DEPARTMENT OF ANATOMY
AND LEGAL MEDICINE
UNIVERSITY OF MÁLAGA

DEPARTMENT OF BASIC MEDICAL SCIENCES
HASSELT UNIVERSITY
BIOMEDICAL RESEARCH INSTITUTE

MEDITERRANEAN INSTITUTE FOR THE
ADVANCE OF BIOTECHNOLOGY AND
HEALTH RESEARCH FOUNDATION

Ph. D. Dissertation

Defective myelination in mice lacking the lysophosphatidic acid receptor LPA1: newcomer models for demyelinating and neuroinflammatory diseases.

Beatriz García Díaz

Málaga, 2010

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Ph. D. Dissertation of BEATRIZ GARCÍA DÍAZ to obtaining the joint academic degree of Doctor at University of Málaga and University of Hasselt.

Málaga 2010

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INVESTIGACIÓN BIOSANITARIA

Defective myelination in mice lacking the lysophosphatidic acid receptor LPA_1: newcomer models for demyelinating and neuroinflammatory diseases.

Memoria de Tesis presentada por Dª BEATRIZ GARCÍA DÍAZ para optar al grado de Doctor por la Universidad de Málaga conjunto con la Universidad de Hasselt

Málaga 2010

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GUILLERMO ESTIVILL TORRÚS (Ph. D.), Senior Researcher from IMABIS Foundation and OSCAR FERNÁNDEZ FERNÁNDEZ (M.D.), Director of the Clinical Neurosciences Institute and Head of Neurology Service at Carlos Haya Hospital, as promoters,

CERTIFY that:

BEATRIZ GARCÍA DÍAZ has carried out the present research work entitled: "*Defective myelination in mice lacking the lysophosphatidic acid receptor LPA_1: newcomer models for demyelinating and neuroinflammatory diseases*" in the IMABIS Foundation under our supervision as Ph.D. work, judging it as deserving of be presented to obtain the degree of Doctor by its scientific merit and rigor. The work has been performed by agreement between the University of Málaga and the University of Hasselt in order to obtain the joint diploma of the degree of Doctor

And for the record, we hereby issue this, at Málaga, at the 1st of February 2010.

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Que Dª BEATRIZ GARCÍA DÍAZ ha realizado en la Fundación IMABIS y bajo nuestra dirección el presente trabajo de investigación titulado: "*Defective myelination in mice lacking the lysophosphatidic acid receptor LPA_1: newcomer models for demyelinating and neuroinflammatory diseases*", y que ha sido objeto de su Tesis Doctoral, considerando que reúne el rigor científico y las condiciones necesarias para optar al grado de Doctor.

Dicho trabajo, a objeto de la obtención del doctorado conjunto ha sido realizado, además, en régimen de cotutela, por convenio, entre la Universidad de Málaga y la Universidad de Hasselt.

Y para que así conste, firmamos el presente certificado en Málaga a 1 de Febrero de 2010.

Fdo. Guillermo Estivill Torrús

Fdo. Oscar Fernández Fernández

UNIVERSIDAD DE MÁLAGA

VICTOR M. SMITH-FERNÁNDEZ (M. D.), Professor in the Department of Anatomy and Legal Medicine at the University of Málaga, tutor,

CERTIFIES that:

The present Ph.D. research work carried out by BEATRIZ GARCÍA DÍAZ and entitled: "*Defective myelination in mice lacking the lysophosphatidic acid receptor LPA_1: newcomer models for demyelinating and neuroinflammatory diseases*", fulfilled beyond the requisites to obtain the degree of Doctor by its scientific content and rigor. The work has been performed by agreement with the University of Hasselt in order to obtain the joint diploma of the degree of Doctor

And for the relevant purposes I hereby certify and sign off in Málaga, at the 1st of February 2010.

Signed: Victor M. Smith

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D. VICTOR M. SMITH-FERNÁNDEZ, Doctor en Medicina y Cirugía, y Profesor Titular del Departamento de Anatomía y Medicina Legal de la Universidad de Málaga, actuando como tutor del presente trabajo,

CERTIFICA:

Que el presente trabajo realizado por Dª BEATRIZ GARCÍA DÍAZ, objeto de su Tesis Doctoral, y titulado: "*Defective myelination in mice lacking the lysophosphatidic acid receptor LPA_1: newcomer models for demyelinating and neuroinflammatory diseases*", reúne el contenido y rigor científico necesarios para optar al grado de Doctor. Dicho trabajo, a objeto de la obtención del doctorado conjunto ha sido realizado, además, en régimen de cotutela, por convenio entre la Universidad de Málaga y la Universidad de Hasselt.

Y para que así conste, firmo el presente certificado en Málaga a 1 de Febrero de 2010.

Fdo. Victor M. Smith-Fernández

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NIELS HELLINGS (Ph. D.), Vice-director and Assistant Professor at Biomedical Research Institute, University of Hasselt, as promoter,

CERTIFIES that:

BEATRIZ GARCÍA DÍAZ has carried out a stage at Biomedical Research Institute in the University of Hasselt under my supervision and as part of the present Ph.D. research work entitled: "*Defective myelination in mice lacking the lysophosphatidic acid receptor LPA_1: newcomer models for demyelinating and neuroinflammatory diseases*", judging it as deserving of be presented to obtain the degree of Doctor by its scientific merit and significance. The work has been performed by agreement between the University of Málaga and the University of Hasselt in order to obtain the joint diploma of the degree of Doctor.

And so I hereby certify, in Málaga, at the 1st of February 2010

Signed: Niels Hellings

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NIELS HELLINGS, Vice-director y Profesor Adjunto del Instituto de Investigación Biomédica de la Universidad de Hasselt , como director,

CERTIFICA:

Que Dª BEATRIZ GARCÍA DÍAZ ha realizado en el Instituto de Investigación Biomédica de la Universidad de Hasselt y bajo su dirección una estancia destinada a la realización de diferentes tareas de investigación constitutivas como parte del presente trabajo de investigación titulado: "*Defective myelination in mice lacking the lysophosphatidic acid receptor LPA_1: newcomer models for demyelinating and neuroinflammatory diseases*", que ha sido objeto de su Tesis Doctoral, estimando que reúne las condiciones y requisitos necesarias para optar al grado de Doctor.
Dicho trabajo, a objeto de la obtención del doctorado conjunto ha sido realizado, además, en régimen de cotutela, por convenio, entre la Universidad de Málaga y la Universidad de Hasselt.

Y para que así conste, firmo el presente certificado en Málaga a 1 de Febrero de 2010.

Fdo. Niels Hellings

I BEATRIZ GARCÍA DÍAZ do hereby declare, that the present Ph.D. research work entitled "*Defective myelination in mice lacking the lysophosphatidic acid receptor LPA_1: newcomer models for demyelinating and neuroinflammatory diseases*" is the outcome of the original research work undertaken and carried out by me under the guidance and supervision of Dr Guillermo Estivill Torrús and Dr Oscar Fernández, at the IMABIS Foundation, acting as tutor from the University of Málaga Dr Victor M. Smith-Fernández from the Department of Anatomy and Legal Medicine at the Faculty of Medicine, as well as at Biomedical Research Institute, University of Hasselt, under supervision of Dr Niels Hellings, with exception of the magnetic resonance spectroscopy, completed at Non-invasive Neurofunctional Evaluation Service of the Alberto Sols Biomedical Research Institute in Madrid, and supervised by Dr Isabel Varela Nieto of the Hearing Neurobiology Research Group.

And so I sign in Málaga, at the 1st of February 2010

Signed: Beatriz García Díaz

Yo, BEATRIZ GARCÍA DÍAZ, declaro que soy autora del presente trabajo de investigación original, a objeto de la realización de tesis doctoral, titulado "*Defective myelination in mice lacking the lysophosphatidic acid receptor LPA_1: newcomer models for demyelinating and neuroinflammatory diseases*", y que lo he realizado en la Fundación IMABIS de Málaga, bajo la dirección del Dr. Guillermo Estivill Torrús y del Dr. Oscar Fernández, actuando como tutor por la Universidad de Málaga el Dr. Victor M. Smith-Fernández, del Departamento de Anatomía y Medicina Legal de la Facultad de Medicina, así como en el Instituto de Investigación Biomédica de la Universidad de Hasselt, en Bélgica, bajo la dirección del Dr. Niels Hellings, a excepción de las pruebas de determinación por espectroscopía por resonancia magnética, realizadas respectivamente en el Servicio de Evaluación Neurofuncional No Invasiva del Instituto de Investigaciones Biomédicas "Alberto Sols" de Madrid por el Grupo de Neurobiología de la Audición de dicho centro dirigido por la Dra. Isabel Varela-Nieto.

Y para que así conste, firmo el presente certificado en Málaga, a 1 de Febrero de 2009.

Fdo. Beatriz García Díaz

Dedicada a mis padres y mis abuelos;
por haberme hecho estar siempre orgullosa de mis raíces,
por creer en mí y por apoyarme en todo momento
incluso cuando no entendían mis absurdos sueños.

Agradecimientos

Desde que el final de esta tesis se empezó a vislumbrar he estado pensado en cómo escribir esta parte. Para mí, esta es la más importante de este trabajo, porque sin todos aquellos que me han rodeado estos cuatro años, dentro y fuera del laboratorio, no hubiese sido capaz de llevarlo a buen puerto.

En primer lugar me gustaría agradecerle a toda mi familia el apoyo y la fé que han tenido en mí desde el principio, incluso cuando la mía propia flaqueaba. Todos sin excepción me han ido dando lecciones de fortaleza, espíritu de trabajo y sacrificio, sin los cuales casi ninguna tarea puede llegar a concluirse con éxito. Debo agradecer a mis abuelos por enseñarme que nunca se ha de tirar la toalla y que siempre se puede poner una sonrisa hasta en los peores momentos. A mis padres, porque aún no compartiendo este "inseguro" camino siempre han estado ahí para todo lo que necesitara: un abrazo, una mano o un empujón. Y a mi hermana y a todo el resto de la familia por no haber dudado de que este día llegaría.

También quiero agradecer a mi familia por extensión: Sandra, Juan Antonio y los peques, por concederme uno de los mayores honores de mi vida, ser madrina de su primogénito, y por abrirme siempre sus puertas cuando los días pintaban demasiado grises; por sus ánimos y sus consejos, y un sinfín de anécdotas juntos.

A todos aquellos que fuera del laboratorio me han acompañado en las risas y en las lágrimas, que me han dado consejo y aliento; y que han seguido a mi lado hasta en los peores momentos donde las alegrías escaseaban. Al trío del Máster, que estos últimos días, a pesar de los agobios, han llenado las tardes de carcajadas, por su ayuda y su comprensión.

Pero también sé que puedo sentirme muy afortunada por mi ambiente de trabajo, por eso no puedo dejar de agradecer a las "niñas" de microscopía, por hacer que dos metros cuadrados sin ventanas puedan convertirse en una caja de risas y compañerismo, convirtiendo la rutina diaria en un placer. Y a todos los miembros de Imabis, por siempre tender una mano para ayudar, aunque sea para quemarse con el nitrógeno líquido, ahogarse en las bases de datos o abandonar lo suyo para explicar cómo funciona un aparato nuevo. A todo el grupo de NFTL y Neurología por ofrecer siempre su ayuda y colaboración sea cuál sea el motivo.

Sin embargo, mi suerte rebasó fronteras; gracias a Patricia que siempre me ha animado a confiar en mi inglés, y a Cristina por ser la primera en venir a rescatarme cuando aún me estaba adaptando. Nunca podré dejar de agradecer a todos aquellos que compartieron mi estancia en Hasselt. Empezando por Niels, quien me dio la oportunidad de vivir una de las mejores experiencias de mi vida, por recogerme en esa estación y acogerme desde entonces. A todos y cada uno de los miembros de uHasselt, porque nunca me sentí sola a 2000 km de casa, por todas la risas, las conversaciones académicas y no académicas, y por todas las invitaciones y ayuda que me proporcionaron desde que pisé la

ciudad hasta que la despedí. A nuestra pandilla internacional, donde colombianos, chilenos, hindúes, italianos, franceses, rusos, belgas y españoles compartíamos nuestro amor por la ciencia y por otras muchas actividades; gracias a todos por hacer que ni el mal tiempo nublara esos meses. Y ante todo me gustaría agradecer a Ambily, por compartir penas y glorias, risas y enfados, y demostrar a todos que puedes encontrar tu alma gemela incluso al otro lado del planeta.

Y volviendo de nuevo a España, no podría dejar de agradecer a mis directores de tesis y mi tutor, Guillermo, Oscar, Niels y Víctor; por confiar en mí y guiarme en estos años. En especial a Guillermo, por aguantar mis nervios e impaciencias y demostrarme que, al final, todo llega. Sin olvidar que este trabajo no hubiese sido posible sin la financiación de la Fundación Ramón Areces, con su convocatoria para la realización de una tesis doctoral.

Y dejando lo mejor para el final, quiero agradecer con todo mi corazón a Jesús, por apoyarme, guiarme, inspirarme y quererme estos últimos años; sin ti este día perdería su valor.

Dedicated to my parents and grandparents;
for making me proud of my roots and believing in me.
For giving me all the support even when
they did not understand my absurd dreams.

Gratitude

Since the end of this thesis was seen, I was thinking of how I was going to write this part. This is the most important part of the work because without all the support during these four years, inside and outside of the laboratory, I would not have been able to carry on.

First of all I would like to thank my whole family, for all the support and faith they have had on me since the very beginning, even when my own was breaking. Each of them has given me lessons of strength, work and sacrifice, without them no task can be successful. I must thank my grandparents for teaching me that I should never give up and I should always have a smile even in the worse moments. To my parents because, although they did not always understand my way, they have always been there for me: for a hand, a hug or a lift. To my sister and the rest of my family because they had no doubt about the arrival of this day.

I also want to thank to my "other family": Sandra, Juan Antonio and the little ones, for honouring me with my godson, and for cheering me up during the worse moments: their advices and great times together.

To all my friends outside the laboratory who were always by me, even in the moments of few smiles, who made me laugh and encourage me to keep going, To my three friends from the Masters that also gave me their support and comprehension.

But also I must feel so fortunate for my working environment, so I must thank the "girls" from microscopy, for making two square meters with no windows be a box of laughs and brotherhood, turning routine into pleasure. And to all the members of Imabis, for always helping me, no matter if it was to be burnt by liquid nitrogen, to dig into databases, or leave their work to explain how the new machines work. To the whole group of NFTL and Neurology to help me and collaborate with me no matter the reason.

Nevertheless, my luck has gone beyond borders; thanks to Patricia that always help me to believe in my English, and Cristina, the one who came to rescue me those first days when I was still afraid of that new world. Furthermore, I could never be grateful enough to those who shared my stay in Hasselt. Niels who gave me the opportunity to have one of the best experiences of my life, thanks for picking up at that station and taking care of me since then. To each one of the members of uHasselt, because I never felt lonely even when I was 2000 km away from home, for all the laughs, academic and not academics chats, and for all the invitations and help that offered me from the moment I arrived to the last one. To our international gang, where Colombians, Chilean, Indians, Italians, French, Russian, Belgians and Spanish shared our passion for Science and for many other activities. Thanks to one and each of them for the best time ever. Ambily, I especially thank you for sharing everything with me, and showing me that you can always find your soul mate even if she comes from the opposite side of the earth.

And coming back to Spain, I must thank my thesis promoters and copromotors: Guillermo, Oscar, Niels and Victor, for trusting in me and guiding me all these years. Especially, I would like to thank Guillermo to support everything and show me that, at the end, you can get anything. This work would not have been possible without the funding from Ramon Areces Foundation.

And I always leave for the end the best, I really want to thank from the bottom of my heart, Jesus, for giving me all the support, inspiring and loving me all these years. Without you, this day would be meaningless.

INTRODUCTION

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1. MULTIPLE SCLEROSIS.

1.1. Overview of the disease.

Multiple sclerosis (MS) is a recurrent inflammatory/autoimmune demyelinating disease of the white matter in the central nervous system (CNS), which usually becomes progressive. In the mid-19th century, MS was a neurological curiosity worthy of single case reports in the daily journals. Even Charcot, in his early descriptions of the disease, said that it seemed rare. At the turn of the 21st century, the disease was more frequently diagnosed when the clinical variations were better understood. Nowadays, MS is recognized as the most common serious neurological disease in young adults living in temperate climates, and has become a high contributor to healthcare spending.

MS is a neurological disease which usually affects young adults. It begins very often with episodic attacks of neurological symptoms, but years later, it enters a progressive phase. Usually, the first episode appears between the ages of 15 and 50 (average age 30), and occurs in about 1 out of 1000 individuals of European ancestry living in temperate climates (Pugliatti et al., 2006). According to actual data, MS occurs because of an interaction between genetic predisposition and environmental factors which "trigger" the disease.

People affected with MS can suffer almost any neurological symptom or sign, among them there might be sensation changing (hypoesthesias and paraesthesias), muscle weakness, muscle spasms, difficulty in moving (Freeman JA, 2001), difficulties with coordination and balance (ataxia) (Freeman JA, 2001), problems in speech (dysarthria), swallowing (dysphagia) (Merson and Rolnick, 1998), visual problems (nystagmus, optic neuritis, or diplopia) (Kaur and Bennett, 2007), fatigue, acute or chronic pain (Pöllmann and Feneberg, 2008; Henze, 2005), bladder and bowel difficulties (Henze, 2005; Andrews and Husmann, 1997), cognitive impairment of varying degrees and emotional symptoms of depression or unstable mood (Bobholz and Rao, 2003; de Seze et al, 2006).

It is important to note that MS has a little effect on life expectancy, however, there is a significant effect on the person's quality of life even in the early stages of disease, and there is often serious disability in the later years. Although people usually learn to cope with the difficulties the disease brings, MS has a major effect on their life plans, employment, and hopes for the future.

The disease may have a number of different courses after the onset of symptoms (detailed in section 1.4). Normally, the early disease shows a relapsing-remitting course, with attacks and remissions of symptoms. Afterwards, many patients will make the transition to a secondary-progressive course. Sometimes, a primary-progressive course, with slow progressive worsening without attacks is the first appearance of symptoms in some MS patients. Recently, a small number of patients whose cases begin as progressive, but who later have one or more acute relapses, have been classified separately. This type has been denominated progressive-relapsing course of disease. Some of the patients develop the so-called benign form characterized for a mild course during many years after the first clinical attack. This benign type is more difficult to define, represents 10 percent of cases, and can only be recognized after several years have passed.

MS affects the CNS myelinated nerves, including the optic nerve as an extension of the brain, but not the peripheral nerves. Physicians can verify the presence and location of the lesions by neurological examination, magnetic resonance image (MRI) and experiences that the patient previously underwent. When clinicians interview patients, it is very common to recognize vague symptoms off and on for many years; in those cases it is evident that MS is the cause.

Despite all efforts employed up to now to understand the causes of MS, theses remain uncertain. So far, it is known MS involves an immune-mediated inflammatory process characterized by breakdown of myelin that surrounds nerve axons in the CNS. This myelin is necessary for the rapid (saltatory) conduction in the myelinated nerves. Immunologically active cells in the blood enter the brain because of a disruption in the blood-brain barrier and cause patchy damage to myelin. These scattered circumscribed lesions, or plaques, provoke the symptoms of MS by slowing or blocking conduction in the nerve axons. These plaques are characterized by inflammation, demyelination, and scarring (gliosis). Currently, we know the axons may remyelinate again, which explains to some extent how remissions may occur after a relapse (attack). However, although remyelinated axons seem to work normally, as the symptoms may have cleared, electrophysiological measurements (evoked potential studies) show that conduction is slower than normal. Perhaps more important in the long-term is the amount of damage that occurs to the axons.

The MRI appearance of many lesions in characteristic areas, and the spinal fluid test (elevated gamma globulin and oligoclonal banding which are caused by inflammation in CNS) are helpful in confirming that MS is present, but do not correlate well with specific symptoms. MRI is a often used tool in the diagnosis and study of many neurological disease, such as MS. The immune reaction in the nervous system is accompanied by inflammation and gliosis, which are evident on MRI scans. So, confirmation of the clinical diagnosis requires correlations with the clinical picture, the presence of characteristic changes on the MRI, evoked potential studies, and spinal fluid analyses.

The earliest investigators recognized that this disease was difficult to treat, especially when the cause was unclear. This is still the case, but drugs are now available that can modify the acute attacks and reduce many of the symptoms. More importantly, for the first time, there are new agents that can reduce the number and severity of the attacks and perhaps slow the progression of the disease.

1.2. History of Multiple Sclerosis.

MS is a disease, known from more than 160 years now. The first clinicopathological description of cases of MS was made by Carswell in 1838; but it was Cruveilhier (1835) who described it more extensively. The classic clinical description and development of diagnostic criteria was reported more than 130 years ago by Charcot, even though, multiple aspects of MS remained an enigma. After decades of intense effort, no cause has so far been clearly identified and the disease remains poorly understood.

Finding the historical roots of MS has been difficult due to the lack of knowledge of clinical-anatomic localisation in neurology, prior to the late 19th century. Two reports from the late 13th century have been described; they were women afflicted with chronic, multifocal, and partially remitting neurological illnesses that might have been MS. These were the cases of Halla, a woman contained in the Icelandic Saga of St. Thorklar (Poser, 1994), and Lidwina van Schiedam, a Dutch woman born April 18, 1380 (Medaer, 1979).

The second case was deeply recorded. Although confounding issues of religious fervor characterized her symptoms, aspect of her case made consider she suffered from MS in almost four decades prior to her death in 1433 (Medaer, 1979). She was the daughter of a laborer, and one of nine children. She developed an acute illnes when she was around 15 year-old, from which she gradually recovered. In February 1396, when she was skating with friends on a frozen canal, she fell and broke some ribs on her right side. After this event, she had difficulty walking and used furniture for support. Healing was slow and it was thought that she had an internal abcess in the area of the fracture. She also developed severe lancinating pains in her teeth, which suggests trigeminal neuralgia. Later, she developed blindness in one eye and light sensitivity in the other. By the age of 19 years, walking was difficult and her right arm was paralyzed. In 1407, she began experiencing "ectasies, and visions in which she participated in the passion of Christ, saw purgatory and heaven, and visited with saints". She took joy from her misery, believing that she was sent to accept suffering for the sins of others. She slowly deteriorated, with increasing paralysis, and developed pressure sores. She lived until April 1433, just short of her 54th birthday. Saint Lidwina was cannonized by Pope Leo XIII in 1890 and now, is the patron saint of sickness and also a patron saint of figure skating.

The most convincing early case of MS was that of Augustus d'Este (Firth D, 1948), an illegitimate grandson of King George III and cousin of Queen Victoria, who kept a diary of his illness. There is no doubt that the cause of the relapsing and remitting neurological symptoms and progressive disease was MS. This is a very precise description of exacerbations including attacks of paraparesis. The diary begins in 1822, he described blurred vision after leaving the funeral of a beloved relative, fortunately, his vision gradually cleared without treatment but symptoms recurred twice during the next few years. He recurrently developed visual loss and diplopia in 1827. Later, he developed numbness in his legs and difficulty walking. In spite of numerous therapies he underwent, none of them had any benefit. The disease went on and the leg weakness continued progressively. He died in 1848. D'Este's diary is of particular interest to make a conclusive diagnosis of MS, characterized by repeated visual loss, diplopia, sensory change, intermittent and progressive paralysis in his legs, bladder difficulty, and impotence. The importance of this diary lies on the fact that it describes a very large number of treatments and remedies current at the time and gives an excellent detailed account of a disease that was undoubtedly MS.

The major advance in our understanding of MS occurred when R. Carswell, a Scottish physician working in Paris published a fascicle describing the pathology of several cases in 1838. Later, in 1842, J. Cruveilhier published other series of cases which included clinical criteria for the diagnosis. This report contains a number of important clinical and histological observations, such as the fact that demyelinated fibers are often perserved, and these fibers are capable of conduction. The development of clinical diagnostic criteria was what established MS as a clinical disease entity (Cruveilhier, 1829-1842).

Although Cruveilhier and Carswell described its anatomopathology, it was Jean Martin Charcot in 1868, a French neurologist at the Salpetrière (Paris), who first described MS, as it is known nowadays. In his report, Charcot noted that, in patients with intermittent episodes of neurologic dysfunction, there was an accumulation of inflammatory cells which appeared perivascularly, both in brain and spinal cord white matter (Charcot, 1868; Charcot, 1877). This fact led him to use the term *sclérose en plaques disseminées*, or multiple sclerosis, as it is called at present. Later, further evidence of an inflammatory nature to this disease was given when Elvin Kabat, in 1948, noticed an increase in oligoclonal immunoglobulin in the cerebrospinal fluid of patients with MS (Kabat et al., 1948; Kabat et al. 1950).

In the 20th century, the emerging epidemiology studies with large population of MS twins demonstrated a strong genetic basis to the clinical-pathologic entity of the disease (Mackay and Myrianthopoulos, 1966; Williams et al, 1980; Ebers et al, 1986; Heltberg and Holm, 1982; Kinnunen et al, 1987; Utz et al, 1993; Mumford et al. 1994; French Research Group on Multiple Sclerosis. 1992). The demonstration of the autoimmune and demyelinating character of MS came in the early 1930s, when Thomas Rivers and colleagues provided the first evidence that immune cells can attack the brain. Rivers, then a virologist at The Rockefeller Institute, and his colleagues injected Rhesus macaques with normal brain extracts from rabbits and showed that most of the monkeys developed acute CNS disease with immune cell infiltration and demyelinating lesions, providing the first hint that myelin was involved in disease induction. Thus, the experimental allergic (now "autoimmune") encephalomyelitis (EAE) model was born (Rivers et al. 1933).

1.3. Anatomopathology

The pathology of MS consists of brain lesions, called *plaques*, these are patches of scar tissue that form in areas where myelin tissue has been destroyed.

Examination of brain tissue of individuals with MS reveals multiple sharply demarcated plaques in the CNS white matter, with relatively little axonal damage. These patches have a predilection to the optic nerves and white matter tracts of the periventricular regions, juxtacortical areas, brain stem, and spinal cord; however they also can be found anywhere in the CNS white or gray matter (Trapp et al. 1998). The size of these plaques can change, usually their diameter is less than about 1, 5 cm, but the coalescence of these lesions can make them bigger. The lesions occur in different parts of the nervous system and are in different stages of activity or maturity.

The lesions in MS are divided into two subtypes according to the stage of the disease: acute and chronic plaques. Acute plaques are mostly present in the first stages of MS while inflammation is the most important phenomenon. In chronic lesions, demyelination, axonal damage and gliosis are the predominant factor with only traces of inflammation. Lesions can vary from acute plaques, with active inflammatory infiltrates and macrophages loaded with lipid and myelin degeneration products, through various degrees of reduced activity to plaques that are active only at their margin, to chronic, inactive demyelinated shrunken glial scars.

1.3.1. Acute plaques

Prineas described that most MS plaques appear to begin with margination and diapedesis of lymphocytes and macrophages forming perivascular cuffs around capillaries and venules (Prineas, 1975; Dow and Berglund, 1942). This is followed by a diffuse parenchymal infiltration by inflammatory cells, edema, macrophage activity with stripping of myelin from axons, astrocytic hyperplasia, and the appearance of increased numbers of lipid-laden macrophages and demyelinated axons. Later, these plaques go on enlarging and coalescing and the initial perivenular distribution starts to disappear. In gray matter plaques, the inflammatory reaction usually is less pronounced. This could be explained by the smaller amount of myelin in these areas and consequently, fewer

macrophages are needed to remove cellular debris. In this context, macrophages strip of myelin from axons provoking the myelin breakdown which characterizes MS. Thus, these lesions are full of macrophages filled with myelin debris and neutral lipid. Apart from those macrophages, other inflammatory cells are involved such as lymphocytes, activated macrophages, microglia and occasionally plasma cells.

MHC class II positive cells have been shown to be the major inflammatory cells in the actively demyelinating central area of acute plaques. These are mostly activated microglia and macrophages with a few T-cells and antibody-producing plasma cells. With regard to T cells, there is a mixture of CD4+ (helper-inducer) and CD8+ (supressor/cytotoxic) lymphocytes that concentrate in the center of the plaque, diminishing in numbers at the lesion border. While lesion ages, the number of T cells increases at the lesion periphery letting the macrophages take their place in the center. The distribution of both subtypes of T lymphocytes is also different, CD4+ cells appear in the plaque margin and extend beyond the margin into periplaque normal white matter, whereas CD8+ cells are confined largely to the plaque margins and perivascular cuffs. Besides, the plaque margins contain numerous oligodendrocytes, astrocytes and inflammatory cells (Traugott et al., 1938; Traugott, 1994; Peterson et al, 2001). As the lesions become more mature, myelin remnants and macrophages progressively disappear from the central part of the plaque, which eventually becomes a gliotic scar. Meanwhile, the plaque margin becomes a hypercellular "glial wall" which contains lymphocytes, oligodendrocytes, and a few macrophages and astrocytes.

Our understanding of MS pathology of has been altered by the reports of Lassman et al. (1998) and Lucchinetti et al. (2000). Their studies with modern staining and labeling techniques demostrated active MS plaques can be classified in four different patterns of demyelination. Those data suggest to consider MS not just a single disease but a group of them with similar clinical and pathologic features.

All of the lesion types have evidence of inflammatory infiltration by macrophages and T lymphocytes, but they are different regarding several aspects, such as myelin protein loss, immunopathologic evidence of complement activation, plaque distribution and patterns of oligodendrocyte destruction. Based on it, four patterns were defined.

Patterns I and II were similar in the sense of active demyelination occurring in association with T-lymphocytes and macrophages as dominating inflammatory factors. Pattern II was distinguished from pattern I by the prominent deposition of immunoglubulins (mainly IgG) and complement C9neo antigen at the active site of myelin destruction. Both patterns, I and II, are typically centred on small venules and veins and have sharply demarcated edges with perivenous extensions but they do not show significant remyelination.

In contrast, the most outstanding characteristic of patterns III and IV is a prominent loss of oligodendrocytes at the active plaque borders. On one hand, pattern III has diffuse lesions with variable inflammation and pronounced microglial activation, resembling a distal oligodendrogliopathy. Although this kind of lesions surround blood vessels they retain a rim of preserved myelin around them. These pattern lesions are also characterized by a striking loss of myelin-associated glycoprotein (MAG). This preferential loss of MAG, in comparison to other myelin proteins, may reflect primary oligodendrocyte injury in demyelinating conditions with evidence of oligodendrocyte apoptosis. Pattern III does demonstrate limited remyelination, and no complement activation.

In contrast, pattern IV lesions do have sharp macrophage borders and evidence of oligodendrocyte degeneration within a rim of normal-appearing white matter along the edge of the demyelinated plaque. There are no oligodendrocytes at the lesion centre, and evidence of repair is minimal. Pattern IV is very different from other lesions since they do not show either MAG loss or complement activation.

In short summary, it seems that myelin is the target in patterns I and II, while patterns III and IV suggest that oligodendrocytes may be the target. In pattern I, demyelination is likely mediated by macrophages, whereas in pattern II, antibody and complement may contribute to demyelination. Then, that patterns I and II could be explained as an autoimmune disease, while patterns III and IV resemble viral, toxic, ischemic, or metabolic models (Lucchinetti et al., 1996; Lucchinetti et al., 2000). Another fact which supports this theory of different pathogenesis of MS is the fact that patterns identified were homogenous within each patient, but heterogeneous between different ones. No heterogeneous patterns were found within the autopsy material of an individual specimen.

1.3.2. Chronic active and inactive plaques.

There are very few cells in mature plaques. This hypocellularity is due to the exit of lipid-laden macrophages and microglia as plaques get older. At the same time, gliosis is increased, followed by gradual shrinkage of the plaque. Then, the activity of the plaque margin can have different fates, either it may continue or relapse, or it may gradually lose inflammatory cells and become inactive.

In chronic inactive plaques occasionally there may be CD4+ and dendritic cells scattered throughout the lesions. At the edges, a few CD4+, CD8+, and dendritic cells, including macrophages and B lymphocytes, may be seen, and these also may occur in small numbers throughout the otherwise normal-appearing white matter (Traugott, 1994).

When a lesion becomes inactive, its margins get very sharp without hypercellularity, just a few widely scattered T-cells, and adhesion molecules or interferon are absent. The main characteristic is a marked gliosis which is revealed by a strong stain for glial fibrillary acidic protein. Inflammatory cells are scarce, and most cells in the plaque are astrocytes. Around the lesions the white matter is not normal. The white matter shows biochemical abnormalities that can be partly explained on the basis of Wallerian degeneration (Prineas et al, 1997) and gliosis. It is remarkable to note that sometimes there are a few nonmyelinating oligodendrocytes seen near the plaque margins and suggested to be responsible for remyelination.

1.3.3. Axonal damage

MS lesions do not just involve myelin breakdown but also considerable axonal interruption, which occurs even in new acute plaques. The presences of the amyloid precursor protein, known to be a sensitive marker of axonal damage, and axonal transections have been considered hallmarks of irreversible axonal degeneration in MS (Ferguson et al., 1997; Trapp et al., 1998). This axonal damage is more patent when in vivo brain magnetic resonance spectroscopy (MRS) is used.

In MS lesions, a significant increase in brain choline (Cho), a compound of cell membranes and myelin, is found. This elevated choline may either indicate degradation or abnormal utilization of choline-derived compounds for membrane and myelin synthesis.

Regarding axonal damage in MS plaques, loss of N-acetyl aspartate (NAA), an aminoacid essentially located in neurons, demonstrates that axons are destroyed (Stefano et al., 2001). A possible role for NAA could be the control of neuronal osmoregulation (Baslow, 2002). A reduction in NAA content is generally viewed as a sign of neuronal injury or loss. It is observed in a wide range of brain pathologies, including neurodegenerative, developmental, and psychiatric disorders.

At the start of axonal damage, demyelinated axon diameters become irregular and later on, their size decrease uniformly. Functionally, axon transport is seriously impaired. If this happens, the amount of NAA could be reduced out of proportion and might well decrease the concentration distal to the site of demyelination. In addition, reactivation of older plaques with demyelination at their margins can be another reason for more axonal destruction.

This axonal damage causes cerebral and corpus callosum atrophy. The combination of myelin and axonal loss, and shrinkage due to scarring provoke atrophy which is commonly seen in advanced disease. Actually, it is believed that axonal and cumulative loss is the main cause for most of the chronic, irreversible disability in MS. Long pathways with several plaques along their course, such as the pyramidal tract and the dorsal columns of the spinal cord, is often very substantial resulting in the frequent symptoms of the disease.

1.3.4. Remyelination

Remyelination is the phenomenon by which new myelin sheaths are generated around axons. This normally happens in the adult CNS, but also can follow the pathological loss of myelin in diseases like MS.

Before the late 1950s, the scientific community accepted that remyelination did not occur and that oligodendroglia, like neurons, is endstage cells, incapable of regeneration. It was in 1961 when Bunge et al. firstly demonstrated, without any doubt, central remyelination could occur in the cat. Afterwards, remyelination was shown in the CNS of essentially every species tested, including tadpole, mouse, rat, guinea pig, rabbit, cat, dog and, certainly, human (Hommes, 1980).

In addition, the shadow plaques show the same characteristics of those of remyelinated areas in experimental animals. These features are an increased number of oligodendrocytes which have been demonstrated to be capable of proliferation (Herndon et al., 1977; Arenella L, Herndon, 1984; Ludwin, 1984), thin myelin sheaths of relatively uniform thickness, and short internodes. This abundant evidence indicates that these are indeed remeylinated fibers.

Remyelination can restore conduction properties to axons (thereby restoring neurological function) and is increasingly believed to exert a neuroprotective role on axons. Remyelination appears in many MS lesions but becomes increasingly incomplete/ inadequate and eventually fails in the majority of lesions and patients (Chari, 2007).

The study of the mechanisms of repair of experimental lesions has demonstrated that remyelination occurs in two major phases. The first one involves the colonization of lesions by oligodendrocyte progenitor cells (OPCs); the second part is the differentiation of these OPCs into myelinating oligodendrocytes contacting demyelinated axons to generate functional myelin sheaths. Potentially with age, because of the progressive loss of OPCs, remyelination can fail at either of these two stages, which probably contributes to the decline in remyelination efficiency during the course of the disease. (Franklin, 2002)

Regeneration of central myelin in some cases is accompanied by Schwann cell invasion of the CNS. Consequently, the peripheral myelin can be seen within a demyelinated or remyelinated area (Itoyama et al., 1983). In pathologic conditions, most of the Schwann cells in remyelinated areas within CNS appear near root entry or exit zones. Breaches in the glial limitans, caused by the demyelinating and inflammatory process, allow Schwann cells to settle in the nerve root or autonomic nerves accompanying cerebral blood vessels, to migrate into the central white matter where they retain their capability to form myelin.

Recurrent demyelination at the plaque margins provokes plaque enlargement with the progression of the pathologic process. Despite remyelination seems to be a frequent event in early MS, its effectivity and frequency decreases as the disease progresses. To achieve experimental remyelination in advanced disease, cell transplantation or promoting endogenous remyelination can be considered. Both strategies require the identification of cells capable of remyelination successes. Several studies about experimental transplantation have been performed providing challenging proof of the repair potential of grafted cells. Schwann cells, oligodendrocytes and their progenitors, olfactory ensheathing cells and more recently embryonic and neural stem cells have been proven to survive, integrate and form myelin after transplantation in the demyelinated CNS. Among these cell types, Schwann cells seem to be the most interesting ones because they enter the CNS spontaneously in neurodegenerative or demyelinating disease and are not a target in MS. According to that, some studies showed vigorous remyelination and restoring of axonal conduction after transplantation in various rodent models of spinal cord demyelination (Baron-Van Evercooren and Blakemore, 2004). They are known to promote axonal regeneration after transplantation in the injured spinal cord (Pearse et al., 2004). Not only myelinating cells, astrocytes have been suggested also to play a positive role by stimulating remyelination and oligodendrocyte development (Williams et al. 2007).

Recently, several lines of evidence support the idea that bioactive lipid sphingosine-1-phosphate (S1P) signaling in oligodendrocytes not only plays an important role during normal CNS development but also offers new and exciting therapeutic avenues to stimulate remyelination in demyelinating diseases like MS (Coehlo et al., 2009) . Thus, FTY720 a sphingosine-1-phosphate (S1P) receptor agonist currently used as a potential immuno-therapy for multiple sclerosis has been shown to induce time-dependent modulation of S1P receptors on human OPCs with consequent functional responses that are directly relevant for the remyelination process (Miron et al., 2008).

1.3.5. Gliosis.

Gliosis is a prominent feature of the MS lesion, but it is considered as a secondary phenomenon. Whenever the CNS is damaged, it undergoes an injury response, usually called reactive gliosis or glial scarring. The response is broadly the same whatever the source of injury, although the details vary somewhat with different types of pathology. The glial reaction to injury includes recruitment of oligodendrocyte precursor cells, stem cells, microglia, and astrocytes. Formation of the glial scar after CNS injury generally occurs over a period of weeks.

Microglia is typically the first cell type to enter the lesion. In the normal brain, they are quiescent with short, branched processes. Following injury, they exhibit various changes, including activation, cell division, and migration to the injury site (Fawcett and Asher, 1999). During activation, microglia display conspicuous functional plasticity, which involves changes in cell morphology, cell surface receptor expression, and production of growth factors and cytokines, and they become, in general, more macrophage like (Streit, 2000). Microglia can be either neurotoxic or neurotrophic.

The final glial scar is made up mainly of a meshwork of tightly interwoven astrocyte processes, attached to one another by tight junctions and gap junctions, and surrounded by extracellular matrix (Fawcett and Asher, 1999). Astrocytes are irregularly star-shaped, background structural cells of the nervous system. Gliosis is usually restricted to the area of demyelination, but it sometimes extends beyond that area. There is no specific way to identify the presence and extent of gliosis in MS lesions through MRI, although T1 weighted images might be more sensitive to gliosis than T2 (Brex et al. 2000).

The role of astrocytes in gliosis is not completely known (Moore, 1998). Since there is evidence that glial scars can inhibit both axon growth and myelination, it is clearly important to know what causes them to form, what cells are involved, why they are inhibitory, and how to manipulate them. Finally, although gliosis is generally considered harmful, there is also evidence that the gliotic ensheathment of demyelinated axons might favor the restoration of nerve conduction (Waxman et al. 1994).

1.4. The course of multiple sclerosis

The course of MS varies from person to person. It is unknown why each person has a different course of symptoms and problems. Whereas one person may have a mild disease that produces little disability over time, another can have a progressive and aggressive form. MS can have different patterns in people of the same family. What type of pattern a person will develop, seems to have nothing to do with anything which can be measured in the body, such a life activities, blood test or genetic studies.

Despite this unpredictability, the courses of MS have been classified into four basic patterns defined by an international survey of MS experts (Fig. 1):

1.4.1. Relapsing-Remitting MS. This form of MS is characterized by clearly defined acute attacks followed either full or partial recovery, but sometimes with remaining symptoms. While there are no new symptoms between disease relapses, the underlying disease process may continue. This is the most common pattern of MS, about 70 percent of patients with MS begin with this pattern.

For a small number of people with relapsing-remitting course, MS has a mild form, and even after 15 years of disease, all neurologic systems remain fully functional. Because of this, the term "benign MS" is used for this subtype of relapsing-remitting MS. Many people with this form of the disease have mostly sensory symptoms. This is the most difficult form of MS to "label" because it requires many years to identify this pattern. As it cannot be reliably identified in the first years of the disease, treatment decisions are not based on the possibility that this course may occur.

Remissions can occur at any time in the course of MS and can last for months or for many years. The reason why they occur is not known, and we are not able to predict in whom or when. Remissions are more common in the early stages of the relapsing-remitting type, and it is quite common to see plateaus in the course, with long periods in which little changes can be seen in the affected person´s symptoms.

The typical pathology of relapsing-remitting MS consists of lesions (plaques) disseminated in location and varying in age, and which can be found wherever there is central myelin. They are present in white and gray matter, but the gray matter lesions are much less obvious. Those latter lesions rarely appear on MRI, possibly because of the relatively small amount of myelin present in grey matter and consequently, a less intense inflammatory reaction. The location of lesions would be expected from the clinical features. Apart from that, secondary demyelinating processes can occur, this consists of demyelination of individual fibers or small groups of fibers and it is best seen in the spinal cord.

1.4.2. Secondary progressive MS: This disease pattern is the one in which the relapsing-remitting form turns in to within 10 to 15 years. Approximately 50 percent of those with a relapsing-remitting course will enter

a progressive phase after those years. Patients may continue to have less frequent acute attacks or may stop having them all together. Disease is marked by a slowly ascending paralysis or, much more rarely, progressive ataxia. It commonly occurs in patients in their 40s or early 50s who have had MS for a number of years.

The pathologic substrate appears to be a progressive loss of axons in the spinal cord which causes atrophy. In addition to chronic inactive or occasionally active plaques, examination of the cord shows demyelination and/or degeneration of multiple individual fibres with a few scattered macrophages along the sheaths (Adams, 1983). These events lead to an inadequate supply of transported materials from the myelin-forming oligodendrocytes which results in inappropriate signal. Certainly such scattered fibre demyelination could be part of a dying axonopathy and then the substrate for the slowly progressive ascending paralysis of secondary progressive MS (Herndon, 2002).

It is not known yet whether new treatments with immunomodulating agents will decrease the number of people who will develop this form of the disease, but that is the aim perspective

1.4.3. Primary Progressive MS: This form of MS is characterized by a slow progression from its onset without attacks, although sometimes occasional plateaus and temporary minor improvements can appear. This course is more commonly seen in men who develop the disease after the age of 40, nevertheless the percent of people initially diagnosed with this type of MS is very low, about 15% of total patients.

In the studies of Lucchinetti et al., all of the cases of primary progressive MS had pattern IV (Lucchinetti et al, 2000). This pattern was the least common in the series of Lucchinetti, comprising 4 percent of the cases studied, and it was the only one associated with a particular clinical disease pattern. This association supports the fact that true primary progressive MS may be a disease distinct from relapsing remitting and secondary progressive MS. In this lesion pattern, inflammation is dominated by T lymphocytes, and there is a distinct lack of macrophages, IgG, and complement deposition. Death of oligodendrocytes occurs in periplaque white matter adjacent to areas of demyelination, but the dying oligodendrocytes do not show features of apoptosis, only DNA fragmentation. There is also a loss in staining for each of the myelin proteins which seems to be essentially simultaneous. In line with all of this, this kind of lesions looks as if it is an oligodendrogliopathy.

1.4.4. Progressive-Relapsing MS: Progressive-relapsing is a rare form of MS and affects around 5% of patients. This form of MS shows progression from onset, but later on, one or more attacks occur.

The results of a MRI study indicate that patients with progressive-relapsing MS are characterized by large or coalescent lesions located predominantly in the periventricular areas. Almost all lesions were found in the parietooccipital region causing significant cognitive dysfunction and severe personality changes. In contrast to patients with relapsing-remitting disease, who present punctiform lesions mainly located in the intermediate and subcortical areas, patients with progressive-relapsing course have significantly less severe cognitive and emotional involvement than the patients with progressive-relapsing disease.

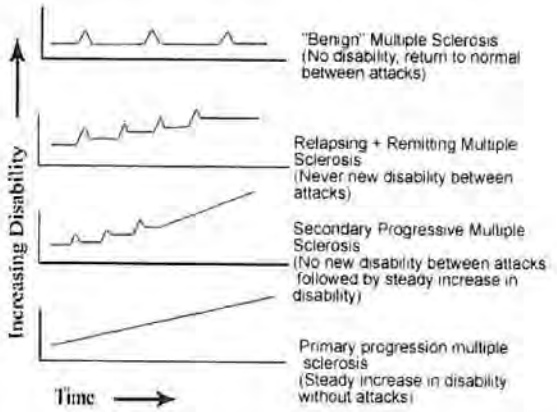


Fig. 1. Diagram representing the different types of multiple sclerosis

1.5. Epidemiology.

Epidemiology is the study of the natural history of disease. Epidemiology studies involve frequency of diseases and their characteristics by race, sex, geography, and other factors. Two main measures used in epidemiological reports are incidence and prevalence. Incidence is the number of new cases per unit of person–time at risk (usually number of new cases per thousand person–years); while prevalence is the total number of cases of the disease in the population at a given time. Thus, prevalence is known to depend not only on incidence, but also on survival rate and migrations of affected people.

Regarding MS, relapsing-remitting MS is the most common course of the disease; about 70% of all patients has this variation, being more common in women than men. The onset of this form occurs by the age of 30 in two-thirds of patients in both sexes (Noseworthy et al., 2000). On the contrary, progressive primary MS is slightly more frequent in men than women and typically begins in midlife.

In general, there is a female preponderance which appears to be increasing. Furthermore, studies in US have demonstrated there is a clear predilection for whites, but other racial groups have shared their geographic distribution though at lower levels.

Studies of MS in migrants from high to lower risk areas indicate the age of adolescence to be critical for risk retention. Those people who migrate at age 15 or older retain the MS risk of their birth place; meanwhile those who do it at younger age acquire the lower risk of their new residence. In addition, other low-to-high studies show that people migrating in childhood or adolescence do in fact increase their risk of MS. The migrant data support the idea that MS is ordinarily acquired in early adolescence, with a lengthy "incubation" or "latent" period between disease onset and symptom onset, and with young children rarely susceptible to this illness.

Despite intensive epidemiological investigations over many decades in many parts of the world, the geographical distribution and the true prevalence and incidence of MS remain uncertain. It has been only in recent years that a clearer understanding of the distribution of MS has emerged.

For years it was believed that the distribution of MS was related to latitude. However, most recent descriptive studies based on more appropriate methods contradicted this accepted belief (Granieri, 1997). Until 1980, European countries from 36° to 46° north latitude were considered to have a much lower prevalence rate of MS, about 5 to 25 cases per 100,000, compared to those Central and North European countries. This theory was based on old surveys done in Italy between 1959 and 1975. New studies have been performed in several South European countries which have reported new data, in fact, showing that MS prevalence is much higher than had been previously believed (Rosati, 1994).

Then, after many reports about this subject, the worldwide distribution was divided into three zones of high, medium, and low prevalence (Kurtzke, 1995). High prevalence areas, with rates of 30 or more per 100,000 population, now comprise almost all of Europe, Cyprus, Israel, Canada and United States, as well as New Zealand and South-Eastern Australia. They also seem to include the easternmost part of Russia. These high regions are bounded by areas of medium prevalence with rates of 5-29 and now mostly 15-25 per 100,000, which then include most of Australia, the southern Mediterranean basin, probably Russia from the Urals into Siberia as well as the Ukraine, South Africa, and perhaps much of the Caribbean region and South America. All other known areas of Asia and Africa and possibly Venezuela and Colombia are low, with prevalence rates under 5 per 100,000 population. Later, the subsequent analysis have evidence an increase of prevalence rates reaching > 100 per 100,000; 50-100 per 100,000; and < 50 per 100,000 for high, medium or low prevalence respectively. At least 23 studies determine a medium-high prevalence for mainland Spain (Fernandez et al., 1994; Modrego and Pina, 2003; Bruck and Stadelmann, 2005; Aladro et al., 2005; Ares et al., 2007).

Therefore, the MS distribution seems to be more complex than it was supposed in the beginning, since great variations between areas at the same latitude as well as within countries have been found. And it must be noted there are highly significant deviations from homogeneity inside each region, and the high-rate areas tend to form clusters or foci.

1.6. Pathogenesis

In spite of many epidemiological, genetic, immunological and other studies, the etiology of MS is still unknown. There have been two hypotheses about the etiology of this disease for years; an environmental hypothesis and a genetic hypothesis.

The environmental hypothesis suggests some environmental factor could be the cause of MS. This is supported by different facts. First, incidence can change in short periods of time, frequency forms foci and the risk of suffering the disease can vary based on migration. Indeed, the study of migrants shows there is a variation in the frequency of disease within genetically homogeneous populations living in different regions (Dean et al., 1976; Kurtzke, Hammond et al., 1988). Together, these data suggest that some aspects of the environment shared at the population level must matter. The most accepted theory is this environmental factor could be a virus infection. This infection would initiate or exacerbate organ-specific autoimmune diseases (Olson et al., 2001). There is growing evidence about possible mechanisms by which some virus infection can trigger autoimmunity, although this putative virus has not been identified yet. Among them are: molecular mimicry, bystander activation, and epitope spreading.

Molecular mimicry involves de novo activation of autoreactive T cells due to the cross-reactivity between self epitopes and viral epitopes during a virus infection (Fujinami and Oldstone, 1985). In case of bystander activation, there is a nonspecific activation of autoreactive T cells in the target organ due to the direct inflammatory and/or necrotic effects of virus infection on tissue (Horwitz et al., 1998). The last mechanism is epitope spreading; this consist of a widening of the immune response initiated by an antigenic epitope to different noncross-reactive epitopes either on the same molecule (intramolecular spreading) or on a different antigenic molecule (intermolecular spreading) (Miller et al., 1997, Vanderlugt and Miller, 2002). These new antigens could have been hidden or cryptic within an unexposed region of the molecule, what prevented from interacting with Abs or lymphocytes. Due to the inflammatory response, those new antigens appear exposed by a conformational change or stereochemical alteration of molecular structure capable to initiate an immune response. Although viral infection is generally implicated as the factor, no evidence of a specific virus's role in MS has been produced (Noseworthy et al., 2000).

The genetic hypothesis is based on the early findings of familial clustering and marked variation in prevalence repeatedly confirmed. Moreover, some ethnic groups are resistent to MS, the risk of suffering the disease increases if relatives also suffer it and some genetic predisposition has been described.

A large database reveals a 20% familial incidence in MS. This genetic predisposition is supported by twin studies. These studies showed concordance rates for monozygotic twins of around 30%, whereas dizygotic twins display 2–4% (Ebers et al., 1986). Furthermore, the probability of transmitting the disease to her children if the mother suffers MS will increase 20- to 40- times, being greater for girls than boys. Other first-degree relatives also have an increased risk of MS (Sadovnick et al., 1988).

Apart from these results, the most recent and largest MS linkage study failed to show any significant association, except for the human major histocompatibility complex (MHC) gene locus, known also as human leukocyte antigens (HLA) system, located on chromosome 6 (Sawcer et al., 2005). The important conclusion obtained from this and previous linkage studies is that MS-related genes have low odds ratios, implying that a large number of different genes might dispose for the same end phenotype in form of MS.

Despite the lack of significant linkage, several genes are more common in MS than in population controls. Jersild et al. (1973) found that the alleles A3, B7 and DR2 occurred twice as commonly in patients with MS, compared to the unaffected population. They also observed that patients who posses both human leukocyte antigen (HLA)-B7 and DR2 had particularly severe disease (Jersild et al., 1973; Berger et al., 1984). Since many genes important in normal immune function and immune-mediated tissue damage, such as tumor necrosis factor (TNF), are located in the region between HLA-B7 and the DR2 locus, several mutations of genes residing in this are being demonstrated as factor for susceptibility as observed for HLA DR2 alleles, DRB1, DQA1 and DQB1 (Fernández et al., 2004; Fernández et al., 2009; Vasconcelos et al., 2009)

Apart from that, other important studies looking for single nucleotide polymorphisms (SNP) in genes outside of the major histocompatibility complex are being studied as well. In this line, allelic association of a polymorphism in the gene encoding the interleukin 7 receptor αchain (IL7R) (Gregory et al., 2007), and within

the interleukin-2 receptorαgene (IL2RA) (Hafler et al., 2007) have been demonstrated as a significant risk factor for multiple sclerosis. Recently, twenty-one SNPs positively associated with MS located at the GFI-EVI5-RPL5-FAM69A locus were analyzed and confirmed EVI5 on chromosome 1 as a novel risk locus (Alcina et al., 2010). Nevertheless, there is no single gene, or combination of genes, implicated specifically in the risk of MS. Furthermore, SNP-based typing over the MHC complex has revealed association only to the class II region and not to other parts, such as class I, III regions and TNF, which have been previously reported to be associated to MS (Lincoln et al., 2005). However, the SNP-based technology does not exclude influences from highly variable loci, such as the class I region. Indeed, with sequence specific MHC class I typing there is an influence from the HLA-A*0201 and HLA-A*0301 alleles (Fogdell-Hahn et al. 2000; Harbo et al., 2004).

Shortly, current hypothesis holds that the disease is caused by an autoimmune process that occurs in genetically predisposed subjects as a consequence of the exposure to several environmental factors.

Although its pathogenesis is incompletely understood, MS is already accepted as an immune-mediated illness. The fact that MS development follows about one-third of acute disseminated encephalomyelitis-complicating infections (Schwarz, 2001; Hartung and Grossman, 2001; Murthy et al., 1999) as well as immunizations (including Semple vaccine, which contains spinal cord and killed virus), suggested an autoimmune origin of this disease. Because of that, EAE has been studied in animal models for decades as a means of understanding the nature of the immune response and also MS pathogenesis. The central role for lymphocytes in the pathogenesis of autoimmune disease was established by successfully transferring EAE from immunized to naive animals using lymph node cells (Patterson, 1960). Moreover, antibody from immunized animals and patients with MS can also induce demyelination in vitro (Bornstein and Appel, 1961; Bornstein and Raine; 1977).

Attention has been centred on the primary role of T cells in the pathogenesis of EAE regardless of the nervous system antigen used to induce disease (Ben –Nun and Cohen, 1982). There is a consensus that T cells are the primary effectors both in MS and EAE (Owens T, Siriam, 1995), even though, B cells, plasma cells, and antibody may also be found in both EAE pathology and MS plaques (Lassman et al, 1998, Lucchinetti et al. 2000). These studies of MS pathology showed that the predominant cells in active lesions are lymphocytes, particularly CD3+ T cells, and macrophages (Lucchinetti et al. 2000).

When antigen is presented by MHC class I- or MHC class II antigen-presenting cells to T cells, an immune response is initiated; aimed to either induce antibody production or a cellular immune response. Then, activated CD4+ cells fall into two functionally distinct classes, Th1 and Th2, each with distinctive lymphokine-production profiles. Following antigenic stimulation, Th1 cells, which are also called CD4+ Th1 cells, produce interleukin (IL)-1, IL-2, interferon (IFN)-γ, and TNF-α and mediate inflammatory pathological processes in immune-mediated tissue damage seen in MS and EAE. In contrast, Th2 cells produce IL-4, IL-5 IL-6, and IL-10 and induce upregulation of antibody production and down regulation of Th1 cellular responses (Adorini and Singaglia, 1997).

An interesting model using MOG to induce EAE in marmosets indicates that antibodies may also mediate demyelination (Massacesi et al., 1995, Uccelli et al., 2003). This was demonstrated by passive transfer of the disease injecting serum from sensitized animals. In this model T cells CD4+ T helper Th2, rather than CD4+ Th1 cells, seem to be the primary mediators of myelin damage in MOG-sensitized marmosets (Uccelli et al., 2003).

Macrophages are the principle source of IL-1, IL-12, and TNF-α and are driven by IL-2 production from antigen-activated CD4+ Th1 cells. Importantly, IL-12 production is IFN-γ dependent and TNF-α production is IL-12 dependent (Yang et al., 2000). Not only are macrophages the principal antigen-presenting cells they also are central effector cells in cell-mediated immunity. In the CNS, microglial cells can function as antigen-presenting cells and exhibit macrophage behaviour. After antigen presentation, CD4+ cells undergo clonal proliferation and recruit other CD4+ cells to participate in the initiation of the cellular immune response. Cytotoxic CD8+ T cells driven by IL-12 may exert their effect directly or target antigen-complexed antibody on target tissue (i.e., antibody-dependent cytotoxicity) (Owens and Siriam, 1995; Liu et al.; 2004). Macrophages may also target these complexes.

To achieve CNS inflammation, immune cells must invade the nervous parenchyma which is isolated from the peripheral circulation by the blood brain barrier (BBB). The BBB is a physical barrier that prevents intravascular cellular elements, antibodies, and other proteins from having free access to the brain and spinal cord. The endothelial cells in the brain and spinal cord possess tight junctions that are impervious to intravascular fluids, as well as to nonactivated cells. These endothelial cells are also surrounded by astrocytic

foot processes, which further support and maintain the integrity of the BBB. Although activated CD4+ cells do cross the BBB, the BBB is an actual physical barrier that may be breached only in an organized and well-orchestrated fashion. The mechanisms of cellular transmigration across the BBB are now well understood (Minagar A, Alexander, 2003; Yednock et al, 1992).

Venulas are the one which control cell migration from blood cells into the nervous system. Attachment requires cellular adhesion molecules and endothelial counter receptors to overcome the considerable shear stresses produced by blood flow. Adhesion molecules on CD4+ cells and macrophages are functional anchors that form stable bonds with their ligands on the vascular wall. In addition to functioning as mechanical anchors, adhesion molecules are tissue-specific recognition molecules (Carlos and Harlan; 1994; Frenette and Wagner, 1996).

Entry of CD4+ cells and macrophages into CNS is accomplished by a series of steps, including tethering or rolling, adhesion (binding), and finally transendothelial migration across the BBB (von Andrian and MacKay, 2000; von Andrian andEngelhardt, 2004). Subsequently, they migrate through the extracellular matrix into the CNS (Fig. 2).

The initial step of tethering is mediated by selectins, through selectin bonds, which leads to rolling (Vestweber D, Blanks, 1999; Takada et al., 1989). These bonds are reversible. Selectins expressed on leukocytes (P-selectin and L-selectin) and endothelium (E-selectin) result in rolling and slowing of the cells. P-selectin and its ligand PECAM-1 appear to have a special role in EAE and MS (Piccio et al., 2002; Minagar et al., 2001). In contrast, L-selectin has a primary role in lymphocyte recruitment for lymphoid tissues. As cells roll and slow down by the interaction of selectins and their ligands, they are able to respond to endothelial cell chemokines.

Specific chemokines are fixed on the endothelial surface and are molecular signals that direct cells to tissues and confer organ specificity with specific adhesion molecules. Chemokines are divided into four families, which are specific for different T-cell subgroups (von Andrian and MacKay, 2000). They have a central role in the egress of specific lymphocytes subgroups into specific target organs. In MS and rheumatoid arthritis, all of the infiltrating Th1 cells express CCR5 and CXCR3 chemokine receptors (Qin et al., 1998). Selectin binding to ligand is an activating signal that induces rapid activation of α4 integrins and β2 integrins (Takada et al., 1989; Hynes, 1987; Hynes, 1992).

To prevent from unbinding on the endothelium, these low-affinity interactions must be supplemented by high-affinity adhesion molecules, these are the integrins. The integrins, including α4β1-integrin (VLA-4), are members of the endothelial immunoglobulin superfamily. The predominant function of the β2-integrin leukocyte function antigen (LFA)-1 and integrins α4 integrins (α4β1-integrin/VLA-4) is to bind to their ligands intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (Hynes, 1987; Hynes, 1992).

On the other hand, intregrins are a large family of cell-surface glycoproteins that mediate intercellular interactions and interactions with matrix protein (Frenette PS, Wagner, 1996; von Andrian and MacKay, 2000; von Andrian and MacKay, 2004). They present high affinity for their ligands, ICAM-1 and VCAM-1 (von Andrian and Engelhardt, 2004; Takada et al. 1989). Under inflammatoy conditions, the integrin heterodimers adopt a high-affinity open molecular conformation and can mediate activation-independent rolling interactions, as well as arrest rolling leukocytes. In MS, the integrins responsible for leukocytes binding are LFA-1, also referred to as CD11aCD18 and α4β1-integrin (VLA-4). In the CNS, α4β1 is of primary importance because VCAM-1 is expressed on CNS at low levels on nonactivated endothelium and at high levels on activated endothelium (Yednock et al, 1992; Kent et al., 1995; Keszthelyi et al, 1996).

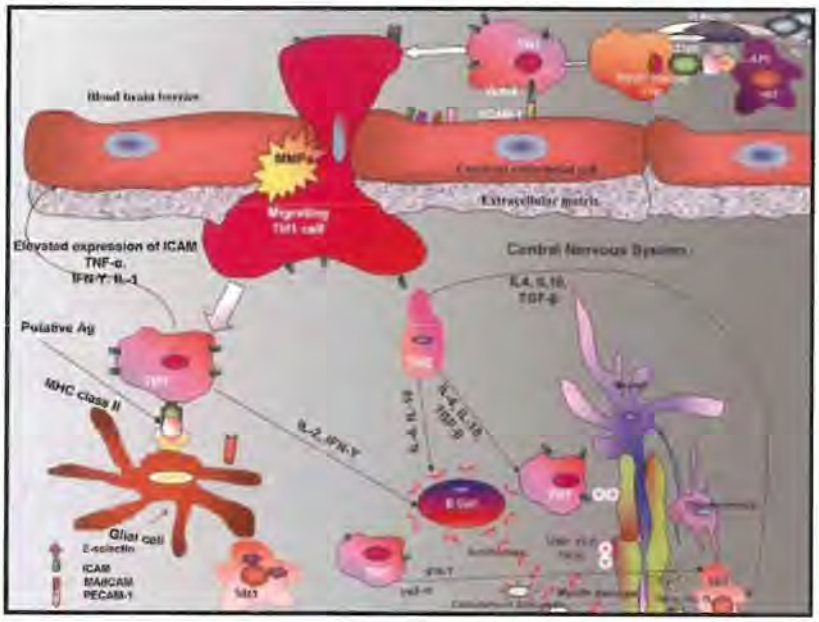


Fig. 2. A model immunopathogenesis of multiple sclerosis. Following exposure to certain environmental antigen(s) in genetically susceptible individuals, myelin-reactive T cells migrate from the peripheral circulation into the central nervous system. Interaction between activated T cells and cerebral endothelial cells leads to upregulation of the adhesion molecules (E-selectin, vascular cell adhesion molecule, intercellular adhesion molecule, mucosal addressin cell adhesion molecule and platelet endothelial cells adhesion molecule). Transendothelial migration of reactive T cells is heralded by the disruption of the blood-brain barrier, which is in part mediated by the activities of the matrix metalloproteinases (MMPs). MMPs digest extracellular matrix and facilitate migration of the activated T cells. Proinflammatory cytokines released by the activated T cells (such as TNF-α and IFN-γ) upregulate the expression of cell-surface molecules on antigen-presenting cells (in figure, glial cells). Binding of a putative multiple sclerosis antigen (e.g. myelin basic protien and myelin oligodendrocyte glycoprotein by the trimolecular complex T cell receptor and class II major histocompatibility molecules on the antigen presenting cells percipitates a massive inflammatory cascade, which leads to production of both pro- and anti-inflammatory cytokines. This inflammatory reaction ultimately results in loss of myelin-oligodendrocyte complexes (Minagar and Alexander, 2005).

2. MORPHOLOGY OF OLIGODENDROCYTE AND MYELIN. SUBSTRATE OF DEMYELINATION.

Macroglia are composed of oligodendrocytes and astrocytes. They account for 95 percent of the glia in the parenchyma of the CNS; microglia make up the remainder. The major function of oligodendrocytes is to form and maintain myelin for the purpose of saltatory conduction. These cells were first described by Robertson in 1899 and 1900 who called these cells mesoglia (Penfield, 1965). Oligodendrocytes and microglia were described in detail by Ramon y Cajal in 1911 but lumped together into category of the "third element". The first accurate description of oligodendroglia as a distinct cell type is generally due to del Rio-Hortega who, in 1921, (Del Rio-Hortega, 1921) modified the silver carbonate method to distinguish oligodendrocytes from microglia.

In fact, the prefix oligo is somewhat of a misnomer. This name was based on the fact that they displayed fewer processes than astrocytes in silver preparations, with technical improvement, an elaborate network of fine processes became evident. Oligodendrocytes, believed to be most numerous in white matter tracts of the CNS aligned in rows between the myelinated fibers, are classically referred to as interfascicular oligodendrocytes. However, oligodendrocytes are surprisingly numerous in many regions of the gray matter, where their cell bodies are often opposed to the perikarya of neurons. Because of this location, they are referred to as satellite or perineuronal oligodendrocytes. The density of these oligodendrocytes can be appreciated with immunocytochemistry or in situ hybridization with the use of oligodendrocyte markers which stain the cell body leaving myelin sheaths unstained.

The complex morphology of oligodendrocytes and their intimate relation to other glia and neurons is showed by the blending of many different techniques. In 1990s, intracellular injections or fluorescent dyes into individual cells showed additional details about the 3-dimensional morphology of oligodendrocytes and astrocytes. Besides, the combination of horseradish peroxidase labeling, which fills the finest processes, and the resolution of electron microscopy has provided information about the relation between structure and function of oligodendrocytes with regard to astrocytes and neurons (Butt and Ransom, 1989, Butt and Ransom, 1993; Butt et al., 1994).

The main function of oligodendrocytes is to allow rapid conduction of axon potentials along axons. But this is not the only one; some other functions associated with specific subtypes of oligodendrocytes have been discovered. Setting an example, a subset of oligodendrocyte progenitors establishes glutamatergic synapses in the hippocampus. The effect of these synapses on oligodendrocytes precursors remains unknown, but they may modulate interactions between neurons and oligodendrocytes via calcium and other ions.

Age-related changes in the number of oligodendrocytes have been difficult to quantify, but several studies have indicated that they slowly increase with age (Ling and Leblond, 1973). They increase dramatically in postnatal development but go on increasing as animals grow old. As oligodendrocytes age, they acquire nuclear filaments and other organelles, but the biochemical significance of these observations is unclear (Mori and Leblond, 1970; Vaughan DW, Peters, 1974; Monteiro et al., 1995).

2.1. Ultrastructure of oligodendrocytes.

Many ultrastructural studies of oligodendrocytes have been carried out in many different species, and they have shown that oligodendrocytes exhibit a wide spectrum of electron densities. In semi- and ultrathin sections, the electron density of mature oligodendrocytes is a remarkable feature, their nuclei and cytoplasm remain electrodense. Their nuclear membranes are rimmed by electrodense clumps of chromatin that extend into the more lightly stained nucleoplasm. On the other hand, the contrast between chromatin and nucleoplasm in gray matter is not as pronounced as in white matter oligodendrocytes, leading to possible misidentification of oligodendrocytes as astrocytes or microglia. In addition, the nuclear membrane of the oligodendrocytes is conspicuously more dilated than that of astrocytes and microglias and abundantly studded with ribosomes. Besides, a scarce proliferation and maturation of glial precursors occurs in the subventricular zone and throughout the white matter in adults (Mori and Leblond, 1970; Paterson et al., 1973). The identification of these cells at the electron microscopic level as multi-, bi-, or unipotential glial precursors is quite hard because many immature glia lack diagnostic features of mature glia.

The morphological characteristics are very helpful to identify oligodendrocyte in micrographs (Fig. 3). With regard to their shapes, the plasma membrane of the oligodendrocytes is often irregular, and has angled borders that adjoint myelinated fibers, astrocytic and microglial processes. When cytoplasm is abundant, it tends to be eccentrically located at one pole of the cell body, with the nucleus at the other one. Rough endoplasmic reticulum is much more abundant than in astrocytes and microglia, and consists of cisternae stacked layer upon layer. The lumen of the endoplasmic reticulum is narrower than that of astrocytes but less so than of microglia, and the membrane is abundantly studded with ribosomes. This abundance of endoplasmic reticulum is due to the massive protein synthesis in these cells, and also free ribosomes can be found extending into the cell distalmost processes, even as far as the node of Ranvier. Because of the presence of free ribosomes, oligodendrocytes processes can be distinguished from small-diameter unmyelinated axons in which ribosomes are absent.

Messenger RNA for myelin basic protein (MBP), one of the two major proteins of myelin, and messages for several other minor components of myelin are transported on free ribosomes into the processes (Barbarese et al., 1999; Gould et al., 1999; Gould et al., 2000). Then, all the translational machinery, including transfer RNA, must be likewise transported down the processes. The Golgi apparatus is also prominent in oligodendrocytes, situated near the nucleus, and consists of four to five cisternae which have a beaded appearance. Mitochondria and microtubules are relatively more abundant in oligodendrocytes than in astrocytes and microglia. Microtubules are randomly oriented in the perikarya but become fasciculated as they enter the oligodendrocytic processes. So undoubtedly, they may play a role in the transport of free ribosomes and the other molecules into the distal processes.

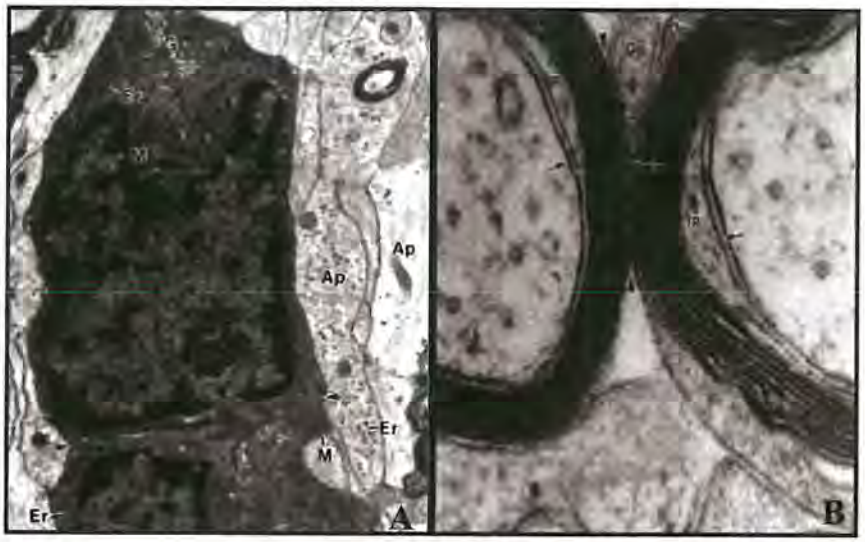


Fig. 3. A: Electron micrograph of oligodendrocyte in white matter of 47-day-old-rat. The electron density of the two oligodendrocyte contrasts with the electron lucency of adjacent astrocytic processes (Ap). Oligodendrocytes are often aligned in rows parallel to myelinated fibers. Golgi apparatus (G), rough endoplasmatic reticulum (Er), mitochondria (M) 13000X. B: High magnification electron micrograh from spinal cord of a 57-day-old postnatal mouse. Several outer (Op) and inner (Ip) tongues of myelin sheaths are present. The trilaminar composition of the plasma membrane can be visualized in several areas (arrows). The inner leaflet of the plasma membrane fuses together to form the major dense line (crossed arrow). The two outer leaflets of the plasma membrane fuse together to form the minor dense line (arrow head). 175000X (Herndon, 2003)

2.2. Lineages of oligodendrocytes and myelinization.

All macroglia and neurons arise from precursor cells in the ventricular layer of the embryonic nervous system. The ventricular layer is a pseudostratified layer of cells that surrounds the ventricles and extends from the rostral tips of the lateral ventricles to the caudal end of the spinal canal. The subventricular layer immediately adjacent to the ventricular layer is also a source of glial precursor cells, especially in adults. Glial precursor cells proliferate in the ventricular layer, migrate laterally from there into the surrounding neuropil, and continue to proliferate and/or differentiate (Skoff and Knapp, 1995; Skoff, 1996). However, glial precursors also migrated rostrocaudally along the neuroaxis for considerable distances (Price and Thurlow, 1988; Walsh and Cepko, 1992; Spassky et al. 1998).

With exception to the olfactory bulb, where OPCs are generated intrinsically, they originate in multiple but discrete areas along the neural tube. Although a proportion of OPCs derive from the dorsal neural tube, most of them develop, under the influence of Sonic hedgehog (Shh) signaling, from a specialized oligodendrogenic domain in the ventricular ventral neuroepithelium, characterized by the expression of the Nkx2.2, Nkx6 and Olig2 transcription factors. In the mouse, the germinal areas for OPCs are located in the ventral neural tube from E9.5 to E10 onwards. Once generated, OPCs proliferate in response to different mitogenic agents, such as PDGF-AA, FGF-2 and VEGF-C, migrate dorsally and ventrally and disperse throughout the prospective grey and white matter to populate the developing CNS prior to initiate their final differentiation into mature oligodendrocytes (Rowitch 2004; De Castro and Bribián 2005). The pattern of OPC migration is not random. OPCs follow precise routes of migration characteristic of each CNS region which suggests the existence of specific combinations of molecular cues that direct their movements.

Around the ventricles and in adjacent white matter tracts, oligodendroglial proliferation occurs simultaneously during postnatal development. An study with oligodendrocyte-specific marker and tritiated thymidine determined which myelin-specific markers are expressed during oligodendroblast development. This study reported that oligodendroblasts express myelin-specific glycolipids, particularly sulfatide but sometimes

galactocerebroside (GalC). These oligodendroblasts were distributed abundantly throughout the white matter in the postnatal brain, suggesting that local proliferation is the major source of mature oligodendrocytes (Skoff et al., 1994).

The maturation of oligodendrocytes precursors into myelinating oligodendrocytes occurs at different time in different fiber tracts during development. A general principle is that myelination of fiber tracts begins with the phylogenetically oldest fiber tracts (Yakovlev and Lecours, 1966). Another rule is considered that myelination take place rostrocaudally in the spinal cord but caudorostrally in the brainstem.

In the mouse, myelination begins in the ventral funiculi at birth, in the optic nerve 5 days postnatally and in the corpus callosum approximately 14 days postnatally. Interestingly, the first myelinated fibers were found adjacent to blood vessels, suggesting that factors from the vascular system accelerate oligodendrocytes differentiation (Skoff et al, 1980). After migration from the ventricular and subventricular zones into the presumptive white and gray matter, the oligodendrocytic precursors undergo a dramatic change in their morphology from bipolar cells to snowflake-shaped cells with multiple radial processes. The processes of these snowflake-shaped cells are likely to be in direct contact with all axons contained within their sphere. The axons "selected" for myelination by an oligodendrocyte are not necessarily the axons closest to the cell body, and all axons touched by oligodendrocytes processes are not ensheathed. It is likely that the radial processes of the oligodendrocyte are already in contact with these axons and that axon-oligodendrocytic contacts trigger myelination (Hardy and Friedrich, 1996; Butt and Ibrahim). Interestingly, one side of an oligodendrocyte may retain the snowflake appearance, whereas the other side has only long processes that have begun to ensheath some axons.

Hardy and Friedrich (Hardy and Friedrich, 1996) found that the first stage of myelin investment consists of the elaboration of a long thin process parallel to the longitudinal axis of the axon. The second stage consists of the spiral ensheathment and longitudinal enlargment of the oligodendrocyte process around the axon. As the oligodendrocyte begins to form more myelin sheaths, it loses a number of radial processes. Morphologically, axons generally attain a certain diameter for myelination to begin, but this diameter-dependent effect is most likely contingent upon biochemical changes in the axon. The appropriate diameter might be accompanied by electrical activity because tetrodotoxin blocked myelination in an in vitro system (Kaplan et al., 1997). Conversely, α-scorpion toxin facilitates myelination by causing repetitive electrical activity (Demerens et al, 1996; Colello et al., 1995).

The development of OPCs from neuroepithelial cells has mostly been studied in the rodent neural tube (Rowitch 2004). In the mouse spinal cord, for example, the first OPCs expressing the platelet-derived growth factor receptor α (PDGFRα) are detected as a narrow band in the ventral neuroepithelium around embryonic day (E)12.5-13, well after neuronal development which begins at E9 (Hardy, 1997; Pringle et al., 1998). However, some proteins that are characteristic of oligodendrocytes and their precursors are expressed earlier than PDGFRα, and may identify the earliest stages of oligodendrocyte lineage specification. These include the Olig1 and Olig2 basic helix-loop-helix gene regulatory proteins, which are expressed in the same region of the spinal cord as PDGFRα at E13, but are expressed as early as E9 (Lu et al., 2000; Zhou et al., 2000). The earliest Olig2-expressing cells in the ventral spinal cord, however, may develop into neurons rather than OPCs (Marquardt and Pfaff, 2001; Mizuguchi et al., 2001; Novitch et al., 2001; Sun et al., 2001; Zhou et al., 2001): if Olig2 is inactivated, neither motor neurons nor OPCs develop in the spinal cord (Lu et al., 2002; Zhou and Anderson, 2002). By E11.5-12, Olig expression is restricted to the same region, where PDGFRα will be expressed and OPCs emerge. Therefore, it is likely that this Olig expression marks the earliest stages of oligodendrocyte specification, 1-1.5 days before PDGFRα expression is detectable (Lu et al., 2000; Zhou et al., 2000). By E13, Olig1, Olig2 and PDGFRα, as well as another OPC marker, the NG2 proteoglycan (Levine and Nishiyama, 1996), are all expressed in the same small region of the ventral spinal cord that generates OPCs (Lu et al., 2000; Pringle et al., 1996; Woodruff et al., 2001; Zhou et al., 2000). OPCs then migrate throughout the CNS, where they proliferate largely in response to PDGF (Fruttiger et al., 1999) and terminally differentiate into oligodendrocytes. The first oligodendrocytes that express galactocerebroside (GalC) in spinal cord appear in the mouse CNS around E17 (Calver et al., 1998).

As myelinogenesis proceeds along a caudorostral gradient in the CNS, occurring at different times and rates among, and within, fiber tracts and cortical areas, their features should be reproduced in cultures initiated from CNS regions. Unlike Schwann cells of the peripheral nervous system, OL cultured in the absence of neurones can express the major glycolipids and proteins of the myelin sheath (Lemke, 1988). These include

GalC (Mirsky et al. 1980), its sulfated ester sulfatide (Singh & Pfeiffer, 1985), 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP; McMorris, 1983; Bansal & Pfeiffer, 1985), myelin basic protein (MBP; Mirsky et al. 1980; Barbarese & Pfeiffer, 1981; Zeller et al. 1985), proteolipid protein (PLP; Macklin et al. 1986; Dubois-Dalcq et al. 1986), and myelin-associated glycoprotein (MAG) (Dubois-Dalcq et al. 1986).

Successive stages of oligodendrocyte development are distinguishable in the rat on the basis of the sequential expression of cell surface antigenic markers (Lee et al., 2000; Baumann and Pham-Dinh 2001; Espinosa-Jeffrey et al., 2009; Fig.4). The earliest is represented by the bipotential O-2A glial progenitor cell, first identified in cultures of postnatal rat optic nerve (Raff et al. 1983). O-2A progenitors can differentiate into either oligodendrocyte or type 2 astrocytes. A second stage in the OL lineage is identified by the appearance of the O4 surface antigen, the earliest surface marker restricted to the oligodendroglial lineage (Sommer & Schachner, 1981, 1982; Schachner et al. 1981). O4+ progenitors define a transitional intermediate between the early bipotential O-2A progenitor stage and the onset of GalC expression (Sommer & Noble, 1986; Dubois-Dalcq, 1987; Levi et al. 1987). A third stage of development is achieved when O4+ progenitors express GalC, the prototypical surface marker for differentiated postmitotic oligodendrocyte (Raff et al. 1978), losing rapidly specific surface gangliosides before synthesizing myelin proteins. The emergence of CNP and MBP in cultured preoligodendrocytes at ages corresponding to postnatal days 7-8 and 10-12, respectively, occurs on a schedule congruent with the onset of rapid accumulation of these markers in the rat cerebrum (Cohen & Guarnieri, 1976; Sprinkle et al. 1978; Bansal & Pfeiffer, 1985). On the other hand, PLP immunoreactivity in newly myelinating cortical tracts appears about postnatal day 10 (Trapp et al. 1987) and there may be a temporal coexpression of PLP and MBP mRNAs in situ (Verity & Campagnoni, 1988).

In brain, expression of DM20 has been detected before PLP as early as E9.5 (Timsit et al. 1995; Peyron et al. 1997). Using IHC with an antibody raised against the 35 amino acids specific for PLP only, it was demonstrated that PLP expression starts later than DM20. Very low levels of PLP were found in rat premyelination oligodendrocytes at P7, during early stages of myelination (Trapp et al. 1997). However, expression of PLP-specific mRNA was reported in P3 rat subcortical white matter using radiolabeled probes (LeVine et al. 1990).

MOG is another surface marker of oligodendrocyte maturation and its presence correlates with late stage of maturation, possibly restricted to the myelinating oligodendrocytes, as shown using the CG4 cell line (Solly et al, 1996). MOG expression is delayed by several days with respect to MBP, and it appears only the myelinating oligodendrocytes, not as same as MBP which can be present on both non-myelinating and myelinating oligodendrocytes. In vitro, immunocytochemical analysis of MOG protein expression, performed on myelinating cultures derived from mouse brain embryos at 15 days of gestation, confirmed the strict restriction of MOG expression to myelinating oligodendrocytes

These observations indicate that an intrinsic genetic program in oligodendrocytes activates myelin gene expression (see Lee et al., 2000 for an excellent review)

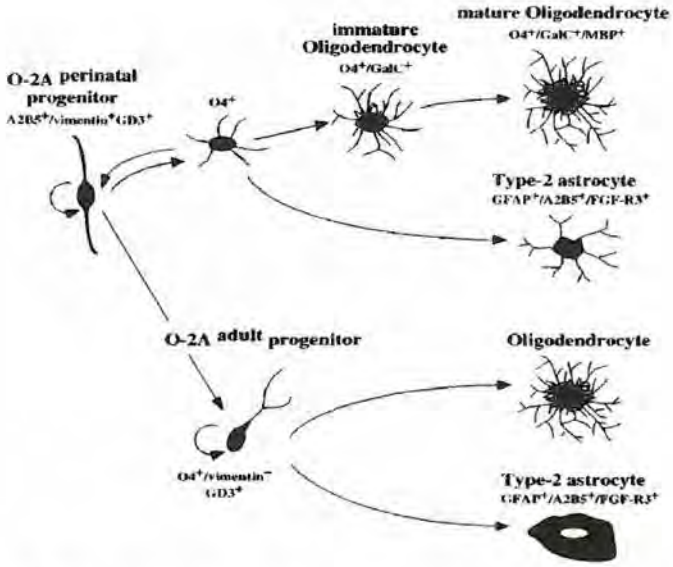


Fig. 4a. O-2A cells develop into oligodendrocytes and type-2 astrocytes. Self-renewing O-2A progenitor cells give rise to either a differentiated progeny or to another class of different precursor cells present in the adult. The differentiation into mature oligodendrocytes is a stepwise process that is marked by the acquisition of specific markers (O4, GalC). At the early "pre-oligodendrocyte stages" that is defined by the expression of O4, cells are not yet terminally differentiated and can be induced to divide and to revert to an O4- stage. Adult O-2A progenitor cells, that can co-exist with perinatal O-2A cells, give rise to the same cell types as their perinatal counterpart. The adult derived type-2 astrocytes show however a different morphology than perinatal derived type-2 astrocytes (Lee et al., 2000).

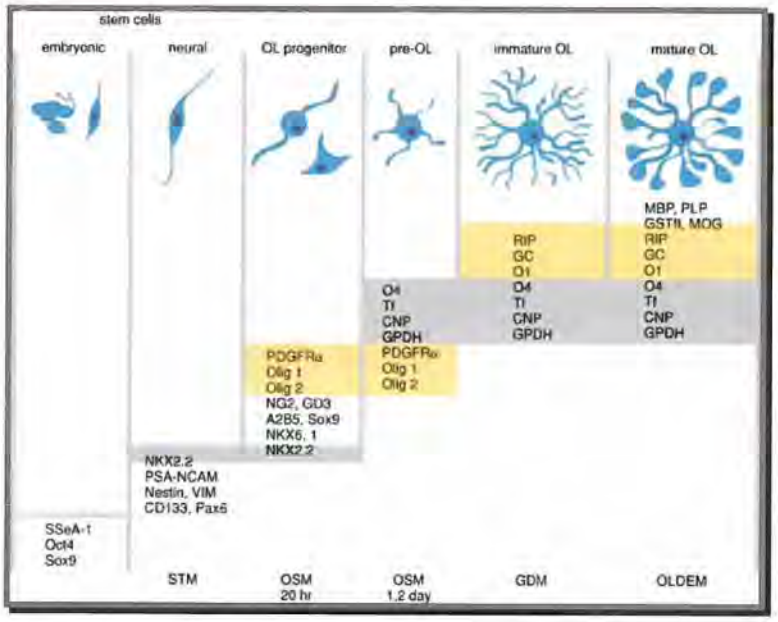


Fig. 4b. Schematic representation of the developmental stages of cells of the oligodendrocyte lineage. Schematic drawing of the morphological and antigenic progression from stem cells to myelinating mature oligodendrocytes, through progenitors, preoligodendrocytes, and immature nonmyelinating oligodendrocytes. Stage-specific markers are boxed. (Espinosa-Jeffrey et al., 2009).

2.3. Myelin

The first description of myelin was reported by Leeuwenhoek who, in 1717, described nervules existing from the spinal cord and surrounded by fatty tunics. The next major advance in the description of myelin was Ehrenberg's teasing out of a single nerve fiber in the 1930s, not only in the peripheral nervous system (PNS) but also in the CNS.

The greek word myelin, or marrow, was used by Virchow in 1858 to refer to the medullary substance between the axon and its external membrane. In 1871 Ranvier wrote that myelin is not a continuous structure along the axon cylinder but interrupted periodically along its length. It was in the 1970s, with the increased resolution of electron microscopy, when accurate descriptions of the complex structure of the myelin sheath were provided. Also in the 1970s, freeze-fracture analyses of myelinated fibers revealed additional structural features of myelinated fibers such as gap junctions.

Myelin consists of the spiral wrapping of plasma membrane by oligodendrocytes or Schwann cells around the axon. A segment of myelin is called an internode and, at the distal ends of each internodal myelin segment, the axon is unmyelinated and named node of Ranvier (Fig. 5). At regular intervals around successive wraps of compacted myelin, this compaction is interrupted by zones of non-compact myelin that form the incisures (or clefts) of Schmidt-Lanterman. Internodes longer than 1 mm have been found in the spinal cord (Murrat and Blakemore, 1980) but the average diameter of an oligodendrocyte perikaryon is 5 to 15 μm, for a 100-fold difference between maximum lengths of the two structures. The successive edges of each loop of the myelin sheet contact the axon and are known as paranodal loops. The zone where these paranodal loops contact the axon is called paranode, and marks a zone of separation of voltage-gated sodium channels at the node from the voltage gated potassium enriched region known as juxtaparanode. The paranodal loops are bridged to the axon by specialized, electrodense adhesions called septate-like junctions that form diffusion barriers between the periaxonal space and the interlamellar and periaxonal spaces of the internode (Bhat et al., 2001, Charles et al., 2002)

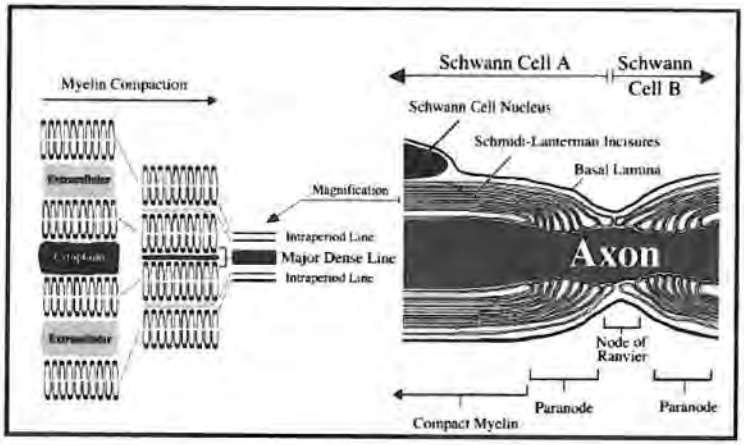


Fig. 5. Schematic longitudinal cut of a myelinated fibre around the node of Ranvier. Diagram of compact myelin, including major dense and intraperiod lines.

Numerous classic and contemporary studies have examined the relation of the length and thickness of the internode to the axon. Correlation of myelin thickness and internodal length to axon diameter are more easily established in the PNS than in the CNS, because individual fibers in the PNS can be teased out and axonal parameters can be measured more easily than in CNS. The classic literature has extensively documented a positive linear correlation between axonal diameter, myelin sheath thickness, and internodal length for the PNS and CNS (Friede RI, Bischhausen, 1980; Smith et al., 1982). While in the CNS this same general relation exists, more recent studies showed the interrelation between these two is not as tightly correlated as in the PNS. Predictably, smaller-diameter fibers have shorter internodes than do larger-diameter fibers, but the internodal

length of these small-diameter fibers was significantly less than plotted by linear regression analyses (Ibrahim et al., 1995).

In the CNS, fine astrocytic processes encircle the bare axolemma of the node of Ranvier (Butt et al., 1994, ffrench-Constant et al., 1986). Three-dimensional recosntructions of these "astrocytic" cells, forming the nodes, extend processes to the pia limitans or blood vessels, according to the traditional theory that all astrocytes contribute processes to form the "blood-brain barrier". That specialized type of astrocyte, the type II astrocyte, generated after oligodendrocytes (Lee et al., 2000) specialized to form the nodal processes. A population of oligodendrocyte progenitors, positive for NG2 but not for carbonic anhydrase or glial fibrillary acidic protein (markers for oligodendrocytes and astrocytes, respectively) were shown to form processes at the node of Ranvier (Butt et al., 1999), in keeping with the hypothesis that these cells are likely to be immature glial precursors.

Adjacent to the node of Ranvier, the compacted layers of myelin membrane become unraveled, the inner leaflets of the fused plasma membranes split, and the intervening space is filled with cytoplasm. This region of myelin is called the paranode and is enriched in many proteins not normally found in the compacted myelin sheath.

A low magnification electron micrograph of internodal compact myelin cut transversely shows alternating layers of light and dark lines. The dark line, termed the major dense line, represents the apposition of the inner leaflets of two plasma membranes in which the cytoplasm between these two has been extruded during myelin formation. The formation of this dark line is best visualized at the outermost or innermost lamella, where the two plasma membranes separate and are filled with cytoplasm. Usually, a thin rim of cytoplasm lies adjacent to the outermost and innermost layers of myelin and are termed the outer (abaxonal) and inner (periaxonal) mesaxons, respectively. The major dense lines are continuous with one another and can be traced as one spiral extending from the outer surface of the myelin sheath to the inner surface adjacent to the axolemma.

On the other side, the light line represents the fusion of the two outer leaflets of the plasma membrane, and their formation can be seen in sections at the outer and inner mesaxons. Very high magnification shows a still more elaborate morphology of the major and minor dark lines. The two outer leaflets do not fuse completely, leaving an intervening gap of 2nm. Similarly, at magnifications greater than $2 \cdot 10^6$ X, the major dense line is composed of two lines representing the fusion of the two inner leaflets of the plasma membrane.

The morphologic complexity of myelin is matched by an asymmetric distribution of myelin proteins and lipids. Compact myelin is composed mainly of PLP and MBP; the inner and outer loops are enriched with MAG, CNPase and MOG, and the paranodal loops are enriched with connexin 32 and myelin-associated oligodendrocytic basic protein (MOBP). Likewise, tight junctions between compact myelin and noncompact myelin are enriched with claudin 11 (Gow et al., 1999; Morita et al., 1999; Bronstein et al., 2000) whereas parnodal/axonal membranes are enriched with neurofascin 155 (Tait et al., 2000). So, the axon at the nodal and paranodal regions must express many specific ion channels and proteins to segregate myelin proteins at paranodes and tight junctions.

2.4. Composition of myelin

Myelin is essentially a highly enriched cell membrane and its composition reveals that it makes up 40 to 50% of the dry weight of the brain, which is about two thirds lipid and one third proteins (Rumsby, 1978).

2.4.1. Lipids. Although the composition of myelin is similar to other cell membranes with respect to its enrichment in lipids, the distribution of them is distinct from that of standard membranes.

The major lipid in myelin is free cholesterol, which comprises about 30% of total lipid by dry weight, hovewer cholesterol esters are not found in normal mature myelin. Phospholipids are collectively the most abundant lipids in CNS myelin, constituting about 40-45% of total lipid by dry weight, and most brain phospholipids are derivatives of phosphatidic acid (discylated glycerol-2-phosphate).

Glycerophospholipid classes are defined by the nature of the compound linked at the third position of the glycerol backbone. The most abundant of these glycerophospholipids in adult human brain are phosphatidyl ethanolamine, phosphatidylcholine (lecithin), phosphatidylserine and phosphatidylinositides. Sphingolipids are distinguished by the substitution of sphingosine, a long chain aminodiol, for the glycerol backbone used by glycerophospholipids. Ceramides are sphingolipids where the amino group of sphingosine is N-acylated with

long-chain fatty acid. The major glycolipid of mammalian CNS myelin is galactocerebroside (GalC), in which galactose is β-glycosidically linked to ceramide. Gal-C constitutes about 16% of total adult human brain lipid. Sulfatide constitutes about 6% of brain lipid and is form galactocerebroside esterified to sulfate at the 3´position of galactose. Other sugars including glucose, N-acetylglucosamine, N-acetylgalactosamine, fucose and other, are conjugated to cerebroside to form other brain glycolipids. Sialic acid, N-acetyl-neuraminic acid (NANA), is an important N-acylated, nine-carbon amino sugar that is linked to glycolipids to form gangliosides. Gangliosides are present in a variety of brain regions as well as in other tissues, and are usually classified according to the number of sialic acid residues in the molecule and their relative migration rates on thin-layer chromatograms.

2.4.2. Proteins. The protein composition is very complex (Fig. 6) with several prominent bands in sodium dodecyl sulfate polyacrylamide gel. Some of the most important proteins are:

2.4.2.1. Proteolipid protein (PLP) and DM20.

It is the major protein in CNS myelin and it has a highly lipophilic character. PLP constitutes half of the proteins in CNS myelin, or roughly one sixth the dry weight of myelin (Tenenbaum and Folch-Pi, 1966; Griffiths et al., 1995; Greer and Lees, 2003). It is one of several brain antigens that can elicit EAE. Its ununsually strong lipid association led to its alternate designation of lipophilin (Gow, 1997).

PLP is a transmembrane protein with four membrane-spanning domains (Laursen et al., 1984; Popot et al., 1991; Inouye and Kirschner, 1994). Its primary structure is remarkably highly conserved, and is identical in humans, mice and, rats. Synthesized as a preprotein with 277 residues, the initiation methionine is cleaved generating the mature protein with 276 residues (29 Kda), highly enriched in hydrophobic amino acids. Both the amino and carboxyl termini lie within the cytoplasm, as does an intracellular loop which is enriched in charged residues. PLP is covalently modified after its synthesis, and several cysteine residues are acylated by fatty acids that probably anchor the protein to the plasma membrane (Pham.Dinh et al., 1991; Scherer et al., 1992; Townsend et al., 1982, Agrawal et al, 1982. Bizzozero et al, 2002). Proteolytic cleavage may also occur in a physiologically regulated manner to release a peptide with mitogenic properties on astrocytes as well as on oligodendrocytes precursors (Yamada et al., 1999).

The *PLP* gene undergoes alternative splicing, generating an additional product, DM20, that lacks 35 residues that lie within the cytoplasmic loop of PLP. Although DM20 appears during embryonic development and is about a equivalent in level to that of PLP in Schawnn cells, PLP predominates in mature CNS myelin, where it is about 10 fold more abundant than DM20 (Pham.Dinh et al., 1991). During development, PLP expression peaks in concert with other major myelin proteins, and depends upon contact with axons (Scherer et al., 1992).

The topology of PLP and its abundance in myelin lead to the speculation that it must act as an adhesion protein, locking together adjacent membrane leaflets in compact myelin, but oligodendrocyte development does not require PLP or DM20 to performance the compaction, as plp deficient mice reveal, so other myelin components must be involved. In those plp knockout mice, with a surprisingly mild null phenotype, a slight "loosening" of myelin lamellae occurs, which does support an adhesive role for PLP. On the contrary, the presence of compact myelin in these animals suggests that other factors are also important for adhesion of myelin leaflets. The expression of other myelin proteins, such as MBP, is not altered when PLP is absent, which means that not an obvious compensatory change in other myelin proteins occurs to soften the absence of this protein.

Other PLP functions have been proposed, including ion channel and glial mitogenic factor (Knapp, 1996). However, the plp knockout studies provide conclusive proof that this protein is necessary for axonal survival (Griffiths et al,, 1998).

2.4.2.2. Myelin basic protein (MBP)

This is the second most abundant protein in CNS myelin, about 30% of myelin protein mass, and the first antigen identified inducing EAE.

MBP is not a transmembrane protein but a hydrophilic protein with a highly basic isoelectric point, and it is probably tightly associated with lipids and/or other myelin proteins. It lies in the thin cytoplasmic space between cell membrane leaflets that constitutes the major dense line of compact myelin (Kies et al., 1972).

MBP is actually a family of related proteins. The MBP gene undergoes a complex pattern of alternative splicing, generating four major products in humans, and five in mice. In mice, their molecular masses are 21.5 kDa (all 7 exons), 18.5 kDa (lacking exon 2), 17.2 kDa (lacking exons 2 and 5), 17.0 kDa (lacking exon 6), and 14 kDa (lacking exons 2 and 6) (de Ferra et al., 1985; Kamholz et al., 1986). Other splice variants occur but are present at very low levels. These differents forms are developmentally regulated, and in the adult brain the 18.5 and 17.2 kDa forms predominate, constituting about 95% of the MBP. Despite the presence of all these isoforms, they are not needed for myelin compaction and maintenance, per se, since mice that express only the 14 kDa form can form normal myelin (Kimura et al., 1989).

Campagnoni et al. (1993) discovered that *MBP* gene is part of a much larger gene which was named Golli-mbp (gene expressed in the oligodendrocyte linage). The expression of the Golli-mbp gene is complex and includes several alternatively spliced transcripts, including some that are found outside of the nervous system, including thymus, where it could potentially play a role as an autoantigen (Pribyl et al., 1993).

The role of MBP in myelin formation is thought to be involved in compactation of myelin and is clearly part of the major dense line of compact myelin. Post-translational modification of MBP, such as methylation and citrullation, may facilitate compactation and interactions with other proteins or oligodendrocyte components (Ulmer and Braun, 1983; Yamamori et al., 1994; Boggs et al., 1997; Boggs et al., 1999).

2.4.2.3. 2′,3′-cyclic nucleotide phosphodiesterase (CNPase)

CNPase constitutes about 5% of CNS myelin protein (Wells and Sprinkle, 1981; Sprinkle et al., 1980; Sprinkle et al. 1983). Although this protein catalyzes the hydrolysis of 2′,3′-cyclic nucleotides to 2′-nucleotides, in vitro it is not known if this activity is present in vivo.

CNPase is one of the so-called Wolfram proteins, it appears as two proteins of about 46 and 48 kDa on SDS gel. This results from differential utilisation of translation initiation sites. Although they are cytosolic proteins, they are associated with membranes. This is because both proteins are isoprenylated at their carboxy termini and are acylated, which provide their association. Moreover, the largest isoform is phosphorylated.

Immunohistochemical studies have demonstrated that 48kDa protein is confined to non-compacted regions of myelin, such as the clefts of Schmidt-Lanterman, the paranodal loops and the inner mesaxon (Braun et al., 1988). Expression of the two protein forms is differentially regulated during development, with only the largest protein expressed in oligodendrocyte precursors (Scherer et al., 1994). CNPase expression is dependent upon contact with axons, similar to structural myelin proteins, such as MBP and PLP, does (Scherer et al., 1992).

The precise role of CNPase in myelin is unclear as far as CNPase knockout mice have ultrastructurally normal myelin, including compact myelin (Lappe-Siefke et al., 2003). Pathologically, the most prominent abnormality in these mice is widespread axonal degeneration, characterized by axonal swellings filled with vesicles, mitochondria and cytoskeletal proteins, and reduction in axon numbers. Thus, CNPase, as well as PLP, must be essential for maintaining axonal integrity, further demonstrating the importance of the dynamic interaction between axons and their ensheathing oligodendrocytes.

2.4.2.4. Oligodendrocyte specific protein (OSP; claudin-11)

OSP is a 22 kDa protein. This is a member of the tetraspanin and claudin family of transmembrane proteins and comprises about 7% of CNS myelin protein, making it the third most abundant myelin protein. According to specific antibodies and ultrastructural studies, OSP appears to form intracellular tight junctions at sites of apposition of noncompact myelin regions, and it is also necessary for formation of tight junctions in Sertoli cells (Gow et al., 1999). In spite of these roles, there is not an apparent deficiency of myelin in mice deficient in OSP.

2.4.2.5. Myelin associated glycoprotein (MAG)

Myelin contains several glycoproteins, the best studied is myelin associated glycoprotein (Quarles, 1997; Quarles, 2002). MAG makes up about 1% of CNS myelin and actually consists of two related proteins of 72 (large or L-MAG) and 67 (small or S-MAG) kDa molecular mass. They are products of alternative splicing of the *MAG* gene. Both are heavily N-glycosylated, with approximately 30% of their mature mass constituted of carbohydrate. MAG is a membrane protein, with a single transmembrane domain and five immunoglobulin (Ig) domains, the first of which has an RGD (arginine-glycine-aspartate) motif characteristic of integrin-mediated cell adhesion molecules. The oligosaccharides in MAG are heterogeneous and complex, most being negatively charged due to sialic acid or sulfate groups, and include the L2/HNK-1 carbohydrate epitope.

MAG is located in the inner mesaxon at the point of axon-glial contact; this structure and localization of MAG seem to be consistent with a putative role in axon-glial signaling. Although in the CNS, MAG is found only in the periaxonal oligodendroglial membrane, in the PNS it is also localized at other non-compacted areas of myelin, i.e. paranodal loops, Schmidt-Lanterman clefts and the outer mesaxons. Based on studies with MAG-null mice, an important signaling function between axons and myelinating glia has been shown, in the CNS and PNS (Montag et al., 1994; Fruttiger et al., 1995). Regarding this, the lack of this glycoprotein displayed some abnormalities in the CNS such as aberrant myelin loops and redundant myelin sheaths, and later developed into dying back oligodendrogliopathy (Lassmann et al., 1997). This implicates MAG is a mediator of an axonal signal that exerts a trophic effect on oligodendrocytes.

On the other hand, MAG also seems to have axonal growth-inhibitory effects in postnatal animals after injury (Mukhopadhyay et al., 1994). The current evidence supports the fact that a complex set of interactions between oligodendrocyte proteins MAG, Nogo66 and -myelin oligodendrocyte glicoprotein (MOG) with the axonal Nogo receptor (NgR) and p75 neurotrophin receptor on lipid rafts can activate the small GTPase Rho, which then leads to axonal growth inhibition.

2.4.2.6. Myelin oligodendrocyte protein (MOG)

MOG is a glycoprotein of 26 to 28 kDa and a minor component of CNS; like MAG, it is a member of the Ig superfamily of proteins (Gardinier et al., 1992, Johns and Bernand, 1999). It has a single Ig domain and a single transmembrane domain. Its localization is restricted to the outer surface of myelin sheaths and oligodendrocytes, suggesting that it may function in mediating signals between the oligodendrocytes and the extracellular space (Matthieu and Amiguet, 1990). MOG was originally identified through its ability to act as a brain antigen (Linington et al., 1984). Both T and B cell-mediated immune responses can be experimentally generated by MOG (Abo et al., 1993, de Rosbo and Ben-Nun, 1998). Hovewer, no naturally occurring or experimentally-generated mutations of MOG have been reported in any species.

2.4.2.7. Myelin/oligodendrocyte specific protein (MOSP)

MOSP is a 48 kDa surface membrane protein with interesting biological properties which is expressed exclusively by oligodendrocytes in the central nervous system (Dyer et al., 1991). The role of this protein was studied by prolonged exposure of cultured oligodendrocyte to anti-MOSP antibodies resulting in a dramatic increase in the number and thickness of microtubules within processes and membrane sheets (Dyer, 1993; Dyer and Matthieu, 1993). This suggests that MOSP plays an important role in membrane/cytoskeleton interactions during the formation and maintenance of CNS myelin.

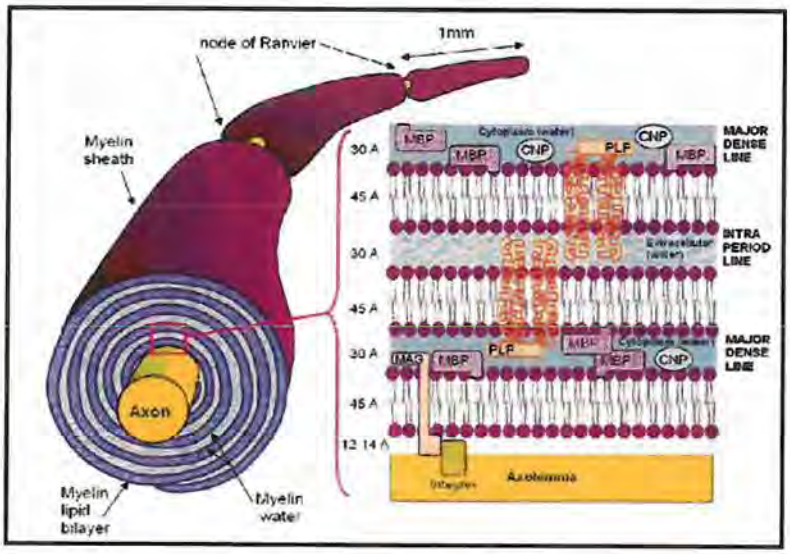


Fig. 6. Myelin structure and protein composition: myelin basic proteins (MBP), proteolipid protein (PLP), 2′,3′-cyclic nucleotide phosphodiesterase (CNP), myelin-associated glycoprotein (MAG) (Laule et al., 2007)

3. ANIMAL MODELS: EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

The mechanism of demyelination in MS is not completely understood. Immune modulating therapy can delay the progression of MS, but there is still no cure for the disease. That is the reason why the development of experimental models for studying its pathogenesis is the most powerful tool. These models are also necessary for testing the safety and efficacy of experimental therapies before using these drugs in clinical trials on human patients.

The main experimental models for MS are divided into immune-mediated models or virus-induced models. In immune-mediated models an autoimmune reaction against myelin is induced by the injection of myelin molecules (or portions of it), in combination with immune boosters (Freund´s adjuvant). The prototype of this category is experimental autoimmune encephalomyelitis (EAE). This model uses a variety of experimental animals including mice, rats, rabbits and even primates. The viral models include several ubiquitous animal viruses such as coronaviruses (mouse hepatitis virus) and enteroviruses (Theiler´s virus) in mice, visna virus in sheep, and distemper virus in dogs. The virus-induced model systems provide both a substrate for studying the pathogenesis of the disease and a hypothetical mechanism for virus-induced autoimmunity in humans.

3.1. Experimental autoimmune encephalomyelitis.

EAE is an animal model of autoimmune disease of the CNS which resembles MS in many aspects. EAE is used in laboratory investigations of MS and also serves as a model for organ specific autoimmune disease in general (reviewed in García-Díaz and Estivill-Torrús, 2008).

EAE was first described over 50 years ago, and it has been a popular and frequently used model. The earliest attempts at producing EAE were efforts to understand the pathogenesis of postrabies vaccination encephalomyelitis. Pasteur´s rabies vaccine consisted of a suspension of desiccated rabbit spinal cords infected with rabies. After a series of injections, occasional patients developed an encephalomyelitis which was distinct from rabies. Early investigators were able to induce a similar encephalomyelitis in rabbits or monkeys by administering repeated injections of neural tissue, thus demonstrating that postrabies vaccine encephalomyelitis probably resulted from the unintentional induction of an autoimmune response against neural antigens (Koritschoner, R and F Schweinburd, 1925; Rivers et al., 1933; Rivers TM and FF Schwentker, 1935).

In these early experiments the incidence of disease was low, and induction of disease required multiple injections over a period of many weeks. The use of an emulsion made of the antigen and complete Freund's adjuvant (CFA), a mixture of mineral oil and Mycobacterium, turned induction of EAE into much simpler and more reliable model. The injection antigen in CFA usually induces a strong and prolonged immune response against the antigen. In 1946, Kabat et al. published a preliminary report of the induction of EAE in 3 of 4 monkeys using three weekly injections of rabbit brain emulsion in Freund's adjuvant (Kabat et al., 1946). Later, in 1949, Olitsky and Yager described the induction of experimental disseminated encephalomyelitis in white mice. As EAE in mouse shared some clinical features with the human disease MS, most prominently paresis/paralysis and ataxia; mouse EAE became the most common model for autoimmune inflammatory disease of the CNS (Olitsky and Yager 1949). EAE has been subsequently induced in many other species, including dogs, cats, rats, mice, sheep, goats, pigs, chickens, and pigeons.

In the beginning, the majority of mice developed neurologic symptoms after three to five injections of brain tissue in CFA and the symptoms began about three weeks after the first injection. Those studies demonstrated that susceptibility to EAE was not homogenous, but this varied widely between strains. Later, Lee and Olitsky demonstrated that separate injections of pertussis vaccine given in addition to antigen in CFA increased the incidence of disease (Lee and Olitsky, 1955). Muñoz and coworkers found that the active ingredient in pertussis vaccine was the toxin, and purified pertussis toxin is now routinely used as an additional adjuvant to EAE in mice (Munoz et al., 1984).

An early focus of EAE research was to identify the encephalitogenic component of the homogenate. At early date, both major structural proteins of myelin, MBP and PLP, appeared to be encephalitogenic (Olitsky and Tal, 1952; Kies and Alvord, 1959; Kies et al, 1960). Many other myelin proteins or peptides derived from myelin proteins have been demonstrated to be encephalitogenic including myelin oligodendrocyte glycoprotein (MOG), myelin-associated oligodendrocytic basic protein, and oligodendrocyte specific protein (Linington C et al., 1993; Amor et al., 1994; Johns et al, 1995; Maeaettae et al, 1998; Stevens et al, 1999). MOG is particularly interesting since it induces relapsing EAE with extensive demyelination. However, not all CNS proteins will induce EAE, the myelin protein 2′,3′-cyclic nucleotide 3′-phosphodiesterase and the stress protein alphaB crystallin did not induce the disease (Maeaettae, MA et al., 1998; Thoua, NM et al., 2000). Since then, multiple T cell epitopes for MBP, PLP, and other encephalitogenic proteins have been reported, and synthetic peptides comprising encephalitogenic T cell epitopes are now standard reagents for inducing EAE. In addition, non-myelin proteins suchs as S100β and GFAP can also cause encephalomyelitis, but the distribution of the lesions is different than that seen with myelin proteins (Kojima et al., 1994; Berger, T et al., 1997).

3.2. EAE Induction.

There are several strategies to induce EAE in animals. Firstly, EAE was induced by immunization of animals with mouse spinal cord homogenate (MSCH) or isolated myelin proteins and peptides, so called active EAE. Alternatively, Weir at al. demonstrated induction of EAE by transferring bone-marrow derived dendritic cells presenting MOG_{35-55} into naive C57BL/6 (Weir et al., 2002). Both immunized animals as well as mice expressing transgenic T cell receptor specific for MBP or other myelin antigens can function as a source of T cells suitable for adoptive transfer of disease (Goverman, 1999; Kuchroo et al., 1991; Brocke et al., 1996). Thus, EAE can be generated by adoptive transfer sensitized lymph node cells (LNCs) from mice or rats which have been immunized (Brocke et al., 1994) with MSCH, MBP, PLP or other CNS antigens (Paterson, 1960; Bernard et al., 1976; Paterson et al., 1975; Pettinelli and McFarlin, 1981). This disease is called adoptive or passive EAE.

Several immunodominant epitopes of myelin components have been characterized, and there are several peptides representing epitopes of MBP, PLP, or MOG widely used for the induction of EAE (Zamvil and Steinman, 1990; Tuohy et al., 1988, Tuohy et al., 1989; Amor et al., Vanderlugt et al., 2000; Kuchroo et al., 2002). However, as mentioned, the encephalitogenicity of myelin-derived peptides is dependent on the expression of particular MHC class II antigens in the appropriate mouse strain, and the same peptides are not be equally effective in all strains of mouse. Thus, induction of disease using PLP peptide $PLP_{139-151}$, MBP peptide MBP_{87-99} or MOG peptide MOG_{92-106} is suitable in SJL ($I\text{-}A^s$) mice, while the N-terminal peptide MBP_{Ac1-11} or MBP_{87-99} are suitable in (PL X SJL)F_1 mice, and MOG_{35-55} is routinely used to induce EAE in C57BL/6 mice. Besides that, in recent study, inoculation of myelin-associated oligodendrocytic basic protein (MOBP) and one

of its derived peptides, $MOBP_{37-60}$, were reported to produce severe clinical and histopathologic signs of EAE in SJL mice (Holz et al., 2000). Moreover, the encephalitogenic potential of synthetic peptides of MAG (peptide MAG_{97-112}) and OSP (peptide OSP_{57-72}) has recently been demonstrated in ABH (H-2Ag7) and SJL mice, repectively, (Morris-Downes et al., 2002).

Typical active immunization protocols require the emulsification of encephalitogens in CFA containing inactived mycobacteria such as Mycobacterium tuberculosis strain H37RA. The induction of active EAE in some rat and mouse models is significantly enhanced by the administration of heat-killed whole organisms of *Bordetella pertussis* (Lee and Olitsky, 1955; Bernand and Carnegie, 1975) or their derived toxin, pertussis toxin (PTX) (Munoz et al., 1984). Antigen concentrations and protocols used for these immunizations vary widely between groups and should be adjusted to each laboratory's own conditions.

In order to choose the proper model for studying each process, that is important to take into account several aspects of the immunization. It should be mentioned the use of CFA in direct immunization may complicate the interpretation of experimental data due to the severe long-term inflammation caused by it. Some of the first observed effects could be attributed to this mode of immunization rather than to the pathogenic process of inflammation and demyelination in the CNS.

On the other hand, induction of EAE by cell transfer allows the separate study of the effector phase of the disease without the influence of immunization and inflammation outside the CNS. More importantly, the prime mediators of this type of disease, myelin antigen-specific T cells, can be studied in detail in vitro by standard immunological techniques such as proliferation assays for the determination of antigen specificity, surface marker analysis by flow cytometry, cyto- and chemokine measurements, and T cell receptor sequencing. In vitro analysis of enriched or cloned antigen-specific autoreactive T cells is essential for our understanding of cellular and molecular properties associated with demyelinating disease.

In transfer protocols, cells can either be directly transferred after dissection of draining lymph nodes of immunized donor animals or be cultured for various time periods with the appropriate antigen to enrich autoreactive cells.

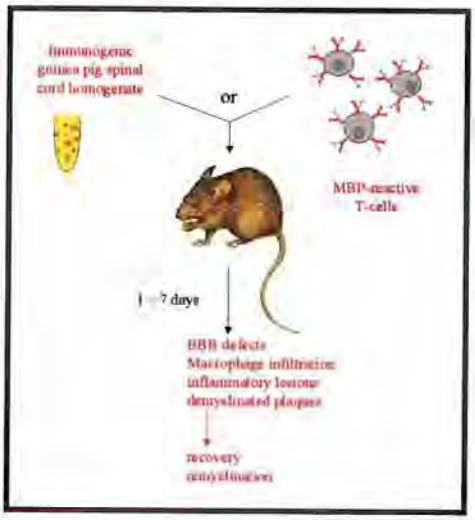


Fig. 7 Scheme of passive and active immunization of EAE in rodents

3.3. Clinical signs and disease course

The clinical signs and disease course of EAE vary depending on the strain and specie of animal, the inciting neural antigen, the adjuvants, the timing, and the dose of antigen and adjuvant. According to this, EAE can take an acute, a chronic, and/or relapsing-remitting course. There is evidence that susceptibility of different animal strains to EAE is linked to MHC class II determinants (Zamvil and Steinman, 1990; Brocke et al., 1994), although non-MHC genes also influence disease expression.

In every case, the first sign of the clinical course are ruffled fur and weight loss. A significant drop in body weight usually precedes clinical signs of EAE for about 1 to 2 days. Then, after a latence period, that can vary between one or two days up to several weeks, animals develop the symptoms which turn out normally as an ascending paralysis. To rate the severity of disease several ordinal scales can be used but the most common is given: 0, animal without deficits; 1, tail weakness; 2, hemi- or paraparesis (incomplete paralysis of one or two hindlimbs); 3, hemi- or paraplegia (complete paralysis of one or two hindlimbs); 4, paraplegia with forelimb weakness or paralysis; 5, moribund or dead animals. Other investigators use similar scales with different numbers of stages or different definitions of stages. But the most important thing is that scoring is performed by an examiner blinded to the experimental protocol and by the same observer throughout the experiment. Although this ascending paralysis is typical in mice, rats, and guinea pigs, most used animals, other species can show different clinical courses. Rabbits may have ataxia and gait difficulties, while monkeys may have visual loss, cranial nerve deficits, and ataxia in addition to paralysis.

The typical course in rodents for actively-induced EAE is the acute, monophasic clinical course. In mouse disease, the onset of EAE lies usually between 7 and 12 days post immunization. This begins with loss of tail tone and progresses to hind limb weakness and sometimes forelimb weakness. Maximal clinical severity is often reached between day 9 and day 14, followed by a remitting disease course. The weakness starts about 9-14 days after injection and worsens over 1 to 4 days. Then, in mice which recover, the symptoms last about 7 days and relapses after recovery are rare, other times relapses can occur depending on the strain. Other different disease courses can also be seen in mouse, including a hyperacute form and relapsing or chronic forms of disease. Several affected mice develop quadriparesis and laboured respiration and may die from the disease. A hyperacute form of disease is usually characterized by a short latent period between injection and symptom onset and a fulminant course with a high mortality.

In contrast, transfer of myelin antigen-specific T lymphocytes in the mouse rarely results in a full recovery and tends to leave long-lasting neurological deficits. Disease starts 6 to 12 days after transfer of encephalitogenic cells. Typically, maximal clinical severity is reached between day 8 and day 14 after transfer. More severe forms of the disease are associated with an earlier onset, a higher disease incidence, higher disease scores, a slower recovery, and a more synchronized course in the different animals.

On the other hand, EAE in the rat is typically an acute paralytic disease from which most animals spontaneously recover. However, also chronic models have been described (Dahlman et al., 1999).

Chronic or relapsing disease has been also described with particular strains of animals and with particular induction regimens.

3.4. Histopathology of EAE

Sensitization with myelin antigens often results in perivenous encephalomyelitis and in some cases, depending on the animal model, in histopathologic changes similar to those seen in MS (Prineas et al., 2002). So, EAE can be considered a useful model for some aspects of MS (Steinman et al., 1984). The pathogenesis of the lesions of EAE is complex but involves two basic processes of demyelination and inflammation.

The characteristic CNS inflammatory lesion in disease such as EAE is the "perivascular cuff", an accumulation of leukocytes in the perivascular space, involving vessels of the neuropil, meninges or choroid plexus. This accumulation of leukocytes involves the processes of leukocyte margination, molecular interactions with endothelia, breakdown of the blood-brain barrier and active egress of leukocytes from the bloodstream to the perivascular space in response to chemokine signalling (Ransohoff 1999, Boztug et al., 2002). Perivascular cuffing may be accompanied by gliosis of adjacent parenchyma, an increased prominence of glial cells (astrocytes, oligodendrocytes or microglia) due to proliferation or cellular hypertrophy. In some models of EAE,

there may be resolution of the inflammatory lesions associated with a period of clinical remission. Currently, it seems that Fas-Fas ligand-mediated apoptosis of the infiltrating lymphoid cells and macrophages, with subsequent phagocytosis of the apoptotic cells by microglia, is involved in this resolution of T lymphocyte-mediated inflammatory lesions (Ouallet et al., 1999; Zipp 2000; Chan et al., 2001).

With regard to demyelination, two kinds of it have been described. On one side, the destruction of a normal myelin sheath resulting in an unsheathed (naked) axon is considered primary demyelination, and is characteristic of MS and some models of EAE. In primary demyelination, there may however be eventual axonal damage following a prolonged period of demyelination (Pivneva et al., 1999). In MS, it is that primary demyelination which occurs since the target of the disease process is the oligodendrocyte-myelin unit. By contrast, secondary demyelination involves primary axonal degeneration with resulting loss of the myelin sheath that cannot be maintained in the absence of the axon.

Otherwise, demyelination is unlikely to be directly mediated by lymphocytes, but it may involve a range of effectors including lymphocyte and macrophage-derived cytokines, oxygen radicals, nitric oxide, matrix metalloproteinases, antibody, complement and phagocytic cells including macrophages and microglia (Smith 1999; Kieseir et al., 1999). Although proliferating glial cells may infiltrate the area of demyelination, the failure of remyelination reflects oligodendrocyte apoptosis and/or failure to renew oligodendrocytes from progenitor cells (Nait-Oumesnar et al., 2000). It is at this point that more studies are required so that remyelination can succed.

Although, the EAE model is very useful for studying demyelinating lesions in MS throughout hindbrain, cerebelum or spinal cord, the elucidation of the pathomechanism(s) leading to cortical demyelination in MS is at present hampered by the lack of useful animal models reflecting human cortical pathogenesis. This can be ascribed to the fact that rodent EAE, the most widely used animal model for MS, rarely affects the brain. Neocortical demyelination reflecting the topographically different cortical lesion subtypes in MS has recently been described in the marmoset EAE model (Pomeroy et al., 2005; Merkler et al., 2006). Lately, a new model to overcome this limitation has been developed in rat. Lesions were targeted to the cerebral cortex by injection of pro-inflammatory mediators in animals that were immunized subclinically with MOG. Merkler et al. (2006) were the ones who generated intracortical and subpial lesions with extensive demyelination and widespread, and transient infiltration by T lymphocytes and macrophages/microglia cells. In cortical lesions, the transient nature of inflammatory infiltration and demyelination in the grey matter contrasts to the lesions reported in white matter of the spinal cord, where dense infiltrates of macrophages/activated microglia persist over an observation period of 4 weeks without significant remyelination (Kerschensteiner et al., 2004a, b). This suggests there must be an extensive remyelinating capacity of cerebral cortex after inflammatory demyelination not found in white matter (Merkler et al., 2006).

Although EAE has been widely employed as an experimental model of MS, it remains an imperfect model system. One major limitation is that EAE is artificially induced rather that spontaneously occurring, hence it has limited use in determining the primary triggers in MS pathogenesis.

An additional limitation is that the majority of EAE models show very little, if any, demyelination with in the CNS and have an acute, monophasic course with spontaneous recovery. This contrasts with the chronic progressive nature of MS, and the demyelination that is a feature of the human disease. Several EAE models have been developed in which the CNS lesions more closely approximate those of MS. For example, EAE generated in Biozzi AB/H mice with spinal cord homogenate (Baker et al. 1990) is characterised by demyelination and displays a relapsing-remitting, chronic time course.

4. LYSOPHOSPHATIDIC ACID.

Lysophospholipids (LysoPLs) are both metabolites in membrane phospholipid synthesis and bioactive molecules capable of a broad variety of biological processes by binding to specific G protein-coupled receptors (GPCRs). The best characterized representatives of signalling LPs are lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P). Their signalling role has been known for decades, but recently new high-affinity receptors for LPA and S1P have been identified improving deeply our comprehension of LysoPL signalling.

The widespread expression of cell surface LP receptors coupled to several classes of G proteins allow regulation of various cellular processes with particular impact on neurogenesis, vascular development, wound healing, immunity, and cancer.

Lysophosphatidic acid (LPA) is a signaling phospholipid with numerous biological actions; its structure is a simple lipid with a phosphate, a glycerol, and a fatty acid, named monoacyl-sn-glycero-3-phosphate (Tokumura, 1995; Moolenaar, 1999; Tigyi and Parrill, 2003) (Fig. 8a). Although its structure is simple it is able to act as a receptor-mediated intercellular lipid mediator in many cellular responses in numerous cell types including cellular proliferation, prevention of apoptosis, cell migration, differentiation, cytokine and chemokine secretion, platelet aggregation, smooth muscle contraction, or neurite retraction. LPA also induces transformation and cellular proliferation of smooth muscle cells (Chun et al., 2002; Ye et al., 2002; Anliker and Chun 2004; Moolenaar et al., 2004; Birgbauer and Chun, 2006; Chun 2005, 2007; Rivera and Chun, 2008).

LPA was firstly researched in 1990, when its ability of being a growth factor for fibroblasts and signals via specific G-protein-coupled receptors (GPCRs; unidentified at the time) was discovered (van Corven et al, 1989; van der Bend et al., 1992; Moolenaar, 1995). Apart from its mitogenic role, LPA induces a long list of cellular responses among them also many non-proliferative effects, such as stimulation of cell migration and survival, neurite retraction and gap junction closure. This extensive list of activities of LPA is due to the great diversity of G-protein-mediated signaling pathways identified (Moolenaar, 1999). LPA not only signals via classic second messenger pathways, it also activates Ras and Rho family GTPases to control cell proliferation, migration and morphogenesis, respectively.

4.1. Distribution and production of LPA

Significant amounts of LPA (~μM level) have been detected extracellularly in biological fluids such as serum (Tigyi and Miledi, 1992; Baker, 2000; Sano et al., 2002; Aoki et al., 2002), saliva (Sugiura et al., 2002), seminal fluid (Hama et al., 2002), follicular fluid (Tokumura et al., 1999), hen egg white (Nakane et al., 2001) and ascites from ovarian cancer patients (Nakane et al., 2001). Among them, serum is the best characterized source of LPA (Tigyi and Miledi, 1992; Baker et al., 2000; Aoki et al., 2002).

The first proposed mechanism of serum LPA production was as a result of blood coagulation. This was suggested by the fact that LPA levels in freshly prepared plasma are much lower than that in serum (Tigyi and Miledi, 1992; Aoki et al., 2002). That part of the serum LPA should be produced by platelets. This production of LPA was first demonstrated by Mauco et al. in human platelets after the cells were treated with exogenous phospholipase C (PLC) from Clostridium welchii (Mauco et al., 1978). They speculated that phosphatidic acid (PA) is rapidly generated in PLC-treated cells and proposed that LPA is converted from PA by phospholipase A-like enzymes (PLA1 or PLA2). Apart from that, two different studies showed that LPA is produced when platelets are activated by thrombin (Gerrard JM, Robinson P, 1989; Eichholtz et al, 1993). Nevertheless the amount of LPA produced by this pathway seemed to be too low to account for the LPA level (μM level) in serum (Gaits et al. 1997)

To know how much of serum LPA is produced by platelet, Aoki et al. used anti-platelet antibody to deplete them in rats. Analysis of serum from these rats revealed that half of the serum LPA is generated in a platelet-dependent pathway, and activated platelets only produced a small part (~10%) of this LPA. However, activated platelets produce and release a large amount of lysophospholipids (LysoPLs) such as lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and lysophosphatidylserine (LPS). On account of this, Aoki et al. (2002) proposed that although activated platelets do not release a large amount of LPA, they do take part into the platelet-dependent pathway supplying LysoPLs, which are produced by PLA1 and PLA2 enzymes expressed by the cells, and subsequently these LysoPLs are converted to LPA by a plasma enzyme, lysophospholipase D (lysoPLD) (Fig. 8b). A similar mechanism had already been proposed in human serum (Sano et al., 2002).

According to this, about half of serum LPA must be produced by a platelet-independent pathway (Aoki et al., 2002). This LPA is generated in plasma when it is incubated for several hours. These latter results clearly indicate that LPA may be produced in a cell-free system, so LPA seemed to be converted from LPC present at high concentrations in plasma (Tokumura et al., 1986). This pathway could explain part of the platelet-independent serum LPA.

Thus at least two pathways are postulated. In serum and plasma (and adipocytes), LPA is mainly converted from LysoPLs and, by contrast, in platelets and some cancer cells, LPA is converted from phosphatidic acid. In each pathway, at least two phospholipase activities are required.

In the first pathway PLA1/PLA2 plus lysoPLD activities are involved where initially LysoPLs are produced by PLA1 and PLA2, and then a plasma enzyme, autotaxin (ATX) with lysoPLD activity produce LPA from these lysophospholipids. In the second pathway phosphatidic acid (PA) is first generated from phospholipids or diacylglycerol by phospholipase D (PLD) or diacylglycerol kinase (DGK) and then deacylated by PLA1 or PLA2 to produce LPA (Fig. 8b) (Aoki et al., 2002; 2008).

On the other side, the amount of LPA produced from PA seems to be much lower, because PA is a minor component of cellular phospholipids in many cell types. However, this pathway might be very important, since it can generate LPA rapidly in response to various stimuli and it may play a role in certain microenvironments. It is significant to realize that the LPA concentration is highly regulated, not only can its production be induced but can also be inhibited by PLA2 inhibitors (Shen et al., 1998; Eder et al., 2000). Those studies noted that agonist of LPA, as phorbol ester (Shen et al., 1998) or LPA itself (Eder et al., 2000), could induce LPA production, and more recently, it was shown that bombesin could stimulate production of LPA, especially 18:1-LPA (Xie et al., 2002).

Furthermore, LPA is an important intermediate product of the synthetic pathway for phospholipids and triacylglycerol in many cell types from various species, including higher and lower organisms, so it can be found inside cells too. This pathway takes place intracellularly in the endoplasmic reticulum or mitochondria, then, an acyltransferase produces LPA by acylation of glycerol 3-phosphate (Dircks L, Sul HS, 1999). This LPA is subsequently converted to PA by LPA acyltransferase and afterwards, to other phospholipids and triacylglycerol.

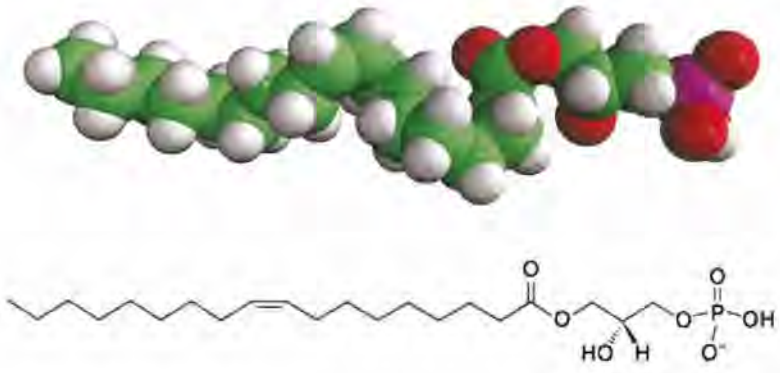


Fig. 8a. Chemical structure of the signaling lysophospholipid LPA

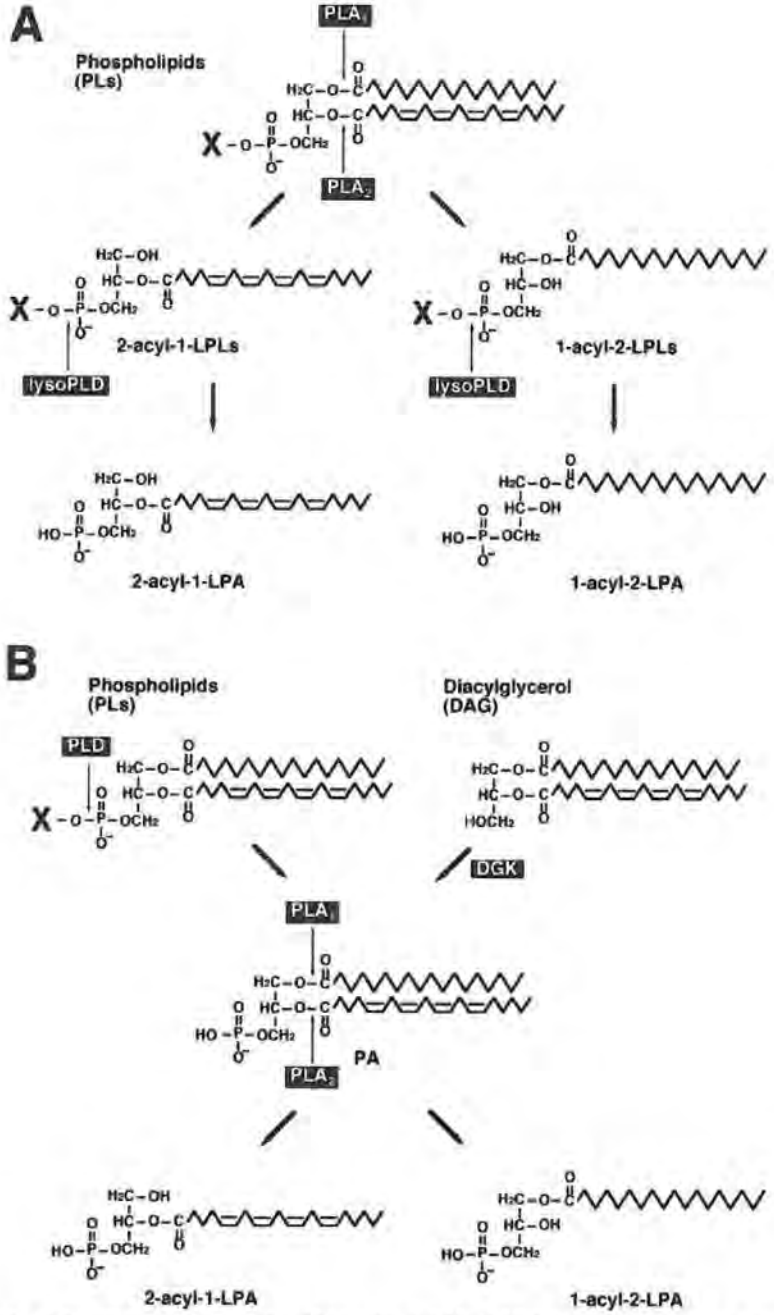


Fig. 8b. Two pathways of LPA production. (A) PLA1/PLA2-lysoPLD pathway and (B) PLD-PLA1/PLA2 pathway. In the PLA1/PLA2-lysoPLD pathway, lysophospholipids (LysoPLs) generated by phospholipase A1 (PLA1) or PLA2 reaction are subsequently converted to LPA by lysophospholipase D (lysoPLD) reaction. In the PLD-PLA1/PLA2 pathway, phosphatidic acid (PA) generated by phospholipase D (PLD) or diacylglycerol kinase (DGK) reaction is subsequently converted to LPA by PLA1 or PLA2 reaction (Aoki et al., 2008).

4.2. LPA species

LPA detected in serum or produced in platelets is not only one molecule but a mixture of various fatty acids. There are LPA species with both saturated fatty acids (16:0, 18:0) and unsaturated fatty acids (16:1, 18:1, 18:2, 20:4), and all of them have been detected in serum, plasma, and activated platelets (Gerrard and Robinson, 1989; Xiao et al., 2000; Baker et al., 2001) (Fig. 9). In activated platelets, several molecular species of LPA with

saturated [stearoyl (18:0), palmitoyl (16:0)] or unsaturated [oleoyl (18:1), linoleoyl (18:2), arachidonoyl (20:4)] acyl chains have been found (Gerrard and Robinson; 1989; Tigyi and Miledi 1992). These diverse fatty acid moieties may be linked to either the sn-1 or the sn-2 position of the glycerol backbone. In addition, a small portion of natural glycerophospholipids contains an ether linkage at the sn-1 position rather than an ester bond (1-alkyl-2-lyso-sn-glycero-3-phosphate and 1-alkenyl-2-lyso-sn-glycero-3-phosphate (alkyland alkenyl-GP)) (Fischer et al., 1998; Sugiura et al., 1999).

Thus, various molecular species of LPA or LPA-like lipids (LPA analogues) are present in vivo. Rat brain was found to contain substantial amounts of potent bioactive lipids lysophosphatidic acid (acyl LPA) (3.73 nmol/g tissue) and lysoplasmanic acid (alkyl LPA) (0.44 nmol/g tissue), being the predominant molecular species of acyl LPA 18:1, 18:0- and 16:0-containing species (Sugiura et al., 1999). Both acyl and alkyl LPAs are suggested to play important physiological roles as intercellular signalling molecules as well as the roles as metabolic intermediates in the nervous system, e.g. for endogenous cannabinoid receptor ligand 2-arachidonoylglycerol synthesis because of the substantial amount of arachidonic acid-containing LPA in brain (Sugiura et al., 1999).

Interestingly, it must be emphasized that these LPA species exhibit differential biological activities (Yoshida et al., 2003; Hayashi et al., 2001; Tokumura et al., 1994; Jalink et al., 1995), possibly because they activate differentially each of the five LPA receptors (LPA_1, LPA_2, LPA_3, LPA_4, and LPA_5) (Bandoh et al., 2000). The structure-activity relationship of these LPA and LPA analogs depends on cells and tissues (Sugiura et al., 1994; Gueguen et al., 1999; Jalink et al., 1995; van Corven et al., 1992; van Corven et al., 1994; Perkins, et al., 1994; Tokumura et al., 1978), but in general, LPA with an unsaturated fatty acid, which is produced by PLA1 isozymes, is biologically more potent than LPA with a saturated fatty acid, which is produced by PLA2 isozymes. For example, LPA with an unsaturated fatty acid induces proliferation and de-differentiation of smooth muscle cells both in vivo and in vitro, whereas LPA with a saturated fatty acid does not (Yoshida et al., 2003; Hayashi et al., 2001).

These observations clearly indicate the biological significance of LPA species in vivo. In addition, the diversity of LPA with respect to its fatty acid means that there are multiple synthetic pathways for LPA.

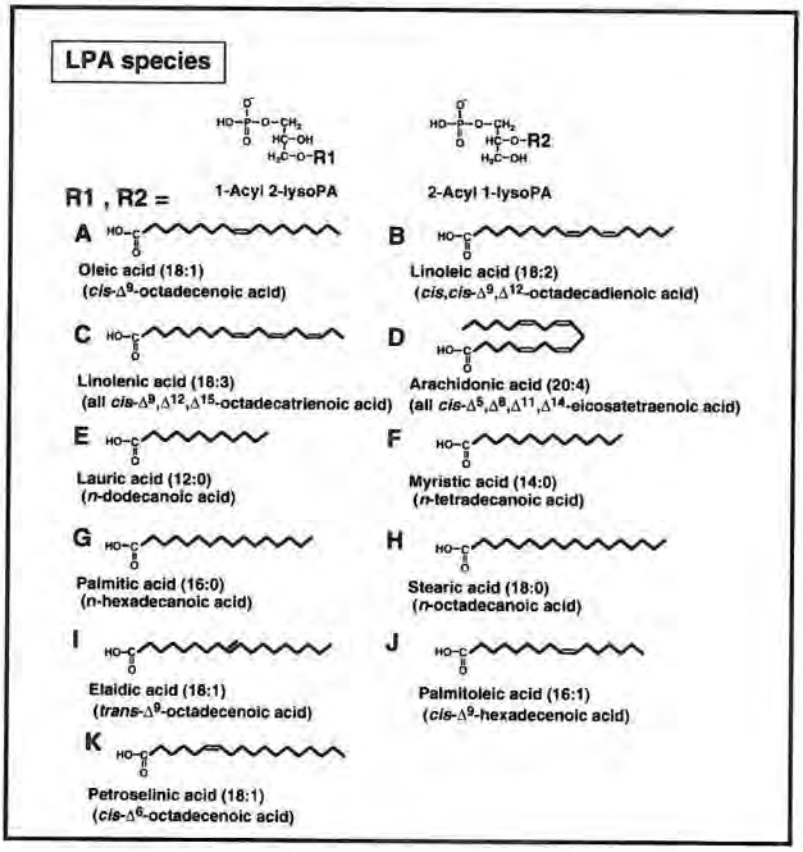


Fig. 9. Different species of LPA (Bandoh et al., 2000)

4.3. Receptors of LPA.

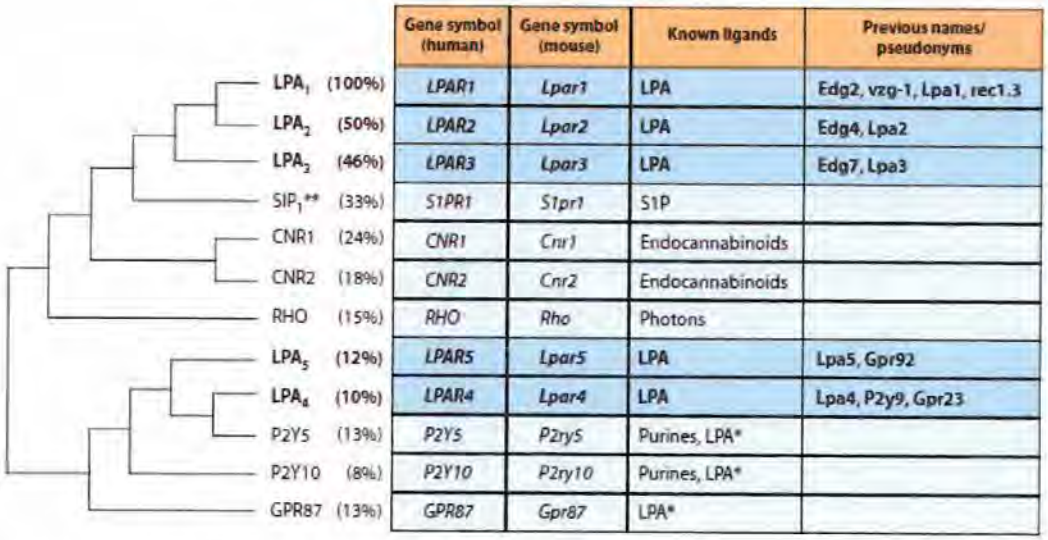
The first LP receptor, ventricular zone gene 1 (*vzg-1*), was identified in 1996 during studies on mammalian neurogenesis in which a cloned orphan receptor was isolated by virtue of its expression in the neurogenic zone of the embryonic cerebral cortex (Hecht et al., 1996; Chun et al., 1999; Chun 1999.). This vzg-1 encodes a GPCR that has the properties of a high-affinity LPA receptor. Based on the nucleotide sequence of vzg-1, immediately several orphan receptor genes or expression sequence tags (ESTs) in DNA sequence databases appeared which most certainly interacted with LPA or similar LP ligands such as S1P.

To date at least six specific LPA receptors (LPA_{1-6}) have been identified (reviewed in Bandoh et al. 2000; Fukushima et al. 2001; Anliker and Chun 2004; Ishii et al. 2004; Aoki et al., 2008; Noguchi et al., 2009; Pasternack et al., 2008; Yanagida et al., 2009; Choi et al., 2010) and other two additional (GPR87 and P2Y10) have been suggested to may be responsive to LPA (Tabata et al., 2007; Murakami et al., 2008).

Three of them, originally named EDG-2/VZG-1/rec1.3, EDG-4 (non-mutant) and EDG-7 are closely related GPCRs, the EDG family (Sanna et al., 2004; Matloubian et al., 2004; Kupperman et al., 2000; MacLennan et al., 2001). These receptors, EDG-2/VZG-1/rec1.3, EDG-4(non-mutant) and EDG-7, were renamed following the guidelines of IUPHAR (Ishii et al., 2002) and received the names of LPA1, LPA_2 and LPA_3 respectively. Recently, a fourth LPA receptor LPA_4/GPR23/P2Y9, was cloned in human. This receptor had only 20–24% amino acid of identity to LPA_1, LPA_2 and LPA_3 which means that LPA_4 is evolutionarily distant from the other LPA receptors. Indeed, LPA_4 is more closely related to nucleotide receptors of the P2Y GPCR family (Candelore et al, 2002). One of the last identified receptor, LPA_5, has also a quite different amino acid sequence which showed just ~35% amino acid identity with the LPA_4 receptor. On the other hand, protein sequence alignment of mouse and human GPR92 (LPA_5) demonstrated 80% identity meaning it is well conserved (Fig. 10a).

In short, phylogenetically, LPA_5 is more related to the fourth receptor LPA_4, meanwhile, the three first receptors are very close to each other (Chang-Wook Lee et al, 2006). Similarly, the last LPA receptor, P2Y5 (Pasternak et al., 2008), namely LPA_6, phylogenetically associates with LPA_{4-5}. Still to be under a more comprehensive analysis, only the LPA_{1-5} receptors will be detailed after.

In addition, in some cell types, LPA-mediated effects were found to be independent of GPCRs. This observation was explained by the demonstration that LPA can also function as a transcellular agonist for the nuclear peroxisome proliferator-activated receptor gamma (PPARγ) (McIntyre et al., 2003).



Gene symbol (human)	Gene symbol (mouse)	Known ligands	Previous names/ pseudonyms
LPAR1	*Lpar1*	LPA	Edg2, vzg-1, Lpa1, rec1.3
LPAR2	*Lpar2*	LPA	Edg4, Lpa2
LPAR3	*Lpar3*	LPA	Edg7, Lpa3
S1PR1	*S1pr1*	S1P	
CNR1	*Cnr1*	Endocannabinoids	
CNR2	*Cnr2*	Endocannabinoids	
RHO	*Rho*	Photons	
LPAR5	*Lpar5*	LPA	Lpa5, Gpr92
LPAR4	*Lpar4*	LPA	Lpa4, P2y9, Gpr23
P2Y5	*P2ry5*	Purines, LPA*	
P2Y10	*P2ry10*	Purines, LPA*	
GPR87	*Gpr87*	LPA*	

Fig. 10a. Phylogenetic relationships among known and proposed human LPA receptors. Non-LPA GPCRs (rhodopsin, S1P1, and cannabinoid receptors) are included for reference. Percent amino acid identity to LPA1 is indicated in parentheses. *Low-affinity or unconfirmed ligand. **There are currently at least five receptor subtypes for S1P (S1P1–5) (Choi et al., 2010).

LPA receptors coupled to members of three major G protein families, the $G_{\alpha}i$, $G_{\alpha}q$, and $G_{\alpha}12$ family (Fig. 10b). LPA_1 and LPA_2 are known to interact with all three G protein families. LPA_3 interacts with $G_{\alpha}i$ and $G_{\alpha}q$, but not with $G_{\alpha}12$ proteins. An exception may be LPA_4 that appears to couple with the fourth subclass, the $G_{\alpha}s$ family. Ligand binding to its receptor can cause conformational changes in this receptor, inducing corresponding response in G protein. This chain of events influences G protein regulated processes: i) Rho activation; ii) activation of phospholipase C (PLC) with subsequent activation of protein kinase C (PKC) and Ca^{2+} mobilization; iii) change in activity of specific mitogen activated protein kinases (MAPK) or phosphoinositol 3-kinase (PI)3K / Akt pathways through either Gi/0 subunits with consequences for cellular proliferation, survival and apoptosis; ii) inhibition/activation of adenylyl cyclase (AC) resulting the activation in cAMP accumulation and to induce Ca^{2+} mobilization. This underlines multiple modes of the effects of LysoPLs and diversity of cell responses to them, which occur via growth stimulating and cytoskeleton dependent pathways (reviewed in Bandoh et al., 2000; Anliker and Chun, 2004; Choi et al., 2008).

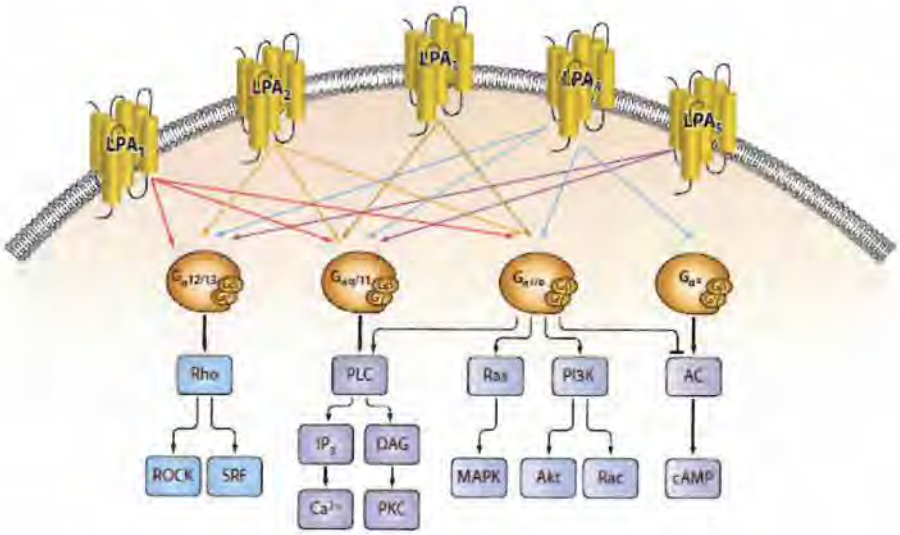


Figure 10b. Signaling pathways activated by the five confirmed LPA receptors (from Choi et al., 2010).

4.3.1. LPA_1 receptor; *Lpar1* gene (formerlylpA1/vzg-1/mrec1.3/edg-2)

Lpar$_1$ was the first LPA receptor gene identified. This receptor was found during developmental studies of the mouse cerebral cortex. These experiments were designed to identify novel GPCR genes associated with neurogenesis (Hecht et al., 1996; Chun et al., 1999; Chun 1999.) and embryonic development and to clear their role in cell proliferation and differentiation in other organisms (Crawford et al, 2003; Brann, 1992).

The protocol used consisted of amplifying GPCR gene fragments by degenerate polymerase chain reaction (PCR) of cDNAs from immortalized mouse cortical neuroblast cell lines (Chun and Jaenisch, 1996). Then, the resulting products were used for in situ hybridization screening to identify genes with enriched expression within the embryonic cortex, particularly within neurogenic regions. This analysis led to the cloning of a then novel orphan receptor gene, *vzg-1*, so named because of its enriched expression pattern within the VZ of the embryonic cerebral cortex (Hecht et al., 1996; Chun et al., 1999; Chun 1999). Nowadays, this name has been replaced by its functional nomenclature name, LPA_1.

The mammalian *Lpar1* gene (human chromosomal locus 9q31.3) encodes for a 41-kDa protein consisting of 364 amino acids with seven transmembrane domains. It shares homology with two known receptors, the cannabinoid and melanocortin receptors (30% and 32% amino acid identity, respectively) but the greatest similarity was shared with an orphan receptor cloned from human endothelial cells called edg-1 [37% amino acid identity] (Hla and Maciag,. 1990).

In adult mice, *Lpar1* is widely expressed with high mRNA levels in testis, brain, lung, heart, spleen and intestine, and moderate levels in kidney, thymus, stomach and muscle but its expression is absent in liver of adult mice (Hetch et al., 1996; Contos et al., 2000; Macrae et al., 1996)). In human adults, the expression is very similar, showing high mRNA expression in brain, heart, colon, small intestine, placenta, prostate, ovary, pancreas, testis and spleen, and lower expression levels in skeletal muscle and kidney. Hardly any LPA_1 mRNA is detected in human lung and thymus and it is completely absent in liver and peripheral blood leukocytes (An et al., 1998b). Moreover, expression of LPA_1 is well known in the mouse nervous system, showing a tight spatio-temporal regulation during development (Contos et al., 2000; Fukushima et al., 2001). While at embryonic stages, during cortical neurogenesis, LPA_1 is predominantly expressed in the VZ (Hecht et al., 1996), shortly before birth, mRNA levels of LPA_1 decrease in the cortex simultaneously with the end of the cortical neuroblast proliferation phase (Hetch et al., 1996). After birth, LPA_1 expression reappears in brain, closely associated with developing white matter tracts and coinciding with the process of myelination showing highest expression between postnatal days 18 and 21 (Hetch et al., 1996; Weiner et al., 1998).

The signaling pathways of LPA_1 involve all three G protein families $G_\alpha i$, $G_\alpha q$ and $G_\alpha 12$ and, as most LysoLP receptors, its general downstream effects include activation of phospholipase C (PLC), Ca^{2+} mobilization, activation of mitogen-activated protein kinase (MAPK) and Adenylyl Cyclase (AC) inhibition. Also Rho is involved in the pathways observed for LPA_1 receptor. Then, activation of PLC, MAPK and Rho via LPA and S1P receptors result in cell proliferation, cell survival and changes in cell morphology such as cell rounding (Lynch and Macdonald, 2002; Fukushima et al., 2002; Anliker and Chun , 2004). Meanwhile, phosphoinositide 3-kinase (PI3K) and its substrate Akt also enhance cell survival (Goetzl et al., 2002; Anliker and Chun, 2004).

LPA_1 have recently emerged as important influence on normal nervous system development (Chun et al., 2002; Anliker and Chun 2004; Moolenaar et al., 2004; Chun 2005, 2007). As mentioned, during development LPA_1 is expressed in neural progenitor cells suggesting a regulatory function in neurogenesis (Hecht et al., 1996; Estivill-Torrús et al., 2008). In vivo LPA_1 expression has been detected not only in neurons (Allard et al., 1998; Tabuchi et al., 2000; Fujiwara et al., 2003; Pilpel and Segal 2006), but also oligodendrocytes (Weiner et al., 1998; Handford et al., 2001), microglia (Moller et al 2001; Tham et al. 2003) and astrocytes (Shano et ál., 2008). Exogenous delivery of LPA has demonstrated LPA_1 receptor-mediated functions including morphophysiological changes in neural progenitors (Dubin et al., 1999; Kingsbury et al., 2003; Fukushima 2004; Fukushima and Morita 2006; Fukushima et al., 2000, 2007). Likewise, LPA delivery to hippocampal neurons is known to increase the tyrosine phosphorylation of FAK (Derkinderen et al., 1998), regulate cell death (Holtsberg et al., 1998a, b), mimic neurotrophic effects (Fujiwara et al. 2003) or mediate synaptic changes associated with spatial memory (Dash et al., 2004). Effects of LPA_1 loss on interneuron-mediated rhythms in vivo (Cunningham et al., 2006) and over-expression gain on synapse formation (Pilpel and Segal 2006) have been reported in the hippocampus. In addition, the presence of modulators of lysophosphatidic acid activity has been also demonstrated in the adult hippocampus (Brauer et al., 2003). At present, null animals have been obtained for most of the known LPA receptors by targeted gene disruption, all of them being mice (Choi et al., 2008). Receptor loss-of-function studies using LPA_1-null or LPA_1/LPA_2-double null mice have suggested centrally mediated behavioral defects and relatively minor morphological cerebral alterations, although the initially generated mutation was associated with ~50% perinatal lethality that may have a CNS component, along with defective olfaction (Contos et al., 2000, 2002; Harrison et al., 2003; Roberts et al., 2005). Recently, the propagation of the original mixed background strain of LPA_1-null mice (Contos et al., 2000) in our laboratories, led to a stable variant of LPA_1-null mice called the "Malaga variant" (reported as "maLPA_1-null" mice). These mutants exhibited improved perinatal viability and showed altered cortical neurogenesis and increased cell death during brain development that caused a reduction of cortical layer cellularity in adults, indicating the action of as yet unidentified genetic modifiers of LPA_1 that influence cortical neurogenesis (Estivill-Torrús et al., 2008). Further studies demonstrated that maLPA_1-null mice display reduced postnatal hippocampal neurogenesis under both basal and environmentally enriched conditions (Matas-Rico et al., 2008). These results correlated with anomalous behavior as showed by impaired spatial memory retention, abnormal use of searching strategies, altered exploration in the open field and increased anxiety-like responses in the elevated plus maze (Santín et al., 2009).

4.3.2. LPA_2 receptor; *Lpar2* gene (formerly,lpA2/edg-4 Nonmutant Form)

When LPA_1 was identified, some other homologous receptors of it were looked for. GenBank searches revealed a novel human genomic sequence, previously named edg-4, which encoded for a GPCR with ~60% amino acid similarity to LPA_1. Afterwards, an isolated mouse homologous for this gene (cDNA and genomic DNA) was characterized (Contos and Chun, 2000), recently called LPA_2.

The *Lpar2* gen predicts an amino acid sequence of 348 amino residues and a calculated molecular mass of 39 kDa. Its structure consists of three exons with an intron inserted within putative transmembrane domainVI, as observed for *Lpar1*, indicating that *Lpar1* and *Lpar2* were derived from a common ancestral gene. An intriguing feature of the *lpar2* gene is the existence of several human variants observed in cancer cells. This feature was evident in a large number of nucleotide variations in 30 untranslated regions (mostly by single nucleotide replacement).

The expression of LPA_2 in adult mice is high in testis and kidney but low in brain, heart, lung, spleen, thymus, and stomach, while hardly any or no LPA_2 transcripts are detectable in liver, muscle and small intestine. Unlike adult brain, embryonic mouse brain showed high levels of LPA_2 mRNA (Contos et al., 2000; Contos and Chun, 2000; Yang et al., 2002), but when this expression was analyzed by northern blot, two *Lpar2* transcripts of distinct size (2.8 kb and 7 kb) were expressed both in embryonic and neonatal mouse brains; but neither of them were detectable in adult brain tissue (Contos et al., 2000). These results were also confirmed by in situ hybridization studies, revealing expression of *Lpar2* in postmitotic neuronal regions (e.g. developing cortical plate) as well as in the VZ of embryonic mice.

In humans, LPA_2 is strongly detected in testis and leukocytes, and likely in mouse. Two species of transcripts were observed in human lymphoid cell lines, although these transcripts differed in size (1.8 kb and 8 kb) from those found in the mouse. Moderate LPA_2 levels are reported in prostate, spleen, thymus and pancreas. In cancer cells, aberrant expression of *lpar*2 has been reported in several cases, suggesting a tumor promoting role (An et al., 1998a; Choi et al., 2010).

As LPA_1 does, LPA_2 also couples to $G_{\alpha}i$, $G_{\alpha}q$ and $G_{\alpha}12$ which mediates LPA-induced PLC activation and leads to intracellular Ca^{2+} increases and inositol phosphate production. Activation of LPA_2 shows also an effect of mitogen-activated protein kinase (MAPK) and Rho, and phosphorylation of Akt, resulting in such processes as cell survival and cell migration. As a consequence, LPA_2 signaling has emerged as a potential factor for cancer metastasis (Yang et al., 2002; Anliker and Chun, 2004; Choi et al., 2010). In all these cases, the estimated 50% effective concentrations to achieve a variety of responses are also, as with LPA_1, in the low nanomolar range. These properties, taken together, demonstrate that LPA_2 is a multifunctional LPA receptor and thus functionally similar to LPA_1.

4.3.3. LPA_3 receptor; *Lpar3* gene (formerly lpA3/edg-7 (edg-5)

In the course of studies on LPA_1 and LPA_2, Bandoh et al. (1999) isolated a new member of the EDG family of G-protein-coupled receptors, EDG7, which showed to act as a cellular receptor for LPA. LPA_3, as it is known now, encodes a 40-kDa GPCR of 353 aa (human) and 354 aa (mouse) with ~60% amino acid similarity to mouse LPA_1 and LPA_2. Mouse LPA_3 is present on chromosome 3, and consists of three exons along with an intron located within transmembrane domain VI, identically placed as in LPA_1 and LPA_2 (Contos et al., 2000). Since the beginning, the high degree of amino acid homology and the conserved location of the intron within transmembrane domain VI, make this gene clearly considered as a member of the same LPA family of receptors. Later, from independent isolation by reverse transcriptase-polymerase chain reaction with degenerate primers from Jurkat T-cell RNA, the same gene was also identified in human (Bandoh et al., 1999).

LPA_3 shows more restricted expression than the two first receptors (Ishii et al., 2004). Human LPA_3 is detectable at highest levels in heart, prostate, pancreas and testis and at moderate levels in lung and ovary (Bandoh et al., 1999; Im et al., 2000). LPA_3 expression was also reported in human brain with particularly strong expression in the amygdala, frontal cortex and hippocampus. In adult mice, strong LPA_3 expression is found in kidney, testis, lung, but lower levels of transcripts are present in small intestine, heart, spleen, thymus, and stomach). Within the mouse brain, LPA_3 expression shows highest expression around birth, whereas expression of LPA_3 is very low during embryonic development and in adult brain. Abundant LPA_3 expression was also

found in rat testis and kidney (Contos et al., 2000). Interestingly, it has been shown that, in the uterus, *Lpar3* is exclusively expressed in the luminal endometrial epithelium being essential for the implantation (Ye et al., 2005) As most LysoPLs receptors, general downstream effects of LPA_3 (Ishii et al., 2000) include PLC activation and Ca^{2+} mobilization, as well as MAPK activation. Moreover, both activation and inhibition of AC have been demonstrated after stimulation of LPA_3. However this receptor differs from the other previous two by interacting with $G_{\alpha}i$ and $G_{\alpha}q$, but not with $G_{\alpha}12/13$ proteins and therefore does not mediate cell rounding in neuronal cells in which $G_{\alpha}12/13$ and Rho are involved. Interestingly, LPA_3 transduce increases in both $[Ca^{2+}]i$ and cAMP level after stimulation, only if LPA contains an unsaturated fatty-acyl chain but not saturated fatty acids(Bandoh et al 1999; Sonoda et al 2002).

4.3.4. LPA_4; *Lpa4* gene (formerly GPR23/P2Y9)

LPA_4 is evolutionarily distant from the other LPA receptors, just with 20–24% amino acid identity to LPA_1, LPA_2 and LPA_3. Indeed, LPA_4 is more closely related to the nucleotide receptors of the P2Y purinergic GPCR family (Janssens et al., 1997; O'Dowd et al., 1997). Although the homology is very low, other techniques such as the use of epitope-tagged LPA_4, pharmacological intervention, LPA concentration-dependent responses and specific membrane binding, or G protein mini-genes have supported enough evidences that LPA_4 is a fourth LPA receptor (Lee et al, 2007).

This receptor is broadly expressed in embryonic tissues, including brain, bone marrow, and embryonic stem cells. In adult mouse, LPA_4 gene expression reveals the highest level in heart, skin, and thymus (Lee et al., 2007, 2008), and contrasts somewhat with the lower expression levels observed in human tissues. This expression was determined both by northern blot and reverse transcription-PCR analysis (Noguchi et al., 2003). Human LPA_4 seems to be very weakly expressed in every tissue examined with the exception of ovary, where LPA_4 expression is strongly upregulated (Noguchi et al., 2003).

LPA_4 activates AC resulting in intracellular cAMP accumulation, via $G_{\alpha}s$, usually not reported for classical LPA receptors, and induces Ca^{2+} mobilization (via both $G_{\alpha}q$- and a $G_{\alpha}i/o$- mediated pertussis toxin-sensitive pathway. Besides, activation of LPA_4 evoked a nonselective cation conductance through similar G protein-mediated pathways as those that modulate calcium signaling. On the other hand, in LPA_4 overexpressing cells LPA_4 stimulation induced $G_{\alpha}12/13$-mediated Rho activation, producing neurite retraction and stress fiber formation (Lee et al., 2007; Yanagida et al., 2007).

4.3.5. LPA_5/ GPR92; *Lpar5* gene

The identification of LPA_4 as a receptor with lower homology to other LPA GPCRs suggests that other dissimilar LPA GPCRs could well exist, as speculated previously (Ishii et al., 2004). Then, a new LPA receptor was identified by screening collections of GPCR cDNAs using reverse transfection and cell-based assays. Lee et al. (2006) identified an orphan receptor, GPR92, which produced LPA-dependent changes in cell shape. GPR92, now named LPA_5, was found to be able to confer LPA responsivity following heterologous expression in B103 cells; such as concentration-dependent neurite retraction, receptor internalization, increased LPA binding, increased cAMP accumulation, intracellular calcium mobilization, and evokement of electrophysiological currents. These responses can be mediated by $G_{\alpha}12/13$, Rho and Gq.

The human LPA_5 belongs to the rhodopsin subfamily of GPCR. Its gene is located on chromosome 12, region p 13.31, and contains an open reading frame, without any intron, that encodes 372 amino acid residues. It is structurally most closely related to LPA_4 displaying 31% homology to LPA_4 and between 21.3 and 22.6% homology with the classic LPA family of receptors (Kotarsky et al., 2006).

LPA_5 is broadly expressed at low levels in embryonic brain, and comparatively high levels in heart, placenta, spleen, brain, lung, and gut. It is also highly expressed in the lymphocyte compartment of the gastrointestinal tract. Moreover it is the most abundant GPCR activated by LPA found in the small intestinal intraepithelial CD8+ cytotoxic T cells (Lee et al., 2006).

The pharmacological binding profile revealed that LPA_5 was unable to discriminate between different LPA analogs varying in the length of their acyl groups. Apart from this, the LPA_5 signaling pattern is similar to that of LPA_4, in which preferentially couple to $G_{\alpha}i$, $G_{\alpha}12/13$ and/or $G_{\alpha}q$ but also possibly to couple to the $G_{\alpha}s$

class of G proteins in HeLa cells. Consequently, LPA_5 activation by high LPA levels leads to increases in PI hydrolysis and cAMP production in a receptor-dependent manner. However, there are still many details of the signal behavior for LPA which must be clarified (Lee et al., 2006).

Table 1

Characteristics of LPA receptors.

Receptor	Synonyms	Gene[a]/location	G-protein coupling	Cellular responses	Physiological/pathological functions
LPA_1	VZG-1 EDG2 Rec1.3 GPR26 GPCR26	*LPAR1*/chr9 (human) *Lpar1*/chr4 (mouse)	$G_{i/o}$, $G_{q/11}$, $G_{12/13}$	Proliferation [20] Survival [70] Stress fiber formation [20] Neurite retraction, cell rounding [3,20,26]	Brain development [14,15] Neuropathic pain [16] Pulmonary fibrosis [90] Renal fibrosis [91]
LPA_2	EDG4	*LPAR2*/chr19 (human) *Lpar2*/chr8 (mouse)	$G_{i/o}$, $G_{q/11}$, $G_{12/13}$	Cell rounding [26]	Vascular injury ($LPA_{1/2}$) [92]
LPA_3	EDG7	*LPAR3*/chr1 (human) *Lpar3*/chr3 (mouse)	$G_{i/o}$, $G_{q/11}$	Neurite elongation [26]	Embryo implantation and spacing [17] Spermatogenesis, male mating activity ($LPA_{1/2/3}$) [18]
LPA_4	GPR23 P2Y9 P2Y5-like	*LPAR4*/chrX (human) *Lpar4*/chrX (mouse)	$G_{i/o}$, $G_{q/11}$, $G_{12/13}$, G_s	Neurite retraction, cell rounding, cell aggregation [28,29]	N/D
LPA_5	GPR92 GPR93	*LPAR5*/chr12 (human) *Lpar5*/chr6 (mouse)	$G_{q/11}$, $G_{12/13}$	Stress fiber formation, neurite retraction, cell rounding [7]	N/D
GPR87[b]	GPR95	*GPR87*/chr3 (human) *Gpr87*/chr3 (mouse)	N/D	Ca^{2+} mobilization [9*]	N/D
P2Y5[b]		*P2YR5*/chr13 (human) *P2yr5*/chr14 (mouse)	N/D	CRE activation [10*]	Hair growth [10*,93]
P2Y10[b]		*P2YR10*/chrX (human) *P2yr10*/chrX (mouse)	N/D	Ca^{2+} mobilization [11*]	N/D

CRE, cyclic AMP response element and N/D, not determined.
[a] Gene names are referred to Human Genome Nomenclature Committee (http://www.genenames.org/) and Mouse Genome Informatics (http://www.informatics.jax.org/).
[b] Preliminary identification as LPA receptors, requiring further confirmation.

Table 1. Scheme of different LPA receptors (Noguchi et al., 2008).

4.4. Lysophosphatidic Acid and Myelination.

Mentioned before, LPA was early shown to induce a great variety of actions, among them cell proliferation, neurite retraction and cell rounding, inhibit differentiation, and disrupt gap-junctional communication (Jalink et al.,1994; Moolenaar, 1995; Moolenaar et al., 1997) In cortical neuroblasts, LPA induces both electrophysiological responses and morphological changes (Dubin et al., 1997, 1999; Fukushima and Chun, 1997), but one of the most outstanding effects is its role in myelination (Weiner et al 1998).

The identification of LPA_1 (Hecht et al., 1996; An et al., 1997), along with the characterization of the related lysophospholipid receptor family (Chun et al., 1998; An et al., 1998a;), has shown that lysophospholipid signaling pathways are involved in the control of nervous system development including formation of myelin.

The LPA_1 receptor was identified as the first G protein–coupled receptor specifically expressed in myelin-forming cells in rodent CNS (Barron et al., 1997; Allard et al., 1998, 1999; Weiner et al., 1998), with a spatio-temporal distribution during postnatal development consistent with a role in myelinogenesis and/or myelin maintenance (Allard et al., 1998). This receptor was also found in myelinated structures in the adult human temporal cerebral cortex (Allard et al 1999).

In rat and human, the presence of LPA_1 was also definitely proven in mature myelinating oligodendrocytes, based on the presence of this LPA receptor colocalized with Luxol Fast Blue-stained myelinated fibers (Handford et al., 2001) or MBP-immunoreactive cells. Numerous LPA_1-immunoreactive fibers, constantly immunostained for MBP, were also identified in the corpus callosum, caudate nucleus, and

cerebral cortex of rat brain. Likewise, in the human brain, the distribution of LPA_1 and MBP proteins share similarities with that observed in the rat (Cervera et al, 2002). The immunolabeling intensity was more or less identical in oligodendrocytes and myelinated fibers, although some oligodendrocyte soma appeared more strongly immunoreactive for LPA_1 than MBP, whereas the immunostaining revealed the opposite in their processes.

At all ages, LPA_1 expression is concentrated in and around developing white matter tracts. Its expression appeared in the hindbrain during the first few postnatal days and expands in a caudal-to-rostral manner over the next 3 weeks, reaching a peak at P18. Double-labeling experiments demonstrated that LPA_1–expressing cells coexpress mRNA PLP but do not show transcripts for GFAP, identifying them as mature oligodendrocytes (Weiner et al 1998).

During the embryonic development of the CNS, a first phase of LPA_1 expression takes place and correlates temporally with the cortical neurogenetic period being the expression restricted to the cortical VZ (Hecht et al., 1996). However at birth, LPA_1 expression by in situ hybridization becomes undetectable in the brain. The second distinct phase of expression begins in the first few postnatal days, with continual rostral expansion over the next 3 weeks. This time, LPA_1 is firstly presented in a population of hindbrain oligodendrocytes. This caudal-to-rostral expansion of the receptor expression correlates with the appearance of differentiated oligodendrocytes as monitored by PLP and MBP mRNA and protein expression (Verity and Campagnoni, 1988; Shiota et al., 1989; Foran and Peterson, 1992), and with the development of observable brain myelination (Jacobson, 1963; Mitrova, 1967; Caley and Maxwell, 1968; Schonbach et al., 1968; Vaughn, 1969; Sturrock, 1980).

Weiner et al (1998) demonstrated by double-labeling studies, that LPA_1–expressing cells coexpress PLP mRNA in all regions and at all ages examined, being the first LPA_1–positive cells shown in the early postnatal hindbrain. These data identify LPA_1 expression as a novel marker for differentiated oligodendrocytes, and suggest that initiation of LPA_1 expression may contribute to the myelinating oligodendrocyte phenotype. This is supported by the observation that LPA_1 expression, as detected by in situ hybridization, is downregulated in the oligodendrocytes of older animals, after myelination has been completed.

The LPA receptors are able to elicit a variety of responses via G-proteins of the Gi/Go class and induce Ca^{2+} signals in different cell types; like Jurkat cells, fibroblasts and astrocytes (Jalink et al., 1994; Goetzl and An, 1998). Studies of LPA-induced $[Ca^{2+}]i$ signals in cultured cells of the oligodendrocyte lineage showed that mature oligodendrocytes (GalC+) but not oligodendrocyte precursors (O4+/ GalC-) developed an $[Ca^{2+}]i$ increase as response to LPA. This $[Ca^{2+}]i$ signal induced by LPA in oligodendrocytes was blocked by preincubation with Pertussis-toxin (PTX) which indicates the involvement of a $G_{\alpha}i$/Go G-protein in the signal transduction of the LPA receptor in oligodendrocytes (Moller et al 1999). These results agree with the ones previously reported by Weiner et al. (1998) where a role of LPA_1 in first stages of myelination was presented.

In the same line, Stankoff et al (2002) demonstrated that LPA_1 receptor is selectively expressed in mature postmitotic oligodendrocytes prior to myelination. These postmitotic mature premyelinating oligodendrocytes consisted of highly arborized cells, expressing all major myelin constituents, like MAG, GalC, CNPase, MBP, and PLP/DM20, but not MOG, which is considered the latest marker of oligodendrocyte maturation (Solly et al., 1996). The transition between a mature nonmyelinating oligodendrocyte to a myelin-forming cell involves a drastic loss of arborization, concomitant with expression of MOG and myelin sheaths elaboration at the extremity of the residual processes. Those data suggest that, LPA_1 must have some role in myelination, and it should intervene at the latest stages of oligodendrocyte maturation, and this may be involved in termination or maintenance of myelination process (Stankoff et al, 2002)

A possible relationship between myelination and the onset of LPA sensitivity was previously suggested by the fact that complex phospholipids make up a considerable portion of myelin (Stoffel W and Bosio, 1997). Lysophospholipids like LPA might be produced during myelin formation and could act as an autocrine feedback signal for oligodendrocytes. LPA could also be released from neighboring axons as a signal to oligodendrocytes to begin myelination.

Other evidence in the involvement of LPA in myelination comes from the studies of Schwann Cells (SC). In SC cell culture, serum promotes cell survival and alters myelin gene expression. SCs in peripheral nerves are spindle-shaped, whereas in cell culture they can assume different shapes. In cell culture, differentiation of SCs is accompanied by a change in cell shape from a flat to a spindle-shaped cell. Serum treatment of SCs induces changes to spindle-shaped cell due to the reorganization of the actin cytoskeleton and the appearance of stress

fibers. The stress fibers form and traverse the length of the cell in bundles. They contain F-actin, myosin, and actin-binding proteins that are anchored to the plasma membrane by focal adhesions composed of F-actin, vinculin, talin, and focal adhesion kinase (Burridge and Chrzanowska-Wodnicka, 1996). After these effects of serum were addressed, Li et al (2003) found that some of them were mediated also by LPA. They found that LPA exerts its effects on SC survival and differentiation using different G proteins coupled to the LPA receptor. The antiapoptotic effects of LPA require the involvement of $G_{\alpha}i$, PI3K, MEK, and MAPK. Additionally, phosphorylation of MAPK was inhibited by pretreatment of SCs with PTX which means MAPK is downstream of $G_{\alpha}i$ and PI3K.

LPA also induced morphological alterations through the expression of myelin protein P0 in SCs. siRNA experiments showed that knockdown of LPA_2 expression (but not LPA_1) inhibited LPA-induced P0 expression, suggesting that LPA_2 mediates this effect (Li et al, 2003). Related to that, pretreatment of SCs with PTX developed an increase of P0 protein expression. This could be explained since P0 expression in SCs is stimulated by cAMP analogs and adenylyl cyclase activators. Then the increase in LPA-induced P0 expression by PTX would involve an increase in intracellular cAMP levels owing to the fact that adenylyl cyclase is negatively regulated by $G_{\alpha}i$.

On the contrary, despite repeated attempts, no action of LPA on oligodendrocyte cell culture survival, maturation, or myelination has been demonstrated (Stankoff et al. 2002) This is particularly intriguing since, as it is mentioned above, LPA increases survival of Schwann cells which are the PNS myelin-forming cell expressing LPA_1 (Allard et al., 1998; Weiner and Chun, 1999). The function of receptor appears restricted to differentiating oligodendrocytes and it seems to be involved in process formation regulating the later stages of oligodendrocyte maturation (Nogaroli et al., 2009) but not having effect on mature oligodendrocytes (Stankoff et al., 2002). Thus, LPA promotes Ca^{2+} mobilization in newly differentiated nonmyelinating oligodendrocytes (Möller et al., 1999) or in precursors differentiated to mature, stimulating process retraction and cell rounding (Dawson et al., 2002) but not in genuine precursors neither oligodendrocytes that have already myelinated axons (Möller et al., 1998; Stankoff et al., 2002). Some of discrepancies of oligodendrocyte responsiveness to LPA found in earlier studies have been recently explained on the basis of recently demonstrated endogenous activity of autotoxin in oligodendrocytes (Nogaroli et al., 2009). Differentiating oligodendrocytes express not only the receptors for extracellular LPA, but also the major enzyme responsible for the production of extracellular LPA, autotoxin. Thus, authors proposed that the so far observed discrepancy after adding LPA exogenously to differentiating oligodendrocyte may at least be in part due to the presence of autotoxin in their extracellular environment with subsequent LPA production and masking the physiological effects of exogenous LPA.

This discrepancy also could well suggest that LPA_1 coupling to second messengers may differ in oligodendrocytes and Schwann cells. Therefore, several studies about the second messengers involved in LPA receptor pathway in oligodendrocytes have been developed.

Regarding these second messengers LPA increases ERK phosphorylation in a number of cellular systems. It was demonstrated that exogenous application of LPA increased pERK activation in oligodendrocytes in a time- and concentration-dependent manner (Yu et al.2004) and this ERK phosphorylation was largely PTX-insensitive. These results support the possibility that G-proteins, such as $G_{\alpha}q$, may play a major role in pERK activation. The lack of effect of PTX sensitivity of LPA- and S1P-induced pERK activation in oligodendrocytes contrasts with the PTX sensitivity in astrocytes, in which LPA forms stress fibers (Rao et al., 2003, Ramakers and Moolenaar, 1998). On the other hand, some results suggested that differentiated oligodendrocytes possess a basal level of PKC and PLC activities, and that pharmacological inhibition of these pathways appears to affect basal ERK phosphorylation. According to that, two types of LPA-induced calcium signal response have been observed in rat mature oligodendrocytes by Fura-2-based single-cell imaging assay (Moller et al., 1999): one with an initial peak and a following decaying plateau (17% out of 71% of responsive cells), and the other with a persistent plateau (54% out of 71% of responsive cells). The partial block of LPA effects by PLC inhibitor (U73122) directly demonstrates PLC activation by LPA and supports the notion that LPA-elicited calcium signals in oligodendrocytes are derived from two routes: calcium influx across the plasma membrane and calcium release from internal stores (Möller et al., 1999). Similar to pERK activation, LPA-induced [Ca2+]i increase in oligodendrocytes is not affected by PTX treatment, implying that G-protein subunits, also likely $G_{\alpha}q$, are involved in LPA-induced calcium signaling.

Lastly, LPA exerts important effects on Olfactory ensheathing cells (OECs) (Yan et al 2004). OECs express LPA receptors, and LPA stimulates the proliferation and migration of this kind of cells. Additionally, it was found that LPA induces actin cytoskeleton reorganization and its stimulation involves Rho/ROCK action, activation of the MAP kinase ERK1/ERK2, and PI-3 kinase-dependent signaling. Moreover, LPA treatment of OECs leads to focal adhesion assembly inducing actin filament redistribution and stress fiber formation. Collectively, these findings suggest that LPA exerts influences on the cytoskeleton to influence cell migration. There is considerable evidence that activity of ERK1/ERK2 MAPK is essential for cell proliferation and differentiation (Hindley and Kolch, 2002) and that LPA can activate Erk1/Erk2 MAPK (Sautin et al., 2001; Baudhuin et al., 2002). The mitogenic signal induced by LPA has been considered to be mediated by these serine/threonine kinases (Nietgen and Durieux, 1998). Yan et al (2004) showed that ERK1/ERK2 MAPK is indeed activated to LPA, and that these pathways play an essential role in LPA effects on proliferation and migration.

4.5. Lysophosphatidic Acid and Inflammation

Inflammation is a complex response that involves cells, plasma components and cellular products, which has the aim of repairing the produced damage. This produced damage may have different causes like infection, trauma, ischemia, necrosis, haemorrhage or disease. Inflammation is the vascular response which leads an increase of the sanguineous flow to the site of inflammation. This increases the capillar permeability by retraction of the endothelium, allowing the exit of molecules and soluble mediators, and lymphocyte migration to the site of the inflammation. This response may exist in any vascularized compartment of the body, including the CNS.

The inflammatory response includes the participation of different cellular types such as neutrophils, macrophages, mast cells, lymphocytes, platelets, dendritic cells, endothelial cells and fibroblasts, among others. During the inflammatory process, chemotaxis is an important event in the recruitment of cells to the site of inflammation. The recruitment of leukocytes implies the presence of chemotactic factors as chemokines, the expression of their receptors in leukocytes, the expression of adhesion molecules in leukocytes and vascular endothelium, the narrow interaction between leukocytes and endothelium, and finally their passage through endothelium to arrive at the site of inflammation (McGeer and McGeer, 2001). Then, inflammation is part of a physiological process that has the aim of repairing the damage. However sometimes, if this process is extended and not controlled, the inflammation causes more damage than repairing it (Nathan, 2002). Actually in the CNS, inflammation is often the cause of damage instead of fulfilling the repairing function as it happens in other compartments of the body. So, neuroinflammation is a complex process that involves cells of the immune system and the CNS and which, unlike other compartments of the body, favours the damage rather than repairing it. In this context, MS is one of the principle diseases caused by neuroinflammation. MS is characterized by a rupture of the bloodbrain barrier, an important mononuclear cell infiltration of the white substance and its subsequent demyelination.

Lipid mediators in inflammation, are mainly derived from activated immune cells like platelets, macrophages, neutrophils, monocytes, eosinophils, mast cells, and some other cells of target organs. Their broad spectrum of primary actions includes vasoconstriction or vasodilation, platelet aggregation and secretion, leukocyte recruitment and activation, modulation of cytokine production by macrophages and lymphocytes, and stimulation of production of other mediators. In this context, one of the most important lipid mediators is LPA, as reviewed by Gräler and Goetzl (2002).

One of the first investigations of LPA-driven effects on lymphocytes was done by Xu et al. in 1995. In those studies, LPA revealed to have supporting and activating effects on proliferating T-cells. These results demonstrated the human Jurkat T-cell line (immortalized line of T lymphocyte cells) treated with LPA, sphingosylphosphorylcholine (SPC) or lysophosphatidylserine (LPS) showed a calcium response. LPA also stimulated proliferation of Jurkat T-cells increasing the amount of interleukin-2 (IL-2) production upon activation with phorbol esters. These results were confirmed with human blood-derived CD4+ T-lymphocytes, where the secretion of IL-2 in human CD4+ T-cells was enhanced up to twofold by mitogen activation (Zheng et al., 2000).

Later, the first analyses of LPA receptors and their responses to LPA were done in cultured lines of T cell lymphomas and lymphoblastomas. It was demonstrated that freshly isolated human blood CD4+ T cells constitutively expressed predominantly LPA_2, but just very low levels of LPA_1. In contrast, CD8+ T-cells expressed both receptors, not showing any dominance of either LPA_1 or LPA_2, leading to a different functional responsiveness compared to CD4+T-cells. So far, only CD4+ T cells, and not CD8+ T cells, demonstrate the inhibition of stimulated generation of IL-2 by LPA (Goetzl et al., 2000)

With the aim of improving the knowledge about the effects of LPA on T cells, Zheng et al. (2000) studied the human blood CD4+ T cell and Jurkat T cell transfectant systems. They transfected those cells with either LPA_1 or LPA_2 and showed that LPA appears to act directly on T cell secretion of IL-2 without major involvement of second or intermediate cytokines or other factors. Thus, the most relevant alteration in the T cells resulting from mitogenic activation is the shift from predominance of LPA_2 to codominance of LPA_2 and LPA_1. The increase of the ratio of LPA_1/LPA_2 is able to shift the effect of LPA from suppression to enhancement of T cell receptor-evoked generation of IL-2.

This study reported that Jurkat T cell transfected, which presented predominantly LPA_1 or LPA_2, behaved differently after mitogen or LPA stimulation. Whereas LPA and anti-LPA_2 antibody suppressed IL-2 after stimulation in LPA_2-predominant Jurkat-T cells and unactivated CD4+ T cells; Jurkat-T cells expressing LPA_1 in the absence of LPA_2 generated IL-2 more remarkably than that of the mitogen-activated set of CD4+ T cells, with LPA_1 and $-_2$ codominant expressions. This may occur, in part, because of the lack of opposition from LPA_2 signals.

Another functional aspect of altered LPA_1 and LPA_2 expression on T-cells was further examined using transfected Jurkat T-cells selectively expressing LPA_1 or LPA_2 in migration assays. In these measures, significant changes in the synthesis of matrix metalloproteinases that are required to cell migrate through a protein matrix were observed. Expression of LPA_2 on Jurkat T-cells resulted in an LPA-dependent upregulation of trans-Matrigel migration that was not seen on LPA_1-expressing Jurkat T-cells. Besides that, only LPA_2-expressing Jurkat T-cells upregulated the expression of matrix metalloproteinases up to fourfold upon LPA-stimulation (Zheng et al., 2001).

The reason why these different effects may occur could be because LPA_1 and LPA_2 differ in their coupling to G proteins and consequent signalling mechanisms. Current evidence suggests that LPA_2 couples most effectively to $G_\alpha q$ and thereby directly activates phospholipase C (PLC) and inositol phosphate signaling of $[Ca^{2+}]i$ increases. Unevenly, LPA_1 is more closely coupled to $G_\alpha i$, thus elicits increases in $[Ca^{2+}]i$ through $G_{\beta/\gamma}$ enhancement of PLC activity, both directly and by augmenting a concurrent lower level of activation of $G_\alpha q$ through LPA_1. Such mechanisms have been already characterized in detail in other systems (Zheng et al,2000). Therefore, certain subsets of T-lymphocytes are able to respond to a specific LPA-stimulus by increased or decreased proliferation depending on their LPA receptor expression pattern.

LPA_1 and LPA_2 have opposite effects on the migratory capacity of T-cells as well as on proliferation. Taken together, LPA supports the migratory capacity and mobility of naive human CD4+ T-cells via LPA_2. Upon mitogen activation, they upregulate the expression of LPA_1 resulting in a switch of their LPA responsiveness towards proliferation.

LPA has also been shown to be a survival factor for serum-deprived murine macrophages. There is evidence that LPA alone is a potent macrophage survival factor and that albumin-bound LPA is a key survival factor in serum. Every survival factor described so far has been proteins or steroid-based lipids. LPA represents a new class of cell survival factors. LPA-mediated survival is dependent on activation of PI3K. One of the downstream mediators of survival signaling by PI3K is the kinase pp70s6k. However inhibition of pp70s6k only partially blocks LPA-mediated survival, so other mediators of PI3K, besides pp70s6k, must be involved in survival signaling by LPA (Koh et al., 1998)

Another effect of LPA in inflammation is promoting wound healing in vivo. This effect makes it be an important growth factor in this environment. When LPA is applied topically during the healing of wounds in rat skin, the immigration of histiocyte-like macrophages increases, but only a modest increase in cell proliferation was seen (Balazs et al., 2000). LPA also led to an increased cell surface expression of E-selectin and vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells. This, at least in part, might contribute to the observed increase in binding of monocytes, neutrophils and human leukemia cells (HL60) (Rizza et al., 1999). Furthermore, it was demonstrated that LPA activates the transcription factor nuclear factor-κB (NF-κB) in endothelial cells followed by increased levels of mRNA transcripts encoding E-selectin, intercellular adhesion

molecule-1 (ICAM-1), IL-8, and monocyte chemoattractant protein-1 (MCP-1) through LPA_1 and LPA_3 (Palmetshofer et al., 1999, Gustin et al., 2008). Taken together, LPA can be considered as an initial signalling molecule for the onset of macrophage infiltration in wound healing.

Moreover, the expression of LPA receptors in human alveolar macrophages was studied by reverse transcription-polymerase chain reaction (RT-PCR) showing mRNA for LPA_1, LPA_2 receptors and, to a lesser extent, for the LPA_3 receptor (Hornuss et al., 2001),

The growth factor-like action of LPA has not only been demonstrated for T-lymphocytes and macrophages but also for B-lymphocytes (Rosskopf et al., 1998). Addition of LPA to human Epstein–Barr virus-immortalized B-lymphoblasts evokes the formation of IP3, which is accompanied by a transient increase in [Ca2+]i, enhanced inositol phosphate formation, MAP-kinase activation, DNA synthesis, and immunoglobulin formation, presumably in a receptor-mediated fashion. Continously, specific transcripts of LPA_1 receptor were demonstrated in these EBV-immortalized B lymphoblasts. Besides, this receptor elicits PTX-sensitive (inhibition of cAMP generation) and PTX-insensitive effects (cell rounding) (Xie et al., 2002), which supports the hypothesis that the LPA receptor couples to more than one G protein (Allende et al., 2003).

Moreover, one enzyme generating LPA, sPLA2, which is induced by proinflammatory cytokines, was found in high concentrations in inflammatory exudates, (Xu, 2002) leading to high concentrations of sPLA2 in septic states. Although data from in vitro studies are difficult to compare with the situation in vivo, one could envisage that LPA is released at sites of local inflammation (in response to cell injury, during wound healing, or from growing tumors) or generalized inflammation (septic states or multiple sclerosis).

Recently, the biological activity of LPA on other types of cells involved in inflammatory processes, such as dendritic cells (DCs), has been analyzed (Phanther et al 2002). In immature DCs, LPA triggered intracellular Ca2+ transients, actin reorganization, and chemotactic migration, but it does not affect the capacity of DCs to internalize proteins or particles. The increase in the $[Ca^{2+}]i$ in response to LPA is due to a mobilization of stored Ca^{2+} via activation of $G_{\alpha}i/o$ proteins. Meanwhile, the mechanism underlying actin reorganization was presumably regulated by interaction of phosphoinositides with the actin-binding proteins which requires activation of $G_{\alpha}i$ proteins, and presumably of small GTP-binding proteins of the rho family (Contos et al., 2000, Swarthout and Walling. 2000). In contrast, in LPS-differentiated DC, LPA-induced Gi/o protein-dependent responses and signaling events were no longer present, although mature DCs still responded to other stimuli. Similar to platelet-activating factor, monocyte chemotactic proteins 1–4, sphingosine-1-phosphate, and adenosine, LPA could be involved in the accumulation of immature DCs at peripheral target sites, then loss of $G_{\alpha}i/o$ protein-dependent sensitivity to LPA during DC maturation could clear the way for MIP-3β-driven migration of DCs to secondary lymphoid organs (Sozzani et al., 2001). In contrast to what happens in T-cells, it was shown that mRNA levels of LPA_1, LPA_2, and LPA_3 are comparable in immature and LPS-differentiated DCs.

In the CNS, the role of antigen-presenting involves microglia cells. To this point, Moller et al. (2001) showed that LPA application to cultured rat and mouse microglia resulted in elevation of intracellular calcium. In this line, the expression of LPA receptors by microglia was reported by Tham et al. (2003), finding that LPA_3 is strongly up-regulated on the rat microglia only after activation by LPS treatment, and even on cells treated, this receptor is still less highly expressed than LPA_1. It is noteworthy that within this study, differences in expression of several LPA receptor subtypes were correlated with differences in the degree of cell activation. As mentioned, LPA_3 was essentially undetectable in acutely isolated cells, was lowly expressed by conventionally cultured cells, and became significantly expressed after LPS treatment. On the other hand, LPA_1 was expressed at higher levels in the cultured cells than in acutely isolated cells, and declined after LPS activation. Prolonged exposure of microglia to LPA produced modest decrements in gene expression of LPA receptor mRNA; in contrast, exposure to LPS, produced robust increases in lpa3 gene expression.

In conclusion, it is clear enough, that LPA must play an important role in the inflammatory process, also in myelination, although its effects and how these one can be modulated to prevent CNS from the damage caused by neuroimflammatory and autoimmune disease should be deeply studied.

LPA receptor	Cell type	Cellular response to LPA
LPA_1	Myeling forming cells in rodent CNS (Barron et al., 1997; Allard et al., 1998; Weiner et al., 1998) Human mature myelinating oligodendrocytes (Cervera et al., 2002) Mature postmitotic oligodendrocyte prior myelination (GalC+/CNPase+/MBP+/PLP+/MOG-) (Stankoff et al., 2002)	Myelinogenesis and/or myelin maintenance ?
	Jurkat cells, fibroblasts and astrocytes (Jalink et al., 1994; Goetzl and An, 1998)	Responses via G-proteins of the Gi/Go class and Ca^{2+} signals
LPA_1	Mature oligodendrocytes GalC+ but not precursors O4+/GalC- (Möller et al., 1999)	Increase $[Ca^{2+}]i$ through Gi/Go G-protein
LPA_1 and LPA_2	Schawnn cells (Li et al. 2003)	Survival and differentantion
LPA_2	Schawnn cells (Li et al. 2003)	Expression of myelin protein P0
LPA_1 (LPA_2 and LPA_3)	Olfactory ensheathing cells (Yan et al., 2004)	Proliferation and migration
LPA_1	Jurkat T-cells and human blood CD4+-T cells (Zheng et al., 2000)	Proliferation and secretion of IL-2
LPA_2	Jurkat T-cells and human blood CD4+-T cells (Zheng et al., 2000)	Migration
LPA_1, LPA_2 (and LPA_3)	Macrophages (Koh et al., 1998, Balazs et al., 2000, Hornuss et al., 2001)	Survival and migration
LPA_1 and LPA_3	Endothelial cells (Rizza et al., 1999, Palmetshofer et al., 1999, Gustin et al., 2008)	Expression of E-selectin, vascular cell adhesion molecule-1 (VCAM-1), factor nuclear factor-κB (NF- κB), intracellular adhesion molecule-1 (ICAM-1), IL-8 and monocyte chemoattractant protein-1 (MCP-1)
LPA_1	Lymphocytes B (Rosskopt et al., 1998, Xie et al., 2002, Allende et al., 2003)	Formation IP3 and inositol phosphate, increase $[Ca^{2+}]i$, MAP-kinase activation, DNA synthesis and immunoglobulin formation
	Immature dendritic cells (Phanther et al., 2002, Contos et al., 2000, Swarthout and Walling, 2000)	Increase $[Ca^{2+}]i$, actin reorganization, and chemotactic migration
$LPA_1$and LPA_3	Rodent microglia (Möller et al., 2001, Tham et al., 2003)	Increase $[Ca^{2+}]i$

Table 2. Scheme of different roles of LPA throught several receptors in main components of neuroinflammation and myelination processes.

HYPOTHESIS AND OBJECTIVES

As it was mentioned, the lysophosphatidic acid (LPA) is involved in many different biological processes. Previous studies have shown that LPA signalling pathway plays an important role in inflammation and myelination, above all through LPA_1 receptor.

In regard with myelination, *Lpar1* is expressed in myelinating cells, such as oligodendrocytes, Schawnn cells and olfactory ensheathing cells, right before myelination and in maturing cells, inducing survival, migration or proliferation depending on the cell types.

On the other hand, LPA, through LPA_1, induces proliferation and secretion of proinflammatory cytokines in CD4+ T cells, survival and migration in macrophages, and it is also involved in the permeability of the blood-brain barrier through the expression of selectins, adhesion molecules and cytokines in endothelial cells. The involvement of LPA_1 in inflammation is also shown by the presence of its signalling pathway in B lymphocytes and microglia.

The relationship between the lysophosphatidic acid (LPA) and both myelination and immune system suggests that LPA could be considered as a regulator of neuroinflammatory demyelinating disease like multiple sclerosis or target of new treatments.

Given the above, the following is hypothesized:

Hypothesis

The LPA_1 receptor is essential for normal myelination and immune response. Thus, its absence in LPA_1-null mice of triggers cellular alterations associated to both central nervous and inflammatory systems, particularly those concerning normal oligodendrocyte development, myelin formation, and the adaptive immune response following induction of autoimmune demyelinating disease.

In order to prove this hypothesis the following primary and secondary objectives were presented:

Objectives

- Analyze the role of LPA in myelination, using an LPA_1 knockout mouse model ($maLPA_1$-null variant). The study will be carried out comparing the phenotypes of wild type and $maLPA_1$-null mice.
  - Study of myelination pattern
  - Study of oligodendrocyte development.
- Analyze the role of LPA in remyelination by intracisternal administration of LPA in neurotoxicant-induced, cuprizone model of demyelination.
- Analyze the role of LPA in the pathogenesis of multiple sclerosis by studying its experimental form in the widely accepted animal model of encephalomyelitis autoimmune experimental (EAE).
  - Study of the differences in the external clinical symptoms and the histology of immune response between wild type and null mice after the induction of the disease.
  - Study of the expression of *Lpar1* and *Lpar2* receptors in peripheral blood mononuclear cells (PBMC) along the clinical course to infer their roles at some stage in the pathogenesis of the disease.
  - Study the effects of intravenous administration of antagonist for LPA_1 receptor in EAE

MATERIAL AND METHODS

1. MICE

Procedures were carried out with males and females of maLPA$_1$ strain: wild-type [ma*lpa*$_1$(+/+), also called also *normal* in this work], LPA$_1$-null heterozygous [ma*lpa*$_1$(+/-)] and LPA$_1$-null homozygous [ma*lpa*$_1$ (-/-), namely null or ma*lpa*$_1$-null (Estivill-Torrús et al., 2008)]. These mice (C57BL/6J × 129X1/SvJ) are affected with a deletion in *Lpar1* gen, exon 3 codifying domains I and VI of LPA$_1$ receptor was removed (Contos et al, 2000).

The maLPA$_1$-null was established as *Málaga* variant of LPA$_1$-null (Estivill-Torrús et al., 2008), this mouse colony arose spontaneously from the initially reported LPA$_1$-null mouse line (Contos et al., 2000) while colony production was being carried on.

The original null mice were generated in a mixed background by targeted gene disruption using homologous recombination and Cre-mediated deletion in a 129X1/SvJ background. Isolating and subcloning exon 3 from 129X1/SvJ, allowed to insert into it neomicine resistence gen flanked with LoxP secuences into a vector. This vector was electroporated into R1 embryonic stem cells and, both homologous recombination and Cre-mediated deletion (system Cre/LoxP) were detected by PCR and confirmed by Southern blot analysis. When a clone was confirmed, it was injected into blastocysts and the chimeric mouse backcrossed with C57BL/6J females (Contos et al., 2000).

Original heterozygous mice, gently given by Dr. Jerold Chun from the Scripps Research Institute in San Diego, California, in constant collaboration with our group, were used for intercrossing as well as with mice generated from one additional backcross. The maLPA$_1$-null mouse was spontaneously derived during the original colony expansion by crossing heterozygous foundation parents (maintained in the original hybrid C57BL/6J × 129X1/SvJ background). Intercrosses were performed with these mice and subsequently backcrossed with mice generated within this mixed background. Targeted disruption of the *Lpar1* gene was confirmed by genotyping (according to Contos et al. 2000), PCR, and immunochemistry (Estivill-Torrús et al., 2008).

Malaga LPA$_1$-null mice carrying and checked for *Lpar*1 deletion displaying reduced size and craniofacial defects. Mice were born at the expected mendelian ratio and survived to adulthood exhibiting increased survival rates and only very reduced suckling defects when compared with original LPA$_1$-null mice.

This colony has been established in the Animal Facility Center of Malaga University for more than eight years and the defects described in the present work have been observed through more than 22 maLPA$_1$-null generations. Every procedure was always in compliance with European animal research laws (European Communities Council Directives 86/609/EU, 98/81/CEE, 2003/65/EC and Commission Recommendation 2007/526/EC) and Spanish National Guidelines for Animal Experimentation and use of genetically modified organisms (Real Decreto 1205/2005 and 178/2004 and Ley 32/2007 and 9/2003).

Research was performed on young mice (postnatal day 10 to P30) and 12 weeks-old male mice for the analysis of the different myelinization patterns, and 6-8 weeks-old female mice for EAE induction,obtained from heterozygous×heterozygous/homozygous maLPA$_1$-null mating and genotyped for *Lpar1* deletion by PCR (Contos et al., 2000) or immunohistochemistry (Estivill-Torrús et al., 2008). Animals were housing in standard conditions of temperature, airing, humidity, lighting (light was timer-controlled to switch ON/OFF automatically following a circadian rhythym of 12h light/dark) and water and food supply.

For cuprizone demyelinating model, 8-weeks old CNP-EGFP mice were used. These are from a transgenic mouse expressing enhanced green fluorescent protein (EGFP) driven by the 2-3-cyclic nucleotide 3-phosphodiesterase (CNP) promoter, and available in the Biomedical Research Institute, University of Hasselt, in Belgium, wherein procedures were carried out under the European and local laws.

1.1. Genotyping

Adult mice from wild-type and LPA$_1$ heterozygous and homozygous females were genotyped for L*par1* deletion by polymerase chain reaction (PCR) using DNA isolated from a small part of the tail according to Contos et al. 2000.

Isolation of DNA from mouse tail was performed by cutting off 4-5 mm of the tail tip with a scalpel and digesting with proteinase K [Roche Diagnostics S.L., Barcelona; shortly, Roche] (20 mg/ml) diluted in STES (comercial buffer STE: 100mM NaCl, 100 mM Tris-HCl ,pH 8.0 solution, 1 mM EDTA, and 0.1% SDS (Sigma-Aldrich Química S.A.; Madrid). After overnight incubation (8-24h) at 45 °C with shaking, a volume of 200 µl 7.5M ammonium acetate (Sigma-Aldrich) was added to each tube and centrifugated at 14000 rpm for 10 min at 4°C. For DNA precipitation supernatants were immediately place on 900 µl of iced cold ethanol. After shaking softly tubes were centrifugated again at 14000 rpm for10 min at 4°C to pellet DNA. After rinsing pellet with 70% ethanol (v/v) they were submitted to centrifugation at 14000 rpm for 5 min at 4°C, ethanol was discarded and DNA pellet was air-dried. Two hours later, DNA was rehydrated using sterilized water and stored for 24 h at 4°C.

DNA quantification was assayed by spectrophotometry, so the presence of proteins could be measured. DNA concentration was determined by measuring absorbance of the sample at 260nm (A260). The ratio of absorptions at 260nm vs 280nm is commonly used to validate the purity of DNA with respect to protein contamination, since protein (in particular, the aromatic amino acids) tends to absorb at 280nm (A280). The method dates back to 1942, when Warburg and Christian showed that the ratio is a good indicator of nucleic acid contamination in protein preparations, to consider a good quality of DNA the ratio must be about 1.8 (Sambrook and Russell, 2001). The ratio A260/280 of samples in the present study was always near this value.

For PCR genotyping of mice, 1 µl (100-200 ng) of DNA was added as template in DNA polymerase comercial mix, BioThermMix™ (Genecraft GMBH, Lüdinghausen, Germany) which includes BioTherm™ DNA polymerasae (0.06 U/µl), 2,5x PCR buffer with 3,75 mM $MgCl_2$, 500 µM each deoxynucleotide triphosphate (dNTP, such as dATP, dTTP, dGTP and dCTP), and stabilizers.

Later, the reaction was developed in a thermocycler (Perkin-Elmer GeneAmp PCR system 2400; PerkinElmer España S.L., Madrid) cycled 35 times (95°C, 30 s; 56°C, 30 s; and 72°C, 2 min) with a final incubation of 72°C for 5 min before cooling to 4°C. Primers (Proligo; Sigma-Aldrich Química S.A.; Madrid) used in this PCR protocol allowed to distinguish different alelles of *Lpar1* since they recognize the flanking sequence of this gen, being these:

513QL, 59- GCCAATCCAGCGAAGAAGTC-39;
vzg.il1, 59-GGTATTCTTAATTCTAGAGGATCAGC-39;
vzg.is2, 59-TATAGGAGTCTTGTGTTGCCTGTCC-39.

The mix for PCR was done in a final volume of 25 µl per tube and its composition as follows:

Reagents per tube	Volume
Sterilize water	11 µl
BiothermMix™	10 µl
Primer 513QL	1 µl
Primer vzg.il1	1 µl
Primer vzg.is2	1 µl
DNA sample	1 µl

1.2. Agarose Gel and Electrophoresis

After PCR, amplified fragments were separated on the basis of size by agarose gel electrophoresis. This was achieved by moving negatively charged nucleic acid molecules through an agarose matrix in an electric field. Agarose gel were made disolving 1,5% powdered agarose (agarose D1, low EEO; Pronadisa; Laboratorios Conda; Madrid) in electrophoresis 1x TBE (Tris/borate/ EDTA) buffer,pH 8.3 (Sigma-Aldrich), adding ethidium bromide (EtBr, Sigma-Aldrich) up to a final concentration of 0.5 µg/ml to make DNA bands visible. Different fragments were resolved applying a current of 70 V after samples were injected with a loading buffer consists of 0,25% bromophenol blue (Sigma-Aldrich) in 40% of sucrose solution (Sigma-Aldrich). In every gel, a control lane of

DNA standards (DNA molecular weight marker, Roche; *8-587 b*) suitable for the weight of the amplified fragments was also run.

According to Contos et al. (2000), the resulting amplified fragments by using ofvzg.is2 and 512QL primers were 348 pb in lengh, this size was obtained from wild-type samples wherein *Lpar1* gen including exon 3 is present (Fig. 11A). Shorter sequence obtained after exon3 deletion in null homozygous mice were detected using vzg.is2 and vzg.il1 primers resulting in 227 pb sized fragments. Samples from heterozygous mice exhibit both fragments (Fig. 11B).

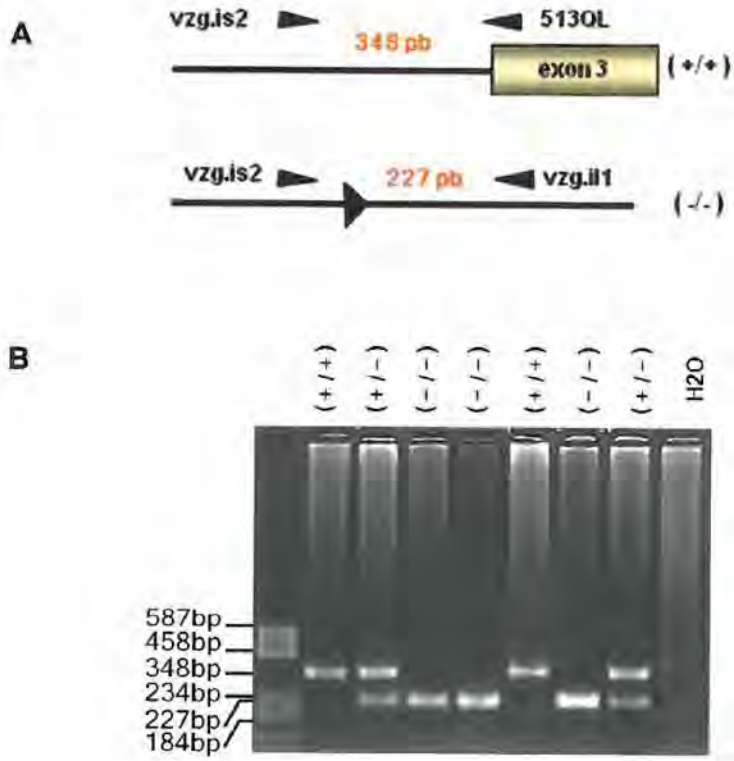


Figura 11. A. Scheme of primer sequences and mapping position used for PCR detection of exon 3 in *Lpar1* gen of wild-type (+/+) and null (-/-) mice. B. Agarose gel electrophoresis of products from genotyping using cited primers. DNA bands corresponding to wild-type (+/+), heterozygous (+/-), and homozygous null (-/-) genotype.

1. MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY

In vivo experiments were performed in accordance with institutional guidelines and accordingly Fernández-Medarde et al. (2009). Spontaneously breathing mice were anesthetized by inhalation in an induction box with oxygen (1 l/min) containing 3 % of isofluorane, and maintained during the experiment employing a mask and 1-2 % of isofluorane in O_2. During measurements animals were placed in a heated probe, which maintained the core body temperature at approx. 37 °C. The physiological state of the animal was monitored by Biotrig physiological monitor (Bruker Medical Gmbh, Ettlingen, Germany) using the respiratory rate and body temperature, which allowed the adjustment of anaesthetic concentration.

^{1}H Magnetic Resonance spectroscopy and Imaging experiments were performed on a Bruker Pharmascan system (Bruker Medical Gmbh) using a 7.0-T horizontal-bore superconducting magnet, equipped with a ^{1}H selective birdcage resonator of 23 mm and a Bruker gradient insert with 90 mm of diameter (maximum intensity 300 mT/m). The magnet was interfaced to a Hewlett-Packard console running Paravision software (Bruker Medical Gmbh) operating on a Linux platform.

T2-weighted (T2-W) spin-echo anatomical images were acquired with RARE a (rapid acquisition with relaxation enhancement) sequence in sagital orientation, using the following parameters: TR (repetition time) = 2500 ms, TE (echo time) = 60 ms, RARE factor = 8, Av = 3, FOV = 2.3 x 2.3 cm, acquisition matrix = 256 x

256 corresponding to an in-plane resolution of 90 x 90 μm2 and slice thickness of 1.00 mm was achieved, taking 10 slices.

The in vivo spectroscopy protocol use PRESS (Point-Resolved Spatially Spectroscopy), combined with VAPOR (variable power pulses with optimized relaxation delays) water suppression, spectra were acquired from a voxel in the central mouse brain, containing the thalamus and surrounding areas. The used parameters were: TR = 3000 ms, TE = 35 ms, Av = 128, volume = 3 mm3. Metabolite concentrations were determined for N-acetylaspartate (NAA), creatine (Cr) and choline (Ch), in vivo values were obtained for the main resonances of NAA at 2.01 ppm, Cr at 3.02 ppm, and Ch at 3.21 ppm,. Peak areas were calculated by using the MestRe-C software, and the area ratios were obtained and statistical analysis was performed with these values.

3. LIGHT MYCROSCOPY SAMPLE PREPARATION

Common buffers

0.2 M *Phosphate buffer (PB), pH 7.4*

For 1 litre of 1x phosphate buffer, 6.90 g of $NaH_2PO_4.H_2O$ (Merck Farma y Química, S.L.; Madrid) and 26.70 g of $Na_2HPO_4.2H_2O$ (Merck) were added to 800ml of destilled water, solution was made adjusting the pH to 7.4 with HCl or NaOH and finally filled up to 1000 ml.

0.1 M *Phosphate-buffered saline (PBS), pH 7.4*

For 1 litre of 1x PBS, 1.90 g of $Na_2HPO_4.2H_2O$, 0.43 g of KH_2PO_4, and 7.22 g of NaCl (all from Merck) were added to 800ml of destilled water, solution was made adjusting the pH to 7.4 with HCl or NaOH and finally filled up to 1000 ml.

3.1. Fixation procedure

The mode of fixation for all immunohistochemistry techniques used in this study was always vascular perfusion of mice followed by immersion of sample in the same fixative (post fixation step).

Animals were perfused through the heart after being given an appropriate amount of anaesthetic. After opening the chest, a needle was inserted into the left ventricule and the right atrium was immediately opened, perfusion started washing circulatory system with phosphate buffer solution (PBS; 0.1 M pH 7,4) during 15 min and, when blood had been cleared from the body, fixative solution continued for 10 min at a very low flow rate given by a perfusion pump (Dinko modelo D-25V; DINTER, S.A. - DINKO Instruments; Barcelona). After perfusion, brain and spinal cord were excised and placed in vials to immersion in the same fixative for 24 or 48 h at 4°C

Different fixative solutions were used in this work depending on the antibody/ marker assayed.

4 % PARAFORMALDEHYDE (4%PFA): Used to fix tissues for immunochemistry which employed antibodies against chondroitin sulfate proteoglycan (NG2) in oligodendrocyte progenitors.

To dissolve paraformaldehyde, distilled water was previously heated to 60°C before adding paraformaldehyde and 0.2M PB, pH 7.4. One or two drops of 2.5% NaOH (Panreac Química S.A.U; Barcelona) were added if neccesary to dissolve it.

4 % PARAFORMALDEHYDE and 0.25% GLUTARALDEHYDE (4% PFA / 1% Glut): this fixative was used when 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP) was studied by immunochemistry.

The preparation was the same as explained above for 4% PFA but 1% of glutaraldehyde stock solution [25% glutaraldehyde (Merck)] was added before its use.

PERIODATE-LYSINE-PARAFORMALDEHYDE (PLP): Used in the fixation of tissues for immunochemistry against lysophosphatidic acid receptor 1 (LPA_1).

The composition of PLP was 4% paraformaldehyde, 0.075M lysine, 0.01M sodium periodate, in PB 0.1M, pH 7.4 (Mclean and Nakane, 1974).

SOMOGYI'S FIXATIVE (Somogyi and Takagi, 1982): This solution was used to fix the animals when histochemistry involves antibodies against: proteolipid protein (PLP), myelin basic proteins (MBP), galactocerebroside (GalC), myelin specific oligodendrocyte protein (MOSP), transcription factor Olig2, glial fibrillary acid protein (GFAP), T cell receptor (TCR or CD3), and integrin αM of microglia (CD11b/c equivalent antibody, OX42).

Its composition was 4% paraformaldehyde, 0.08% glutaraldehyde, and 15% picric acid solution saturated aqueous, in PB 0.1M, pH 7.4

3.2. Embedding and sectioning

3.2.1. Agar embedding for vibratome sections

Most of immunohistochemical techniques were carried out on free-floating vibratome cut slices after samples were embedded in agar. For this purpose, after postfixation, tissues were embedded in 4% neutral agar (Sigma-Aldrich) in distilled water allowing it to cool until solid. Tranverse sections of approximately 50 µm thick were cut serially. Free-floating sections were obtained and stored in in PBS 0.1M, pH 7.4, with 0.01% sodium azide, at 4°C.

3.2.2. Cryoprotection and sectioning with freezing microtome

After dissecting out brain and spinal cords were washed in PBS and cryoprotected by incubation in 30% sucrose in PBS (Panreac Química S.A.U; Barcelona) overnight at 4°C. Slices (50 µm) were obtained using a freezing microtome, washed several times in ice-cold PBS and stored also free-floating in PBS 0.1M pH 7.4 with 0,01% sodium azide at 4°C.

3.2.3. Paraffin embedding and processing

Paraffin sections were used to measure apoptosis by employing TUNEL assay.

After fixation, the tissue block was infiltrated in paraffin with an automatic carousel-type tissue processor (120P model; MYR S.L.; Tarragona) following a standard dehydration protocol using ethanol: [70% (v/v), 2 x 1h; 80% (v/v), 1h, 96% (v/v), 2 x 1h; 100% (v/v), 2 x 1h], xylene (2 x 1.5h), and paraffin (2 x 1.5h). Paraffin block was obtained using a paffarin embedding station (EG1150H model; Leica Microsytems; Leica Microsistemas SA; Barcelona). Then blocks were cut in a microtome to the desired thickness (approximately 10 µm) and affixed onto the slide. Tissue sections were mounted on poly-L-lysine coated slides. Once mounted, the slides were allowed to dry to remove any water that may be trapped under the section by leaving them at room temperature overnight or, alternatively, incubation at 60°C for a few hours.

Before use, sections were deparaffinized and rehydrated placing the slides in a rack, and performing the following washes: xylene (2 x 15 minutes), 100% ethanol (2 x 15 minutes), 96% ethanol (2 x 15 minutes), 70 % ethanol (10 minutes), and final distilled water to rinse.

4. HISTOCHEMICAL TECHNIQUES

4.1. Apoptosis detection

Apoptosis is a physiological mechanism of cell death which involves the fragmentation of a cell into membrane-bound particles. The process of apoptosis is involved in a variety of normal and pathogenic biological events, both during development and in adulthood. In view of the biological importance of apoptosis, there exists a need for methods to specifically detect cells undergoing apoptosis and those which have suffered apoptotic cell

death. These methods are crucial to the identification, characterization, and diagnosis of diseases distinguished by abnormal apoptosis.

The method used in this work to analyze apoptotic cells was the commercial In Situ Cell Death Detection Kit, AP (Roche) which uses paraffin sections fixed from samples fixed with commercial solution of 30-36% formaldehyde (Panreac). Eight animals from each genotype were used to achieve this aim.

❖ Procedure

After dewaxing and rehydrating tissue section according to standard protocols previously described, they were incubated for 15-30 min at 21–37°C with a proteinase K working solution [10-20 µg/ml in 10 mM Tris/HCl, pH 7.4-8] and rinsed twice for 5 min with 0.1M PBS, pH 7.4. Later, after drying area around sample, 50 µl of TUNEL reaction mixture were added on the sample [Terminal deoxynucleotidyl transferase from calf thymus (EC 2.7.7.31), recombinant in E. coli, with nucleotide mixture in reaction buffer] and incubated 1h at 37°C in a humidified atmosphere in the dark. Finally sections were washed four times with PBS 0.1M, pH 7.4 (5 min each one).

As positive control, slides were incubated prior to labeling procedures with DNase I recombinant [3000 U/ml– 3 U/ml in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl 2 1 mg/ml BSA] for 10 min at 15–25°C to induce DNA strand breaks. As negative control, label solution (without terminal transferase) was used instead of TUNEL reaction mixture during the incubation.

After this step, samples could be analyzed in a drop of PBS under a fluorescence microscope at this state using an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (green).

To signal conversion for analysis in light microscope, the area around sample was dried and 50 µl converter-AP were added on sample [anti-fluorescein antibody, Fab fragment from sheep, conjugated with alkaline phosphatase (AP)] incubating slides in a humidified chamber for 30 min at 37°C again. Sections were rinsed three times with PBS before adding 50–100 µl substrate solution and incubating for 10 min, 15-25°C in the dark and washed several times with PBS after incubation.

Optionally, nuclei could be stained before dehydratation following a conventional hematoxylin staining protocol.

Lastly, slides were rinsed with PBS, dehydrated with ethanol [70% (v/v), 5 min; 96% (v/v), 2 x 5 min; 100% (v/v), 2 x 10 min], cleared in xylene (2 x 10 min), and mounted with DPX (Sigma-Aldrich) to be analyzed under light microscope with a digital camera attached (Olympus BX51, Olympus DP70; Olympus España S.A.; Barcelona). The number of apoptotic cells in the cerebral cortex of each genotype was estimated using a informatic programme, Image J versión 1.37v (National Institute of Health, United States; available at http://rsbweb.nih.gov/ij/). Statistical analyses using the t-student test were performed with at least six animals per group, using the software program SPSS statistics 17.0 Values of $p < 0.05$ were considered to be significant.

4.2. Immunohistochemistry

4.2.1. Antigens analyzed and antibodies used for detection

αM integrin, equivalent CD11b/c (OX42). Integrin αM is a cell adhesion molecule that acts as a receptor for cell surface ligands such as intracellular adhesion molecules (ICAMs) or soluble ligands. Integrin αM combines with Integrin β2 to form a leukocyte-specific integrin referred to as macrophage receptor 1 (Mac-1) or inactivated-C3b (iC3b) receptor 3 (CR3). Integrin αM/β2 is important in the adherence of neutrophils and monocytes to stimulated endothelium, and also in the phagocytosis of complement coated particles. CD11b antigen is present on macrophages, granulocytes, natural killer cells, blood monocytes and microglia.

The antibody used for its detection was mouse monoclonal anti-OX42 (Ref. 1211, Abcam plc, Cambridge, Reino Unido)

CD3 antigen (antigen of cluster of differentiation 3) is a protein complex expressed in T lymphocytes and composed of four distinct chains. In mammals, the complex contains a CD3γ chain, a CD3δ chain, and two CD3ε chains. These chains associate with a molecule known as the T cell receptor (TCR) and the ζ-chain to

generate an activation signal in T lymphocytes. The TCR, ζ-chain and CD3 molecules together comprise the TCR complex.

The antibody used for its detection was rabbit policlonal anti-CD3 (Ref. 5690, Abcam plc)

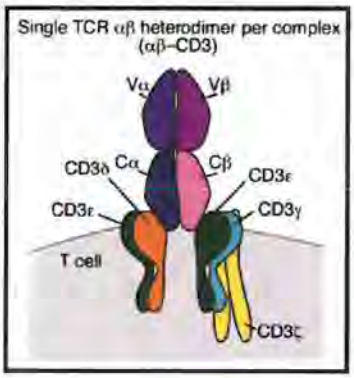


Figura 12. Scheme of T-cell receptor and its subunits.

CNPase (2', 3'-cyclic nucleotide 3'-phosphodiesterase) is present in very high levels in brain and peripheral nerve. This enzyme is found almost exclusively in oligodendrocytes and Schwann cells, the cells that form myelin in the central and peripheral nervous system, respectively. Therefore, CNPase has been very useful as a marker for these particular cell types.

The antibody used for its detection by immunochemistry and western-blot was mouse monoclonal anti-CNP (C5922; Sigma-Aldrich).

Galactocerebroside or galactosylceramide (GalC) is a type of cerebroside consisting of a ceramide with a galactose residue and it is a major galactosphingolipid of myelin which plays a role in myelination. Galactosylceramides is an early marker that remains present on the surface of mature oligodendrocytes in culture (Raff et al., 1979) and in vivo (Zalc et al., 1981). Some polyclonal IgG antibodies to galactocerebrosides alter the organization of oligodendroglial membrane sheets, Dyer and Benjamins (1988) demonstrated long-term exposure of oligodendroglia to anti-GalC causes extensive contraction of membrane sheets with accompanying reorganization of the underlying cytoskeleton.

The antibody used for its detection was mouse monoclonal anti-GalC (Ref. MAB342; Chemicon, Millipore Iberica S.A.U.; Madrid)

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is thought to be specific for astrocytes in CNS (Eliasson et al., 1999). Later it was shown that GFAP also is expressed by other cell types in CNS-ependymal cells (García et ál., 2004). GFAP is thought to help to maintain astrocyte mechanical strength, as well as the shape of cells but its exact function still remains poorly understood. It is commoly used as astrocyte marker.

The antibody used for its detection was rabbit policlonal anti-GFAP (Ref.: Z0334; Dako Diagnósticos S.A.; Barcelona)

Golgi phosphoprotein 4 (Golph4). This protein, also called GPP130, is a ubiquitous homodimeric type II integral membrane protein with a short, 12-amino acid, cytoplasmic tail (Linstedt et al., 1997). The membrane proximal region is strongly predicted to form a coiled-coil stem structure and contains distinct Golgi and endosomal targeting determinants that mediate Golph4 bypass pathway trafficking and targeting (Puri et al., 2002).

The antibody used for detection of lysosomes and late endosomes was rabbit policlonal anti-Golph4 (Ref. 28049; Abcam plc.).

Lysosome associated membrane protein (LAMP1) is a transmembrane glycoproteins that is localized primarily in lysosomes and late endosomes. Newly synthesized molecules are mostly transported from the trans-Golgi network directly to endosomes and then to lysosomes. A second pathway involves the LAMPs being delivered from the Golgi to the cell surface, and then along the endocytic pathway to the lysosomes. A minor pathway involves transport via the plasma membrane. Upon stimulation, a rapid translocation of intracellular LAMPs to the cell membrane is dependent on a carboxyl-terminal tyrosine based motif (YXXI). If there is a disturbance in this spacing, lysosome localization of LAMP1 is abolished and the mutant protein then cycles between the membrane and the endosome (Rohrer et al. 1996).

The antibody used for detection of lysosomes and late endosomes was rabbit policlonal anti-LAMP1 (Ref. 24170; Abcam plc.).

Lysophosphadic acid receptor-1 (LPA_1). LPA_1 represents the first lysophospholipis receptor identified. Its responses are attributable to the activation of specific, seven-transmembrane domain G protein-coupled receptors (GPCRs), which induce diverse cellular responses including proliferation, adhesion, migration, morphogenesis, differentiation and survival. In both humans and mouse, adult expression is widespread and includes most major tissues (Anliker and Chun, 2004; Ishii et ál., 2004).

The antibody used for its detection was rabbit policlonal anti-LPA_1 (Ref.: PA1-1041; ABR-Affinity Bioreagents; Colorado, United States)

Myelin Basic Proteins (MBP) is a protein believed to be important in the process of myelination of nerves in the central nervous system (CNS) and it was initially sequenced in 1979 after isolation from myelin membranes. MBP is not a single protein but a very diverse pool of them in CNS with several splice variants being expressed and a large number of post-translational modifications on the protein, which include phosphorylation, methylation, deamidation and citrullination. The gene for MBP is on chromosome 18 and the protein localizes to the CNS and to various cells of the hematopoietic system.

The antibody used for its detection by immunochemistry was rat monoclonal anti-MBP (Abcam, ab7349-2) and by western-blot analysis, rabbit polyclonal anti-MBP (Ref.: AB980; Millipore).

Myelin/oligodendrocyte specific protein (MOSP) is a surface membrane protein expressed exclusively by oligodendrocytes in the central nervous system (Dyer et al., 1991). MOSP has interesting biological properties which were studied by prolonged exposure of cultured oligodendrocyte to anti-MOSP. Its role seems to be to increase the number and thickness of microtubules within processes and membrane sheets (Dyer, 1993. Dyer and Matthieu, 1993). This suggests that MOSP plays an important role in membrane/cytoskeleton interactions during the formation and maintenance of CNS myelin.

The antibody used for its detection was mouse monoclonal anti-MOSP (IgM) (Ref. MAB 328; Millipore Iberica S.A.U)

NG2 or Chondroitin sulfate proteoglicano. NG2 proteoglycan is an integral membrane chondroitin sulfate proteoglycan with a core protein of 260 kDa. The cells which express this protein have extensive arborizations of their cell processes and are found ubiquitously long after oligodendrocytes are generated. They do not express antigens specific to mature oligodendrocytes, astrocytes, microglia, and neurons, suggesting that they are a novel population of glial cells which undergoes proliferation with morphological changes in response to stimuli such as inflammation or demyelination (Dawson et al., 2000).

The antibody used for its detection was rabbit policlonal anti-NG2 (Ref. AB 5320; Millipore Iberica S.A.U)

Oligodendrocyte lineage transcription factor 2(olig2). Olig2, a basic helix-loop-helix transcription factor, is an essential regulator of ventral neuroectodermal progenitor cell fate. Olig2 is a recently identified transcription factor involved in the phenotype definition of cells in the oligodendroglial lineage. The expression of its transcript has been demonstrated in a restricted domain of the spinal cord ventricular zone that sequentially generates motoneurons and oligodendrocytes.

The antibody used for its detection was rabbit policlonal anti-Olig2 (AB 9610; Millipore Iberica S.A.U).

Protein Disulphide Isomerase (PDI) is involved in disulphide-bond formation and isomerization, as well as the reduction of disulphide bonds in proteins. PDI, which catalyses disulphide interchange between thiols and protein dilsulphides, has also been referred to as thiol protein-disulphide oxidoreductase and as glutathione insulin transhydrogenase because of its role in reduction of disulphide bonds. The highly conserved sequence Lys-Asp-Glu-Leu (KDEL) is present at the carboxy-terminus of PDI and other soluble endoplasmic reticulum (ER) resident proteins. The presence of carboxy-terminal KDEL seems to be necessary for ER retention and appears to be sufficient to reduce the secretion of proteins from the ER. Then, PDI is used as a good marker of ER.

The antibody used for detection of ER was rabbit policlonal anti-PDI (Ref. 2672; Abcam plc.).

Proteolipid protein (PLP and DM20), also called lipophilin, PLP is the most abundant protein of myelin. It is a 4 transmembrane domain protein which binds strongly to other copies of itself on the extracellular side of the membrane. In a myelin sheath, as the layers of myelin wraps come together, PLP will bind itself and tightly hold the cellular membranes together. PLP and DM20 are produced from alternatively spliced mRNAs from the primary PLP gene transcript. The gene *PLP1* that codes for PLP is on the X chromosome.

The antibody used for its detection by immunochemistry and western-blot was mouse anti-PLP (Ref.:MAB 388; Millipore Iberica S.A.U)

PRIMARY ANTIBODY	SUPPLIER	ISOTYPE AND CLONALITY	DILUTION	SOLVENT
Anti-αM Integrin (OX42)	Abcam, Ref : 1211	IgG, monoclonal, raised in mouse	1:200	Rabbit PST
Anti-CD3	Abcam, Ref : 5690	IgG, polyclonal, raised in rabbit	1:100	Swine PST
Anti-CNPase	Sigma, Ref.: C5922	IgG, monoclonal, raised in mouse	1:500	Rabbit PST
Anti-GalC	Chemicon, Ref.: MAB 342	IgG, monoclonal, raised in mouse	1:500	Rabbit PST
Anti-GFAP	Dako, Ref.: Z0334	IgG, policlonal, raised in rabbit	1:1000	Swine PST
Anti-Golph4	Abcam, Ref.: 28049	IgG, policlonal, raised in rabbit	1:400	Swine PST
Anti-LAMP1	Abcam, Ref.:24170	IgG, policlonal, raised in rabbit	1:400	Swine PST
Anti-LPA$_1$	Affinity Ref.: PA 1-1041	IgG, policlonal, raised in rabbit	1:200	Swine PST
Anti-MBP	Abcam, Ref.: 7349-2	IgG, monoclonal, raised in rat	1:200	Rabbit PST

Anti-MBP	Chemicon, Ref.: AB 980	IgG, polyclonal, raised in rabbit	1:1000	Swine PST
Anti-MOSP	Chemicon, Ref.: MAB 328	IgG, monoclonal, raised in mouse	1:500	Rabbit PST
Anti-NG2	Chemicon, Ref.: AB 5320	IgG, polyclonal, raised in rabbit	1:500	Swine PST
Anti-Olig2	Chemicon, Ref.: AB 9610	IgG, polyclonal, raised in rabbit	1:750	Swine PST
Anti-PDI	Abcam, Ref.: 3672	IgG, polyclonal, raised in rabbit	1:100	Swine PST
Anti-PLP	Chemicon, Ref.: MAB 388	IgG, monoclonal, raised in mouse	1:100	Rabbit PST*

Table 3. Primary antibodies used in immunohistochemical studies.

Solution of primary and secondary antibodies were made either on PST, i.e., 0.1M PBS, pH 7.4, with 2.5% serum (rabbit, swine, goat; as it corresponds or specified; Biolink2000 S.L.; Barcelona), and 0.5% Triton TX100 (Panreac Química S.A.U.), either PBS-TX100 (same as described but without serum).

4.2.2. Secondary antibodies and substrates.

A secondary antibody is an antibody used to bind to primary ones, so the secondary antibody must be raised against the specific type of immunoglobulin and the animal species in which the primary antibody has been raised. This second layer antibody can be labelled with a fluorescent dye or an enzyme which make them useful for detection. In this study three different kinds of secondary antibodies were used.

The first kind of antibody was labelled with fluorescent dyes, these antibodies were visualized directly using fluoresence or confocal microscopy, illuminating slides with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit longer wavelengths of light (of a different color than the absorbed light).

Other antibodies were labelled with an enzyme, alkaline phosphatase. This enzyme deposited a colored reaction product in the presence of the appropriate substrate, such as NCIP, which has a blue colour.

The most common procedure was the use of a biotinylated secondary antibody. Subsequent high affinity coupling with Extravidin®-peroxidase (a modified form of affinity purified avidin coupled to peroxidase; Sigma-Aldrich) peroxidise and 3,3'-diaminobenzidine (DAB) reaction produce a brown staining wherever primary and secondary antibodies are attached.

Negative controls were developed by omission of primary antibody using diluting buffer (solvent) instead of the corresponding primary antibody.

SECONDARY ANTIBODY	SUPPLIER	ISOTYPE AND CLONALITY	DILUTION	SOLVENT
Biotinylated Anti-mouse IgG	Dako, Ref : E0464	IgG, polyclonal, raised in rabbit	1:800	Goat PST

Biotinylated Anti-rabbit IgG	Dako, Ref.: E0353	IgG, polyclonal, raised in swine	1:800	Goat PST
Biotinylated Anti-rat IgG	Serotec star 80B	IgG, polyclonal, raised in goat	1:800	Goat PST
Biotinylated Anti-mouse IgM	Dako Ref.:E0465	IgM, polyclonal, raised in rabbit	1:200	Goat PST
Alkalyne Phosphatase-Conjugated Anti-rabbit IgG	Sigma-Aldrich Ref.:A3687	IgG, polyclonal, raised in goat	1:100	TBS
Anti-rabbit IgG Alexa Fluor® 488	Invitrogen, Ref.: A11034	IgG, policlonal, raised in goat	1:5000	PBS-TX100
Anti-mouse IgG Alexa Fluor® 555	Invitrogen, Ref.: 21424	IgG, policlonal, raised in goat	1:5000	PBS-TX100
Anti-rat IgG Alexa Fluor® 568	Invitrogen Ref.: A11077	IgG, polyclonal, raised in goat	1:5000	PBS-TX100
OTHER REACTIVES	**SUPPLIER**	**TYPE**	**DILUTION**	**SOLVENT**
Extravidin®	Sigma-Aldrich, Ref : E 2886	Peroxidase conjugated	1:1000	PBS/ Tris-PBS
DAB	Sigma-Aldrich,	–	0,1%	PBS/ Tris-PBS
NBT/BCIP	Roche Ref:11681451001	–	1:500	0.1 M Tris-HCl, pH 9.5 (20°C), 0.1 M NaCl, 0.05 M $MgCl_2$

Table 4. Secondary antibodies and other reactives used in immunohistochemical studies

4.2.3. Immunohistochemistry on vibratome/ freezing microtome free-floating sections.

All procedures were developed using free-floating sections at room temperature with gently shaking, unless otherwise specified.

- Immunohistochemical staining using the peroxidase method was carried out as follows:

After washing in 0.1M PBS, pH 7.4, during 10 min, free-floating sections were processed for inactivation of the endogenous peroxidase by incubation in a solution of 3% H_2O_2 (Merck) and 10% (v/v) methanol (Merck) during 30 min in dark. Sections were washed three more times in 0.1M PBS, pH 7.4 (10 min each) before blocking endogenous biotin with a commercially available kit (Zymed® Laboratories Inc.; California, Estados Unidos). Then, slices were exposed to PST diluted primary antibody (see table 3) overnight. After washing three times with 0.1M PBS, pH 7.4, slices were incubated with PST diluted secondary antibody (see table 4) for 2 h . Subsequent three PBS washes preceded the incubation with Extravidin®-peroxidase solution in 0.1M PBS, pH 7.4, for 1h in dark. Finally and after washing extensively three times with PBS, slices were incubated in peroxidase substrate DAB solution containing 0.05% DAB (3,3'-diaminobenzidine-

tetrahydrocholoride) and 0.03% H_2O_2 in 0.1M PBS, pH 7.4, during 15-25 min in dark. Substrate solution was prepared right before using it. The staining reaction was stopped with distilled water and, after several washes, floating sections were mounted in poly-lysine coated slides and allowed to air-dry. Finally, they were dehydrated with ethanol [70% (v/v), 5 min; 96% (v/v), 2 x 5 min; 100% (v/v), 2 x 10 min], cleared in xylene (2 x 10 min), and mounted with DPX (Sigma-Aldrich) as usual to be analyzed under light microscope

- Double-immunohistochemical staining using fluorescent-labeled antibodies was used to colocalize proteins inside the cells by confocal microscope or to show the spatial relationship between two different immunoreactive cells by fluorescent microscope. The protocol was developed as follows:

Incubation with primary antibodies (sequentially or simultaneously) was the same as above described for the peroxidase method, washing with 0.1M, PBS pH 7.4, between steps. After second primary antibody incubation, slices were three times washed in PBS and incubated with the two secondary antibodies labelled with different fluorochromes and diluted in PBS containing 0,05% TX-100 (see Table 4). Incubation was carried out for 3h at room temperature in obscurity. Slices were washed exhaustively, mounted with Fluoromount™ (Sigma-Aldrich), an aqueous-based mounting medium designed for the preservation of fluorescence-stained, and observed under fluorescence with a BX51 microscope, equipped with a Olympus DP70 camera.

- Double staining was also developed using peroxidase and alkaline phosphatase based methods to label two different cellular constituents in a tissue section since the brown peroxidase reaction product contrasts well with the blue alkaline phosphatase product. For this purpose, initial primary antibody detection was carried out as previously described. After visualizing of DAB reaction, slices were washed in destilled wateruntil being sure no DAB solution remains and they were processed for the second staining, all carried out at room temperature. Accordingly slices were first profusely washed in tris-buffered saline (TBS) containing 0.05 M Tris-HCl and 0.15 M NaCl, pH 7.5,

Before incubating with primary antibody solution (see table 3) overnight. Slices were then exposed to a 2 mM levamisole solution in TBS to block endogenous alkaline phosphatase.

After washing in TBS, sections were incubated with the corresponding alkaline phosphatase-conjugated secondary antibody for 3h. In this case, secondary antibody was diluted in TBS. For detection of the color reaction it was used a NBT/BCIP solution (Roche Diagnostics GmbH; Penzberg, Germany) containing 18.75 mg/ml NBT (Nitro blue tetrazolium chloride) and 9.4 mg/ml BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) in 67% DMSO (v/v). Slices were incubated in the staining solution, prepared by adding 200 µl of the stock solution to 10 ml 0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5. After visualizing the colour, the reaction was stopped with distilled water. Sections were mounted onto slides, allowed to air-dry and dehydrated using subsequent washes in ethanol and xylene, as detailed above. Development of intense blue colour was achieved by lengthening of 96° ethanol step for 3-4 h.

Analysis were performed by a light microscope Olympus BX51 using objective lens UPLSAPO 4x, 10x, 20x, 40x, 100x of 0.16, 0.4, 0.75, 0.90, and 1.40 numeric aperture, respectively, and a photo digital camera Olympus DP70 (Olympus España S.A.; Barcelona) mounted on it to capture the images. When an estimation of the number of cells was needed, Image J version 1.37v (National Institute of Health, USA; http://rsbweb.nih.gov/ij/) was used, taking area samples of 200 µm x 200 µm in cerebral cortex. In case of stereological studies, the procedure will be detailed later.

Immunohistochemistry analysis was developed using six animals per genotype and statistical studies were based on t-student test or ANOVA which were carrying out with Sigma Stat 3.0 and Sigma Plot 8.0.

5. STEREOLOGICAL QUANTIFICATION

The number of oligodendrocytes in the motor cortex of mice was calculated by stereology.

Stereology (from Greek stereos = solid) was originally defined as 'the spatial interpretation of sections' and deals with a body of methods for the exploration of three-dimensional space, when only two-dimensional sections through solid bodies or projections on a surface are available. It is an interdisciplinary field that is largely concerned with the three-dimensional interpretation of planar sections of materials or tissues.

Contributions from statistics, statistical geometry, and topology have all played a role in the development of stereology.

The disector principle is the first theoretically unbiased method used to estimate total number of objects per unit of volumen (numerical density, N_v) on tissue sections. The disector is a 3D-geometric probe for counting numbers of objects (cells) with a probability that is unaffected by the size, shape, or orientation of the objects. In combination with precautions to avoid edge effects, the disector method permits total number of cells to be estimated without assumptions, models, or correction factors. Practical applications include counting objects with two physical planes (physical disector) (Sterio, 1984), two optical planes (optical disector) (Gundersen and Jensen, 1985, 1987, Gundersen et al., 1988; West, 1993), and optical planes in conjunction with the fractionator sampling scheme (optical fractionator) (Gundersen et ál., 1988; West et al., 1991; Begega et al., 1998).

The original formulation of the disector method was designed for scanning tissue using a pair of thin sections, a process that has come to be known as the physical disector. The approach was termed physical because the disector counting is done on actual (physical) sections. The optical disector, first proposed by Gundersen in 1986, is an extremely efficient modification of the dissector principle for counting objects on relatively thick sections. Finally, the optical fractionator is a combination of the optical disector and the fractionator sampling scheme, first proposed by West et al. 1991.

5.1. Optical disector.

The optical disector uses thin focal plane optical scanning to count objects as they come into focus within the known volume of the disector. The section must be thick enough to permit inclusion of a disector of sufficient heigh to count the objects of interest. The actual height of the disector is determined by the size of the objects to be counted. Then, with the addition of a microcator, a device for accurate measurement of stage movement in the z-axis, the user can optically section through a known distance in the z-axis and count the number of objects that comes into focus only the first time once.

This method requires determination of the volume containing the cells of interest, termed the "reference volume" (*Vref*), and the density (N_V) of the cells within that volume. The total number of cells (N) is calculated as the product of the reference volume containing the oligodendroglial population in question and the numerical density of those neurons in that volume (Bonthius et al., 1992; West and Gundersen, 1990).

$$N = Nv \times Vref$$

Estimates of *Vref* were obtained according to the principle of Cavalieri (Sterio, 1984; Gundersen, 1986; Uylings et ál., 1986). Using the Cavalieri method, an estimate of the volume of a structure of arbitrary shape and size may be obtained efficiently and with known precision. The object under study is intercepted by a series of parallel planes a distance t apart, and the corresponding cross-sectional areas are estimated by point counting. To remove the influence of line thickness, a test point was defined consistently as the upper right point of intersection between outer test-lines in the point counting grid. Point counts are converted into section areas by multiplying the total number of counted points, $\sum P$, by the area per test point $a(p)$. The Cavalieri volume of brain compartments is finally estimated by multiplying the distance between sections, t, by their total cross-sectional area:

$$V_{ref} = t \times a(p) \times \sum P$$

The total number of objects counted in a known volume of the reference space (Nv), also known as the numerical density, can be estimated by the sum of the objects counted [$\sum Q^-$] divided by the total volume of the disector probes ($\sum V_{samp}$),

$$Nv = \sum Q^- / \sum V_{samp}$$

where:

$$\sum V_{samp} = n \times Vol_{dis} = n \times [a(frame) \times h]$$

and:

Nv	= estimate of total number per unit of volumen (μm^3)
$\sum Q^-$	= sum of objects counted
$\sum V_{samp}$	= total volume of disector probes (μm^3)
n	= total number of disectors sampled
a (frame)	= area of the disector frame (μm^3)
h	= height of the disector probe (μm^3)
Vol_{dis}	= volumen of a single disector (μm^3) = [a (frame)h]

Additionally, coeficient of error (*CE*) was calculated according to Begega et al. (1998) who proposed an estimation of variations dued to the counting of particles in a section, considering this could be the principal source of error within the total *CE*.

$$CE = \frac{\sqrt{(3A + C - 4B)/12}}{\sum P_i}$$

When CE on the volumen estimation is calculated, $\sum Pi$ is the sum of the numbers of counted points in the chosen sections for the analysis; $A = \sum Pi2$; $B = \sum Pi \times P(i+1)$; $C = \sum P(i) \times P(i+2)$ being, Pi = counted points (oligodendrocytes).

When CE of oligodendrocyte number is calculated, $\sum Pi$ is substituted by $\sum N$, being the sum of the number of oligodendrocyte counted in each sections; and $A = \sum Qi^2$ refers to the square number of oligodendrocyte in each section; $B = \sum Qi \times Q(i+1)$; $C = \sum Q(i) \times Q(i+2)$.

Finally, coefficients of total variation (*CV*), defined as the standard deviation of a group of values divided by their mean (*CV* = standard deviation/mean)

Then these two sources of variation, intraindividual (*CE*) and interindividual (*CV*) were estimated in every animal and values were in the range of 0.05-0.1.

5.2. *Optical fractionator.*

The optical fractionator is a combination of the optical disector and fractionator sampling, a scheme involving the probing of a known fraction of the tissue (West et al., 1991). Three sampling fractions are used with the optical fractionator method. First, the section sampling fraction (*ssf*) represents the proportion of microscopical sections of the entire, serially sectioned brain structure that is sampled for evaluation. The area sampling fraction (*asf*) corresponds to the proportion of the sectional area that is investigated within the sampled sections. And finally, the thickness sampling fraction (*tsf*) captures the part of the investigated cross-sectional area of the sampled sections. The estimated total number of particles (*N*) of a brain structure in an animal is obtained by multiplying the reciprocals of the fractions with the total particle count ($\Sigma Q-$) per brain structure, obtained with the optical disectors (West et al., 1991)

$$N = \Sigma Q- \cdot (1/ssf) \cdot (1/asf) . (1/tsf)$$

The size of the region of interest (*ROI*) is implicitly determined from the combination of these fractions. This means that, with the optical fractionator technique, no information on the size of the *ROI* or the magnification of the microscope is needed. This also implies that this counting technique is independent of e.g. swelling and/or shrinking of the tissue during processing (Howard and M.G. Reed, 1998; West et al., 1991).

5.3. Stereology equipment

The stereology equipment used consist of a microscope BX51 (Olympus España S.A.; Barcelona) attached to a computer and a video camera (CCD-iris, Sony España, Barcelona). A random set of sampling frames with a known area (a_{frame}) was generated for each section using the C.A.S.T. Grid (Olympus; Albertslund, Denmark). The microscope stage was driven by a pair of stepping motors with preset steps of known length in the x- and y-

directions and a microcator was used to measure stage movements in the z-axis with a resolution of 0,5 µm. The microscope objective lens were UPLSAPO 4x, 10x, 20x, 40x, 100x of 0.16, 0.4, 0.75, 0.90, and 1.40 of numeric aperture, respectively.

The motor cortex was delineated according to Paxinos and Franklin (2001) stereotaxic coordinates, from 2.46 mm to -1.34 mm, with 10x objective lens. Using 100x oil immersion objective, the informatic program CAST Grid generated randomly sampling frames where stained oligodendrocytes were present.

5.4. Stereological procedure

Stereological techniques were developed to estimate the number of oligodendrocytes and preoligodendrocyte. These two stages of oligodendrocyte lineage were stained with GalC and NG2 respectively.

Six animals of each genotype were analyzed by sterology. Mice were perfused, postfixed and embedded as it was detailed before. The sectioning was serially done at 50 µm and one out of four series per animal was analyzed, so these sections were sampled every 200 µm. Immunostaining was also performed following the same protocol used to study myelinization patterns and exposed previously.

Stereological data were tested for significant differences between groups by ANOVA or Kruskal-Wallis one-way analysis of variance. Statistical analyses were performed with at least six animals per group, using the software program SPSS statistics 17.0 Values of $p < 0.05$ were considered to be significant.

6. ELECTRON MICROSCOPY

Three-months-old mice were used in this study. Mice were perfused transcardially with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M PBS phosphate buffer, pH 7.4 (Karnovsky, 1965). Brains were dissected out, postfixed overnight in the same solution and agar-embedded as it was mentioned before. Slices of the brain cortex, 1-2 mm thick, were obtained, postfixed in 1% osmium tetroxide during 1 h at 4°C in dark. They were rinsed with PBS, dehydrated with ethanol and acetone and finally oriented and embedded in Araldite epoxi resine up to polymerization 48h at 60°C.

Semithin sections, 1 µm thick, were stained with 1% toluidine blue. Ultrathin ultramicrotomesections were fished in copper grids, selecting the region under study such as corpus callosum and cortex, and counterstained with 0,1% lead citrate and 1% uranyl acetate before observation throught electron microscopy (Phillips CM200).

7. IMMUNOBLOTTING OR WESTERN BLOTTING

Since its introduction in 1979 (Towbin et al., 1979), protein blotting has become a routine tool in research laboratories and it is traditionally used to detect low amounts of proteins in complex samples. Shortly, in this technique, proteins are transferred from an electrophoresis gel to a support membrane and then probed with antibodies. This combines the resolution of electrophoresis with the specificity of immunoassays allowing individual proteins in complex mixtures to be detected and analyzed. In this work, this technique was developed to quantify the expression of different myelin proteins in oligodendrocytes of the cerebral cortex.

7.1. Tissue preparation

Myelin was obtained from the cortex of eight animals of each genotype: wild-type, heterozygous and homozygous null. Animals were sacrificed by cervical dislocation method and skulls were removed to extract the brains and these were frozen on dry ice immediately. Brains were stored at -80°C until dissectioning.

Dissection of the cerebral cortex was performed on ice, and two areas containing upper (I-III) and lower (IV-VI) cortical layers were separated to prepare the extracts. Brains were dissected transversally to isolate the different groups of layers and to detach them from corpus callosum. Samples were kept on ice as far as possible.

Myelin of the cortex samples was purified by sucrose gradient centrifugation (Norton and Poduslo, 1973). Accordingly, cortex was homogenized in 2 ml of 0.35 M sucrose and 5 mM EGTA using a motor-driven pellet pestle (Sigma-Aldrich). The suspension was layered on top of the same volume of 0.85 M sucrose and 5 mM EGTA, and centrifuged at 10000g for 20 min at 4°C. The myelin-containing interphase was collected with a bent Pasteur pipette, diluted with three volumes of distilled water and sedimented at 10000g for 30 min. The pellet was again washed twice with distilled water. The isolated myelin was resuspended in 20 mM Tris-HCl and then stored in aliquots at –20°C.

7.2. Protein Quantification.

Bradford assay was used to measure the protein concentration in the myelin extract. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The binding of the dye to protein involves strong noncovalent interactions including electrostatic, between amino and carboxyl groups, as well as van der Waals forces. Because all proteins possess amino and carboxyl groups and participate in both electrostatic and van der Waals interactions, this method is largely insensitive to protein structure at any level. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range (Bradford, 1976).

The method can be used developing a standard curve for known concentrations of any abundant protein. The linear relationship between the absorbance and known protein concentration can then be used to determine the relative protein concentration of any given sample.

In the current study, the Bradford dye was mixed with known concentrations of a standard protein, in this case bovine serum albumin (BSA), in a linear range of 0.1-1,4 mg/ml. The absorbance of each standard was measured at 595 nm. The straight-line relationship between absorbance and concentration fitted by the least squares method. The concentration of unknown samples will be determined using the slope and intercept from the derived equation.

The assay was performed in 96-well plates, adding 5 µl of myelin samples in each well (diluting them if it was necessary to fit into standard curve) and 250 µl of Bradford reagent (Sigma-Aldrich). Each sample was allowed to incubate at room temperature for 10 minutes and no longer than half-hour before being measured. The absorbance of each standard and sample was measured at 595 nm against a blank that was composed of 5 µl of water and 250 µl of Bradford reagent. The absorbance was measured by a spectrophotometer (VERSAmax, MDS Analytical Technologies; Molecular Devices Corp.; California, United States), obtaining the standard curve with the software programme SOFTmax® Pro versión 3.2.1 (Molecular Devices Corp.).Absorbance data were substituted in the equation obtained from the standard curve for protein quantification.

7.3. Gel electrophoresis

The proteins of every sample were separated using discontinous polyacrylamide gel electrophoresis and buffers load with sodium dodecyl sulfate (SDS), this technique is called SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). SDS-PAGE maintains polypeptides in a denatured state and thus allows separation of proteins by their molecular weight. Samples proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel.

The myelin proteins studied by western-blotting analysis were MBP, PLP/DM20 and CNPase. Proteins were separated according to their apparent molecular weight (MBP: 21,5 KDa; 18,5 KDa; 17,2 KDa; 17,0 KDa; and 14 KDa ; PLP: 29 Kda; DM20:20 KDa; CNP1: 48 KDa, and CNP2: 46 KDa) in a 12% resolving polyacrylamide gel under denaturing conditions with SDS.

The equipament used to perform the electrophoretic procedure was Mini-PROTEAN® 3 system and the reagents and accesories provided by the same supplier (Bio-Rad Laboratories S.A., Barcelona).

Stacking gel (4% acrylamide/ bisacrylamide 37:1) and acrylamide gel (15%; acrylamide:bisacrylamide 37:1) were prepared following the manufacturers protocol of the commercial reagents for stacking and resolving gel. Accordingly, 25 ml of stacking gel (4%) were obtained mixing 2.5 ml 40% Acrylamide/Bis solution, 6.3 ml 0.5 M Tris-HCl pH 6.8, 250 µl 10% SDS and 15.9 ml destilled water, adding 25 µl tetramethylethylenediamine,

125 µl 10% ammonium persulphate solution and 100 ml of resolving gel (15%) were made by 37.5 ml 40% Acrylamide/Bis solution, 25 ml 1.5 M Tris-HCl pH 8.8, 1 ml 10% SDS and 45 ml destilled water, adding 50 µl tetramethylethylenediamine, 500 µl 10% ammonium persulphate solution.

After resolving gel polymerized, stacking gel was poured on top of it and a gel comb (forming the wells and defining the lanes where proteins, sample buffer and ladders placed) was inserted.

After leaving to polymerize, 5µl of myelin protein was loaded per well with sample buffer (3.55 ml bi-destilled water, 1.25 ml 0.5 M Tris-HCl, pH 6.8, 2.5 ml glycerol, 2.0 ml 10% (w/v) SDS, 0.2 ml 0.5%(w/v) bromophenol blue, to prepare a 9.5 ml volume; all reagents from Sigma-Aldrich) and ran the proteins through the stacking gel at 80-100V, then turned up the power increasing the voltage until ~200 V (approximately 60 mA) for one gel, the electrophoresis was displayed during 35 min. A prestained weight marker was also loaded onto one of the wells. Inner compartment was full of buffer (Tris 25mM pH 8.3, glycine 192 mM and 0.1% SDS; Bio-Rad Laboratories S.A), and some at the bottom as well..

Efficiency of electrophoresis was reported in some gels by staining with Coomassie® blue dye and incubating in Coomassie solution [50 % (v/v) methanol, 10 % (v/v) acetic acid, 0.125% Coomassie R-250 in methanol; all reagents from Sigma-Aldrich] for 2-4 h at room temperature. Visualization of the protein bands was achieved, in case, after several washes with destaining solution [10% (v/v) methanol in water with 10% (v/v) acetic acid] from 2-3 h up until overnight.

7.4. Protein Transfer

After electrophoresis, transfer of proteins from gel to membrane was carried out by directing an electric field across the thickness of the gel and on to the membrane. The method used for that aim in this work was wet (or tank) blotting, in which the gel-membrane sandwich was submerged in transfer buffer, using a membrane of polyvinylidene difluoride (PVDF) and the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories S.A).

PDVF membrane was previously activated for 10 s by placing immersed in 100% methanol. Afterwards, membrane and gel were equilibrated in transfer buffer for 10 min (25 mM Tris, 192 mM glycine, 20 % methanol and 0.25% SDS, pH 8.3; Bio-Rad Laboratories S.A.).

Then, a sandwich with moisten fiber pads, some pieces of wetted filter paper (all soaked in transfer buffer) and the membrane put on gel without bubbles was prepared to place it into the transfer chamber, with membrane closest to the positive electrode (anode). The electrotransfer was performed overnight at 50 V, at 4 °C. Alternatively, it can be done at 200mA 1.5h at room temperature with icepacks in apparatus. Additionally, transferred gel was stained, as described, with Coomassie® Blue to verify completeness of transfer.

7.5. Immunobloting

The reason for transferring proteins to membranes from gels is so as to be able to get at them more efficiently with various probes, as polyacrylamide is not particularly amenable to the diffusion of large molecules. The attachment of specific antibodies to specific immobilised antigens can be readily visualised by indirect enzyme immunoassay techniques.

The immunoassay for myelin protein was performed by blocking the transfer membrane with 2.5% BSA in 0.1M PBS pH 7.4 during 1h (every step was at room temperature with gentle shaking unless otherwise specified). After blocking, membranes were incubated with the corresponding antibodies overnight, the same one used for immunostaining and specific for MBP, PLP/DM20 and CNPase (see table 3). Antibodies were diluted in PBS with 0.2% (v/v) Tween® 20 (Sigma-Aldrich) (PBST), either 1:7000 for MBP and PLP/DM20 detection, or 1:2300 for CNPase revealing. Membranes were washing three times with PBST, 5 min each, incubated in appropriate biotynilated secondary antibody also diluted in PBST 1:2000, washed again, and subsequent exposed to a 1:2000 solution of Extravidin® (Sigma-Aldrich) in PBST during 1h in dark.

For band detection by the colorimetric method, membranes were incubated in 4-chloro-1-naphthol (Opti4CN™, Ref.: 170-8235; Bio-Rad Laboratories S.A) following the manufacturer protocol. After a while, bands were visualized according to the corresponding molecular weight and reaction was stopped with distilled water.

Membranes were allowed to air-dry and the expression of each protein was quantified by densitometry with OptiQuant v.4.00 software programme (Packard Instruments-Co. Illinois, United States). Statistical analyses to compare the average of expression between groups were performed using the t-student test and the software program SPSS statistics 17.0. Sample sizes were no lower than ten animals per group, Values of $p < 0.05$ were considered to be significant.

8. GENE EXPRESSION BY REAL TIME POLYMERASE CHAIN REACTION (RT-PCR)

Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA, p.e. SYBR® Green. The fluorescence of the reporter dye increases as the product accumulates with each successive cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase. If a graph is drawn between the log of the starting amount of template and the corresponding increase the fluorescence of the reporter dye fluorescence during real time PCR, a linear relationship is observed.

The genes of interest whose expressions were measured by RT-PCR were: *Lpar1, Lpar2, Lpar3, Lpar4, Lpar5*, being their expressions normalized to that for Glyceraldehyde-3-phospate dehydrogenase (*GAPDH*) and *β-actin*, acting as housekeeping genes. No differences were found when both actin and GADPH were used, thus for the present study, GADPH expression has been considered as referred

Every solution and material, which was used, was always RNAase-free following standard protocols of sample manipulation and cleaning of work surface.

8.1. Primer design

Primer3 software version 0.3.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design the individual primers sets used. There were some essential parameters required for optimal design of primers, such as product size range between 150 and 250 base pair (bp), melting temperature range between 57 and 63°C, G-C percentage ranges between 40 and 60% and the primer size between 18 and 23 nucleotides.

Once the primer pairs were designed and chosen, an alignment of sequences were performed through the mouse database by Nucletotide BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) to discriminate against those pairs which could amplify cDNA from other genes during the PCR.

Primers were supplied by Proligo (Proligo; Sigma-Aldrich).

8.2. Sample preparation

Quantification of LPA receptor mRNA expression by RT-PCR was studied from two different samples, brains from wild type and null mice, and peripheral blood mononuclear cells (PBMC) of EAE mice.

Brain samples were dissected from different interesting zones, such as callosum corpus, striatum, cerebral cortex or hippocampus, where the expression of the five LPA receptors were determined. Animals needed to this study were sacrificed by cervical dislocation method; brains were removed from the skulls and frozen immediately in dry ice. Then, they were stored at -80°C until their use.

Different regions were rapidly dissected out on ice, 1 ml of Trizol® (Roche Diagnostics) was added and carefully homogenized. Sampling for RT-PCR expression was as result of means of all animals analyzed. These were centrifuged 12000g at 4°C for 10 min and pellet discarded

The expression of *Lpar1* and *Lpar2* were also measured in PBMC of mice which showed clinical signs of EAE. These samples were obtained in different days after immunization, and were chosen as function of clinical symptoms present.

The blood from mice was collected in heparinized tubes of 3ml by transcardiac perfusion with saline serum. PBMC isolation was performed by Ficoll density gradient using a solution containing polysucrose (Ficoll

400) and sodium diatrizoate, Histopaque®-1077 (Sigma-Aldrich) . The standard protocol is as follows: anticoagulated blood was diluted 1:1 with saline and completely layered on 3ml Ficoll. Samples were centrifuged for 25 min at 400 g and 20°C without applying brake. The PBMC interface was carefully removed by pipetting and was washed twice with saline serum by centrifugation, first time for 10 min at 400g and the second one for 10 min at 200g

Once PBMC are isolated, they were resuspended in 1 ml of Trizol® to extract RNA.

8.3. RNA extraction

Trizol® method was used for RNA isolation. This is the procedure originally described by Chomczynski and Sacchi (1987).

Firsly, 200 µl of chloroform was added to the Trizol® samples, shaking with vortex for 25 s and centrifuged at 13000 rpm for 15 min at 4°C. Aqueous phase, where RNA is present, was removed carefully into a clean eppendorf tube and added 500 µl of isopropanol. It is important to be careful not to contaminate the sample with the interphase where DNA and proteins are. Then, mixing by gentle inversion and letting sit at -20°C to precipitate RNA for 20 min and centrifuging at 13000 rpm, 10 min at 4°C. Supernatant was discarded and pellet washed with 500 µl cold 75° ethanol, centrifuging again 5 min at 10000 rpm, 4°C. Ethanol was discarded and pellets were air dried. Finally, RNA pellets were resuspended in 15 µl DEPC water.

8.4. RNA Quantification

RNA quantification was assayed by spectrophotometry as DNA quantification was done. RNA concentration was determined by measuring absorbance of the sample at 260nm (A260) in spectrophotometer (Biotech Photometer, UV 1101, WPA), and the ratio of absorptions at 260/280nm was used to assess the purity of RNA with respect to protein contamination. Samples were always diluted to obtain an absorbance between 0.1-1 which is considered reliable. The ratio A260/280 of our samples was always near 1.8 denoting certainly a good quality.

8.5. Reverse Transcription

Reverse transcriptase was discovered by Howard Temin at the University of Wisconsin-Madison, and independently by David Baltimore in 1970 at MIT. In reverse transcription, a DNA polymerase enzyme transcribes single-stranded RNA into double-stranded DNA (complementary DNA or cDNA)

The synthesis of cDNA was carried out with a thermocycler (Mastercycler gradient, Eppendorf) from 1µg of total RNA. The enzyme reverse transcriptase MMLV (Sigma-Aldrich) used random primers to synthesize cDNA from each RNA of the samples.

The procedure was as follows: mix 1µl of comercial random primers (Roche Diagnostics), 1 µg of total RNA from the samples and DEPC (Diethyl pyrocarbonate; Sigma-Aldrich) -treated water up to a volume of 10 µl. This mix was heated at 70°C during 10 min to let primers align and denaturalize possible secondary structures. Later, adding to each sample: 2 µl of 10X buffer (Sigma-Aldrich), 1 µl 10mM dNTP (Roche Diagnostics) 0,5 µl of RNase inhibitor (Roche), 5,5 µl of DEPC-water and 1 µl of MMLV reverse transcriptase (Sigma-Aldrich), this was incubated for 10 min at 25°C, 1h at 44 °C and finally, 10 min at 92 °C. Once the reaction finished, cDNA was stored at -20°C.

8.6. Real Time PCR

RT-PCR can be used to measure changes in expression levels using two different methods: absolute and relative.

Absolute quantitation, using competitive RT-PCR, measures the absolute amount of a specific mRNA sequence in a sample. Dilutions of a synthetic RNA (identical in sequence, but slightly shorter than the endogenous target) are added to sample RNA replicates and are co-amplified with the endogenous target. The

PCR product from the endogenous transcript is then compared to the concentration curve created by the synthetic "competitor RNA".

Relative quantitation compares transcript abundance across multiple samples, using a co-amplified internal control for sample normalization. Results are expressed as ratios of the gene-specific signal to the internal control signal (housekeeping). This yields a corrected relative value for the gene-specific product in each sample. These values may be compared between samples for an estimate of the relative expression of target RNA in the samples.

In this work, relative quantitation has been used to measure the expression of LPA receptors, and they have been normalized to two constitutive or housekeeping genes: GAPDH and β-actine.

The Real-Time PCR System used was LightCycler® (Roche Diagnostics), an carousel/based thermal cycler platform with fluorescence detection system; and PCR products were detected fluorescently, using the intercalating fluorescent dye SYBR® Green I (Roche LightCycler FastStart DNA Master SYBR® Green I). When the SYBR® Green I dye intercalates into dsDNA, its fluorescence increases greatly. During the different stages of PCR, the intensity of the fluorescent signal will vary, depending on the amount of dsDNA present. In the elongation phase, more dsDNA is formed and more SYBR® Green I dye can intercalate. The fluorescence is measured at the end of each elongation phase to monitor the PCR, SYBR® Green absorbs at 497 nm and emits at 530nm.

For SYBR® Green based amplicon detection, it is important to run a dissociation curve following the real time PCR. This is due to the fact that SYBR® Green will detect any double stranded DNA including primer dimers, contaminating DNA, and PCR product from misannealed primer. Every piece of dsDNA has a melting point (*Tm*) at which temperature 50% of the DNA is single stranded. The temperature depends on the length of the DNA, sequence order, G:C content and Watson-Crick pairing. When DNA-binding dyes are used, as the fragment is heated (0,5°C/cycle; from 56°C to 98°C), a sudden decrease in fluorescence is detected when *Tm* is reached due to dissociation of DNA strands and release of the dye (measure by the thermocycler every 0,5°C). This point is determined from the inflection point of the melting curve or the melting peak of the derivative plot.

By viewing a dissociation or melting curve, you ensure that the desired amplicon was detected. Then, contaminating DNA or primer dimers would show up as an additional peak separate from the desired amplicon peak (Fig 13). When dimers anneal the peak appears with a lower temperature.

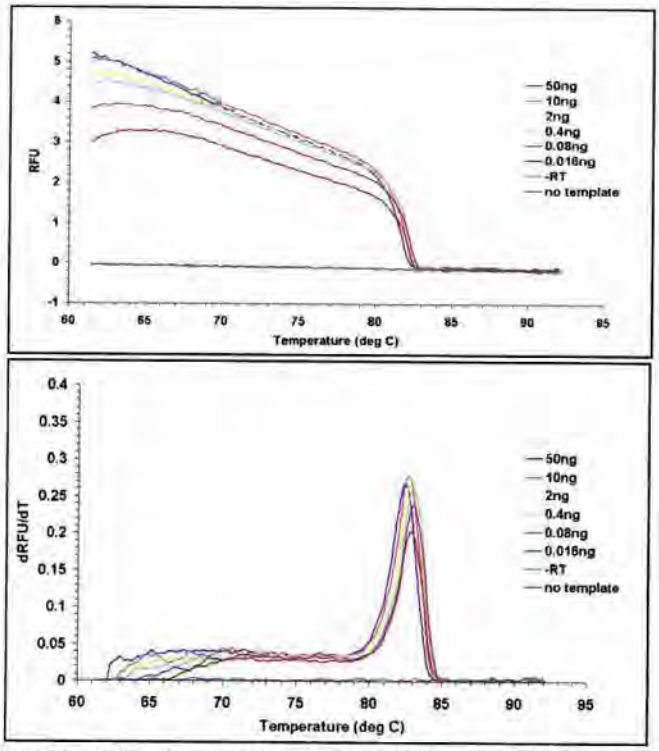


Figure 13. Melting Curve and derivative Melting Curve for Standard Curve Samples in Real Time for GAPDH Endogeneous Control

The determination of the amounts of DNA was by comparing the results to a standard curve produced by real-time PCR of serial dilution of a known amount of DNA. As mentioned above, to accurately quantify gene expression, the measured amount of RNA from the gene of interest is divided by the amount of RNA from a housekeeping gene measured in the same sample to normalize for possible variation in the amount and quality of RNA between different samples. This normalization permits accurate comparison of expression of the gene of interest between different samples, provided that the expression of the reference gene used in the normalization is very similar across all the samples.

The slope of the standard curve also provides useful information about the reaction because it reflects the amplification efficiency (*Eff*) (Fig. 14). Eff can be calculated by the formula:

$$Eff = 10(-1/slope) - 1$$

The efficiency of the PCR should be 90 - 100% (– 3.6 > slope > – 3.1).

Figure 14. Slope of the standard curve which reflect amplification efficiency (Eff).

Amplification was performed with about 1 ng cDNA and specific primer pairs for Lpar1, Lpar2, Lpar3, Lpar4, Lpar5 (in figures named as LPA1, LPA2, LPA3, LPA4, LPA5), *GAPDH* and *β-actin*.

For the PCR reaction of each sample was mixed 5,4 µl of distilled water RNAase-free, 1,3 µL of MgCl2, 0,2 µl of each forward and reverse primers, 1 µl of Fast SYBR® Green Master Mix and 2 µl of cDNA.

The amplification programme was similar for every gene, only the annealing temperature and time was specific for each prime pair:

- ✓ Denaturalization: 15 min at 95°C
- ✓ Amplification 40 cycles: 30 s at 95°C, annealing time and temperature, and 18 s at 72°C
- ✓ Melting: from 65°C to 95°C (but LPA2, 98°C) increasing the temperature 0,1°C/s
- ✓ Cooling: 30 s at 40°C

Primer pair used were:

Lpar1 forward: gaggaatcgggacaccatgat; and reverse: acatccagcaataacaagaccaatc,
Annealing time and temperature: 5s at 68°C

Lpar2 forward: gaccacactcagcctagtcaagac; and reverse: cttacagtccaggccatcca,
Annealing time and temperature: 5s at 69°C

Lpar3 forward: agggctcccatgaagctaat; and reverse: ttcatgacggagttgagcag,
Annealing time and temperature: 5s at 69°C

Lpar4 forward: aacctggccctctctgattt; and reverse: cgatcggaagggatagacaa,
Annealing time and temperature: 10s at 62°C

GAPDH forward: gccaaggtcatccatgacaact, and reverse: gaggggccatccacagtctt
Annealing time and temperature: 10 s at 68°C

βactin forward: agccttccttcttgggtatg; and reverse: cttctgcatcctgtcagcaa,
Annealing time and temperature: 10s at 68°C

After PCR, the amplification products were separeted by electrophoresis in 2% agarose gel to check the size of the samples and the absence of other contaminating fragments.

Statistical analyses to compare the average of expression between wildype and null mice were performed using the t-student test and the software program SPSS statistics 17.0. Sample sizes were no lower than ten animals per group, Values of $p < 0.05$ were considered to be significant.

9. ENCEPHALOMYELITIS AUTOIMMUNE EXPERIMENTAL

Wildtype and maLpal-null 6- to 8-week-old female mice were immunized with 300 µg of MOG peptide, sequence from 35 to 55 aas, MOG_{35-55} (synthesized by GenScript Corp., USA) emulsified in complete Freund's adjuvant (CFA) through two inoculations.

This CFA was prepared mixing 20 ml IFA (Incomplete Freund Adjuvant, DIFCO 263910, BD Diagnostics, Sparks, MD, USA) with 100 mg M.tuberculosis H37 Ra (killed and desiccated, DIFCO 231141, BD Diagnostics). Then, MOG_{35-55} stock was diluted with PBS and mixed in equal volumes with CFA to make an emulsion.

The final concentration of MOG_{35-55} was 200 µg per 100 µL emulsion for each mouse in the first inoculation and the half amount in the second inoculation, seven days later. It was important to take care of not introducing air bubbles in the sryinge and keep it at 4°C until the injection. The stability of the emulsion was tested by adding one drop of emulsion into a beaker of water: if the emulsion wass stable, the drop remained as solid clump, which slowly dissipated. The volume of the mixture was injected subcutaneously in the flanks in two injections for each mouse.

Pertussis toxin (300 ng/mouse; Invitrogen Corp., Carlsbad, California, USA) was injected by peritoneal injections immediately and 48 hours after MOG_{35-55} inoculations. Pertussis toxin (PTX) is a major virulence factor of Bordetella pertussis which exerts a range of effects on the immune system, including the enhancement of IgE, IgA and IgG production, delayed-type hypersensitivity reactions, and the induction of experimental autoimmune diseases. Ryan et al. (1998) showed that PTX could potentiate antigen-specific T cell proliferation and the secretion of IFN-gamma, IL-2, IL-4 and IL-5 when injected with foreign antigens. They found that PTX stimulated the production of IFN- gamma and IL-2 by naive T cells and IL-1 by macrophages. Therefore potentiation of distinct T cell subpopulations may have resulted in part from the positive influence of IFN-gamma on the development of Th1 cells and the co-stimulatory role of IL-1 for Th2 cells.

The mice were observed over a period of 60 days for clinical signs which started between 15 and 20 days postimmunization (dpi), and scores were assigned based on the following scale according Fuller et al., (2004) and Brown et al., (2007):

Clinical score standards

0.0 Normal mouse: no overt signs of disease.

0.5 Partial paralysis of the tail: flaccidity and absence of curling in the distal half of the tail when mouse is picked up.

1.0 Complete paralysis of the tail: complete flaccidity of the whole tail and absence of curling at the tip of the tail when mouse is picked up.

1.5 Weakness in one hind limb: mouse cannot hang itself by one hind foot (not by the joint of the hind limb), however, can carry out this action in the other hind foot.

2.0 Weakness in both hind limbs: mouse cannot hang itself by neither hind foot; and a waddling gait, the objective sign being that, in walking, mouse cannot raise up its rump completely by the hind limbs.

2.5 Partial hind limb paralysis: complete paralysis in one hind limb or partial paralysis in both hind limbs. The objective sign of partial paralysis is that mouse can move the limb to some extent fruitlessly; and the objective sign of complete paralysis is that mouse cannot move the limb at all, and drags the hind limb during walking. Mice at this stage are given water every day and food every other day on the cage floor.

3.0 Complete hind limb paralysis: complete paralysis in both hind limbs however intact forelimb functions monitored by the ability of hanging itself by either forelimb. Mice at this stage or even worse stages (see below) are given water and food every day on the cage floor.
3.5 Partial forelimb paralysis: complete paralysis in one forelimb or partial paralysis in both forelimbs.
4.0 Complete forelimb paralysis: complete paralysis in both forelimbs. Moribund state.
5.0 Death by EAE; sacrifice for humane reasons.

Note: Some mice may not fulfill the standards above in a uniform order, i.e., the objective sign of the higher score may be obtained by the mice without all symptoms of the lower score. In this case, one can make some compromise between these two standards, for example, if the mouse shows partial (Score 2.5) but not complete (Score 3.0) hind limb paralysis, however with weakness or partial paralysis in its forelimbs (Score 3.5), one can come to a compromising Score 3.0.

Mice were sacrificed in different days depending on the state of the disease it was wanted to study.

9.1. LPA_1 antagonist administration

VPC 32183 is devoid of agonist activity at the human LPA_1, LPA_2 and LPA_3 receptors and, presumably, other mammalian LPA receptors; the molecule behaves as a competitive antagonist at the LPA_1 and LPA_3 receptors (Heasley et al., 2004).

VPC 32183 (S), (S)-Phosphoric acid mono-{2-octadec-9-enoylamino-3-[4-(pyridine-2-ylmethoxy)-phenyl]-propyl} ester (Ammonium Salt) (857340; Avanti Polar Lipids, Alabaster, Alabama, USA) was dissolved in 3% free-fatty acid BSA (FFA-BSA; Sigma-Aldrich) in saline. VPC32183 was diluted to a concentration of 5 µM and 100 µl were injected intravenous in the tail vein. In nontreated control mice only vehicle injections were performed (3% FFA BSA in saline).

10. CUPRIZONE MODEL FOR DEMYELINATION

The cuprizone model followed the standard protocol applied in the recent years. 8-weeks-old CNP-EGFP mice were fed with 0.3% cuprizone (w/w) (bis-cyclohexanone oxaldihydrazone; Sigma-Aldrich, St. Louis, MO) for 6 weeks. Animal chow was ground to powder, and cuprizone was added as previously described (Hiremath et al., 1998). Animals were provided the cuprizone-containing chow ad libidum in a bowl inside their cages (food levels were checked daily). The administration of cuprizone caused cell death of the oligodendrocytes, which lead to subsequent demyelination.

Animals showed no severe side effects of this treatment, although an altered gait, ruffled fur, and lethargic movements were common observations. Cuprizone fed mice exhibited weight loss, which was reversed when the cuprizone diet was discontinued. From visual observations, the cuprizone-treated animals appear to have a lower activity level compared with the control ones. Initially, they go through a period of hyperactivity and decreased anxiety behaviour (after 3 weeks of cuprizone treatment) and then developed motor dysfunction (after 5 weeks). The motor dysfunctions seem to persist even 6 weeks after withdrawal of the neurotoxin.

Animals were daily observed to evaluate their wellness, being sacrificed in case it would be necessary.

10.1. LPA administration.

Non hydrolyzable LPA, C18:1, (1-O-9-(Z)-Octadecenyl-2-Hydroxy-*sn*-Glycero-3-Phosphate, 857230P, Avanti Polar Lipids) was dissolved in 3% free-fatty acid BSA (FFA-BSA; Sigma-Aldrich) in saline. LPA was diluted to a concentration of 5 µM and 20 µl were injected intracisternally, i.e. in the cisterna magna. In nontreated control mice only vehicle injections were performed (3% FFA BSA in saline).

Intracisternal (cisterna magna) injections were performed acutely in mice under anesthesia. Mice were anesthetized with ketamine-xylazine (Sigma-Aldrich) intraperitoneally (Ketamine:Xylazine; 80-120 mg/kg:10-16 mg/kg; 20-40 min of anaesthesia).

The effect of LPA in myelination was evaluated in two different times of injection. In one group, LPA and BSA injection were performed after 6 weeks of cuprizone treatment and at the time of the neurotoxin withdrawal. Animals were sacrificed five days later.

In a second group, cuprizone treatment was stopped four days before LPA injections. In this case, the withdrawal of neurotoxin allowed spontaneous remyelination which could be increased by the effect of LPA. LPA was injected at the day in which spontaneous remyelination is described to start, day 4 after withdrawal. Euthanasia of the mice was carried out two days after injection.

RESULTS

1. INFLUENCE OF LPA_1 RECEPTOR IN MYELINATION

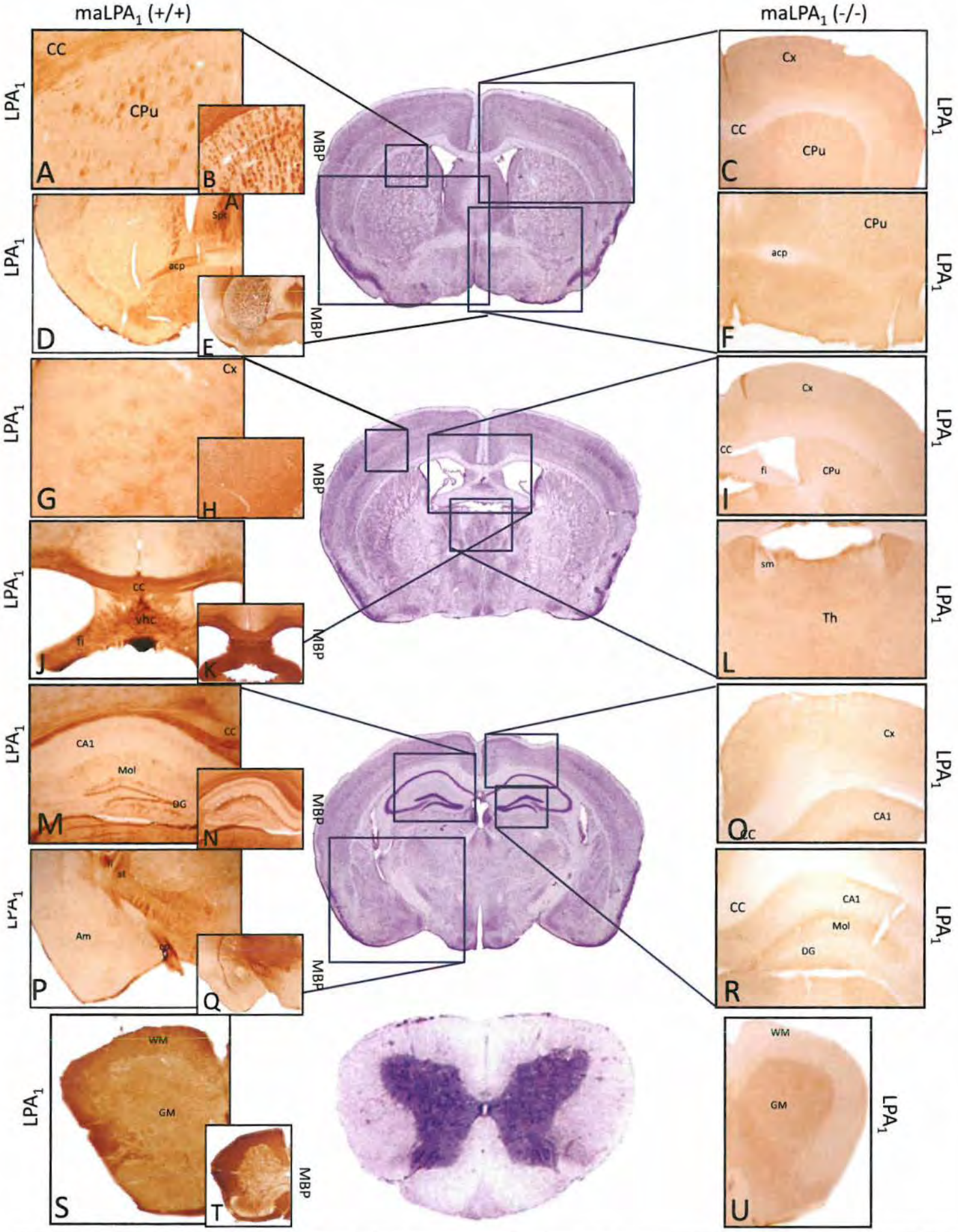
1.1. Expression of LPA_1 receptor in the Central Nervous System; presence in myelin and oligodendrocytes.

LPA_1 receptor has been demonstrated to be involved in central nervous system (CNS) physiology in many studies (Chun et al., 2002; Anliker and Chun 2004; Moolenaar et al., 2004; Chun 2005, 2007; Estivill-Torrús et al., 2008; Matas-Rico et al., 2008; Santín et al., 2009; Choi et al., 2010). These works including myelinating cells function as well (Weiner et al., 1998; Stankoff et al., 2002; Nogaroli et al., 2009). However, despite they are essential to understand LPA_1receptor -mediated mechanisms, in vivo requirement of LPA_1 has not been yet demonstrated. With the purpose of understanding properly potential alterations caused by the absence of LPA_1, the expression of this receptor was studied by immunohistochemistry in in adult and postnatal day 30 (P30) mice, in coincidence this last, with the peak of LPA_1 expression and myelination process (Weiner et al., 1998). Our results showed, first, both, adult and P30 brains, depicting a similar pattern of immunoreactivity. As expected, LPA_1 expression in normal maLPA$_1$ (+/+) mice (Fig.15A, D, G, J, M, P, S) contrasted to the lack of signal in maLPA$_1$-null (-/-) (Fig.15C, F, I, L, O, R. U), corroborating the absence of LPA_1 receptor in this genotype.

The immunoreactivity of LPA_1 in brain and in spinal cord located in similar distribution to that observed for myelin markers, e.g. MBP (Fig. 15B, E, H, N, Q and T). According to this, the staining was more evident in myelinated structures, such as corpus callosum (Fig. 15A, J, M), external capsule, anterior (Fig. 15D) and posterior commissures, fimbria (Fig. 15J) and spinal cord white matter (Fig. 15S) among others. These results agreed with those already described by Handford et al. (2001), in which they noted the distribution of LPA_1 receptor within adult rat brain showed prominent expression in white matter tract regions and closely correlated with areas positive for Luxol® fast blue stain, i.e. myelin.

Aiming to prove that LPA_1 is present in myelin, a double fluorescent immunostaining assay was performed to confirm the coexpression of LPA_1 and one of the major and representative myelin CNS proteins, such as PLP, through the brain and the spinal cord (Fig.16). These experiments showed that the expressions of LPA_1 and PLP coincide in most of the myelinated structures, like corpus callosum (Fig. 16C), caudate-putamen (Fig. 16F) or white matter of spinal cord (Fig. 16I) among many others.

In parallel, although antibody binding was more evident in white matter tracts, immunoreactivity showed some cells also expressing LPA_1 in adult brain. In addition of those previously described by our research group as neuronal precursor cells in hippocampus (Fig. 15M) (Matas-Rico et al., 2008) some scattered cells were also found in other regions of the brain, like cerebral cortex (Fig. 15D), striatum, thalamus, brainstem, and grey matter of spinal cord, as well as myelinated fibers (Fig. 15A, G, J, M, P and S). Double labelling for detection of LPA_1 receptor and transcription factor Olig2, as oligodendroglial lineage marker (Zhou et al., 2000, 2001) allowed to detect many of thesecells in cerebral cortex (Fig. 17A), corpus callosum (Fig. 17B), caudate-putamen (Fig. 17C) or thalamus (Fig. 17D) demonstrating them as oligodendrocytes.



5. Expression pattern of LPA_1 in wildtype and maLPA1-null mice. Immunohistochemical detection of LPA_1 receptor confirmed its presence in wildtype mice (A, D, G, J, and O) and its absence in maLPA$_1$-null mice (C, F, I, L O, R and U) through CNS. Areas of intense immunoreactivity coincided with myelinated structures stained by MBP (B, E, H, K, and U) such as anterior comisure (acp), corpus callosum (cc), fimbria of the Hippocampus (fi), posterior comisure (cp), striatum (st), ventral hippocampal commisure (vhc) and matter of spinal cord (WM); and positive cells were also found in the dentate gyrus of the Hippocampus (DG), cerebral cortex (Cx) thalamus (Th) and grey matter of spinal cord Amygdala (Am), caudate-putamen (CPu), Septum (Stp), stria medialis (sm), lateral habenula (LHb), molecular layer of the dentate gyrus (Mol). Abbreviations as cited hereafter.

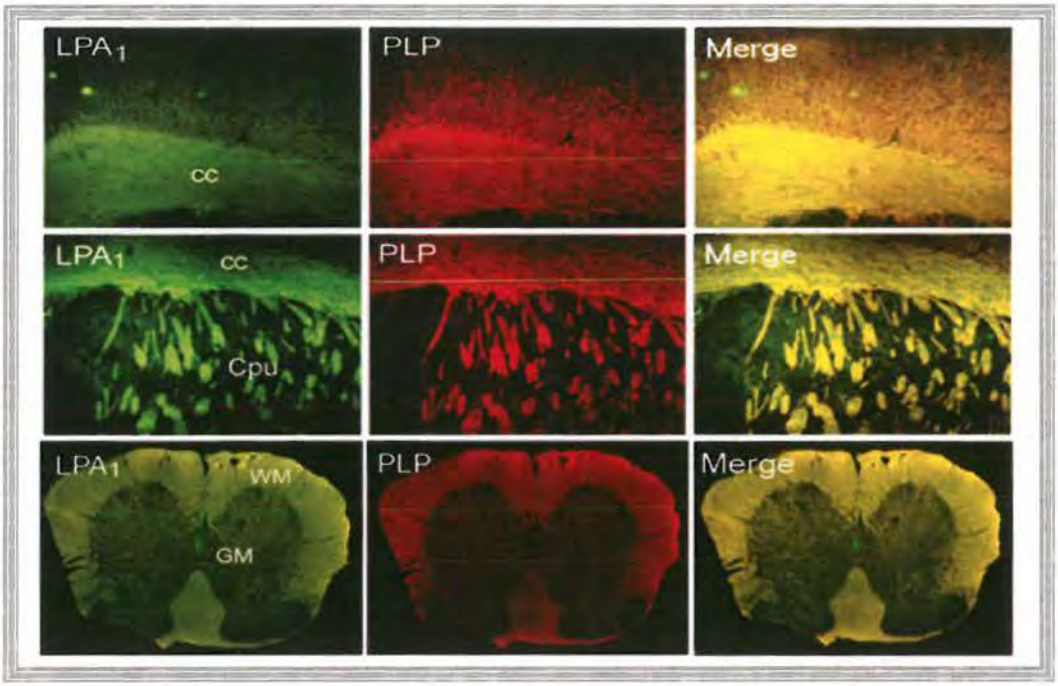


Fig. 16. Colocalization of LPA_1 and PLP in myelinated structures. Descriptive images of double immunofluorescence labelling for LPA_1 receptor (A,D,G) and the myelin protein PLP (B,E,H) in wild-type maLPA_1. Colocalization in myelin is indicated when structures appear yellow in the Merge (C,F,I).

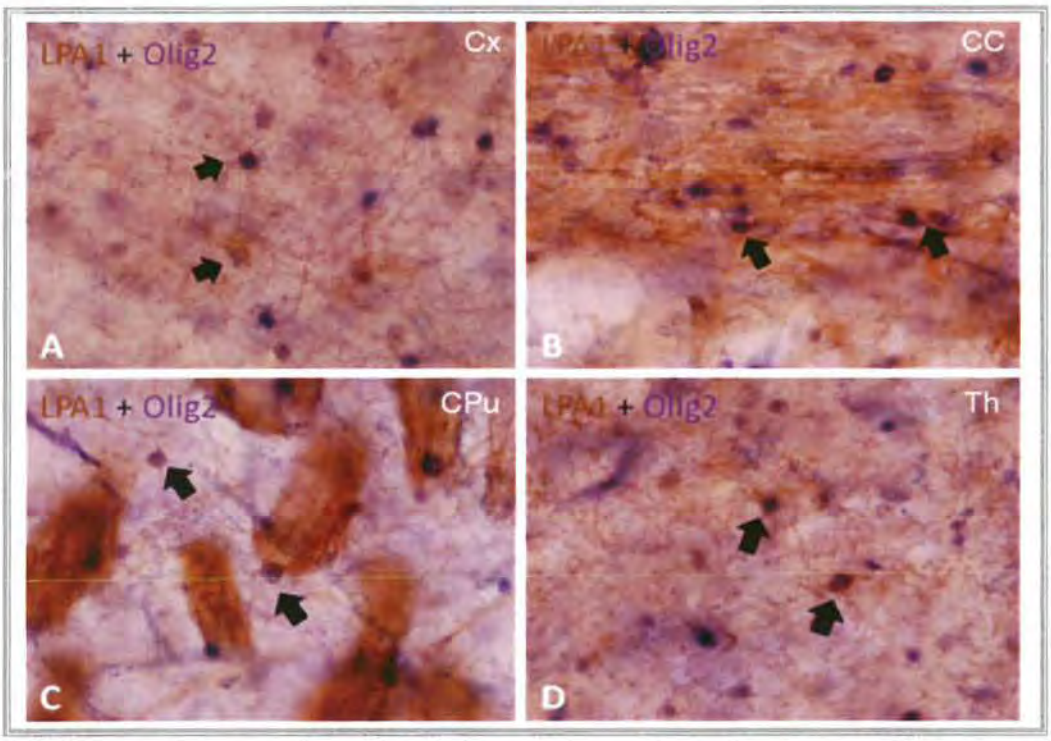


Fig. 17. Coexpression of LPA_1 and Olig2 in some cells through different regions of the brain. Double-immunostaining experiments employing peroxidase method to reveal LPA_1-positive cells (detected with DAB substrate, brown precipitate), and alkaline phosphatase method to reveal Olig2-immunorreactive cells (detected with BCIP substrate, blue precipitate) demonstrated that LPA_1 is expressed by oligodendrocytes. Cells coexpressing both LPA_1 and Olig2 could be found in the cerebral cortex (Cx)(A), corpus callosum (cc)(B), caudate putamen (CPu)(C) and thalamus (Th)(D)

1.2. Effects of LPA_1 absence on other LPA receptors gene expression: no evidence of mechanisms of genetic compensation.

Disruption of gene function using knockout method can be covered by proportional response of other gene expressions, where levels of expression are altered actively and in a proportional fashion with respect to the gene being knocked out. Several examples can be found where a gene is up-regulated following knockout of its paralog, suggesting an active compensatory mechanism to confer robustness and maintain function.

This gene expression compensation by upregulation of similar genes in knockout animals is a frecuent mechanism and it should be taken into account. Then, since homologous genes were more likely than nonhomologous genes to overlap in function, the expression of the other four LPA receptors was studied in both wildtype and maLPA$_1$-null mice. This study was aimed to clarify if the effects showed later were owed to the lack of LPA$_1$ or they could be influenced by compensatory transcriptional effects of the other LPA receptors. In all cases, the expressions were normalized to a housekeeping gen expression, using in this case GAPDH.

First of all, *Lpar1* gene expression was measured by RT-PCR (Fig. 18) to confirm the results showed by immunochemistry (Fig 15). According to the previously reported staining, no expression was obtained in null mice verifying the targeted disruption of *Lpar1* and the subsequent absence of LPA$_1$. In normal mice, the highest expression was provided by corpus callosum, where myelin is more abundant. Expressions in striatum and hippocampus showed significant levels too, due to the myelinated fiber bundles along the striatum and hippocampal neural precursors respectively. Lower LPA$_1$ expression was detected in the cerebral cortex, noting the presence of cells and fibers which expressed this receptor in the mentioned area, as it was visualized by immunostaining (Fig. 15G).

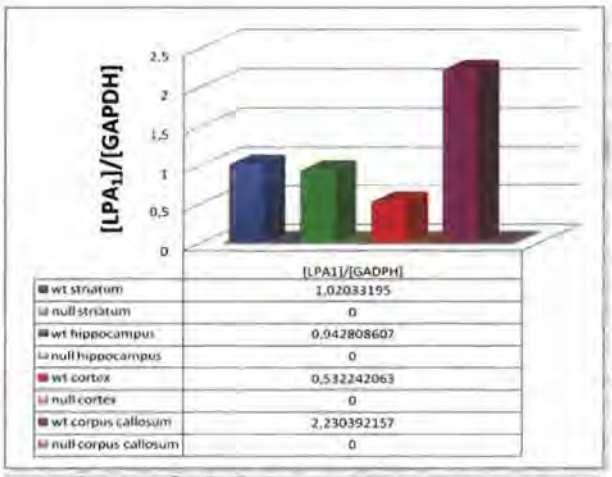
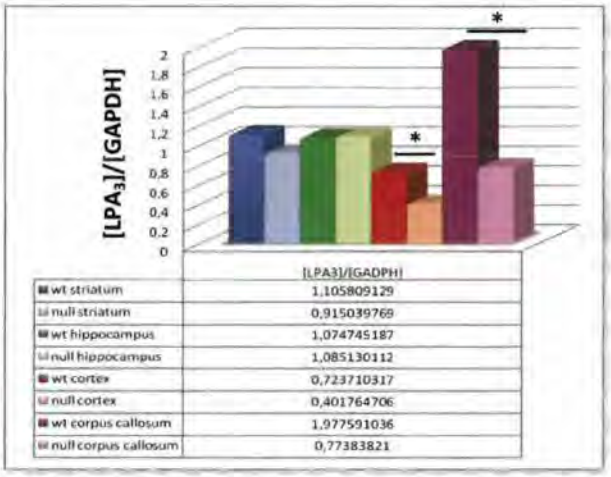


Fig.18. Relative RT-PCR analysis of *Lpar1* expression normalized to that of GAPDH. *Lpar1*expression was only detected in wild type samples, and not in null mice. The transcript levels were higher in corpus callosum and striatum due to its expression in myelinated fibers. LPA$_1$ was also expressed in hippocampus and cerebral cortex at lower levels. *Lpar1-5* genes expressed in figures as LPA1-5 henceforth.

Since *Lpar2* gene shows the major identity with *Lpar1* sequence, it was considered the first candidate for compensation. *Lpar2* transcripts were detected by RT-PCR at very low levels in every tissue assayed and in both genotypes. Despite *Lpar2* is expressed in adult brain (Contos et al.2000), its expression was too low to be taken into account, and no significant differences were observed between normal mice and those in which LPA$_1$ was absent (data not shown).

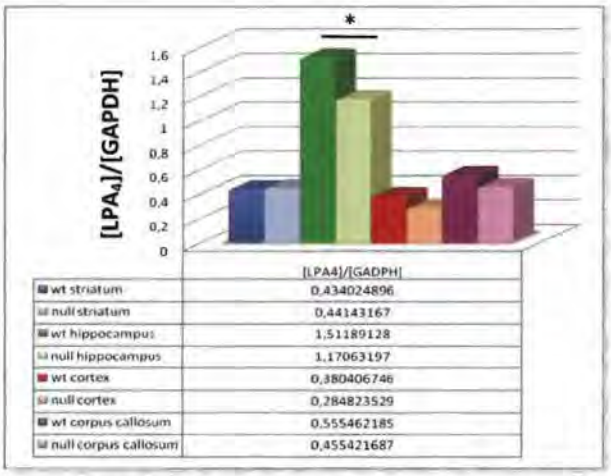
By contrast, when the expression of the other LPA receptor gene, *Lpar3*, was determined, some differences were showed. Neither striatum nor hippocampus demonstrated any alteration in *Lpar3* transcript levels when LPA$_1$ was lacking. However, there was a slight but significant less expression of *Lpar3* in cerebral cortex of knockout animals. This decrease was more pronounced in corpus callosum, where *Lpar3* level in null mice turned out to be only 40 per cent of the transcripts in wild type animals (Fig.19).



	[LPA3]/[GADPH]
wt striatum	1,105809129
null striatum	0,915039769
wt hippocampus	1,074745187
null hippocampus	1,085130112
wt cortex	0,723710317
null cortex	0,401764706
wt corpus callosum	1,977591036
null corpus callosum	0,77383821

Fig.19. Relative RT-PCR analysis of *Lpar3*expression normalized to that of GAPDH. LPA_3 expression decreased slightly in striatum of null mice but without statistical relevance, and showed a significant drop in cerebral cortex and corpus callosum in those animals. Transcript levels in both genotypes remain equal (* P< 0.05).

LPA_4 and LPA_5 somewhat divergent from LPA_{1-3} receptor EDG family were also considered for expression because of their shared signalling pathways and ligands. Significance and differential expression was not found between genotypes for *Lpar4* gene in striatum, cerebral cortex and corpus callosum. Nevertheless, there was a trend towards lower *Lpar4* levels in the animals in which Lpar1 was not expressed. Unlike, hippocampus showed a significant decrease in the expression of this receptor parallel to the absence of LPA_1 (Fig. 20).



	[LPA4]/[GADPH]
wt striatum	0,434024896
null striatum	0,44143167
wt hippocampus	1,51189128
null hippocampus	1,17063197
wt cortex	0,380406746
null cortex	0,284823529
wt corpus callosum	0,555462185
null corpus callosum	0,455421687

Fig. 20. Relative RT-PCR analysis of *Lpar4* expression normalized to that of GAPDH. A trend towards a decrease was observed in *Lpar4*expression when LPA_1 was lacking, however this tendency was not statistical significant except for hippocampus (* P< 0,05).

Quantification of relative*Lpar5* transcript levels showed very stable amounts in the different regions analysed with regard to both genotypes. Likewise, the same slight tendency towards decrease was observed, not showing significant differences in any region (Fig.21).

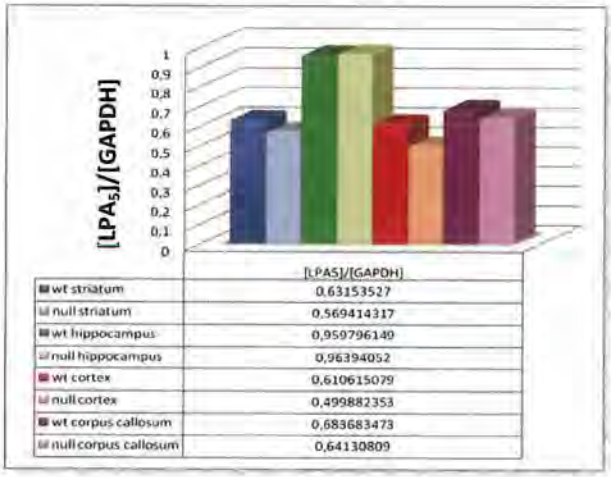


Fig. 21. Relative RT-PCR analysis of *Lpar5* expression normalized to that of GAPDH. *Lpar5*expression was not altered in null mice, only a slight trend for reduction was observed which was not significant (* P< 0,05).

In summary, a clear but no significant trend towards a reduced expression of LPA receptors was observed in null mice. However, only *Lpar3* and *Lpar4* showed a decrease in the transcript levels, being these restricted to cerebral cortex and corpus callosum, in the case of *Lpar3* and hippocampus, for *Lpar4 expression*. These data suggest that transcriptional compensation may not play an important role in maintaining robustness when *Lpar1* is knocked out.

1.3. Myelination pattern of mice lacking the LPA_1 receptor.

1.3.1. The lack of LPA_1 increased choline levels as measured by magnetic resonance spectroscopy.

Magnetic resonance spectroscopy (MRS) is a non-invasive technique that provides useful information on brain chemistry. MRS offers the opportunity to investigate changes in the metabolite composition of different brains in vivo. Proton metabolites detectable in this study with MRS were N-acetylaspartate (NAA), essentially found in neurons, phosphocreatine (Cre), considered an index of neuronal density, and choline (Cho), constituent of membranes and myelin.

The choline peak correlates with cellular density and with choline-containing compounds that participate in phospholipid metabolism. An increase in choline peak usually is associated with increased turnover of myelin. Since membrane phospholipids are released during active myelin breakdown, a higher peak of choline suggests active demyelination as well, whilst decrease is associated with hypomyelination (Bonavita et al., 1999; Simone et al., 2001; Khiat et al., 2007). NAA is a neuronal marker, and a decrease in NAA levels is associated with neuronal and axonal loss.

In this study, MRS was performed at different ages in mice of both genotypes wild type and malpa_1-null (Fig.22). Metabolites analyzed were N-acetylaspartate (NAA), choline (Cho) and creatine (Cre) and their ratios were generated by automated procedures to detect the location of NAA, Cho and Cre peaks.

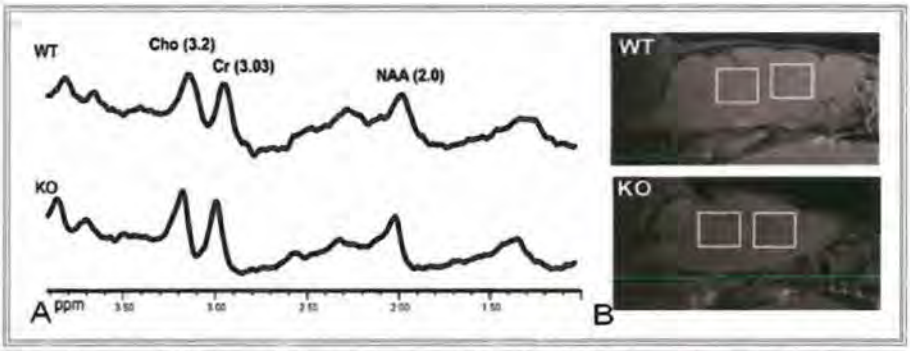


Fig. 22. A. Curves from proton magnetic resonance spectroscopy (MRS) of the brain of a normal (wt) and a knockout (ko) mouse, showing peaks of the metabolites N-acetyl aspartate (NAA), creatine (Cr) and choline (Cho). B. Sagital images of the brain regions in which MRS was performed. MRS curves of normal and null mice demonstrated an increase in the height of Cho peak in those mice in which LPA_1 was lacking.

MRS was performed at different ages showing a temporal gradual significant increase in choline levels, referred to creatinine, of LPA_1-null brains as compared to wild type mice The youngest mice studied were 30 days-old (P30). From that age, the spectra showed an increase in choline in null mice by comparison with normal mice. This trends towards increase in choline levels went on at the following ages (P60-P140), showing significant statistical difference in all of the cases (*P < 0.05) (Fig. 23).

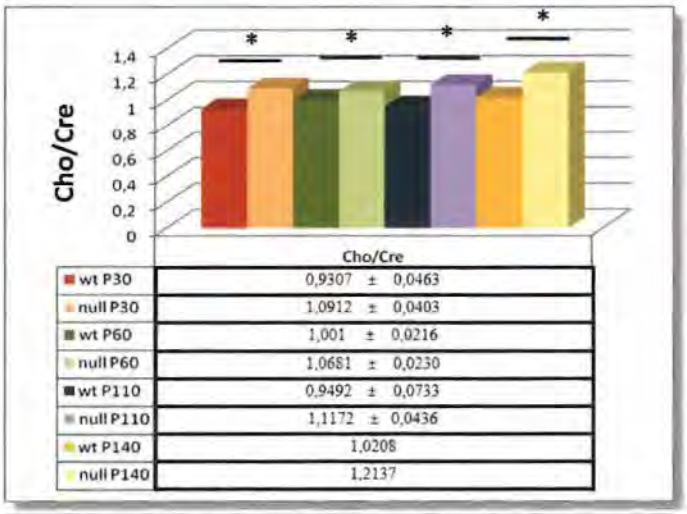


Fig. 23. Cho/Cr ratio in the brain of wild type (wt) and null mice (null) at different ages. Null mice showed higher choline / creatine ratios than those in which LPA_1 was expressed. These differences were statistical significant in all the ages (Data expressed as mean ± SEM; * P< 0,05).

This increase in the peak of choline, which suggested myelination alteration, was not accompanied withchanges in the NAA / Cr ratios suggesting that axons were relatively unaffected. Not only no reduction in NAA was showed, but a slight increase in null mice NAA levels at some ages. This rise was no significant in the younger mice, becoming more important at P110 and P140 in which seemed to be statistical significant (*P < 0.05) (Fig. 24).

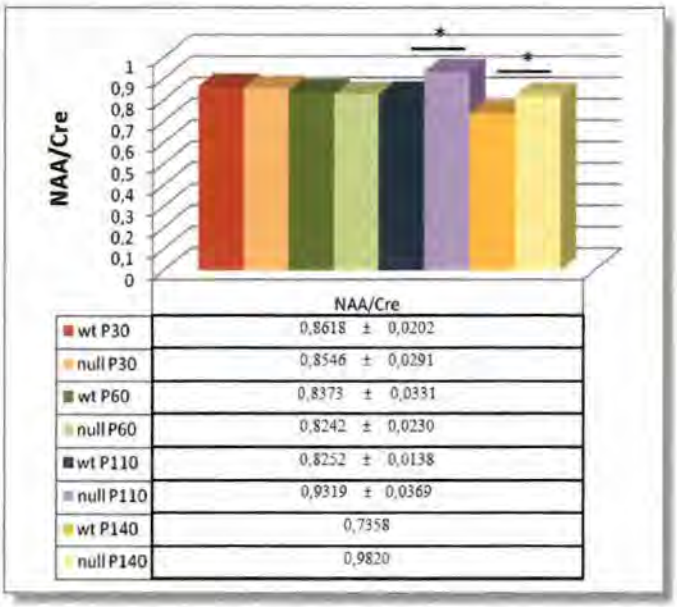


Fig. 24. NAA/Cr ratio in the brain of wild type (wt) and null (null) mice at different ages. Both genotypes presented the same level of NAA at younger ages, showing a minor increase in null mice at P110 and P140 ages where differences were statistical significant (Data expressed as mean ± SEM; * P< 0,05).

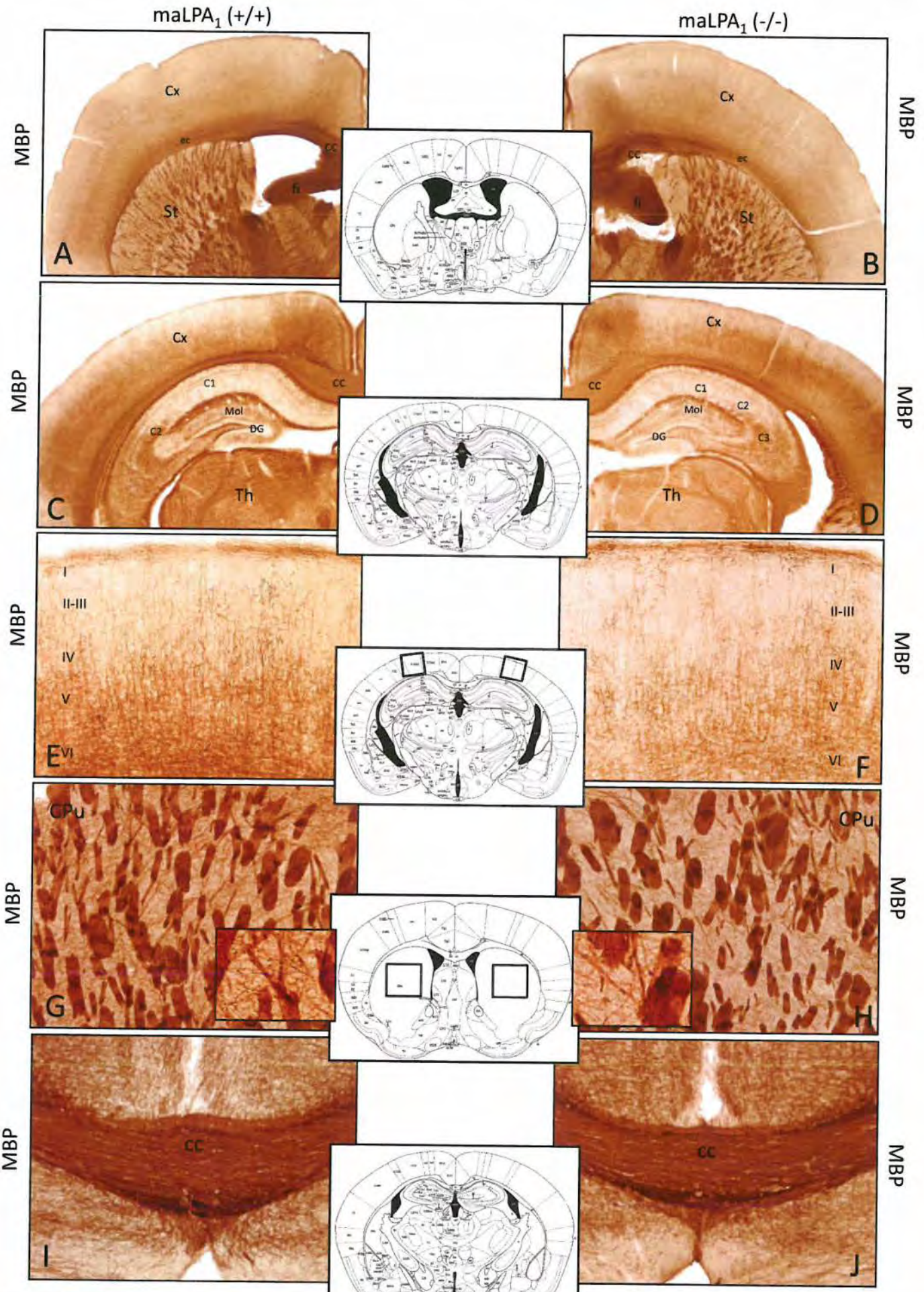
Then, although the higher levels of Cho in maLPA$_1$-null mice were consistent with a turnover in myelin and demyelination in this genotype, the NAA levels do not seem to suggest neuronal loss when LPA$_1$ is lacking.

1.3.2. LPA$_1$ absence results in abnormal myelination in brain not linked to defective oligodendrocyte precursors.

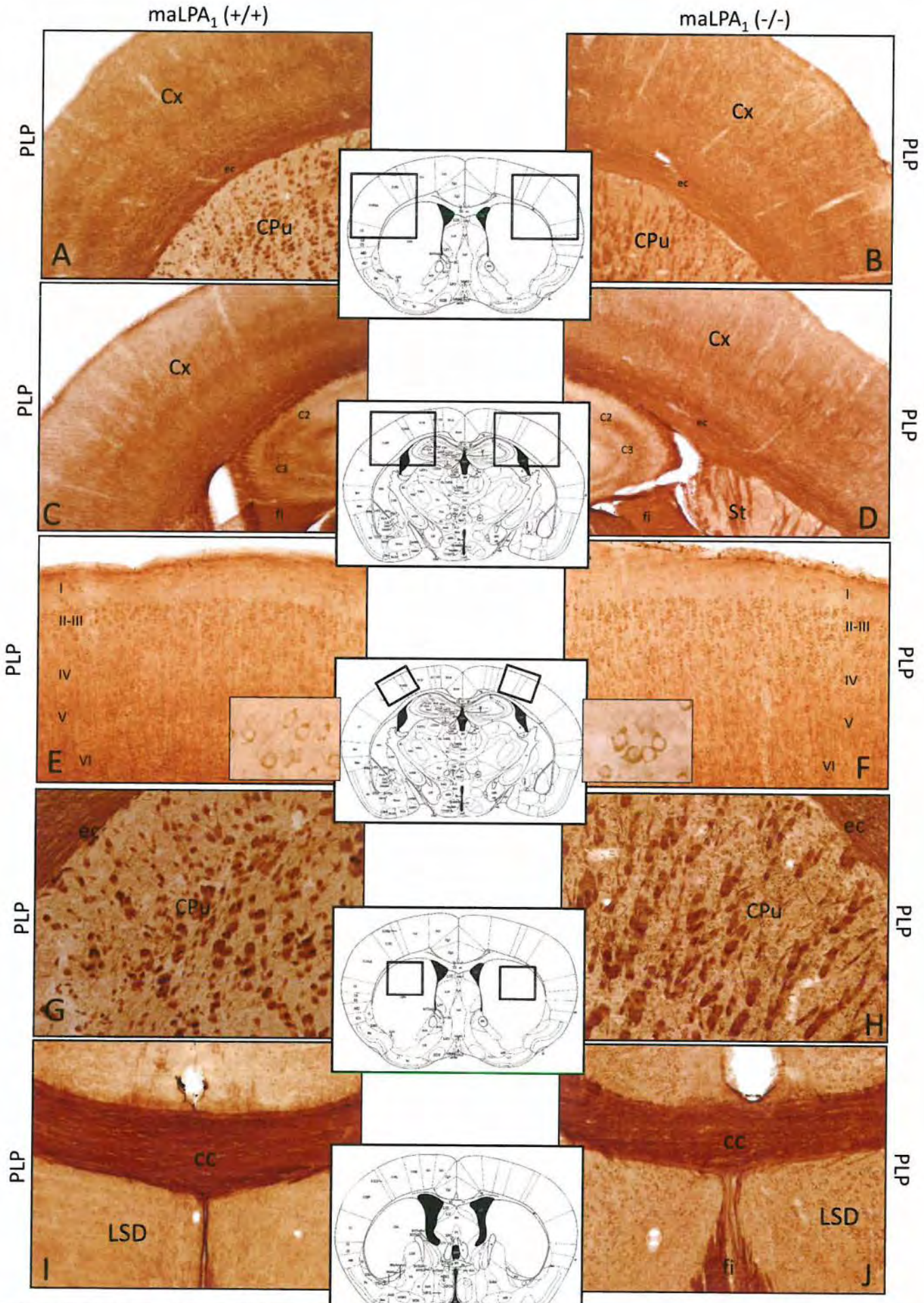
1.3.2.1. Immunohistochemistry for myelin proteins: Myelin Basic Protein , Proteolipid Protein and 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase.

The requirement of LPA$_1$ receptor mediated pathway in myelination was studied by comparison of the myelin pattern in adult normal and maLPA$_1$-null mice. Wild type mice were used as control of normal myelin presence. This pattern was evaluated according the expression by immunohistochemistry of the most abundant proteins in myelin sheaths: Myelin Basic Proteins (MBP, Fig.25), Proteolipid Protein (PLP, Fig. 26) and 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase, Fig. 27).

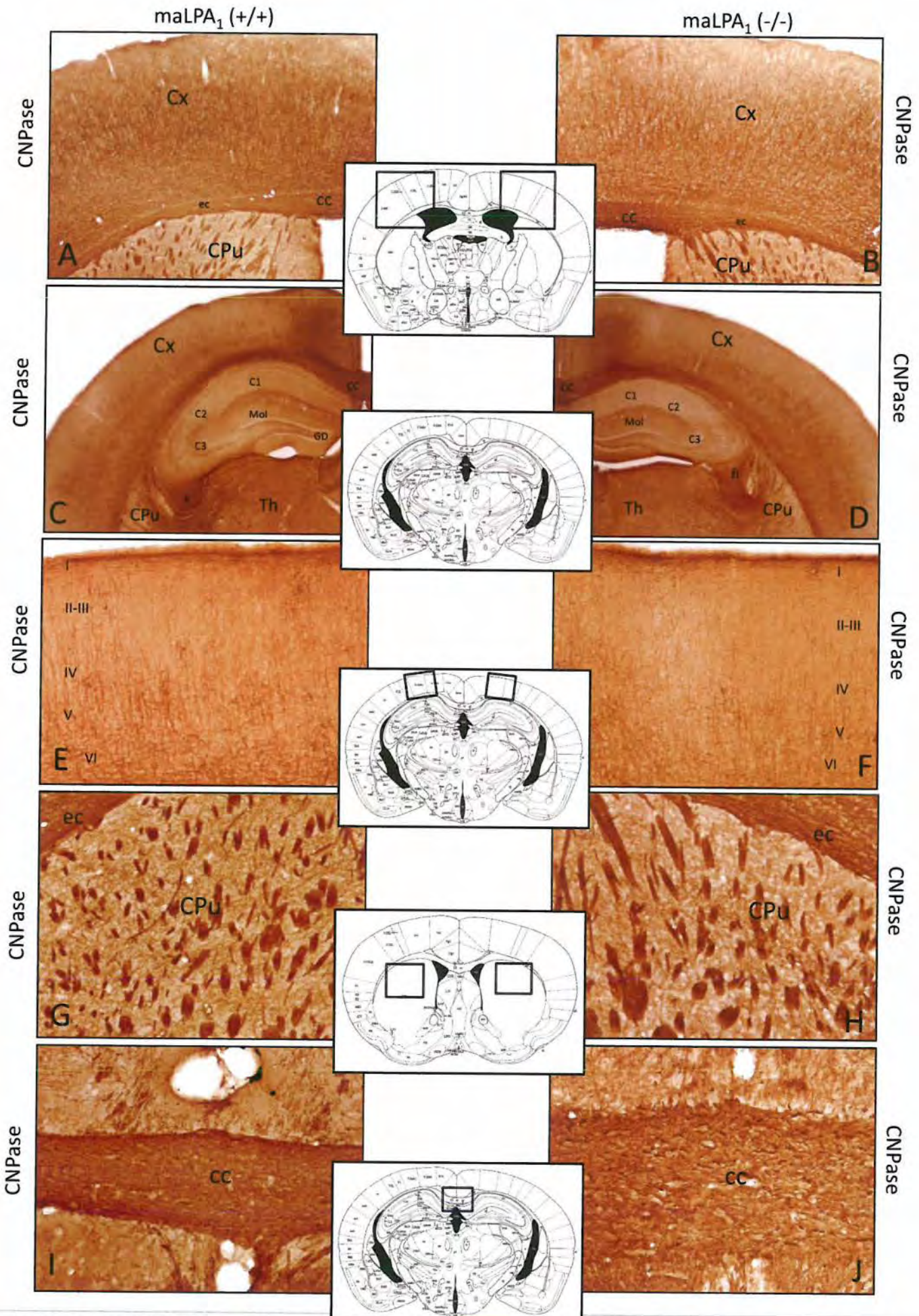
MBP is a group of hydrophilic proteins that may function to maintain the correct structure of myelin, interacting with the lipids in the myelin membrane by electrostatic and hydrophobic interactions. Various forms of MBP exist which are produced by the alternative splicing of a single gene; these forms differ by the presence or the absence of short (10 to 20 residues) peptides in various internal locations in the sequence. MBP staining was assayed to evaluate the myelinated fibers throughout the brain. At a single glance, not great differences in the staining were seen between both genotypes (Fig. 25A, B, C, D). MBP signal appeared in the myelinated structures such as corpus callosum, fimbria, external capsule or thalamus, with aparently the same aspect in wild type and null mice. Looking in better detail those less dense areas exhibiting fewer or more spaced myelinated fibers, a decrease in the number of these fibers was then clearly observed in null mice. This reduction of MBP-positived fibers was more evident in cerebral cortex (Fig. 25E, F) than in other regions of the brain. The most superficial layers in the cortex (I-IV) showed the major and better detectable decrease in the density of immunorreactive fibres, although the inner layers also depicted this alteration. Caudate putamen also exhibited decrease in the number of fiber bundles, being noticeable the reduction in the small fibers which surrounded them (Fig. 25G, H). Since corpus callosum is one of the most important myelinated structuresubstantial



. 25. **Expression of Myelin Basic Protein (MBP) throughout the brain in normal and maLPA$_1$-null mice.** Although, in a general view, no important 'erences were observed in MBP stained cortical coronal sections between both genotypes , at bigger magnification it could be seen a reduction in the ısity of positive fibers in the cerebral cortex and caudate putamen in null mice (F, H). Schemes indicating staining image, and hereafter.



ig. 26. Expression of Proteolipid Protein (PLP) throughout the brain in normal and maLPA$_1$-null mice. PLP staining in null animals differed from normal nice in the density of positive cells in cortex and caudate putamen , which turned out to be lower in the absence of LPA$_1$ (F, H); in contrast, the nmunoreactivity of PLP in the oligodendrocyte soma increased in knockout (F, H,J).



g. 27. Expression of 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase) throughout the brain in normal and maLPA$_1$-null mice. At first glance, no etectable differences were observed between normal and null mice, however detailed capture evidenced a decrease in the density of positive fibers in ıe cerebral cortex and caudate putamen when LPA$_1$ was lacking (F, H).

differences were expected, however, light microscopy observation did not demonstrate any remarkable defect from LPA_1 lacking (Fig. 25I, J).

Together with MBP, other myelin proteins were analysed by immunostaining, such as PLP. PLP (also named lipophilin) is the major myelin protein from the central nervous system (CNS). It probably plays an important role in the formation or maintenance of the multilamellar structure of myelin. PLP is a highly conserved hydrophobic protein which seems to contain four transmembrane segments, two disulphide bonds and which covalently binds six palmitate groups. Its detection by immunochemistry showed the same pattern observed for MBP (Fig. 26). It was analysed in the principle myelinated structures, like corpus callosum, fimbria, external capsula, striatum and cortex (Fig. 26A, B, C, D). In all those areas, the same effects of LPA_1 absence became evident: whereas the major structures did not reveal any significant differences due to the lack of LPA_1, those regions wherein the fiber density was lower, such as most superficial layers of cerebral cortex or caudate putamen showed a detectable decrease in the number of PLP-immunoreactive fibers (Fid. 26E, F, G, H). The reduction in the number of PLP-positive fibers was again more remarkable in the upper layers of the cerebral cortex (I-IV) although it was also visible in the inner ones. This drop in the stained fiber density was reflected in striatum as well. Meanwhile, corpus callosum did not demostrated any effect caused by the absence of LPA_1.

PLP is a myelin protein which is present in the sheaths, as it is translated inside RER. Thus immunostaining defined the fiber pattern though some cytoplasms appeared also slightly stained (26I, J). In addition, thinner sectioning of brain slices allowed detecting PLP immunoreactivity in the cytoplasm of cells (Fig. 26E, F, and inserts). Combination of both procedures showed that in wild type mice PLP immunoreactivity drew the fibers mainly, whilst soma appeared slightly positive (Fig. 26E, G, I). In contrast, in null mice, the number of immunorreactive fibers were lower and it was accompanied of a strong signal in oligodendrocyte body (Fig. 26F, H, J). This increase in the cytoplasmic staining of PLP was more remarkable in the upper layer of the cortex (Fig. 26F) although observed throughout the whole brain (Fig. 26H, J).

Analysis of myelinated fibers was completed for CNPase. CNPase, a member of the 2H phosphoesterase superfamily, is the third most abundant myelin protein in the CNS. It catalyzes the hydrolysis of 2',3'-cyclic nucleotide to produce 2'-nucleotide in vitro, however, this enzymatic activity may not be relevant to its function in vivo. CNP is expressed by oligodendrocytes and sent to the myelin sheaths during the development where it is firmly associated with tubulin. In adult brain, CNPase immunoreactivity pattern is quite similar to MBP(Fig. 27). Similarly, at first glance,differences in CNPase positive fibers were barely detected in null mice (Fig. 27A, B, C, D). Nevertheless, once again, detailed observation through cerebral cortex or caudo putamen areas demonstrated a reduction of positive fibers in those animals lacking the receptor (Fig. 27F, H). Regarding the cortex, the reduction in the number of CNPase-immunoreactive fibers was noticeable in the upper layers (I-IV) being also seen in the inner ones but in a lesser extent (Fig. 27F).

1.3.2.2. Evaluation of differential myelin protein expression by Western Blot analysis.

The altered myelination pattern observed in null animals was corroborated by western blot analysis of myelin extracts, obtained after dissection of cerebral cortex, for a better quantification. Cortical upper layers (I-III), where the major differences were more detectable, were separated from the inner ones (IV-V), in which a higher number of myelinated fibers were present, and both so processed for protein extraction. The VI layer was excluded to prevent sampling from corpus callosum contamination.

Before western blotting, a resulting control polyacrylamide gel was stained by standard Coomasie® blue method to prove similarity of total protein per lane (Fig.28). Band pattern did not showsignificant differences after comparing the three genotypes, wild typel, heterozygous and homozygous null.Every line showed a similar group of bands corresponding to each protein which myelin sheaths consists of. Likely, the lack of LPA_1 does not alter the composition of myelin protein pool drastically.

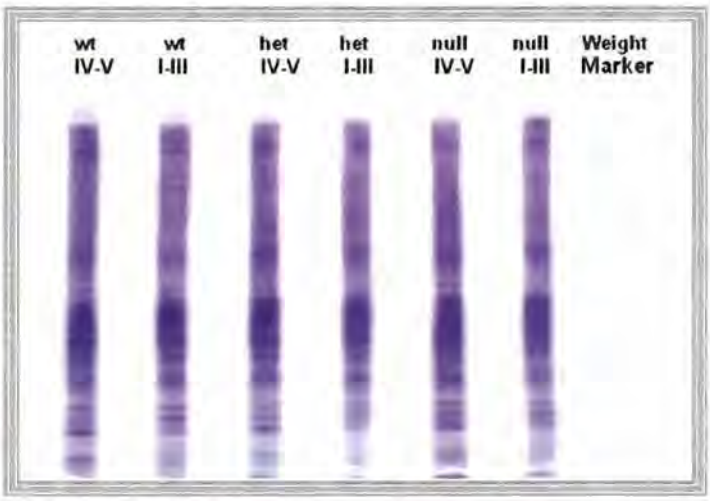


Fig. 28. SDS-PAGE analysis of myelin protein extracts stained with Coomasie® blue. Wild type (wt), heterozygous (het) and homozygous null protein extracts from dissected upper (I-III) and inner cortical layers (IV-V) loaded and submitted to electrophoresis in a 15% polyacrylamide gel with SDS; . Weight marker lane loaded with SDS molecular weight standards (30-200 kDa; Sigma-Aldrich).

Western blot analysis of the same myelin proteins previously considered for immunohistochemistry revealed a decrease in the relative amounts of MBP, PLP/DM20 and CNPase proteins in absence of LPA_1 (Fig. 29).

MBP detection showed five isoforms which constitute the pool of these basic proteins. The apparent molecular weight of the five bands agreed with the different splice variants described in mouse (21,5 kDa; 18,5 kDa; 17,2 kDa; 17 kDa; and 14 kDa). These isoforms were present in all genotypes, more abundant in the inner layers (IV-V) than in the upper ones (I-III), and significantly reduced in null mice, whereas heterozygous genotype showed an intermediate expression. Besides this general effect, it was noted that in null mice, the difference between the levels of expressions of MBP proteins in upper and inner layers was not so significant as compared to that observed in wildtype and heterozygous samples.

The analysis of the expression of PLP (29 kDa), and its isoform DM20 (21 kDa), presented the same alterations associated to the loss of LPA_1. Both proteins expression was again more abundant in the superficial layers that in the deeper ones and significantly reduced in null mice in comparison to those in normal mice. Although this reduction was more remarkable in PLP, DM20 isoform also showed a decrease. Heterozygote pattern demostrated a halfway pattern which was slightly more similar to null mice.

Lastly, CNPase proteins, 46 and 48 kDa, appeared as one single band in the gel and showed comparable significant reduction of expression in absence of LPA_1.

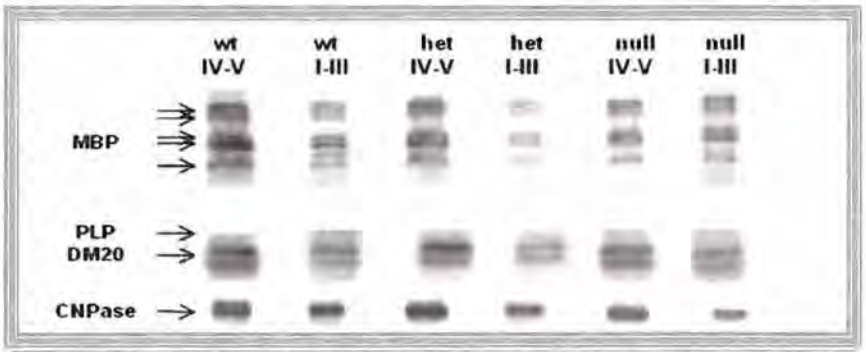


Fig.29. Western blot analysis of myelin extract from dissection of cerebral cortex layers, with antibodies for different myelin proteins: myelin basic proteins (MBP), proteolipid protein (PLP) and its isoform DM20, 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase).

In all cases, the expression of each protein was quantified by measuring the optical density (OD) of the band . to evaluate statistically the deficient myelin pattern due to LPA_1 absence. , as it is showed below in the table (Fig. 30).

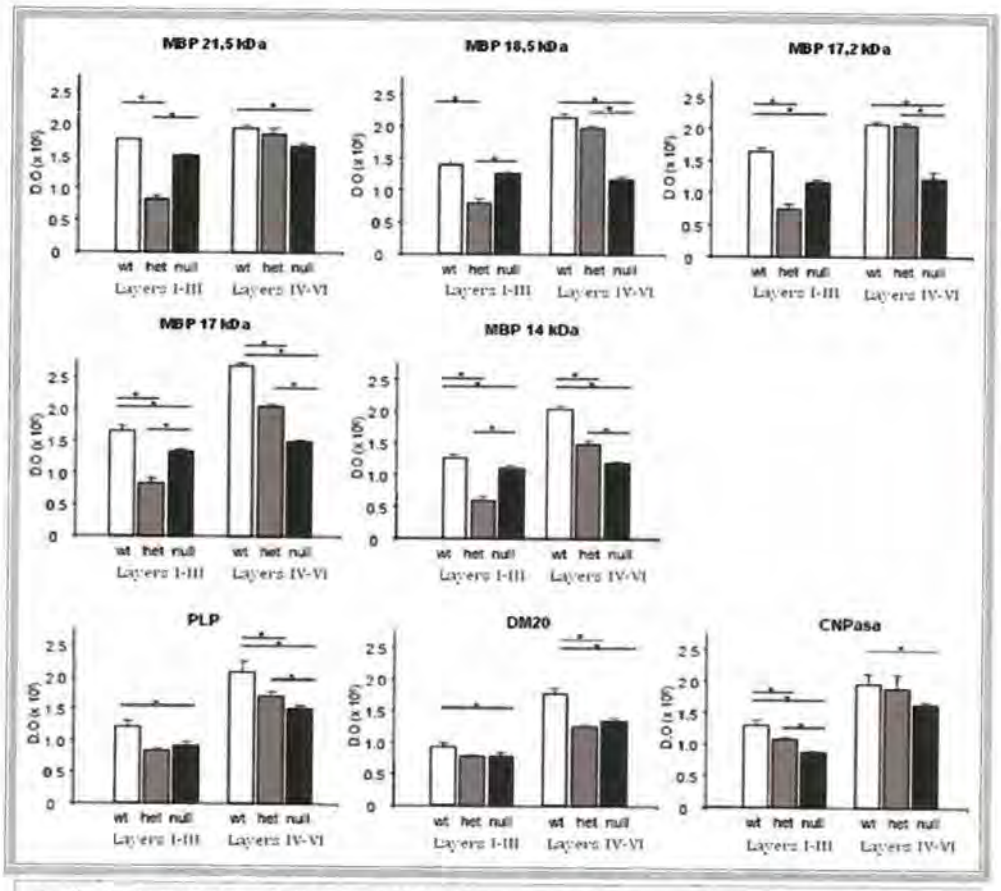


Fig.30 . Analysis of the optical density (OD) for each myelin protein band. Bands which showed significant differences were marked with asterisk (ANOVA, * = P < 0,05; Data expressed as mean ± SEM; n = 3).

Taken together the immunostaining and western blot results, the loss of LPA_1 turned into a decrease in the number of myelinated fibers as well as into an alteration in the composition of these ones. The amount of every protein analysed was reduced with regard to the total of proteins when LPA_1 receptor was lacking.

1.3.2.3. *Immunohistochemistry for mature (GalC) and precursor (NG2) oligodendrocytes.*

Inadequate myelination of brain fibers could be associated with a failure of oligodendrocyte precursors to differentiate into mature, myelin-producing cells. In order to better understand which role LPA_1 plays in oligodendrocyte differentiation or myelination, the effect of LPA_1 absence was not only studied regarding the density of myelinated fibers, but also with regard to the number of myelinating cells.

Oligodendrocyte precursor cells (OPCs) differentiate during the first postnatal weeks to give rise to the oligodendrocytes that synthesize myelin around the axons. OPCs are characterized by the presence on their surface of NG2, a high-molecular-weight chondroitin sulphate proteoglycan (Dawson et al., 2000). On the other hand, mature oligodendrocytes are marked by galactocerebroside (GalC), which is the major glycolipid in myelin.

GalC immunohistochemistry stained a high number of perineuronal oligodendrocytes in both adult normal and null mice, showing also some intrafascicular ones (Fig. 31A, B, C, D). GalC-positive cell density was observed in different regions of the brain, comparing null mice to those in which LPA_1 receptor was expressed. Apparently, the number of mature oligodendrocytes decreased in absence of the receptor in all the

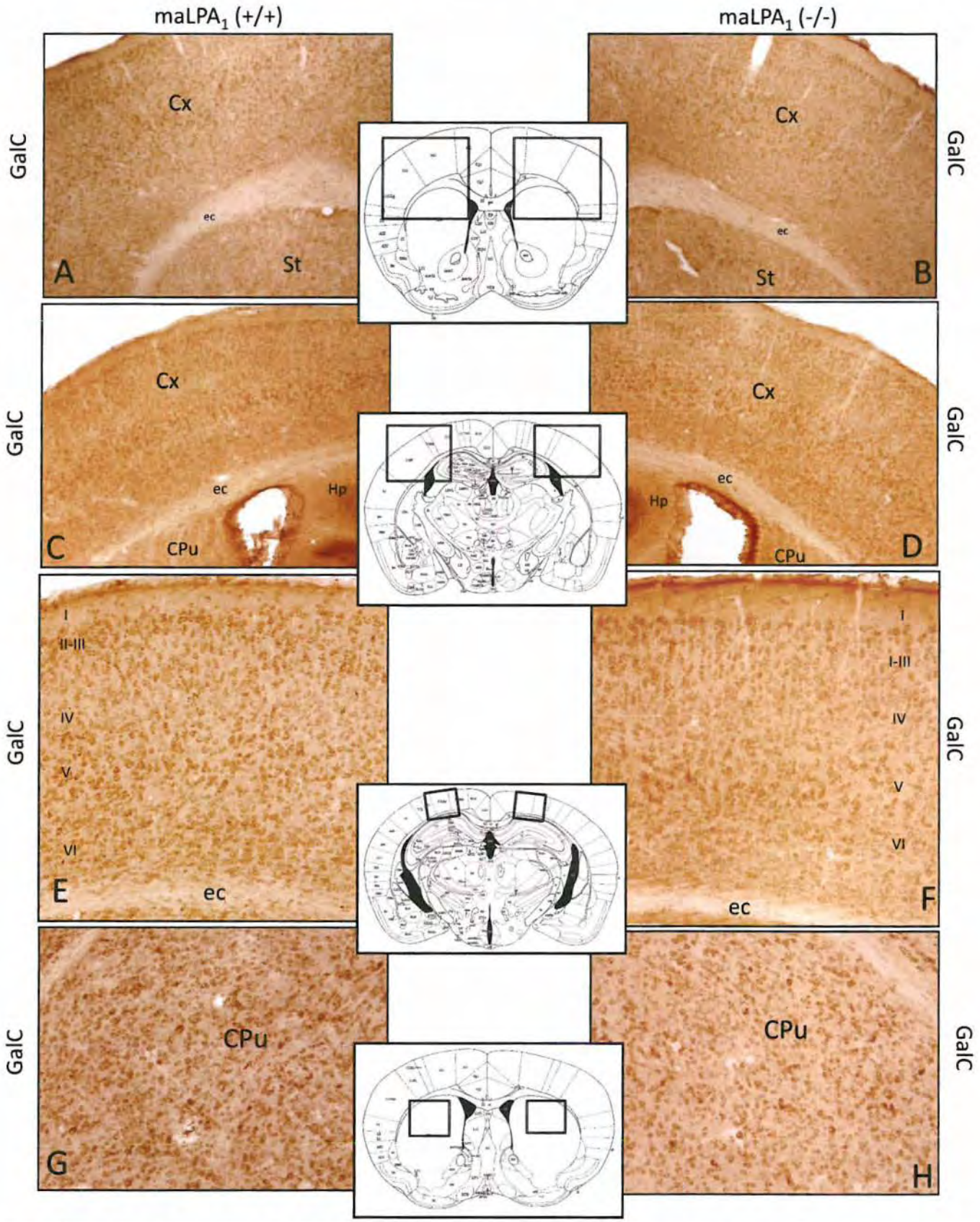


Fig. 31. Expression of Galactocerebroside (GalC) throughout the brain in normal and maLPA$_1$-null mice. Differences were barely observed with low magnifications between normal and null mice (A, B, C, D). Nevertheless, higher magnification showed a decrease in the number of mature oligodendrocyte (GalC+) in the cerebral cortex and caudate putamen when LPA$_1$ was lacking (F, H) with respect to wild to phenotype.

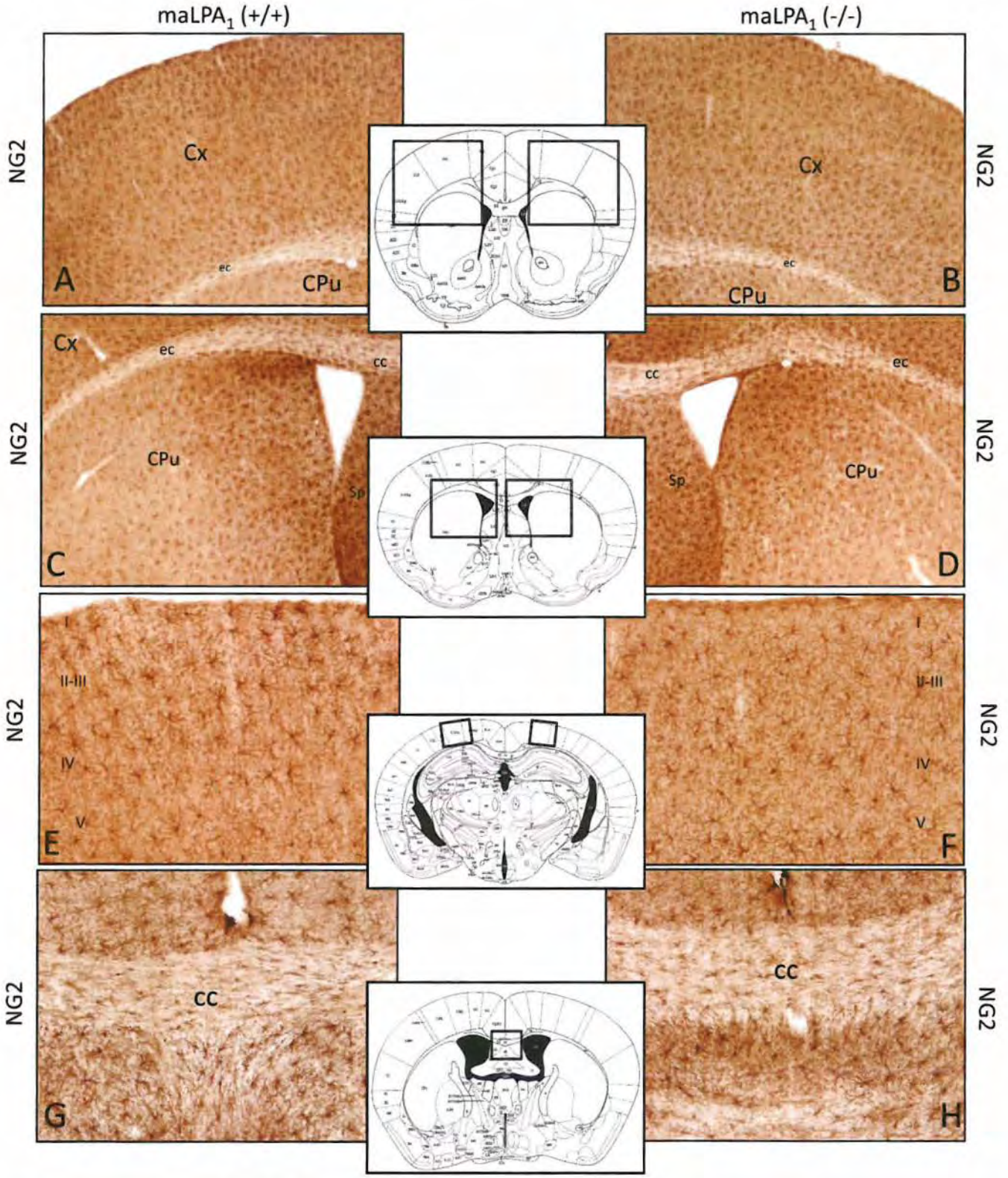


Fig. 32. Expression of Proteoglycan NG2 throughout the brain in normal and maLPA$_1$-null mice. There were no differences in the number , distribution or shape of the oligodendrocyte precursor s in presence or absence of LPA$_1$ receptor in any of the regions studied, such as cerebral cortex (A, B, E, F), caudate putamen (C, D) or corpus callosum (G, H) among others.

structures seen (Fig. 31B, D, F, H). This effect was more noticeable in the two areas in with less density of myelinated fibers, that is, cerebral cortex and caudate putamen (Fig. 31E, F, G, H). In the cortex, the higher reduction in the oligodendrocyte number was showed in the upper layers (I-IV) (Fig. 31F), as same as observedin myelin markers. Nevertheless, in the other two inner layers (V and VI), the decrease was also significant. Likewise, GalC-positive cells appeared more scarces in striatum of null animals (Fig. 31H).

So far, the lack of LPA_1 seems to induce a decrease in myelination due to a reduction in the number of cells which produce the myelin sheaths, mature oligodendrocyte. However, this lower number of mature oligodendrocytes did not correlate with a fewer number of their precusors, NG2-positive.

Immunostaining for NG2, the earliest marker for oligodendrocyte linage, was studied to evaluate the effect of the lack of LPA_1 in oligodendrocyte precursors. At this point, no differences were found between both genotypes (Fig. 32). The density and shape of these oligodendrocyte precursors were analysed throughout the brain, and no alterations either in the number or shape were found in absence of LPA_1. NG2 patterns in wildtype and knockout were indistinguishable in all the areas studied, such as cerebral cortex (Fig. 32A, B, E, F), caudate putamen, capsula externa, septum (Fig. 32C, D), striatum, or corpus callosum (Fig. 32G, H).

1.3.2.4. Quantification of precursor (NG2) and mature oligodendrocyte (GalC) density by stereological analysis.

The analysis by immunohistochemistry revealed a decrease in the number of GalC-positive (+) cells throughout the brain, unlike, no differences were showed in the density of oligodendrocyte precursors (NG2+).

These effects of the lack of LPA_1 were quantified by stereological anaylisis. To perfom this study a specific area was chosen as representative of what could be happening in the rest of the brain. Chosen region was the primary and secondary cerebral motor cortex (M1 and M2).

Regarding mature oligodendrocytes, the reduction of its density was significant, turning out to be almost 30 per cent less in null mouse than in normal animals. In contrast, NG2+ cells did no show any decrease in its density, remaining identical in both genotypes (Fig. 33).

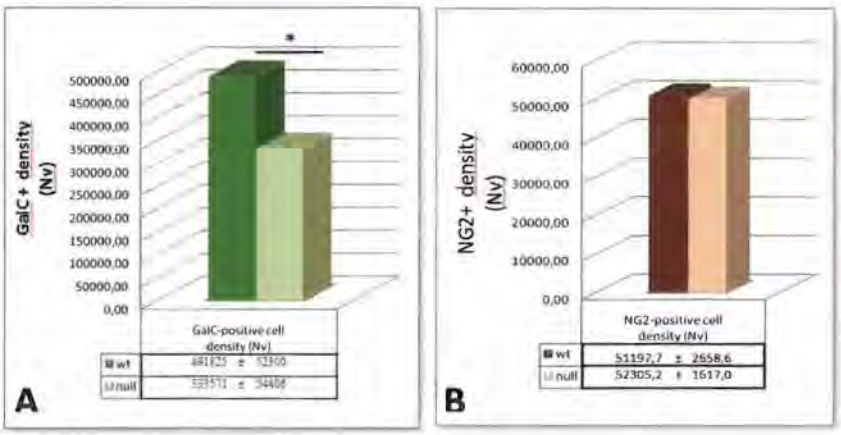


Fig. 33. Quantification of the density (Nv) of immunoreactive cells for galactocerebroside GalC (A) and proteoglycan NG2 (B) in the cerebral motor cortex of normal (wt) and LPA_1-null mice (null). Quantification by stereological analysis showed a significant decrease of 30% in the number of mature oligodendrocyte (GalC+) in LPA_1 absence, (Data expressed as mean ± SEM; n = 4; * P < 0,01). However, no differences were evident with regards to the number of oligodendrocyte precursor cells (NG2+) in presence or absence of LPA_1.

1.3.2.5. Electron microscopy analysis of myelinated fibers in absence of LPA_1 receptor.

Despite no differences were seen by immunostaining in corpus callosum, its ultrastructure was analysed with the aim of clarifying if LPA_1 absence does not provoke any alterations or if these ones were attenuated by the high amount of fibers in that region. By electron microscopy, the ultrastructure of the fibers were studied, as well as the characteristics of their sheaths, their organization and their density.

Transversal ultrasections of corpus callosum area showed that the number of the myelinated axons per section in null mice was lower than in animals expressing LPA_1 (Fig. 34A, B). In absence of LPA_1, bigger spaces were found among the fibers (arrow tips), which demonstrated the same reduction in myelinated fibers as that observed by immunohistochemistry in other structures of the brain, like cerebral cortex, striatum or caudate putamen. This indicated that the effects caused by the lack of LPA_1 also occurred in corpus callosum. The fibers sections were counted in several samples revealing about 20 per cent reduction in number in absence of LPA_1 receptor. Then, as a possible reason for no shown evident immunohistochemical alterations could be well explained by the gross abundance of myelinated fibers in this region, which partially hid the deffects caused by the absence of LPA_1.

Apart from the reduction in the fiber density, other consequences of the lack of LPA_1 were reported. Thus null fibers displayed in a general disorganization pattern, mostly showing irregular shapes, in contrast to that observed in normal mice. This anomalous profile was observed in both transversal (Fig. 34A, B) and longitudinal planes (Fig. 34C, D), and it was accompanied by an altered distribution. Hence whilst wild type myelin fibers fibers oriented parallel constituting bundles, null mice fibers laid out quite randomly through corpus callosum, being able to be found with different orientations in the same plane (Fig. 34D).

On the contrary, although some differences in the thickness of the sheaths were expected, axonal dimater and myelin thickness seemed not affected, remaining the number of lamella and their compactation equal in both genotypes.

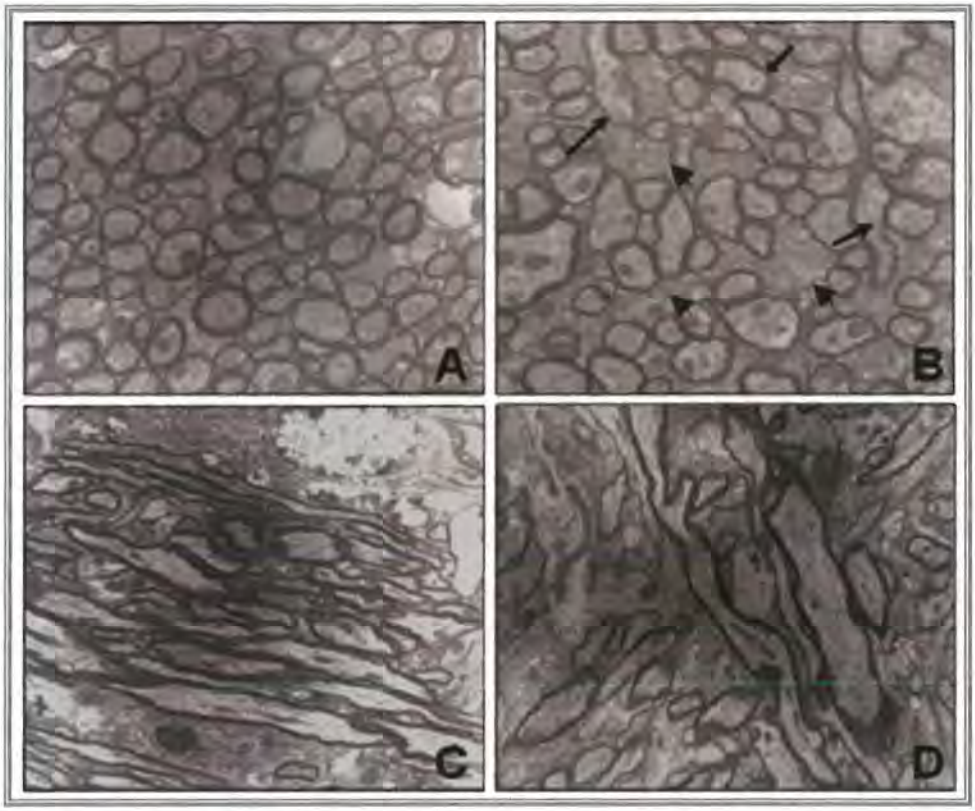


Fig. 34. Transmission electron micrograph of myelinated axons in corpus callosum of normal and null mice. Transversal sections of corpus callosum of normal (A, C) and knockout (B, D) mice revealed there is a lower number of fibers when LPA_1 is lacking, showing bigger spaces among the fibers (arrow tips). Sections showed more irregular shapes in null animals (arrows; B, D) and altered distribution which caused fibers do not group themselves properly into bundles but in a more randomly organization.

1.4. The lack of LPA_1 receptor generates anomalous trafficking of proteolipid protein susceptible to induce apoptosis in oligodendrocyte.

As it was mentioned before, there was a significant reduction in the number of fibers labelled with PLP throughout the brain in animals without the receptor (Fig. 26). However, despite this decreased in PLP expression along the fibers, the body cells in null mice showed an increase in the immunoreactivity by PLP (Fig. 26F, H, J). Most of oligodendrocytes of wild type animals revealed a minor staining, which seems to be distributed to the outer parts of the body cells (Fig. 26E). In contrast, PLP reflected to be more spread around all the soma of myelinating cells in null mice. These different patterns due to their genotypes contrasted with the staining of PLP in P10 where oligodendrocytes revealed highly signal by this protein in both normal and null mice (not shown).

Then in adult, PLP-positive cells were divided in two categories depending on their immunoreactivity. On one hand those cells in which PLP immunoreactivity was less intense and tipically located in the outer cytoplasm were called normal PLP-positive cells, since this kind of labelling was the most frecuent in normal mice. On the other, cells which presented a strong immunostaining fullfilling the cell body, named as intense PLP-positive cells in this work. These were also found in wild type mice although they were much more numerous in absence of LPA_1. Both types of PLP-immunoreactive oligodendrocytes were quantified in the cerebral motor cortex.

The total number of total PLP-positive oligodendrocytes showed a reduction in adult null mice and in coincidence with previosuly demonstrated by GalC staining. This significant drop in the density of oligodendrocyte labelled with PLP coincided with that in GalC-positive cells, about 30 per cent. At once, the number of as intense PLP-positive fraction of oligodendrocytes significantly increased in the null mice (Fig. 35).

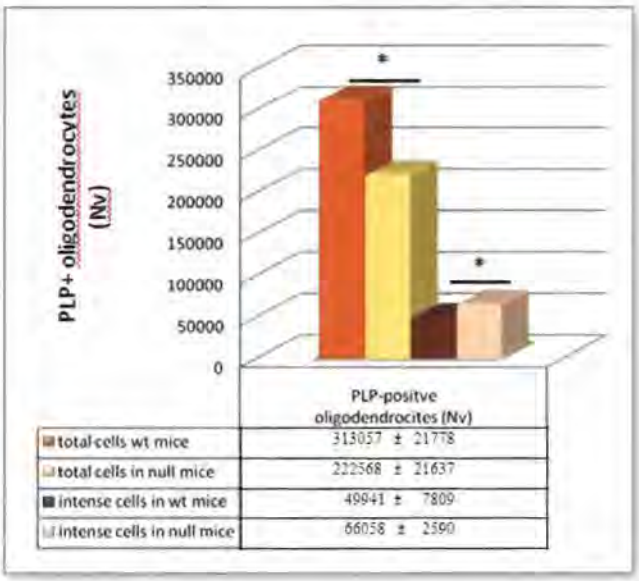


Fig. 35. Quantification of the density (Nv) of immunoreactive cells for proteolipid protein in the motor cerebral cortex of normal (wt) and null-LPA_1 mice. Quantification analysis showed a significant decrease of 30% in the total number of oligodendrocyte (PLP+) in LPA_1 absence. However, the number of intensive PLP-positive cells increased in null mice. (Data expressed as mean ± SEM; n = 4; * P < 0,01)

PLP, unlike MBP or CNPase, is translated into the rugose endoplasmic reticulum (RER), from there, transported through the Golgi apparatus (GA) and sorted to the plasma membrane where the translated protein is inserted into myelin rafts. In order to see where along this pathway PLP is retained in null mice, several double fluorescent immunostainings were performed to indentify these three compartments.

Protein disulfide isomerase (PDI) is an enzyme localized in the endoplasmic reticulum which catalyzes the formation and breakage of disulfide bonds between cysteine residues within proteins as they fold. Therefore, it is normally used as a marker of RER (Wang et al., 1998;). PLP and PDI colocalized in cortical oligodendrocytes from mice lacking LPA_1 as it can be visualized by confocal microscopy (Fig. 36L). This result suggested that part of the PLP protein kept trapped in RER in null oligodendrocytes (Fig. 36K), whereas in normal mice PLP was no longer present in this organelle after synthesized (Fig. 36C). Actually, low or discrete staining of PLP remained in the wild type oligodendrocyte after the peak of myelination(Fig.36B).

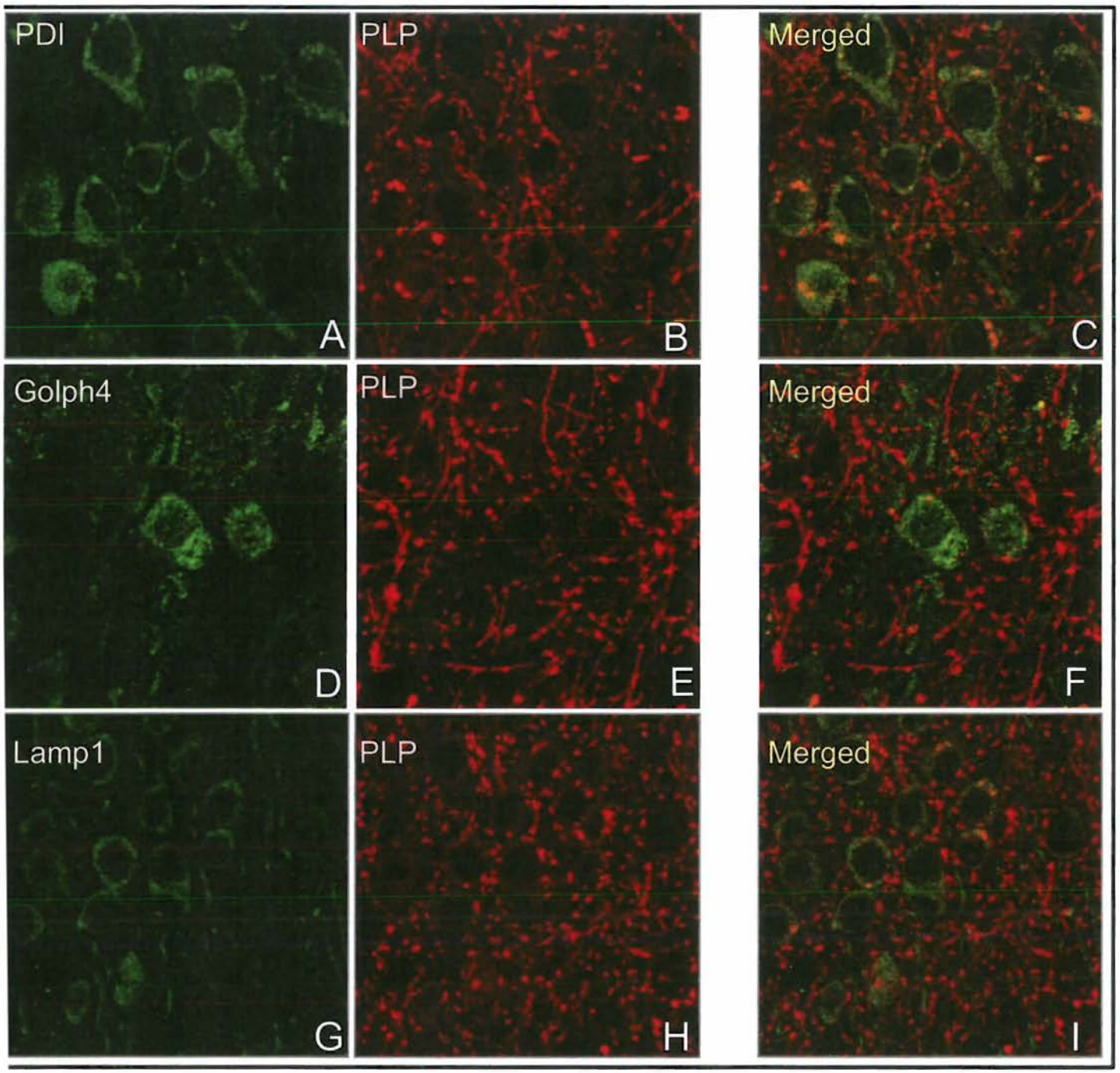
Golgi was marked with Golph4, a transmembrane protein localized to the cis-Golgi, where it is targeted by virtue of its lumenal coiled-coil domain. Golph4 has been a revealing marker of early Golgi vesicle trafficking (Linstedt et al., 1997). Its staining in null mice showed that PLP colocalized with this antibody in some points (Fig. 36O). Nevertheless, while in some cells the overlayed image showed PLP kept in the GA (Fig. 36O, asteriscs) in other cells colocalization was not absolute (Fig. 36O, arrows), suggesting that PLP could stay in Golgi-network temporarily and be recycled continously. Colocalization with PLP was not observed in wild type oligodendroglial cells.

The last marker used for double staining was Lamp1. Lamp1 is a type I transmembrane glycoprotein that is localized primarily in lysosomes and late endosomes (Rohrer et al., 1996). Newly synthesized molecules are mostly transported from the trans-Golgi network directly to endosomes and then to lysosomes. Both Once again and in contrast with wild type, PLP and Lamp1 immunorreactivity mostly colocalized in LPA_1-null oligodendrocytes (Fig. 36R). Lysosome, which can be rather precisely defined , is now known to link the pathways of biosynthesis and degradation of protein, secretion, turnover of membranes, and endocytosis and to carry out multifunctional roles in sorting, processing and degradation. So at this point, it is unknown if the localization of PLP in these vesicles is due to either normal transport towards the membrane or as part of some degradation pathways provoked by the protein acumulation.

Taken all this together, it is logical to think that this increase in the amount of protein present in the membrane system of the oligodendrocyte could induce some kind of deleterious effect to the cells. As an example, overexpression of some myelin proteins can trigger ER stress-induced apoptosis in the transgenic cells (Lin and Popko, 2009)..

In this line, apoptosis was measured in the cortex by TUNEL assay (Fig.37). Besides the staining, the apoptotic cells were clearly defined by morphological changes. Firstly, cells exhibit normal size for the nucleus and cell body while there were numerous darkly stained bodies within the nucleus (clumping of nuclear chromatin). Later, degenerating cells show an extensive and tight condensation of nuclear materials and cytoplasm into a darkly stained small ball (pyknosis). The following stage consists of smaller degenerating cells with morphologic breaking up of the condensed nucleus and cytoplasm. Diverse morphological profiles were found at this type (disassembly of pyknotic cells). Finally, the condensed nucleus and cytoplasm are disassembled into many progressively smaller degenerating particles of debris (degenerating debris). All these phases were found in both genotype (Fig. 37A, B).

Degenerating cells were counted in the motor cortex of normal and null mice. Based on this count, the number of apoptoctic cells turned out to be higher when LPA_1 was lacking, about 15 per cent more of condensed cells (Fig. 38). This difference was significant, demostrating the loss of LPA receptor induces some kind of stress in the cells causing apoptosis.


PDI
A
PLP
B
Merged
C
Golph4
D
PLP
E
Merged
F
Lamp1
G
PLP
H
Merged
I

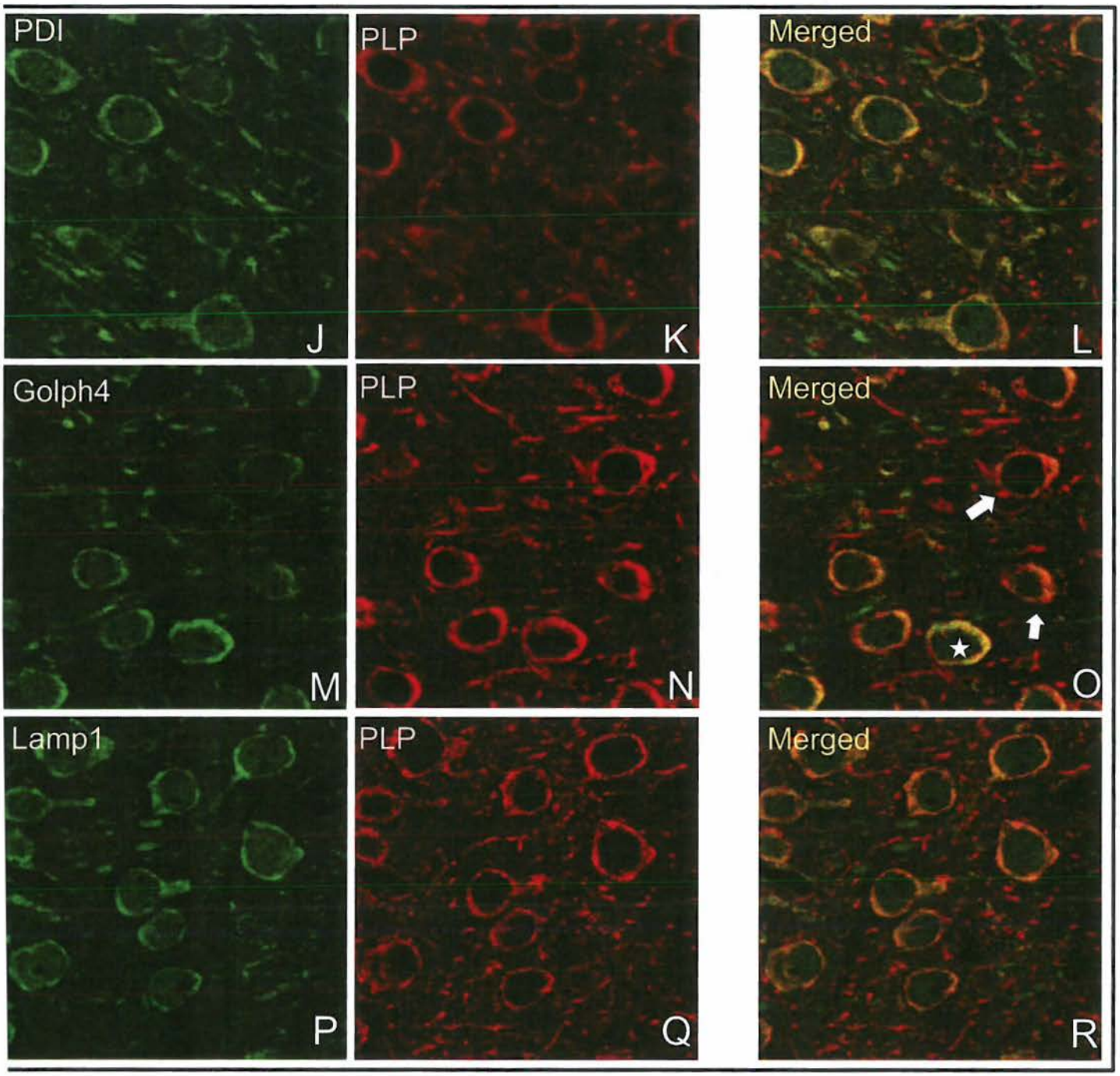


Fig. 36. Confocal images of double fluorescent immunostaining for PLP (B, E, H, K, N, Q), RER marker PDI (A, J); Golgi marker Golph4 (D, M) or endosomal/lysosomal sytem marker Lamp1 (G, P) and merged after combining (C, F, I, L, O, R) in coronal sections from normal (A-I) and null mice (J-R) cerebral cortex. In normal mice oligodendrocytes PLP did not colocalize with any of the markers (C, F, I). In contrast, PLP mostly colocalized with PDI (L), Goplh4 in some cells (O, asterisk) and Lamp1 (R) in absence of LPA_1.

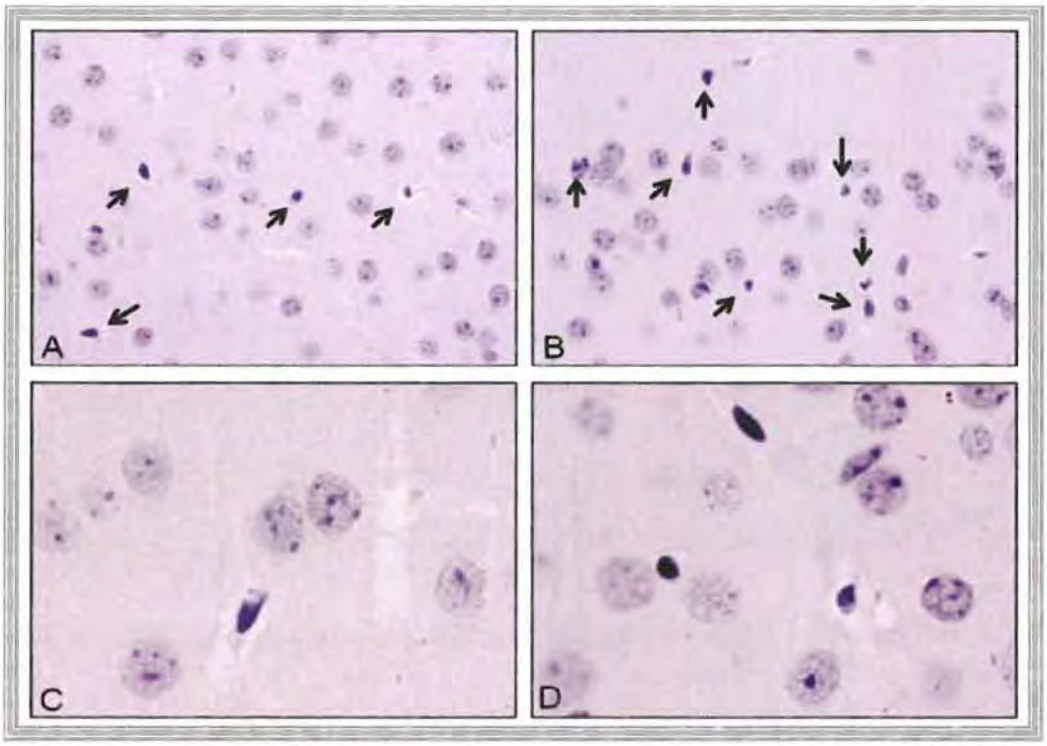


Fig. 37. Apoptotic cells detected by TUNEL assay in wild type (A, C) and knockout mice (B, D). Cells stained by TUNEL showing morphological changes (arrows) due to the normal process of apoptosis were observed in both genotypes, although its number seemed to be higher in the cerebral cortex of null mice.

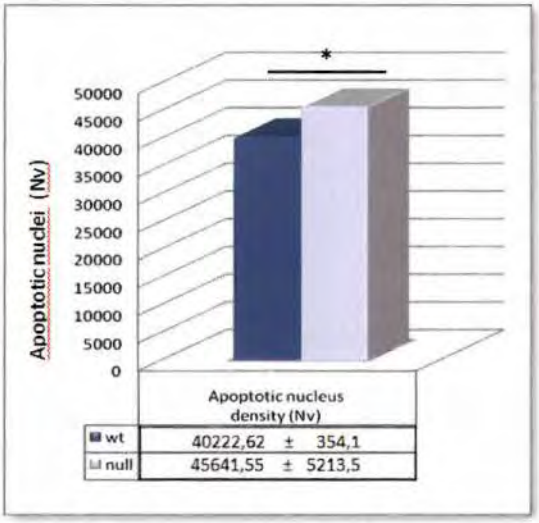


Fig.38 . Quantification of the density (Nv) of apoptotic cells stained by TUNEL assay in the cerebral motor cortex of normal (wt) and LPA_1-null mice. Quantification analysis showed a significant increase of 15% in the total number apoptotic cells in absence of LPA_1. (Data expressed as mean ± SEM; n = 4; * P < 0,01)

1.5. Defects of myelin pattern were showed in maLPA$_1$-null mice after the peak of myelination.

All of these alterations were studied in adult mice, however myelination is a process which starts after birth and develops during the first month of life. A possible explanation for much of these LPA$_1$-dependent defects is to be a consequence of anomalous oligodendroglial development. Thus, in order to discriminate their origin as well as the role of LPA in myelination the same studied as that performed in adults was achieved in two different younger stages. The first chosen age, postnatal day 10 (P10), was right before the peak in the expression of LPA$_1$ (P18), which coincides with the moment of the most active myelinization (Weiner et al., 1998). The other stage of interest was that when the process of myelination had just finished, P30 (Nakahara et al., 2001). The study was focused on the alterations on the cerebral cortex, due to it was in that region where the reduction of myelinated fibers and oligodendrocytes was more evident in adult mice, although other areas were also observed.

The differences in myelination pattern between both genotypes, normal and null, by P30 (Fig. 39) were quite similar to those reported throughout adult brain. Regarding cerebral cortex, lower densites of MBP- (Fig. 39A, B), PLP- (Fig. 39C, D) or CNPase-positive fibers (Fig. 39E, F) were showed in null mouse respecting wild type genotype, mainly in the upper layers. Some cells more intensely stained by PLP were also observed in null mice, as same as it happened in adults. Likewise, the number of oligodendrocyte labelled with GalC decreased in this region (Fig. 39G, H), although the number of oligodendrocyte precursor cells (OPC) (NG2-positive) remained equal, confirming the results obtained in adult mice (Fig. 39I, J).

In contrast, before myelination onset, no significant differences were showed in P10 null mice by comparison with wild type mice (Fig. 40). The amount of fibers stained by MBP (Fig. 40A, B) and CNPase (Fig. 40E, F) were very low and only found near corpus callosum. No fibers were yet present in the cortical upper layers. PLP staining was also different from that observed in adult, that is, soma cells expressed this protein without being sent to their process yet and the number of these PLP-positive cells was similar in both genotypes (Fig. 40C, D). Mature oligodendrocytes were also stained by GalC (Fig.40G, H) and counted by stereology, demostrating that presence or absence of LPA$_1$ did not affect to GalC-immunorreactive cell density (Fig.41A). In this line, the number of OPCs (Fig. 40I, J) were also counted not showing any variation either (Fig. 41B).

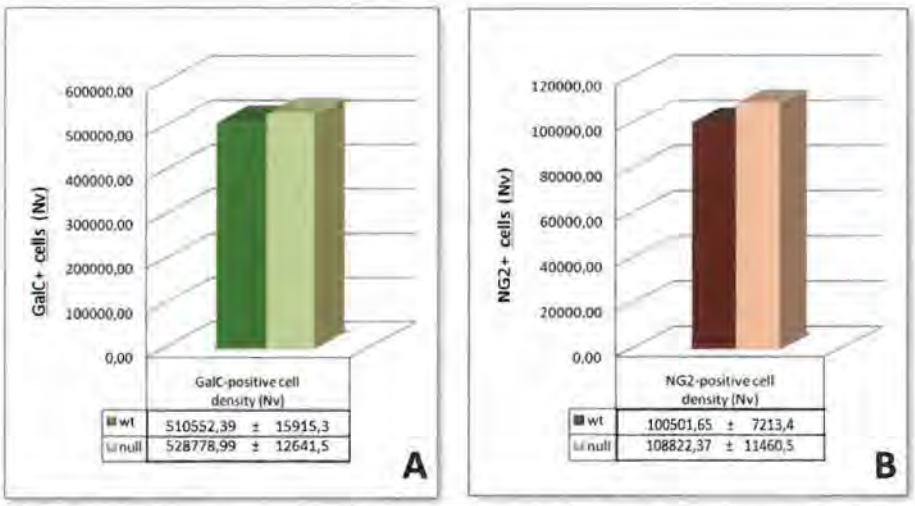
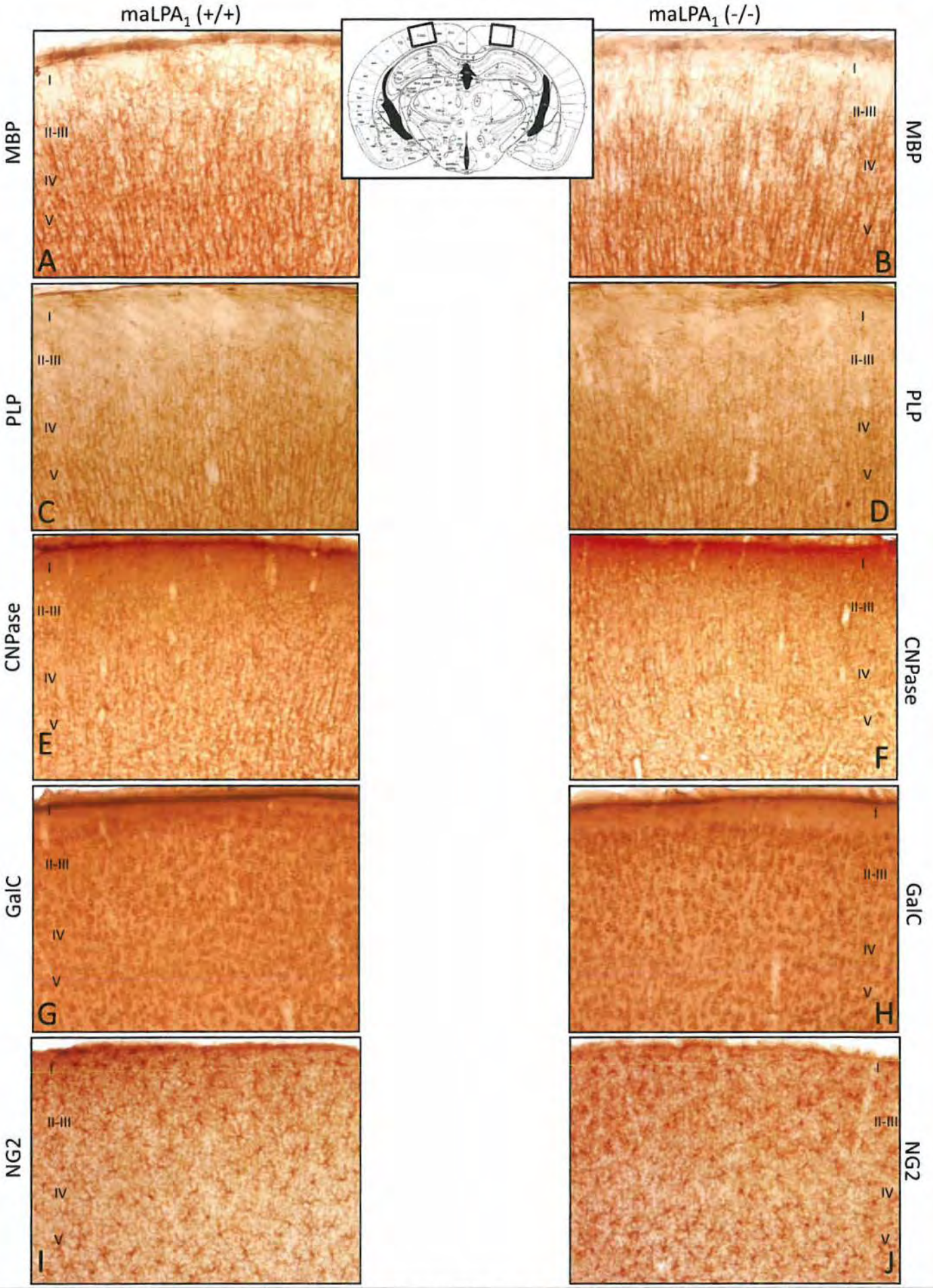


Fig. 41. Quantification of the density (Nv) of immunoreactive cells for galactocerebroside GalC (A) and proteoglycan NG2 (B) in P10 motor cortex of wild type (wt) and LPA$_1$-null mice. Quantification by stereological analysis did not show any significant variation in the number of mature oligodendrocyte (GalC+) or oligodendrocyte precursor cells (NG2+) in absence of LPA$_1$. Data expressed as mean ± SEM.



39. Expression of Myelin Basic Proteins (MBP), Proteolipid Protein (PLP), 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase), Galactocerebroside C) and proteoglycan NG2 in cortical coronal sections from P30 wild type and maLPA$_1$-null mice. A reduction in the density of MBP- , PLP- and CNPase- ive fibersin null mouse was reported (B, D, F), together with a fewer number of mature oligodendrocyte GalC+ (H). Unlike, the number of dendrocyte precursors was not altered by the absence of LPA$_1$.

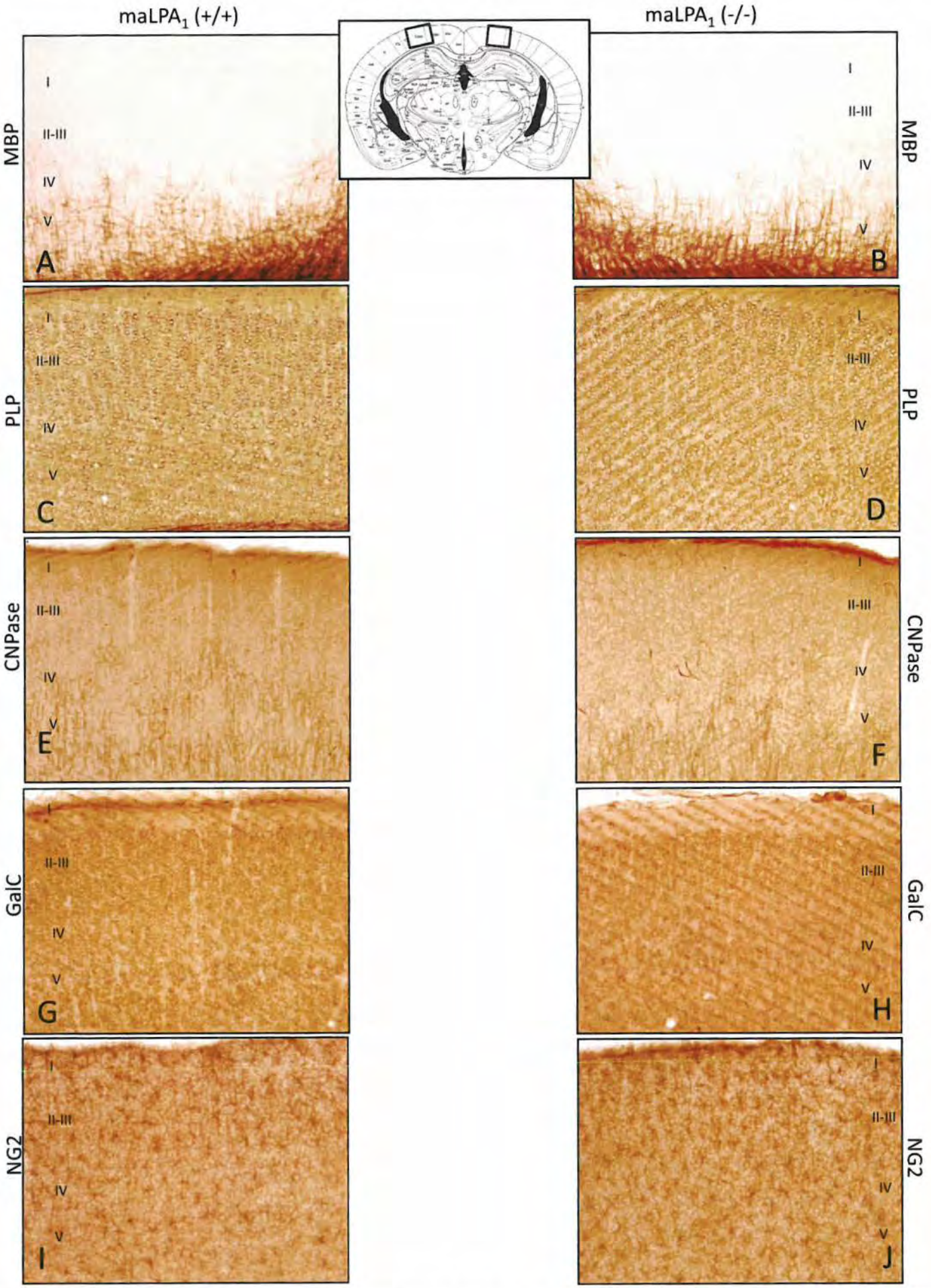


Fig. 40. Expression of Myelin Basic Protein (MBP), Proteolipid Protein (PLP), 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNPase), Galactocerebroside (GalC) and proteoglycan NG2 in cortical coronal sections from P10 wild type and maLPA$_1$-null mice. No differences were showed between both genotypes regarding myelinated fibers, oligodendrocytes or their precursors.

1.6. Effects of LPA in remyelination after cuprizone treatment.

Numerous studies have pointed out the role of LPA in myelination. The spatio-temporal distribution during postnatal development of its main receptor in CNS, LPA_1, is consistent with this role. Moreover, among LPA observed effects are included to induce neuroblast proliferation and migration (Contos et al., 2000; Fukushima, 2004; Fukushima et al., 2007), process retraction of oligodendrocyte precursors (Dawson et al., 2003), and to promote an increase in the area occupied by the oligodendrocyte process network by stimulating the extension of membranous structures (Nogaroli et al, 2009). In this line, our studies with maLPA_1-null mice lacking LPA_1 receptor have demonstrated that LPA is necessary for the correct myelination of the fibers. All these facts point out that LPA must play an important role in myelination.

Initially, oligodendrocyte precursors require a few dynamic processes for migration throughout the developing CNS. Later, after differentiation, mature oligodendrocytes extend many more stable processes, which myelinate axons (Baumann and Pham-Dinh 2001; Miller 2002).Then, formation and extension of these membrane processes are fundamental characteristics of oligodendrocytes in the CNS to ensheath nerves. Understanding how an oligodendrocyte regulates such changes in cellular morphology and defining the signalling molecules involved therefore is of interest and may have relevance for understanding why remyelination fails in demyelinating diseases, such as multiple sclerosis (Franklin 2002).

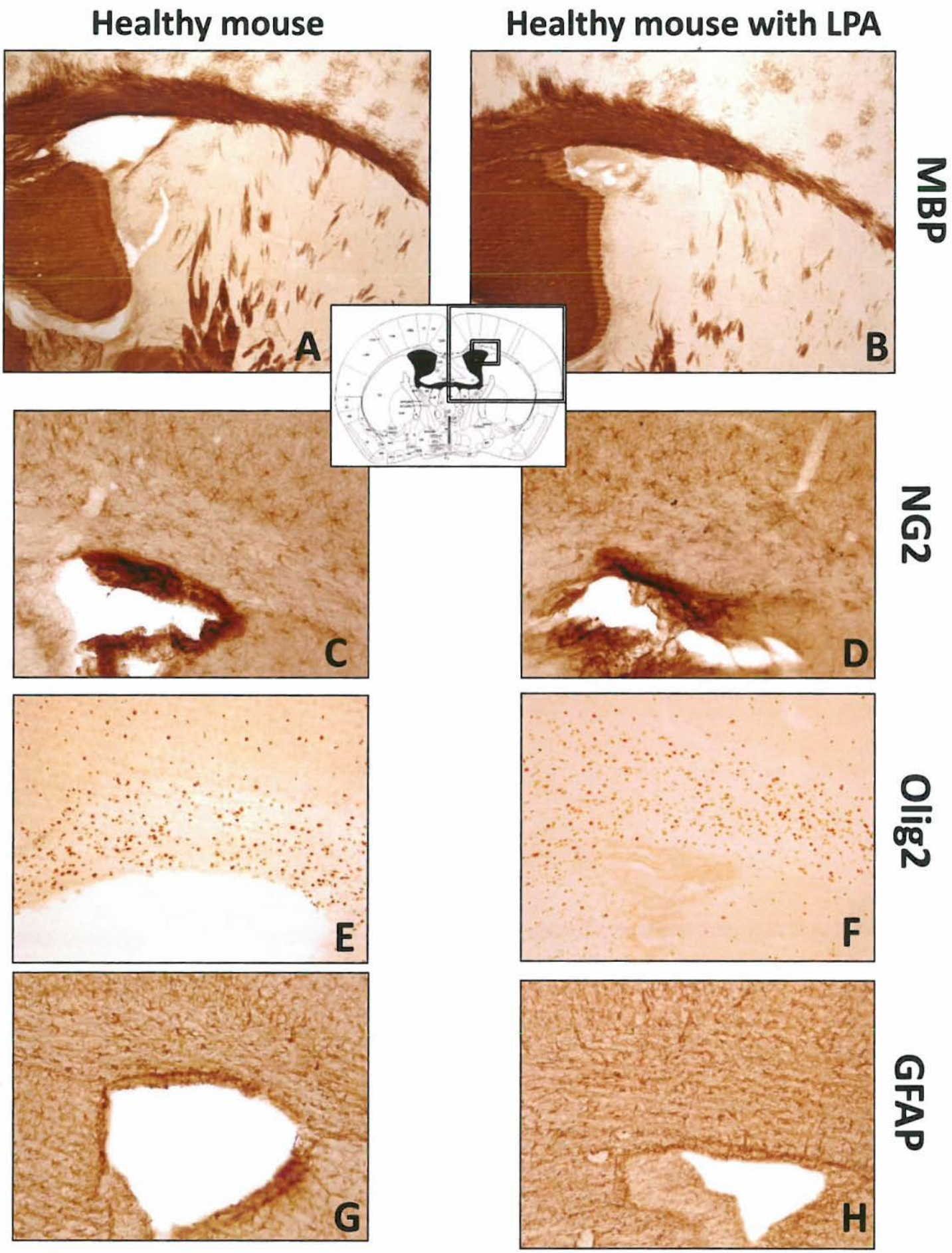
Due to the fact that LPA through its receptor LPA_1 is involved in both processes required for myelination, i.e. migration of oligodendrocyte precursors and formation of myelin processes, it might be considered as a good candidate to improve remyelination. Although LPA is non essential for myelination, it is worth to put forward a question, whether it can induce remyelination after fibers had been demyelinated.

Destruction of the myelin sheath of the central nervous system (CNS) is prominent in the physiopathology of many clinically relevant disorders. In addition to mouse models such as that inducing experimental autoimmune encephalomyelitis (EAE) for studying demyelination and remyelination, one of the best-characterized demyelinating mouse model is that of mice with 0.2% cuprizone to the diet (Matsushima and Morell, 2001). Several hypotheses have been proposed but it is still unclear why cuprizone, a copper chelator molecule, specifically affects oligodendrocytes. The physiopathology of the cuprizone model has been extensively evaluated with respect to variations in the cuprizone treatment time and dose, the identification of the fiber tracts that demyelinate, and the identification of the cellular types that are affected. Intoxication of young mice with 0.2% of cuprizone causes metabolic perturbation and death of mature oligodendrocytes which leads to a decrease in myelin proteins such as MBP and MAG (Hiremath et al., 1998; Mason et al., 2000a; Morell et al., 1998). This causes extensive demyelination of several well-myelinated tracts such as those of corpus callosum and the cerebellum (Blakemore, 1973). The first response to demyelination is an activation of microglia/macrophages (Arnett et al., 2003; McMahon et al., 2002) and reactive astrocytes (Hiremath et al., 1998), which occurs 1–2 weeks following cuprizone administration. As a response to demyelination and glial cell activation, at 3–4 weeks of cuprizone treatment, oligodendrocyte progenitors begin to accumulate within the lesion and become mature oligodendrocytes in 2 weeks, with remyelination occurring over the subsequent 4 weeks (Morell et al., 1998; Mason et al., 2000b; Mason et al., 2001) if, following 6 weeks of treatment, the cuprizonen is removed from the diet.

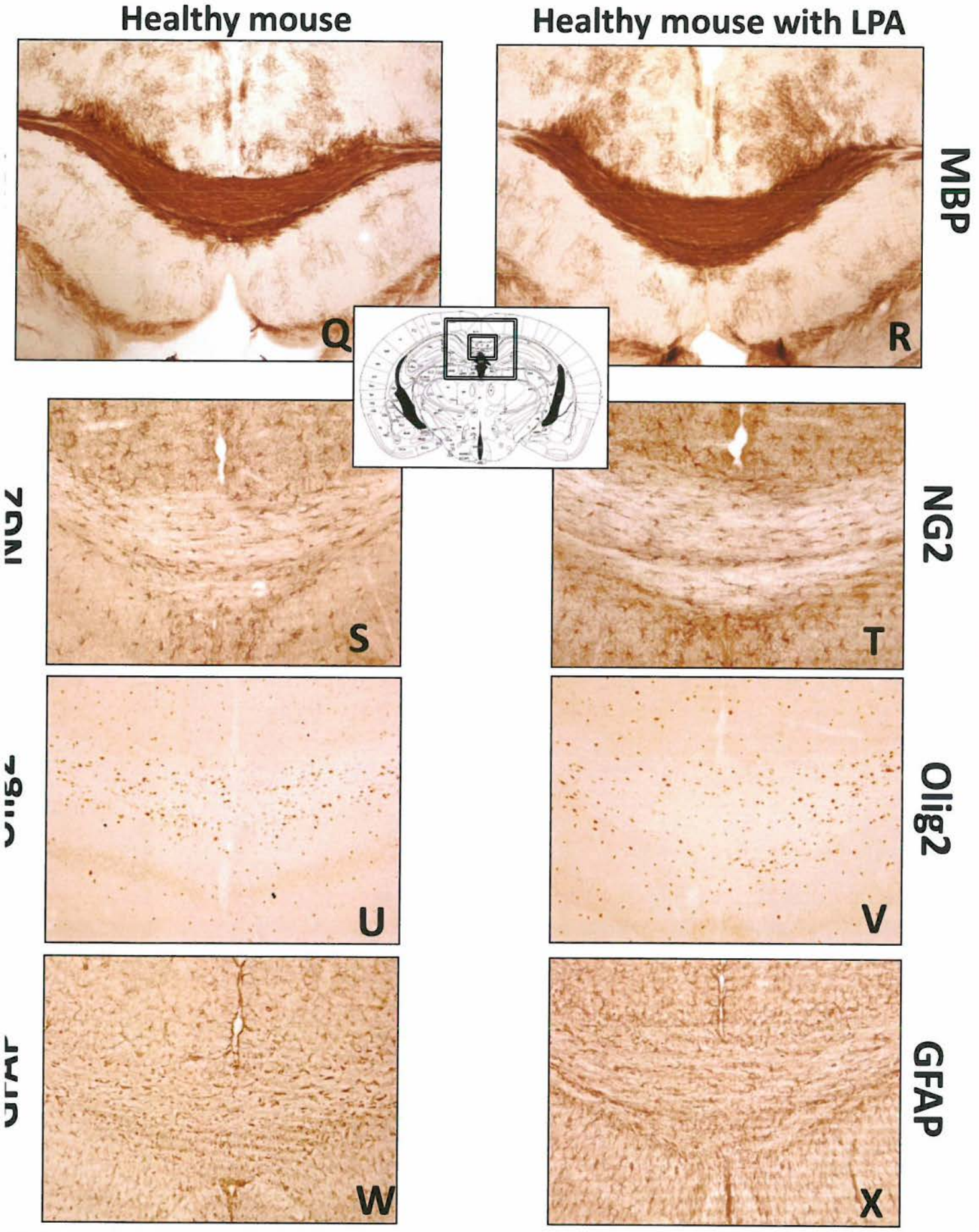
To this aim, non-hydrolizable LPA (C18:1) was intracisternally injected in 0.3% cuprizone-treated CNP-EGFP mice as demyelinating model and devepeloped in the Biomedical Research Institute in the University of Hasselt under Dr. Niels Hellings supervision, to provide insight into LPA role in remyelination.

Before evaluating its effects on remyelination LPA was intracisternally injected in healthy animals as safety assay control and for searching of possible side effects in normal situation . LPA did not cause itself any morphologically detectable changes in myelin. Thus, in absence of treatment, no differences in the myelin pattern were observed in CNP-EGFP healthy mice (Fig.42). Neither myelin fibers labeled with MBP (Fig.42A, B, I, J, Q, R), nor oligodendrocyte precursors labeled with NG2 (Fig.42C, D, K, L, S, T) or Olig2 (Fig.42E, F, M, N, U, V) nor GFAP-labelled astrocytes, (Fig.42G, H, O, P, W, X) showed any visible alteration after LPA administration. This indicates that, at doses used (5 µM range) intracisternal administration of LPA has not determining effects in healthy situations, neither stimulating proliferation nor inducing toxicity in oligodendrocytes or astrocytes.

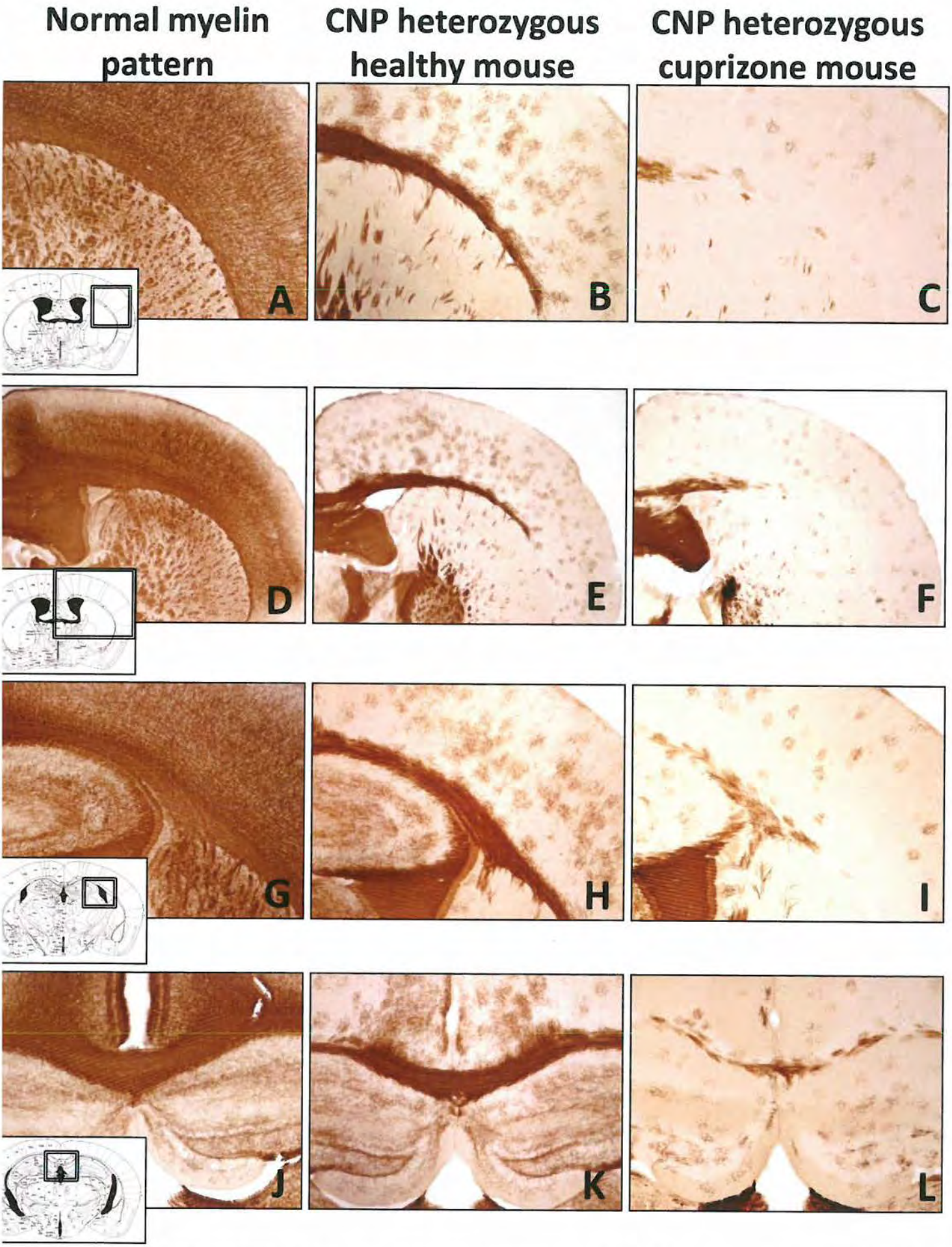
The evaluation of the animals in this case was done two days after the administration of the lipid. Since LPA stops inducing its effects eighteen hours after being administrated when exogenously added in culture (Dawson et., 2003), no longer time seemed to be suitable to observe some effects. At this time, an increased in the number of Olig2 expressing cells should had been showed in case LPA had induced oligodendrocyte proliferation, however this effect was not observed. Likewise, no increase in the number of astrocytes as GFAP-


Healthy mouse
Healthy mouse with LPA
MBP
A
B
NG2
C
D
Olig2
E
F
GFAP
G
H

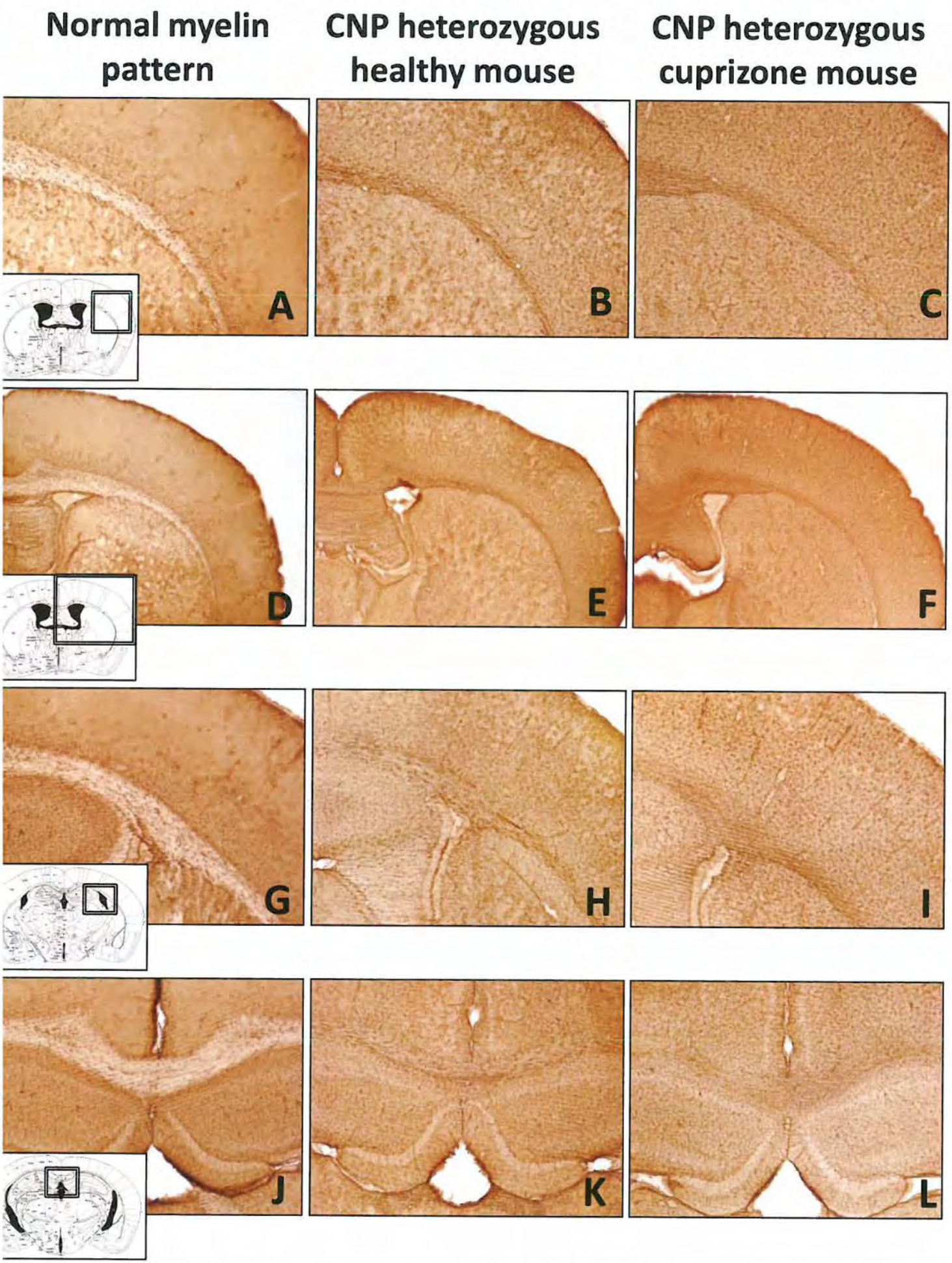
Healthy mouse
Healthy mouse with LPA
MBP
NG2
Olig2
GFAP
I
J
K
L
M
N
O
P



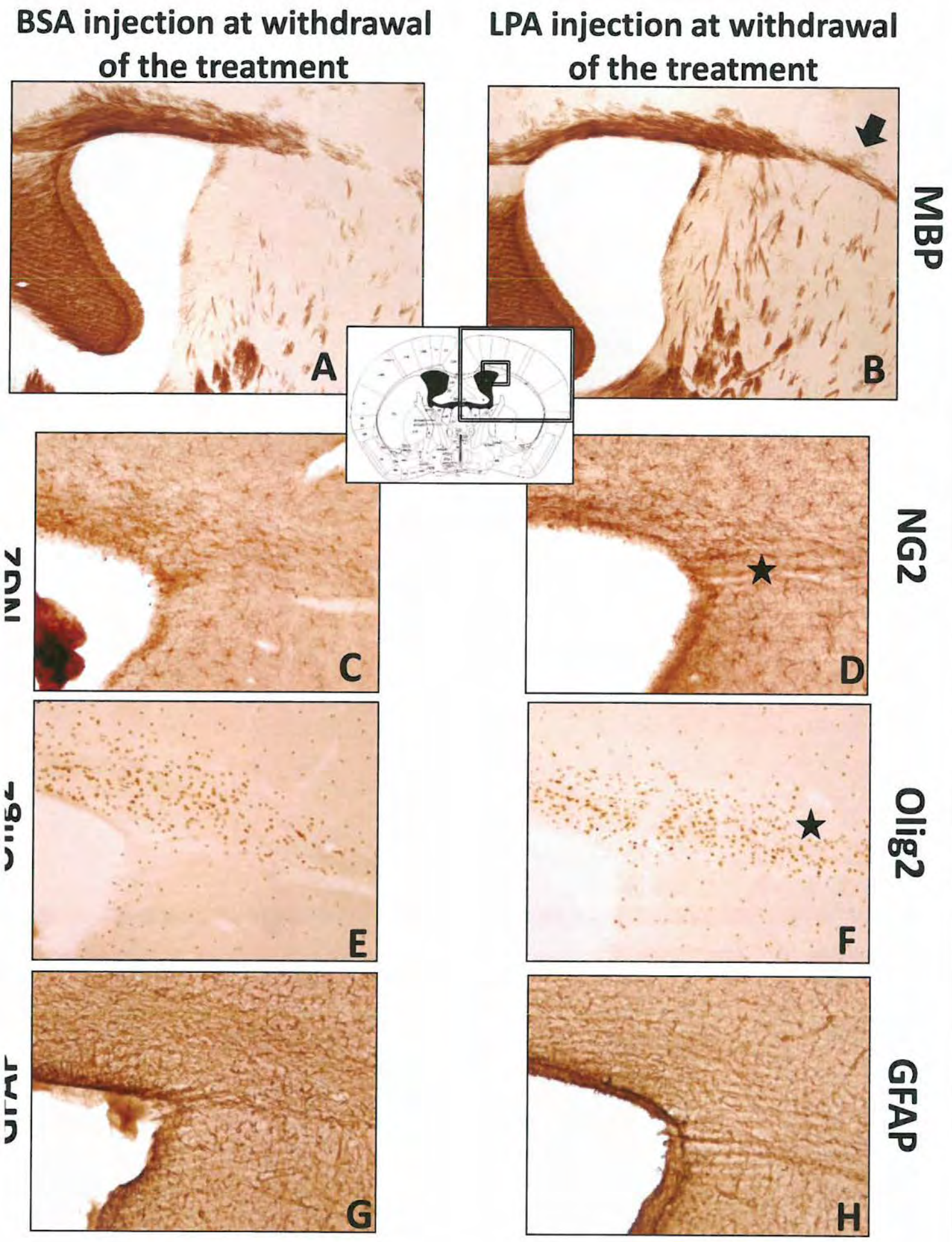
42. Immunoshistochemical analysis of Myelin Basic Protein (MBP), proteoglycan NG2, transcription factor Olig2 and Glial fibrillary acidic protein (GFAP) ıealthy CNP mice and animals treated with LPA intracisternally injected. LPA does not seem to affect the myelin pattern or proliferation of ɔdendrocytes or astrocytes in healthy mice.



43. Expression of Myelin Basic Protein (MBP) throughout the brain in mice of normal genotype (A, D, G, J), healthy heterozygous CNP mice (B, E, H, K) CNP mice under cuprizone treatment (C, F, I, L).



44. Expression of Glial fibrillary acidic protein (GFAP) throughout the brain in mice of normal genotype (A, D, G, J), healthy heterozygous CNP mice (B, E,) and CNP mice under cuprizone treatment (C, F, I, L).


BSA injection at withdrawal of the treatment
LPA injection at withdrawal of the treatment
MBP
NG2
Olig2
GFAP
A
B
C
D
E
F
G
H

BSA injection at withdrawal of the treatment

LPA injection at withdrawal of the treatment

MBP

NG2

Olig2

GFAP

I

J

K

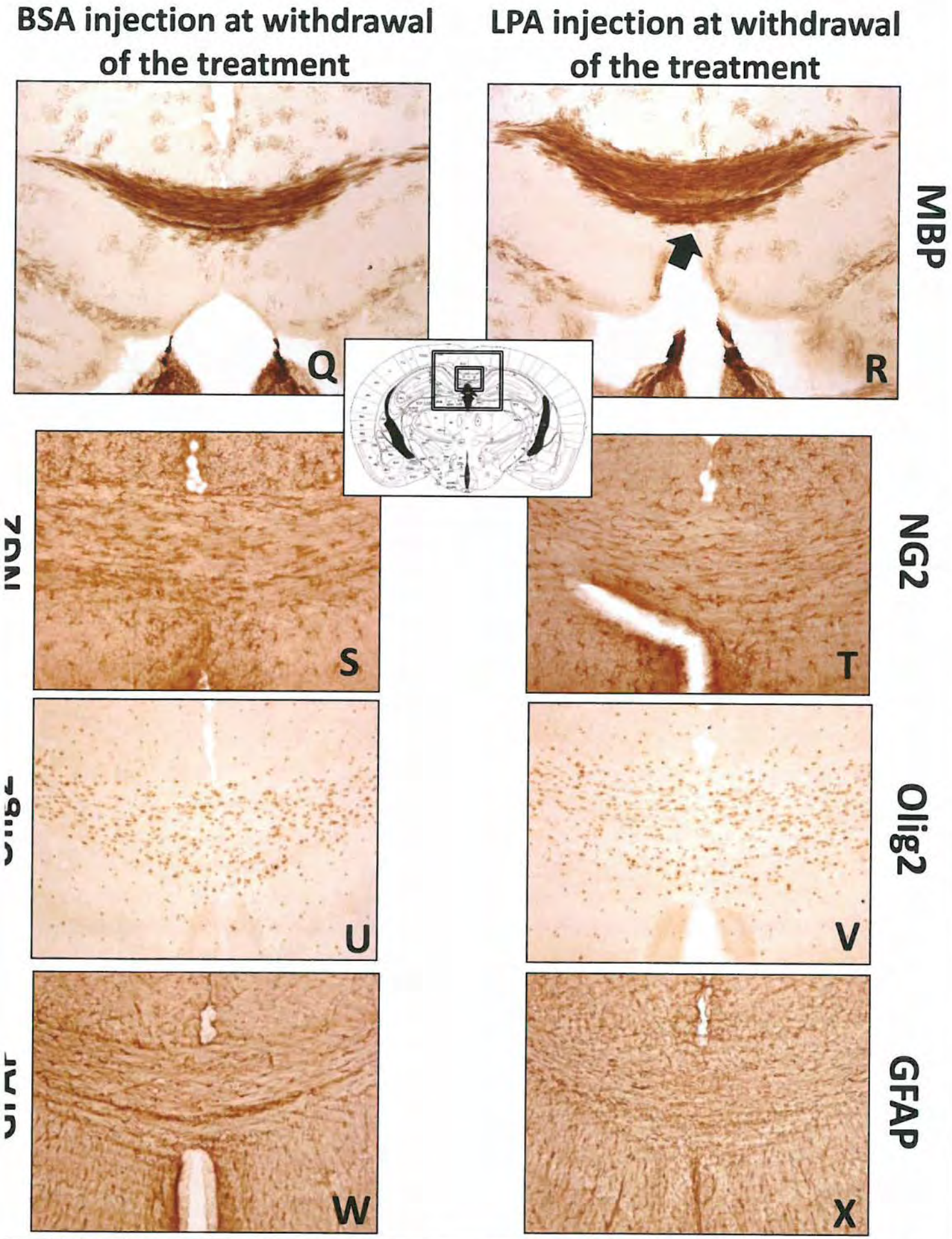
L

M

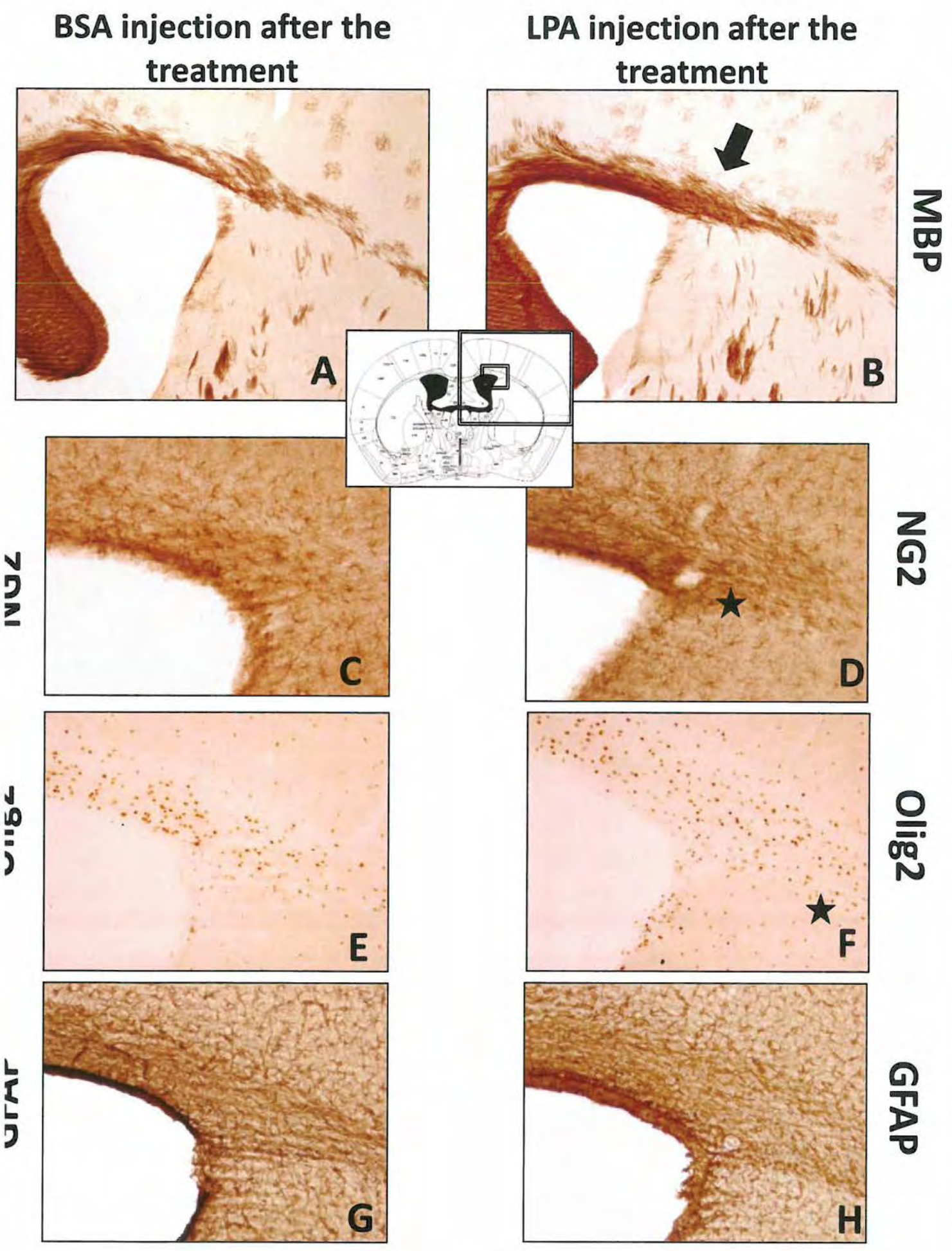
N

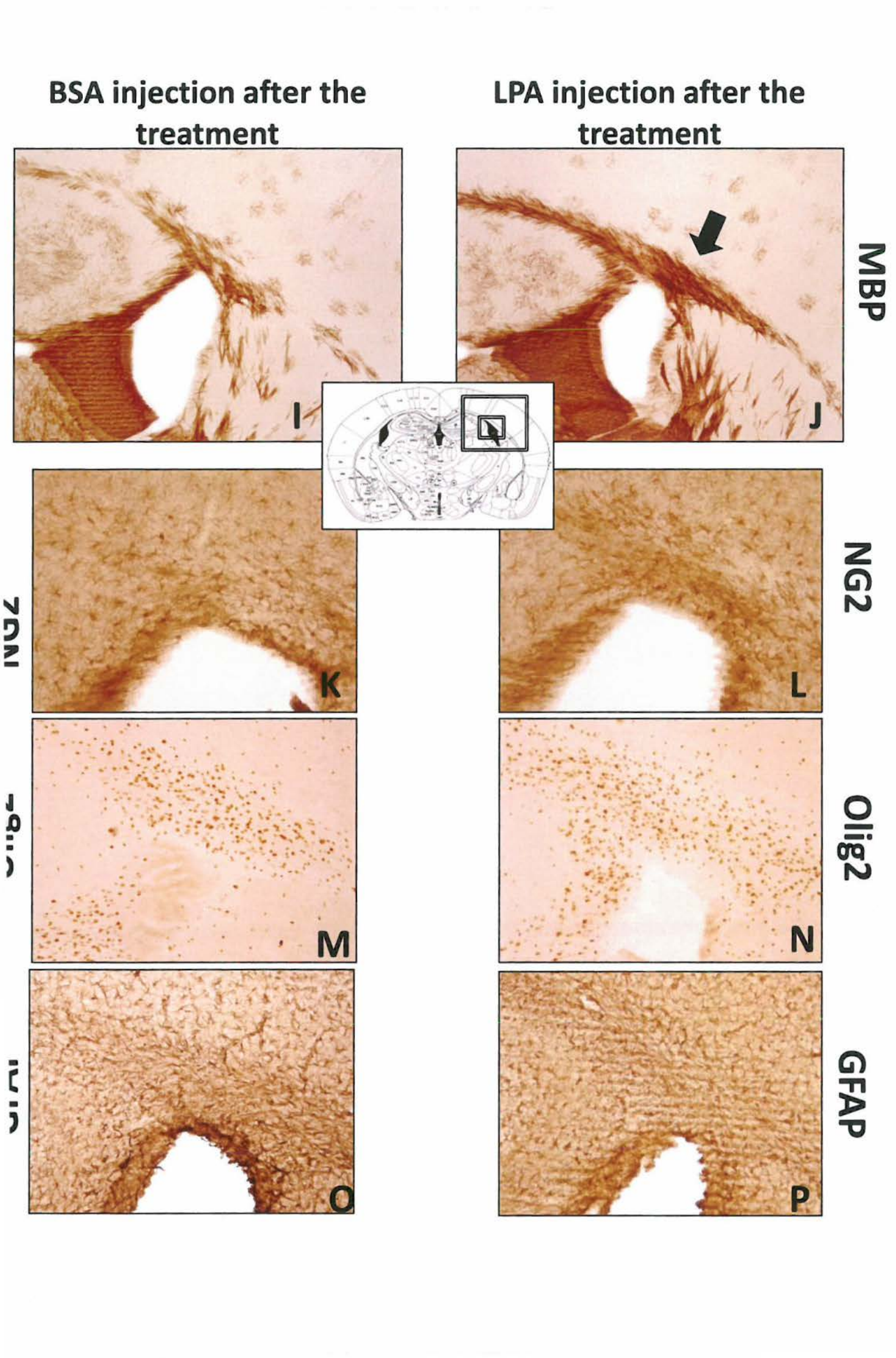
O

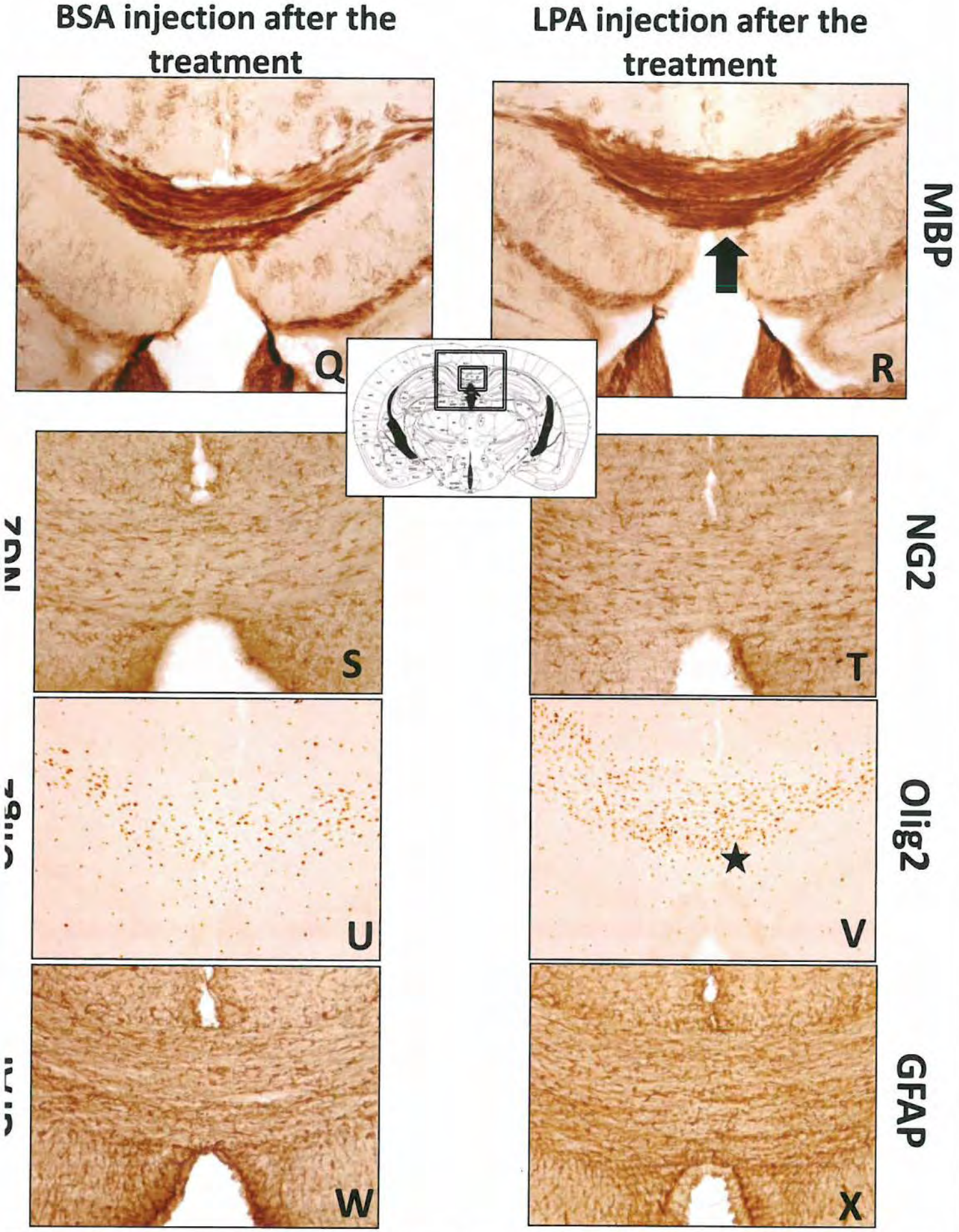
P



45. Immunoshistochemical analysis of Myelin Basic Protein (MBP), proteoglycan NG2, transcription factor Olig2 and Glial fibrillary acidic protein (GFAP) uprizone-treated CNP mice injected with BSA or LPA at the withdrawal of the treatment. LPA injections increased slightly remyelination (arrows) and odendrocyte proliferation (stars) in some regions .


BSA injection after the treatment
LPA injection after the treatment
MBP
NG2
Olig2
GFAP
A
B
C
D
E
F
G
H


BSA injection after the treatment
LPA injection after the treatment
MBP
NG2
Olig2
GFAP
I
J
K
L
M
N
O
P



46. Immunoshistochemical analysis of Myelin Basic Protein (MBP), proteoglycan NG2, transcription factor Olig2 and Glial fibrillary acidic protein (GFAP) uprizone-treated CNP mice injected with BSA or LPA after stopping the treatment. Remyelination was increased (arrows) as well as oligodendrocyte iferation (stars) when LPA was injected four days after the cuprizone treatment was finished .

positive cells was detectable, despite of previous works reporting LPA effects on astrocyte proliferation(Zeng and Zhang, 2007).

Evaluation of the cuprizone treatment efficacy was assessed by immunohistochemistry showingboth demyelination (loss of MBP label) (Fig. 43) and gliosis (extensive GFAP labelling) (Fig.44). Intracisternal injections ofBSA (vehicle) and LPA were performed at two different times to analyze the potential remyelinating effects of LPA, the day of treatment withdrawal from diet, and four days later.

When LPA was injected at the day of cuprizone withdrawal, no major differences were observed in remyelination (Fig.45A, B, I, J, Q, R). Only some regions, e.g. corpus callosum, seemed to exhibit an increase in the density of myelinated fibers (Fig. 45B, J, R) which correlated with a slightly higher number of oligodendrocyte precursors (Fig.45D, F, L, N). Nevertheless, LPA seemed not to have any effect in astrocytes and no signs on gliosis was observed in the LPA treated animal in comparison with BSA control ones (Fig. 45H, P, X).

In contrast, mice treated with LPA four days after the cuprizone treatment stopped, revealed an important increase in the number of myelinated fibers in corpus callosum (Fig. 46B, J, R). Not only MBP immunoreactivity was raised, but also NG2- and Olig2-positive cells were more numerous (Fig. 46D, F, L, N, S, T, U, V). In some areas, the effect pointed out more to an increase in the membranous extensions (Fig. 46L) than to a higher number of nuclei. Whereas, in other regions, the most outstanding effect of LPA was the presence of greater number of oligodendrocyte precursors instead of extensive staining of membranous structures (Fig.46T, V).

2. INFLUENCE OF LPA_1 RECEPTOR IN INFLAMMATORY PROCESSES

2.1. Absence of LPA_1 ameliorates the clinical symptoms of experimental autoimmune encephalomyelitis.

Multiple sclerosis (MS) represents the prototypic inflammatory autoimmune disorder of the central nervous system and the most common cause of neurological disability in young adults, exhibiting considerable clinical, radiological and pathological heterogeneity. A better understanding of the immunopathological processes underlying this disease have recently led to the design of numerous novel therapeutical approaches.

Like MS, the animal model experimental autoimmune encephalomyelitis (EAE) is characterized by CNS inflammation and demyelination and can follow a relapsing–remitting or chronic disease course. LPA has been involved in both, myelination and inflammation, as introduced, and from present results of this work. Thus, in the search for, not only new regulatory pathways to be considered as targets in the developing treatments but also for the development of animal models that could well serve for the analysis of neuroinflammatory and demyelinating mechanisms, we studied the effect of LPA_1 absence in EAE, particularly concerning neuroinflammatory events.

As it was explained in the introduction, LPA is clearly involved in inflammatory process. Lysophospholipids have the capacity to evoke and modulate immune responses by attracting and activating T-cells, B-cells and macrophages directly; and influencing their interactions with other cell types. Immune cells express multiple subsets of lysophospholipid receptors, which are critical for specific cellular responses such as proliferation and migration that are fundamental to immunity. LPA also regulates endothelial cells participating in inflammation processes via interactions with endothelial differentiation gene (Edg) family G protein-coupled receptors. In this sense, LPA_1 receptor is present in several types of immune system cells, like T lymphocytes, B lymphocytes, macrophages and microglia, as well as in endothelial cells. Then, its absence should report some kind of effect in response to inflammation. Since EAE is an autoimmune disease, it was used to study the role of LPA through LPA_1 in the immune system by analyzing the different clinical scores showed both genotypes.

EAE was induced under same conditions in both genotypes, normal and null for LPA_1. Then, the clinical courses in these mice were compared, showing some differences along the disease. The figure below depicts the mean clinical score of two groups of five animals per each genotype. Same similar results were obtained in three more independent experiments as well.

The first difference found regarding the loss of LPA_1 was a delay of a few days in the appearance of the symptoms developed by null mice by comparison with wild type animals (Fig. 47). This early effect due to the lack of LPA_1 was maintained along the disease ameliorating the score of the EAE. Despite this reduction in the severity of the clinic signs, both presented relapsing-remitting course with two or three relapses in the first fifty days, which turned into a more stable course without important variations on the symptoms. Then, the disease from that point was considered stabilized although small recovers or worsening could be eventually seen. At this stage of the disease, null mice showed less severe score and in some cases they were almost completely recovered exhibiting only some weakness in the tail (score 1).

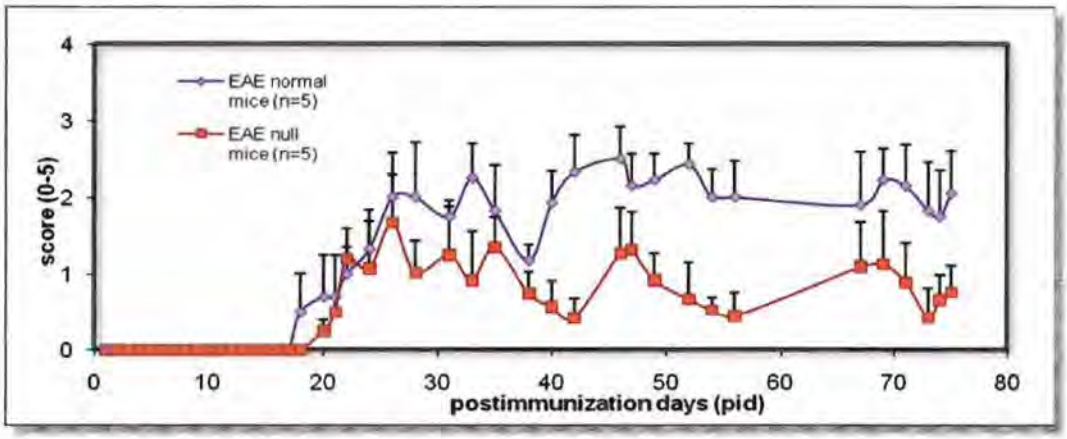


Fig.47. Experimental autoimmune encephalomyelitis (EAE) clinical signs were monitored in normal and LPA_1-null EAE-induced mice. The graph shows the development of EAE according to clinical scores (n = 5; Data expressed as mean ± SEM; SEM as bar indicated) in 1 out of 3 independent experiments. The lack of LPA_1 seemed to delay and ameliorate the symptoms after the immunization with MOG_{35-55}/CFA and pertussis toxin in all the experiments analyzed.

2.2. Expression of LPA_1 receptor increases during inflammatory processes in PBMCs and correlates with cellular infiltration and inflammation.

The fact that the absence of LPA_1 could ameliorate the EAE symptoms suggested that LPA could be involved in some role during the development of the disease. In this line, the study of Zheng et al. (2000) threw some light about the possible role of LPA_1 and LPA_2 in EAE. Accordingly, their results showed that mitogen activation of CD4+ T cells increase their LPA_1 expression in detriment of LPA_2. This shift of the expression of LPA receptors in activated CD4+ cells results in an increase of LPA-induced IL-2 secretion, a proinflammatory cytokine. Unlike, LPA induces the opposite effect, a decrease of IL-2 secretion, in inactivated lymphocytes which predominantly expressed LPA_2.

In agreement with this concept, in this study the expressions of *Lpar1* and *Lpar2* were measured in peripheral blood mononuclear cells (PBMCs) of control and EAE wild type mice by real time PCR and normalized to the expression of GAPDH as housekeeping gene. Levelsof relative expression were correlated with clinical score and histological evidence of EAE.

2.2.1 An increase in the expression of *Lpar1* in detriment to the *Lpar2* transcript levels seems to correlate to the initiation of EAE relapses.

Control animals were immunized with saline instead of MOG myelin peptide and using both pertussis toxin and Freund's complete coadjuvants.. No EAE symptoms were observed in these animals, showing normal gait and strength in tail and limbs. As it was described in the literature, these mice presented low *Lpar1* levels in PBMCs but, in contrast, the expression of *Lpar2* was high, resulting in a small *Lpar1*/ *Lpar2* ratio.

Analysis of the expressions of these two receptors in all mice in which EAE was induced by MOG_{35-55}, showed clear differences. In this first analysis, animals were not subdivided by their scores. Then, the levels of *Lpar1* transcripts increased almost three-fold in those animals developing the disease, while *Lpar2* expression dropped slightly (Fig. 48). Both the increase of *Lpar1* levels in EAE animals and the reduction of *Lpar2* transcripts were statistically significant.

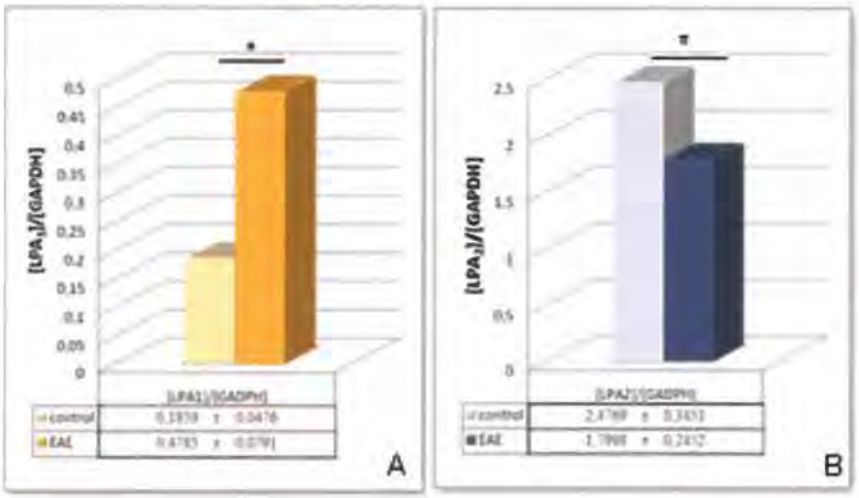


Fig. 48. Relative RT-PCR analysis of *Lpar1* (A) and *Lpar2* (B) expression normalized to that of GAPDH in EAE and control mice. The expression of *Lpar1* increased up to 3-fold in EAE mice. However, only a trend to decrease was observed in *Lpa2* in those animals, without statistical significance (* P< 0,05).

Despite this lack of significance in the fall of $Lpa2_2$ transcript levels during the clinical course, the *Lpa1*/ *Lpa2* ratio was measured in EAE mice and compared with control animals. In this case, these value demonstrated again to be determined by immunization because it resulted in a three-fold augmentation in immunized animals respecting control mice (Fig. 49).

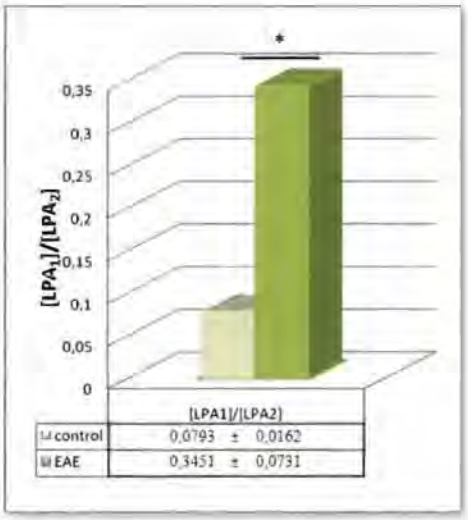


Fig. 49. Ratio of *Lpar1/ Lpar2* gene expression in EAE and control mice. Relative expression was measured byRT-PCR. The value of this ratio increased more than 3-fold in EAE mice (Data expressed as mean ± SEM; * P< 0,05).

Nevertheless, although the differences were significant in *Lpar1* expression and *Lpar1/ Lpar2*ratio, the intragroup variability was very high among immunized mice. This could be due to the fact that these animals were not sorted by their scores.

Trying to reduce the variability of *Lpar1* and *Lpar2* expressions into the EAE group, these values were analyzed together with their clinical courses. Owing to the need of sacrificing the animals to obtain enough blood for the test, the course of the disease couldn't be followed after it, however, the progression of the previous days may give some hints about which could have been the evolution of the disease after that.

As the expression of these two receptors could change along the disease in correlation with the moments of more active inflammatory processes, levels of expression were grouped according either their scores or duration of symptoms. This analysis showed that the corresponding increase and reduction of *Lpar1* and *Lpar2* transcription respectively were both more significant when mice had just started the relapse, so that, at the time of more active inflammatory processes. Likewise, after animals spent several days with the same symptoms or, after relapses, when the disease turned out a chronic course, LPA_2 returned to a normal values and LPA_1 expression decreased slightly.

Regarding this, the expressions of the receptors were sorted in those from animals exhibiting an *active EAE* course, which presented the symptoms of an early or new relapse, and those from animals displaying symptoms of a *stabilized EAE* course, so considered when animals prseented the same symptoms for at least three consecutive days. As it is showed in the figure, mice with *active EAE* demonstrated a higher increase in the *Lpar1* expression, being this slightly reduced when the clinical course reached the plateau. In the same reasoning, only mice exhibiting *active EAE* showed a decrease of *Lpar2* transcripts, regaining the normal values for *Lpar2* some days after relapse (Fig. 50).

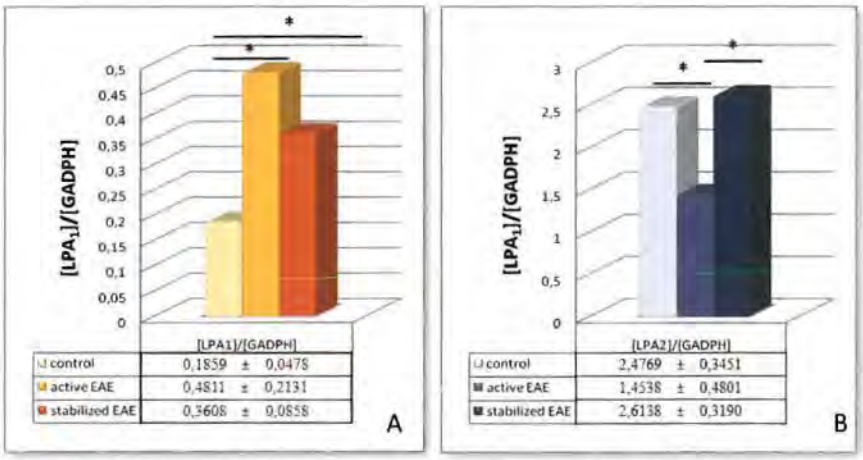


Fig. 50. Relative RT-PCR analysis of *Lpar1* (A) and *Lpar2* (B) expression normalized to that of GAPDH. The expression of *Lpar1* increased in EAE mice exhibiting *active EAE*. being this reduced after symptoms settled. Unlike, *Lpar2* transcript levels only decreased in those animals which recently started the relapse, returning to normal values when EAE was stabilized (Data expressed as mean ± SEM; * P< 0,05).

According to this, the ratio *Lpar1/ Lpar2* resulted as a good indicator of the state of the disease. At the onset of disease, when symptoms appeared and/or while EAE score/symptomatology increased, the ratio display the highest levels. Later, with the ongoing symptoms, these values decreased, remaining higher than in control animals but noticeably lower than in the mice with active disease (Fig. 51).

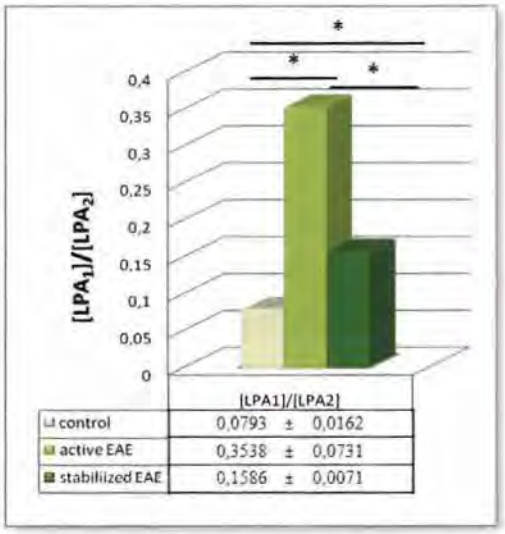


Fig. 51. Ratio of *Lpar1/ Lpar2* gene relative expression in EAE and control mice. The value of this ratio increased more than 4-fold in mice which have just presented a new relapse (*active EAE*). Whereas, in *stabilized EAE* mice this ratio decreased being still higher than in control animals (Data expressed as mean ± SEM; * P< 0,05).

In this analysis, the score the animals reached in every relapse was not taken into account. Nevertheless, a trend towards higher expression levels of *Lpar1* was observed when mice showed more severe symptoms. Since the expression of these receptors was measured in PBMC and not in immune cells inside CNS, it is reasonable to think the altered expression could be anticipated moderately to the symptom appearance. Then, this correlation between *Lpar1* receptor levels and symptoms severity could not be proved so far, due to the need of sacrificing of animals at the moment the expressions wanted to be analyzed. This implies that the score, those mice could have reached, were not seen. On the other hand, the same tendency was not observed with LPA_2 levels, no correlation was seen between its reduction and the severity of the symptoms.

2.2.2. The altered expression of LPA receptors seems to correlate to the infiltration of immune cells in the Central Nervous System.

The expression of both receptors was not only compared with the clinical courses but also with inflammation. This inflammation was evaluated through infiltration of T lymphocytes and macrophages, and presence of activated microglia. Infiltrates of T cells was revealed by CD3-immunostaining, whereas the presence of macrophage/microglia was immunodetected by the presence of OX42 antigen. Microglia constitute the resident macrophages of the brain, and in normal adult CNS are generally small-bodied cells with multiple branched extensions, called ramified (or resting). However, when microglia is activated undergo morphological changes exhibiting an ameboid shape with few processes. Both CD3 and OX42 staining demonstrated immune cell infiltration and microglia activation correlated with the EAE score of each individual.

In control animals, no infiltration of T lymphocytes (CD3-positive) or macrophages/activated microglia (OX42-positive) was found neither in brain (Fig. 52) nor spinal cord (Fig. 53). Only some OX42-positive cells were found in spinal cord of control mice (Fig. 53D, H), although since their shapes were ramified, they were considered inactivated microglia.

EAE animals were sacrificed at several stages of the disease showing different scores. In those animals, their inflammatory infiltrates were analyzed together with the expressions of both LPA receptor genes to draw a possible correlation.

In mice diagnosed with *active EAE*, which had just showed the symptoms before being sacrificed, the inflammatory infiltrates were extensive in brain and spinal cord, varying according to severity of the symptoms. In the brain, infiltration was localized above all in corpus callosum and other periventricular zones near choroid plexus where immune cells may have crossed the blood brain barrier. A great number of T cells were also found around some blood vessels in telencephalon and brainstem. In those regions, OX42-positive cells were abundant and showed the activated ameboid morphology. Same infiltration was found along the spinal cord, more evident in caudal than rostral levels. In these animals, as above mentioned, *Lpar1* expression was increased, the higher the more severe clinical signs were showed. Likewise, *Lpar2* decreased resulting in a high *Lpar1*/ *Lpar2* ratio.

In contrast, mice diagnosed with *stabilized EAE* and sacrificed after few days withsymptoms, showed less CD3-positive cells than observed in mice at the onset of symptoms. According to this, the amount of microglial cells was also scarce and they appeared scattered throughout the CNS. In some cases, their morphology remained ameboid, although a group of ramified OX42-positive cells appeared near foci of inflammation. This indicated a reduction of activated microglia in favour of such unactivated (or resting). Taken this together with the reduction of T cell number, it indicated that the inflammatory process started to diminish. In these animals, the *Lpar1* expression levels were lower than in the group stated before, although they remained higher than in control animals without EAE. On the other hand, *Lpa2*went back to the normal values, being indistinguishable from those measured in healthy mice. As a result, the ratio *Lpar1* / *Lpar2* showed higher values than in control mice but lower than those observed for mice at the onset of relapses.

In order to illustrate with some examples these processes, several individual cases with different scores will be presented here. In all of them, the expression of LPA receptor genes was correlated to the extent of the inflammation and clinical course.

The figures 54 and 55 show histological evidences of inflammation in coronal sections of a mouse with quite benign course of the disease. Regarding score, this animal only exhibited some weakness in the tail and always showed normal gait, so it barely scored 1 (limp tail). At the moment it was sampled, the symptoms had

just started to be present. The expression of *Lpar1* receptor normalized to the housekeeping gene in this situation resulted to be similar to the control animals (*Lpar1/GAPDH*: 0,071), whereas the level of *Lpar2* decreased considerably (*Lpar2/GAPDH*: 0,523). When the ratio of both receptors was calculated, it demonstrated a moderate value, according to a mild state of the encephalomyelitis (*Lpar1/ Lpar2*: 0,136).

In the brain, infiltrated CD3-positive cells were present in corpus callosum (Fig. 54A) which spread out along the septum. Apart from these structures, habenulas also showed asymmetric T lymphocyte infiltration (Fig. 54C), and also fewer T cells appeared in the optic tract (Fig. 54E) and more caudally in the dorsal mesencephalon (Fig. 54G). All those areas correlated with a higher presence of OX42-immunorreactive cells with ameboid morphology (Fig. 54B, D, E, G).

The spinal cord also showed histological evidences of inflammation (Fig. 55). In this case, infiltrates laid out around the vessels which surround the cord (Fig. 55G), and as it was expected by the mild symptoms, the number of T cells (Fig. 55A, C, E, G) and activated microglia (Fig. 55B, D, F, H) was moderate. However, although OX42-positive cells were no very abundant, all of them presented the ameboid morphology typical of activated cells (Fig. 55H).

Another immunized mouse followed a slightly more severe clinical course (Fig. 56), this time showing complete limp tail (score 1). These symptoms were reached the day before the animal was sacrificed, not having exhibited more than a partial weakness in the tail until that day. Corroborating what was mentioned before, the expression of *Lpar1* reached a bit higher level (*Lpar1/GAPDH*: 0,118) always in detriment of *Lpar2* transcription (LPA_2/GAPDH: 0,371), turning out in an increase of the ratio LPA_1/LPA_2, which was 0,319.

Histological slices demonstrated many places in which some infiltration of T lymphocytes occurred, such as septum (Fig. 56A), motor cortex (Fig. 56C), corpus callosum (Fig. 56E), somatosensorial cortex, optic tract (Fig. 56G), hypothalamus and mesencephalon. Paralelly, ameboid-shaped microglial cells were observed in all these regions (Fig. 56B, D, F, H).

In contrast, mice which had been showing the same symptoms for several days, or were recovering from more severe scores, revealed different expression levels of LPA receptors despite the clinical signs were similar to those mentioned before. A mouse which suffered hind limb paresis the day before to be sacrificed, but which recovered to a normal gait exhibiting only limp tail (score 1) the day sampled, was taken here as an example. Then, the *Lpar1* transcript level turned out to be very low (*Lpar1/GAPDH*: 0,038) while the expression of *Lpar2* also was near control values (*Lpar2/GAPDH*: 0,890), so the ratio was also in normal range (*Lpar1/ Lpar2*: 0,043).

When the sections were analyzed by immunostaining, scarce T lymphocytes were observed (Fig.57). Some of them were still present in optic tractus (Fig. 57E, G) and lateral cerebral peduncle (Fig 57G) whereas in the habenulas (Fig. 57C) or mesencephalon these cells were absent. In contrast to the previous example, where most of the OX42-immunoreactive cells showed ameboid morphology, this mouse also presented fewer cells resembling activated microglia, but many others with ramified shaped (Fig. 57D, H). These latter cells were observed at certain distance of the sites in which inflammation was common, such as habenulas (Fig. 57D). Since this morphology was characteristic of resting microglia, this suggested the inflammatory processes were remitting in those cases, but the symptoms remained while the damage caused was repairing.

As it was expected by the symptoms, the inflammation in the spinal cord in those two animals was more profuse than in the first one. Numerous T cells, which were extravased from different vessels surrounding the spinal cord, invaded the white matter (Fig. 58A, C, E and G). The same way, OX42 positive cells occupied extensive areas near the vessels (Fig. 58B, D, H), and sometimes covered the whole slice, mainly in the caudal levels (Fig. 58F).

The animals with more severe symptoms, reaching 3 of score on the clinical scale, also showed differences in LPA receptor expression and in histological inflammatory pattern based on the moment in which the symptoms were acquired; either just before the samples were taken (Fig. 59) or after they were maintained for several days before sampling (Fig. 60). In the first case, the transcript level of *Lpar1* was higher (*Lpar1/GAPDH*: 0,670) than in the second one (*Lpar1/GAPDH*: 0,391). And this same difference was reflected in the ratios; being 0,241 and 0,171 respectively. Despite these two increases in the *Lpar1* expression, the ratio *Lpar1/ Lpar2* did not result so raised. This was due to the fact the expression of *Lpar2* in both cases was high as

well. Since mice needed at least two or three days to reach the score 3, this could mean *Lpar2* only needed this time to return to normal levels. This fact suggested that its presence may constitute a mean to control the relapses of the disease.

Likewise, the histological analysis of the slices showed the same tendency. The animal which had just reached the score 3 showed a higher number of inflammatory cells present throughout the telencephalon and mesencephalon (corpus callosum (Fig. 59A), motor cortex, prefrontal cortex, caudate putamen (Fig. 59C), cerebral peduncles (Fig.59E), hypothalamus). T lymphocytes and microglia occupied a big extension, and these latter cells depicted again the typical ameboid morphology of activated cells. Furthermore, an important number of dilated vessels was observed, around which the infiltrates were settled (Fig. 59C, E).

On the contrary, when mice showed the clinical signs for some days, the number of T cells (CD3-positive) along the brain decreased (Fig. 60A, C, E and F) and ramified (resting) immunoreactive OX42 cells appeared (Fig.60B, D, H).

The spinal cord did not present any difference between those animals. The inflammation on both cases was profuse and these mice demonstrated some important T cell infiltrates (Fig. 61A, C, E, and G) which after crossing the endothelium invaded most of the white matter in the spinal cord. In this line, the immunoreactivity of OX42 also increased, covering the whole slice in every level analyzed (Fig. 61B, D, F and H), and making difficult to distinguish the individual cells.

Despite the numerous infiltrates present in the cerebral parenchyma of the immunized mice, none of them showed demyelinating plaques in the brain. The presence of some possible damage in the myelin was evaluated by immunostaining for one of the most abundant myelin proteins: MBP. This immunostaining was performed in every studied animal, and even those which showed severe symptoms (score 3) did not demonstrated any signs of myelin damage in the telencephalon. As an example of this lack of brain demyelination, the same mouse which recently scored 3 (Fig. 59), is presented here (Fig. 62B, D, F and H). Equivalent levels were chosen to demonstrate that, even in the regions in which extensive T cells and microglia infiltrations were revealed, the myelinated fibers remained intact and indistinguishable from control mice (Fig. 62A, C, E and G).

A prominent feature of brain injury and demyelination is astrocytosis. For this reason, the behavior of astroglia was also studied in those mice. The same animal as before is showed. As in many other individuals, an increase in the number of astrocytes near the site of inflammation was observed (astrocytosis) (Fig. 63B, D, F and H) when it is compared to healthy animals (Fig. 63A, C, E and G). This increase in GFAP-immunoreactivity in astrocyte cell bodies and processes means that remyelination may have occured when inflammatory cells invaded some specific region in the brain. Since a high regenerative capacity of cerebral regions was already demonstrated (Merkler et al., 2006), this astrocytosis might be the prove of an extensive and fast remyelination masking the presence of putative demyelinating plaques in those brain mice.

Nevertheless, due to the fact that inflammation was more profuse in the spinal cord, or maybe the remyelination was also less successful, extensive demyelination was observed in this structure. All the animals, even from score 1, showed demyelinating plaques in the spinal cord. This demyelination was more outstanding in the caudal levels and more abundant in mice with higher score. As an example of this effect, MBP stained slices of a mouse with hind limb paralysis (score 3) is showed here. In those pictures, unmyelinated patches were detected in the white matter along the spinal cord (Fig. 64B, D, F and H), in contrast to the strong immunoreactivity observed in those areas of control animal (Fig. 64A, C, E and G).

Like in brain, inflammation correlated to an increase of GFAP immunoreactivity and astrocytosis in the spinal cord. Here, the same animal as before is presented and compared with healthy mice (Fig. 65). A higher number of astrocytes with more processes were observed covering the regions in which the inflammation occurred. In animals in which inflammation was profuse, astrocytosis occupied the whole spinal cord. In contrast in control mice, astrocytes were only seen in the pial surface and around the vessels, being GFAP-immunoreactivity very low.

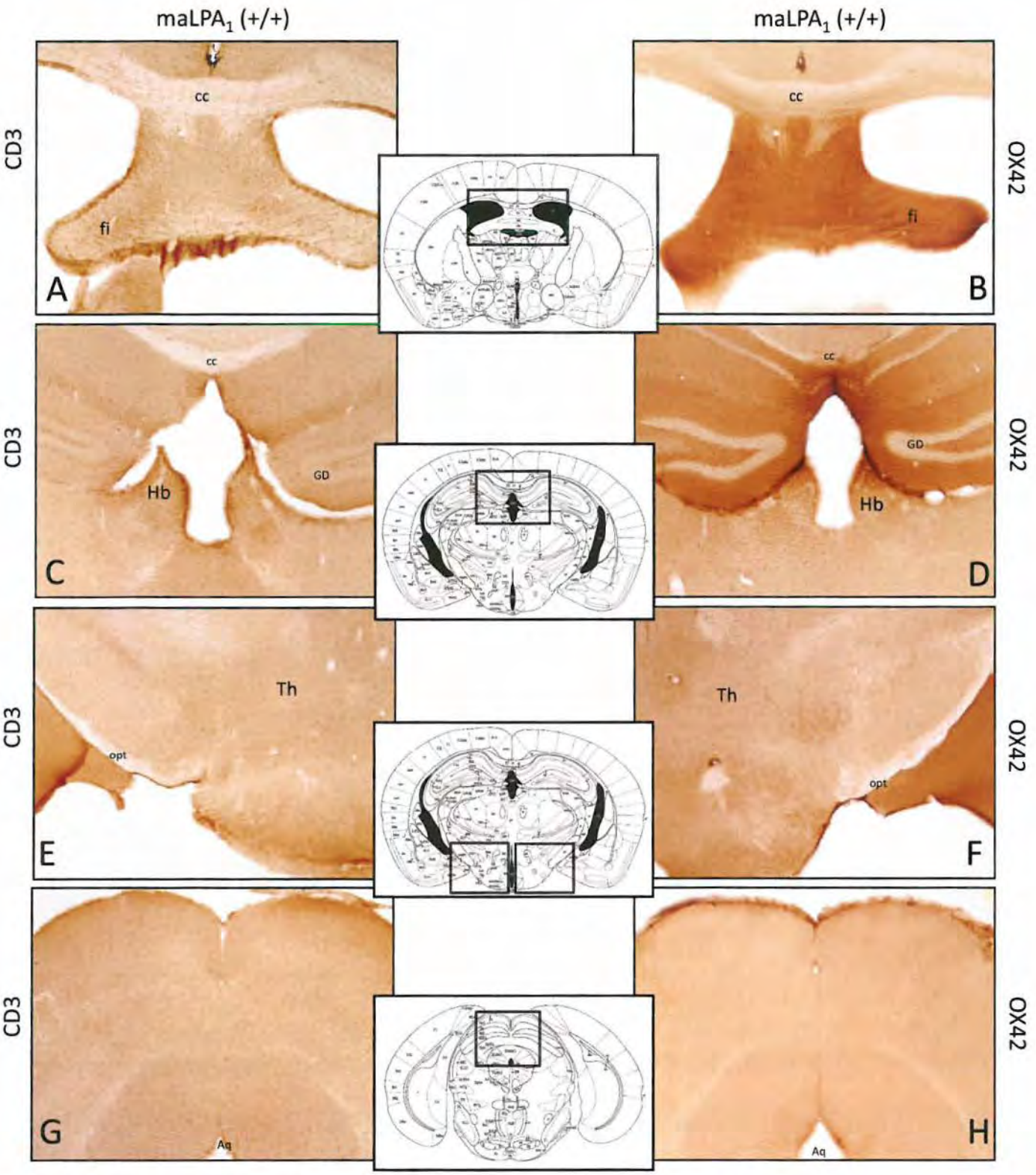
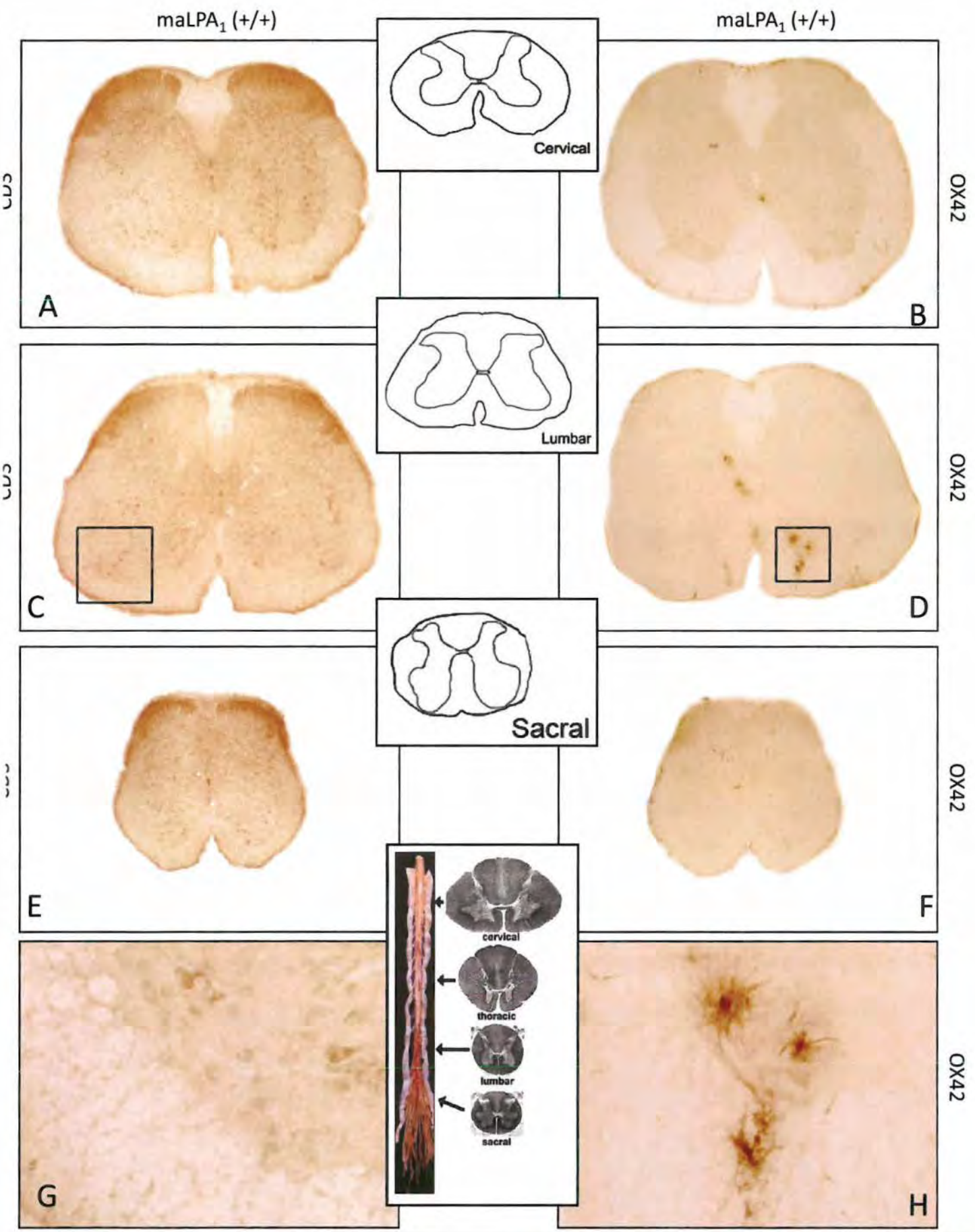


Fig. 52. Immunoshistochemical analysis of inflammation in coronal brain sections of control EAE mice. The presence of T cells was depicted by CD3 staining, whereas macrophages/microglia were marked by OX42. In control mice, no positive cells to neither of these proteins was found, demonstrating there was not inflammation. As it was mentioned in the literature, the expression of *Lpar1* was low in these mice (*Lpar1 /GAPDH*: 0.1859) and *Lpar2* levels were high (*Lpar2/GAPDH:* 2.2696), resulting in a very low ratio (*Lpar1/Lpar2*: 0.0793)



ig. 53. Immunoshistochemical analysis of inflammation in coronal spinal cord sections of control EAE mice. The resence of T cells was depicted by CD3 staining, whereas macrophages/microglia were marked by OX42. In control mice, o positive cells to CD3 was found, and only few unactivated OX42-immunoreacitve cells appeared in some levels. This howed there was not inflammation. The expression of *Lpar1* was low in these mice (*Lpar1 /GAPDH*: 0.1859) and *Lpar2* evels were high (*Lpar2/GAPDH:* 2.2696), resulting in a very low ratio (*Lpar1/Lpar2*: 0.0793)

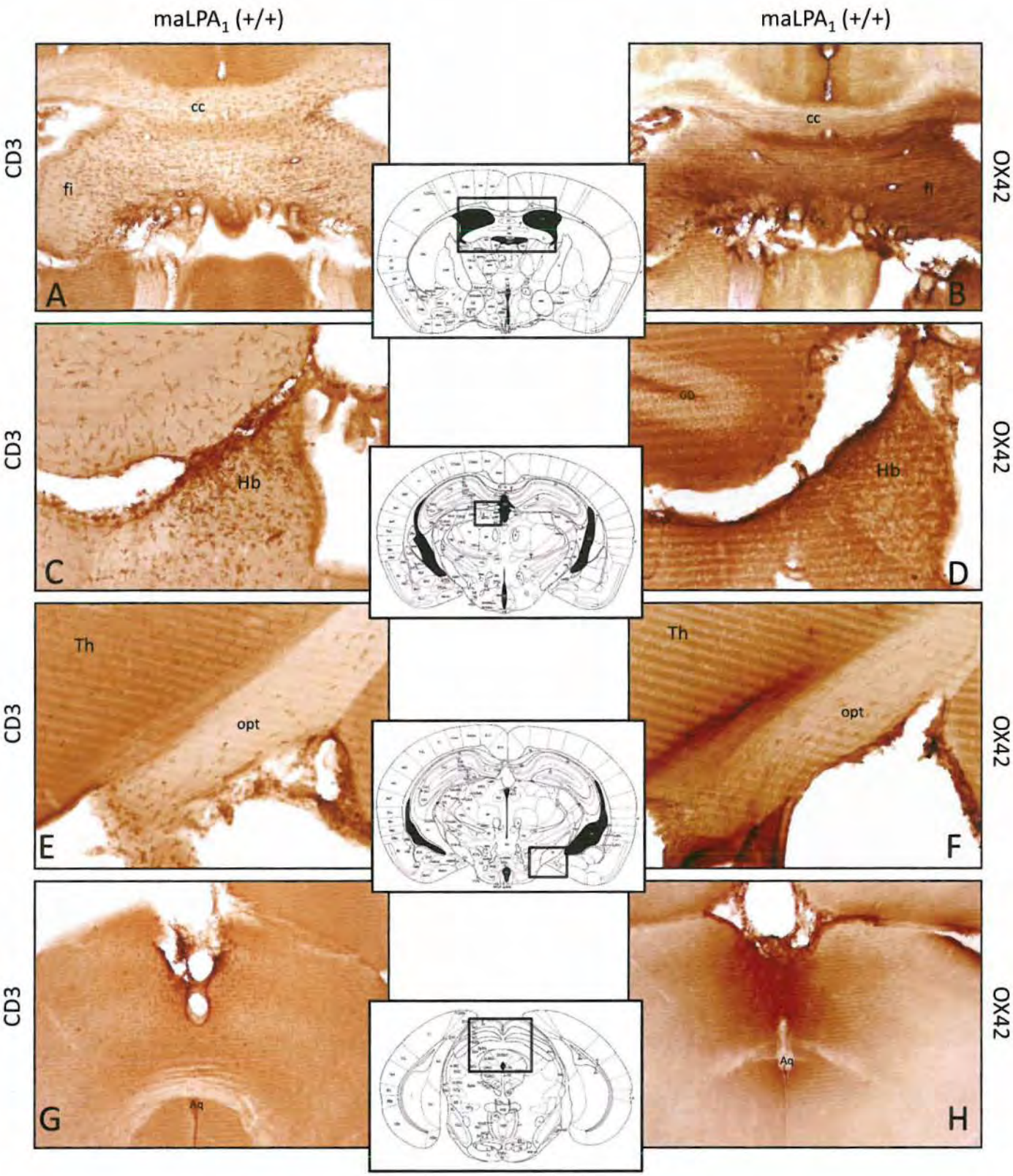
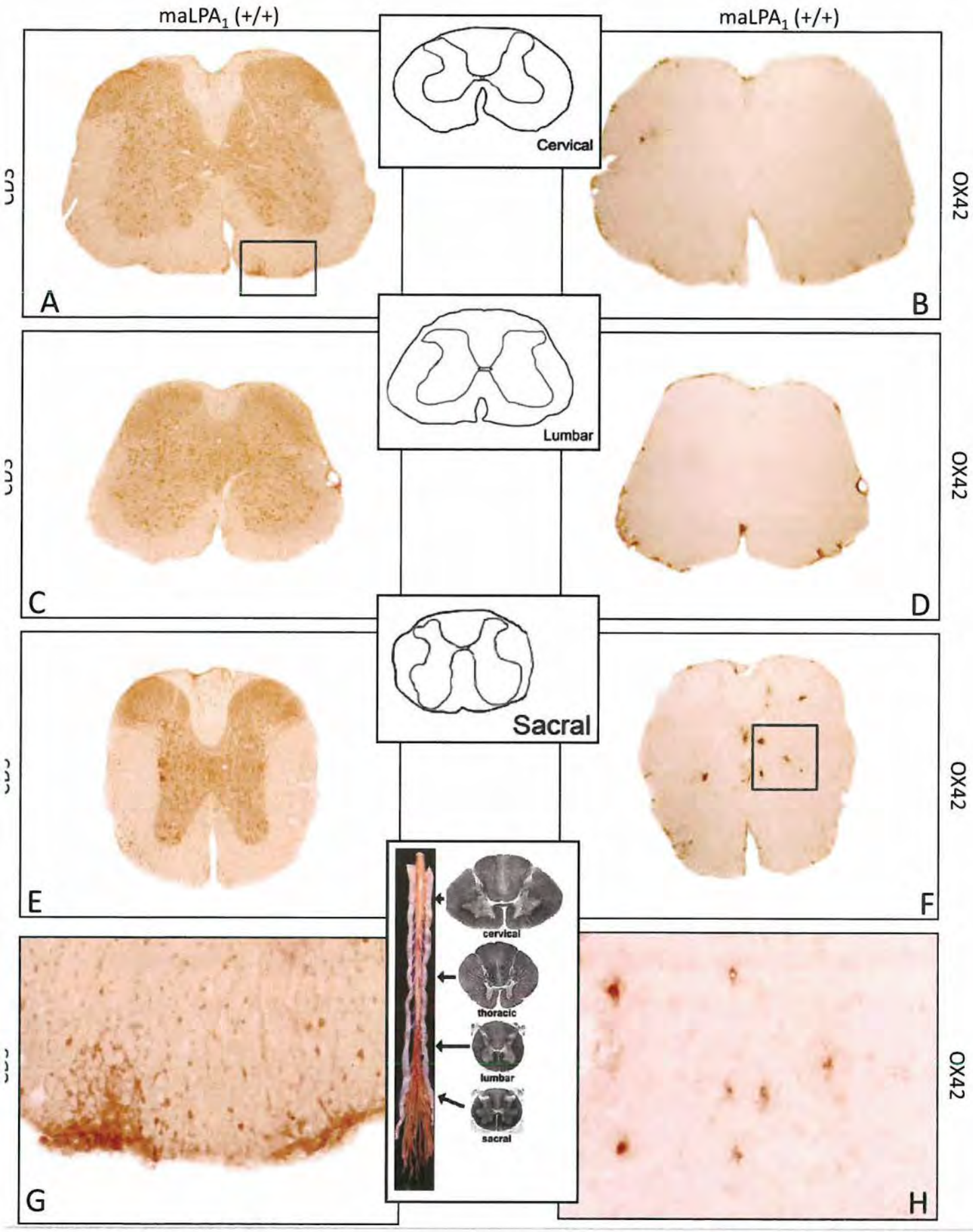


Fig. 54. Immunohistochemical analysis of inflammation in coronal brain sections of EAE mice showing a mild course of the disease (score lower than 1). The presence of T cells was depicted by CD3 staining, whereas macrophages/microglia were marked by OX42. Infiltrated T cells were found in corpus callosum (cc) (A), fimbria (fi) (A), septum, habenula (Hb) (C), optic tractus (op) (E) and brainstem (G). Immunoreactivity to OX42 correlated to these areas (B, D, F, H). The expression of *Lpar1* was low in these mice (*Lpar1 /GAPDH*: 0.0713) but *Lpar2* levels decreased (*Lpar2/GAPDH:* 0.523), resulting in a moderate ratio (*Lpar1/Lpar2*: 0.136)



. 55. Immunoshistochemical analysis of inflammation in coronal spinal cord sections of EAE mice showing a mild course the disease (score lower than 1). The presence of T cells was depicted by CD3 staining, whereas macrophages/microglia re marked by OX42. According to the mild symptoms, few T cell infiltrates were found surrounding the vessels (A, C, E, G) d the number of OX42-positive cells was also low, in this case showing an ameboid shape. *Lpar1* expression was low in this ce(*Lpar1/GAPDH:* 0.0713)but *Lpar2* level decreased (*Lpar2/GAPDH*:0.523), resulting in moderate ratio (*Lpar1/Lpar2*: 0.136).

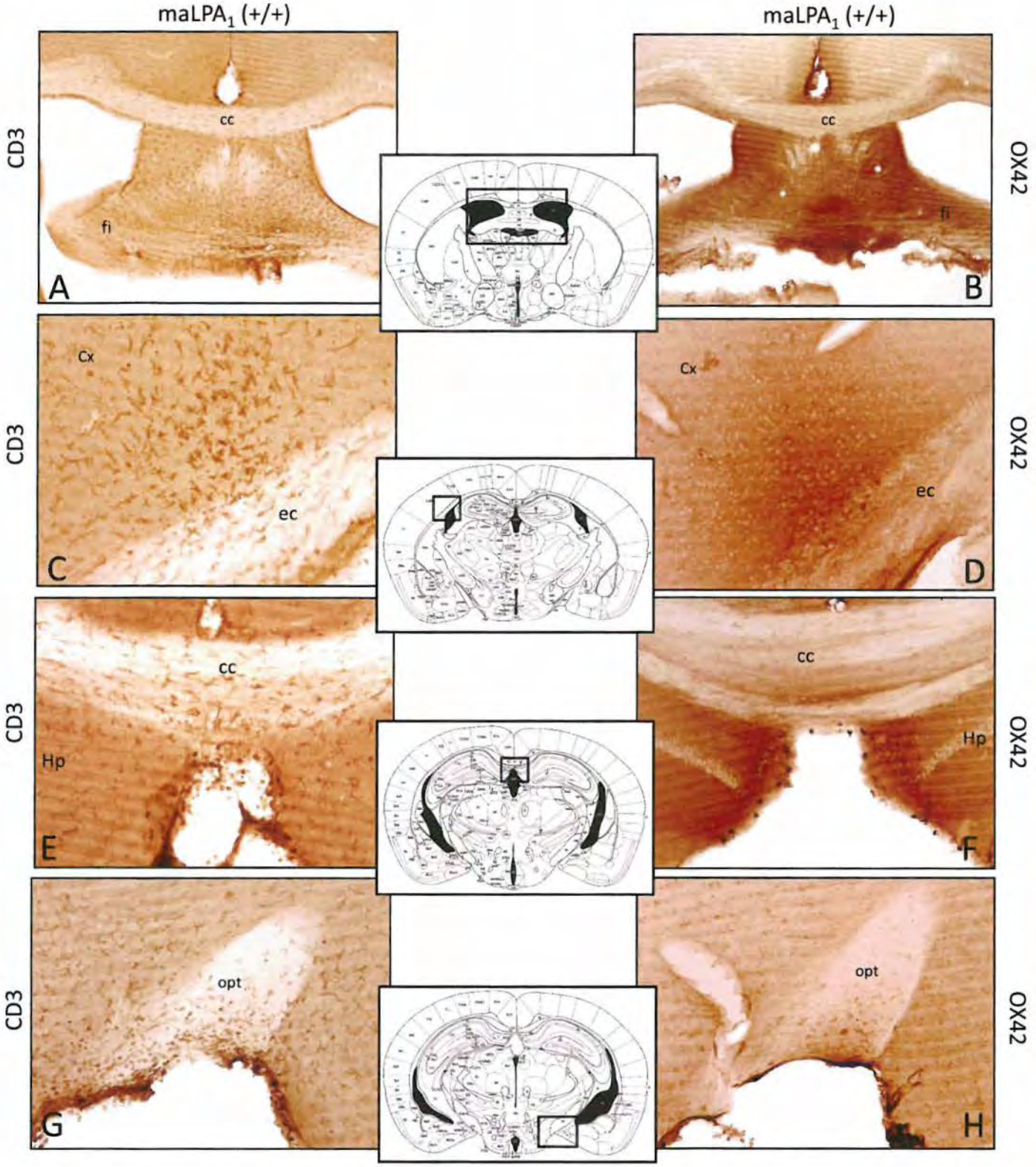
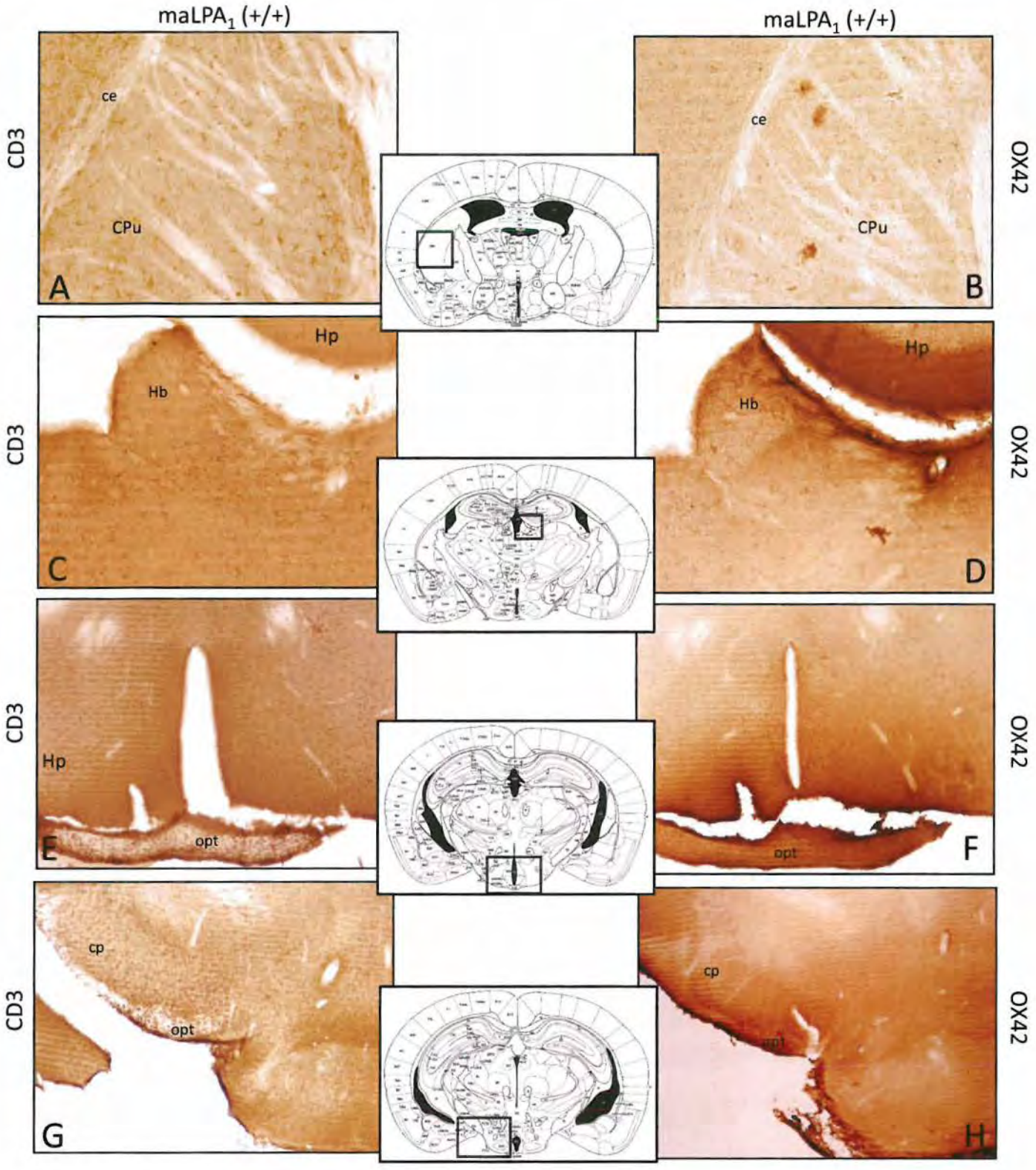
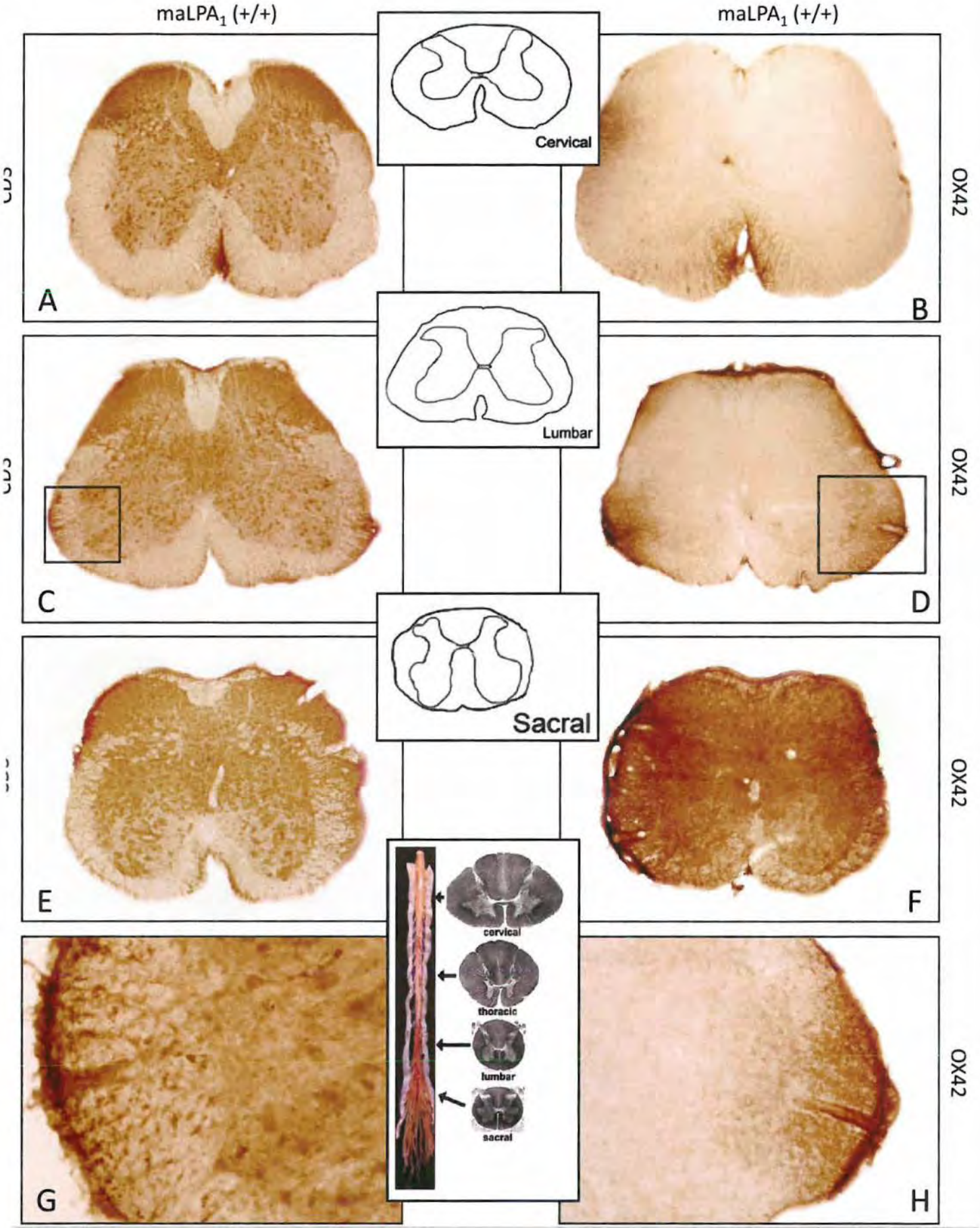


Fig. 56. Immunoshistochemical analysis of inflammation in coronal brain sections of an *active* EAE mouse showing score 1. The presence of T cells was depicted by CD3 staining, whereas macrophages/microglia were marked by OX42. Infiltrated T cells were found in fimbria (fi) (A), cerebral cortex (Cx)(C), corpus callosum (cc) (E) and optical tract (opt) (G). Ameboid OX42-positve cells appeared in the same areas (B, D, F, H). LPA_1 expression increased slightly in this mice (*Lpar1/GAPDH:* 0.118) and *Lpar2* level decreased (*Lpar2/GAPDH*:0.371), resulting in moderate ratio (*Lpar1/Lpar2*: 0.319).



ig. 57. Immunoshistochemical analysis of inflammation in coronal brain sections of an EAE mouse showing score 1 ıfter recovering form more severe symptoms. CD3 staining revealed the presence of T cells, whereas OX42 antibody narked macrophages/microglia. T cell infiltrates were scarce, only some cells were present in optical tract (opt) (E, G) ınd cerebral peduncles (cp) (G). Ameboid OX42-positive cells appeared in these same areas (F, H) but some of ramified nicroglia were showed in the areas there is no inflammation anymore, such as habenulas (D) or caudate putamen (Cpu) B). *Lpar1* expression was lower than in the previous animal, (*Lpar1/GAPDH*: 0.038) and *Lpar2* level returned to higher evels (*Lpar2/GAPDH*: 0.890), decreasing the ratio (*Lpar1/Lpar2*: 0.043).



58. Immunoshistochemical analysis of inflammation in coronal spinal cord sections of EAE mice showing score 1. The sence of T cells was depicted by CD3 staining, whereas macrophages/microglia were marked by OX42. The histological dences of inflammation in spinal cords were similar in animals with *active* or *stabilized* EAE showing score 1. In these cases, ammation was more extensive, numerous T cells were showed surrounding the vessels (A, C, E, G) which correlated with 42-positive cells, showing again an ameboid shape.

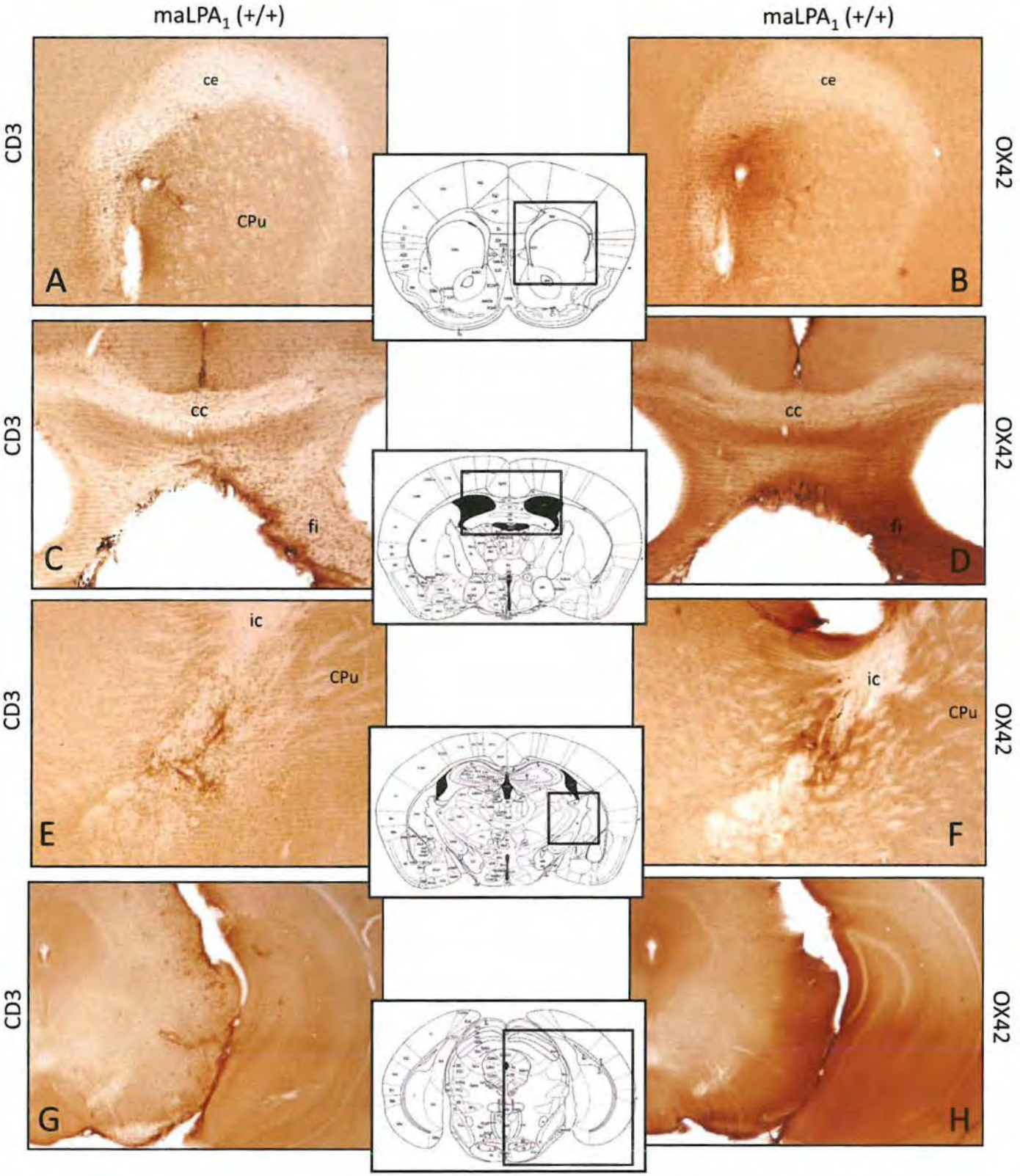
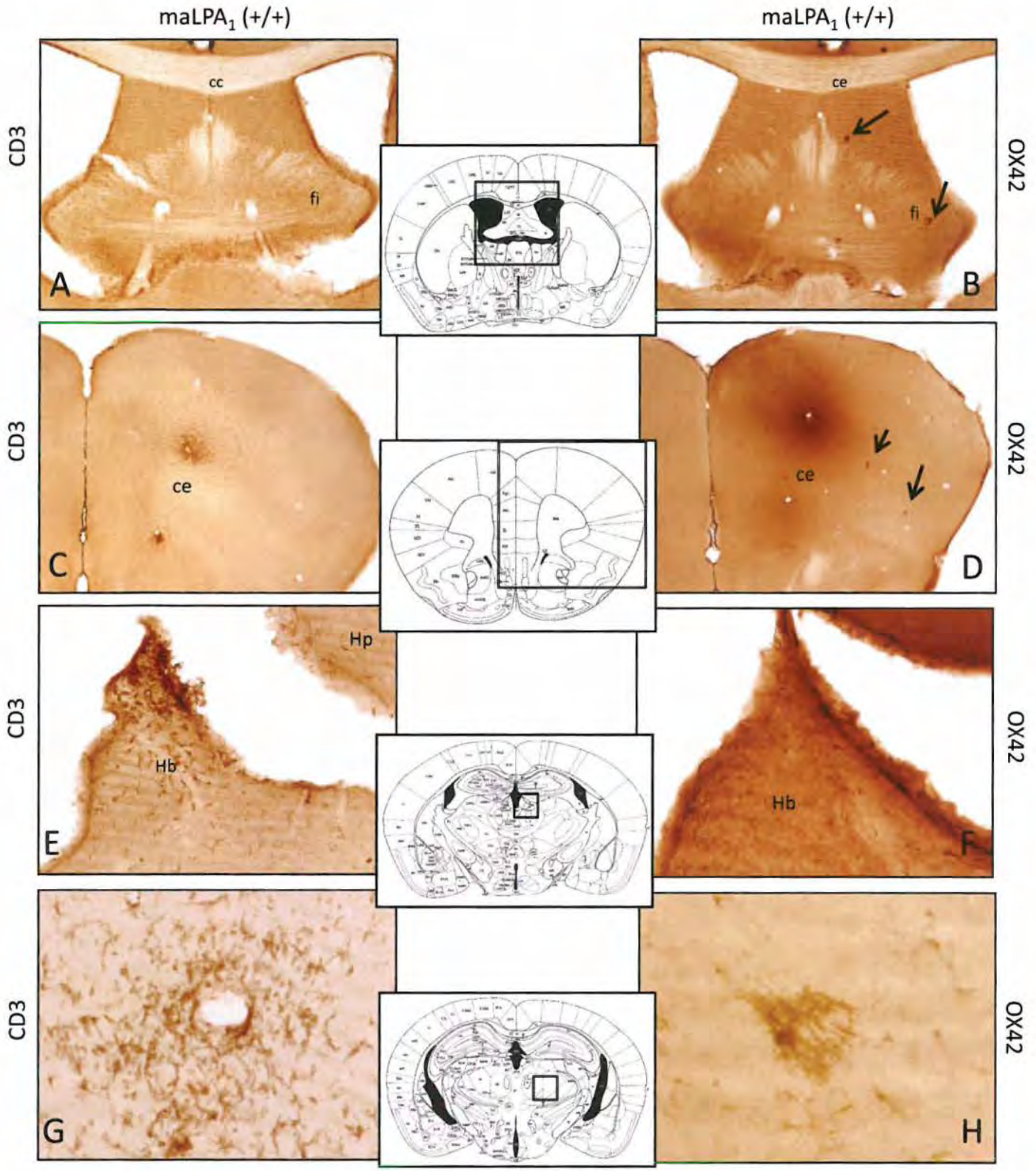
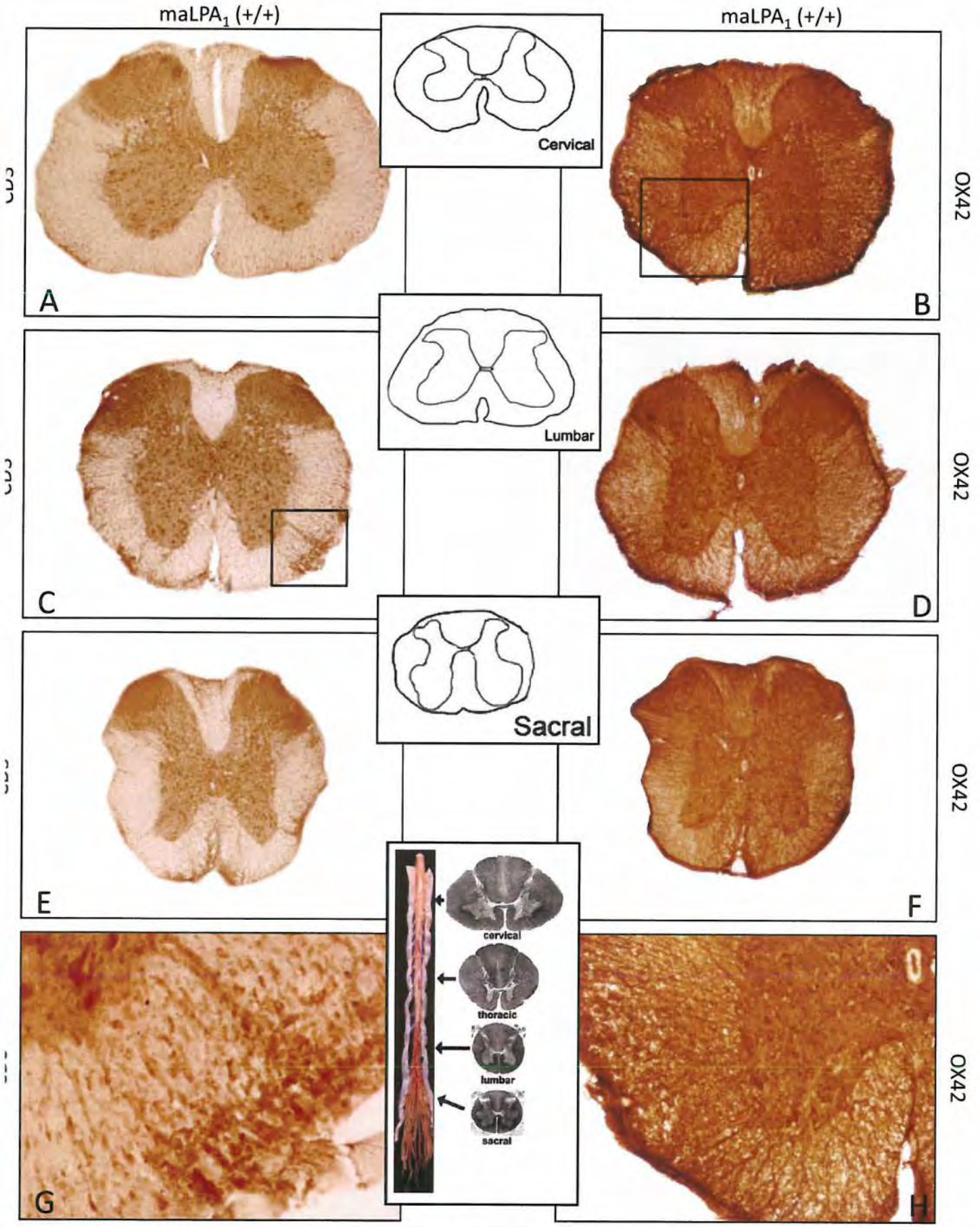


Fig. 59. Immunoshistochemical analysis of inflammation in coronal brain sections of an *active* EAE mouse showing score 3. The presence of T cells was depicted by CD3 staining, whereas macrophages/microglia were marked by OX42. Infiltration was profuse around dilated vessels, finding T cells in caudoputamen (Cpu) (A), corpus callosum (cc) (B), fimbria (fi) (B), internal capsule (ic) (E), and mesencephalon (G). Ameboid OX42-positve cells appeared in the same areas (B, D, F, H). *Lpar1* level was very high (*Lpar1* /GAPDH: 0.670) although *Lpar2* expression showed normal value (LPA_2/GAPDH: 2.776), resulting in moderate ratio (*Lpar1*/ *Lpar2*: 0.241).



ig. 60. Immunoshistochemical analysis of inflammation in coronal brain sections of an EAE mouse showing score 3 luring several days. CD3 staining revealed the presence of T cells, whereas OX42 antibody marked nacrophages/microglia. The number of infiltrated T cells was low, only some cells remained surrounding some vessels C, G) and in the habenulas (Hb) (E), whereas corpus callosum (cc) and fimbrias (fi) didn´t show any infiltration. Ameboid)X42-positive cells were found in the sites in which T cells appeared (D, F, H) but also ramified cells were present in any ther areas (arrows, B, D, H). *Lpar1* expression was lower than in the previous animal, (*Lpar1 /GAPDH*: 0.391) and *Lpar2* lso demonstrated normal levels (*Lpar2*/G*APDH*: 2.290), decreasing the ratio (*Lpar1* / *Lpar2*: 0.171).



. 61. Immunoshistochemical analysis of inflammation in coronal spinal cord sections of EAE mice showing score 3. The esence of T cells was depicted by CD3 staining, whereas macrophages/microglia were marked by OX42. The same as with ore 1, histological evidences of inflammation in spinal cord couldn´t distinguish animals with *active* or *stabilized* EAE. nflammation was profuse, a high number of T lymphocytes infiltrated the white matter (A, C, E, G) which correlated with 42-positive cells, covering almost completely the area of the slices.

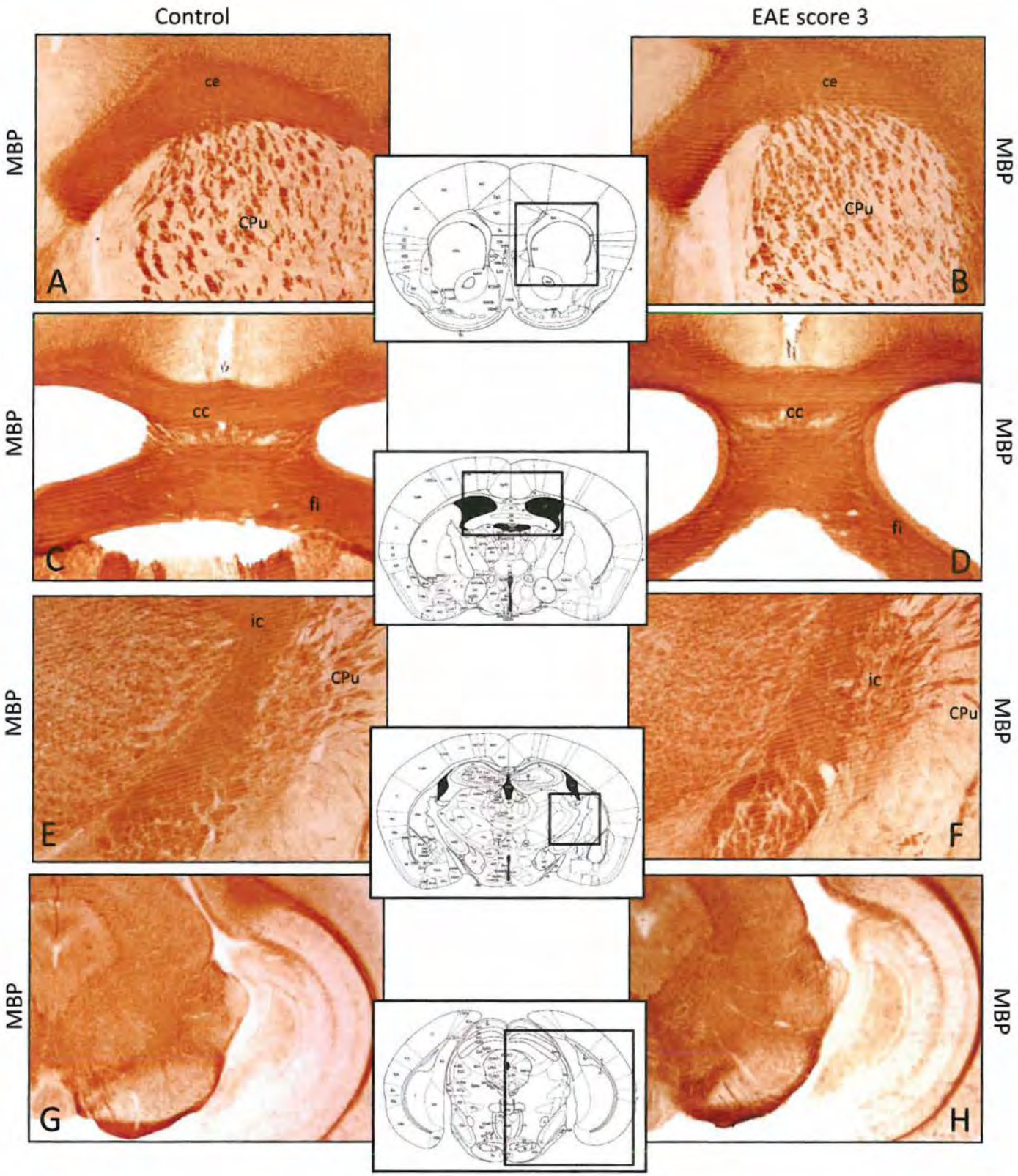


Fig. 62. Immunostaining of myelin basic protein (MBP) in brain of control and an immunized mouse which showed *active* EAE with scored 3. No signs of demyelination were observed in the brain of EAE mice, even when this one demonstrated a profuse infiltration.

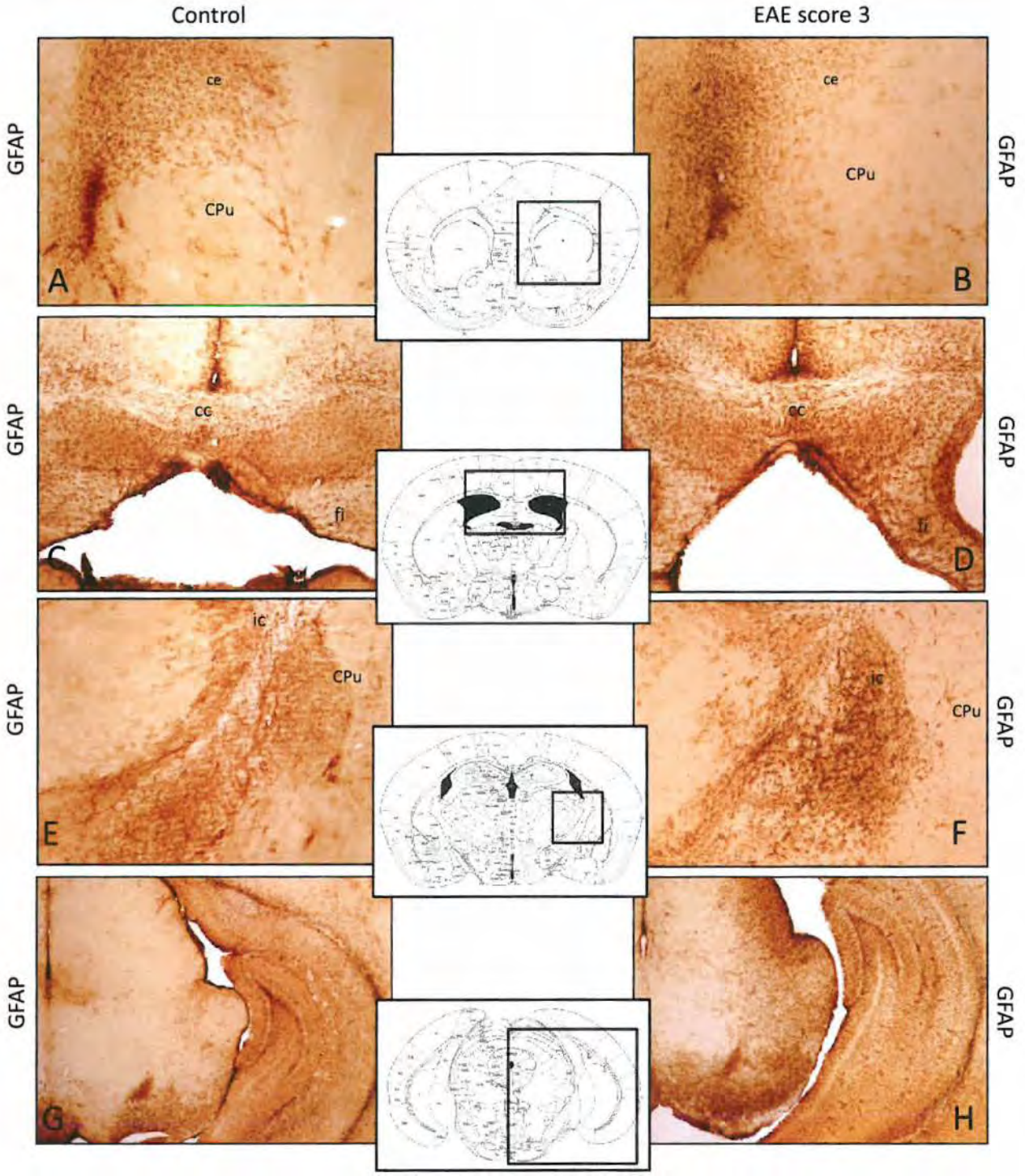


Fig. 63. Immunostaining of glial fibrillary acid protein (GFAP) in brain of control and an immunized mouse which showed *active* EAE with scored 3. An increase of GFAP immunoreactivity (astrogliosis) was observed in those areas in which infiltrates were found.

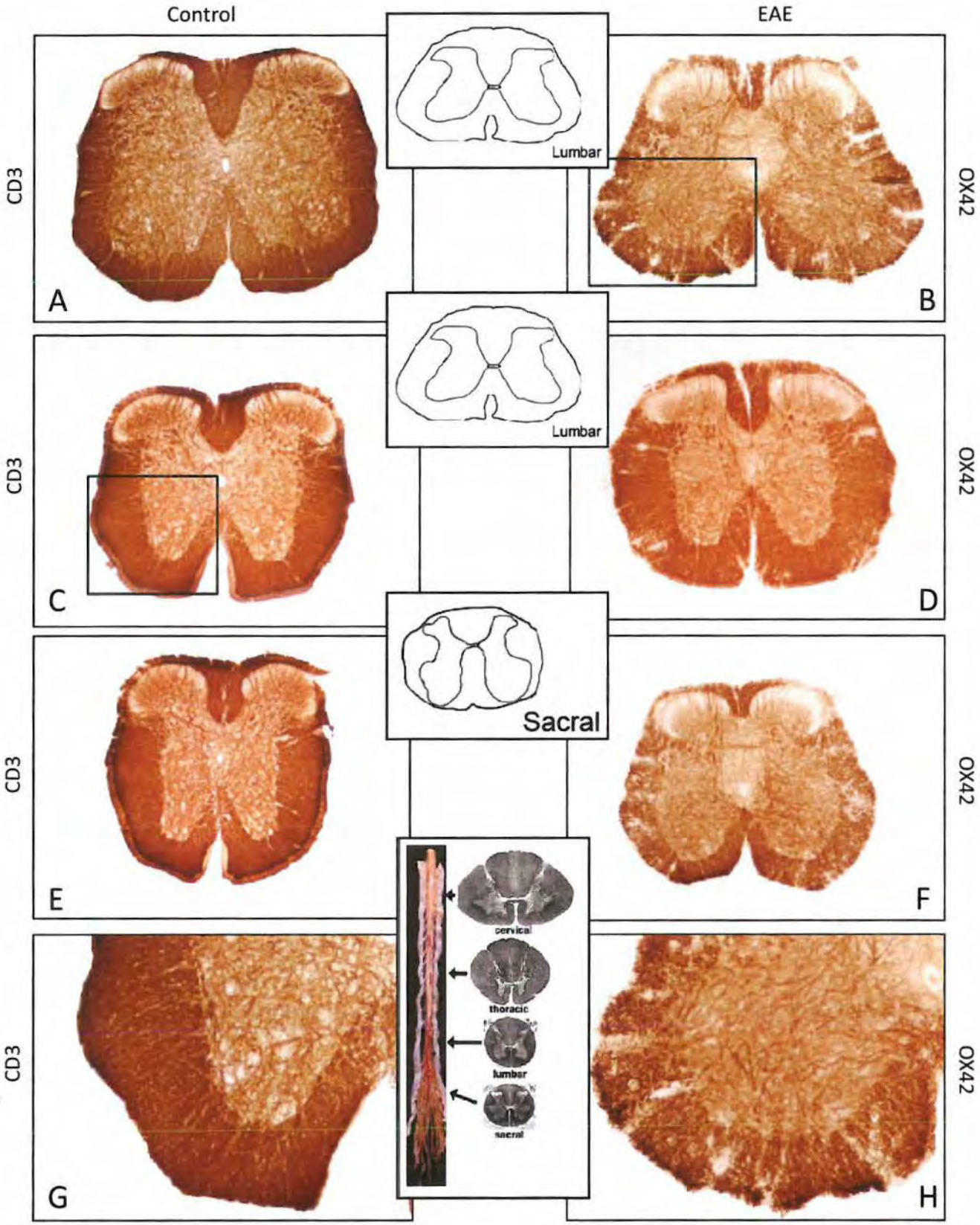


Fig. 64. Immunostaining of myelin basic protein (MBP) in spinal cord of control and an immunized mouse which showed *active* EAE with scored 3. Numerous demyelinating plaques were observed in different levels which correlated with the areas in which infiltrates were found.

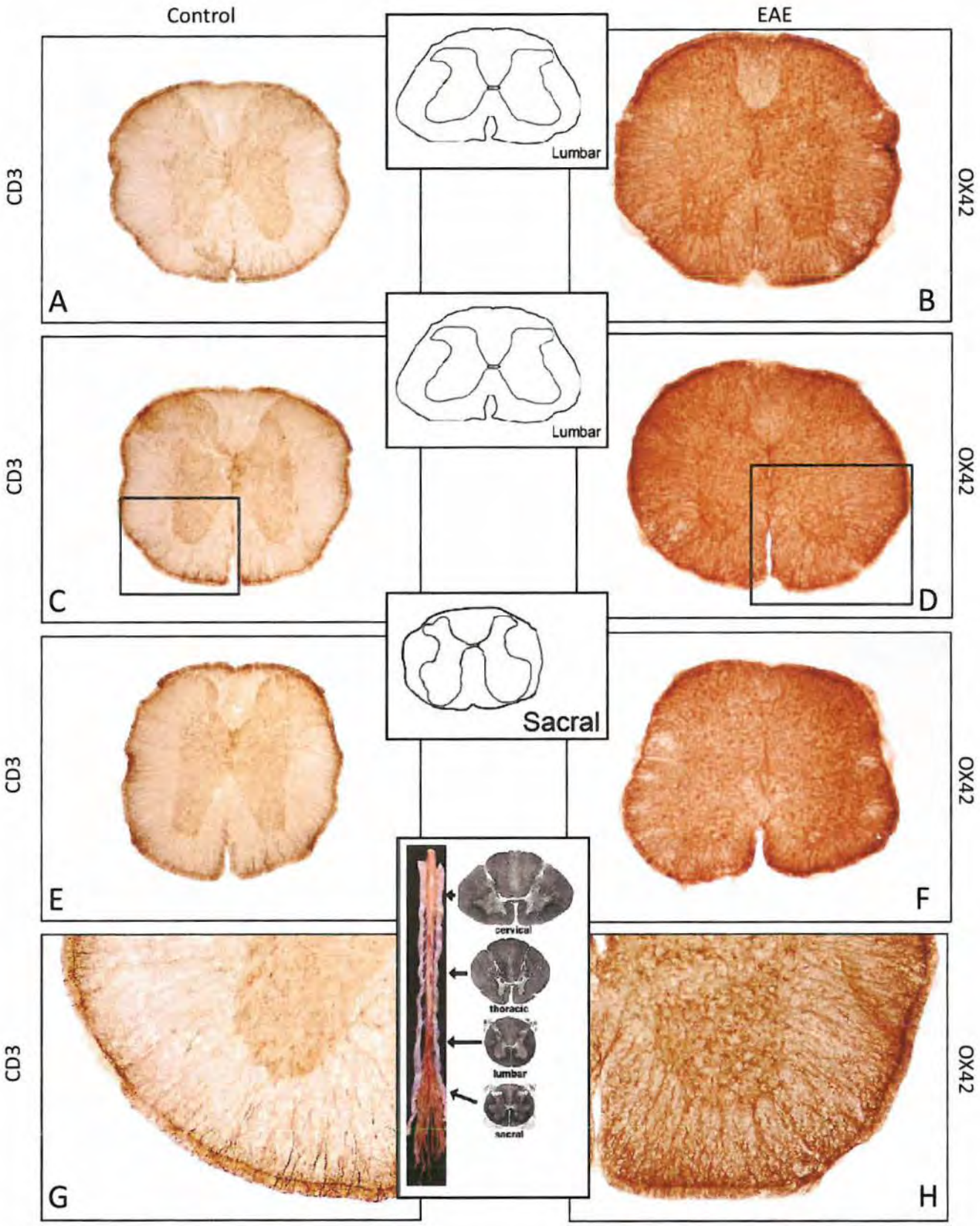


Fig. 65. Immunostaining of glial fibrillary acid protein (GFAP) in spinal cord of control and an immunized mouse which showed *active* EAE with scored 3. An increase of GFAP immunoreactivity (astrogliosis) was observed in all the slices in which infiltrates were found.

2.3. LPA_1 is necessary for the normal clinical course of the encephalomyelitis but not for the infiltration of the immune cells.

Histology evidences of the disease were studied in both genotypes. Since knockout mice showed less severe symptoms along the clinical course, it could be thought that the lack of LPA_1 may have some effects in the immune cell infiltrates of null mice.

Double immunostaining for T lymphocytes (CD3-positive) and myelinated fibers (MBP-positive) were performed in spinal cords of wild type and null mice. Animals with similar scores were evaluated to see whether or not the infiltrates were equivalent in both genotypes. These results demonstrated that there were no differences between the presence and the absence of LPA_1 (Fig. 66), since in both animals T cells could reach the white matter and provoke demyelination in the spinal cord.

The study pointed out that clinical scores were the visible result of the presence of T lymphocytes and microglia in the CNS. Therefore, the more benign course was the outcome of less infiltration of these immune cells. From these results LPA through LPA_1 receptor in inflammation seemed to be involved in the recruitment of inflammatory cells into the CNS, which results in the corresponding amelioration of symptoms and score. Once these cells went inside the cerebral or spinal parenchyma, the absence of LPA_1 did not reflect any difference.

2.4. Intravenous injections of LPA_1 receptor antagonist delay the appearance of the symptoms and ameliorate the clinical score.

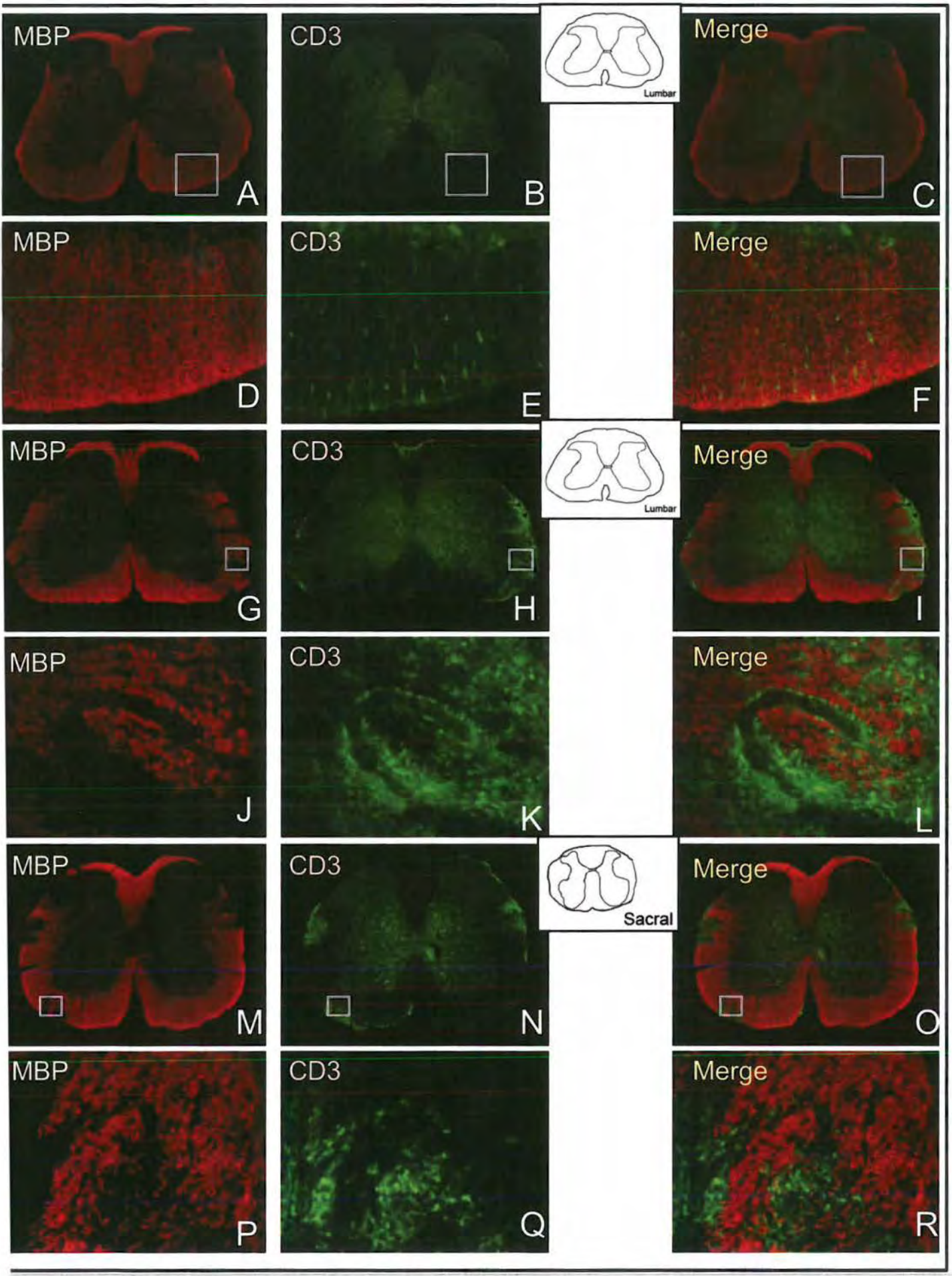
So far, the presence of LPA_1 has showed to be related to more severe symptoms and to the initiation of inflammatory processes. According to literature, expression of LPA_1 is necessary to make LPA induce IL-2 secretion in lymphocytes, and as said in this work, its expression correlates with the beginning of relapses.

Consequently, blocking of LPA_1-mediated signaling pathway should interfere somehow the clinical course of EAE. For that reason, an antagonist of this receptor VPC 32183 was injected intravenous trying to stop the initiation of the disease or delaying the appearance of the symptoms. Although this antagonist blocks primarily the LPA_1 receptor, it also exerts its effect partially on LPA_3 (Heasley et al., 2004). Since LPA_3 is involved in the lymphocyte extravasation, partial blockage of this receptor could also help the amelioration of the encephalomyelitis.

The administration was chosen to be intravenous because of the increase in LPA_1 expression observed in blood, and by the fact that, once the cells were inside the CNS, infiltrates and score correlated similarly in presence or absence of LPA_1.

Initially, the antagonist was injected only once at the onset of the disease, day 14. At that day, none of the animals showed any symptoms and mice grouped randomly.

Intravenous administration of VPC 32183 delayed the severity of symptoms in four days as compared to the vehicle injection (3% BSA). However, after that period, the clinical course became worse, regaining the severity of the control group. Moreover, a slight increase it could be seen in the course of treated mice from 30 pid, although it was not significant (Fig. 67).



5. Double fluorescent immunostaining for MBP (A, D, G, J, M and P) and T lymphocytes, CD3 (B, E, H, K, N and Q) in the spinal cord of control (A, B, C, D, F), wild type EAE (G, H, I, J, K and L) and maLPA$_1$-null EAE mice (M, N, O, P, Q and R). Healthy mice don´t show either any infiltrates of T cell or elinating plaques. In both genotypes EAE mice showed T cell infiltrates in white matter triggering demyelination, however no histological differences found regarding the presence or absence of LPA$_1$.

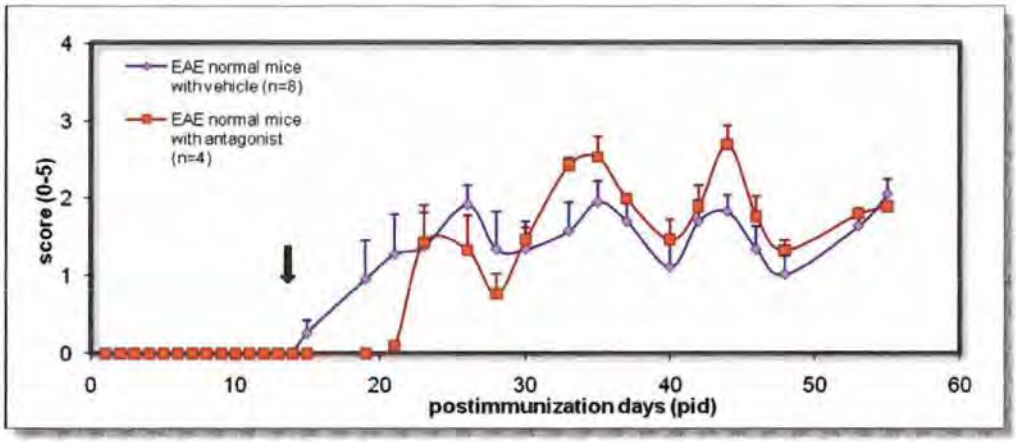


Fig. 67. Experimental autoimmune encephalomyelitis (EAE) clinical signs were monitored in normal EAE mice injected with vehicle and LPA_1 receptor antagonist. The graph shows that detection of clinical symptoms is delayed in several days after antagonist administration as compared with vehicle injection (pid 14, arrow). However, as time passed, the clinical course turned out to be the same as in control group. (Data expressed as mean ± SEM; SEM as indicated in bars; in 1 out of 3 independent experiments.

Due to the fact of its lipidic structure, VPC32183 could be easily metabolized in the blood stream, explaining a decrease in its levels its temporary effects. Consequently a protocol consisting of continuous injections every week was done to elucidate whether a certain maintenance and availability could contribute to ameliorate the disease.

The first injection was performed at 14 pid to prevent from beginning the symptoms manifestation. After first dose, two additional more per week were administrated resulting in a decrease of the clinical scores.

This improvement lasted until the mice reached the plateau phase in which mice did not need additional doses and symptoms remained less severe (Fig. 68).

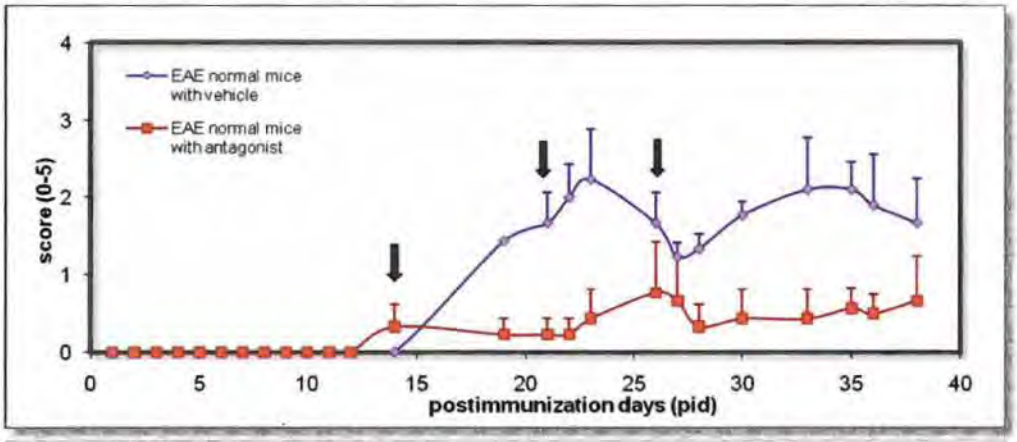


Fig. 68. Experimental autoimmune encephalomyelitis (EAE) clinical signs were monitored in normal EAE-induced mice injected with vehicle and LPA_1 receptor antagonist. The graph shows the symptoms are ameliorated in comparison to the vehicle after repeated antagonist injection (pid 14, arrow) (Data expressed as mean ± SEM) in 1 out of 3 independent experiments.

DISCUSSION

1. INFLUENCE OF LPA THROUGH THE LPA RECEPTOR LPA_1 IN MYELINATION.

One of the main objectives of this work has been to determine the role of LPA in myelination process through its most important receptor in the central nervous system, LPA_1. To this point, possible differences in myelin pattern, as well as in oligodendroglia, have been studied comparing normal mice, which express LPA_1 receptor, and their littermate lacking of it.

As previous studies already showed, LPA_1 expression correlates to a large extent with the expression of several myelin proteins. This correlation suggested that LPA could be involved in some important roles in myelination. Although LPA_1 is also expressed in other cellular types in the brain, such as neural precursors or neurons (Allard et al., 1998; Tabuchi et al., 2000; Fujiwara et al., 2003; Pilpel and Segal 2006; Matas-Rico et al., 2008), astrocytes (Shano et ál., 2008) or microglia (Moller et al 2001; Tham et al. 2003), its presence in oligodendrocytes is more predominant (Weiner et al., 1998; Handford et al., 2001). Many of these functions are expected based on in vitro findings, but there are still discrepancies between in vitro and in vivo studies and no obvious defects in oligodendrocyte development or myelination were reported with the loss of receptor so far.

Thus, this is the first time that an in vivo necessity of LPA_1 is demonstrated for myelination. Our results show that LPA_1-mediated signalling is important during normal myelination, revealed by the *Malaga* maLPA1-null variant (Estivill-Torrús et al., 2008) derived from the original (Contos et al. 2000). The absence of LPA_1 in the $maLPA_1$-null mice results in a reduction of the number of mature oligodendrocytes, decrease of the myelinated fiber density and altered composition of myelin. Nevertheless, this effect is not due to any deficiency during the development of their precursors, but it seems to be related to some alterations in the maturation process of them. The results presented here pointed out that LPA does have a significant role in formation and stabilization of myelin sheaths. Thus, one of the main finding of this study is that there is an essential in vivo requirement of LPA_1 for normal myelination, as shown through the analysis of $maLPA_1$-null mice.

1.1. The absence of LPA_1 alters the expression of other LPA receptors in the adult brain

The analysis of gene expression for the different LPA receptors corroborated the targeted deletion of *Lpar1* in $maLPA_1$-null mice. In order to elucidate whether any mechanism of compensation was involved, the gene expression corresponding to the other four receptors (*Lpar2*, *Lpar3*, *Lpar4* and *Lpar5*) was measured. These results showed that the absence of LPA_1 did not alter significantly the expression of those genes, excluding some specific areas

In agreement with most studies, RT-PCR analysis revealed that LPA receptors are expressed in many cerebral areas. Normally, when some genomic sequences are knocked out or deleted, other closely related genes with similar functions increase their expressions in an attempt of the system to compensate this deficit. Despite this, $maLPA_1$-null mice did not show any increase in the expression of other LPA receptors which could compensate the absence of LPA_1. However, a general tendency to decrease the expressions of those other receptors was observed in null mice. In most of the cases, these differences were not significant, although both LPA_3 and LPA_4 did show some important reductions in several regions.

Regarding LPA_3, recent studies have shown the role of LPA_3, as well as LPA_1, in the differentiation of oligodendrocytes from stem cells (Svetlov et al., 2008). Moreover, its presence was reported in rat cortical oligodendrocytes in culture by Yu et al. (2004), suggesting that they must have some role in those cells. Taken all this together, it seems important to note that it was in cerebral cortex, and above all in corpus callosum, where the reduction of LPA_3 was more significant and in coincidence with those regions where a decrease in the number of mature oligodendrocytes and myelinated fibers, and also an alteration in electron microscopy were observed later.

On the other hand, LPA_4 seems to be involved in neurogenesis (Yanagida et al., 2007), role which has been given to LPA_1 receptor as well (Kingsbury et al., 2003; Fukushima, 2004; Fukushima et al., 2007; Estivill-Torrús et al., 2008, Matas-Rico et al., 2008). When LPA_1 was missing, LPA_4 expression also decreased in the region where neurons are generated in adults, that was, hippocampus. However, its expression did not seem to reveal a significant alteration in other areas such as cortex, striatum or corpus callosum.

Then, the absence of LPA_1 reduces LPA_3 and LPA_4 expression in those regions in which they carry out some similar role to the former. Unlike this, LPA_2 receptor (almost lacking in adult brain) and LPA_5 did not demonstrate any significant variation of their transcript level in adult brains.

On the other hand compensation not only imply LPA signaling through other LPA receptors, but also related signaling pathways like S1P signaling, which induced similar actin reorganization in both wild-type and

LPA_1-null mouse; and/or the action of peptide growth factors (e.g., neuregulins) on their cognate receptors. As above mentioned, many of the expected functions for LPA_1 based on in vitro findings were disappointing as well as those based on gain of function even using double mutants (Choi et al., 2008). In addition, despite of well described numerous studies showing an association between LPA_1 and myelination, they oddly did not showed LPA-induced changes in morphology or physiological mechanisms of oligodendrocytes (Moller et al., 1999; Dawson et al., 2003; Cervera et al., 2002; Stankoff et al., 2002). In this sense, many of neural noticeable defects observed in maLPA1-null mice and hidden in original LPA_1-null mouse can be attributed to the presence of unknown modifiers on a single mutant allele, a common fact in studies addressing the spontaneous variance of mutant phenotypes. Genetic modifiers, such as other genes, viruses, or retrotransposons may contribute to the new phenotype and identifying them exceeds the aims of the present studies. However, because $maLPA_1$-null mice have a highly penetrant and reproducible phenotype, this should serve toward understanding and identifying interacting genes or non gene elements in future studies. Nogaroli et al. (2008) demonstrated clearly LPA-dependent effects on myelinating cells, and proposed the presence of endogenous activity of autotoxin in oligodendrocytes. Like this, many factors should be considered as influencing signaling pathways and gene expression as well, some of them epigenetic. Moreover, gene expression at specific stages of oligodendrocyte differentiation and maturation is also under epigenetic regulation (Copray et al., 2009).

1.2. The absence of LPA_1 alters formation of myelin sheaths and oligodendrocyte survival.

1.2.1. Effects of LPA in myelin formation.

The non-invasive studies through magnetic resonance spectroscopy (MRS) showed that choline levels increase in those mice lacking LPA_1 receptor.

Choline and its metabolites (acetylcholine, glycerophosphocholine, phosphocholine and phosphatidilcholine) are involved in synthesis and degradation of cellular membrane. Thus, their elevation is related to an increase of this membrane metabolism, either synthesis or degradation. An increase in the choline/ creatine ratio has been interpreted as evidence of demyelination because of the abundance in choline-containing compounds in myelin with the subsequent release of phosphocholine and glycerophosphocholine during the active demyelination or of inflammatory cell membrane turnover because choline-containing compounds are also abundant in cell membranes (Bonavita et al., 1999; Khiat et al., 2007). However, choline-containing compounds are considered as turnover products of cell membranes, and an increase of choline seems to be related mainly to inflammatory and glial cell proliferation rather than to myelin destruction (Simone et al., 2001). This observation had been also confirmed by histopathological and spectroscopic correlative study in EAE showing high levels of choline associated with inflammation and not with demyelination (Brenner et al., 1993).

The rise of the choline peak in the $maLPA_1$-null animals suggests that there is an alteration in this process, which could be due to either an aberrant synthesis or an increase of membrane degradation and consequent demyelination. The results of this work pointed out that the lack of LPA_1 might have a deleterious effect in the CNS membrane production.

In accordance with this, the immunohistochemical studies of different myelin proteins, such as MBP, PLP and CNPase, showed a reduction in the density of fibers positive to these proteins throughout telencephalon when LPA_1 receptor was lacking. These results suggested that the rise of choline peak might be due to an increase in the degradation of myelin membranes, and not in its synthesis. On the other hand, this increase is typical of diseases in which myelin degradation (demyelination) occurs, like in acute plaques of multiple sclerosis, what leads to support the same hypothesis.

Normally, an increase in the choline levels correlates with a drop in NAA peak, a neural metabolite, suggesting a secondary neurodegeneration. However, null mice did not show any reduction of the NAA levels, but an increase in older ages.. NAA is involved in many processes including fatty acid synthesis after transported to oligodendrocytes early at postnatal development, enhancing mitochondrial energy production from glutamate, and also a direct precursor for the enzymatic synthesis of the neuron specific dipeptide N-acetyl-aspartyl-glutamate (NAAG), one of most concentrated neuropeptide in the brain (Neale et al. 2000) synthesized enzymatically from NAA and glutamate and hydrolyzed on astrocytes together with glutamate (Moffett et al., 2007). The reason why NAA increases with age in null mice still needs to be studied but LPA_1 absence not only affect many of these CNS components i.e. oligodendrocytes, neurons and astrocytes, but also glutamatergic pathways (Palomino-Barrigas, 2009) suggesting this a possible indirect effect on NAA levels not associated to axonal damage or neuronal loss.

Besides that, the analysis of myelin extracts through western blot revealed that the absence of the LPA_1 signaling pathway turns out to alter the composition of lamella. Myelin membranes showed lower proportions of three main proteins, MBP, PLP and CNPase. The reduction of these protein expressions correlates with previous studies which demonstrated a role for LPA in the induction of oligodendroglial processes associated to the formation of membranous structures and a rise in MBP transcription during oligodendrocyte differentiation (Nogaroli et al., 2009). Likewise, a perfect correlation between the expression of LPA_1 and PLP was already showed, which indicates that LPA might also have some effect in the expression or translation of this protein (Weiner et al., 1998). According to this, the absence of LPA effect could alter the presence of PLP in myelin as it is observed in null mice. In this line, Matsushita et al. (2005) also observed that LPA inhibits the apoptosis of oligodendrocyte precursors and induces maturation and expression of some oligodendrocyte proteins in culture, such as O4, CNPase and GalC. So taking all this together, the absence of this LPA receptor in oligodendrocytes could affect either the expression of these proteins or their sorting to myelin sheaths. This would explain the altered composition of myelin when LPA_1 is lacking.

An altered composition of the main myelin proteins in fibers, which are involved in its sheath formation, should show some variations at ultrastructural levels. MBP and PLP are two important proteins which form the major dense line and intraperiod line, respectively. Nevertheless only MBP is necessary for the formation of compact myelin (Readhead and Hood, 1990), whereas the absence of PLP only shows slight defects in the sheaths (Rosenbluth et al., 2006). When thickness and compactation of lamella were studied in $maLPA_1$ colony, no significant differences were observed between mice with and without the receptor. The fact that null mice do not show any alteration in myelin compactation suggests that LPA may not be necessary to sheath formation and its effect could be more focus on the maturation of oligodendrocytes instead.

Although the compactation of myelin remains quite normal, important differences were observed by electron micrographs. As it was already demonstrated by immunological analysis, the number of myelinated fibers was lower. In addition to it, their orientation seemed to be altered above all in corpus callosum where fibers, instead of being aligned, presented a quite random organization. Moreover, transversal sections of fibers were not circular as in normal mice, but they depicted irregular shapes. These abnormal-shaped sections can be observed in remyelinating process, as well as in animals with an incomplete differentiation of their oligodendrocyte precursors (Baas et al, 2002). The morphology of these fibers suggests that the presence of LPA_1, that is the effect of LPA, might be to induce the complete oligodendrocyte maturation. Therefore, the presence of LPA_1 is important but not essential for formation of compact myelin sheath

Cervera et al. (2001) observed a weaker immunoreactivity for LPA_1 in myelinating fibers compared to oligodendrocyte soma suggesting that LPA_1 expression was lost when myelination occurs. Alternatively, they suggest that LPA_1 expression could only play a role at the level of the oligodendrocyte soma and would not be involved in myelination itself, but rather in oligodendrocyte maintenance. That would be in accordance with data reporting apoptosis in myelinating peripheral cells of LPA_1-null mice (Contos et al., 2000) but in contrast with LPA_1 reactivity found in fibers.

Interestingly, they attributed the weaker immunoreactivity in oligodendrocyte processes to the fact that the immunoreactive epitope could be masked in myelinating fibers due to presence of LPA_1 receptor in the most internal parts of the myelin sheath, directly contacting axons. This would indicate a function of LPA_1 in the axon-oligodendrocyte chemical communication, in consistence with the LPA_1 appearance at a very late stage of the oligodendrocyte lineage, after and before that of MBP and MOG, respectively, which indicates that LPA_1 is not involved in the earliest events of myelinogenesis. According to this hypothesis, LPA_1 would be a receptor for an axon-derived signaling molecule, which is necessary for correct functioning of oligodendrocytes and appropriate formation of the myelin sheaths by oligodendrocytes (Demerens et al., 1996).LPA could well interact this way as release by postmitotic neurons (Fukushima et a., 2000) or as secreted by hydrolysis of phospholipids that are highly enriched in neuronal membranes. Actually, we cannot exclude any of proposed roles since our data support both, an involvement in oligodendrocyte maintenance and functioning as well as for myelin formation.

1.2.2. Effects of LPA in oligodendrocyte survival.

The reduction in null mice of the density of fibers immunoreactive to the studied proteins was also accompanied by a lower number of mature oligodendrocytes, galactocerebroside-positives. This drop of myelinating cell density could be explained by two different ways: either by some deficiencies in proliferation of oligodendrocyte precursors, or by an alteration in their maturation process leading to death. An increase in the death of oligodendrocyte lineage cells also may contribute to the decreased number in oligodendrocytes and their precursors in these mutant mice. Both effects, proliferation and induction on maturation, had already been described as roles of LPA (Ankiler and Chun, 2004; Nogaroli et al., 2009).

In order to distinguish between both hypotheses, oligodendrocyte precursor cells (NG2-positive) were studied. Even so, this result implies caution since some adult NG2 or Olig2 positive cells exhibit different fates other than oligodendroglial. Although the progenitors characterized by Olig2 or NG2 are certainly involved in oligodendrogliogenesis during development, they have recently also been implicated in the generation of various other lineages. The transcription factor Olig2 is a necessary regulator of oligodendrocyte and motoneurons development, and Olig2-expressing cells also generate cholinergic neurons, ependymal cells, as well as some astrocytes during development (Dimou et al., 2008). Likewise some glial cells that express NG2 and platelet-derived growth factor receptor-α are found throughout the mature CNS can generate both oligodendrocytes and some cortical projection neurons in the adult brain (Kang and Bergles, 2008).

Our work did not show any differences in the number, morphology or distribution of these precursors comparing both genotypes. Hence the signaling pathway of LPA_1 does not seem to be involved in oligodendrocyte precursor proliferation, but it might be in their maturation

In short, the results of this study point out that the role of LPA must be related to the process of oligodendrocyte maturation. According to this, LPA_1 could be involved in the transport of myelin proteins to the sheaths.

1.2.3. Effects of LPA in myelin protein sorting.

When LPA_1 is lacking, PLP protein does not reach its proper place in the fibers. In null animals, this lipoprotein translated in endoplasmatic reticulum remains in the cellular body. Then, PLP can be found in different cellular compartments, such as reticulum itself; endosomal/lysosomal system; and in lesser extent, Golgi apparatus. This low amount of PLP in the Golgi means that its presence in cisternae is only a transitory step of its sorting.

Finding this protein in those three compartments, as well as less quantity in myelinated fibers when LPA_1 is absent, suggests that there is some kind of alteration in its transport to myelin, or maybe in assembly. Both explanations would prevent proteins from being inserted in myelin rafts and lamella. Thus, either because of a defective transport to the sheaths, or because of an altered maturation or folding, PLP protein gets stuck in endoplasmatic reticulum and cellular membranes, which could be included in endosome and lysosome to its subsequent degradation.

Myelinating cells, produce an enormous amount of plasma membrane during the myelination process making them particularly susceptible to disruptions of the secretory pathway. The secretory pathways for proteins and lipids, including the rough endoplasmic reticulum, smooth endoplasmic reticulum and Golgi apparatus, are well developed in oligodendrocytes. Evidence is accumulating that oligodendrocytes, rank among the cells that are most sensitive to the disruption of the secretory pathway. Endoplasmic reticulum stress, initiated by the accumulation of unfolded or misfolded proteins, activates the unfolded protein response, which adapts cells to the stress. If this adaptive response is insufficient, the unfolded protein response activates an apoptotic program to eliminate the affected cells (Faitova et al., 2006; Szegezdi,et al. 2006). Recent observations suggest that endoplasmic reticulum stress in myelinating cells is important in the pathogenesis of various disorders of myelin, including Charcot-Marie-Tooth disease, Pelizaeus-Merzbacher disease and Vanishing White Matter Disease, as well as in the most common myelin disorder, multiple sclerosis (Lin and Popko, 2009).

In the same idea, Bauer et al. (2002) showed that the impairment of PLP transport from the cell body to the processes in rats overexpressing PLP interfered with the translocation of other membrane proteins such as MAG or MOG for CNS but not MBP for peripheral. Interestingly, based on differential disturbances, authors suggest that gray matter oligodendrocyte differ from white matter oligodendrocytes in their capacity to stabilize metabolic disturbances by an unfolded protein response, being formers more susceptible to reticular stress. In this case, the endoplasmic reticulum of these metabolically disturbed oligodendrocytes revealed extreme swelling of the cisternae, and intense expression of the ER chaperone molecule BiP/GRP78 and folding enzyme protein disulfide isomerase (PDI). PDI intense labeling seems thereby to be a common for endoplasmic reticulum disturbances, as showed in our results. However, by contrast, not morphological disturbances were

found in maLPA$_1$-null oligodendrocytes (not shown). We should consider, accordingly, that, in our study PLP accumulation has been showed at cortical level whilst ultrastructural analysis was carried out on callosal areas. Thus the absence of more evident ultrastructural oligodendroglial alterations in null mice may well need further analysis.

Although the total absence of PLP in knockout mice has shown surprisingly few defects and normal myelin compactation (Rosenbluth et al., 2006), mutations in its aminoacid sequence have been described as new models of a demyelinating disease, named Pelizaeus-Merzbacher (McLaughlin et al., 2006). These mutations produce aberrant isoforms which prevent the normal processing of the protein, mainly due to some misfolding. This abnormal folding causes protein gets stuck in reticulum where it is translated. This protein accumulation in that organelle induces certain cellular stress which decreases the expression of other myelin proteins such as MBP, MAG or CNPase, and can even induce oligodendroglial apoptosis (Koeppen and Robitaille, 2002).

What is happening in those animal models could throw a light on what might be happening in mice lacking LPA$_1$. However, since PLP protein is not altered in maLPA$_1$ null animals, LPA might be involved in oligodendrocyte maturation process through its transport to myelin sheaths. The absence of the main LPA receptor in these cells during maturation would affect the proper localization of PLP, causing the observed stuck protein in soma of knockout mice and some cellular stress. Nevertheless, since null mice showed this protein along the myelinated fibers, the transport of PLP towards fibers might not be regulated only by the LPA$_1$ signaling pathway but by any other factors. In this sense and taking up again one of mentioned hypothesis, axonal plasticity has been recently demonstrated to induce changes in myelin composition (Drøjdahl et al., 2010) affecting axon diameter, myelin thickness, and number of oligodendrocytes, and serving as stimulus to oligodendrocytes to engage actively in the myelination processes. This make quite attractive a possible scenary where LPA modulates, through LPA$_1$, both plasticity (as demonstrated) and myelination in a bidirectional way.

On the other hand, TUNEL assays also revealed a rise in the number of picnotic nuclei in those regions of null animals where the reduction of myelinated fibers and mature oligodendrocyte was more outstanding, that is, in cerebral cortex. This corroborated the increase in the peak of choline which means more membrane degradation, maybe caused by the presence of these apoptotic cells. This rise in apoptosis could be due to the stress caused by PLP accumulation in the oligodendrocytes which would induce it.

All this together would lead to the observed reduction in the density of oligodendrocyte as well as a lower myelination of the fibers in adult mice.

1.3. The defective myelin pattern is observed only after the peak of LPA$_1$ expression in the postnatal murine brain, coinciding with the peak of myelination

The expression of LPA$_1$ had already been shown to coincide with the moment of most active myelination. The peak of myelination occurs about postnatal day 18 (P18) when the expression of this LPA receptor is the highest (Weiner et al., 1998). This was one of the first results which led to suggest the putative role of LPA in myelin formation.

When myelin pattern was studied before the peak of myelination and the strongest expression of LPA$_1$, at P10, no differences were observed in animals lacking the receptor. At this age, fibers were not completely myelinated and oligodendrocytes were still inmature. Then, the density of both fibers and galactocerebroside-positive oligodendrocytes showed the same pattern in normal and null mice, which was characterized by a very low number of fibers. These fibers were present in the proximal area to corpus callosum, although oligodendrocytes expressing galactocerebroside were already observed throughout the cortex.

At this point, it is important to stress the presence of intense PLP-immunoreactivity in the cellular bodies of both genotype oligodendrocytes, as it was observed in null-LPA$_1$ adult mice. This could suggest that previous to the LPA$_1$ expression, both genotypes keep this lipoprotein in the soma before initiating myelination of the fibers.

When the same immunohistochemical studies were performed in older aged animals (P30), the same differences as in adults were observed. That was, a lower density of myelinated fibers as well as a reduced number of mature oligodendrocytes. Likewise, the differences regarding PLP staining also corroborated the ones showed in adults. While in normal mice most of PLP labelling was in myelin sheaths, in null animals it was localized in the cellular bodies.

Although this work was focused in the cerebral cortex mainly, where the same differences were more outstanding; these alterations were observed in the whole telencephalon.

Once more, the results indicated the role of LPA in the transport of PLP protein to the myelin sheaths as the most suitable explanation of the obtained data. The lack of LPA_1 receptor at the moment of myelination would decrease the sorting of these proteins to the lamella, which would remain in the reticulum and plasmatic membranes. This stuck protein might cause some cellular stress which would reduce the expression of other myelin proteins and could induce apoptosis. All this would lead to a drop in the number of oligodendrocyte and then, the density of myelinated fibers.

1.4. LPA administration increases remyelination after cuprizone treatment

After pathological processes characterized by myelin damage, there can be a highly effective regenerative process, in which a population of adult neural stem or progenitor cells, OPCs, is rapidly activated and mobilized to generate new myelinating oligodendrocytes. The coordinated sequence of events leading to the proliferation and maturation of oligodendrocytes precursors results in remyelination of the fibers Although recent studies indicate the presence of extensive remyelination in the CNS of patients with multiple sclerosis, they also illustrate that remyelination is not an inevitable consequence of demyelination and that axons can persist in a chronically demyelinated state, in which they are vulnerable to atrophy (Patrilios et al., 2006; Patani et al., 2007). Thus, promoting remyelination remains an important therapeutic objective. As a proportion of non remyelinating lesions in multiple sclerosis contain OPCs that fail to differentiate into myelinating oligodendrocytes, an understanding of the mechanisms which control OPC differentiation would be crucial.

Our data presented here indicate, for first time, that the lipid signaling molecule LPA may facilitate endogenous remyelination being this of extraordinary relevance for development of new therapies.

This study has shown that injections of this lipid could improve the normal remyelination processes which take place after the withdrawal of the neurotoxic effect of cuprizone.

Although in both cases LPA injections increased remyelination, this effect was more outstanding when LPA was injected four days after stopping the treatment..Previous studies (Dawson et al., 2003) showed LPA in cell culture medium can induce effects during 18 hours, suggesting that after this period LPA have been metabolised or inactivated. However, in our approach a non-hydrolizable LPA moiety was used. Thus, differences should be attributed to time for remyelination. Nogaroli et al. (2009) already described the effect of LPA in premyelinating oligodendrocyte inducing the extension of the processes. So, this could explain that remyelination increased more when LPA is injected several days after the withdrawal, in which more premyelinating oligodendrocytes are present. If LPA is injected earlier at withdrawal of cuprizone, not spontenous remyelination would have started yet and LPA could be metabolised before inducing its effect.

Despite LPA-treated mice showed an increase of oligodendrocyte precursors, NG2-and Olig2- positive cells, in some areas, injections of this lipid in healthy animals revealed LPA is not enough by itself to induce proliferation. Then, LPA is maybe not enough to promote this effect, although it can increase it if proliferation is already going on. However, recently, Narayanan et al. (2009) have demonstrated that Olig2 is not only essential for the oligodendrocyte specification and differentiation but also in myelination via transcriptional regulation of myelin genes per se, inducing Sox10 and Nkx2.2 expression, thereby increasing myelin gene transcription, and doing more relevant the increase in Olig2 positive cells after LPA administration in our murine cuprizone model.

In addition many factors are also involved. In demyelinated young brains, new myelin synthesis is preceded by downregulation of oligodendrocyte differentiation inhibitors and neural stem cell markers, and this is associated with epigenetic mechanisms such as recruitment of histone deacetylases (HDACs) to promoter regions (Copray et al., 2009). Thus as a possible explanation for not to promote remyelination itself, some of these remyelination mechanisms could well be only possible after the existence of a pathological state implying changes in gene transcription.

Apart from this, LPA has not shown any effect in gliosis or astrocyte proliferation, indicating this lipid can affect only to the oligodendrocyte lineage.

Shortly, although more experiments should be carried out in order to determine doses, temporal window and effect of other structurally related molecules, our data point out that LPA seems to be able to increase remyelination and led the forthcoming experiments.

2. EFFECTS OF LPA IN THE INFLAMMATORY PROCESSES

2.1. The absence of the LPA_1 receptor reduces the severity of the symptoms in encephalomyelitis autoinmune experimental (EAE).

The observed defects of myelination in mice lacking LPA_1 receptor could have suggested a quite more severe clinical course of these animals during encephalomyelitis autoinmune experimental (EAE) because of generating an environment permissive to make more dramatic the pathological course and consequences on demyelination. However, the response after immunization was ameliorated in comparison with normal genotype. This difference in the severity of EAE symptoms despite the myelin defects revealed an important role of LPA in inflammatory process.

On the other hand, MOG induced EAE used for this study develops on a mixed C57BL/6J × 129X1/SvJ background and exhibit a relapsing-remitting course where unlike other chronic EAE models, inflammation take priority over myelin loss, differing with observed in chronic EAE based on C57BL/6J background where myelin loss, and axonal damage/loss are sustained and worsen in the later stage (Berard et al., 2010). To avoid less severe EAE course on 129 background (Slavin et al., 1998), many transgenic mouse strains are derived initially from 129 mice and are then backcrossed with C57BL/6 to provide transgenic strains that are susceptible to MOG_{35-55} peptide-induced disease. In this case, a minimum of at least six backcrosses used to be performed before the offspring are used in experimental studies, and controls must include wild-type littermates. In this sense maLPA1 colony used for this study backcrossed frequently and 22 generations were done.

The first distinctive feature in the development of the disease under LPA_1 absence was the appearance of symptoms about three days later than in normal animals. This indicates that LPA_1 signaling pathway might be involved in the onset of the disease.

Likewise, once the clinical course was developed, the severity of the symptoms was also reduced when LPA_1 receptor was absent, being clear its role in the progression of the inflammatory response as well. These results corroborate previous studies which suggest LPA has a role in the response of T lymphocytes. This response would be modified by the expression of two kinds of LPA receptors, LPA_1 in activated and LPA_2 in inactivated T cells (Zheng et al., 2000). Whereas LPA has an anti-inflammatory effect through the LPA_2 receptor, inhibiting the expression of the proinflammatory cytokine IL-2; the expression shift towards LPA_1 gives to LPA a proinflammatory role. This LPA through LPA_1 induces IL-2 production. Because of the lack of the latter receptor, LPA_1, in T lymphocytes, LPA would not induce this proinflammatory effect in these cells what could be the reason for the delay and amelioration of the symptoms in knockout mice.On the other hand, the presence of LPA_2 receptor, without the opposite effects of its counterpart LPA_1, could stop the development of the disease. Then, the late onset of the encephalomyelitis in null mice could be owed to a decrease in the inflammatory response by the anti-inflammatory effects of LPA_2.

In addition to it, other effects of this receptor could also be involved in the progression of EAE, such as migration. Although the migration of T lymphocytes seems to be regulated by LPA_2 receptor (Zheng et al., 2001), previous studies have shown that LPA_1 and LPA_3 are involved in the regulation of the endothelial cells during inflammatory processes (Lin et al, 2007). When these two receptors are activated by LPA in endothelial cells, the expressions of some inflammation-related genes are induced, such as ICAM-1, IL-1β, IL-8 or MCP-1. These factors are involved in the adhesion of immune cells to the vascular endothelium promoting its transvasation.

Therefore, the lack of one of these receptors could have a deleterious effect in the cellular infiltration into the central nervous system, and as a result, a delay in the onset of the clinical course.

Nevertheless, the presence of infiltrates in null mouse brains states that LPA_1 is not essential for immune cells to pass through the blood brain barrier (BBB), since its absence does not prevent the cells from reaching the CNS. This could be explained by some compensatory effect of LPA_3 which is also involved in transvasation.

2.2. The expression of Lpar1 increased in PBMCs during the inflammatory process and correlates with cellular infiltrates and inflammation.

As it was already mentioned, some studies with cells in culture showed there is a shitf in the expression of LPA_1 and LPA_2 receptors when CD4+ T lymphocytes are activated. The expression of LPA_2 in these cells decreases in favour of a rise in LPA_1 transcript levels (Zheng et al., 2000).

This work has demonstrated that an alteration of both LPA receptor expressions does also occur in vivo, in normal mice suffering EAE. This study was carried out measuring the expression of LPA_1 and LPA_2 in peripheral blood mononuclear cells (PBMC), in which the percentage of CD4+ lymphocytes is high, about 40%. Then, the levels of both transcripts were studied in several moments along the course of the disease. However, due to the fact that animals must have been sacrificed to obtain enough quantity of sample, the clinical course of each mouse could not been followed after getting the blood, to give them some predictive values.

The expression studies showed LPA_1 receptor increased in PBMC when animals were suffering encephalomyelitis, that means, when the inflammatory process were highly present in those mice. Although this raise in LPA_1 expression was observed in all animals with EAE, the increase in transcript levels was more outstanding in the beginning of relapses, when the symptoms had just started and an important number of infiltrates was observed in CNS. Likewise, the raise of LPA_1 levels was correlated with a reduction in the expression of LPA_2. This drop was more important at the start of the symptoms as well.

On the other hand, when these clinical signs were maintained for several days, LPA_2 expression values went back to normal levels while LPA_1 transcripts remained slightly higher than control. In those cases, remitting inflammation was present in the brain with less infiltrates and presence of resting ramified macrophages.

This shift of expressions, more relevant in the first days of relapses, suggested once more LPA must be involved in the initiation of the inflammatory processes. As these alterations in the immune cells were observed when they were still in the circulatory system, the role of LPA through LPA_1 pathway seems to be related to the initial activation of the cells before extravasation from the blood stream to inflammation foci.

Due to the opposite effects of LPA_2 and LPA_1, that is, due to the capacity of LPA_2 to inhibit the production of IL-2 induced by LPA_1, the new increase in LPA_2 expression after the first days of relapse could be the clue to contain the progression of the inflammation and thwart the proinflammatory effects of LPA through LPA_1.

Moreover, the increase in the values of LPA_1 has been related to more severity of the symptoms. A general tendency to higher LPA_1 levels was observed in those mice in which the clinical course was more serious. However, because the receptor expression levels were measured in blood, it is reasonable to think these were previous to symptom appearing. Nevertheless, the need to sacrifice the animals to obtain the samples did impossible to know the score the mice could reach. This could be the reason which would explain the non-perfect correlation between high LPA_1 levels and more severe courses.

Taken all this together, this study has shown that LPA_1 and LPA_2 variations confer to their ratio an indicative value of the state of the disease. In case of control animals, the almost absent expression of LPA_1 and the high LPA_2 levels turn out in a very low ratio. In turn, mice suffering from EAE show different results. Those which were starting some relapses show higher values of the ratio LPA_1/LPA_2, correlating with more active inflammation and more severe symptoms. As the symptoms progress during the relapse, the levels of LPA_2 transcripts go back to their normal ones reducing the ratio. At the end of this period, the transcription of LPA_1 also decreases, turning the ratio into control levels.

Thus, the value of LPA_1/LPA_2 seems to be a good marker to follow the progression of the inflammation: high in first stages, moderate when symptoms have been stabilized and very low when there is no inflammation.

Histological analysis was performed in parallel with the transcription studies. These results corroborated the role of LPA_1 and LPA_2 expressions, and their ratio, as a marker of the course of the disease. The immunohistochemical study demonstrated a correlation between the levels of LPA_1 and the infiltrates in the CNS. The number of T cells and activated macrophages was high in those animals which showed an increased LPA_1 transcription.

When LPA_1 levels were moderates, infiltrates were observed surrounding choroid plexus, around ventricles and near major blood vessels; and in more caudal regions of the spinal cord. These moderate values were showed by mice with mild forms of the encephalomyelitis. However, in cases in which LPA_1 increased, normally related to more severe signs, both T lymphocytes and macrophages occupied more extent areas, being present near smaller vessels as well. These macrophages, when LPA_1 was high, showed ameboid morphology typical of activated microglia.

The previous analysis demonstrated some remission of the inflammatory processes when the symptoms were maintained during several days. This remission was visualized as a lower number of T cell infiltrates. Likewise, the presence of macrophages was also reduced and often observed around the inflammatory foci with

ramified morphology. This shape, characteristic of resting macrophages, corroborate that inflammation remits after several days without worsening the symptoms. In those cases, the levels of LPA_1 expression decreased partially. Then, the values remained slightly higher levels than the controls but lower than in the first days of the relapses.

On the contrary, the alteration in the values of LPA_2 followed the opposite tendency. Whereas in the first moments of the relapses the transcription of LPA_2 was reduced, this increased quickly one or two days after the symptoms started. This made the ratio LPA_1/LPA_2 did not correlate exactly with the inflammatory processes. That was because many infiltrates were still present in the beginning of the inflammation after LPA_2 went back to its normal levels while LPA_1 expression remained high. This could be due to the need of the LPA_2 expression to counteract the effects of LPA_1 over the interleukin production, and so that, to stop the progression of the inflammation.

This far, the ratio LPA_1/LPA_2 seems to be a good marker of the state of the disease which correlates quite well with its clinical course and its histological reflection. However, more studies are needed to understand completely the role of each receptor along the inflammatory processes.

2.3. Intravenous injections of LPA1 receptor antagonist delay the appearance of the symptoms and ameliorate the clinical score.

In recent years, large efforts have been undertaken to establish new therapeutic options for the treatment of MS. So far, all of these strategies more or less specifically target subsets of the immune response in MS, including not only activation and expansion of T cells, their circulation and transmigration over the blood–brain barrier but also other cell types such as B cells and probably also natural killer cells.

From previous numerous works and data from present study LPA_1 is demonstrated to be related to more severe symptoms and to the initiation of inflammatory processes at the beginning of relapses. Even though further studies are necessary we showed in this preliminary assay that blocking of LPA_1 receptor results in ameliorating the EAE symptoms and clinical course.

It seems premature to determine the clinical relevance of these observations since a more extensive study will be necessary but, undoubtedly it could be consider a first step in the searching of therapeutical strategies.

The increasingly well studied lysophospholipids known LPA or, closely related S1P garner interest per year. The current therapies available for MS work by an immunomodulatory action, preventing T-cell- and macrophage-mediated destruction of brain-resident oligodendrocytes and axonal loss. Recently, FTY720 (fingolimod) was shown to significantly reduce relapse rates in MS patients and is currently in Phase III clinical trials. This drug attenuates trafficking of harmful T cells entering the brain by regulating sphingosine-1-phosphate (S1P) receptors (Brinkmann et al., 2002; Dev et al., 2008; Chun and Hartung, 2010)

Interestingly FTY720 has been recently suggested to act in part by reducing LPA concentration through the inhibition of ATX.

Finally, the existence of an experimental model, not only the LPA1-null mouse to validate the contribution of LPA_1 receptor but also the experimental induction of a demyelinating and neuroinflammatory pathology (EAE model) are two helpful methodological approaches in order to analyze and characterize the physiological role of LPA_1 receptor in the myelination as well as in the experimental pathology where both, demyelination and inflammatory events are present.

Because of the identified influence of LPA in the differentiation of myelinating cells, the $maLPA_1$-null mouse characterization here detailed concerns to myelinating glia, specifically expressing LPA_1 in corresponding way to myelination processes. Thus $maLPA_1$-null mouse could be well considered as new highly attractive model for the study of demyelinating diseases.

Besides the new data regarding the involvement of LPA_1 receptor pathways in the course of demyelinating and neuroinflammatory diseases, LPA stimulation of endogenous remyelination after cuprizone treatment gives way to therapeutical approaches no previously thought. The relevance of the results can also easily be evident because of the two other main achievements, functional for EAE studies and, likewise, as putative for multiple sclerosis, first, the proposal of the ratio between the LPA receptors expression as a new disease marker; second, the observed less severity of symptoms after administration of antagonist of LPA_1 receptor.

CONCLUSIONS

- The LPA_1- signalling pathway is directly involved and required for a normal myelination as showed by the subsequent alterations observed in the mice lacking this receptor and affecting quantity, quality and organization of myelinated fibers.

- The presence of LPA_1 has an important role in trafficking PLP from soma to myelin sheet. The absence of this receptor sticks this protein in the endoplasmic reticulum that correlates with cellular loss in null mice and suggests stress-induced apoptosis.

- Accordingly to proposed role for LPA_1 in myelinization and maintenance of oligodendrocyte viability, intracisternal administration of LPA following cuprizone-induced demyelination promotes oligodendrocyte proliferation and remyelination processes.

- LPA signalling pathway, through LPA_1 and LPA_2 receptors, contributes to the ongoing of the inflammatory processes in EAE as suggested by the observed modulation of their expressions in PBMCs during the disease. In absence of LPA_1, EAE develops later and has a significantly more benign course corroborating the involvement of the receptor in EAE pathogenesis.

- Likewise, the periodical intravenous administration of LPA_1 antagonist resembled the effects of the absence of receptor in maLPA_1-null mice with induced EAE.

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Abbreviations

AC: Adenylyl cyclase

AP: Alkaline phosphatase

ATX: Autotaxin

BBB: Blood brain barrier

BSA: Bovine serum albumin

cc: Corpus callosum

CFA: Complete Freund's adjuvant

Cho: Choline

CNP: 2',3'-Cyclic Nucleotide 3'-Phosphohydrolase

CNS: Central Nervous System

CPu: Caudate putamen

Cre: Creatine

Cx: Cerebral cortex

DAB: 3,3'-diaminobenzidine

DEPC: Diethyl pyrocarbonate

DGK: Diacylglycerol kinase

dNTP: Deoxynucleotide triphosphate

EAE: Experimental allergic encephalomyelitis

ec: External capsule

EGFP: Enhanced green fluorescent protein

EtBr: Ethidium bromide

fi: Fimbria

GA: Golgi apparatus

GalC: Galactocerebroside

GAPDH: Glyceraldehyde-3-phospate dehydrogenase

GFAP: Glial fibrillary acidic protein

Golph4: Golgi phosphoprotein 4

GPCR: G protein-coupled receptor

Hb: Habenule

HLA: Human leukocyte antigen

ICAM: Intercellular adhesion molecule

IFN: Interferon

IHC: Immunohistochemistry

IL: Interleukin

ko: Knockout

LAMP1: Lysosome associated membrane protein

LNC: Lymph node cell

LPA: Lysophosphatidic acid

LPA_1: Lysophosphatidic acid receptor 1

LPA_2: Lysophosphatidic acid receptor 2

LPA_3: Lysophosphatidic acid receptor 3

LPA_4: Lysophosphatidic acid receptor 4

LPA_5: Lysophosphatidic acid receptor 5

LPA_6: Lysophosphatidic acid receptor 6

Lpar1: Lysophosphatidic acid receptor 1 gene

Lpar2: Lysophosphatidic acid receptor 2 gene

Lpar3: Lysophosphatidic acid receptor 3 gene

Lpar4: Lysophosphatidic acid receptor 4 gene

Lpar5: Lysophosphatidic acid receptor 5 gene

LPC: Lysophosphatidylcholine

LPE: Lysophosphatidylethanolamine

LPS: Lysophosphatidylserine

LysoPLs: Lysophospholipids

M1 and M2: Motor cerebral cortex

MAG: Myelin-associated glycoprotein

MAPK: Mitogen activated protein kinases

MBP: Myelin basic protein

MHC: Major histocompatibility complex

MMP: Metalloproteinases

MOBP: Myelin-associated oligodendrocytic basic protein

MOG: Myelin oligodendrocyte glycoprotein

MOSP: Myelin/oligodendrocyte specific protein

MRI: Magnetic resonance image

MRS: Magnetic resonance spectroscopy

MS: Multiple sclerosis

MSCH: Mouse spinal cord homogenate

NAA: N-acetyl aspartate

NANA: N-acetyl-neuraminic acid

NG2: Chondroitin sulfate proteoglycan

OEC: Olfactory ensheathing cells

olig2: Oligodendrocyte lineage transcription factor2

OPC: Oligodendrocyte progenitor cell

ot: Optic tractus

PA: Phosphatidic acid

PB: Phosphate buffer

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PDGFRα: Platelet-derived growth factor receptor α

PDI: Protein Disulphide Isomerase

PFA: Paraformaldehyde

pid: Postimmunization day

PKC: Protein kinase C

PLA: Phospholipase A-like enzymes

PLC: Phospholipase C

PLD: Phospholipase D

PLP: Proteolipid protein

PNS: Peripheral nervous system

PP-MS: Primary Progressive Multiple Sclerosis

PR-MS: Progressive-Relapsing Multiple Sclerosis

PTX: Pertussis toxin

RER: Rough endoplasmic reticulum

RR-MS: Relapsing-Remitting Multiple Sclerosis.

RT-PCR: Real time polymerase chain reaction

S1P: Sphingosine 1-phosphate

SC: Schwann cells

Shh: Sonic hedgehog

SNP: Single nucleotide polymorphisms

SP-MS: Secondary progressive Multiple Sclerosis

St: Striatum

TBS: Tris-buffered saline

TCR: T cell receptor

Th: Thalamus

TNF: Tumor necrosis factor

VCAM: Vascular cell adhesion molecule

VZ: Ventricular zone

RESUMEN en ESPAÑOL

INTRODUCCIÓN

La esclerosis múltiple (EM) es la enfermedad neurológica crónica más frecuente en adultos jóvenes caracterizada por neuroinflamación, desmielinización y degeneración axonal en grado variable y en cuya patogenia parecen estar involucrados una predisposición genética y un componente ambiental, presumiblemente una infección de origen vírico, desencadenante del proceso. Aunque no se conoce bien su etiología, se conoce que en la EM está implicado el sistema inmunológico, presumiblemente por medio de un proceso autoinmune donde juegan un papel fundamental los linfocitos T. Así, la alteración parece estar causada por la existencia de células T CD4+ autorreactivas que reconocen a los antígenos de la mielina, generando una respuesta inflamatoria conducente a la propia destrucción mielínica. (Martino y Hartung 1999; Sospedra y Martín 2005) El daño producido en el sistema nervioso parece, sin embargo, estar mediado también por otros componentes del sistema inmune, sistema del complemento, células T CD8+, anticuerpos específicos, entre otros (Sospedra y Martín 2005; Ziemssen y Ziemssen 2005).

Buena parte de los conocimientos de la EM en relación a los mecanismos celulares que tienen lugar durante el curso de la enfermedad, así como los encaminados a determinar su patogenia han venido dados por las investigaciones realizadas en el, hasta la fecha, su más validado modelo experimental animal por medio de la inducción de encefalomielitis autoinmune experimental.

La encefalomielitis autoinmune experimental (EAE) es una enfermedad autoinmune neuroinflamatoria desmielinizante mediada por células T y macrófagos, inducida en animales bien a través de la inmunización activa contra antígenos de la mielina del sistema nervioso central (SNC) ,o bien, a través de la transferencia de células T colaboradoras de tipo 1 (CD4+) activadas y específicas para estos antígenos, siendo, al día de hoy, empleada como modelo experimental para el estudio de la EM humana por compartir con ella numerosas características clínicas y patológicas, con manifestación de infiltración celular, desmielinización multifocal y pérdida axonal (Swanborg 1988; Bashir y Whitaker 2002).

A pesar de los avances realizados hasta la fecha se desconocen muchos de los aspectos de la patogenia de la EM así como de aspectos concretos de los mecanismos reguladores de la enfermedad. El objeto de este estudio es conocer el papel de un mediador lipídico, el ácido lisofosfatídico, relacionado de manera clara con buena parte de los componentes de respuesta celular afectados en el

curso de la EM y que podría desempeñar un papel regulador en los procesos de mielinización y de respuestas proinflamatorias involucrados en la EM.

El ácido lisofosfatidico (LPA, de *lysophosphatidic acid*) es un fosfolípido endógeno bioactivo que actúa como mensajero intercelular en numerosos tipos celulares a través de receptores específicos (LPA_{1-5}) acoplados a proteína G (Tigyi et al. 2003) e induciendo respuestas biológicas variadas, proliferación, diferenciación, supervivencia y quimiotaxis, entre otras. Se han demostrado diferentes respuestas biológicas inducidas por LPA en un amplio rango de células nerviosas y que incluyen movilización de calcio, estimulación de la proliferación celular, inhibición de la supervivencia celular, cambios morfológicos y de conductancia iónica así como activación de diferentes cascadas de señalización, observándose también buena parte de estas respuestas en células no neurales. (Moolenar et al. 2004; Chun 2004). Igualmente y, por ello, se ha sugerido su implicación como molécula reguladora en diversas patologías (rev. en Sengupta et al. 2004).

La primera parte de este estudio se centra en aumentar el conocimiento acerca del papel que desempeña esta molécula lipídica en los procesos de mienilización. Ya se ha sugerido un importante papel del receptor del ácido lisofosfatidico LPA_1 en la regulación del desarrollo de la mielinización. Weiner et al. (1998) demostraron una correlación cercana al 100% entre la expresión del receptor LPA_1 y el inicio de la expresión de la proteína proteolipídica (PLP), una proteína constituyente de la mielina y expresada exclusivamente en oligodendrocitos maduros. Son, precisamente, los oligodendrocitos maduros, mielinizantes, y no los precursores, no mielinizantes, los que responden a la administración de LPA, medida ésta en aumento de la concentración de calcio intracelular (Möller et al. 1999). Diferentes estudios han demostrado la expresión receptor LPA_1 en cerebro adulto en los tractos mielínicos y en dichas células mielinizantes (Weiner et al. 1998; Allard et al. 1999; Handford et al. 2001). Cervera et al. (2002) demostraron que el receptor LPA_1 se expresaba los tractos mielínicos del cerebro adulto, en el soma de los oligodendrocitos y en sus fibras mielinizantes viendo, además, que éste colocalizaba con la expresión de la proteína básica para la mielina en rata y en el cerebro humano. Los mismos autores observaron que la expresión del receptor era mayor en los oligodendrocitos que en las fibras mielinizantes, en relación inversa a la de la proteína mielínica. La función del receptor parece estar ligada a las últimas fases de maduracion oligodendrocítica y podría estar implicada en la terminación o mantenimiento del proceso de mielinización (Stankoff et al. 2002). Así mismo experimentos recientes han demostrado que la administración de LPA a

oligodendrocitos en cultivo estimula la expansión de sus procesos membranosos (Nogareli et al., 2009).

Si bien hasta la fecha los efectos descritos mediados por LPA se deben a la administración exógena del LPA, no se conoce aún con exactitud el significado funcional de los mecanismos de señalización mediados por LPA en el sistema nervioso. Su producción, por parte del organismo depende principalmente de la acción de las fosfolipasas A1, A2, D y lisofosfolipasa D, que se encargan de su génesis a nivel plasmático y sérico (Aoki 2004). La disponibilidad cerebral de LPA puede ser regulada no sólo a nivel cerebral, sino desde la propia concentración sérica. Las altas concentraciones de LPA en suero (Baker et al. 2000; Aoki et al. 2002) han permitido postular (Weiner et al. 1998) alguna de las acciones potenciales del LPA sobre los oligodendrocitos habida cuenta de las acciones mediadas in vitro por el suero a través de alguno de sus componentes de naturaleza probablemente lipídica y en relación a la supervivencia de las células mielinizantes (Louis et al. 1992) o al incremento de los niveles de galactocerebrósidos en éstas (Bologa et al. 1988). En las mismas hipótesis se contempla la participación de dicho fosfolípido y otros estructuralmente semejantes en la constitución lipídica de la cubierta mielínica, representada en más del 75%, en el SNC y el sistema periférico desde donde podrían actuar como señal autocrina de control negativo para los oligodendrocitos (Weiner et al 1998; Möller et al 1999).

La segunda parte de este estudio se ha centrado paralelamente en papel del LPA sobre otros tipos celulares, aquellos implicados en los procesos inflamatorios y que también se ven afectados en el desarrollo de la EM.

Numerosos estudios han tratado el papel del LPA en los procesos inflamatorios, a nivel general, demostrando su mediación en la supervivencia de las células T, así como en la incorporación de macrófagos (rev. en Gräler et al. 2002). El papel que pueda desempeñar el LPA en la respuesta inmune en la EM adquiere más relevancia si consideramos que los linfocitos T (Goetzl et al. 2000) y los macrófagos (Hornuss et al. 2001) expresan diferentes receptores para LPA (Chinetti et al. 1998; Cippitelli et al. 2003). Aunque se ha observado que existen receptores para LPA tanto en células CD4+ como CD8+, son las primeras las que presentan una respuesta funcional ante la presencia de LPA (Goetzl et al., 2000). En la misma línea Zheng et al. (2000) mostraron que existía una expresión diferencial de receptores para LPA en células CD4+ según estuviesen o no activadas por mitógeno. En este estudio se observó que las células CD4+ inactivas presentan un predominio del receptor LPA_2 siendo el LPA_1 casi

inexistente, en cambio, cuando estas son activadas por mitógeno la expresión del receptor LPA_2 disminuye, aumentando el tipo LPA_1. Este cambio cualitativo y cuantitativo en los receptores para LPA tiene su reflejo en el efecto que produce en las mismas su presencia en el medio. Mientras en células T inactivas, en las cuales predomina el receptor LPA_2, el LPA produce una disminución en la expresión de IL-2; en las células CD4+ activadas la expresión de esta citoquina se ve fuertemente aumentada en presencia de LPA. Esta citoquina proinflamatoria ha sido motivo de estudio debido a que sus niveles se encuentran elevados tanto en el suero como en LCR de pacientes con esclerosis múltiple (Sivieri et al., 1998) así como también se ha relacionado un polimorfismos en el promotor del receptor soluble de IL-2 con una mayor susceptibilidad para adquirir la enfermedad (Matesanz et al., 2004).

Paralelamente la disponibilidad cerebral de LPA puede ser regulada no sólo a nivel cerebral, donde está presente junto a sus rutas metabólicas (Sugiura et al 1999), sino desde la propia concentración sérica. Así, ante situaciones experimentales de daño cerebral vascular se genera desde las plaquetas activadas un incremento de LPA sérico a concentraciones similares a las requeridas para su efecto exógeno experimental (Tigyi et al. 1995) promoviendo un incremento de la permeabilidad vascular que permitiría su difusión hacia el parénquima cerebral a la concentración efectora (Schulze et al. 1997). Además de este aumento de la permeabilidad vascular el LPA a través de dos de sus receptores, LPA_1 y LPA_3, induce la expresión de genes relacionados con la inflamación en células endoteliales humanas tales como la IL-1ß, IL-8 y MCP-1 (Lin et al., 2007), interviniendo en la quimiotaxis de las células del sistema inmune a través de la barrera hematoencefálica.

Basándose en todo ello el estudio del papel que juega el LPA en la EM, fundamentalmente a través de su principal receptor, LPA_1, parece adquirir una importante relevancia no sólo en controlar los procesos de maduración oligondendrocitaria y mielinización sino que también puede estar implicado en la respuesta de las principales células causantes de la autoinmunidad en la EM, las células T CD4+, así como en su movilidad a través de la barrera hematoencefálica.

HIPÓTESIS Y OBJETIVOS

Hipótesis: El receptor LPA_1 es esencial para la mielinizacion normal y la respuesta inmune. Por lo tanto, su ausencia en los ratones nulos para LPA_1 provoca alteraciones celulares asociadas tanto al sistema nervioso central como al sistema inflamatorio, afectando por ello al desarrollo normal de los oligodendrocitos, formación de mielina y a la respuesta inmune adaptativa tras la inducción de una enfermedad autoinmune desmielinizante.

Con el fin de demostrar dicha hipótesis se han planteado los siguientes objetivos primarios y secundarios:

- Analizar el papel del LPA en mielinización, usando el modelo de ratones *knock-out* para LPA_1 (variante *Málaga* nula para LPA_1 ó $maLPA_1$-nulonulo). El estudio se llevará a cabo comparando los fenotipos de los ratones normales y $maLPA_1$-nulos
  - Estudio de los patrones de mielinización
  - Estudio del desarrollo de los oligodendrocitos
  - Administración de análogos de LPA en un modelo de desmielinización tóxica por administración de cuprizona.

- Analizar el papel del LPA en la patogénesis de la esclerosis múltiple mediante el estudio de su modelo experimental ampliamente aceptado de encefalomielitis autoinmune experimental (EAE).
  - Estudio de las diferencias en los síntomas clínicos externos y la histología de la respuesta inmune entre los ratones normales y los ratones nulos tras la inducción de la enfermedad.
  - Estudio de la expresión de los receptores LPA_1 y LPA_2 en células mononucleares de sangre periférica (PBMC) a lo largo del curso clínico para inferir sus papeles en algunos estados de la patogénesis de la enfermedad.
  - Estudio de los efectos de la administración intravenosa de agonistas vs. antagonistas para los receptores de LPA en la EAE.

MATERIAL Y MÉTODOS

En el presente trabajo se han desarrollado una serie de técnicas con el fin de poder estudiar los objetivos anteriormente planteados. Estas técnicas han consistido en el genotipado y mantenimiento de la colonia de ratones maLPA$_1$-nulo que han constituido los sujetos de estudio; preparaciones histológicas para su análisis por microscopía óptica convencional y de fluorescencia (fijación de muestras, inclusión y corte, y técnicas inmunohistoquímicas); cuantificación estereológica, procesamiento y observación por microscopía electrónica; electroforesis, electrotransferencia e inmunotinción de proteínas; evaluación de la expresión de genes mediantes *PCR* a tiempo real (*RT-PCR*); inducción de los modelos animales estudiados: encefalomielitis autoinmune experimental y modelo de desmielinización tóxica por cuprizona, así como la administración de agonistas o antagonistas de los receptores de LPA.

RESULTADOS y DISCUSIÓN

Influencia del receptor LPA$_1$ en mielinización

Expresión del receptor LPA$_1$ en el sistema nervioso central de los ratones normales.

En una primera aproximación se ha estudiado la expresión del receptor de LPA en el sistema nervioso central con el fin corroborar su presencia en todas estructuras mielinizadas (cuerpo calloso, estriado, corteza, tálamo y sustancia blanca medular entre otras) como era ya referido en la bibliografía, además de poder contrastar la misma con su completa ausencia en los ratones nulos para el receptor.

La deleción del receptor LPA$_1$ no muestra mecanismos de compensación por parte de los otros receptores de LPA.

Mediante estudios de expresión por *PCR* a tiempo real se muestra la ausencia de LPA$_1$ en los ratones nulos, corroborando los resultados inmunohistoquímicos, y una expresión ligeramente alterada de los otros cuatro receptores de LPA$_{(2\text{-}5)}$. En ninguno de los casos la ausencia de LPA$_1$ parecía verse compensada por una sobreexpresión de algunos de los otros receptores, si bien, en ciertas estructuras ocurre una disminución paralela de alguno de ellos. LPA$_3$ muestra un descenso de expresión en la corteza y el cuerpo calloso de los ratones nulos, mientras que LPA$_4$ disminuye su expresión en el hipocampo. Cabe

destacar que la alteración de sus expresiones aparece en las estructuras en las que LPA_1 y el otro receptor posible de LPA comparten parcialmente alguna función.

Patrón de mielinización en ratones carentes de LPA_1.

Tras una primera aproximación no invasiva mediante espectometría por resonancia magnética se observó que los ratones nulos mostraban un aumento en los niveles de colina en el cerebro lo que parece indicar algún tipo de alteración en el metabolismo de los componentes de sus membranas celulares.

La carencia del receptor LPA_1 en oligodendrocitos parece jugar un papel importante en la mielinización y en su maduración. Esto se ha puesto de manifiesto mediante el estudio de los patrones mielínicos y de las poblaciones oligodendrogliales.

Así, se observa que la población de oligodendrocitos maduros (GalC+, PLP+) esta disminuida en los ratones carentes del receptor mientras que las células precursoras de oligodendrocitos (NG2+) no han demostrado seguir la misma tendencia. Paralelamente las fibras que expresan las principales proteínas que constituyen la mielina (MBP, PLP, y CNPasa) también presentan una disminución de su densidad en los ratones nulos.

Mediante los estudios de *westernblot* en mielina se demuestra que, además de la disminución en el número de fibras observado por inmunohistoquímica, tiene lugar una alteración en la constitución de dicha mielina. Esto se debe a una disminución cuantitativa de las principales proteínas constituyentes de la mielina (MBP, PLP y CNPasa). Esta disminución ocurre principalmente en la corteza, donde se ha centrado el estudio, aunque también puede apreciarse en el estriado.

Sin embargo, esta disminución de las proteínas de la mielina no parece afectar a la ultraestructura o compactación de las vainas tras su observación a microscopia electrónica. Paralelamente, a dicho nivel, sí que se observa que la ausencia del receptor altera la simetría radial y organización de las fibras, siendo esta alteración más apreciable en el cuerpo calloso. Las fibras presentan un aspecto más laxo, en lugar de formar paquetes fibrilares, y no se orientan uniformemente sino que entrelazan sus direcciones. Esta configuración de las fibras a nivel ultraestructural recuerda a la observada en algunas patologías en las que ocurre una maduración incompleta de los oligodendrocitos o una remielinización tras alguna lesión.

Por otro lado, basándonos en los estudios de estereología, se hace evidente que el LPA a través de su receptor de membrana LPA_1 interviene de forma decisiva en la oligodendroglía del ratón adulto, disminuyendo hasta un 30% el número total de oligodendrocitos en corteza cuando se elimina dicho receptor.

Los defectos en el patrón de mielina se observan tras el pico de mielinización.

Estas alteraciones se analizaron en distintos momentos del desarrollo, en ratones postnatales de 10 días de edad (P10) y ratones adultos jóvenes de un mes de edad (P30), es decir, antes y después del momento de máxima expresión de LPA_1 en los tractos mielínicos (P16). Se observó que mientras en adultos jóvenes las diferencias seguían el mismo patrón que en adultos, existiendo una disminución en la densidad de fibras mielinizadas así como en el número de oligodendrocitos, los ratones nulos a estadio P10 no presentaban ninguna diferencia con sus homólogos normales. Estos datos apoyan la hipótesis de partida de nuestro estudio, ya que las alteraciones se producen una vez desaparecidas las etapas de mayor expresión del receptor durante el desarrollo oligodendrocitario.

La ausencia del receptor LPA_1 genera un tráfico anormal de la proteína PLP lo cual puede producir apoptosis en los oligodendrocitos.

En los ratones que no presentan el receptor se observa una acumulación de proteína PLP en el cuerpo celular en lugar de distribuirse hacia las vainas. Esta proteína parece encontrarse repartida entre el retículo endoplasmático principalmente, donde se traduce, el aparato de Golgi, donde madura, y sistema endosomal, donde podría llevarse a cabo su degradación.

Aún no está clara la razón o el mecanismo por el que se produce la disminución de oligodendrocitos en los individuos adultos carentes del receptor sin embargo, los resultados presentados en este trabajo parecen apuntar a un aumento de su apoptosis debido a una acumulación de la proteína PLP en el cuerpo celular. Esta acumulación sería debida a una alteración en el proceso de maduración del oligodendrocito mediante el cual la proteína PLP no sería correctamente transportada a la vaina de mielina. Esto explicaría el hecho de que en estadios tempranos en los que aún no se ha producido el transporte de dicha proteína a las vainas de mielina, las diferencias entre ratones normales y nulosson prácticamente inexistentes. Mientras que, una vez transcurrida esta etapa,

cuando, como resultado de la falta del receptor, se altera el transporte de PLP y éste, en lugar de ser transportado a su lugar de destino, comienza a acumularse en los cuerpos celulares, se puede originar un estrés en el retículo que terminaría en la muerte de la célula. Esta teoría se ve apoyada por el aumento de apoptosis que se observa en la corteza de los ratones nulos.

El hecho de que los ratones carentes del receptor LPA_1 no muestren una disminución en la población de oligodendrocitos inmaduros en migración, NG2+, lleva a pensar que el efecto de este mediador lipídico se centra en la maduración de estas células, interviniendo en el transporte de la proteína PLP a su correcta localización celular para llevar a cabo su función, y no en su proliferación.

Efectos del LPA en remielinización tras tratamiento con cuprizona.

Tras haber mostrado la necesidad de la señalización mediada por el receptor LPA_1 para la maduración y supervivencia de los oligodendrocitos, así como para una correcta mielinización, cabía esperar que el tratamiento con análogos de LPA en modelos en los que ha tenido lugar un proceso de desmielinización favoreciera la normal remielinización de las fibras. En este caso el modelo a estudiar fue el de desmielinización tóxica por cuprizona, y fue llevado a cabo en ratones heterocigotos, de una cepa diferente, que expresaban la proteína verde fluorescente bajo el promotor de la proteína oligodendrocitaria CNP.

Los resultados muestran que la administración central delanálogo de LPA, (octadecil-LPA en forma de sal amónica no hidrolizable; Avanti Polar Lipids Inc) favorece los procesos de remielinización que tienen lugar tras detener el tratamiento con el neurotóxico. Este efecto es mayor cuando la administración ocurre cuatro días después de detener la intoxicación, es decir, cuando la remielinización espontánea es mayor.

Influencia del receptor LPA_1 en inflamación.

La ausencia del receptor LPA_1 genera un cuadro de menor severidad del curso clínico en la encefalomielitis autoinmune experimental.

La inmunización de la cepa maLPA$_1$-nulo estudiada con el péptido de mielina MOG_{35-55} genera un curso clínico de tipo remitente-recurrente en los primeros 40 días de desarrollo de la enfermedad, pasando, después de esta fase, a

una evolución progresiva de la misma en la que los síntomas no desarrollan cambios significativos.

Cuando se comparan el cuadro clínico de los ratones inmunizados se observan que en aquellos que no presentan el receptor LPA_1 existe un retraso en la aparición de los síntomas entre dos y cuatro días respecto a lo observado en sus consanguíneos normales. Además, la gravedad de los síntomas alcanzados durante los brotes en fase remitente-recurrente es también menor en los ratones nulos, y por tanto muestran una menor gravedad cuando evolucionan a la fase progresiva.

La expresión del receptor LPA_1 incrementa durante los procesos inflamatorios en la célulasmononucleares de sangre periférica (PBMC) y se correlaciona con la infiltración celular en el sistema nervioso central y la inflamación.

Durante el desarrollo de la enfermedad se recogieron muestras de sangre de los ratones en distintos estadios y grados de severidad de los síntomas. En las PBMC obtenidas de dichas muestras se midieron la expresión de los receptor LPA_1 y LPA_2 así como de GADPH, como gen constitutivo para normalizar la expresión de los receptores estudiados.

Se observó, como ya habían mostrado estudios previos en cultivos de linfocitos T CD4+ (Zheng et al., 2000), que la expresión del receptor LPA_1, casi inexistente en ausencia de síntomas, aumentaba considerablemente cuando éstos se desarrollaban y que dicha expresión se correlacionaba con su gravedad. Paralelamente a este aumento de la expresión de LPA_1 tenía lugar una disminución de la expresión de LPA_2, receptor éste que presentaba sus valores más altos en los individuos controles sanos. En ambos casos, tanto el aumento de LPA_1 como la disminución de LPA_2 se presentaban con mayor intensidad al inicio de los brotes, haciendo que la proporción de expresión o *ratio* LPA_1/LPA_2 fuera máxima en dichos momentos.

Al correlacionar los estudios de expresión de los receptores de LPA en sangre con el análisis histológico del sistema nervioso central, se observó que el aumento de los niveles de expresión de LPA_1 en sangre se correlacionaba con un mayor número de infiltrados celulares, tanto de linfocitos T CD3+, como de macrófagos y microglía activada con morfología ameboidea. De manera consecuente, cuando dichos estudios se realizaban en animales en fase progresiva o estabilizada de la enfermedad, en los cuales los valores de expresión de LPA_1

eran menores, los referidos a LPA_2 elevados, y los síntomas no mostraban una alteración significativa, el número de células T observadas en el parénquima cerebral era también muy reducido.

Basándonos en estos resultados podría concluirse que el valor del *ratio* de las expresiones de LPA_1 y LPA_2 podría tener un valor como marcador del estado en el que se encuentra la enfermedad.

Estos datos junto al desarrollo de un curso clínico más suave en carencia del receptor apuntan a que el LPA, a través de LPA_1, juega algún papel de importancia en la patología de la encefalomielitis.

La presencia de la vía de señalización mediada por LPA_1 es necesaria para el desarrollo de un curso clínico normal pero no para la infiltración de células inmunes.

Paralelamente a los estudios de expresión de los receptores se analizaron histológicamente las diferencias entre los animales normales y los animales nulos a LPA_1 a lo largo del curso clínico.

La evaluación de los síntomas se correlacionaba con la histología, presentándose un mayor infiltrado de macrófagos y linfocitos T en aquellos individuos que mostraban más gravedad en los síntomas y pudiendo encontrarse, además, placas desmielinizantes, aunque su presencia se observó únicamente en la médula espinal y no en el telencéfalo. Estas características también se reflejaban en los ratones carentes del receptor, los cuales, al presentar una menor severidad en los síntomas, mostraban menor número de infiltrados y de placas desmielinizantes. Sin embargo, cuando se comparaban ambos genotipos con sintomatología similar, no se observaron diferencias significativas en el grado de infiltración linfocitaria. Esto hace pensar que, si bien el receptor LPA_1 puede jugar un papel importante en la activación o iniciación de los procesos inflamatorios, cuya ausencia retrasaría y reduciría su gravedad, su presencia no es completamente necesaria, ya que dicha inflamación también puede desarrollarse en su ausencia.

Inyecciones intravenosas de agonistas para el receptor LPA_1 retrasan la aparición de los síntomas y disminuyen la gravedad del curso clínico.

Como último punto de este trabajo y con el fin de mostrar la implicación del receptor LPA_1 en el desarrollo y evolución de la enfermedad se procedió a la

administración de antagonistas para dicho receptor en ratones normales con EAE inducida, lo cual, de acuerdo con la hipótesis previamente formulada acerca de su implicación en la enfermedad, debería modificar el curso clínico.

Para ello se llevaron a cabo dos experimentos, los cuales se repetieron al menos tres veces obteniéndose resultados esimilares. En una primera aproximación se utilizaron ratones normales a los que se les indujo la EAE y se les inyectó de manera intravenosa un antagonista para el receptor LPA_1 (VPC 32183 (S); Avanti Polar Lipids Inc.) antes de la aparición de los síntomas. En este caso, la presencia de dicho antagonista consiguió retrasar ligeramente la aparición de los síntomas de uno a tres días, y generar un cuadro similar a lo observado en el curso clínico mostrado por los ratones nulos. Sin embargo,a los cuatro y cinco días de la administración los animales comenzaban a agravar sus síntomas hasta ser éstos indistinguibles de los mostrados por los ratones a los que únicamente se les había administrado el vehículo.

Estos resultados sugerían que la reversión de los efectos del antagonista podía deberse a una degradación del lípido, por lo que, en una siguiente aproximación, se administró el fármaco de manera periódica cada 5 días. En este caso, los ratones tratados no mostraron un agravamiento del curso clínico y mantuvieron de manera prolongada este estado, a la par que en los controles sin tratamiento la enfermedad evolucionaba a una forma progresiva.

CONCLUSIONES

- La ruta de señalización de LPA_1 está directamente implicada y es necesaria para la correcta mielinización de las fibras, como se muestra por las alteraciones observadas en los ratones carentes de este receptor, y que afectan a la cantidad, calidad y organización de las fibras mielinizadas.

- La presencia de LPA_1 tiene un papel importante en el tráfico de la proteína PLP desde el soma celular hasta su destino final en la vaina de mielina. La ausencia de este receptor provoca una acumulación de la proteína en el retículo endoplasmático que se correlaciona con pérdida celular y sugiere una apoptosis de los oligodendrocitos inducida por estrés.

- De acuerdo al papel propuesto para LPA_1 en la mielinización y el mantenimiento de la viabilidad de oligodendrocitos, la administración intracisternal de LPA tras desmielinización inducida por cuprizona promueve la proliferación de oligodendrocitos y los procesos de remielinización endógena.

- La ruta de señalización mediada por los receptores LPA_1 y LPA_2, contribuye al desarrollo de los procesos inflamatorios en la EAE como sugiere la modulación correlacionada de sus expresiones en las células mononucleares de sangre periférica durante el curso de la enfermedad. En ausencia de LPA_1, la patología se desarrolla mas tarde y presenta un curso significantemente más benigno, corroborando la implicación del receptor en la patogénesis de la EAE.

- De manera similar, cuando, tras la inducción de EAE en animales normales, la vía de señalización por LPA_1 está bloqueada, no por la ausencia del receptor, sino por la administración intravenosa de un antagonista de LPA_1, se obtienen resultados similares que muestran cuadros clínicos más benignos de la patología.