistatin in the MPTP animal model. We are investigating the differential susceptibility of the nigrostriatal dopaminergic pathway to MPTP in the absence of cortistatin and the effect of MPP^+ on the *in vitro* mesencephalic dopaminergic neurons isolated for cortistatin deficient mice. A previous study from our group demonstrated that the absence of this neuropeptide in nociceptive neurons exacerbated pain responses to inflammatory stimuli (297), suggesting that endogenous cortistatin play an important role in the function of some neurons. In addition, taking into account that glial cells from mice lacking cortistatin showed an exacerbated immune response in an inflammatory context (unpublished preliminary data), we will further examine the MPTP sensing of the glial niche in the absence of this neuropeptide and the relevance for the disease.

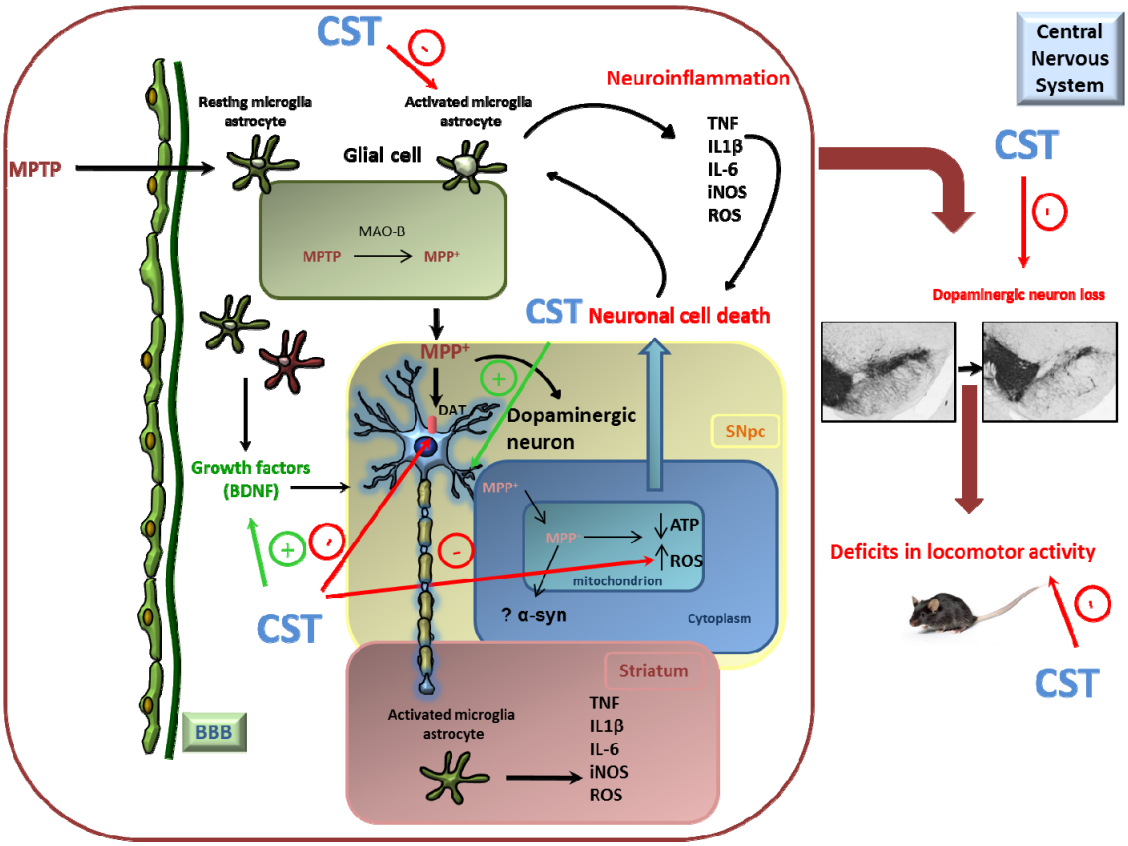

Figure 5.2: Conclusions II. Effect of cortistatin in Parkinson's disease. The treatment with cortistatin reduced the loss of dopaminergic neurons in the SNpc and their projections to the striatum caused by MPTP injection. This effect correlated with an improvement in the locomotor activity. Furthermore, in an in vitro model of PD, cortistatin decreased cell death of dopaminergic neurons caused by MPP+. Moreover, cortistatin diminished the presence and activation of microglia cells and astrocytes in the SNpc of MPTP-mice, suggesting a role of this neuropeptide in the regulation of neuroinflammation associated to this disorder. Furthermore, cortistatin induced the expression of neurotrophic factors and regulated the expression of DAT. We also observed that cortistatin modulated the immune response of glial cells in the presence of α-synuclein, as extracellular stimuli.

6. CONCLUSIONS

1. A short-term systemic treatment with adrenomedullin reduces clinical severity and incidence of chronic experimental autoimmune encephalomyelitis (EAE). The effect of adrenomedullin is exerted at multiple levels affecting both early and late events of the disease.

    a. Adrenomedullin reduces the appearance of inflammatory infiltrates in the spinal cord and the subsequent demyelination and axonal damage.

    b. Adrenomedullin down-regulates the production of several inflammatory mediators in peripheral lymphoid organs and central nervous system.

    c. Adrenomedullin decreases the presence/activation of encephalitogenic Th1 and Th17 cells in the periphery and in the central nervous system without affecting the polyclonal immune response.

2. The treatment with adrenomedullin generates a long lasting protective effect in EAE even when the peptide is administered in animals with fully established disease. Adrenomedullin induces immune tolerance in EAE mice, by increasing the number of IL-10-producing regulatory T cells and generating dendritic cells with a tolerogenic phenotype.

3. The treatment with adrenomedullin favoured an active program of neuroprotection and remyelination modulating the activity of the glial niche.

    a. Adrenomedullin promotes the expression of neurotrophic factors in EAE mice.

    b. Adrenomedullin inhibits the production of pro-inflammatory mediators released by the glial niche and increases the survival of oligodendrocytes exposed to oxidative stress.

c. Treatment with adrenomedullin protects from the demyelination induced by the toxin lysolecithin by increasing the number of remyelinating oligodendrocyte precursor cells in the lesioned area.

4. Cortistatin has a therapeutic effect in a murine model of Parkinson's disease induced by the neurotoxin MPTP.

   a. Systemic administration of cortistatin protects from dopaminergic loss in the striatum and substantia nigra, and rescues from locomotor deficits caused by MPTP.

   b. Treatment with cortistatin regulates the expression of relevant factors in the pathogenesis of Parkinson's disease, such as brain-derived neurotrophic factor.

   c. Cortistatin regulates neuroinflammation associated to MPTP-induced parkinsonism by reducing the activation of microglia and astrocytes in vivo.

   d. Cortistatin directly protects dopaminergic neurons from cell death induced by MPP+ neurotoxin and modulates the inflammatory phenotype of glial cells activated by alpha-synuclein monomers.

7. CONCLUSIONES

1. El tratamiento sistémico con adrenomedulina durante un breve periodo de tiempo reduce la severidad y la incidencia de la forma crónica de encefalomielitis autoinmune experimental (EAE). Adrenomedulina actúa a varios niveles, afectando a eventos tempranos y tardíos de la enfermedad.

   a. Adrenomedulina reduce la aparición de infiltrados inflamatorios en la médula espinal, afectando la subsiguiente desmielinización y daño axonal.

   b. Adrenomedulina reduce la producción de diferentes mediadores inflamatorios en órganos linfoides secundarios y en el sistema nervioso central.

   c. Adrenomedulina disminuye la presencia y activación de células Th1 y Th17 encefalitogénicas en la periferia y en el sistema nervioso central, sin afectar a la respuesta inmunitaria policlonal.

2. El tratamiento con adrenomedulina genera un efecto protector en EAE que se mantiene en el tiempo incluso cuando el tratamiento es administrado en animales con la enfermedad plenamente establecida. Adrenomedulina induce tolerancia inmunológica en ratones con EAE incrementando el número de células T reguladoras secretoras de IL-10 y generando células dendríticas con un fenotipo tolerogénico.

3. El tratamiento con adrenomedulina favorece un programa de neuroprotección y remielinización modulando la actividad del nicho glial.

   a. Adrenomedulina promueve la expresión de factores neurotróficos en ratones con EAE.

   b. Adrenomedulina inhibe la producción de mediadores pro-inflamatorios secretados por las células gliales e incrementa la supervivencia de oligodendrocitos expuestos a estrés oxidativo.

c. El tratamiento con adrenomedulina protege de la desmielinización focal inducida por la toxina lisolecitina incrementando el número de oligodendrocitos en la zona lesionada.

4. Cortistatina tiene un efecto terapéutico en el modelo experimental de enfermedad de Parkinson inducido por MPTP.

   a. La administración sistémica de cortistatina protege de la pérdida de neuronas dopaminérgicas en la sustancia negra pars compacta y de sus proyecciones en el estriado, mejorando los déficits locomotores causados por MPTP.

   b. El tratamiento con cortistatina regula la expresión de factores relevantes en la patogénesis de la enfermedad de Parkinson, como BDNF.

   c. Cortistatina modula la neuroinflamación asociada al modelo de MPTP, reduciendo la activación de microglía y astrocitos.

   d. Cortistatina protege directamente de la muerte de neuronas dopaminérgicas inducida por la neurotoxina MPP^+ y modula el fenotipo inflamatorio de células gliales activadas con monómeros de α-sinucleína.

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Adrenomedullin protects from experimental autoimmune encephalomyelitis at multiple levels

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ARTICLE INFO

Article history:
Received 6 September 2013
Received in revised form 22 November 2013
Accepted 29 November 2013
Available online 7 December 2013

Keywords:
Autoimmunity
Regulatory T cells
Dendritic cells
Glial cells
Neuropeptide
Adrenomedullin
Multiple sclerosis

ABSTRACT

Adrenomedullin is a neuropeptide known for its cardiovascular activities and anti-inflammatory effects. Here, we investigated the effect of adrenomedullin in a model of experimental autoimmune encephalomyelitis (EAE) that mirrors chronic progressive multiple sclerosis. A short-term systemic treatment with adrenomedullin reduced clinical severity and incidence of EAE, the appearance of inflammatory infiltrates in spinal cord and the subsequent demyelination and axonal damage. This effect was exerted at multiple levels affecting both early and late events of the disease. Adrenomedullin decreased the presence/activation of encephalitogenic Th1 and Th17 cells and down-regulated several inflammatory mediators in peripheral lymphoid organs and central nervous system. Noteworthy, adrenomedullin inhibited the production by encephalitogenic cells of osteopontin and of Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), two critical cytokines in the development of EAE. At the same time, adrenomedullin increased the number of IL-10-producing regulatory T cells with suppressive effects on the progression of EAE. Furthermore, adrenomedullin generated dendritic cells with a semi-mature phenotype that impaired encephalitogenic responses *in vitro* and *in vivo*. Finally, adrenomedullin regulated glial activity and favored an active program of neuroprotection/regeneration. Therefore, the use of adrenomedullin emerges as a novel multimodal therapeutic approach to treat chronic progressive multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is a disabling demyelinating disease of the central nervous system (CNS) affecting more frequently young people. Although the mechanisms of disease pathogenesis remain unclear, MS is considered as an archetypal neuroinflammatory and autoimmune disease in which Th1 and Th17 CD4 cells reactive to components of the myelin sheath infiltrate the CNS parenchyma, release proinflammatory cytokines and chemokines, and further promote inflammatory cell infiltration and activation (Goverman, 2009; Steinman, 1996). The production of inflammatory mediators such as cytokines and free radicals by infiltrating immune cells and resident glial cells plays a critical destructive role in the process of demyelination and contributes to oligodendrocyte loss and axonal degeneration. Moreover, evidence indicates that a deregulation in the mechanisms involved in the maintenance of immune tolerance, especially those affecting regulatory T (Treg) cells, critically contributes to the establishment and progression of the autoimmune response (Viglietta et al., 2004). Although available therapies based on immunosuppressive agents reduce the relapse rate or delay disease onset, they do not suppress progressive clinical disability. Therefore, we need novel multistep therapeutic approaches that prevent the inflammatory and autoimmune components of the disease, restore immune tolerance and promote and/or allow mechanisms of neuroprotection and neuroregeneration.

Adrenomedullin is a 52-aa neuropeptide belonging to the family of calcitonin-gene related peptide (CGRP) that was initially discovered through its vasodilatory activity and cardiovascular protective actions (Kitamura et al., 1993). Recent evidence indicates that adrenomedullin is a major regulator of the immune response. Besides its presence in the CNS and cardiovascular system (Hinson et al., 2000), adrenomedullin and its receptors are expressed by macrophages, monocytes, T cells and dendritic cells (DCs), mainly in response to inflammatory and immune stimulation (Elsasser and Kahl, 2002; Ishimitsu et al., 1998; Kubo et al., 1998a,b; Rullé et al., 2012; Yang et al., 2001). Moreover, adrenomedullin shows potent

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http://dx.doi.org/10.1016/j.bbi.2013.11.021

anti-inflammatory activity by downregulating the production of a wide panel of inflammatory mediators by activated macrophages, microglia and DCs (Consonni et al., 2011; Gonzalez-Rey et al., 2006a, 2007a; Rullé et al., 2012; Wong et al., 2005; Wu et al., 2003). In addition, adrenomedullin is able to inhibit antigen-specific Th1 responses and increase the number of Treg cells *in vivo* (Gonzalez-Rey et al., 2006b, 2007a). Several studies demonstrated that treatment with adrenomedullin protected from experimental sepsis, inflammatory bowel disease and arthritis (Cui et al., 2005; Gonzalez-Rey et al., 2006a,b, 2007a; Koo et al., 2001; Okura et al., 2008; Zudaire et al., 2006). In view of these findings, the aim of this study was to investigate the potential therapeutic effect of adrenomedullin in an animal model of experimental autoimmune encephalomyelitis (EAE) that mimics chronic progressive MS, characterized by the worst clinical prognosis and lack of effective treatment (Steinman, 1999).

2. Methods

2.1. Induction and treatment of experimental autoimmune encephalomyelitis (EAE)

To induce chronic EAE, female C57BL/6 mice (8 weeks old, Charles River) were immunized subcutaneously (s.c). with 200 μg of myelin oligodendrocyte protein (MOG_{35-55}, MEVGWYR SPFSRVVHLYRNGK, GeneScript) emulsified in complete Freunds adjuvant (CFA) containing 400 μg of *Mycobacterium tuberculosis* H37 RA (Difco). Mice also received intraperitoneal (i.p). injections of 200 ng of pertussis toxin (Sigma) on days 0 and 2. Treatment consisted in the i.p. injection of adrenomedullin (1 nmol/day, American Peptides) or Phosphate buffered saline (PBS, controls) for 5 consecutive days after disease onset in animals with a clinical score of 0.5–1 (onset) or with a clinical score of 1–1.5 or >2 (acute phase). Mice were scored daily for signs of EAE according to the following clinical scoring system (Miller et al., 2010): 0, no clinical signs; 0.5, partial loss of tail tonicity; 1, complete loss of tail tonicity; 2, flaccid tail and abnormal gait; 3, hind leg paralysis; 4, hind leg paralysis with hind body paresis; 5, hind and fore leg paralysis; and 6, death. All experiments with animals were performed in accordance the European ethical guidelines and approved by the Animal Care Unit Committee IPBLN-CSIC (# protocol SAF2010-16923).

2.2. Tissue collection and cell isolation

Spleen, draining lymph nodes (DLNs: cervicals, inguinals and axillaries), brain and spinal cord were removed at various timepoints from mice with EAE that were treated with PBS or with adrenomedullin for 5 consecutive days after the onset of disease (with a clinical score between 1 and 2). Single-cell suspensions were obtained from spleen or pooled DLNs and used for flow cytometry analysis, determination of autoreactive responses and adoptive transfer of EAE as described below. Brain and spinal segments of the cervical and lumbar regions were prepared separately and used for RNA isolation, protein extraction and histopathological analysis as described below. Brain and spinal cord mononuclear cells were isolated by enzymatic tissue digestion and gradient centrifugation as previously described (Kong et al., 2011) and used for flow cytometry analysis and determination of autoreactive responses as described below. Proteins were extracted from cervical and lumbar segments of spinal cord and brain by homogenization (50 mg tissue/ml) in lysis buffer (50 mM Tris–HCl, pH 7.4, 0.5 mM Dithiothreitol and 10 μg/ml of protease inhibitors phenylmethylsulfonyl fluoride, pepstatin and leupeptin). Samples were centrifuged (20,000*g*, 15 min, 4 °C) and the supernatants were assayed for cytokine contents using sandwich ELISA following manufacturer's recommendations (BD Bioscience and Peprotech), and for adrenomedullin levels using a competitive ELISA (Phoenix Pharmaceuticals).

2.3. Histopathological analysis of EAE

For light microscopy, cervical and lumbar spinal cord segments were fixed in buffered 10% formalin for 48 h and processed for paraffin inclusion and sectioning. Transversal sections (4-μm thickness) were stained with luxol fast blue, cresyl violet and hematoxylin following the technique described by Kluver and Barrera (1953) and analyzed for the presence of areas of demyelination and cell infiltration using a light microscope (Olympus).

For immunofluorescence staining, cervical and lumbar spinal cord segments were fixed in 4% paraformaldehyde pH 7.4 for 4–8 h at 4 °C, equilibrated in 30% sucrose for 24 h, and embedded in OCT. Transversal cryosections (10-μm thickness) were blocked with 10% goat serum in PBS-T (PBS + 0.2% Triton X-100) for 30 min at 22 °C, incubated with FITC-labeled anti-CD4 mAb (2.5 μg/ml, BD Bioscience), PE-labeled anti-CD45 mAb (1 μg/ml, BD Bioscience) or anti-Iba1 Ab (1 μg/ml, Wako) for 18 h at 4 °C, followed by incubation with Alexa Fluor 546-labeled anti-rabbit Ab (2 μg/ml, Invitrogen). Nuclear staining was performed with Hoechst (Sigma). Between steps, samples were extensively washed with PBS-T. Samples were observed in a fluorescence microscope (Olympus IX81).

For immunohistochemistry, spinal cord sections were obtained as described for paraffin processing followed by incubation steps with peroxidase blocking reagents, heat-treated in 1 mM EDTA pH 8.0 at 95 °C during 20 min for antigenic unmasking, and incubated for 30 min at room temperature with polyclonal anti-Myelin Basic Protein Ab (Ab980, Millipore). The immunohistochemical study was done on an Autostainer480 (Thermo Fisher Scientific Inc) using the polymer-peroxidase-based method and developed with diaminobenzidine. We used a non-specific peroxidase-conjugated IgG anti-rabbit serum as a negative control of isotype. Nuclei were hematoxylin-counterstained.

2.4. Flow cytometry analysis

For FoxP3 staining, spleen cells, DLN cells and brain/spinal cord mononuclear cells were isolated from C57Bl/6 mice with EAE at the peak of the disease and incubated with FITC-labeled anti-CD25 and APC-labeled anti-CD4 mAbs (4–5 μg/ml, BD Bioscience) for 8 h at 4 °C. After extensive washing, cells were fixed/permeabilized (eBioscience), stained with PE-labeled anti-FoxP3 Abs (4–5 μg/ml, eBioscience) for 30 min at 4 °C and analyzed in a FACScalibur flow cytometer (BD Bioscience). We used isotype-matched Abs as controls, and Mouse BD Fc Block (BD Bioscience) to avoid nonspecific binding to Fc-receptors.

For intracellular analysis of cytokines, brain/spinal cord mononuclear cells, spleen cells and DLN cells were isolated at the peak of the disease and stimulated at 10^6 cells/ml with Phorbol-12-myristate-13-acetate (PMA, 25 ng/ml) plus ionomycin (500 ng/ml) for 8–12 h, in the presence of 3 μM monensin for the last 6 h. Cells were stained with APC-anti-CD4 mAb (4 μg/ml) for 1 h at 4 °C, fixed/saponin permeabilized with Cytofix/Cytoperm (BD Bioscience), stained with FITC- and PE-conjugated anti-cytokine specific mAbs (4 μg/ml, BD Pharmingen) for 30 min at 4 °C, and analyzed in a FACScalibur flow cytometer.

2.5. Determination of autoreactive response

Brain/spinal cord mononuclear cells and spleen and DLN cells (10^6/ml) recovered from the C57Bl/6 mice at peak of clinical EAE

(18–20 days postimmunization) were stimulated in complete medium (RPMI 1640 containing 10% Fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) with 15 μM MOG_{35-55}. In brain/spinal cord cell cultures, plastic-adherent C57Bl/6 splenocytes (2×10^5/ml) were added to cultures as antigen presenting cells. Cell proliferation was evaluated after 72 h by adding 2.5 μCi/ml [^{3}H]-TdR during the last 8 h of culture and determining cpm incorporation in a Microbeta counter 1450. After 48 h, cytokine and chemokine contents in culture supernatants were determined by sandwich ELISAs. Concanavalin A (Con A, 2.5 μg/ml) was employed as control for non-specific polyclonal stimulation. To investigate the direct effect of adrenomedullin on autoreactive responses, the peptide (100 nM) was added to cultures of brain/spinal cord mononuclear and spleen/DLN cells isolated from untreated EAE mice and stimulated with 15 μM MOG_{35-55}.

To assay the capacity to transfer immune tolerance, spleen and DLN cells recovered from untreated and adrenomedullin-treated C57Bl/6 mice at the peak of disease (18 days postimmunization) were T-cell enriched by plastic adherence (2 h, 37 °C) and the non-adherent cells were injected i.p. (10^7 cells/mouse) into EAE mice (clinical score 0.5–1). Where indicated, isolated CD4 cells were depleted of CD25 population (>99% depletion) before transfer, by using immunomagnetic beads (Miltenyi Biotech) following the manufacturer's recommendations and injected i.p. (15×10^6 cells/mouse) into EAE mice at disease onset.

2.6. Determination of autoantibodies

We used ELISA to determine the specific anti-MOG Ab responses. Maxisorb plates (Millipore) were coated overnight at 4 °C with MOG_{35-55} (10 μg/ml) in 0.1 M biphosphate buffer (pH 9.6), blocked with PBS/10% FBS and incubated for 2 h at 37 °C with serial dilutions of sera obtained at the disease peak by cardiac puncture. Biotinylated anti-IgG1 or anti-IgG2a Abs (2.5 μg/ml) (Serotec) were added for 1 h at 37 °C. After washing, the plates were incubated with Streptavidin–Horseradish Peroxidase (HRP), developed with ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] and the absorbance was determined using a spectrophotometer.

2.7. DC isolation and treatments

DCs were differentiated from bone marrow cells obtained from femurs and tibiae of C57Bl/6 mice. Bone marrow cells (2×10^6) were incubated in Petri dishes in complete medium containing 20 ng/ml of rmGM-CSF (PreproTech). At day 6 of culture, non-adherent cells were collected (routinely containing 80–90% $CD11c^+$ cells) and stimulated with bacterial lipopolysaccharide (LPS, 1 μg/ml) and pulsed with MOG_{35-55} (20 μg/ml) in the absence (DCs-control) or presence (DCs-adrenomedullin) of adrenomedullin (100 nM). After 48 h of culture, flow cytometry analysis for CD40, CD80 and CD86 was performed as described previously (Chorny et al., 2005) and the content of cytokines in the culture supernatants was determined by ELISA. After 24 h of culture, MOG-pulsed DCs-control or DCs-adrenomedullin (2×10^5/ml) were added to DLN cells (10^6/ml) isolated from EAE mice at peak of disease and restimulated with MOG_{35-55} (15 μM) or Concanavalin A (2.5 μg/ml) to determine the proliferative response and production of cytokines as above. Alternatively, MOG-pulsed DCs-control or DCs-adrenomedullin (2×10^6/mouse) were injected i.p. in mice with EAE at disease onset and the progression of the disease was evaluated as described above.

2.8. Neuron and glial cell isolation and culture

Primary mixed neuron-glia, microglia, astrocytes and oligodendrocytes were obtained from brains of newborns (postnatal days 1–3, P1–P3) of naïve C57Bl/6 mice as previously described (Souza-Moreira et al., 2013; see also Supplementary data). Cell cultures were used 10–14 days after plating. When indicated, cytokine contents were determined by ELISA in supernatants and nitric oxide production was determined by measuring oxidized nitrite amounts in culture supernatants by using the Griess reagent (Gonzalez-Rey et al., 2006a). Oligodendrocyte cell death caused by oxidative stress was assayed by the MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method after 24 h of culture.

2.9. Determination of gene expression of neurotrophic factors by real-time PCR

Total RNA was isolated using Tripure (Roche) from spinal cord segments. After DNase I treatment, RNA (1 μg/sample) was reverse transcribed using RevertAid First Strand cDNA Synthesis kit (Fermentas) and random hexamer primers. cDNA was used for quantifying gene expression of activity-dependent neuroprotective protein (ADNP) and brain-derived neurotrophic factor (BDNF) by real-time quantitative RT-PCR (60 °C as annealing temperature) by using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. We used beta-actin for normalization and estimated fold change expression with Delta-Delta Ct method.

2.10. Statistical analysis

All data are expressed as the mean ± SEM. In all *in vivo* animal studies, we calculated that we required a minimum of 8–12 mice per group in order to have 80% power of detecting approximately a 30% change, assuming a standard deviation of 30% at a significance level of $\alpha = 0.05$. It has been our experience that this is sufficient to detect clinically and statistically significant differences with repetitive studies. As clinical scores to assess EAE are non-linear we used non-parametric statistical analysis. These include Mann–Whitney U test or the Kruskal–Wallis test when comparing more than two groups. We use these tests for detecting statistical differences between the individual EAE scores on a specific day for the adrenomedullin-treated and untreated EAE mice. In addition, their scores over each day of the experiment were compared by two-way ANOVA or one-way ANOVA (for more than two groups) with appropriate post tests. The rest of the experiments were statistically analyzed by unpaired *t*-test and by the non-parametric Mann–Whitney test and we evaluate if the normalizing transformations were effective by comparing results between parametric and non-parametric analysis. We used Mann–Whitney test when we found different results. We considered significance at $p < 0.05$. All statistical analyses were performed using the Graph-Pad Prism software.

3. Results

3.1. Treatment with adrenomedullin reduces severity and incidence of chronic EAE

We investigated the effect of the treatment with adrenomedullin in a model of chronic progressive EAE induced by MOG_{35-55} in C57BL/6 mice (Baxter, 2007). Without treatment, these mice developed moderate to severe clinical symptoms without subsequent recovery (Fig. 1). Systemic administration of adrenomedullin after the onset (Fig. 1A) or during the effector phase of the disease (Fig. 1B and C), greatly reduced disease incidence and severity

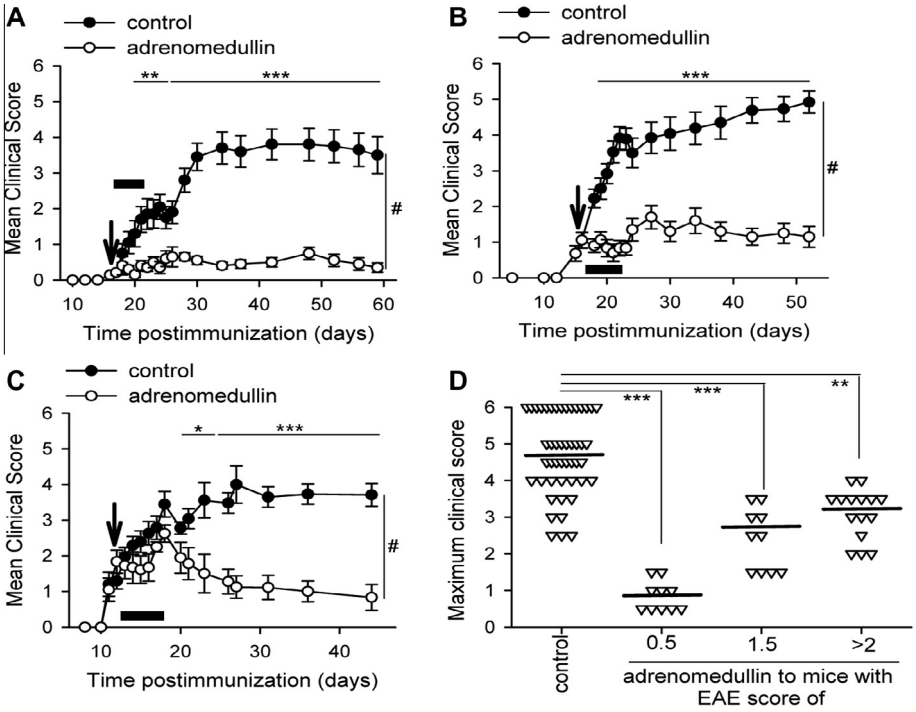

Fig. 1. Treatment with adrenomedullin reduces severity of chronic EAE. Chronic progressive EAE was induced in C57BL/6 mice by immunization with MOG_{35-55}. Mice were treated i.p. with PBS (control) or adrenomedullin (1 nmol/day, black bars) for 5 consecutive days at disease onset (A, arrow indicated adrenomedullin administration started at clinical score <1) or at two points during the acute phase in the therapeutic regimen (B, arrow indicated adrenomedullin administration started at clinical score 1–2; C, at clinical score >2). Data represent the mean clinical score ± SEM (A–C) or maximum peak of disease per animal (D, mean: horizontal line). $n = 10$ mice/group in A; $n = 13$ mice/control group and 10 mice/adrenomedullin group in B; $n = 24$ mice/control group and 15 mice/adrenomedullin group in C; all performed in three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control with Mann–Whitney test (A–C) and Kruskal Wallis Test (D). $^{\#}$ $p < 0.001$ with ANOVA t-test.

(Fig. 1; Table 1). It is remarkable that most of the adrenomedullin-treated EAE mice displayed mild symptoms and a significant number recovered completely and were entirely asymptomatic 30–40 days after disease onset (Table 1). Interestingly, a short treatment with adrenomedullin was enough to generate a long lasting protective effect (Fig. 1).

3.2. Adrenomedullin reduces the inflammatory infiltration and demyelination in the CNS of mice with EAE

We next investigated the mechanisms underlying the beneficial effects of adrenomedullin during the effector phase of EAE in mice with established clinical signs (scores between 1 and 2). The pathology of MS and EAE is characterized by focal areas of inflammatory infiltration and demyelination with oligodendrocyte depletion (Baxter, 2007; Steinman, 1996). Histopathologic analysis of spinal cords in both lumbar and cervical regions showed that treatment with adrenomedullin drastically decreased the number of plaques with inflammatory infiltrates and areas of demyelination (Fig. 2A). Immunofluorescence evaluation of CNS infiltrates in animals with EAE revealed that the inflammatory cells ($CD45^+$) close to the perivascular area were mostly $CD4^+$ cells and $Iba1^+$ macrophages (Fig. 2B). Adrenomedullin decreased the numbers of $CD45^+$, $CD4^+$, and $Iba1^+$ cells (Fig. 2B).

Table 1
Effect of adrenomedullin in chronic progressive EAE.

Timing of adrenomedullin administration in EAE	Incidence[a]				Mortality	CDI[b]
	None (%)	Mild (%)	Moderate (%)	Severe (%)		
1. Disease onset						
Control ($n = 10$)	0	0	70	30	20	76.2 ± 9.6
Adrenomedullin ($n = 10$)	30	70	0	0	0	17.6 ± 1.7***
2. Early acute phase						
Control ($n = 13$)	0	0	54	46	46	103.6 ± 7.8
Adrenomedullin ($n = 10$)	10	80	10	0	0	33.1 ± 4.5***
3. Advanced acute phase						
Control ($n = 24$)	0	0	54	46	29	82.5 ± 7.1
Adrenomedullin ($n = 15$)	13	86	0	0	0	41.2 ± 4.1**

Chronic progressive EAE was induced in C57BL/6 mice by immunization with MOG_{35-55}. Immunized mice were treated i.p. for 5 days with PBS (control) or with adrenomedullin (1 nmol/day) starting at the onset of clinical signs (with a clinical score of 0.5) or at two points during the acute phase of the disease (early acute phase with a clinical score of 1–2; or advanced acute phase with a clinical score >2). The number of animals per group used in each condition is shown in parenthesis. Data represent results from three independent experiments.

[a] Disease incidence (expressed as percentage) is graded as severe (clinical score: >4), moderate (clinical score: 2–4), mild (clinical score: <2) or none (no clinical signs) at day 36.

[b] Cumulative Disease Index (CDI) is the mean of the sum of the daily disease scores for each animal between days 20 and 44 postimmunization.

** $p < 0.005$ vs. control.

*** $p < 0.0001$ vs. control.

The reduction of CNS infiltrating cells in the adrenomedullin-treated EAE mice correlated with the decrease in the amounts of inflammation-related cytokines and chemokines, including IL-6, IL-17, IFNγ, CCL5, CCL2 and CXCL10 in spinal cords (Fig. 3). Moreover, treatment with adrenomedullin reduced the contents of GM-CSF and osteopontin in the CNS of EAE mice (Fig. 3), two inflammatory markers that play a major role in the induction and progression of EAE and MS (Codarri et al., 2011; Chabas et al., 2001; Jansson et al., 2002; Murugaiyan et al., 2008, 2010).

3.3. Adrenomedullin modulates the autoimmune component of EAE in the periphery

In EAE, autoreactive IFNγ-producing Th1 cells and IL-17-secreting Th17 cells infiltrate CNS and promote disease progression (Hu and Ivashkiv, 2009; Jager et al., 2009; Stromnes et al., 2008). Therefore, we investigated whether adrenomedullin could ameliorate EAE by reducing encephalitogenic T-cell responses and/or their migration to CNS. Lymphocytes from DLNs and spleen of EAE mice showed marked MOG-dependent proliferation and production of IFNγ, IL-2 and IL-17 (Fig. 4A). However, T cells from adrenomedullin-treated mice proliferated much less and did not produce Th1 and Th17 cytokines in the MOG-specific recall response (Fig. 4A). Interestingly, adrenomedullin treatment also reduced the capacity of spleen cells to produce GM-CSF and osteopontin upon MOG-restimulation (Fig. 4A). The effect of adrenomedullin was mostly antigen specific since polyclonal T cell activation with Concanavalin A resulted in similar proliferation and cytokine secretion (except for IFNγ) in both groups (Fig. 4A). Moreover, adrenomedullin treatment reduced the number of effector T cells secreting IFNγ, but not IL-17 in DLNs and spleen, and at the same time elevated the percentage of IL-4- and IL-10-expressing CD4 cells (Fig. 4B). These results indicate that adrenomedullin administration during the effector phase of EAE partially inhibits autoreactive Th1- and Th17-cell activation and clonal expansion in the periphery. In order to evaluate whether this action is exerted directly on lymphoid cells, we assayed the *in vitro* effect of adrenomedullin on MOG-induced proliferation and cytokine production by DLNs isolated from EAE mice. Adrenomedullin deactivated MOG-specific recall responses *in vitro* (Supplementary Fig. 1). Finally, because high levels

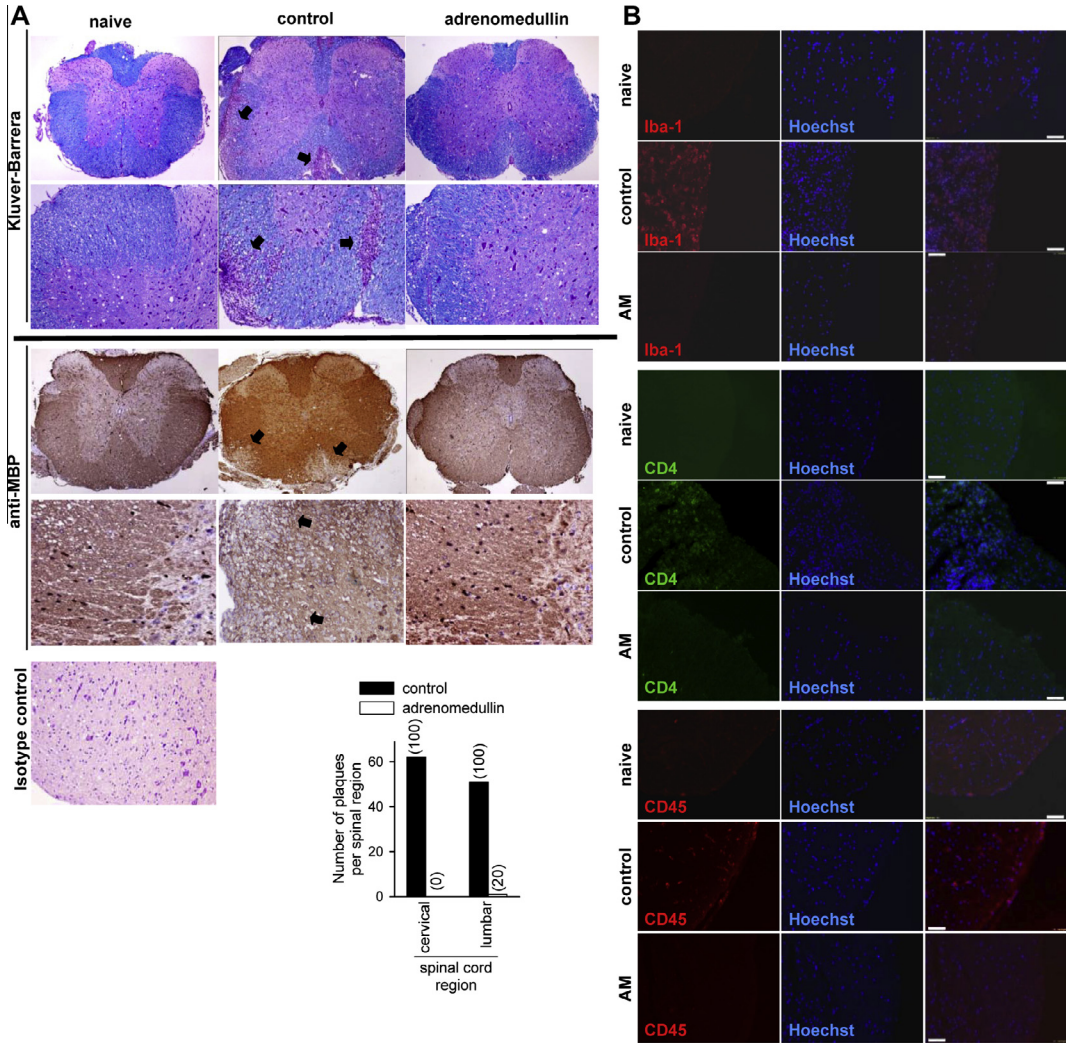

Fig. 2. Adrenomedullin reduces inflammatory infiltration and demyelination in CNS of mice with EAE. Mice with MOG_{35-55}-induced chronic EAE were treated with PBS (control) or with adrenomedullin for 5 days starting at disease onset (6/group, two independent experiments). Naïve animals were used as negative controls. (A) Transverse sections of spinal cord randomly selected at the peak of clinical disease were stained with Kluver-Barrera or immunostained for myelin (anti-MBP). Original magnification for upper images, 40×; lower, 100× for Kluver-Barrera images and 200× for anti-MBP images. Arrows point to areas of demyelination and inflammatory infiltration. A section of spinal cord of naïve mice stained with isotype control antibody was included to assess non-specific immunostaining (magnification, 200×). The mean number of plaques of demyelination in the lumbar and cervical regions and the incidence of occurrence of plaques (in parenthesis) was determined. (B) The phenotype of the infiltrating cells in the lumbar region of spinal cord was assayed by immunofluorescence for CD45, CD4 and Iba1. AM: adrenomedullin. Scale bars: 50 μm.

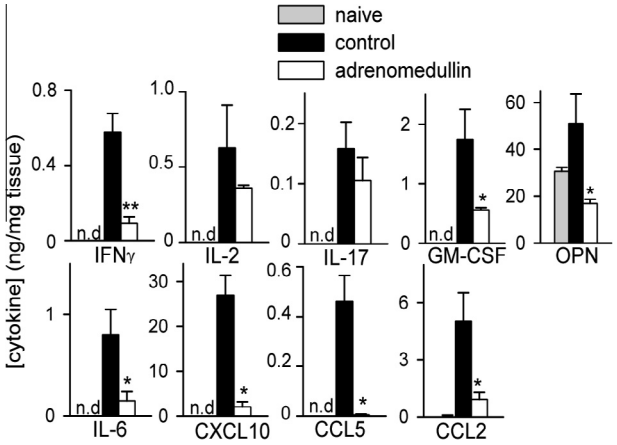

Fig. 3. Adrenomedullin reduces the production of inflammatory markers in the CNS of EAE mice. Mice with MOG_{35-55}-induced chronic EAE were treated with PBS (control) or adrenomedullin for 5 days at the disease onset (clinical score 1–2). Levels of inflammatory cytokines, chemokines, GM-CSF, and osteopontin (OPN) were determined by ELISA in protein extracts purified from spinal cord at peak of the disease. Samples from naïve mice were used as reference (n.d.: under detection limit). $n = 6$ mice/group, two independent experiments. * $p < 0.05$; ** $p < 0.01$, vs. control with Mann–Whitney test.

of circulating antibodies directed against myelin antigens invariably accompany the development of MS and EAE (Owens, 2003), and are major factors in determining susceptibility to EAE (Steinman, 1999), we next examined the effect of adrenomedullin treatment on the serum levels of MOG-specific IgG autoantibodies. Adrenomedullin reduced the ratio between MOG-specific IgG2a and IgG1 isotypes, generally reflective of Th1 and Th2 activities, respectively (Fig. 4C).

3.4. Adrenomedullin impairs the encephalitogenic response in the CNS

We next investigated whether, beside its effect in the periphery, the treatment with adrenomedullin also impaired the encephalitogenic response in the CNS. In agreement with data obtained from histopathology (Fig. 2B), flow cytometry analysis of infiltrating mononuclear cells isolated from the brains of EAE mice showed that adrenomedullin administration resulted in significantly lower numbers of infiltrating CD4 T cells, particularly of IFNγ- and IL-17-producing Th1 and Th17 cells (Fig. 5A). We next examined the encephalitogenic capacity of the few infiltrating T cells found in the CNS parenchyma of adrenomedullin-treated EAE mice. As expected, infiltrating mononuclear cells isolated from brain and spinal cord of untreated EAE mice showed robust proliferation (Fig. 5B) and a Th1/Th17 cytokine profile upon *ex vivo* restimulation with MOG (Fig. 5C). In contrast, CNS mononuclear cells from adrenomedullin-treated EAE mice neither proliferated nor produced Th1/Th17 cytokines in the MOG-specific recall response, although they did respond to polyclonal activation (Fig. 5B and C). Interestingly, addition of adrenomedullin to cultures of MOG-stimulated CNS mononuclear cells isolated from EAE mice reduced proliferation (not shown) and the production of IL-2 and IL-17, but not of IFNγ (Supplementary Fig. 2). These results support that adrenomedullin could regulate the encephalitogenic response of infiltrating immune cells in the CNS parenchyma during the progression of EAE.

3.5. Adrenomedullin increases the number of functional Treg cells in EAE

Evidence supports that Treg cells confer significant protection against EAE by deactivating autoreactive T cells and their homing to the CNS (Bynoe et al., 2003; Kohm et al., 2002). Therefore, we next evaluated the capacity of adrenomedullin to generate Treg cells during EAE. We found that adrenomedullin injection increased the number and percentage of IL-10-secreting $CD4^+CD25^{+-}$ $FoxP3^+$ Treg cells in DLNs and spleens of EAE mice (Fig. 6A). In the CNS, despite lower numbers of CD4 T cells, adrenomedullin increased significantly the percentage of $FoxP3^+$ cells (Fig. 6B).

To evaluate the suppressive capacity of Treg cells generated by the treatment with adrenomedullin *in vivo*, we examined their

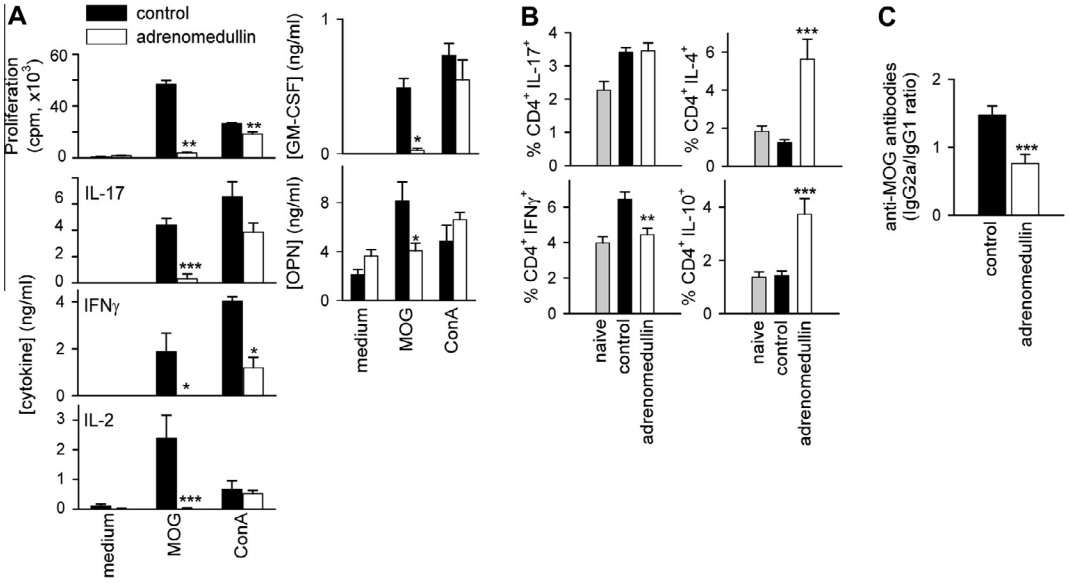

Fig. 4. Adrenomedullin ameliorates EAE severity by downregulating the peripheral encephalitogenic Th1/Th17 response. Mice with MOG_{35-55}-induced chronic EAE were treated with PBS (control) or adrenomedullin for 5 days at disease onset (clinical score 1–2). (A) Adrenomedullin-treated EAE mice showed reduced encephalitogenic Th1/Th17-mediated responses in lymphoid organs. Proliferation and cytokine production by DLN cells isolated at peak of the disease and stimulated with medium, the encephalitogenic antigen (MOG_{35-55}) or a polyclonal stimulus (Con A). We obtained similar results with spleen cells (data not shown). $n = 6$ mice/group, two independent experiments. (B) Adrenomedullin treatment regulated the number of Th1, Th17 and Th2 cells in EAE mice. Spleen cells isolated at peak of the disease were assayed for intracellular cytokine expression by flow cytometry in the CD4 gated population. We used naïve mice as basal controls. We obtained similar results with DLNs (data not shown). $n = 6$–8 mice/group, three independent experiments. (C) MOG-specific IgG1 and IgG2a serum levels (peak of disease). $n = 14$ mice/group, three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control with Mann–Whitney test (A) and *t*-test (B and C).

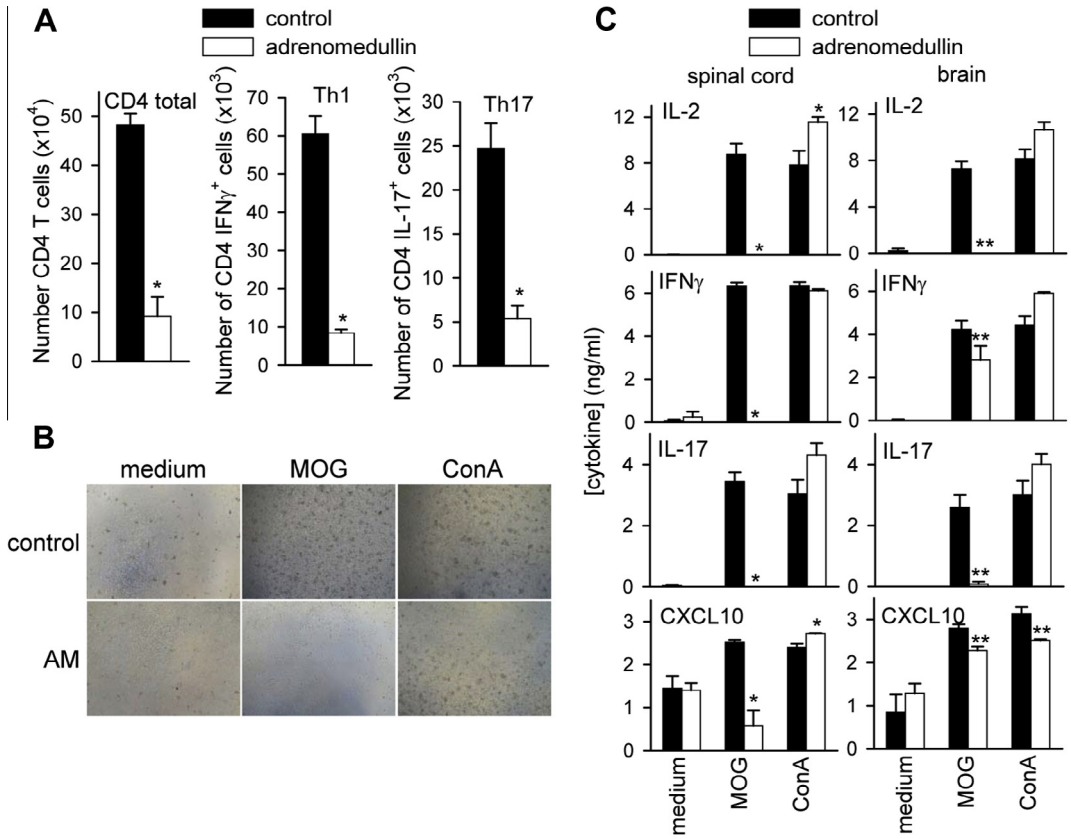

Fig. 5. Treatment with adrenomedullin impairs the encephalitogenic response in the CNS of mice with EAE. Mice with MOG_{35-55}-induced chronic EAE were treated with PBS (control) or adrenomedullin for 5 days at disease onset (clinical score 1–2). (A) Phenotypic analysis by flow cytometry of brain infiltrating mononuclear cells isolated at the peak of disease. Results show total numbers of CD4 T cells, $CD4^+IFN\gamma^+$ cells or $CD4^+IL\text{-}17^+$ cells per brain. $n = 6$ mice/group, two independent experiments. (B) Proliferation (depicted by the formation of cell clusters of activation; original magnification, 100×) and (C) cytokine production by mononuclear cells isolated from brains and spinal cords at peak of the disease and restimulated with MOG_{35-55} or with a polyclonal stimulus (ConA). $n = 6$–8 mice/group, two independent experiments. AM: adrenomedullin. * $p < 0.05$; ** $p < 0.01$ vs. control with Mann–Whitney test.

potential protective effect in the progression of EAE. Thus, injection of T cells from spleen/DLNs of adrenomedullin-treated EAE mice, but not of untreated EAE mice, into EAE mice ameliorated clinical symptoms (Fig. 6C, left panel). Moreover, depletion of $CD25^+$ T cells prior to transfer abrogated the protective effect *in vivo*, suggesting that the capacity to generate tolerance resides in the $CD4^+CD25^+$ Treg population (Fig. 6C, right panel).

3.6. Adrenomedullin generates dendritic cells that suppress the encephalitogenic response in vitro and in vivo

A recent report demonstrated the capacity of adrenomedullin to regulate DC function by inducing a semi-mature phenotype (Rullé et al., 2012). Because DCs play a major role in antigen presentation during autoimmune responses, we investigated how adrenomedullin may modulate DCs in the scenario of EAE. We first confirmed that addition of adrenomedullin during the process of activation/maturation of DCs with LPS reduced the expression of the costimulatory molecules CD40 and CD80, and the production of inflammatory cytokines such as TNFα, IL-12 and IL-6, inducing IL-10 secretion (Supplementary Fig. 3). MOG-pulsed DCs activated in the presence of adrenomedullin also impaired the proliferative response and the production of IL-2 and IL-17, but not of IFNγ, by MOG-activated DLN cells isolated from EAE mice (Fig. 7A). Interestingly, they did not affect the polyclonal activation of T cells (Fig. 7A). Noteworthy, MOG-pulsed DCs exposed to adrenomedullin kept the suppressive activity *in vivo*, because they reduced disease severity and incidence following administration to EAE mice (Fig. 7B). These data suggest that adrenomedullin could generate antigen-specific responses during EAE progression by modulating DC function.

3.7. Adrenomedullin down-regulates inflammatory mediators by glial cells and promotes neuroprotective responses

We next investigated whether, besides its immunoregulatory activity, adrenomedullin exerts an active protective effect in CNS by directly acting on resident cells. Because local production of cytotoxic factors by activated microglia and astrocytes in an inflammatory milieu critically contributes to MS/EAE pathology, by inducing demyelination, oligodendrocyte loss and axonal degeneration (Goverman, 2009; Owens, 2003; Steinman, 1996), we investigated the effect of adrenomedullin on the production of inflammatory mediators by activated glial cells. This neuropeptide reduced the production of inflammatory cytokines like TNFα, IL-12 and IL-6 from LPS-activated astrocytes, microglia and neuron-glia cultures (Fig. 8A) and of nitric oxide by LPS/IFNγ-activated microglia (nitrite: 1.4 ± 0.1 μM by untreated cells vs. 0.5 ± 0.2 μM by adrenomedullin-treated cells, $p < 0.005$). Notably, adrenomedullin decreased cell death induced by oxidative stress in precursor and mature oligodendrocytes (Fig. 8B). In agreement with this protective response, we observed that treatment with adrenomedullin significantly increased the expression of neuroprotective factors, like brain-derived neurotrophic factor (BDNF) and activity-dependent neuroprotector protein (ADNP) in the CNS of EAE mice (Fig. 8C). The neurotrophic factors are involved in processes of

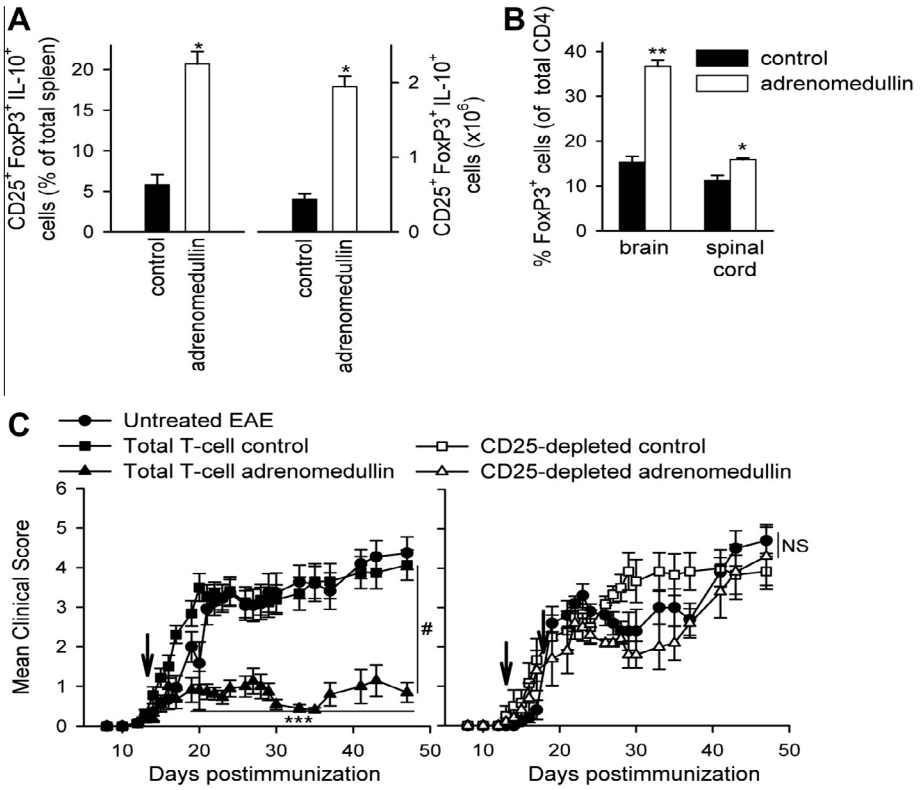

Fig. 6. Treatment with adrenomedullin increases the number of Treg cells which suppress EAE progression. Mice with MOG_{35-55}-induced chronic EAE were treated with PBS (control) or adrenomedullin for 5 days at disease onset (clinical score 1–2). (A) Adrenomedullin induces the emergence of peripheral Treg cells in EAE mice. Percentage and number of $CD4^+CD25^+FoxP3^+IL\text{-}10^+$ Treg cells in spleens isolated at peak of the disease. $n = 18$ mice/group, three independent experiments. (B) Adrenomedullin increases the percentages of Treg cells in the CNS inflammatory infiltrates. Spinal cord and brain mononuclear cells isolated at peak of the disease were analyzed for the presence of $CD4^+CD25^+FoxP3^+$ Treg cells by flow cytometry and expressed as percentage of $FoxP3^+$ cells in the CD4 population. $n = 10$ mice/group, two independent experiments. (C) Treatment (arrow) of EAE mice with CD4 T cells or CD25-depleted CD4 T cells isolated from spleen/DLNs of EAE mice that were previously treated with PBS (control) or adrenomedullin. Untreated EAE mice were used as reference. $n = 7$–9 mice/group, two independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. control with Mann–Whitney test (A and B) and Kruskal–Wallis test (C). $^{\#}p < 0.01$; NS: not significant vs. control with Kruskal–Wallis test.

remyelination, axonal growth and neuroregeneration (Braitch et al., 2010; Lewin and Barde, 1996; Linker et al., 2010). All together these data suggest that adrenomedullin could promote protective responses in EAE by inducing CNS neurotrophic factors, by downregulating the destructive inflammatory response mediated by resident glial cells and by directly preventing oligodendrocyte cell loss.

3.8. EAE mice have reduced levels of adrenomedullin in the CNS

Several studies report that immune cells including lymphocytes, macrophages and DCs secrete adrenomedullin following activation (Elsasser and Kahl, 2002; Ishimitsu et al., 1998; Kubo et al., 1998a,b; Rullé et al., 2012; Yang et al., 2001). Similarly, microglia and astrocytes increase adrenomedullin production upon LPS stimulation (Fig. 9A). We assessed whether the levels of adrenomedullin changed during EAE progression. Surprisingly, we found that the content of adrenomedullin in the CNS decreased in EAE mice (Fig. 9B), supporting an inverse correlation between EAE progression and endogenous adrenomedullin production.

4. Discussion

In this study, we report that the neuropeptide adrenomedullin provides a highly effective therapy for chronic EAE. Treatment with adrenomedullin reduced the level of inflammatory infiltrates in the CNS and subsequent demyelination and axonal damage typical of EAE. This therapeutic effect is associated with a striking reduction of the two deleterious components of the disease, namely the autoimmune and neuroinflammatory responses.

Our data indicate that treatment with adrenomedullin decreased the presence of encephalitogenic Th1 and Th17 cells in the periphery and CNS. This effect is mostly exerted by regulating the encephalitogenic sensitization in the peripheral immune compartment. Adrenomedullin reduced the number of Th1 and Th17 cells in lymphoid organs of EAE mice and specifically impaired MOG-specific Th1 and Th17 recall responses, while at the same time, it increased the number of Th2 cells, as reflected by a class switch in autoantibodies. Importantly, this treatment was antigen-specific and it did not result in general immunosuppression, since the response to a polyclonal T cell stimulation remained mostly unaffected in the adrenomedullin-treated EAE mice. Our data indicate that adrenomedullin could regulate the self-reactive T cell responses directly in the peripheral lymphoid organs. Whether this effect is exerted directly on T cells, affecting their activation, clonal expansion or differentiation, or indirectly through the modulation of antigen presenting cells, remains to be determined. Our data could support either possibility. We have previously found that adrenomedullin inhibited the activation of T cells in the absence of antigen presenting cells (unpublished data). Here we demonstrate that adrenomedullin regulates DC function by generating a semi-mature phenotype with the capacity to impair the encephalitogenic response. The effect on DCs could partially explain the fact that adrenomedullin inhibited the T cell response in an antigen-specific manner.

Beside its effect in the peripheral immune compartment, our *in vitro* experiments suggest that adrenomedullin could exert

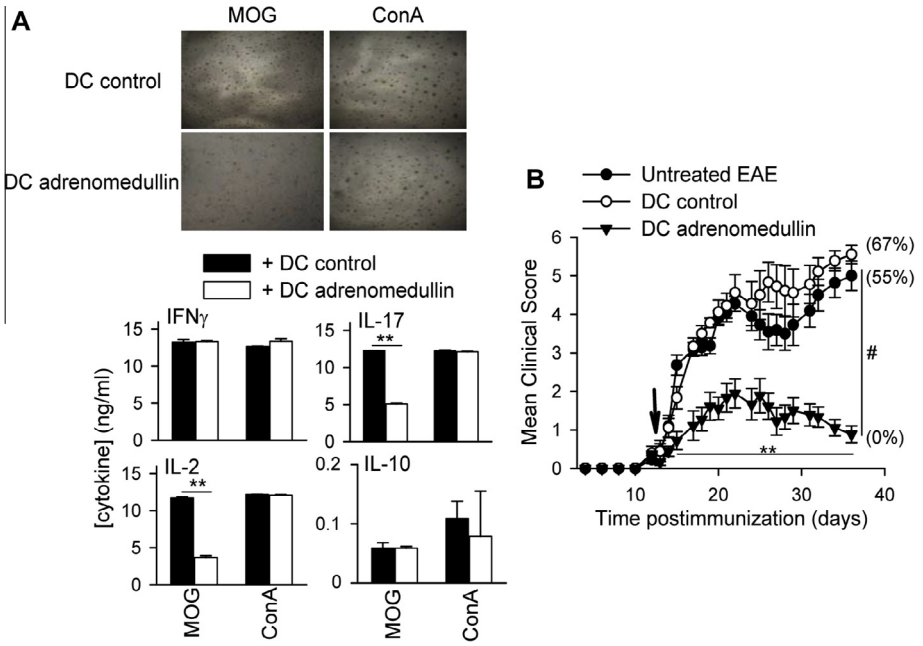

Fig. 7. Adrenomedullin generates dendritic cells (DCs) with the capacity to deactivate the encephalitogenic response *in vitro* and *in vivo*. (A) DCs matured with LPS and pulsed with MOG_{35-55} in the absence (DC-control) or presence of adrenomedullin (DC-adrenomedullin) were co-incubated with DLN cells isolated from mice suffering EAE and stimulated with MOG_{35-55} or Concanavalin A. Cell proliferation (evidenced by the presence of clusters of activation in the culture; original magnification, 100×) and the production of cytokines were determined 48 h later. $n = 5$, in duplicate. (B) DCs matured with LPS and pulsed with MOG_{35-55} in the absence (DC-control) or presence of adrenomedullin (DC-adrenomedullin) were administered i.p. to EAE mice at disease onset (arrow). Untreated EAE mice were used as reference. Numbers in parenthesis represent the mortality rate in each group. $n = 6–8$ mice/group, two independent experiments. ** $p < 0.01$ vs. control with Mann–Whitney test; $^{\#}$ $p < 0.001$ vs. control with Kruskal–Wallis test.

direct actions on infiltrating encephalitogenic T cells in the CNS parenchyma following traffic through the compromised blood–brain-barrier. In this case, whereas adrenomedullin fully suppressed MOG-specific Th17 responses, it failed to inhibit the production of IFNγ. For over a decade, Th1 cells were thought to be the driving force behind MS neuroinflammation (Hu and Ivashkiv, 2009). However, this traditional view has been challenged by studies describing exacerbated EAE development in animals deficient in IFNγ, which supports a protective role for endogenous IFNγ (Chu et al., 2000; Ferber et al., 1996). While this remains the subject of some debate, evidence now indicates that IL-17 producing T cells are critical for EAE (Komiyama et al., 2006; Park et al., 2005; Reboldi et al., 2009; Stromnes et al., 2008), and that T cell infiltration and inflammation in the CNS in EAE occur only when Th17 cells outnumber Th1 cells (Stromnes et al., 2008). Therefore, the potent suppressive effect of adrenomedullin on the activation of peripheral and central encephalitogenic Th17 cells might be an important component in its protective effect in EAE. Although we previously reported the inhibitory effect of adrenomedullin on Th1 responses in experimental colitis and arthritis (Gonzalez-Rey et al., 2006b, 2007a), the present study is the first demonstrating the impairment of self-reactive Th17 responses by this neuropeptide. Of relevance is also the fact that adrenomedullin downregulated the production of GM-CSF and osteopontin by encephalitogenic T cells, since both cytokines serve a nonredundant function in the initiation of autoimmune inflammation and in the generation of Th1 and Th17 cells in EAE and MS (Codarri et al., 2011; Chabas et al., 2001; Jansson et al., 2002; Murugaiyan et al., 2008, 2010).

On the other hand, the increase in the number of IL-10-producing $CD4^+CD25^+FoxP3^+$ Treg cells in lymphoid organs and CNS could partially explain the antigen specificity of the long lasting protective response generated by adrenomedullin, and the fact that adrenomedullin administration subsequent to the activation/differentiation of antigen-specific effector Th1/Th17 cells still inhibited the inflammatory phase of EAE. In agreement with the present study, we previously described the generation of $CD4^{+-}$ $CD25^+$ Treg cells by adrenomedullin in animal models of rheumatoid arthritis and inflammatory bowel disease (Gonzalez-Rey et al., 2006b, 2007a). Further investigations are necessary to determine whether adrenomedullin affects the expansion of already existing Treg cells or their *de novo* generation. Other neuropeptides, such as vasoactive intestinal peptide and urocortin, increased Treg cells by promoting their generation from the non-Treg compartment through direct actions on T cells and indirectly on tolerogenic DCs (Gonzalez-Rey et al., 2007b). A recent study demonstrates that stimulation of DCs with adrenomedullin induce the expression of indoleamine 2,3-dioxygenase and the expansion of $FoxP3^+$ Treg cells *in vitro* (Rullé et al., 2012). Here, we found that DCs stimulated and pulsed with MOG in the presence of adrenomedullin are capable to transfer tolerance to mice with EAE, although it remains to be determined whether they induce antigen-specific Treg cells *in vivo*. The IL-10-secreting CD4 Treg cells increased by adrenomedullin in EAE mice could represent a subtype of Tr1-like cells generated by tolerogenic DCs (Maldonado and von Andrian, 2010).

Regarding the inflammatory response in EAE, it is evident that the regulation of a wide spectrum of inflammatory mediators by adrenomedullin has an advantage over therapies directed against single mediators. The reduction of inflammation in CNS in adrenomedullin-treated EAE mice is associated with a decrease in the levels of chemokines in CNS parenchyma. This is especially relevant for chemokines such as CXCL10 (chemotactic for Th1 cells), CCL5 (for T cells) and CCL2 (for macrophages and T cells) that contribute to MS neuropathology (Goverman, 2009; Owens, 2003). Whether the effect on chemokines is exerted at local level or as a consequence of the peripheral action of adrenomedullin on IL-17 or GM-CSF (which play major roles in the initial recruitment of inflammatory cells to the CNS parenchyma in EAE), remains to be determined. A recent study reporting the capacity of adrenomedullin

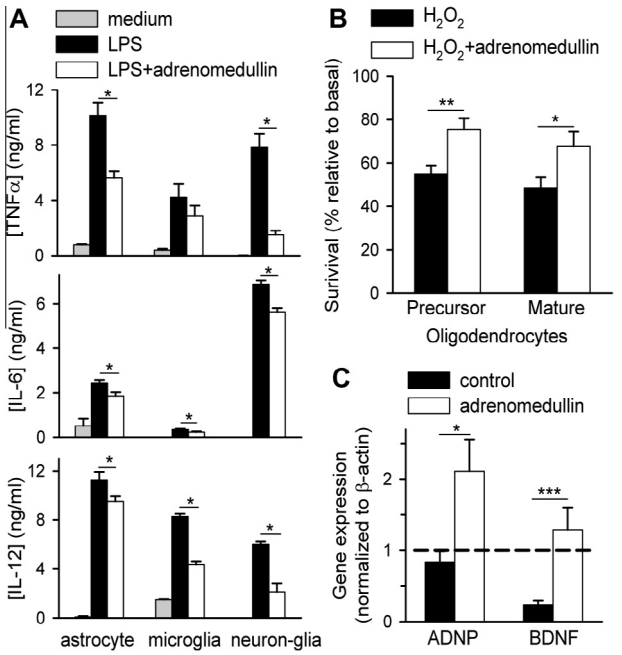

Fig. 8. Adrenomedullin promotes neuroprotective responses in EAE mice. (A) Adrenomedullin downregulates the inflammatory response of CNS resident cells. Microglia, astrocytes and mixed neuron-glia isolated from newborn mice were cultured in medium or stimulated with LPS in the absence or presence of adrenomedullin (100 nM) and cytokine contents in culture supernatants were determined 24 h later. $n = 4$, in duplicates. (B) Adrenomedullin protects oligodendrocytes from oxidative-induced cell death. Cell survival of precursor and mature oligodendrocytes cultured for 24 h with 200 μM H_2O_2 in the absence or presence of 100 nM adrenomedullin. $n = 3$, in duplicates. (C) Adrenomedullin increases neurotrophic factors. BDNF and ADNP gene expression (relative to β-actin) in spinal cords isolated from EAE mice treated with PBS (control) or adrenomedullin at disease onset. Dashed line corresponds to expression in naïve mice normalized to 1. $n = 8$ mice/group, three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control with Mann–Whitney test.

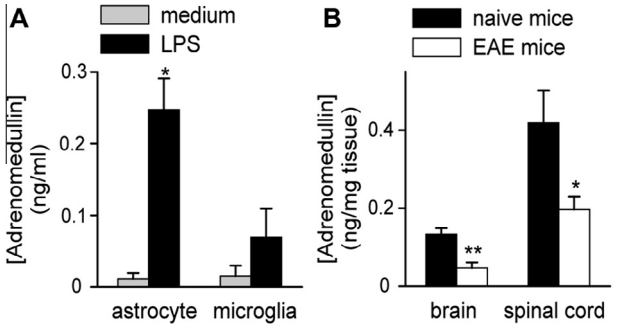

Fig. 9. EAE mice show decreased CNS adrenomedullin content. Levels of adrenomedullin in culture supernatants of LPS-activated astrocytes and microglia (24 h) (A, $n = 5$, in duplicates) and in the brains and spinal cords of naïve and EAE mice (B, $n = 5$–8 mice/group, in duplicates). * $p < 0.05$ vs. medium with Mann–Whitney test; * $p < 0.05$; ** $p < 0.01$ vs. naïve with t-test.

to inhibit the secretion of CCL2 and CCL3 by activated microglia *in vitro* (Consonni et al., 2011) supports a local effect of adrenomedullin. Moreover, the present study and others (Consonni et al., 2011) demonstrate that adrenomedullin might exert its anti-inflammatory action locally by downregulating the production of cytokines and nitric oxide by astrocytes and microglia. The effect on resident inflammatory cells, together with the inhibitory action of adrenomedullin on infiltrating macrophages (Gonzalez-Rey et al., 2006a, 2006b), probably contributes to the protection against oligodendrocyte/neuronal cell loss and axonal damage in this inflammatory milieu.

Attention has recently focused on regenerative mechanisms as targets for MS therapy, especially in the secondary progressive phase of the disease. Notably, the delayed treatment with adrenomedullin induced complete recovery in a significant number of animals, suggesting a role of adrenomedullin in repair and/or neuroregeneration. Supporting this, we found that adrenomedullin protected oligodendrocytes from cell death in an oxidative milieu. Moreover, adrenomedullin increased the local levels of BDNF and ADNP, which induce axonal outgrowth, remyelination, and rescue of degenerating neurons (Braitch et al., 2010; Lewin and Barde, 1996; Linker et al., 2010).

How does adrenomedullin regulate such a wide spectrum of immune mediators? The answer to this question could lie in the fact that adrenomedullin is the endogenous ligand of the calcitonin-related-like receptor (CRLR). CRLR forms complexes with various receptor activity-modifying proteins (RAMP1, RAMP2 and RAMP3) and the binding specificity for the ligand and the activity of the receptor depend on the RAMP subtype associated to CRLR (Gibbons et al., 2007). In contrast to CGRP which specifically binds to the CRLR-RAMP1 complex, adrenomedullin binds preferentially to CRLR associated to RAMP2 and RAMP3 (Hinson et al., 2000). Because CRLR-RAMP2/3 complexes are coupled to Gαs proteins, adrenomedullin signals through the elevation of cAMP and activation of protein kinase A, an intracellular pathway generally involved in the downregulation of inflammatory mediators, inhibition of Th1 responses, generation of Treg cells and induction of tolerogenic DCs (Torgersen et al., 2002; Taskén and Stokka, 2006; Klein et al., 2012). Noteworthy, macrophages, lymphocytes and DCs specifically express CRLR-RAMP2/3 complexes, which are differentially up-regulated during inflammatory processes (Ono et al., 2000), or during the differentiation and maturation of DCs (Rullé et al., 2012; EG-R, unpublished data).

What is the role of endogenous adrenomedullin in EAE? Evidence suggests that adrenomedullin is an endogenous immunomodulatory factor since both immune and glial cells produce adrenomedullin in response to inflammatory-related substances, cytokines and oxidative stress (Elsasser and Kahl, 2002; Ishimitsu et al., 1998; Kubo et al., 1998a, 1998b; Rullé et al., 2012; Yang et al., 2001; see also Fig. 9A). Adrenomedullin is increased in several inflammatory conditions, including rheumatoid arthritis, systemic lupus erythematosus and systemic sclerosis (Chosa et al., 2003; Mak et al., 2006; Mok et al., 2007; Yudoh et al., 1999). However, we found an inverse correlation between EAE severity and adrenomedullin levels in the CNS. Because adrenomedullin deficiency is embryonic lethal due to severe prenatal vascular failures (Shindo et al., 2001), cell- and tissue-conditional knockout mice are required to study the role of endogenous adrenomedullin in EAE. A recently generated brain conditional knockout mouse demonstrated the role of adrenomedullin in the differentiation of adult neural stem/progenitor cells and the development of oligodendrocytes (Vergaño-Vera et al., 2010). Finally, it was demonstrated that carriers of a single nucleotide polymorphism in the proximity of the adrenomedullin gene have lower levels of circulating peptide (Cheung et al., 2011; Martinez-Herrero and Martinez, 2013). Therefore, it should be interesting to investigate whether this single nucleotide polymorphism and low circulating adrenomedullin correlates with the susceptibility to suffer the disease or with its severity in patients with MS or other autoimmune encephalomyelitis.

5. Summary and conclusions

Our findings suggest that adrenomedullin may be a therapeutic option in MS. This novel therapeutic strategy is targeted to the inhibition of various neuropathological components of the disease,

while promoting long-lasting antigen-specific suppressive responses through the induction of Treg cells and tolerogenic DCs and mounting an active program of neuroprotection. This multimodal action represents a therapeutic advantage over current treatments. The ability of adrenomedullin upon delayed administration to ameliorate ongoing disease also fulfils an essential prerequisite for its development as a therapeutic agent for MS. Translation of our findings to clinical practice could be imminent since adrenomedullin-based treatments have been proven safe and effective in several human pathologies, including inflammatory disorders (Nagaya et al., 2000) and EAE is an established preclinical model for MS therapies. Finally, our study supports that adrenomedullin is a key player in the bidirectional communication between the neuroendocrine and immune systems.

Competing financial interest

Authors declare no competing financial interest.

Acknowledgments

This work was supported by Grant SAF2010-16923 from Spanish Ministry of Science and Innovation (to EGR) and the NIH grant 2RO1AI47325 (to DG). Support was also provided through Fondo Social Europeo (to MM, program JAE-DOC) and FPU grant from Spanish Ministry of Education (to MP).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2013.11.021.

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RESEARCH ARTICLE

Mesenchymal Stem Cells Induce the Ramification of Microglia Via the Small RhoGTPases Cdc42 and Rac1

Veronika E. Neubrand, Marta Pedreño, Marta Caro, Irene Forte-Lago, Mario Delgado, and Elena Gonzalez-Rey

Activated microglia play a central role in the course of neurodegenerative diseases as they secrete cytotoxic substances which lead to neuronal cell death. Understanding the mechanisms that drive activation of microglia is essential to reverse this phenotype and to protect from neurodegeneration. With some exceptions, evidence indicates that changes in cell morphology from a star shape to a round and flat shape accompany the process of activation in microglia. In this study, we investigated the effect of adipose-tissue-derived mesenchymal stem cells (ASCs), which exert important anti-inflammatory actions, in microglia morphology. Microglia exposed to ASCs or their secreted factors (conditioned medium) underwent a cell shape change into a ramifying morphology in basal and inflammatory conditions, similar to that observed in microglia found in healthy brain. Colony-stimulating factor-1 secreted by ASCs played a critical role in the induction of this phenotype. Importantly, ASCs reversed the activated round phenotype induced in microglia by bacterial endotoxins. The ramifying morphology of microglia induced by ASCs was associated with a decrease of the proinflammatory cytokines tumor necrosis factor-α and interleukin-6, an increase in phagocytic activity, and the upregulation of neurotrophic factors and of Arginase-1, a marker for M2-like regulatory microglia. In addition, activation of the phosphoinositide-3-kinase/Akt pathway and the RhoGTPases Rac1 and Cdc42 played a major role in the acquisition of this phenotype. Therefore, these RhoGTPases emerge as key players in the ramification of microglia by anti-inflammatory agents like ASCs, being fundamental to maintain the tissue-surveying, central nervous system supporting state of microglia in healthy conditions.

GLIA 2014;62:1932–1942

Key words: cell shape, neurodegeneration, neuroinflammation, colony stimulating factor-1

Introduction

Microglia are the resident immune cells of the central nervous system (CNS). They are activated once they detect any CNS lesion or dysfunction. During this activation process, microglia migrate to the insult site, secrete a variety of inflammatory cytokines and chemokines, and perform phagocytosis (Jonas et al., 2012), similarly to classically activated macrophages (Mosser and Edwards, 2008). As cytotoxic substances secreted by overactivated microglia lead to neuronal cell death, microglia play a central role in the course of neurodegenerative diseases, such as multiple sclerosis, Alzheimer's, and Parkinson's diseases (Block et al., 2007). Therefore, it is essential to understand the mechanisms of activation and deactivation processes at the molecular level to limit a possible overactivation during neurodegenerative diseases and to return activated microglia back to noninflammatory states as they are found in a healthy CNS.

Interestingly, the activation of microglia can often be followed morphologically as they change from a resting star-shaped form to a round and flat shape when activated (Hanisch and Kettenmann, 2007). However, this view has been revised recently (Perry et al., 2010). As activated microglia can adopt many different phenotypes depending on the disease and other systemic influences, the morphology alone seems not to be sufficient to specifically identify these diverse phenotypes and functions. In this sense, some studies revealed that ramified microglia (historically characterized as "resting") can exert different functions and that microglia are able to

View this article online at wileyonlinelibrary.com. DOI: 10.1002/glia.22714

Published online July 3, 2014 in Wiley Online Library (wileyonlinelibrary.com). Received Oct 10, 2013, Accepted for publication June 18, 2014.

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respond without being morphologically activated (Cunningham et al., 2005). Therefore, caution should be taken to directly infer function from preconceived morphological categories.

Nevertheless, any kind of morphological cell shape change underlies a rearrangement of the actin and microtubule cytoskeleton, which allows them to migrate to the site of injury or inflammation and perform efficient phagocytosis (abd-el-Basset and Fedoroff, 1995; Cross and Woodroofe, 1999). The family of small RhoGTPases regulates these processes, but their role in microglia is largely unknown. Among the best characterized small RhoGTPases are Cdc42, which is involved in filopodia formation, and Rac1, being responsible for lamellipodia formation. Filopodia are actin bundles, ordered in a parallel manner, whereas lamellipodia are crosslinked actin filaments forming a meshwork. Both structures are indispensable for a variety of cellular functions in different cell types, such as cell migration, neuronal outgrowth, and dendritic spine development.

In contrast, mesenchymal stem cells (MSCs) isolated from many adult tissues have recently emerged as potent immunomodulatory cells with therapeutic applications in regenerative medicine (Phinney and Prockop, 2007) and in the treatment of inflammatory and autoimmune disorders (Uccelli and Prockop, 2010). Thus, MSCs derived from bone marrow and adipose tissue were shown to ameliorate experimental autoimmune encephalomyelitis and protected neurons from neuroinflammation in experimental models of brain ischemia and brain injury (Constantin et al., 2009; Uccelli et al., 2011; Zappia et al., 2005; Zhang et al. 2005). Among their multimodal actions, MSCs seem to modulate microglia activation (Kim et al., 2009; Zhou et al., 2009) although the mechanisms involved are largely unknown.

In this study, we exploited the potential anti-inflammatory properties of ASCs in glial cells to investigate their effect on the morphological changes that microglia undergo in healthy and inflammatory conditions and to identify the intracellular signaling factors involved in this process.

Materials and Methods

Cell Isolation and Cultures

Primary microglia were prepared from P0 to P2 newborn C57Bl/6 mice. Dissected brains were discarded of olfactory bulb, cerebellum, hindbrain, and meninges and then homogenized in microglia growth medium consisting in DMEM (Invitrogen), supplemented with 10% of fetal bovine serum (FBS, Gibco), 10% of horse serum, and 1% of penicillin/streptomycin (Gibco), using a Pasteur pipette. Brain homogenates were centrifuged and the resulting cells were plated in poly-D-lysine-coated flasks and incubated with microglia growth medium for 10–14 days at 37°C and 5% of CO_2. Microglia were harvested on a shaker for 3 h at 200 rpm and plated on poly-D-lysine-coated coverslips at a density of 16,000 cells/cm^2 or tissue culture six-well plates in microglia growth medium at a density of 33,000 cells/cm^2. After 24 h, microglia growth medium was removed and fresh mouse MesenCult growth medium (Stem Cell) supplemented with mouse mesenchymal supplement (Stem Cell), herein referred as control medium, or by ASC-conditioned medium (CM) were added to plates. We did not observe any difference in growth or cell shape in microglia grown with control medium compared with the standard microglia culture medium (data not shown). To confirm the purity of the microglia culture, cells were stained with a rat anti-CD11b antibody (clone M1/70, BD Biosciences), a microglial marker (Kettenmann et al., 2011), mouse anti-GFAP antibody (Molecular Probes), a marker for astrocytes, mouse anti-βIII-tubulin antibody (Covance) as a neuronal marker and rabbit anti-Olig-2 antibody (Millipore) to stain oligodendrocyte precursor cells. As a result, we observed that our primary microglia cultures consisted out of 99% of CD11b-positive cells with a contamination of <0.02% cells of astrocytes and oligodendrocyte precursor cells. No neurons were identified in these cultures. In addition, microglia were cultured in control medium in the absence or presence of transwell inserts plated with ASCs (see below). When indicated, lipopolysaccharide (LPS, serotype 055:B05 at 0.1 μg/mL, Sigma) was added to the microglia culture.

ASCs were isolated from adipose tissue of adult C57Bl/6 mice as described previously (Anderson et al., 2013). These cells showed a fibroblast-like morphology and differentiation capacity to the adipocytic and osteocytic lineages, and expressed the phenotype MCH-II$^-$ CD14$^-$CD18$^-$CD31$^-$CD34$^-$CD45$^-$CD80$^-$CD117$^-$CD144$^-$ CD13$^+$CD44$^+$CD29$^+$CD54$^+$CD73$^+$CD90$^+$CD105$^+$CD106$^+$CD 166$^+$. ASC (2×10^4) were plated in transwell inserts (Millipore), which were transferred to 24-well plates containing microglia plated on coverslips at a cell density of 16,000 cells/cm^2. Cells were cultured in control medium for 48 h and microglia were analyzed for their shapes as described below. To determine gene and protein expression, ASCs (2×10^5) were plated in six-well transwell inserts and then cocultured in control medium with microglia plated in six-well plates at a cell density of 33,000 cells/cm^2. At different time points, inserts were removed and total RNA and proteins of microglia were isolated as described below. Cell supernatants were collected to determine the amounts of tumor necrosis factor-α (TNF-α) as described below.

ASC CM was collected from passage 2 until passage 6 of ASC cultures, which were plated at a cell density of 15,000 cells/cm^2, grown for 2 days before collecting their supernatant, and then stored at −20°C. Before use, the ASC CM was quickly thawed and passed through a 0.2 μm filter. When indicated, ASC CM was depleted of various cytokines by incubating with anti-CSF-1 and anti-interleukin-6 (IL-6) antibodies (10 μg/mL, both from BD Biosciences) for 20 min prior to its addition to microglia cultures.

In some experiments, microglia were incubated with the inhibitors PD 98059 (50 μM, Invitrogen) and LY 294002 (10 μM, Invitrogen) in microglia growth medium for 30 min, then the medium was removed and cells were incubated in ASC CM supplemented

with the inhibitors at the same concentrations. After 24 h of culture, microglia were fixed and evaluated for cell shape as described below.

For transfection, microglia (1.2×10^5 cells in suspension) were nucleofected with plasmids expressing GFP-tagged wild-type and dominant negative (DN) mutants of Cdc42 and Rac1 (Estrach et al., 2002) using the Amaxa nucleofector Small Cell Number (SCN) Kit according to the manufacturer's instructions with the SCN programme 6 and plated on coverslips coated with poly-D-lysine in microglia growth medium. After 2 h, medium was replaced with fresh growth microglia medium. In brief, 24 h later, microglia were incubated with ASC CM for 48 h and analyzed for changes in cell shape. Although the transfection efficiency for GFP was 20%, it decreased to <0.1% for the GFP-tagged wild-type and DN mutants of Cdc42 and Rac1.

Determination of Cell Shape and Form Factor

Microglia plated on coverslips were fixed for 20 min in 3.5% of paraformaldehyde, permeabilized in Triton X-100 and immunostained with the primary rat anti-CD11b antibody (clone M1/70, BD Biosciences) for 30 min at room temperature. After three washes with PBS, samples were incubated with Alexa Fluor488-conjugated secondary antibody (Molecular Probes) for 30 min at room temperature and then mounted with Mowiol (Sigma). Images were acquired with 10, 40, or 60× objectives on an Olympus IX 81 fluorescence microscope. To determine the form factor, images were analyzed using Image J. Each image was processed by the median filter at a radius of 8 pixels, and then a black and white threshold image was generated. Cell surroundings were drawn by the wand tracing tool and the area and the perimeter of each cell was determined. Cells touching the borders of the image were excluded from the quantification procedure. The form factor was calculated using the formula $4\pi \times \text{area}/(\text{perimeter})^2$ as described previously (Wilms et al., 1997). Form factors close to 1 correspond to round cells and values approaching 0 indicate highly ramified cells. At least 102 cells per condition were analyzed in at least three independent experiments, except for the transfected cells (experiments shown in Fig. 5B), where owing to the low transfection efficiency, 45–69 cells per mutant were analyzed in four independent experiments.

ELISA

TNF-α levels in culture supernatants were determined using a specific sandwich ELISA that uses capture/biotinylated detection antibodies from BD Pharmingen according to the manufacture's recommendations.

Western Blot Analysis

Microglia were grown in microglia growth medium, then the medium was changed to control medium (medium), control medium + LPS (LPS), ASC-CM, ASC conditioned medium + LPS (CM+LPS), ASC plated in transwell inserts (ASC), and ASC plated in transwell inserts + LPS (ASC+LPS) for the indicated time periods. For the detection of activity-dependent neurotrophic protein (ADNP) and brain-derived neurotrophic factor (BDNF), cells were incubated for 24 h in 2 μM monensin (Sigma) in the indicated medium. Subsequently, they were harvested with cold lysis buffer consisting in 10 mM of Tris-HCl, pH 8.0, 150 mM of NaCl, 1% Nonidet-P40, 1 mM of EDTA, 10 mM of NaF, 1 mM of Na_3VO_4, a cocktail of commercially available protease inhibitors (Sigma, containing 104 mM AEBSF, 80 μM Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin, and 1.5 mM Pepstatin A) and phosphatase inhibitors (PhosStop from Roche, acting against a wide spectrum of phosphatases, including acid and alkaline phosphatases, serine/threonine phosphatases PP1, PP2A, and PP2B and tyrosine protein phosphatases PTP). After centrifugation for 15 min at 14,000 rpm, the protein concentration of the supernatants was determined by Bradford assay (Bio-Rad) and the samples were prepared for SDS-PAGE with Laemmli SDS sample buffer. After semi-dry blotting, PVDF membranes were blocked with 5% of bovine serum albumin (BSA) in TBS and incubated 8–10 h at 4°C with the primary antibodies, including rabbit anti-Akt, rabbit anti-phospho-Akt, rabbit anti-Cdc42, rabbit anti-phospho-Erk1/2, rabbit anti-Erk1/2 (all from Cell Signalling), mouse anti-Rac1 (BD Transduction laboratories), mouse anti-Arginase-1 (BD Transduction Laboratories), rabbit anti-GAPDH (Sigma), rabbit anti-BDNF (Peprotech), rabbit anti-ADNP (Antibody-Online), or mouse anti-α-tubulin (Sigma) antibodies diluted in the blocking solution. Horseradish peroxidase-conjugated secondary antibodies (DakoCytomation) and ECL (GE Biotech) were used for detection. If necessary, membranes were stripped with stripping buffer (100 mM β-mercaptoethanol, 2% of SDS, 62.5 mM Tris, pH 6.8) for 30 min at 55°C.

RNA Extraction and qRT-PCR

Total RNA was extracted using Tripure (Roche) from microglia plated in six-well plates. After DNase I treatment (Sigma), RNA (1 μg/sample) was reverse transcribed using RevertAid First Strand cDNA Synthesis kit (Fermentas) and random hexamer primers. The cDNA was analyzed by qPCR in triplicates on an i-Cycler (Bio-Rad) with the iQ™ SYBR®Green Supermix (Bio-Rad) and 2.5 pmol of the following primers: mouse TNF-α forward, AAC TAG TGG TGC CAG CCG AT; mouse TNF-α reverse, CTT CAC AGA GCA ATG ACT CC; mouse β-actin forward, AAT CGT GCG TGA CAT CAA AG; mouse β-actin reverse, ATG CCA CAG GAT TCC ATA CC; mouse BDNF forward, CCC TCC CCC TTT TAA CTG AA; mouse BDNF reverse, GCC TTC ATG CAA CCG AAG TA; mouse ADNP forward, AGA AAA GCC CGG AAA ACT GT; mouse ADNP reverse, AAG CAC TGC AGC AAA AAG GT; mouse IL-6 forward, TGC TGG TGA CAA CCA CGG CCT; mouse IL-6 reverse, GGC ATA AAC GCA CTA GGT TTG CCG A; mouse FGF2 forward, GCG ACC CAC ACG TCA AAC TA; mouse FGF2 reverse, CCG TCC ATC TTC CTT CAT AGC; mouse glial cell-derived neurotrophic factor (GDNF) forward, TCC AAC TGG GGG TCT ACG G, mouse GDNF reverse, GCC ACG ACA TCC CAT AAC TTC AT. After 42 cycles, the Ct values were determined. To normalize the samples, ΔCt between the gene of interest and β-actin Ct values was calculated. The *x*-fold difference in expression between the different treatments was then determined by subtraction of the ΔCt values and called ΔΔCt. Finally, the total change was calculated as $2^{-\Delta\Delta ct}$ and the relative amount compared with medium-treated cells was deducted.

RhoGTPase Activation Assays

To determine Cdc42 and Rac1 activation, we performed a GST-pulldown using the CRIB domain of Pak1 as described previously (Briancon-Marjollet et al., 2008). Microglia were treated with the indicated conditions and scraped with ice-cold lysis buffer (25 mM Hepes-NaOH, pH 7.5, 1% Nonidet-P40, 10 mM $MgCl_2$, 100 mM NaCl, 5% glycerol, 100 mM PMSF, phosphatase inhibitors (PhosStop from Roche)). Lysates were centrifuged for 30 s at 14,000 rpm, the supernatant incubated with 20 µg GST-CRIB beads for 30 min at 4°C and washed with binding buffer (25 mM Hepes-NaOH, pH 7.5, 0.5% Nonidet-P40, 30 mM $MgCl_2$, 40 mM NaCl, and 1 mM dithiothreitol). Finally, beads were resuspended in 12 µL of sample buffer, loaded on an SDS-PAGE together with 15 µL of the cell lysate to determine the total amount of Cdc42 or Rac1 per sample, and analyzed by Western blot as described above.

Phagocytosis Assay

Nile Red FluoSpheres (1-µm diameter microspheres, Life Technologies) were washed in distilled water, pelleted (10,000*g*, 15 min, room temperature) and coated by incubation in 3% of BSA containing 20 mM of sodium phosphate buffer, pH 7.4, at room temperature for 15 min on a rotating wheel. Subsequently, the microspheres were washed with the phosphate buffer containing 1% of BSA and resuspended in the same buffer. Before starting the phagocytosis assay, the microspheres were opsonized with inactivated FBS (Gibco) for 1 h at 37°C and then added (10^9 microspheres/mL) to microglia. The microspheres were homogenously distributed throughout each well by gentle movements of the plate. After 1 h of incubation at 37°C, the medium containing nonphagocytosed microspheres were removed and the cells were washed twice with PBS prior to their fixation with 3.5% of paraformaldehyde in PBS for 20 min at room temperature. The number of microglia that phagocytosed and the number of phagocytosed microspheres per cell were determined on an Olympus IX 81 fluorescence microscope.

Statistical Analysis

All data are expressed as the mean ± SEM. Statistical analysis was carried out with two-way ANOVA followed by Student's *t*-test. We assumed significance at $P < 0.05$.

Results

ASCs Induce Microglia Ramification

As morphological changes in microglia cells can correlate with their activation state (Hanisch and Kettenmann, 2007; Kettenmann et al., 2011; Perry et al., 2010), we first investigated the immune-modulatory effect of ASCs on the cell shape of microglia in basal noninflammatory conditions. After 48 h, cells were fixed and changes in microglia morphology were assessed by staining with the microglia marker CD11b (Fig. 1A). To avoid cell-to-cell contact, ASCs and microglia were cocultured in a transwell system separated by a semipermeable membrane. The presence of ASCs in the culture induced a drastic change in the microglia shape, characterized by the appearance of spindle-like extensions (Fig. 1A). The fact that we observed these effects in a transwell system suggested that soluble factors produced by ASCs might mediate the induction of microglia ramification. Indeed, CM collected from ASC cultures increased the ramification of microglia. Next, we evaluated whether the action of ASCs in microglia morphology was also observed in an inflammatory milieu. As reported previously (abd-el-Basset and Fedoroff, 1995; Wilms et al., 1997), the bacterial endotoxin LPS induced a round and flat shape in microglia, and both ASCs and their CM prevented this cell shape change (Fig. 1A). Even more important was that ASC CM reversed the inflammatory phenotype acquired by microglia previously exposed to LPS (Fig. 1B).

In summary and based on the criteria for morphological categories of microglia described by Karperien et al. (2013), we observed in our experiments three morphological types: (1) Cells that were grown in medium were classified as ramifying/bushy, which are characterized by numerous short and thick processes, arranged in thick bundles around swollen somata, hereafter called "bushy" microglia. (2) ASCs/CM induced a ramifying/hypertrophied phenotype, which shows frequently elongated, larger somata, thicker primary, and retracting secondary processes, hereafter referred to as "ramifying" microglia. (3) LPS-stimulated microglia were unramified/amoeboid, bearing no processes with a large round to variable shaped cell body. Interestingly, in our *in vitro* experimental set-up, we did not observe the ramified complexity as usually seen *in vivo*, an observation also mentioned by others (Karperien et al., 2013; Kettenmann et al., 2011).

For the quantification of our results, we decided to use the form factor among other available methods (Karperien et al., 2013; Soltys et al., 2001), because it was previously shown that it discriminates well between bushy, hypertrophied, and unramified cells (Soltys et al., 2001; Wilms et al., 1997). For example, a form factor with the value of 1 corresponds to a round cell, whereas values tending to 0 indicate ramified cells (Wilms et al., 1997). The quantification of our experiments with the form factor revealed that bushy medium-treated cells showed a value of 0.37 ± 0.02, that unramified LPS-stimulated cells had a form factor of 0.47 ± 0.02 and that the incubation with ASCs or their CM significantly reduced the form factor of microglia in both basal and inflammatory conditions (Fig. 1).

The ASC-Induced Ramification of Microglia is CSF-1 Dependent

The previous results indicate that the ramification of microglia was induced by a soluble factor secreted by the ASCs rather than direct cell–cell contact. Therefore, we next investigated whether some of the molecules secreted by ASCs, among them the colony-stimulating factor-1 (CSF-1, also known as macrophage-CSF or M-CSF) and the cytokine IL-6 (M.D. unpublished data) were involved in microglia cell shape changes induced by ASCs. We found that the depletion of CSF-1, but not of IL-6, in ASC CM reversed almost

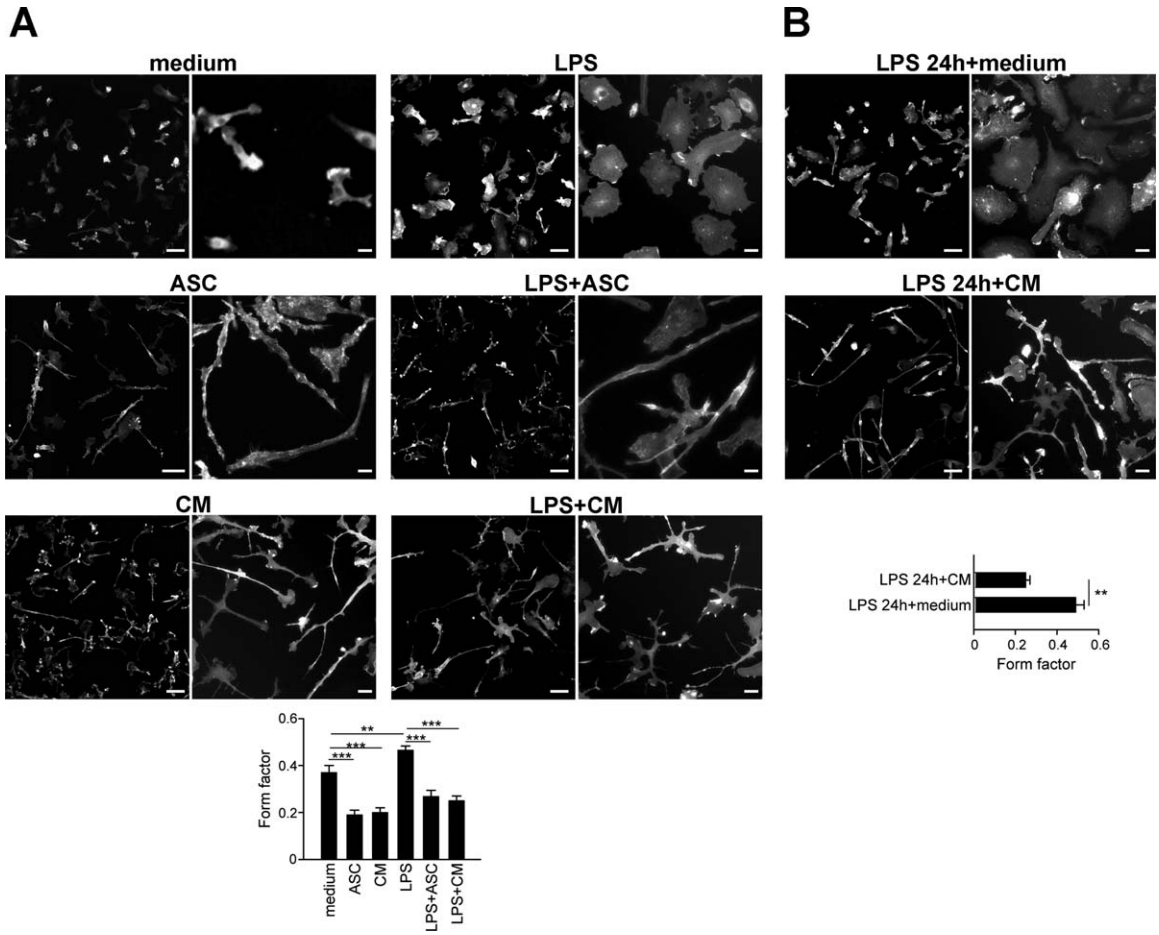

FIGURE 1: ASCs induce cell shape changes in microglia. (A) Microglia cells were incubated in control medium (medium) or activated with LPS in the absence or presence of ASCs (in transwell inserts). When indicated, microglia were incubated with CM collected from ASC cultures. After 48 h of incubation, cells were fixed and immunostained for the microglia marker CD11b. Similar results were obtained after 72 h of incubation. Quantification of the cell shape was performed by calculating the form factor = $4\pi \times$ area/(perimeter)^2. (B) Microglia were incubated for 24 h with LPS and then incubated with control culture medium (medium) or ASC-CM for further 24 h. Cells were CD11b-immunostained and the form factor was calculated in both conditions. Data are mean ± SEM and 102–212 cells per condition in at least three independent experiments were quantified. **$P < 0.01$, ***$P < 0.001$. Scale bars, 50 μm in left-panel pictures and 10 μm in right-panel pictures, for each treatment.

completely the induction of microglia ramification observed previously (Fig. 2), suggesting a major involvement of CSF-1 produced by ASCs in the change of microglia morphology. Indeed, microglia cells treated with recombinant CSF-1 showed a ramifying phenotype and a decreased form factor (Fig. 2). We also observed that indomethacin, an inhibitor of the cyclooxygenase enzyme responsible for the prostaglandin E2 production, did not affect the degree of ramification of ASC-treated microglia (data not shown), indicating that prostaglandin E2 is not essential for the ASC-induced microglia ramification.

Ramifying Microglia Induced by ASC Showed a Noninflammatory Phenotype

The initial hypothesis of our study is that ramified microglia exert anti-inflammatory functions as opposed to round proinflammatory microglia stimulated with LPS. Although the microglia shape might be an indicator of its function, the activation state of microglia should be confirmed by additional experiments. Therefore, we investigated whether, besides changes in microglia morphology, ASCs or their CM were able to regulate other factors related to the inflammatory response of microglia. Thus, we found that treatment with ASC CM prevented LPS-induced expression of the inflammatory cytokines IL-6 and TNF-α in microglia (Fig. 3A and B), suggesting that ASCs induce an anti-inflammatory state in microglia. We next tested whether the microglia generated in the presence of ASC CM were simply in a quiescent unresponsive state or whether they were capable of producing the factors involved in regenerative processes. Thus, we observed that microglia treated with ASC CM produced between 2.5- and 11-fold more neurotrophic and neuroprotective factors,

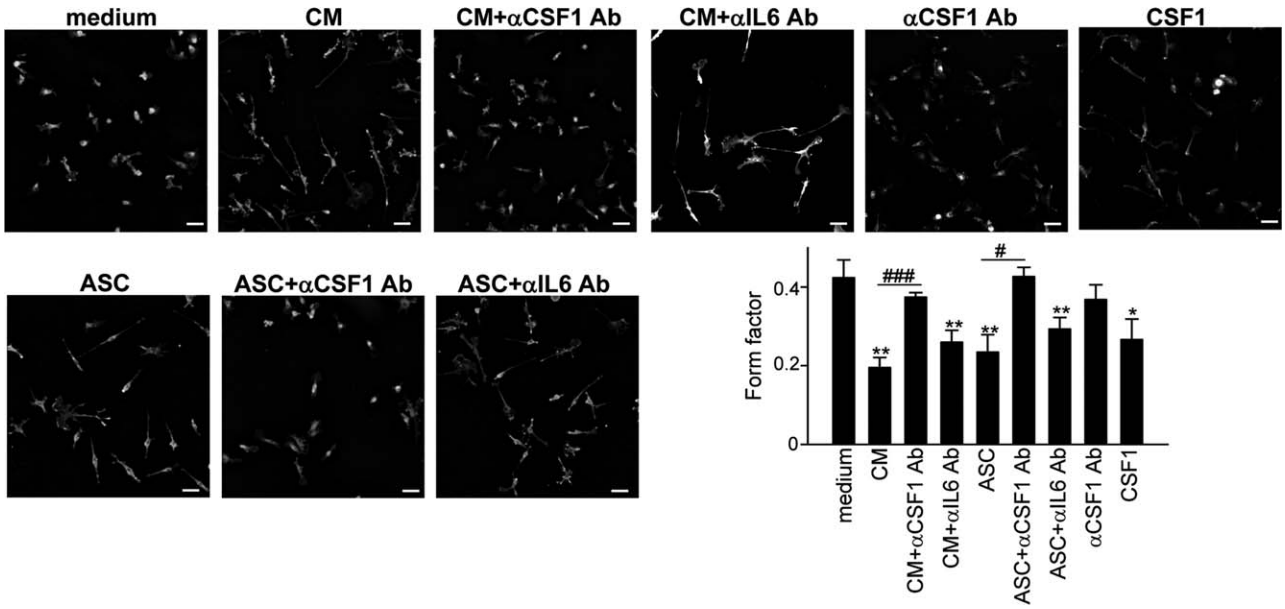

FIGURE 2: Microglia cell shape changes induced by ASCs depend on the production of CSF-1. Microglia cells were incubated in control medium (medium), with ASCs (in transwell inserts) or with CM collected from ASC cultures in the absence or presence of neutralizing antibodies against IL-6 or CSF-1. When indicated, recombinant CSF-1 (10 ng/mL) was added to microglia cultured with control medium. After 48 h of culture, cells were CD11b-immunostained and the cell shape was quantified by calculating the form factor in all conditions. Data are mean ± SEM, and 107–249 cells per condition in at least three independent experiments were quantified. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus medium; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$. Scale bars: 50 μm.

such as ADNP, BDNF and basic fibroblast growth factor (FGF2) than microglia incubated with control medium (Fig. 3C and D). Although ASC CM alone did not affect the expression of GDNF in microglia (Fig. 3C), it avoided the LPS-induced decrease of this neurotrophic factor (36 and 92% of GDNF expression compared with 100% in medium-treated microglia for LPS- and LPSCM-treated cells, respectively, P 0.04). In addition, microglia cultured in the presence of ASCs or their CM were able to produce high levels of arginase-1, a marker for alternatively activated and anti-inflammatory macrophages (Munder, 2009), in both basal and inflammatory conditions (Fig. 3E). Finally, we evaluated the phagocytic activity of microglia incubated with ASC and their CM. As shown in Fig. 3F, treatment with ASC and CM increased dramatically the phagocytic capacity of microglia for inert microspheres in comparison to microglia incubated with control medium (ASC and CM increased both the number of phagocytosing cells and the number of microspheres per cell). These data support the idea that ASC-treated microglia are not just in a quiescent state, but have rather activated a cell program promoting neuroprotective activities.

ASC CM Induced Signaling Pathways Involved in the Regulation of Microglia Morphology

We next investigated the intracellular pathways that could be involved in the induction of the phenotype observed in microglia generated in the presence of ASC CM. Evidence from the literature indicates that the phosphoinositide-3-kinase (PI3K)/Akt-dependent signaling pathway promotes anti-inflammatory properties in microglia (Tarassishin et al., 2011), and stimulates actin polymerization and phagocytosis (Song et al., 2012). In addition, the morphological change between different microglia phenotypes underlies a rearrangement of the actin cytoskeleton (abd-el-Basset and Fedoroff, 1995; Cross and Woodroofe, 1999), and the small RhoGTPases Rac1 and Cdc42 regulate these processes. Furthermore, the activation of Rac1 and Cdc42 partially depends on the activity of PI3K. We found that treatment with ASC CM rapidly activated Akt (measured as its phosphorylated form) in microglia in both basal and inflammatory conditions (Fig. 4A). In addition, as CSF-1 activates not only the PI3K pathway, but also extracellular-signal-regulated kinases 1/2 (ERK1/2) (Bourette and Rohrschneider, 2000; Pixley, 2012) in macrophages and other cell types in which ERK1/2 activation regulates lamellipodia formation and cell polarity, we tested whether this would also be the case in our experimental system. Indeed, we found a strong ERK1/2 phosphorylation upon ASC CM treatment in microglia (Fig. 4A). By using a pulldown system to detect the GTP-bound active form of the RhoGTPases, we observed that ASC CM activated rapidly both Rac1 and Cdc42 GTPase activities, mainly in basal conditions (Fig. 4B and C).

To confirm the involvement of these pathways in the phenotype induced in microglia by ASCs, we first inhibited the PI3K activity using LY 294002 and the ERK1/2 activity

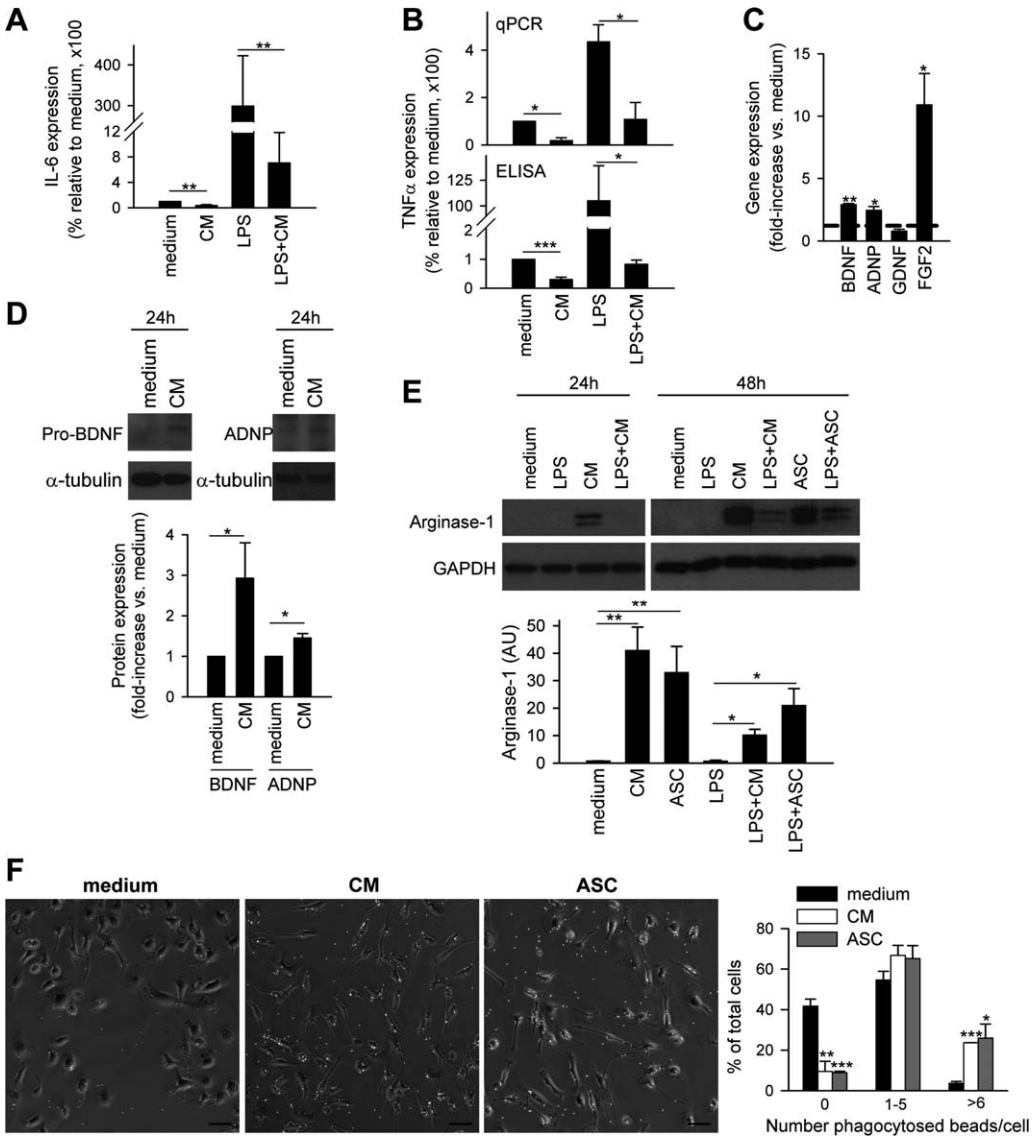

FIGURE 3: Microglia induced by ASC show noninflammatory, neuroprotective properties and increased phagocytic capacity. (A and B) Microglia cells were incubated in control medium (medium) or in CM collected from ASC cultures in the absence or presence of LPS. After 24 h, gene expression for IL-6 and TNF-α was quantified by qRT-PCR and normalized to β-actin levels. Culture supernatants were assayed for the content of TNF-α using a specific ELISA. Results are expressed as relative amount to microglia treated with control medium. Data are mean ± SEM of three (for TNF-α ELISA) four (for TNF-α qRTPCR) or five (for IL-6) independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (C and D) Microglia cells were incubated in control medium or in CM. After 24 h, gene expression for BDNF, GDNF, FGF2, and ADNP was quantified by qRT-PCR and normalized to β-actin levels. Protein expression for BDNF and ADNP was determined by Western blot analysis and normalized to α-tubulin levels. Results are expressed as fold-change to microglia treated with control medium (dashed line in C). Data are mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$. (E) Microglia cells were incubated in control medium or in CM, or coincubated in transwells with ASC in the absence or presence of LPS. At the indicated times, the protein expression of Arginase-1 was determined by Western blot analysis. Quantification corresponds to 48-h cultures and is expressed in arbitrary units (AU). Data are mean ± SEM of four independent experiments. *$P < 0.05$, **$P < 0.01$. (F) Microglia were cultured in control medium, with ASCs (in transwell inserts) or in CM for 48 h and then subjected to a phagocytosis assay using fluorescent Nile Red FluoSpheres. Data are mean ± SEM of three independent experiments with 281–423 cells being analyzed per condition. The quantification shows the percentage of cells in the culture that did not phagocyte (0 beads) or phagocytosed few (1–5 beads) or many (>6 beads) microspheres. Scale bars: 50 μm. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

using the MEK inhibitor PD 98059 in ASC CM-treated microglia. Figure 5A shows that the PI3K/Akt inhibitor blocked significantly the microglia ramification induced by ASC CM. However, the MEK inhibitor only slightly reversed the effect of ASC CM (Fig. 5A).

In addition, we investigated the involvement of Cdc42 and Rac1 by nucleofecting microglia with DN mutants of these RhoGTPases and then treated them with ASC CM. We observed that the expression of DN mutants of Cdc42 and Rac1 inhibited the ramification of microglia induced by ASC

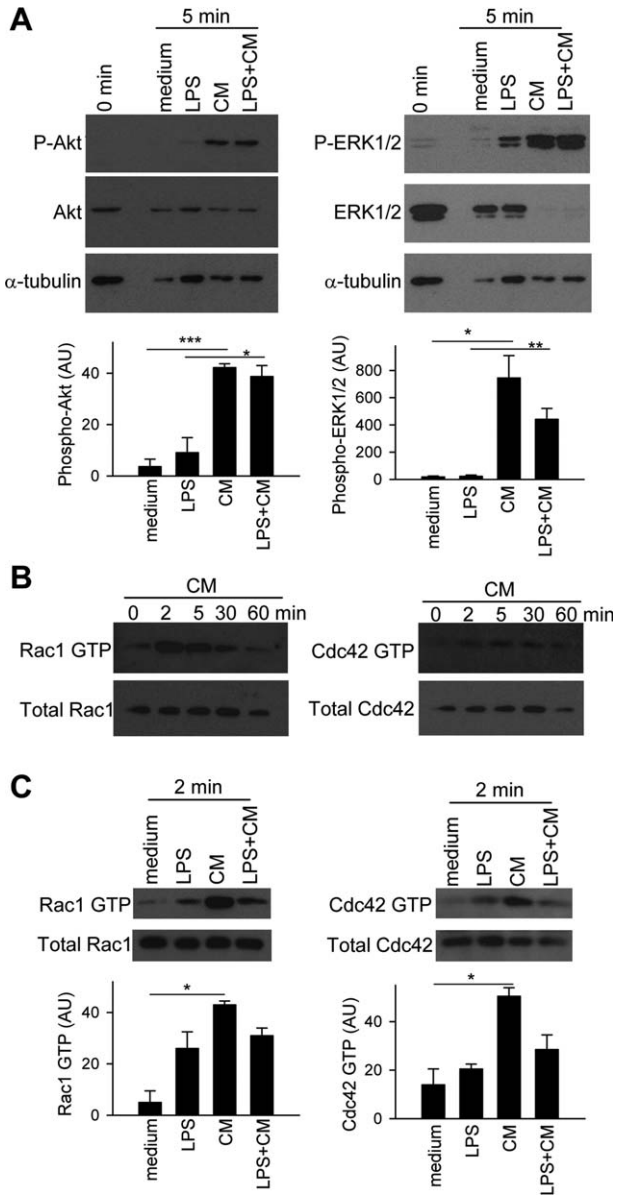

FIGURE 4: Signaling molecules activated upon ASC CM treatment. Microglia cells were incubated in control medium (medium) or in ASC-CM in the absence or presence of LPS at the indicated time points. The time point at 0 min corresponds to cells growing in microglia growth medium. (A) After 5 min of incubation, the activation of Akt and ERK1/2 was assayed by quantifying the levels of phosphorylated-Akt and phosphorylated-ERK1/2 by Western blot analysis, expressed as AU normalized to total Akt and ERK1/2, respectively. Data are mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. (B and C) At the indicated time points, cell lysates were assayed for the activation of the small RhoGTPases Rac1 and Cdc42 as described in the Materials and Methods section and normalized to total Rac1 and Cdc42 amounts in cell lysates. Data are mean ± SEM of three independent experiments. *$P < 0.05$.

CM (Fig. 5B). These findings suggest that activation of PI3K/Akt, Cdc42, and Rac1 is essential for the generation of ramified, surveying microglia by ASCs and play a major role in the transition between activated and resting microglia.

Discussion

In a healthy brain, microglia are found in a highly ramified morphology, surveying and supporting continuously the surrounding tissue (Hanisch and Kettenmann, 2007). Although it is now clear that any brain insult, inflammation, or infection leads to microglia activation to secrete inflammatory factors, it has been less studied how or through which factors microglia can return to their originally ramified phenotype with their tissue supporting properties. The previous studies indicate that MSCs can influence microglia toward that direction (Kim et al., 2009; Zhou et al., 2009), but no detailed investigation on their morphology nor on intracellular molecules involved in the cell shape change has been performed. In this study, we showed that ASCs induce in microglia a cell shape change into a ramifying morphology, even in the presence of inflammatory stimuli. It is noteworthy that ASCs were able to reverse the round, flat microglia phenotype acquired in an inflammatory milieu. As we show in our study that ASCs were able to setback an inflammatory microglia phenotype into a ramifying, anti-inflammatory one, they are the ideal tool to study the molecular mechanism behind this reversion.

In agreement with its noninflammatory supporting function, microglia exposed to ASCs did not express the proinflammatory cytokines TNF-α and IL-6, even in the presence of bacterial mediators of inflammation. Far to be in an unresponsive quiescence, the microglia generated by ASCs were in an active state characterized by the expression of neurotrophic factors and a strong phagocytic activity, which play an important role in the supportive activity of resting microglia in healthy CNS. In addition, we found that ASCs induced the expression of Arginase-1 in microglia, a marker for alternatively activated or regulatory macrophages, which downregulates inflammation during late stages of adaptive immunity (Mosser and Edwards, 2008). Although microglia can adopt a variety of different phenotypes (Perry et al., 2010) and thus a classification in "M1" or "M2-like" microglia might not be correct, ASC treatment seems to generate a kind of anti-inflammatory regulatory or alternatively activated microglia, which could be similar to "M2-like" microglia/macrophages as opposed to "M1-like" microglia/macrophages, representing classically activated microglia/macrophages with a proinflammatory profile (Mosser and Edwards, 2008). The induction of this phenotype in microglia in the CNS could play a major role in the protective effect shown by ASC-based treatments in many neurodegenerative disorders that course with neuroinflammation.

Consisting with the previous studies, these cell shape changes are likely to be mediated via the signaling pathways initiated mainly by PI3K/Akt, and maybe by ERK1/2 (Song et al., 2012; Tarassishin et al., 2011). We also observed that the small RhoGTPases Rac1 and Cdc42 play a fundamental

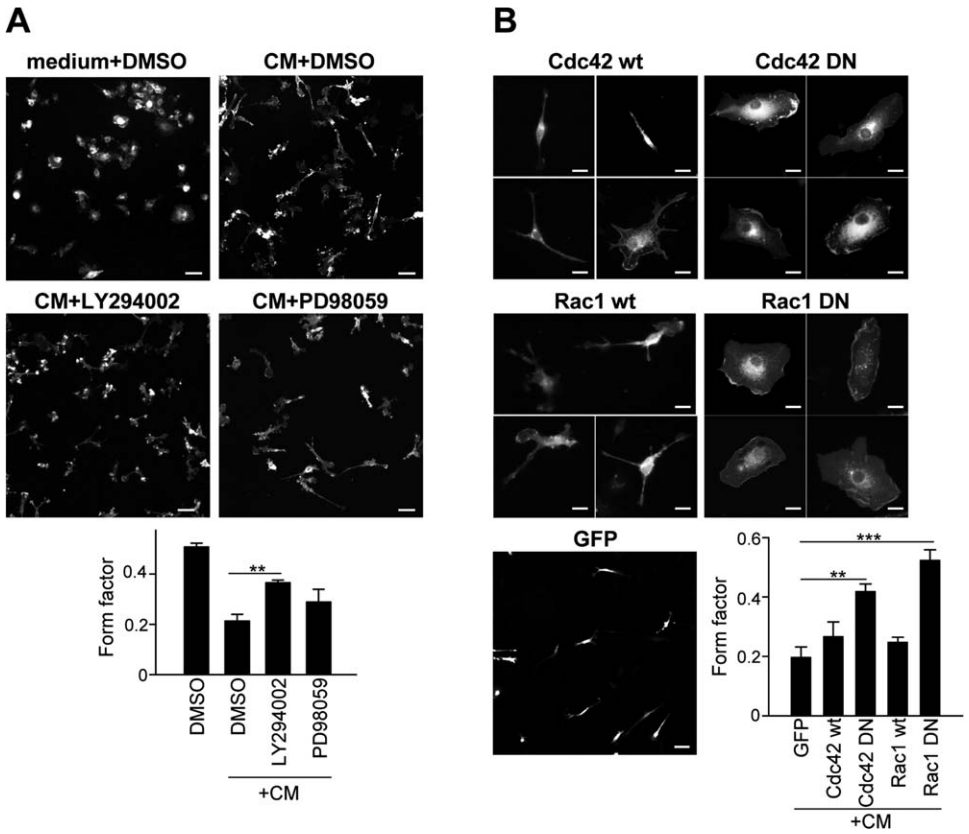

FIGURE 5: Activation of PI3K/Akt, Cdc42, and Rac1 is involved in the ASC-induced microglia cell shape changes. (A) Microglia cells were incubated in control medium (medium) or in ASC-CM containing DMSO in the absence or presence of the PI3K inhibitor LY 294002 (10 μM) or the MEK inhibitor PD 98059 (50 μM) for 24 h. Then cell shape was analyzed by CD11b-immunostaining and quantified by calculating the form factor. Data are mean ± SEM of four independent experiments with 106–131 cells being analyzed for each condition. **$P \leq 0.01$. Scale bars: 50 μm. (B) GFP-tagged wild-type (wt) and DN mutants of Cdc42 and Rac1 were nucleofected into microglia cells and then treated with ASC-CM for 48h. Cell shape changes were quantified by calculating the form factor. Data are mean ± SEM of four independent experiments with 45–69 cells being analyzed for each mutant. Scale bars are 10 μm for RhoGTPases and 50 μm for GFP. **$P < 0.01$ and ***$P < 0.001$.

role in the ramification of microglia induced by ASCs. Both events, activation of PI3K and these small RhoGTPases, are probably related. In parallel to the activation of Akt, PI3K phosphorylates phosphoinositol, generating phosphatidylinositol-(3,4,5)-triphosphate (PIP3) (Hawkins et al., 2006), which is an important lipid mediator, recruiting proteins containing Pleckstrin-Homology (PH) domains to the membrane, such as Akt and PH domain containing Rho guanine nucleotide exchange factors (RhoGEFs), the activators of RhoGTPases (Rossman et al., 2005). Indeed, the prerequisite for a successful GDP exchange on small RhoGTPases is their membrane recruitment (Rossman et al., 2005). Thus, the generation of PIP3 is an important event in activating RhoGTPases via their corresponding RhoGEF. Once activated, Rac1 and Cdc42 can recruit their effector proteins (Ridley, 2011) and induce actin polymerization in the form of lamellipodia and filopodia, respectively. This actin polymerization would lead to drastic changes in the cell morphology, inducing the ramification of microglia and stimulating their phagocytic activity. Like in neurons where both the actin cytoskeleton and the signaling molecules act in concert for successful axonal and dendritic growth (Huber et al., 2003), it is likely that also in microglia a concerted action of actin remodeling and gene expression is necessary to generate ramifying, anti-inflammatory microglia.

Our study clearly demonstrates that the effect of ASCs on microglia shape is fully mediated by soluble trophic factors produced by the mesenchymal cells. Among the main factors produced by ASCs, we found that CSF-1 is necessary and sufficient for the induction of ramification in microglia, whereas IL-6 and prostaglandin E2, which were previously involved in the anti-inflammatory action of these cells (Anderson et al., 2013; Gonzalez-Rey et al., 2010), are not essential in this process. CSF-1, initially described as a major growth factor for macrophages (Pixley and Stanley, 2004), is now recognized as a cytokine that plays an essential role in the differentiation, growth, and motility of microglia *in vitro* and *in vivo*. CSF-1 appears to be important for maintaining a normal number of microglial cells in the brain (Erblich et al., 2011; Giulian and Ingeman, 1988; Wegiel et al., 1998). In

addition, CSF-1 was found to induce ramification of human fetal microglia *in vitro* (Liu et al., 1994). In macrophages and other cell types, binding of CSF-1 to its receptor leads to phosphorylation of PI3K and ERK1/2, and subsequently to activation of small RhoGTPases, to actin polymerization, changes in cell morphology and migration (Bourette and Rohrschneider, 2000; Lo et al., 2008; Martin et al., 2003; Pixley, 2012; Sampaio et al., 2011; Smith et al., 2008). These findings support our data describing that CSF-1 produced by ASCs is involved in the induction of the ramified morphology in microglia and links CSF-1 to the intracellular signaling observed during this process.

Conclusions

In summary, we provide for the first time a comprehensive study of the parameters that drive the microglial shape changes induced by MSCs, with the aim to elucidate the factors required to reprogram proinflammatory damage-inducing microglia back to their tissue-surveying, CNS supporting function. These factors represent possible drug targets for the treatment of neurodegenerative diseases in order to prevent further neuronal cell death induced by inflammatory microglia.

Acknowledgment

Grant sponsor: Spanish Ministry of Science and Innovation; Grant number: JCI-2008-01843, SAF2010–16923; Grant sponsor: Excellence Grants of Andalusian Government.

The authors thank G. Robledo and J. Campos-Salinas (IPBLN, CSIC) for technical help in tissue culture and cell shape determination, respectively.

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Ann. N.Y. Acad. Sci. ISSN 0077-8923

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Neuroimmunomodulation in Health and Disease*

Lulling immunity, pain, and stress to sleep with cortistatin

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Cortistatin is a neuropeptide isolated from cortical brain regions, showing high structural homology and sharing many functions with somatostatin. However, cortistatin exerts unique functions in the central nervous and immune systems, including decreasing locomotor activity, inducing sleep-promoting effects, and deactivating inflammatory and T helper (T_H)1/T_H17–driven responses in preclinical models of sepsis, arthritis, multiple sclerosis, and colitis. Besides its release by cortical and hippocampal interneurons, cortistatin is produced by macrophages, lymphocytes, and peripheral nociceptive neurons in response to inflammatory stimuli, supporting a physiological role of cortistatin in the immune and nociceptive systems. Cortistatin-deficient mice have been shown to have exacerbated nociceptive responses to neuropathic and inflammatory pain sensitization. However, a paradoxical effect has been observed in studies of immune disorders, in which, despite showing competent inflammatory/autoreactive responses, cortistatin-deficient mice were partially resistant to systemic autoimmunity and inflammation. This unexpected phenotype was associated with elevated circulating glucocorticoids and anxiety-like behavior. These findings support cortistatin as a novel multimodal therapeutic approach to treat autoimmunity and clinical pain and identify it as a key endogenous component of the neuroimmune system related to stress responses.

Keywords: neuropeptide; inflammation; autoimmune; pain; stress

Introduction

Cortistatin (CST) is a cyclic peptide discovered almost 20 years ago in the rat cerebral cortex and hippocampus.[1] CST is synthesized as two mature Cys-Cys loop peptides by the activity of a prohormone convertase: CST-17 and CST-29 in humans, and CST-14 and CST-29 in mice and rats. CST shows high structural homology to somatostatin; thus, CST-14 shares 11 of its 14 residues with somatostatin-14 and displays an amino acid sequence (FWKT) identical to somatostatin in the portion of the molecule interacting with somatostatin receptors.[1–4] Such identity accounts for the fact that CST binds to all of the somatostatin receptor subtypes (sstr1 to sstr5) with similar affinity to that of somatostatin. Consequently, CST shares some pharmacological and functional properties with somatostatin, including the inhibition of neuronal activity and cell proliferation and the regulation of endocrine and exocrine secretions.[3] However, CST also has many properties that are distinct from somatostatin, such as the induction of slow-wave sleep, reduction of locomotor activity, inhibition of vascular calcification, and regulation of smooth muscle cell function.[1,3,5,6] These CST-like functions seem to be dependent on the two residues (P and K) immediately adjacent to the Cys-Cys loop. Moreover, studies of binding and intracellular calcium release demonstrate the capacity of CST to activate G protein–coupled receptors other than somatostatin receptors, including the ghrelin/growth hormone secretagogue receptor 1 (GHSR1), Mas-related gene X2 receptor (MgXR2), and truncated variants of sstr5.[2,7–10] Furthermore, studies using antagonists for GHSR1 suggest that the GHSR1 is mainly involved in many functions of CST, not shared by somatostatin, in the vascular and

doi: 10.1111/nyas.12789

immune systems (see discussion later).[5,6] Finally, because some CST-like functions are carried out in cells and tissue localizations where none of these receptors are present, and MgXR2 is only expressed in human cells, it has been speculated that an unidentified CST-selective receptor likely exists.

CST was initially identified in scattered gamma-aminobutyric acid (GABA)ergic interneurons of the cerebral cortex and hippocampus[3,11,12] and is absent in most peripheral tissues. However, CST is moderately expressed in various human and murine cells, including endothelial and endocrine cells, peripheral nociceptive neurons, and smooth muscle and immune cells.[6,8,13–16] The fact that muscle cells, endothelium, nociceptors, and immune cells also express sstr, GHSR1, and MgXR2 (in human nociceptors) supports an endogenous role of CST in vascular activity, pain sensitization, and immune responses.[6,8,13–16] This review focuses on the role played by CST in immune responses and in the bidirectional communication that exists between the immune and nervous systems.

CST is a potent anti-inflammatory factor

Although the expression of somatostatin in the immune system was previously postulated by various authors, two seminal studies performed by Dalm *et al.* clearly demonstrated that CST, but not somatostatin, is produced by various immune cells, including lymphocytes, monocytes, macrophages, and dendritic cells (DCs).[13,14] Even more important, they reported that the levels of CST correlate with the differentiation and activation state of immune cells and are paralleled by the levels of its receptors in these cells.[14,17,18] This suggests that CST might be a major endogenous regulatory factor in the immune system. In fact, several studies demonstrated that CST exerts a general anti-inflammatory action *in vitro* and *in vivo*. For example, CST impaired the production of a wide panel of inflammatory cytokines (including tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-1β, and IL-12)), chemokines, and nitric oxide by macrophages activated with bacterial endotoxin.[19,20] At the same time, CST was able to stimulate the production of the anti-inflammatory cytokine IL-10 by macrophages.[19,20] Similar anti-inflammatory responses were observed for CST in activated synovial cells isolated from arthritic mice.[21] Interestingly, CST showed higher efficiency than somatostatin in all these responses.[19–21] Moreover, only CST inhibited the production of inflammatory prostanoids and cytokines by microglia, a population of central nervous system resident–like cells.[22–24]

Several pharmacological studies suggested that the anti-inflammatory effects of CST seem to be mainly mediated through its binding to both GHSR1 and somatostatin receptors expressed in inflammatory cells,[19–21] although the involvement of MgXR2 or the truncated variants of sstr5 remains unknown. Moreover, the molecular mechanisms involved in the regulation of such a spectrum of immune mediators by CST have not been studied in detail.

On the basis of these findings, various studies investigated the effect of CST in immune disorders mediated by exacerbated inflammatory responses. The first evidence of the anti-inflammatory action of CST *in vivo* was reported in models of septic shock and sepsis.[19] Septic shock is a systemic response to severe bacterial infections (generally Gram-negative bacteria) and is the most common cause of death in intensive care units. In response to bacterial endotoxins, the immune system induces a hyperactive response through the production of high amounts of endogenous proinflammatory cytokines and mediators.[25,26] The overproduction of inflammatory mediators generates a systemic response, which affects body metabolism, vascular permeability, the coagulation system, and cardiac function, leading to tissue damage and potentially multiple-organ failure and eventually death. Treatment with CST improved survival in an experimental model of septic shock syndrome induced by the systemic administration of the endotoxin lipopolysaccharide (LPS) in mice and in two "true infection" models closer to sepsis in humans induced by polymicrobial infection caused by cecal ligation and puncture and by *Escherichia coli* injection.[19] In these experimental models, CST prevented the histopathological signs associated with septic shock, including inflammatory cell infiltration, and disseminated coagulation in various vital organs. CST exerted its therapeutic effect through a decrease in the local and systemic levels of many inflammatory mediators, including cytokines (TNF-α, IL-6, IL-1β, interferon γ (IFN-γ), and IL-12), chemokines, and acute-phase proteins (serum amyloid A).[19] This effect seems to be mediated

through deactivation of resident and infiltrating macrophages in peritoneal and target organs. The effect of CST in the production of high-mobility group box 1 (HMGB1), a constitutive DNA-binding protein considered a necessary and sufficient late mediator of multiple organ failure in sepsis,[27] has not yet been demonstrated. However, other related neuropeptides, such as ghrelin, urocortin, and vasoactive intestinal peptide (VIP), inhibit the secretion of HMGB1 by activated macrophages and the systemic levels of HMGB1 by septic mice.[28,29] Therefore, it is plausible that CST, at least through the GHSR1, could regulate this factor. Finally, ghrelin, VIP, or urocortin have been shown to have direct antimicrobial actions *in vitro* and *in vivo*, on the basis of their structural conformation and sequence.[28,30–32] However, CST does not show properties characteristic of host antimicrobial peptides and failed to kill a variety of bacteria (unpublished observations). Interestingly, combined injection of CST with VIP or urocortin showed synergistic protective effects in experimental endotoxemia,[19] supporting the concept of combination therapies in sepsis, as used in other complex disorders such as AIDS and cancer.

CST induces tolerance in autoimmune disorders

Besides its effects in innate immunity, CST is able to modulate the adaptive immune response, mainly by regulating the activation and differentiation of T lymphocytes at multiple levels (Fig. 1). The presence of CST inhibits the differentiation of T_H1 and T_H17 responses and favors T_H2-mediated responses.[18,19,22] Again, these immunomodulatory effects are mediated through its binding to GHSR1 and somatostatin receptors. In fact, studies show that somatostatin and ghrelin partially mimick the effects of CST in T cell function.[15,16,19,33,34] Under these perspectives, CST emerged as an attractive candidate to treat immune pathologies with excessive T cells responses, such as autoimmune disorders (Fig. 1). Recent studies examining CST in preclinical models of autoimmunity have shown the clinical potential of therapies based on the use of this neuropeptide in disorders such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and multiple sclerosis (MS), as discussed later.

Inflammatory bowel disease

IBD is a family of chronic and relapsing diseases of unknown ethiology, which clinically appear as two phenotypes: Crohn's disease and ulcerative colitis. Both disorders are characterized by dysfunction of mucosal T cells against endogenous antigens, altered cytokine production, and chronic cellular inflammation, which ultimately leads to mucosal damage of the colon and distal small intestine.[35] The clinical symptoms are characterized by weight loss, abdominal pain, rectal bleeding, diarrhea, and other extraintestinal manifestations. Evidence demonstrates the efficiency of CST in the treatment of colitis induced by intracolonic injection of 2,4,6-trinitrobenzene sulfonic acid (TNBS),[20] an experimental model that mimics many of the clinical, histopathological, and immunological features of Crohn's disease.[36] A single injection of CST at the onset of the disease has been shown to ameliorate the clinical and histopathologic severity of inflammatory colitis.[20] CST abrogated body weight loss and reduced diarrhea, intestinal inflammation, and the mortality caused by this syndrome.[20] It was noteworthy that the delayed administration of CST ameliorated ongoing disease, which is attractive for any newly proposed therapy. The therapeutic effect was associated with downregulation of various inflammatory mediators in colonic mucosa and with impairment in T_H1-driven autoimmune responses in colon and draining mesenteric lymph nodes.[20] Moreover, initial treatment with CST prevented recurrence of the disease after giving a second dose of TNBS, suggesting the induction of a kind of immune tolerance in these animals. The induction of this immune tolerance could be related to the fact that the treatment with CST increased the number of IL-10–secreting T regulatory (T_{reg}) cells in colonic mucosa,[20] a cell subtype that plays a key role in the control of tolerance in the intestine.[37] It remains to be determined whether this effect is because of a direct action on the generation of these IL-10–secreting cells, as occurs with other neuropeptides,[38] or a consequence of a promotion of recruitment of these cells to the colonic mucosa. Because the protection of colitis development by CST was partially reversed by somatostatin and ghrelin receptor antagonists, CST could be exerting its therapeutic effect on IBD through both receptors.[20] In agreement, ghrelin also inhibited the progression of

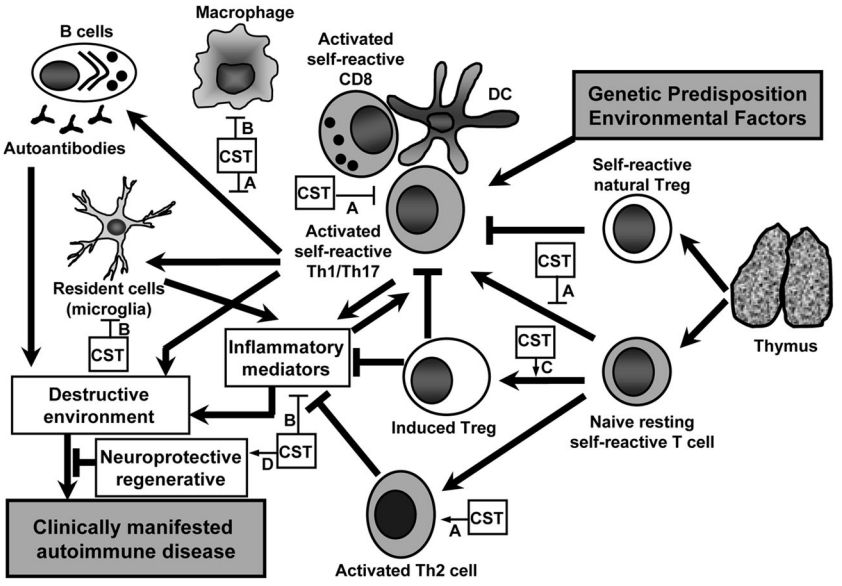

Figure 1. Cortistatin (CST) reinforces tolerance in autoimmunity at multiple levels. The initial stages of autoimmune disease occur in peripheral lymphoid organs; are associated with initiation of effector immune responses to self-components, which involve the development of self-reactive T_H1 and T_H17 cells by dendritic cells (DCs) presenting self-antigens; and occasionally occur as a byproduct of unchecked nonspecific inflammation or overly vigorous responses to chronic infections. Moreover, T_H1 cells promote the secretion of autoantibodies by self-reactive B cells and the deposition of immune complexes and activation of complement in the target tissue. Progression of the autoimmune response involves the entry of self-reactive T_H1/T_H17 cells into the affected organ, release of proinflammatory cytokines and chemokines, and subsequent recruitment and activation of inflammatory cells (macrophages and neutrophils). Later events are associated with evolving immune responses and the destructive inflammatory environment, in which inflammatory mediators (cytokines, oxygen and nitrogen reactive species, free radicals, and extracelluar matrix–degrading enzymes) produced by infiltrating cells and resident cells (i.e., microglia or synovial cells) collaborate in the tissue destruction. Regulatory T (T_{reg}) cells naturally occurring in the thymus or T_{reg} cells induced in the periphery are key players in maintaining immune tolerance by their suppression of self-reactive T cells and inflammatory responses. An imbalance of T_{reg} cells versus T_H1/T_H17 cells, or of anti-inflammatory cytokines versus proinflammatory factors, is the cause of autoimmune disorders. In order to restore tolerance on autoimmune diseases, CST reinforces various mechanisms. (A) CST decreases T_H1/T_H17 cell functions through direct actions on differentiating T cells. As a consequence, the infiltration/activation of neutrophils and macrophages and deposition of immune complexes are avoided, and the inflammatory and autoimmune responses are inhibited. (B) CST inhibits the production of inflammatory cytokines, chemokines, and free radicals by macrophages and resident cells, avoiding the inflammatory response and its cytotoxic effect against self-tissue components. (C) CST favors the generation of peripheral T_{reg} cells that suppress activation of self-reactive T cells. (D) CST induces a program of neuroprotection and/or neuroregeneration, which is critical in neuroinflammatory conditions such as multiple sclerosis. Arrows indicate a stimulatory effect. Back-crossed lines indicate an inhibitory effect.

TNBS-induced colitis, mimicking the mechanisms showed by CST,[34] and CST was significantly more efficient in inhibiting intestinal inflammation than somatostatin or its stable analogue, octreotide.[20]

Rheumatoid arthritis

RA is a chronic autoimmune disease, which affects all joints in the body, characterized by destruction of cartilage and erosion of bone as a consequence of an inflammatory milieu.[39] Several studies point to a pathogenic role in RA for cytokines and chemokines produced by T_H1 and T_H17 cells, which are reactive to components of the joint and promote the infiltration and activation of inflammatory cells (neutrophils and macrophages) in the synovium.[39] Although current therapies based on immunosuppressive agents that inhibit the inflammatory component of RA have been proven effective in delaying disease progression (i.e., erosions and deformity), they have some side effects.[39] Therefore, new therapeutic tools aimed at preventing the inflammatory and autoimmune components of the disease are desirable. Treatment with CST significantly reduced the severity and incidence of established

collagen-induced arthritis.[21] CST abrogated joint swelling and destruction of cartilage and bone, reduced production of various inflammatory cytokines and chemokines in affected joints, decreased collagen-specific T_H1 cell responses in joints and lymph nodes, and induced the generation of T_{reg} cell–producing IL-10 and transforming growth factor β1.[21] Through the use of specific antagonists, it was demonstrated that CST exerted its effects on synovial cells through both somatostatin and ghrelin receptors,[21] and CST showed higher efficiency than the two related peptides in protecting against experimental arthritis.[21] Interestingly, CST was markedly decreased in the joint synovium of rats with arthritis, and activation of human fibroblast-like synovial cells with TNF-α decreased the production of CST.[40] Similarly, CST expression was significantly downregulated by TNF-α in human cells of vertebral discs.[41] These findings suggest an inverse correlation between joint inflammation and CST levels.

Multiple sclerosis

MS is a disabling, inflammatory, demyelinating disease of the central nervous system, in which T_H1 and T_H17 cells reactive to components of the myelin sheath of nerves infiltrate the parenchyma of brain and spinal cord, and release inflammatory mediators (cytokines and free radicals that contribute to demyelination, oligodendrocyte loss, and axonal degeneration) and chemokines (promoting inflammatory cell infiltration).[42] Moreover, deregulation in the mechanisms involved in the maintenance of immune tolerance, especially those affecting T_{reg} cells, seems to play a critical role in the establishment and progression of the autoimmune response.[43] Current therapies inhibit the inflammatory component of MS, and although they reduce disease relapse or delay disease onset, they do not significantly suppress progressive clinical disability; new multistep therapeutic approaches are needed to prevent the inflammatory and autoimmune components of the disease and, at the same time, to promote mechanisms of regeneration and restoration of immune tolerance. A recent study reported that the systemic injection of CST ameliorated both chronic and relapsing-remitting experimental autoimmune encephalomyelitis (EAE) in mice,[24] which clinically and histopathologically resembles two forms of human MS. Treatment with CST after the onset of the disease reduced the appearance of inflammatory infiltrates in the cervical and lumbar segments of the spinal cord and the subsequent demyelination and axonal damage typical of EAE.[24] These effects were accompanied by a decrease in the presence of encephalitogenic T_H1 and T_H17 cells in the central nervous system,[24] which occurs mainly by regulating encephalitogenic sensitization in the peripheral immune compartment and favoring T_H2-driven protective responses. Moreover, CST was able to reduce the production of inflammatory mediators and nitric oxide by activated microglia and astrocytes,[24] supporting an effect of the neuropeptide at the local level in resident cells.

Importantly, treatment with CST did not result in a general immunosuppression. The administration of CST to mice with EAE increased the repertory of antigen-specific $CD4^+CD25^+FoxP3^+$ T_{reg} cells in lymphoid organs,[24] an effect that could partially explain the specificity of antigen of the long-lasting protective response generated by CST treatment. Similarly to autoimmune conditions, CST increased the generation of $CD4^+CD25^+$ T_{reg} cells in mice subjected to allogeneic skin transplantation.[44] It remains unsolved whether this increase in T_{reg} cells is a consequence of an effect of CST on the expansion of already existing T_{reg} cells or on new generation of T_{reg} cells in the periphery. Other neuropeptides, such as VIP, urocortin, and adrenomedullin, with protective effects in autoimmunity,[45] increased the repertory of T_{reg} cells by promoting their generation from the non-T_{reg} compartment through direct actions on T cells and indirect actions on tolerogenic DCs.[46,47]

In addition to its effect in the inflammatory and self-reactive response in EAE, CST seems to regulate an important aspect of the disease that has lately received special attention, namely regenerative mechanisms as targets for therapy in MS, particularly in the secondary progressive phase of the disease. Interestingly, delayed treatment with CST has been shown to lead to complete recovery in a significant number of mice,[24] suggesting a role of CST in repair, neuroregeneration, or both. Indeed, CST was able to protect oligodendrocytes from cell death caused by oxidative stress, as occurs in MS.[24] Moreover, CST increased the local levels of neurotrophic factors (e.g., brain-derived neurotrophic factor (BDNF) and activity-dependent neuroprotective protein (ADNP)), which are critical in the

induction of axonal outgrowth, remyelination, and rescue of degenerating neurons.[48,49] This effect was mainly exerted on neurons and glial cells.[24]

Therefore, the injection of CST emerges as a novel treatment strategy for MS by impairing the different neuropathological components of the disease while restoring long-lasting immune tolerance and mounting an active program of neuroprotection. This multimodal action may have a therapeutic advantage over current treatments.

CST and stress/anxiety behavior

Now, an important question is, What is the role of endogenous CST in the regulation of the immune response? Evidence suggests that CST is an endogenous immunomodulator. First, it had been demonstrated that various immune cells produce CST in response to inflammatory/immune stimulation.[11,12] However, others have reported that inflammatory stimulation decreases the expression of CST by synovial cells and vertebral disc cells.[40,41] In any case, the presence of specific receptors for CST in the same immune cells that produce it suggests an autocrine/paracrine action of CST in this scenario. Second, a negative correlation exists between CST levels in affected organs (spinal cord and joint) and the severity of experimental arthritis and EAE.[24,40] Moreover, a deficiency of CST in the retina of diabetic patients with retinopathy correlated with increased retinal neurodegeneration and glial activation.[50] Consistent with this finding, macrophages, T cells, and microglial cells isolated from CST-deficient mice showed exacerbated inflammatory and immune responses upon stimulation,[24] suggesting again that a lack of CST predisposes to stronger responses to immunostimulation. These findings support the idea that endogenous CST might normally provide protection against autoimmune and neurodegenerative pathologies. However, whereas CST-deficient mice developed exacerbated inflammatory/immune responses locally in experimental models of monoarticular arthritis and neurogenic inflammation,[24,51] they showed paradoxical responses against autoimmunity and systemic inflammation. Thus, a lack of CST surprisingly conferred certain resistance to EAE, IBD, and sepsis, with a delayed onset and mild clinical profiles.[24] Despite this, the levels of autoantibodies and antigen-rechallenge experiments indicated that CST-deficient mice with EAE exhibited robust encephalitogenic T_H1/T_H17 cell responses,[24] supporting the existence of a compensatory mechanism in CST-deficient mice other than an intrinsic defect in immune cells. Interestingly, the case of CST is not unique, and VIP-deficient mice were almost completely resistant to EAE and sepsis.[52–54] Although the mechanisms involved in this unexpected effect of a lack of VIP are still unknown, some evidence supports the partial involvement of an altered glucocorticoid system in the phenotype observed in CST-deficient mice.[24]

Glucocorticoids are well-known immunosuppressors with potent and widespread actions in different cells of the immune system. Two different studies reported elevated levels of corticosterone in mice lacking CST.[24,55] Indeed, pharmacological blocking of glucocorticoid receptors partially reversed the resistance to EAE and sepsis observed in CST-deficient mice.[24] It has been postulated that these abnormal glucocorticoid levels might be a consequence of altered activity in the hypothalamic–pituitary–adrenal (HPA) axis, because CST-deficient mice showed elevated levels of adrenocorticotropic hormone (ACTH) produced by the pituitary gland.[56] The HPA axis is generally activated in response to psychological and physical stressors, and a correlation has been shown between stress, states of immunosuppression, and altered HPA axis activity.[56] Interestingly, exacerbated anxiety-like behavior in the open-field and elevated plus-maze tests has been shown in animals lacking CST.[24] How CST regulates this neuroimmune axis is still unknown and under investigation. Evidence suggests that CST could act at multiple levels as an endogenous brake for the HPA axis. For example, systemic treatment with CST decreased ACTH and cortisol levels in patients with Cushing's disease.[57] Moreover, CST inhibited the secretion of ACTH by pituitary cell cultures,[55] supporting a direct action of CST in the pituitary gland. However, CST also inhibited the secretion of corticotropin-releasing hormone (CRH) by hypothalamic and hippocampal cultures,[58] two brain areas where CRH controls HPA-mediated responses, supporting its action at these brain levels as well. Indeed, CST is produced by hippocampal interneurons.[1,3] However, other systems involved in the regulation of stress and anxiety, namely adrenergic and cholinergic systems, cannot be ruled out,[56] mainly considering that CST impairs cholinergic function in the brain.[1,3]

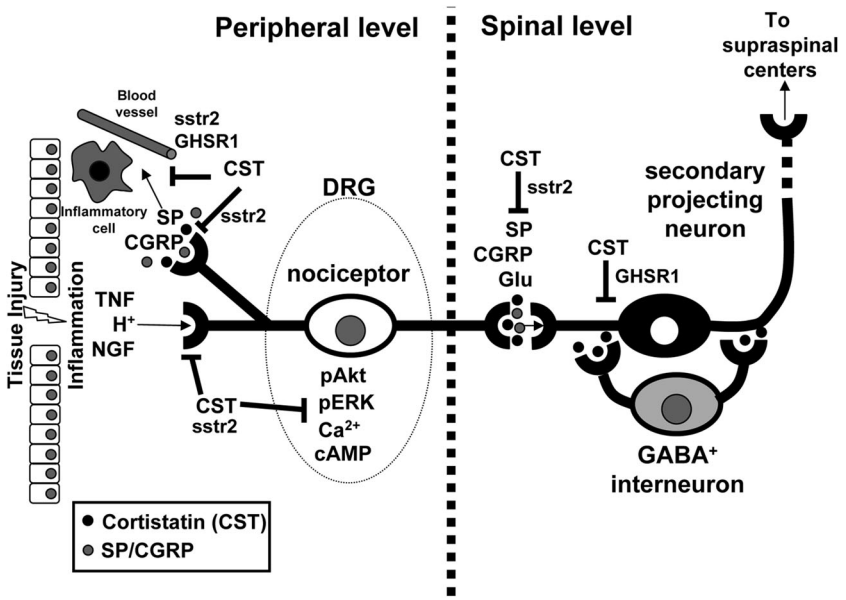

Figure 2. The role of cortistatin (CST) in the murine nociceptive system. Pain is induced by inflammatory mediators released in injured tissue, such as peptides (nerve growth factor, NGF), cytokines (TNF-α), and protons (H^+, acidic inflammatory milieu) acting on nociceptors in peripheral nerve terminals, via interaction with cell-surface receptors. Each of these mediators initiate a cascade of intracellular signaling pathways, including protein kinase A–cAMP and Ca^{2+}-dependent kinases (Akt and ERK) that decrease the threshold or excite the terminals of nociceptors by activation of cation channels. An alteration in the excitability of nociceptors transmits afferent messages through the dorsal root ganglia (DRG) to the spinal cord dorsal horn via release of excitatory neurotransmitters (glutamate, Glu) and the neuropeptides substance P (SP) and calcitonin gene–related peptide (CGRP) by the central terminal, subsequent activation of secondary spinal neurons, and from there to supraspinal centers. Concomitant to the release of excitatory peptides, nociceptors respond to noxious and inflammatory stimuli secreting CST in peripheral and central terminals. By activating Gαi-coupled receptors (mainly somatostatin receptor sstr2) and blocking cAMP-, Ca^{2+}-, and Akt/ERK–mediated signaling, CST can counteract increases in the excitability of nociceptors, inhibit the release of nociceptive peptides, and produce analgesia at both peripheral and spinal levels. At the spinal level, CST is also produced by GABAergic inhibitory interneurons and can exert inhibitory responses in secondary projecting neurons in the superficial level (lamina II and III, via sstr2) and deep level (lamina IV–VI, via ghrelin receptor GHSR1) of the spinal dorsal horn. Finally, CST released by efferent peripheral terminals can counteract local neurogenic inflammation (and persistent inflammatory pain) induced by substance P and CGRP by deactivating inflammatory cells and nociceptors (release of CGRP).

CST is an endogenous analgesic factor

Pain is unequivocally and largely linked to inflammation, and clinical pain represents a serious public health issue associated with inflammatory disorders. Substantial evidence supports the role of CST as a neuropeptide produced by the peripheral arm of the nociceptive pathway involved in defining pain perception in physiological and inflammatory states.[14,51,59,60] Three different studies recently provided proof of principle for the therapeutic relevance of CST in clinical pain.[14,51,59] In response to tissue injury or peripheral inflammation, nociceptive neurons release CST at the local level through peripheral terminals and at the spinal level through central terminals.[14] This release presumably occurs concomitantly with pronociceptive excitatory neuropeptides (calcitonin gene–related peptide and substance P) and neurotransmitters (glutamate) in order to counterbalance pain sensitization, swelling, and other deleterious changes that are subsequent to inflammation (Fig. 2). Moreover, nociceptors produce CST at the spinal level in response to central sensitization with inflammatory and algogenic factors.[14,51] The endogenous production of CST is important for tuning the nociceptive response to inflammation, since CST-deficient mice have been shown to respond with exacerbated and longer hyperalgesic responses to chronic inflammation, arthritis, and surgery.[14,51] Important from a therapeutic point of view is that, besides its effect on

acute pain and hyperalgesia, CST ameliorated tactile allodynia,[14,51] which is one of the most debilitating manifestations of pain and difficult to treat with conventional analgesics in clinically relevant pathological conditions such as arthritis, nerve injury, and postoperative conditions. Of note, the effect of CST in allodynia pertained not only to primary hyperalgesia in the injured/inflamed tissue but also alleviated secondary mechanical hyperalgesia in places distantly located from the affected member.[14,51] This is an effect that can only be explained through a central mechanism,[61,62] suggesting that the inhibitory activity of CST at peripheral nociceptors is not only linked to an impairment in excitatory drive into the spinal cord, but CST is also interfering with neuronal plasticity processes involved in secondary hyperalgesia at the central level. Indeed, the analgesic effects of CST seem to be exerted at both peripheral and spinal levels, with a predominant involvement of sstr subtype 2 (sstr2) at the periphery and of both sstr2 and GHSR1 at the spinal level (Fig. 2). Its capacity to activate both types of receptors could explain the more pronounced analgesic effects observed for CST in comparison to ghrelin, somatostatin, or analogues.[59,63–65]

Concluding remarks

The findings reviewed above indicate that CST, initially isolated as a neuropeptide involved in regulation of locomotor activity and sleep induction, is an endogenous factor, which is able to calm immune, stress, and nociceptive responses, at multiple levels. In the immune system, CST acts in a redundant manner to regulate the ratio between proinflammatory and anti-inflammatory factors, and the balance between autoreactive T_H1/T_H17 cells and protective T_{reg} cells (Fig. 1). On the basis of these characteristics, CST emerges as a promising factor in designing therapies for the treatment of chronic inflammatory diseases, such as sepsis, RA, MS, and Crohn's disease. CST could show therapeutic advantages over agents directed only against one component of these diseases, since it is able to regulate in a pleiotropic manner both inflammatory and autoimmune responses while inducing/restoring immune tolerance at the same time. This is in agreement with the strategy based on combination therapies proposed by other autoimmunity researchers. Moreover, the fact that CST induces a positive program of neuroprotection and neuroregeneration is also valuable in neuroinflammatory conditions, especially in advanced stages of the disease. On the other hand, by acting at both spinal and peripheral levels, CST exerts potent analgesic effects in many inflammatory conditions, emerging as an attractive candidate to treat clinically relevant pain, especially secondary hyperalgesia and allodynia (Fig. 2). Finally, evidence indicates that CST could be acting as an endogenous brake for stress/anxiety responses and could be a critical component of the language that immune and neuroendocrine systems speak to regulate homeostatic responses. However, caution should be applied when extending these findings to human diseases because most studies that have described the therapeutic potential of CST were performed using animal models and because notable differences exist between rodents and humans in the expression of different subtypes of CST receptors, especially in sstr expression. In any case, we are optimistic about the translation of CST use to human pathologies, as has occurred in Cushing's disease patients.[57] Functional and pharmacological studies indicate that CST exerts most of its effects through somatostatin and ghrelin receptors, and both peptides have been successfully used clinically for other pathologies. In this regard, we must be careful to conserve the exclusive CST-like structure (distinct from that of somatostatin and ghrelin) in order to design more stable and effective analogues for this neuropeptide.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Economy and Competitiveness and the Excellence Program from Junta de Andalucia.

Conflicts of interest

The authors declare no conflicts of interest.

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The Journal of Immunology

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J Immunol 2013; 191:2144-2154; Prepublished online 5 August 2013;
doi: 10.4049/jimmunol.1300384
http://www.jimmunol.org/content/191/5/2144

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Paradoxical Effect of Cortistatin Treatment and Its Deficiency on Experimental Autoimmune Encephalomyelitis

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Cortistatin is a cyclic-neuropeptide produced by brain cortex and immune cells that shows potent anti-inflammatory activity. In this article, we investigated the effect of cortistatin in two models of experimental autoimmune encephalomyelitis (EAE) that mirror chronic and relapsing-remitting multiple sclerosis. A short-term systemic treatment with cortistatin reduced clinical severity and incidence of EAE, the appearance of inflammatory infiltrates in spinal cord, and the subsequent demyelination and axonal damage. This effect was associated with a reduction of the two deleterious components of the disease, namely, the autoimmune and inflammatory response. Cortistatin decreased the presence/activation of encephalitogenic Th1 and Th17 cells in periphery and nervous system, and downregulated various inflammatory mediators, whereas it increased the number of regulatory T cells with suppressive effects on the encephalitogenic response. Moreover, cortistatin regulated glial activity and favored an active program of neuroprotection/regeneration. We further used cortistatin-deficient mice to investigate the role of endogenous cortistatin in the control of immune responses. Surprisingly, cortistatin-deficient mice were partially resistant to EAE and other inflammatory disorders, despite showing competent inflammatory/autoreactive responses. This unexpected phenotype was associated with elevated circulating glucocorticoids and an anxiety-like behavior. Our findings provide a powerful rationale for the assessment of the efficacy of cortistatin as a novel multimodal therapeutic approach to treat multiple sclerosis and identify cortistatin as a key endogenous component of neuroimmune system. ***The Journal of Immunology*****, 2013, 191: 2144–2154.**

Multiple sclerosis (MS) is a disabling inflammatory, autoimmune demyelinating disease of the CNS. Although the mechanisms of disease pathogenesis remain unclear, MS is considered as archetypal CD4 Th1/Th17 cell–mediated autoimmune disease in which Th1 and Th17 cells reactive to components of the myelin sheath, infiltrate CNS parenchyma, release proinflammatory cytokines and chemokines, and promote inflammatory cell infiltration and activation (1–3). Inflammatory mediators such as cytokines (i.e., IFN-γ, IL-17, and TNF-α) and free radicals, produced by infiltrating cells and resident microglia, play a critical role in demyelination, contributing to oligodendrocyte loss and axonal degeneration. Moreover, a deregulation in the mechanisms involved in maintenance of immune tolerance, especially those affecting regulatory T cells (Tregs), seems to critically contribute to establishment and progression of the autoimmune response (4). Although available therapies based on immunosuppressive agents inhibit the inflammatory component of MS and either reduce the relapse rate or delay disease onset, they do not suppress progressive clinical disability. This illustrates the need for novel multistep therapeutic approaches to prevent the inflammatory and autoimmune components of the disease and to promote mechanisms of regeneration and restoration of immune tolerance.

Cortistatin is a recently discovered neuropeptide that shows a remarkable sequential resemblance with somatostatin (5). Although it shares many functions with somatostatin, especially concerning the regulation of hormone secretion and neuronal activities (6), cortistatin exerts unique functions in the CNS and immune system. Thus, cortistatin, but not somatostatin, decreases locomotor activity, shows potent sleep-promoting activities, and deactivates inflammatory and Th1-driven responses in experimental sepsis, arthritis, and colitis (5, 7–10). Besides its release by cortical and hippocampal interneurons, cortistatin is produced by macrophages and T cells in response to inflammatory and immune stimulation (11), supporting a physiological role of cortistatin in the immune system. In this study, we investigated the potential therapeutic effect of cortistatin in two murine MS models and the role played by endogenous cortistatin in the control of inflammatory and autoimmune responses by using mice deficient for cortistatin.

Materials and Methods

Peptides and animals

Female SJL/J and C57BL/6 mice 8 wk old were obtained from Charles River. Mice lacking the gene for cortistatin (cortistatin-deficient [$CST^{-/-}$])

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Received for publication February 8, 2013. Accepted for publication June 27, 2013.

This work was supported by Spanish Ministry of Science and Innovation Grants SAF2007-60101 and SAF2010-16923 (to E.G.-R.) and Fondo Social Europeo (to M.M.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: ACTH, adrenocorticotropic hormone; ADNP, activity-dependent neuroprotective protein; BDNF, brain-derived neurotrophic factor; CLP, cecal ligation and puncture; CRH, corticotropin-releasing hormone; $CST^{-/-}$, cortistatin-deficient; DLN, draining lymph node; DRG, dorsal root ganglion; EAE, experimental autoimmune encephalomyelitis; HPA, hypothalamic-pituitary-adrenal; MOG, myelin oligodendrocyte glycoprotein; MPO, myeloperoxidase; MS, multiple sclerosis; PLP, proteolipid protein; Treg, regulatory T cell; wt, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1300384

and for somatostatin were generated in a C57BL/6 background (12), bred in-house, and matched by sex and weight with wild-type (wt) C57BL/6 mice. All experiments with animals were performed in accordance with the European ethical guidelines and approved by the Animal Care Unit Committee from the Institute of Parasitology and Biomedicine Lopez-Neyra-Consejo Superior Investigaciones Cientificas (protocol SAF2007-60101; SAF2010-16923). Proteolipid protein ($PLP_{139–151}$, HCLGKWLGHPDKF) and myelin oligodendrocyte glycoprotein ($MOG_{35–55}$, MEVGWYRSPFSRVVHLYRNGK) peptides were purchased from GeneScript and mouse cortistatin-29 from American Peptides.

Induction and treatment of experimental autoimmune encephalomyelitis

To induce chronic experimental autoimmune encephalomyelitis (EAE), we immunized C57BL/6 mice s.c. with 200 μg $MOG_{35–55}$ emulsified in CFA containing 400 μg *Mycobacterium tuberculosis* H37 RA (Difco). Mice also received i.p. injections of 200 ng pertussis toxin (Sigma) on days 0 and 2. Treatment consisted of the i.p. injection of cortistatin (1 nmol/d) or PBS (controls) for 5 consecutive days after disease onset in animals with a clinical score of 0.5–1 (onset) or of 2 (acute phase). In some animals, we applied a preemptive regimen of treatment consisting of 9 i.p. injections of cortistatin (1 nmol/d) starting 4 d before immunization. To study the role of endogenous cortistatin, we challenged $CST^{-/-}$ mice for EAE as described for wt C57BL/6 mice. For the relapsing-remitting EAE model, SJL/J mice were immunized s.c. with 150 μg $PLP_{139–151}$ emulsified in CFA containing 400 μg *M. tuberculosis* H37 RA and treated i.p. for 5 d with cortistatin (1 nmol/d) or PBS (controls) starting after the first disease peak. Mice were scored daily for signs of EAE according to the following clinical scoring system: 0, no clinical signs; 0.5, partial loss of tail tonicity; 1, complete loss of tail tonicity; 2, flaccid tail and abnormal gait; 3, hind-leg paralysis; 4, hind-leg paralysis with hind-body paresis; 5, hind- and fore-leg paralysis; and 6, death.

Tissue collection and cell isolation

At various time points after immunization, spleen, draining lymph nodes (DLNs: cervicals, inguinals, and axillaries), brain, and spinal cord were removed. Single-cell suspensions were obtained from spleen or pooled DLNs and used for flow cytometry analysis, determination of autoreactive responses, and adoptive transfer of EAE as described later. Brain and spinal segments of the cervical and lumbar regions were prepared separately and used for RNA isolation, protein extraction, and histopathological analysis as described later. Proteins were extracted from cervical and lumbar segments of spinal cord and brain by homogenization (50 mg tissue/ml) in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5 mM DTT, and 10 μg/ml proteinase inhibitors PMSF, pepstatin, and leupeptin). Samples were centrifuged (20,000 × *g*, 15 min, 4°C), and the supernatants were assayed for cytokine contents using sandwich ELISA following manufacturer's recommendations (BD Bioscience and PeproTech), and for cortistatin levels using a competitive ELISA (Phoenix Pharmaceuticals).

Histopathological analysis of EAE

For light microscopy, cervical and lumbar spinal cord segments were fixed with buffered 10% formalin for 48 h and processed for paraffin inclusion and sectioning. Transversal sections (4-μm thickness) were stained with Luxol fast blue, cresyl violet, and hematoxylin following the Klüver-Barrera technique (13) and were analyzed for the presence of areas of demyelination and cell infiltration using a light microscope (Olympus).

For immunofluorescence staining, cervical and lumbar spinal cord segments were fixed in 4% paraformaldehyde pH 7.4 for 4–8 h at 4°C, treated with in 30% sucrose for 24 h, and embedded in OCT in liquid nitrogen. Transversal cryosections (10-μm thickness) were blocked with 10% FBS in PBS-T (PBS + 0.2% Triton X-100) for 30 min at 22°C, incubated with FITC-labeled anti-CD4 mAb (2.5 μg/ml; BD Bioscience), PE-labeled anti-CD45 mAb (1 μg/ml; BD Bioscience), or anti-Iba1 Ab (1 μg/ml; Wako) for 18 h at 4°C, followed by incubation of Alexa Fluor 546–labeled anti-rabbit Ab (2 μg/ml; Invitrogen). Nuclear staining was performed with Hoechst (Molecular Probes). Between steps, samples were extensively washed with PBS-T + 1% FBS. Samples were observed in a fluorescence microscope (Olympus IX81).

For immunohistochemistry, spinal cord sections were obtained as described for paraffin processing followed by blocking steps with peroxidase blocking reagents, heat-treated in 1 mM EDTA buffer pH 8 at 95°C during 20 min for antigenic unmasking, and incubated for 30 min at room temperature with polyclonal anti-Myelin Basic Protein Ab (Master Diagnostica). The immunohistochemical study was done on an Autostainer480 (Thermo Fisher Scientific Inc) using the polymer-peroxidase–based method and developed with diaminobenzidine. Nuclei were hematoxylin counterstained.

Flow cytometry analysis

For Foxp3 staining, spleen and DLN cells were isolated for C57BL/6 mice with EAE at the peak of the disease and incubated with FITC-labeled anti-CD25 and allophycocyanin-labeled anti-CD4 mAbs (4–5 μg/ml; BD Bioscience) for 8 h at 4°C. After extensive washing, cells were fixed/permeabilized (eBioscience), stained with PE-labeled anti-Foxp3 Abs (4–5 μg/ml; eBioscience) for 30 min at 4°C, and analyzed in a FACSCalibur flow cytometer (BD Bioscience). We used isotype-matched Abs as controls, and mouse BD Fc block to avoid nonspecific binding to FcRs.

For intracellular analysis of cytokines, spleen and DLN cells were isolated at the disease peak and stimulated at 10^6 cells/ml with PMA (25 ng/ml) plus ionomycin (500 ng/ml) for 8–12 h, in the presence of 3 μM monensin for the last 6 h. Cells were stained with allophycocyanin–anti-CD4 mAbs (4 μg/ml) for 1 h at 4°C, fixed/saponin permeabilized with Cytofix/Cytoperm (BD Bioscience), stained with FITC- and PE-conjugated anti–cytokine-specific mAbs (4 μg/ml; BD Pharmingen) for 30 min at 4°C, and analyzed in a FACSCalibur flow cytometer.

Determination of autoreactive response

Spleen and DLN cells (10^6/ml) recovered from the C57BL/6 mice at the peak of clinical EAE (18–20 d postimmunization) were stimulated in complete medium (RPMI 1640 containing 10% FBS, 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) with 15 μM $MOG_{35–55}$. Cell proliferation was evaluated by adding 2.5 μCi/ml tritiated thymidine during the last 8 h of culture (72 h) and determining cpm incorporation in a Microbeta counter 1450. After 48 h, cytokine and chemokine content in culture supernatants were determined by sandwich ELISAs. We used cell activation with anti-CD3 (1 μg/ml) plus anti-CD28 (2.5 μg/ml) mAbs as controls of nonspecific polyclonal stimulation.

Adoptive transfer of EAE

Spleen and DLN cells recovered from untreated and cortistatin-treated C57BL/6 mice at the peak EAE (18 d postimmunization) were T cell enriched by plastic adherence (2 h, 37°C) and then injected i.p. (10^7 cells/mouse) into C57BL/6 mice suffering from EAE at the disease onset (clinical score, 0.5–1) to assay the capacity to transfer immune tolerance. When indicated, $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were isolated (purity >98%) before transference by using immunomagnetic beads (Miltenyi Biotech) following the manufacturer's recommendations and then injected i.p. (15×10^6 $CD25^-$ or 1.5×10^6 $CD25^+$ cells/mouse) into mice with EAE at the disease onset.

To transfer adoptively EAE to naive C57BL/6 mice, we mixed spleen and DLN cells from C57BL/6 or $CST^{-/-}$ mice isolated at the peak of the disease (17 d postimmunization) and then restimulated ex vivo in complete medium in the presence of 15 μM $MOG_{35–45}$ for 72 h. Cells recovered after culture were injected i.p. (12×10^6 cells/mouse) into naive C57BL/6 or $CST^{-/-}$ mice and the evolution of the disease was assessed as described earlier.

Determination of autoantibodies

We used ELISA to determine the specific anti-MOG Ab responses. Maxisorb plates (Millipore) were coated overnight at 4°C with $MOG_{35–55}$ (10 μg/ml) in 0.1 M biphosphate buffer (pH 9.6), blocked with PBS/10% FBS, and incubated for 2 h at 37°C with serial dilutions of sera obtained by cardiac puncture at the disease peak. Biotinylated anti-IgG1 or anti-IgG2a Abs (2.5 μg/ml; Serotec) were added for 1 h at 37°C. After washing, plates were incubated with streptavidin-HRP, developed with ABTS, and absorbance was determined in a spectrophotometer.

Neuron and glial cell isolation and culture

Primary mixed neuron-glia, microglia, astrocytes, and oligodendrocytes were obtained from brains of newborns (postnatal days 1–3) of naive C57BL/6 and $CST^{-/-}$ mice following the protocol described for rats (14), adapted for mice. Purity of the different glial populations was determined before culture by immunofluorescence: microglia cultures were >95% $Iba1^+$, astrocyte cultures were >99% $GFAP^+$, and cultures of oligodendrocyte precursors were >85% Olig-2^+. Our neuron-glia cocultures consisted of 27.28 ± 1.8% neurons, 43.9 ± 2.4% astrocytes, and 8.9 ± 1.6% microglia (mean ± SEM). The cell cultures were used 10–14 d after the plating.

Microglia and astrocytes were cultured in DMEM/2% FBS or activated with LPS (0.1 μg/ml) or LPS (0.1 μg/ml) plus IFN-γ (500 U/ml) in the absence or presence of 100 nM cortistatin. After 24–48 h, cytokine con-

tents were determined by ELISA in supernatants, and NO production was determined by measuring oxidized nitrite amounts in culture supernatants by using the Griess reagent (8). Oligodendrocyte precursors were incubated in free-serum DMEM/Nutrient Mixture F-12 (Life Technologies) supplemented with Apo-transferrin (25 μg/ml), biotin (10 nM), sodium selenite (30 nM), putrescine (1 μg/ml), insulin (5 μg/ml), hydrocortisone (20 nM), progesterone (20 nM), penicillin (100 U/ml), streptomycin (100 mg/l), basic fibroblast growth factor (5 ng/ml), platelet-derived growth factor (5 ng/ml), and BSA (0.1%). Mature oligodendrocytes were generated by incubation of precursors with T3 hormone (30 nM) for 3–5 d. Oligodendrocyte cell death was caused by oxidative stress by incubation with 200 μM H_2O_2 in the absence or presence of 100 nM cortistatin, and cell survival was assayed by the reduction of MTT after 24 h of culture.

Neuron-glial cocultures (5×10^5 cells/ml) were incubated in DMEM/10% FBS or stimulated with LPS (0.1 μg/ml) in the absence or presence of 100 nM cortistatin, and expression of neurotrophic factors was determined by real-time PCR as described later in RNA samples isolated after 6 h of culture.

Primary sensory neurons were isolated from lumbar dorsal root ganglia (DRGs) of 4-wk-old C57BL/6 mice as described previously (15). Isolated neurons were cultured in F12-defined medium (Invitrogen) supplemented with 10% FBS and stimulated with LPS (1 μg/ml) in the absence or presence of cortistatin (100 nM). After 24 h, NO and cytokine levels were determined in supernatants as described earlier.

RNA isolation and RT-PCR assay

We assayed gene expression of cytokine, neurotrophic factors and cortistatin by semiquantitative RT-PCR. Total RNA was isolated using Tripure (Roche) from brain and spinal cord segments. After DNase I treatment, RNA (1 μg/sample) was reverse transcribed using RevertAid First Strand cDNA Synthesis kit (Fermentas) and random hexamer primers. Semiquantitative PCR was performed using Taq polymerase (Biotools) and the specific primers and conditions depicted in Supplemental Table I. Amplified PCR products were resolved in a 2% agarose gel and densitometric analysis was performed and normalized by β-actin y/o hypoxanthine guanine phosphoribosyl transferase (HPRT) expression. We also assessed activity-dependent neuroprotective protein (ADNP) and brain-derived neurotrophic factor (BDNF) gene expression by real-time quantitative RT-PCR (60°C as annealing temperature) by using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions, using β-actin for normalization and estimating fold change expression with Delta-Delta Ct method.

Induction of inflammatory models

Polyarticular arthritis was induced in C57BL/6 wt and $CST^{-/-}$ mice by s.c. immunization with 200 μg chicken type II collagen (CII; Sigma) emulsified in CFA containing 200 μg *M. tuberculosis* H37 RA, and s.c. boosting with 100 μg CII in CFA. Mice were injected i.p. with 50 μg LPS 26 d later to synchronize arthritis and were monitored for signs of arthritis by evaluating paw swelling (measuring thickness in both hind paws with a caliper) and clinical score (0, no swelling; 1, slight swelling and erythema; 2, pronounced edema; 3, joint rigidity) in each limb.

Sepsis was induced by cecal ligation and puncture (CLP). Cecum of anesthetized C57BL/6 wt and $CST^{-/-}$ mice was ligated 5.0 mm from the cecal tip, punctured once with a 22-gauge needle, and the stool then extruded (1 mm). Animals were monitored for survival. Serum was obtained by cardiac puncture, and peritoneal exudates and lungs were collected 18 h after surgery.

To induce experimental colitis, we repetitively injected C57BL/6 wt and $CST^{-/-}$ mice intrarectally with 2,4,6-trinitrobenzene sulfonic acid (TNBS, 3 mg, in 50% ethanol; Sigma) once every 7 d for a period of 3 wk. Animals were monitored for the appearance of diarrhea, body weight loss, and survival.

Local persistent inflammation was induced by intraplantar injection of carrageenan (200 μg/20 μl; Sigma) in left hind paw of C57BL/6 and $CST^{-/-}$ mice, and paw swelling was measured using calipers. For cytokine determination in inflamed paws, protein extracts were isolated by homogenization of skins (50 mg tissue/ml) in tissue lysis buffer (in 50 mM Tris-HCl, pH 7.4, with 0.5 mM DTT, and 10 μg/ml proteinase inhibitors) dissected 2 h after carrageenan injection. Samples were centrifuged at 20,000 × *g* for 15 min at 4°C, and the supernatants were assayed for cytokine contents by sandwich ELISAs as described earlier. Neutrophil infiltration was determined by measuring myeloperoxidase (MPO) activity in the paw after 4 h (8).

Anesthetized C57BL/6 and $CST^{-/-}$ mice were injected intra-articularly with 10 μg CFA (10 μl in left knee) and saline (10 μl in right knee) to induce monoarticular inflammation. Knee diameter was measured with calipers at different times and used as an index of joint swelling. MPO activity and TNF-α levels were determined in the knee synovial fluid extracted 24 h after CFA injection. Knees obtained at day 10 were fixed, decalcified, embedded in paraffin, and processed for histopathological analysis (15).

Glucocorticoid receptor blocking

To investigate the involvement of glucocorticoids in the phenotype observed in $CST^{-/-}$ mice, we blocked glucocorticoid receptors with RU-486 (Sigma) in wt and $CST^{-/-}$ mice subjected to the various inflammatory models. RU-486 dissolved in ethanol was diluted in PEG400 at a ratio 1:1 before administration. We used ethanol:PEG400 (1:1) as vehicle control. In the EAE model, $CST^{-/-}$ and wt mice were injected s.c. with vehicle or RU-486 (1 mg, 3 times/wk, for 3 wk) starting the day of immunization. In the sepsis model, RU-486 (0.6 mg) or vehicle was injected s.c. immediately after CLP and 24 h later.

Behavioral tests

To measure anxiety-like behavior, we used individually housed male and female $CST^{-/-}$ and wt littermates. We handled the animals on alternate days 1 wk before testing and carried out the tests during the light phase of the light/dark cycle as described previously (16). In brief, mice were placed in the center of the plus maze platform and videotaped for a period of 5 min; we then determined the number of entries into and the time spent on the open arms. Moreover, mice were placed in the center of the open-field apparatus (Plexiglas box 40 × 40 × 35 cm divided in outer and inner squares) and allowed to explore the whole field for 3 min. We monitored the behavior by determining time spent in the inner and outer squares, ambulation (number of squares crossed), defecation, rearings, and time spent grooming.

Infection with Leishmania major

C57BL/6 wt and $CST^{-/-}$ mice were injected s.c. in the left hind footpads with 10^4 *L. major* purified metacyclic promastigotes isolated from stationary cultures by negative selection with peanut agglutinin (17). Disease progression was monitored by measuring the inflammation edema with a plethysmometer (IITC Life Sciences) and the area of the cutaneous lesion of the infected footpad using a caliper, in comparison with the values obtained in the uninfected contralateral footpad. Parasite burden was determined 6 wk postinfection by the presence of amastigotes in homogenates of footpad using a limiting dilution assay (18).

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was carried out with two-way ANOVA followed by Bonferroni multicomparison test or Student post hoc test. We assumed significance at $p < 0.05$.

Results

Treatment with cortistatin reduces EAE severity

We investigated the effect of the systemic administration of cortistatin in two models of EAE that mirror different clinical characteristics of MS (19). Chronic progressive EAE induced by $MOG_{35–55}$ in C57BL/6 mice is a model that mimics 20% of clinical MS. Without treatment, these mice developed moderate (27%) to severe (63%) clinical signs, and they never recovered from the disease (Fig. 1A–C). Pre-emptive and delayed treatment with cortistatin after the onset or during the effector phase of the disease greatly reduced incidence and severity (Fig. 1A–D, Table I). Remarkable are the facts that most of the cortistatin-treated EAE mice displayed mild symptoms and a significant number of them completely recovered and were entirely asymptomatic 20–30 d after disease onset (Fig. 1C, 1D; Table I). Interestingly, a short treatment with cortistatin was enough to generate a long-lasting protective effect (Fig. 1).

In the majority of MS patients, clinical disease follows a relapsing-remitting course. In a model of relapsing-remitting EAE induced by $PLP_{139–151}$ in SJL/J mice, a short systemic treatment with cortistatin after the onset of clinical symptoms substantially reduced clinical severity (Fig. 1E, Table I).

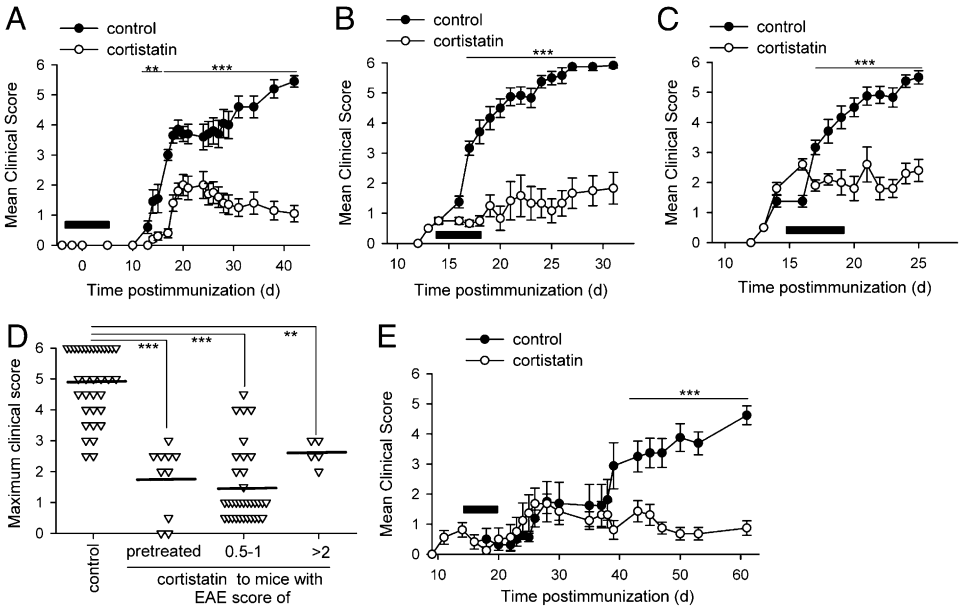

FIGURE 1. Treatment with cortistatin reduces EAE severity. Chronic progressive EAE was induced in C57BL/6 mice (16–18/group) by immunization with $MOG_{35–55}$ (**A**–**D**) and relapsing-remitting EAE in SJL/J mice (8/group) with $PLP_{139–151}$ (**E**). Mice were treated i.p. with PBS (control) or cortistatin (1 nmol/d, black bars) starting 4 d before immunization in a pre-emptive regimen (A), at disease onset (B, E, clinical score 1), or at the acute phase of the disease in a therapeutic regimen (C, clinical score 2). Data represent the mean clinical score (± SEM) (A–C, E) or maximum peak of disease per animal (D, mean: horizontal line). $^{**}p < 0.01$, $^{***}p < 0.005$ versus control. Similar results were obtained in three (A–C) or two (E) independent experiments.

Cortistatin modulates the inflammatory and autoimmune components of EAE

We next investigated the mechanisms underlying the amelioration of chronic EAE after cortistatin treatment. The pathology of MS and EAE features focal areas of inflammatory infiltration and demyelination with oligodendrocyte depletion (1, 19). Histopathologic examination of spinal cords confirmed that the beneficial actions of cortistatin were due to a decrease in inflammatory infiltrates and in the subsequent demyelination and axonal loss (Fig. 2A). Evaluation of CNS infiltrates in EAE mice revealed that the inflammatory cells ($CD45^+$) close to the perivascular area were mostly $CD4^+$ cells and Iba-1^+ macrophages (Fig. 2B). Cortistatin significantly decreased the number of all these infiltrating cell populations and probably of activated $Iba1^+$ microglia (Fig. 2B).

The reduction of inflammatory infiltration in CNS of cortistatin-treated EAE mice correlated with the decrease in the expression of inflammation-related genes including IL-12, TNF-α, IL-6, IL-17, and IFN-γ, and the chemokines Rantes, MCP-1, and IP-10 (Fig. 3A, Supplemental Fig. 1A). Interestingly, despite the lower cell infiltration, cortistatin did not reduce levels of anti-inflammatory cytokines such as IL-4, TGF-β, or IL-10 in the CNS (Fig. 3A, Supplemental Fig. 1A).

In both EAE and MS, autoreactive Th1 and Th17 cells, producing IFN-γ and IL-17, respectively, infiltrate the CNS and promote the disease, whereas treatments that induce a skewing toward an IL-4–dominated Th2 response generally suppress EAE (20). Cortistatin could ameliorate EAE by reducing encephalitogenic T cell responses and/or their migration to the CNS. Therefore, we determined the proliferation and cytokine profile of peripheral T cells from cortistatin-treated EAE mice. Lymphocytes derived from DLNs of EAE mice showed marked MOG-dependent proliferation and production of IFN-γ, IL-2, and IL-17, whereas T cells from cortistatin-treated mice proliferated much less and did not produce Th1 and Th17 cytokines in the MOG-specific recall response (Fig. 3B). The effect was Ag specific because T cell activation with anti-CD3/anti-CD28 Abs resulted in similar proliferation and cytokine secretion in both groups (Fig. 3B). Moreover, cortistatin treatment did not reduce the number of effector T cells secreting IFN-γ and IL-17 in DLNs and spleen, although it elevated the percentage of IL-4–expressing CD4 cells (Fig. 3C). These results indicate that cortistatin injection during the effector phase of EAE partially inhibits autoreactive Th1 and Th17 cell activation and clonal expansion in the periphery. This effect seems to be exerted directly on lymphoid cells because cortistatin deactivated MOG-specific recall responses in vitro (Supplemental Fig. 1B). Notably, cortistatin treatment of EAE mice also reduced the capacity of activated lymphoid cells to produce GM-CSF (Fig. 3D), a cytokine mandatory for EAE induction (21).

Table I. Effect of cortistatin in chronic progressive EAE and remitting and relapsing EAE

	Incidence (%)[a]					
	None	Mild	Moderate	Severe	Mortality	CDI[b]
CP-EAE						
Control	1/19 (5%)	1/19 (5%)	5/19 (27%)	12/19 (63%)	12/19 (63%)	97.9 ± 11.2
Cortistatin	9/18 (59%)	4/18 (22%)	3/18 (17%)	2/18 (11%)	0/18 (0%)	34.0 ± 6.1**
RR-EAE						
Control	0/8 (0%)	0/8 (0%)	4/8 (50%)	4/8 (50%)	1/8 (12%)	38.4 ± 8.4
Cortistatin	5/8 (62%)	3/8 (37%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	20.5 ± 6.6*

Chronic progressive EAE (CP-EAE) was induced in C57BL/6 mice by immunization with $MOG_{35–55}$, and relapsing and remitting EAE (RR-EAE) was induced in SJL/J mice by immunization with $PLP_{139–151}$. Immunized mice were treated i.p. for 5 d with PBS (control) or with cortistatin (1 nmol/day) starting at the onset of clinical signs (clinical score, 1). $n = 3$ independent experiments for CP-EAE; $n = 1$ experiment for RR-EAE.

[a]Disease incidence is graded as severe (clinical score, >4), moderate (clinical score, 2–4), mild (clinical score, <2), or none (no clinical signs) at day 36 for CP-EAE and at day 50 for RR-EAE.

[b]Clinical disease index (CDI), the mean of the sum of the daily disease scores.

$^*p < 0.005$, $^{**}p < 0.0001$ versus control.

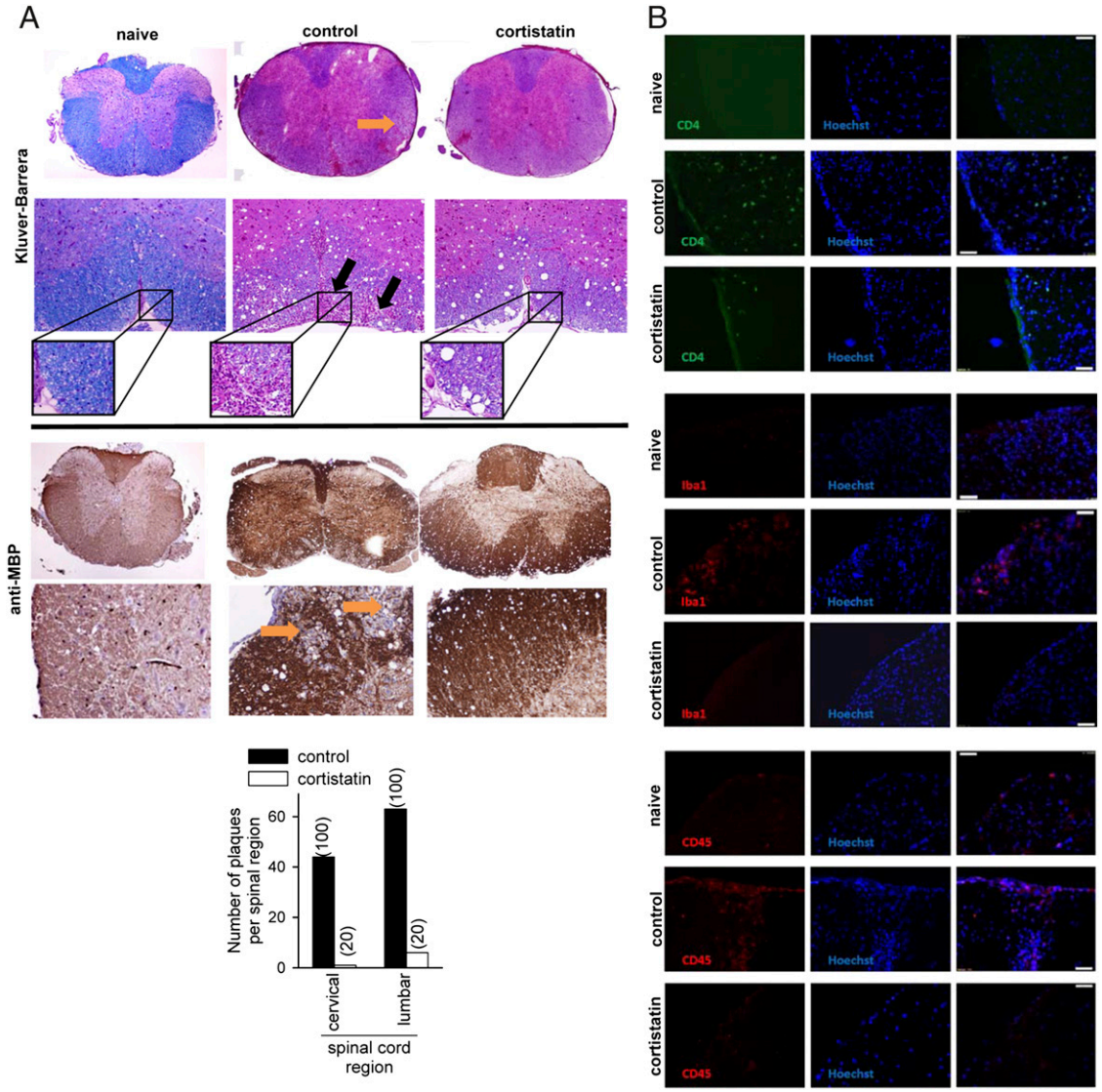

FIGURE 2. Cortistatin reduces inflammatory infiltration and demyelination in the CNS of mice with EAE. Mice with $MOG_{35–55}$–induced chronic EAE were treated with PBS (control) or cortistatin for 5 d starting at the disease onset (5/group, $n = 2$). Naive animals were used as negative controls. (**A**) Transverse sections of spinal cord randomly selected at the peak of clinical disease were stained with Klüver–Barrera (original magnification for upper images, ×40; middle images, ×100; detail, ×200) or immunostained for myelin content (anti-MBP, original magnification for upper images, ×40; lower, ×200). Arrows point to areas of demyelination and inflammatory infiltration. The mean number of plaques of demyelination in the lumbar and cervical regions, and the incidence of occurrence of plaques (in parentheses) were determined. (**B**) The phenotype of the infiltrating cells in the lumbar region of spinal cord was assayed by immunofluorescence for CD45, CD4, and Iba1. Scale bar, 50μm.

High levels of circulating Abs directed against myelin Ags invariably accompany the development of MS and EAE, and are major factors in determining susceptibility to the disease (3). Cortistatin regulated serum levels of MOG-specific IgG, particularly reducing the ratio between IgG2a and IgG1, generally reflective of Th1 and Th2 activities, respectively (Fig. 3E).

Cortistatin induces the emergence of functional Tregs in EAE

We next evaluated the capacity of cortistatin to generate Tregs during EAE, because Tregs confer significant protection against EAE by promoting Th2 protective responses and deactivating autoreactive T cells and their homing to CNS (22, 23). We found that cortistatin injection increased the percentage of IL-10–secreting CD4 cells and $CD4^+CD25^+Foxp3^+$ Treg in DLNs and spleens of mice suffering from EAE (Fig. 3F). This increase in Tregs could be functionally related with the suppression of encephalogenic responses and the induction of immune tolerance, because injection of T cells from spleen/DLNs of cortistatin-treated, but not of untreated EAE mice, into diseased mice alleviated their clinical signs (Fig. 3G). Experiments of cell depletion before transference suggested that the capacity to generate tolerance resides in the $CD4^+CD25^+$ Treg population (Fig. 3G, *right panel*). Moreover, injection of $CD4^+CD25^+$ T cells isolated from cortistatin-treated EAE mice into diseased mice significantly protected from EAE development, being more efficient cell by cell than $CD4^+CD25^+$ T cells isolated from untreated EAE mice (Supplemental Fig. 1C).

Cortistatin regulates CNS resident glial cells and promotes neuroprotective responses

We next investigated whether, besides its immunoregulatory activity, cortistatin exerts an active protective effect in the CNS directly acting on resident cells. We observed that cortistatin treatment significantly increased the expression of neuroprotective

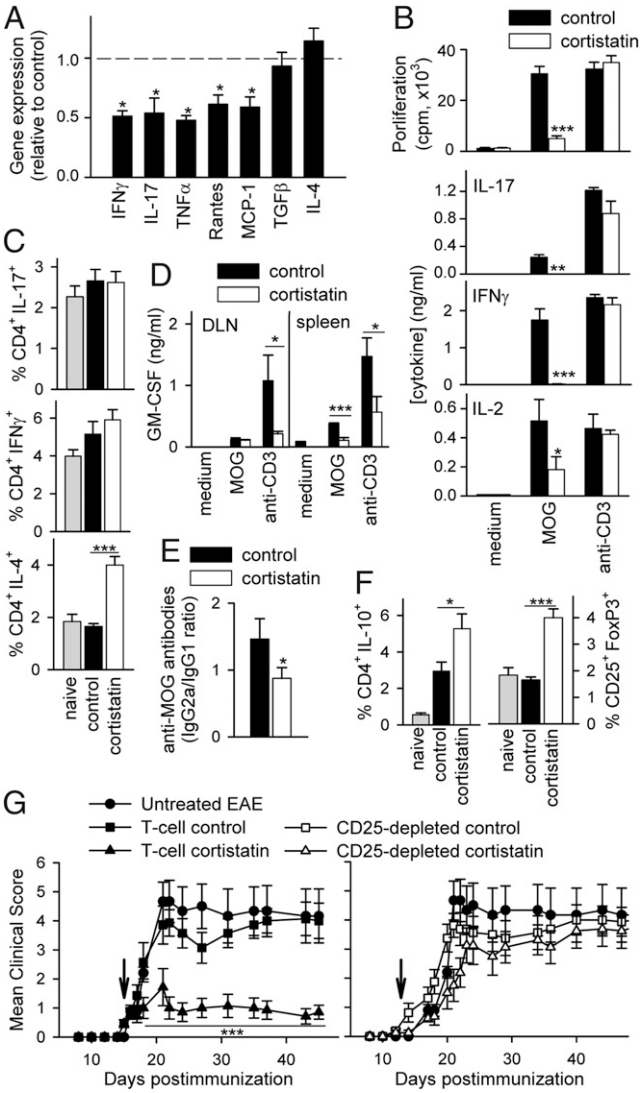

FIGURE 3. Cortistatin alleviates EAE severity by modulating both inflammatory and autoimmune components of the disease. Mice with $MOG_{35–55}$–induced chronic EAE were treated with PBS (control) or cortistatin for 5 d at the disease onset. (**A**) Expression of inflammatory cytokines and chemokines determined by RT-PCR in spinal cords at the disease peak (6/group, $n = 2$). (**B**) Cortistatin decreases peripheral encephalitogenic Th1/Th17 responses. Proliferation and cytokine production by DLN cells isolated at EAE peak and stimulated with medium, the encephalitogenic Ag ($MOG_{35–55}$), or a polyclonal stimulus (anti-CD3/CD28 Abs). We obtained similar results with spleen cells (6/group, $n = 2$). (**C**) Cortistatin affects Th1, Th17, and Th2 numbers in lymphoid organs. Spleen cells isolated at EAE peak were assayed for intracellular cytokine expression by flow cytometry in the CD4 population. We used naive mice as basal controls. We obtained similar results with DLNs (6–8/group, $n = 3$). (**D**) Cortistatin reduces GM-CSF production upon restimulation (6/group, $n = 2$). (**E**) MOG-specific IgG1 and IgG2a levels in sera collected at the disease peak (14/group, $n = 3$). (**F**) Cortistatin induces the emergence of Tregs in EAE. Percentage of $CD4^+IL\text{-}10^+$ and $CD4^+CD25^+Foxp3^+$ Tregs in DLN cells isolated at EAE peak (18–20/group, $n = 3$). (**G**) Treatment (arrow) of EAE mice with T cells or CD25-depleted CD4 cells isolated from spleen/DLNs of EAE mice that were previously treated with PBS (control) or cortistatin (7–9/group, $n = 2$). We used untreated EAE mice as reference. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0001$ versus control.

factors, such as BDNF and activity-dependent neuroprotector protein (ADNP), in the CNS of EAE mice (Fig. 4A), which are involved in processes of remyelination, axonal growth, and neurogeneration (24–26). Because neurotrophic factors (especially ADNP) may be produced by resident cells (neurons and glia) and infiltrating T cells (26), we investigated whether cortistatin exerted this effect at central and/or peripheral levels. Treatment with cortistatin increased ADNP levels in spleen/DLNs of EAE mice, mainly on MOG recall responses (Fig. 4B, *left* and *middle panels*). This effect could be mediated directly on T cells because cortistatin elevated ADNP expression in spleen/DLN cell cultures (Fig. 4B, *right panel*). Contrary to ADNP, we did not detect BDNF in spleen/DLNs of untreated or cortistatin-treated EAE mice (data not shown). Supporting its effect at the CNS level, cortistatin increased the expression of ADNP and BDNF in neuron-glia cocultures in both basal and inflammatory conditions (Fig. 4C).

Local production of cytotoxic factors by activated microglia and astrocytes in an inflammatory milieu critically contributes to the pathology of MS and EAE by inducing demyelination, oligodendrocyte loss, and axonal degeneration (1, 3). Cortistatin reduced the production of inflammatory cytokines such as TNF-α, and of IL-6 and NO by activated astrocytes and microglia, respectively (Fig. 4D). However, cortistatin did not affect significantly the production of inflammatory mediators by LPS-activated primary mouse DRG neurons (Fig. 4D) and rat PC12 neurons (data not shown). Notably, cortistatin decreased cell death induced by oxidative stress in precursor and mature oligodendrocytes (Fig. 4E). These data suggest that cortistatin could promote protective responses in EAE by inducing neurotrophic factors at both central and peripheral levels, by downregulating the destructive inflammatory response mediated by resident glial cells and directly avoiding oligodendrocyte loss.

Paradoxical effect of cortistatin deficiency in EAE and other inflammatory disorders

Once the protective action of cortistatin in EAE was established, we asked about the role played by endogenous cortistatin in the regulation of inflammatory and autoimmune responses in EAE. We found that the expression of cortistatin expression in the CNS of mice inversely correlated to the clinical severity of EAE (Fig. 5). As expected, macrophages, microglia, and lymphocytes deficient in cortistatin responded with exacerbated responses to inflammatory and T cell stimulation (Fig. 6A; Supplemental Fig. 2A), supporting an autocrine/paracrine effect of cortistatin. Surprisingly, the induction of EAE in $CST^{-/-}$ mice showed a paradoxical response. $CST^{-/-}$ mice challenged for the induction of EAE showed delayed disease onset (11.7 ± 0.8 d for wt versus 15.3 ± 1.1 d for $CST^{-/-}$, $p < 0.05$) and reduced clinical disease index (CDI) (CDI: 79.2 ± 10.3 for wt versus 39.9 ± 6.4 for $CST^{-/-}$, $p < 0.005$) compared with wt mice (Fig. 6B). Moreover, $CST^{-/-}$ mice showed less demyelination and spinal cell infiltration than wt mice, which correlated with decreased CNS expression of inflammatory mediators, especially chemokines, and elevated neurotrophic factors and Th2 cytokines in these animals (Supplemental Fig. 2B–D). However, mice that lacked somatostatin, a peptide structurally related with cortistatin, experienced development of exacerbated EAE signs (Supplemental Fig. 2E).

We next investigated whether the unexpected effect of cortistatin deficiency in the development of EAE was due to a defect in the autoreactive response. $CST^{-/-}$ mice generated similar levels of autoantibodies than wt mice, and immune cells from $CST^{-/-}$ mice with EAE strongly responded to MOG restimulation, even with higher Th1 and Th17 autoreactive responses (Fig. 6C; Supplemental Fig. 2F, 2G). The encephalitogenic capacity of T cells of $CST^{-/-}$ mice was confirmed in experiments of adoptive transfer of EAE to naive mice. Transfer of spleen/DLN cells from $CST^{-/-}$ mice restimulated ex vivo with MOG caused an EAE more severe and with an earlier onset in the recipient mice than

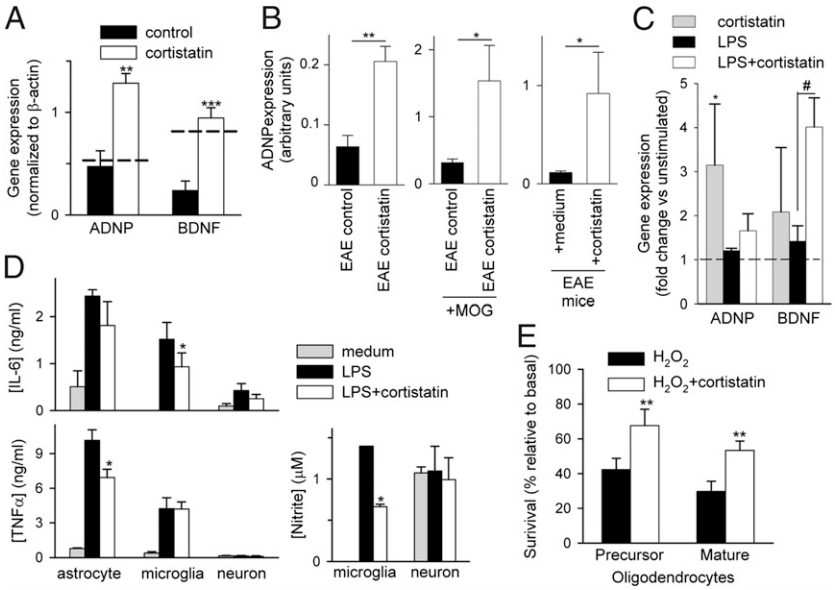

FIGURE 4. Cortistatin promotes neuroprotective responses in mice with EAE. (**A** and **B**) Cortistatin increases the production of neurotrophic factors in EAE. BDNF and ADNP gene expression (relative to β-actin) in spinal cords (A) and spleen/DLNs (B) isolated from chronic EAE mice treated with PBS (control) or cortistatin at disease onset. Dashed line in (A) corresponds to expression in naive mice. Spleen/DLN cells were restimulated ex vivo with MOG (B, *middle panel*) or incubated with 100 nM cortistatin (B, *right panel*) for 24 h. 4–5/group, $n = 2$. (**C**) Cortistatin induces the expression of neurotrophic factors in neuron-glial cocultures. Mixed neuron-glial cultures were stimulated with LPS with or without cortistatin (100 nM) for 6 h, and BDNF and ADNP expression was determined by real-time quantitative RT-PCR and expressed as fold change relative to unstimulated cells (dashed line). $n = 3$, in triplicates. (**D**) Cortistatin regulates the inflammatory response of CNS resident cells. Microglia and astrocytes isolated from naive newborn mice and sensory neurons isolated from adult mouse DRGs were cultured in medium or stimulated with LPS in the absence or presence of cortistatin (100 nM) and cytokine, and NO contents in culture supernatants were determined 24 h later. $n = 3$, in duplicates. (**E**) Cortistatin protects oligodendrocytes from oxidative-induced cell death. Cell survival of precursor and mature oligodendrocytes isolated from naive mice cultured for 24 h with 200 μM H_2O_2 in the absence or presence of 100 nM cortistatin. $n = 3$, in duplicates. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$ versus control or unstimulated cells; $^{\#}p < 0.05$ versus LPS-stimulated cells.

that induced by transfer of cells from wt mice (Fig. 6D). Interestingly, recipient $CST^{-/-}$ mice were mostly resistant to adoptive transfer of EAE with encephalitogenic cells, suggesting that the effector phase of the disease is affected (Fig. 6D).

We further studied whether this paradoxical effect observed in $CST^{-/-}$ mice was exclusive of EAE or it could be extended to other inflammatory conditions, in which cortistatin-based therapies were proved effective (8–10). $CST^{-/-}$ mice showed delayed disease onsets and initial lower severity in collagen-induced polyarthritis and experienced less severe chronic colitis than wt mice (Fig. 6E, Supplemental Fig. 3A). Similarly, lack of cortistatin significantly protected mice from sepsis (Fig. 6F). Despite septic $CST^{-/-}$ mice showing lower systemic inflammation and histopathologic signs than wt mice, macrophages isolated from both mice at the disease peak produced similar levels of IL-6, TNF-α, and IP-10 ex vivo (Fig. 6F, Supplemental Fig. 3B, 3C). Surprisingly and contrary to that observed in systemic inflammatory conditions, $CST^{-/-}$ mice were more susceptible to suffering local inflammation caused by intraplantar carrageenan injection (Supplemental Fig. 3D) and by monoarticular knee arthritis (Fig. 6G). These results indicate that cortistatin deficiency differentially affects systemic and local inflammatory responses. Although the intrinsic deficiency of cortistatin in immune cells predisposes to stronger self-reactive and inflammatory responses, a compensatory, probably systemic, mechanism impairs the development of EAE and other inflammatory disorders in these animals.

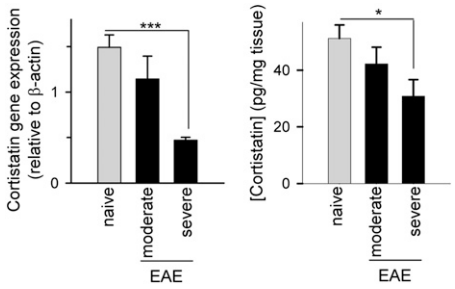

FIGURE 5. Cortistatin content in the CNS inversely correlates with EAE severity. Cortistatin expression (RNA and protein) in brains of naive and EAE mice with different clinical scores. $n = 5–8$, in duplicates. $^*p < 0.05$, $^{***}p < 0.0001$.

$CST^{-/-}$ mice show increased glucocorticoids, states of anxiety, and infection susceptibility

Characterizing the endocrine system of $CST^{-/-}$ mice, we found recently that lack of cortistatin significantly affects the functionality of the hypothalamic-pituitary-adrenal (HPA) axis (12). The systemic levels of the glucocorticoid corticosterone in $CST^{-/-}$ mice in basal conditions are markedly elevated (Fig. 7A). High glucocorticoid levels, as a consequence of an altered HPA axis, are frequently related to altered states of anxiety and immunosuppression (27). We evaluated the behavior of $CST^{-/-}$ mice in basal conditions and observed that $CST^{-/-}$ mice entered less and spent less time in the open arms of the elevated plus-maze, and explored longer in peripheral areas of the open field than did wt mice (Fig. 7B). Both behaviors are indicative of states of anxiety. Moreover, we found that the systemic immunosuppressive state observed in $CST^{-/-}$ mice increased susceptibility to infection. In a model of cutaneous leishmaniasis, $CST^{-/-}$ mice had higher edema and parasite burden in the infected paw than wt mice and showed cutaneous lesions (Fig. 7C).

We finally investigated the effect of blocking the glucocorticoid system in the response of $CST^{-/-}$ mice to EAE and sepsis. Sys-

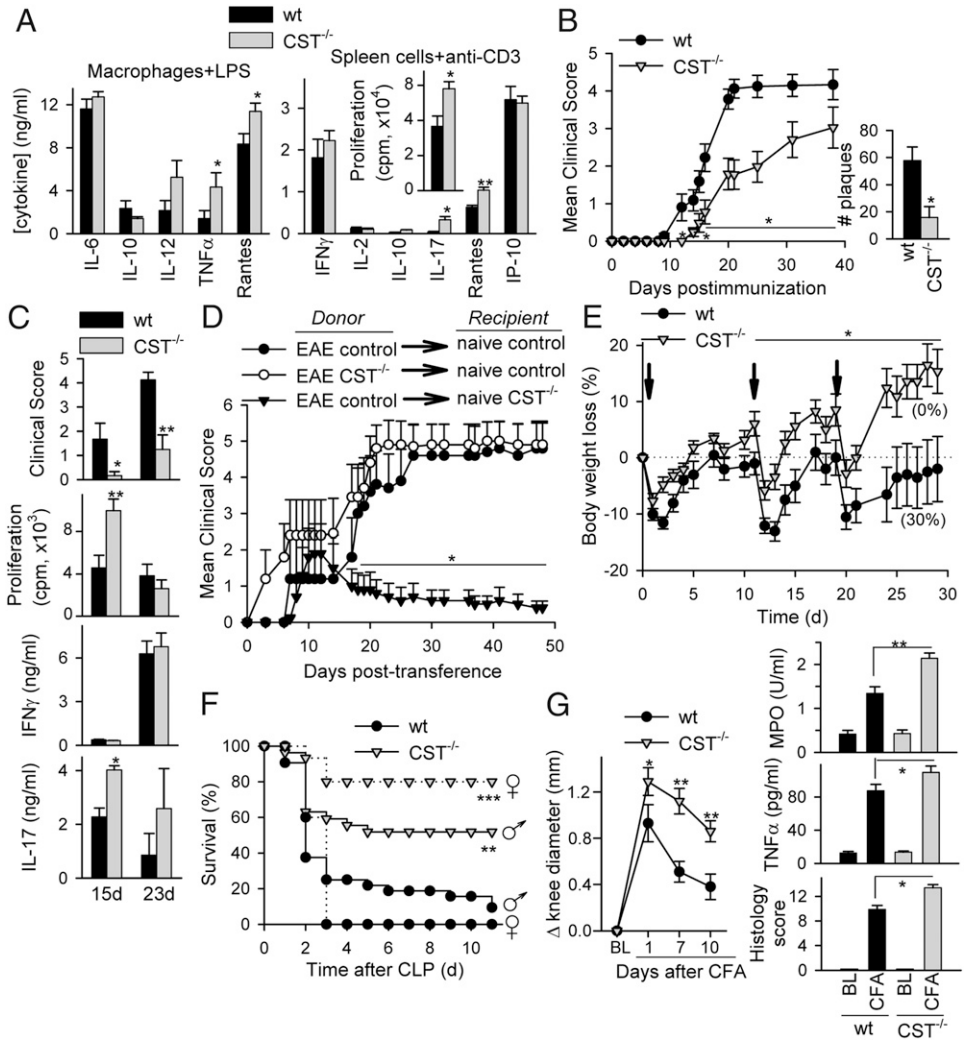

FIGURE 6. Paradoxical effect of cortistatin deficiency in EAE and other inflammatory diseases. (**A**) Proliferation (inset) and cytokine production by macrophages and spleen cells (similar results with lymph node cells) isolated from naive wt or CST$^{-/-}$ mice stimulated with LPS or anti-CD3/CD28 Abs (8–13/group, $n = 2$). (**B**) Clinical evolution of MOG-induced chronic EAE in wt and CST$^{-/-}$ mice (25–35/group, $n = 4$). The number of demyelinating plaques per animal was evaluated at day 23. (**C**) Proliferation and cytokine production by DLN cells (similar results with spleen cells) isolated at various times from wt and CST$^{-/-}$ mice (four per group) suffering chronic EAE restimulated with MOG_{35-55}. Clinical score shows the state of mice before cell isolation. (**D**) Clinical evolution after adoptive transfer into naive wt or CST$^{-/-}$ mice (5–10/group, $n = 2$) of spleen/DLN cells isolated from wt and CST$^{-/-}$ mice at the EAE peak and restimulated ex vivo with MOG. (**E**) Body weight loss and mortality (in parentheses) after repetitive intrarectal 2,4,6-trinitrobenzene sulfonic acid infusions (arrows) to wt and CST$^{-/-}$ mice (10/group). (**F**) Mortality by sepsis caused by CLP in wt and CST$^{-/-}$ mice (27–35/group, $n = 3$, males and females evaluated separately). (**G**) Knee arthritis was induced in wt and CST$^{-/-}$ mice (6/group), and disease evolution was followed by measuring knee swelling (diameter), MPO activity, and TNF-α content in synovial fluids (at 24 h) and histopathology (inflammatory infiltration, synovial growth, cartilage/bone erosion) in knee sections (at 10 d). $*p < 0.05$, $**p < 0.005$, $***p < 0.0001$ versus wt mice.

temic injections of the glucocorticoid receptor antagonist RU-486 partially reversed the phenotype observed in CST$^{-/-}$ mice in both conditions (Fig. 7D). However, pre-emptive cortistatin injection did not reverse this resistance, probably because cortistatin treatment is itself protective (Supplemental Fig. 3E).

Discussion

The initial stages of MS and EAE involve multiple steps that can be divided into two main phases: early events associated with initiation and establishment of autoimmunity to myelin sheath components, and later events associated with the evolving destructive inflammatory responses. Progression of the autoimmune response involves the development of reactive Th1 and Th17 cells with encephalitogenic potential, their entry into the CNS, and further recruitment of inflammatory cells through multiple mediators (1, 3). In this study, we report that the neuropeptide cortistatin provides a highly effective therapy for chronic and relapsing-remitting EAE. The therapeutic effect of cortistatin is associated with a striking reduction of the two deleterious components of the disease, namely, the autoimmune and inflammatory responses. As a consequence, cortistatin reduced the appearance of inflammatory infiltrates in the CNS and the subsequent demyelination and axonal damage typical of EAE.

Our data indicate that treatment with cortistatin decreased the presence of encephalitogenic Th1 and Th17 cells in the periphery and CNS. This effect is mostly exerted by regulating the encephalitogenic sensitization in the peripheral immune compartment. Importantly, this treatment did not result in a general immunosuppression. Cortistatin did not affect the number of Th1 and Th17 cells in lymphoid organs or the response to a polyclonal stimulation, but it specifically impaired the activation of MOG-specific Th1 and Th17 responses; at the same time, it favored

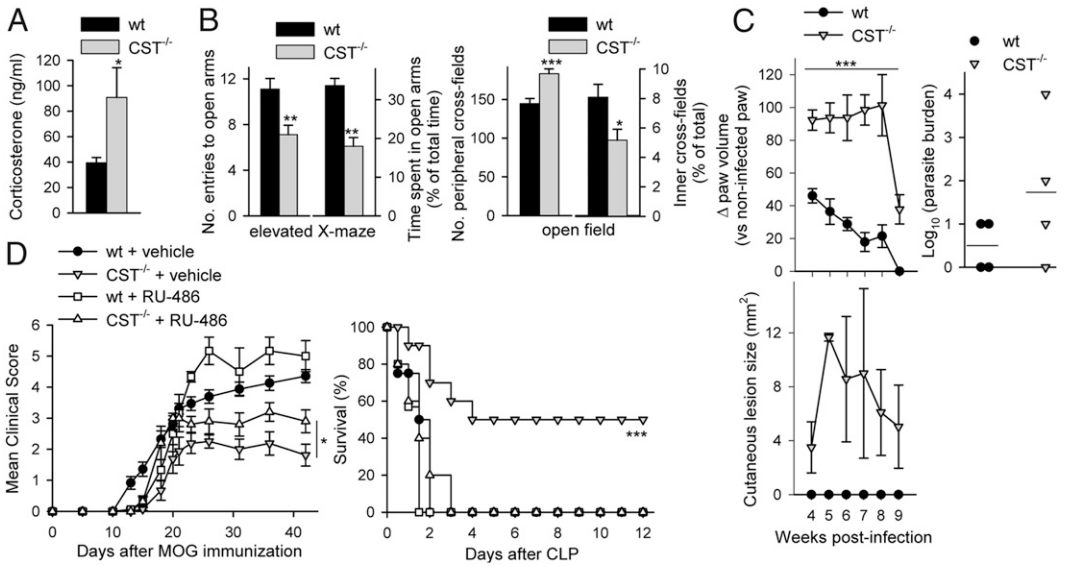

FIGURE 7. $CST^{-/-}$ mice have elevated circulating glucocorticoids, a chronic state of anxiety, and increased susceptibility to infection. (**A**) Serum corticosterone levels in wt and $CST^{-/-}$ mice (five per group). (**B**) $CST^{-/-}$ mice show a chronic state of anxiety. $CST^{-/-}$ and wt mice (10–19/group, $n = 2$) were subjected to two behavioral tests: the elevated plus maze that measures the number of entries into and the time spent on the open arms (for 5 min), and the open field that determines the time exploring peripheral or central zones (for 3 min). (**C**) Susceptibility to leishmaniasis in $CST^{-/-}$ mice. wt and $CST^{-/-}$ mice (10–14/group) were infected s.c. with *L. major* in the plantar hind paw. Disease progression was followed by measuring paw swelling (increase of volume versus noninfected paw) and lesion size at different times, and by determining the parasite burden in the paw at the end of the study. (**D**) Effect of the glucocorticoid receptor antagonist RU-486 in the progression of EAE (18–26/group, $n = 3$) and sepsis (16–22/group, $n = 3$) in wt and $CST^{-/-}$ mice. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$.

Th2 responses, which is reflected by a class switch in autoantibodies. The potent suppressive effect of cortistatin on the activation of encephalitogenic Th17 cells might be an important component in its protective effect on EAE, because of the critical role that Th17 cells play in the disease effector phase in EAE and MS (28, 29). Of relevance is also the fact that cortistatin downregulated the production of GM-CSF by activated T cells, because it serves a nonredundant function in the initiation of autoimmune inflammation in EAE regardless of Th cell polarization (21).

The increase in the repertory of $CD4^+CD25^+Foxp3^+$ Tregs in lymphoid organs could partially explain the specificity of Ag of the long-lasting protective response generated by cortistatin treatment, and that cortistatin injection inhibits events in the inflammatory phase of EAE after the activation/differentiation of Ag-specific effector Th1/Th17 cells. In agreement with our results, a recent study described the generation of $CD4^+CD25^+$ Tregs by cortistatin in a model of allogeneic skin transplantation (30). However, to our knowledge, our work is the first to demonstrate the involvement of functional Tregs in the therapeutic action of cortistatin in autoimmunity. Further investigation will determine whether this increase in Tregs is a consequence of an effect of cortistatin on the expansion of already existing Tregs or on de novo generation of peripheral Tregs. Other neuropeptides, such as vasoactive intestinal peptide, urocortin, and adrenomedullin, with protective effects in autoimmunity (31), increased the repertory of Tregs by promoting their generation from the non-Treg compartment through direct actions on T cells and indirectly on tolerogenic dendritic cells (31). It remains also unknown whether the population of IL-10–secreting CD4 T cells increased by cortistatin corresponds to a subtype of Tr1-like cells (32) and whether it plays any role in its protective effect in EAE.

Regarding the inflammatory response in EAE, it is evident that the regulation of a wide spectrum of inflammatory mediators by cortistatin could suppose an advantage over other therapies directed against a single mediator. The reduction in the inflammatory infiltration of CNS observed in the cortistatin-treated EAE mice seems to be associated with the decrease in the levels of chemokines in CNS parenchyma. This is especially relevant for chemokines such as IP-10 (chemotactic for Th1 cells), Rantes (for T cells), and MCP-1 (for macrophages and T cells) that contribute to MS neuropathology (1, 3). Interestingly, cortistatin did not decrease MDC-1, which is chemotactic for Th2 cells. As a consequence, cortistatin increased the rate of Th2 versus Th1/Th17 cytokines in the CNS of EAE mice, which is protective for the disease. Whether the effect on chemokines is exerted at the local level or as a consequence of the peripheral action of cortistatin (i.e., on Th17 or GM-CSF) is unknown. The fact that cortistatin failed to inhibit Rantes secretion by activated glial cells in vitro argues against a local effect of cortistatin in the chemokine repertory. However, we found that cortistatin might exert its anti-inflammatory action locally by downregulating the production of cytokines and NO by astrocytes and microglia. This effect on resident inflammatory cells, together with the inhibitory action of cortistatin on infiltrating macrophages (9), probably contributes to the protection against oligodendrocyte/neuronal loss and axonal damage in this inflammatory milieu.

However, attention has recently focused on regenerative mechanisms as targets for therapy in MS, especially in the secondary progressive phase of the disease. Notably, the delayed treatment with cortistatin induced a whole recovery in a significant number of animals, suggesting a role of cortistatin in repair, neuroregeneration, or both. In this article, we found that cortistatin protected oligodendrocytes from cell death in an oxidative milieu as occurs in EAE. Moreover, cortistatin increased the local levels of BDNF and ADNP, which induce axonal outgrowth, remyelination, and rescue of degenerating neurons (24, 25). For ADNP, this effect is exerted on both peripheral lymphoid cells and resident CNS cells. However, the increase in BDNF could mainly depend on a central action of cortistatin on neuron-glia cells.

Which is the role of endogenous cortistatin in this scenario? Evidence suggests that cortistatin is an endogenous immunomodulator: immune cells produce cortistatin in response to inflammatory/immune stimulation (11); cortistatin produced by macrophages and lymphocytes plays an autocrine/paracrine regulatory role in the immune response, as lack of cortistatin predisposes to stronger response to immunostimulation (Fig. 6A); an inverse correlation exists between EAE severity and the levels of cortistatin in the CNS (Fig. 5); and a deficiency of cortistatin in the retina of diabetic patients with retinopathy correlated with increased retinal neurodegeneration and glial activation (33). These findings suggest that endogenous cortistatin might normally provide protection against autoimmune and neurodegenerative pathologies. Indeed, we found exacerbated local inflammatory responses in $CST^{-/-}$ mice. However, lack of cortistatin surprisingly conferred certain resistance to EAE and other systemic inflammatory pathologies, with delayed onset and mild clinical profile. Despite this, the levels of autoantibodies and Ag-rechallenge experiments indicated that MOG-treated $CST^{-/-}$ mice exhibited robust Th1/Th17 cell responses. Moreover, adoptive transfer of the disease with lymphocytes from immunized $CST^{-/-}$ mice to naive recipients supports their encephalitogenic potential. This suggests a compensatory mechanism in $CST^{-/-}$ mice other than an intrinsic defect in immune cells. Although unpredictable, the case of cortistatin is not unique. Mice that lacked the immunomodulatory neuropeptide vasoactive intestinal peptide were almost completely resistant to EAE (34), although they generate Th1 with stronger encephalitogenic capacity than wt mice.

We present evidence that supports the partial involvement of an altered glucocorticoid system in the paradoxical phenotype observed in $CST^{-/-}$ mice challenged through induction of EAE and other pathologies. Glucocorticoids are well-known immunosuppressive factors with widespread actions in different components of the immune system. The elevated levels of corticosterone found in $CST^{-/-}$ mice could partially explain their resistance to systemic immune responses. These abnormal glucocorticoid levels might be consequence of an altered HPA axis (i.e., elevated adrenocorticotropic hormone [ACTH] levels) (12), which is reflected by an exacerbated anxiety-like behavior in these animals. Numerous studies correlate stress to states of immunosuppression and alteration in the HPA axis (27). Indeed, $CST^{-/-}$ mice were more susceptible to infection by the intracellular parasite *L. major*, which depends on fully functional Th1-driven inflammatory responses to be eliminated. How cortistatin regulates this neuroimmune axis is still unknown. However, evidence indicates that cortistatin could act at multiple levels as an endogenous brake for the HPA axis. Thus, systemic injection of cortistatin decreased the levels of ACTH and cortisol in patients with Cushing disease (35), and it inhibited ACTH production in murine and monkey pituitary cultures (12), supporting a direct pituitary effect. However, the fact that cortistatin inhibited the secretion of corticotropin-releasing hormone (CRH) by tissue cultures of hypothalamus and hippocampus (36), two brain areas where CRH functions are associated with the control of endocrine HPA-mediated responses and with the pathogenesis of anxiety/depressive disorders, respectively, supports its action at these brain levels. Moreover, because cortistatin is produced by hippocampal interneurons (7), a possible interplay between CRH and cortistatin is expected. Besides glucocorticoids, we cannot rule out the involvement in the establishment of the phenotype observed in $CST^{-/-}$ mice of other factors related to stress and anxiety, namely, adrenergic and cholinergic systems (27, 37–40), especially considering that cortistatin is a negative regulator of acetylcholine in the brain (5, 7). Moreover, the anti-inflammatory ghrelin and the proinflammatory prolactin are elevated and decreased, respectively, in $CST^{-/-}$ mice (12). Besides a direct effect on the immune response (41), an elevated ghrelin level could affect the activity of HPA axis in $CST^{-/-}$ mice because ghrelin stimulates the production of hypothalamic CRH and pituitary ACTH (42, 43).

In summary, we are proposing a novel treatment strategy for MS that is targeted to the inhibition of the different neuropathological components of the disease, whereas restoring long-lasting immune tolerance and mounting an active program of neuroprotection. This multimodal action would suppose a therapeutic advantage versus current treatments. Its ability, on delayed administration, to ameliorate the ongoing disease also fulfills an essential prerequisite for a therapeutic agent for MS. This work also demonstrates that cortistatin from the immune source plays a critical role in the tuning of the immune responses in health and disease, and that cortistatin is a key player in the bidirectional communication that exists between the neuroendocrine and immune systems, which together define the final immune response of our body.

Acknowledgments

We thank Dr. C. Guaza (Institute Cajal, Consejo Superior Investigaciones Cientificas, Madrid, Spain) for guidance in the isolation of glial cells and Dr. M. Delgado (Institute of Parasitology and Biomedicine, Consejo Superior Investigaciones Cientificas, Granada, Spain) for assistance in the induction of the experimental animal models.

Disclosures

The authors have no financial conflicts of interest.

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