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PhD PROGRAM IN NEUROSCIENCE
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PhD Thesis:

“D-Aspartate treatment stimulates differentiation of oligodendrocyte precursors, prevents demyelination and accelerates remyelination in the cuprizone mouse model of Multiple Sclerosis”

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Abbreviations

AC	Adenylate Cyclase
APC	Antigen-Presenting Cell
BBB	Blood-Brain-Barrier
bHLH	Basic Helix–Loop–Helix
BMPs	Bone Morphogenetic Proteins
CaCA	Calcium-Cation-Antiporters
CC	Corpus Callosum
CIS	Clinically Isolated Syndrome
CNPase	2',3'-Cyclic Nucleotide-3-Phosphodiesterase
CNS	Central Nervous System
CSF	Cerebrospinal fluid
CXCL13	C–X–C motif Chemokine 13
D-Ala	D-Alanine
D-Asp	D-Aspartic Acid
D-Ser	D-Serine
DDO	D-Aspartate Oxidase
EAE	Experimental Autoimmune Encephalomyelitis
EB	Ethidium Bromide
EBV	Epstein-Barr Virus
EP	Evoked Potential
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FLC	Free Light Chains
GalC	Galactosylceramide
GPCR	G protein-coupled protein
HHV-6	Human Herpes Virus type 6
HLA	Human Leukocyte Antigen
HPLC	High Performance Liquid Chromatography
HPV	Human Papilloma Virus
Id2/4	Inhibitor of DNA binding 2/4
IGF1	Insulin-Like Growth Factor 1
iGluR	ionotropic Glutamate Receptor
IHC	Immunochemistry
K2P	Potassium Channel Two-Pore domain
KA	Kainate
LFB	Luxol Fast Blue
LFB-PAS	Luxol Fast Blue-Periodic Acid Schiff
LPC	Lysolecithin
MAG	Myelin Associated Glycoprotein
MAPK	Mitogen Activated Kinase
MBP	Myelin Basic Protein
mGluR	metabotropic Glutamate Receptor
miRNA	microRNA

MOG	Myelin Oligodendrocyte Glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
NCX	Na^{+}/Ca^{2+} exchanger
NfL	Neurofilament Light Chain
NMDAR	N-methyl-D-Aspartate Receptor
NT-3	Neurotrophin-3
OCBs	Oligoclonal Band(s) of IgG
OPC	Oligodendrocyte Precursors Cell
P60	Postnatal day 60
PDGF	plateled-derived growth factor
PDGF-αR	PDGF-α receptor
PI_3K	Phosphoinositide-3 Kinase
PKC	Protein Kinase C
PL	Plaque
PLC	Phospholipase C
PLP	Proteolipid Protein
PMA	Phorbol-Myristate-Acetate
PNS	Peripheral Nervous System
PPMS	Primary Progressive Multiple Sclerosis
PPWM	Periplaque White Matter
PRMS	Progressive Relapsing Multiple Sclerosis
RRMS	Relapsing-Remitting Multiple Sclerosis
Shh	Sonic Hedgehog
SPMS	Secondary Progressive Multiple Sclerosis
Tcf/LEF	T-cell factor/Lymphoid Enhancer Factor
TGF-β	Transforming Growth Factor-β
TH	Thyroid Hormone
TM	Transmembrane
VDCC	Voltage-Gated Calcium Channel
YY1	YinYang1

SUMMARY

Emerging evidence support a role for some D-Aminoacids as neurotrasmitters and neuromodulators, since they are found in mammalian tissues and also in the central nervous system (CNS) (Hashimoto and Oka, 1997). They play important roles in some physiological processes, including dendritic morphology, synaptic plasticity and cognition (Wolosker *et al.*, 2008; Billard, 2012). Among D-Aminoacids, recent studies suggest that D-Aspartic acid (D-Asp), a newly discovered agonist for NMDA receptors, play a role in NMDA receptor-dependent processes such as synaptic plasticity and memory (Errico *et al.*, 2015). The D-Asp was described in the multi-lamellar membrane that insulate axons (Fisher *et al.*, 1986) and its effects on the hormone biosynthesis and release have been largely explored in the years (Gold and Voskuhl 2009; Nuñez *et al.*, 2000; Cerget *et al.*, 2006).

The exact mechanism of myelination process is still unknown, but emerging studies demonstrated the importance of intracellular changes in $[Ca^{2+}]_i$ levels during myelination and remyelination processes (Soliven *et al.*, 2001). Indeed, differentiation of oligodendrocyte precursors cells (OPC) and remyelination are associated with NMDARs-dependent $[Ca^{2+}]_i$ changes (Martinez-Lozada *et al*., 2014). A recent work performed by our research group demonstrated that $[Ca^{2+}]_i$ signaling mediated by the Na^+/Ca^{2+} exchanger NCX3 plays an important role during oligodendrocytes differentiation and myelin formation (Boscia *et al.*, 2012; Casamassa *et al.*, 2016).

In the present study, we investigated the effects of D-Asp during the OPC differentiation and remyelination by using both *in vitro* and *in vivo* techniques. *In vitro*, we evaluated the effects of D-Asp exposure both in human oligodendrocyte MO3.13 cell line and rat primary OPC, exposed to different concentrations of D-Aspartic acid (10-100-200 μM). Quantitative RT-PCR analyses showed that 10-200 μM D-Asp exposure for 3 days, upregulated, in a concentration-dependent manner, both the myelin markers CNPase and MBP and NCX3 transcripts in human oligodendrocytes M03.13 progenitors. The transcripts increase were significantly prevented by the NMDA receptor antagonist 10 μM MK-801 and the two NCX3 blockers, 30nM YM-244769 and 100nM BED. In accordance, microfluorimetric studies demonstrated that 100μM D-Asp administration induced an initial calcium peak of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ followed by an oscillatory $[Ca^{2+}]_i$ pattern both in oligodendrocyte MO3.13 progenitors and rat primary OPC. The NMDA antagonist 10μM MK-801 completly suppressed $[Ca^{2+}]_i$ oscillations but only partially affected the first $[Ca^{2+}]_i$ peak. Similar effects were observed in presence of the two selective blockers for NCX3, 30nM YM-244769 and 100nM BED. In addition, electrophysiological recordings performed

in oligodendrocytes M03.13 progenitors showed that the current elicited by 100 µM D-Asp stimulation were dependent by AMPA activation, since the AMPA receptor inhibitor 10µM DNQX significantly prevented D-Asp induced inward currents.

Our *in vitro* results suggest that D-Asp stimulates oligodendrocyte development through a mechanism involving calcium signaling through the glutamate receptors AMPA and NMDA and the Na^{+}/Ca^{2+}exchanger NCX3.

Next, we investigated the effects of D-Asp administration in an *in vivo* model of demyelination/remyelination, the cuprizone mouse model. D-Asp was given during cuprizone feeding (demyelination), or after cuprizone withdrawal (remyelination). In both conditions, D-Asp treatment improved motor coordination performance in the beam balance and rotarod test. When given during demyelination D-Asp prevented MBP loss and reduced inflammation, as revealed by Western Blot analysis of MBP, Iba1 and GFAP proteins and quantitative coexpression analysis of MBP with the axonal marker NF200. Finally, electron microscopy performed on *corpus callosum* sections showed that D-Asp treatment accelerates remyelination in cuprizone mice, as demonstrated by the increased number in myelinated axons if compared to untreated cuprizone mice.

Collectively, our results show that treatment with D-Aspartate, by influencing calcium signaling in oligodendrocytes, might produce beneficial effects during demyelination and remyelination processes.

CHAPTER I

INTRODUCTION

I. INTRODUCTION

The biology of myelin remained for long times unexplored, since where an increasing interest in its physiological role newly aroused (Boullerne, 2016). Myelin is the protective sheath formed by oligodendrocyte processes which envelop axons in the nervous system as long spiralized wire, necessary to increase velocity of nerve impulse conduction, with a unique architecture in lipid and protein composition.

Myelin disorders include any pathological condition of the nervous system in which myelin is injured in terms of composition, shape or relative amount. Myelin disorders can be divided into three groups: 1) hypomyelinating diseases; 2) demyelinating diseases; 3) dysmyelinating diseases.

In *hypomyelinating diseases*, myelin is never made in sufficient amounts as consequence of genetic disorders as occurs in leukosdystrophyes or *in utero* infections (Barkovich and Deon, 2016).

Demyelinating diseases are characterized by the loss of the myelin sheath insulating the nerves, although an healthy myelin is initially formed. The most common disorders of this group include Multiple Sclerosis, some autoimmune or genetic disorders. The etiology of demyelination could refer also to an external event such as febrile episodes, head trauma, infectious, stroke or exposure to toxic chemical (Love, 2006; Popescu and Lucchinetti, 2012).

Dysmyelinating diseases are clinical conditions characterized by a defective structure and function of the myelin sheath because myelin is incorrectly formed. These groups of disorders manifest early in life and are genetically determined. Unlikely to demyelinating diseases, this group of disorders does not include visible lesions. These disorders include leukosdystrophyes, (including Pelizaeus–Merzbacher disease, Canavan disease, phenylketonuria) and also schizophrenia (Kramer *et al.*, 2006; Matalon *et al.*, 2006; Tkachev *et al.*, 2007). The myelin alterations cause in return deficiencies in superior functions including cognition, sensation and movement, contributing to complex neurological disorders outcome.

1. MULTIPLE SCLEROSIS

Discovered in 1849, Multiple sclerosis (MS) is one of the most common chronic inflammatory neurodegenerative disease affecting the white matter of central nervous system (CNS) and spinal cord characterized by inflammation, demyelination and axonal degeneration (Trapp *et al.*, 1998).

Among Caucasians in the temperate zone, MS is the third most frequent neurological disease (Compston and Coles, 2008). As widespread disabling neurological condition of young adults between age of 20 to 40, the disease presents a chronic evolution with different progression stages in which an abnormal immune system response against self components of the myelin affect its own integrity. The exact etiology of the disease remains still unknown despite various immunological, viral, genetic and environmental events could trigger or make individuals more susceptible to the onset of the disease. (Compston and Coles, 2008).

Typical histological hallmarks of the neuropathological alterations in MS are the demyelination-associated plaques (Lucchinetti *et al.*, 2000). Heterogeneous demyelination may be observed all around the brain nerve fibers, including spinal cord and optic nerve. This condition affects the brain functions associated to injured fibers resulting in a progressive disability of cognitive and sensory-motor skills, with consequent significant costs for society. Currently, no definitive MS treatment is yet available.

The first case in the history of MS is reported by Sir Augustus d'Esté's (1794-1848), descendent of the House of Este (North-Eastern Italy) (Landtblom *et al*., 2010). In his diaries the young Augustus at the age of 28 reported bilateral optic neuritics as consequence of rubella infection. The course of his disorder, which could not be diagnosed during his lifetime, evolved with the progressive occurrence of a number of other invalidating symptoms. Extremities weakness, fatigue, vertigo, tremors and numbness obliged him to the wheelchair. The disease secondarily progressed with paraparesis, sphincter incontinence, urinary problems and impotence (Reynolds, 2004).

The first dissertation about MS was written in 1868 by the french neurologist Jean Marie Charcot and entitled *"La Sclérose en Plaques"* (Charcot, 1868; Clanet, 2008). Professor Charcot studied 34 cases of MS, whom 9 were young men. Patients also displayed cognitive impairment related to worsening in speech, memory and attention (Sherwin *et al*., 1957).

1.1 Epidemiology

MS is an unpredictable disease difficult to diagnose. According to the last estimates extracted from the Atlas of MS 2013, MS affects more than 2.3 million of people worldwide. The numbers could be higher in consideration of a diffuse mis-diagnosis. The most important epidemiologic parameters that might help to investigate the cause and occurrence of the disease are the incidence and the prevalence of cases. The incidence of the disease is the number of new cases during one year, which still remains not fully accurately write down. The prevalence are the number of cases at one specific time or geographic area (Figure 1).

Patients are diagnosed between the ages of 20 and 40, despite cases in young children and in the mature age are also reported. MS is at least two to three times more common in women than in men, suggesting that hormones may also play a significant role in determining susceptibility to MS.

While MS is present in all regions of the world, MS prevalence is higher in North Americans and Caucasians of Northern European ancestry. Thus, MS is more common in areas farthest from the equator but this is not a general rule since the prevalence may differ significantly among ethnic groups previously migrated and living in the same geographic area regardless of distance from the equator (Atlas of MS, 2013). These findings suggest a complex relationship between environmental and genetic factors in determining who develops MS. Several studies have also provided support for the opinion that MS is caused by early exposure to some environmental trigger in genetically susceptible individuals (Compston and Coles, 2008). The increase in worldwide prevalence of MS between 2008 and 2013 (from 2.1 to 2.3 millions of MS people) (Figure 1) might be partially attributed to the lengthening of life expectancy and to the better MS incidence reported (Browne *et al.*, 2014).

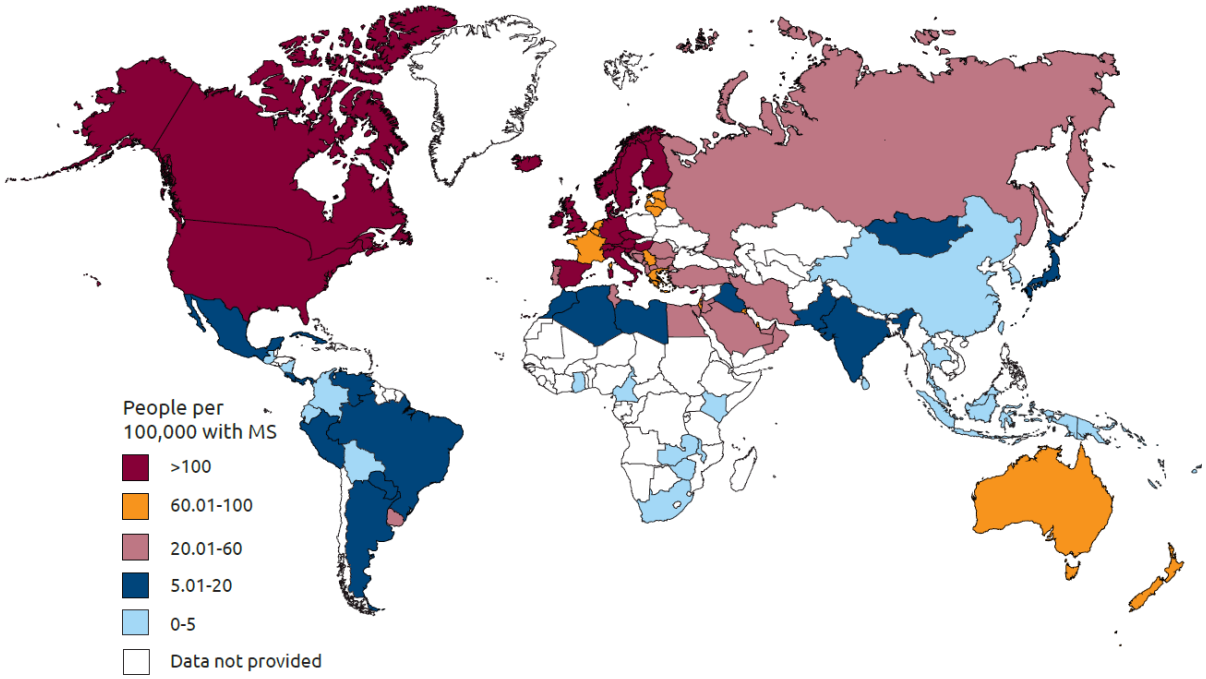

Figure 1. Prevalence of MS estimated by country. Atlas of MS international Federation (from Atlas of MS 2013, Multiple Sclerosis International Federation 2013).

1.2 Histological hallmarks

The term "*multiple sclerosis"* refers to "multifocal" or "multiple scars" named "sclerae" known as lesions or plaques that mainly develop in the white and grey matter of the brain and spinal cord (Compston and Coles, 2002).

The microscopic examination of post-mortem MS brains reveals the presence of easily distinguishable plaques in the white matter having a size extension in the range of few millimeters to a few centimeters (Sarbu *et al.*, 2016). Plaques are the result of a complex myelin damage characterized by inflammation, demyelination, oligodendrocyte loss and astrogliosis. Remyelinated shadow plaques may be also observed, but when axons are severely injured, remyelination fails (Compston and Coles, 2008; Stadelmann *et al.*, 2011).

1.3 Pathological mechanisms

The exact etiology of MS remains still unknown. Several pathological mechanisms have been proposed over time. Either predisposing genetic factors and other environmental triggers, such as biological agents (virus and bacteria), heavy metals and poisoning, might induce a self-sustaining autoimmune disorder that leads to recurrent immune attacks the CNS (Compston and Coles, 2008)(Figure 2).

MS is believed to result from a cellular-mediated autoimmune response caused by autoreactive lymphocyte T cells that recognize self-components of myelin. The first pathogenic event of MS is the break of the immunological tolerance that allows the activation, in the peripheral blood, of specific naïve T cells against myelin antigens. In parallel, antigen-activated B cells proliferate and differentiate into antibody-secreting plasma cells. Activated T lymphocytes express molecules on their surface, such as the integrin α4 β1 (VLA-4) binding the VCAM-1 on the brain vascular endothelium, that allow extravasation through the BBB and their invasion of the CNS. In the CNS, microglia may act as antigen-presenting cells (APC) and they contribute to amplify the inflammatory response. Activation of T and B cells and their extravasation within the CNS induces a cascade of events including the release of cytokines, chemokines, and antibodies against myelin antigens, thus amplifying the inflammatory response with recruitment of other CNS inflammatory cells, including microglia and astrocytes. Lymphocytes and macrophages operate in synergy leading to myelin injury and, consequently, to neuronal and axonal degeneration (Goverman, 2011) (Figure 2).

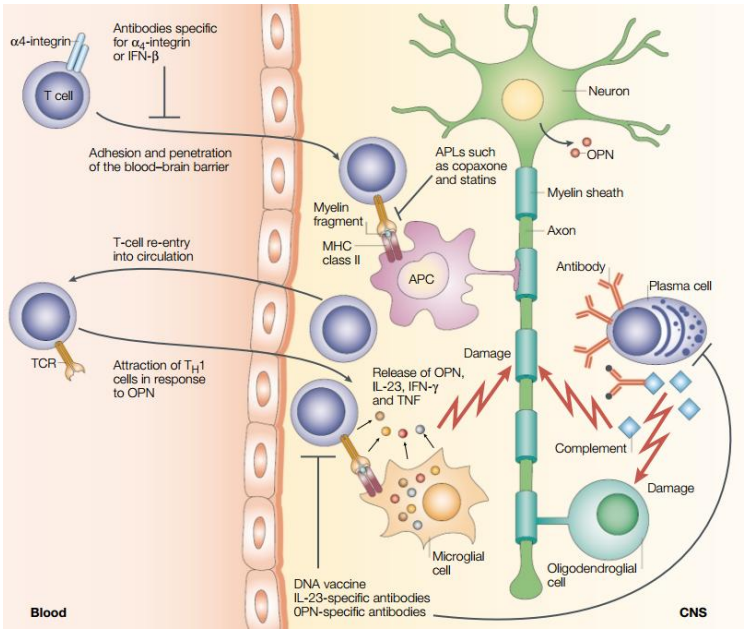

Figure 2. The auto-immune mediated pathophysiological mechanism in multiple sclerosis. T cells, B cells and antigen-presenting cells (APCs), including macrophages, enter the CNS and attacks the myelin forming cells, the oligodendrocytes (From Steinman and Zamvil, 2003).

1.4 Classification and histological heterogeneity of MS lesions

Despite the recent advances in Magnetic Resonance Imaging (MRI) studies, it is still difficult to define the entity of MS lesions. Classical histological stainings such as Hemotoxilin & Eosin, Luxol Fast Blue (LFB), immunohistochemical (IHC) and Human Leukocyte Antigen (HLA)-DR antigens analysis of MS biopsies allow the classification of white matter lesions in active, chronic active, or chronic inactive, based upon pattern and extent of inflammation (Bo *et al*. 1994; Trapp *et al.* 1998; van der Valk and De Groot 2000):

- Active: macrophages infiltrate through the lesion.
- Chronic active: macrophages infiltrate at the border but not within the center of the lesion.
- Chronic inactive: minimal macrophage infiltration through the lesion.

Histopathological studies from Lucchinetti *et al.* (2000) evidenced more in detail the heterogeneous complexity of active lesions which can be further classified in 4 major groups or patterns according to observations of the immune-mediated inflammation, localization and myelin injury type (Lucchinetti *et al.*, 2000; Lassman *et al.*, 2001) (Figure 3).

Pattern I and II lesions (15% and 58% of MS biopsies, respectively) display active demyelinating lesions with T lymphocyte and macrophage-dominated inflammation and diffuse IgG reactivity in tissue and in astrocytes cytoplasm, due to the blood-brain-barrier (BBB) damage. In pattern II lesions the Ig reactivity is more pronounced with degenerating myelin debris within macrophages and a prominent deposition of IgG and complement at sites of active myelin destruction. Pattern I and II active lesions are typically noticed on small veins and venules.

Pattern III lesions (26% of MS biopsies) also contained an inflammatory infiltrate of T lymphocytes, macrophages and activated microglia. Lesions are frequently found on the myelin edges around inflamed vessels which borders result ill-defined. Lesions lack of Ig and complement deposition. Myelin sheaths are almost deficient in MAG proteins content but not in the other proteins such as CNPase, MBP and PLP. Within plaques, oligodendrocytes are apoptotic. A pronounced loss in oligodendrocytes is found at the border of the active lesion, whereas oligodendrocyte presence is almost excluded in the inactive center. Remyelinated shadow plaques are not found.

Pattern IV lesions are the most rare as they are present in almost 1% of MS biopsies. T lymphocytes and macrophages infiltration is well evident while IgG deposition and complement activation are not found. Oligodendrocyte death is evident in periplaques region where DNA fragmentation occurs. Myelin antigens (MAG, MBP, PLP, CNP, and MOG) are similarly distributed through the lesions. Almost total loss of oligodendrocytes in active and

inactive lesions was associated with deficient remyelinated shadow plaques (Lucchinetti *et al.*, 2000; Popescu *et al.*, 2013).

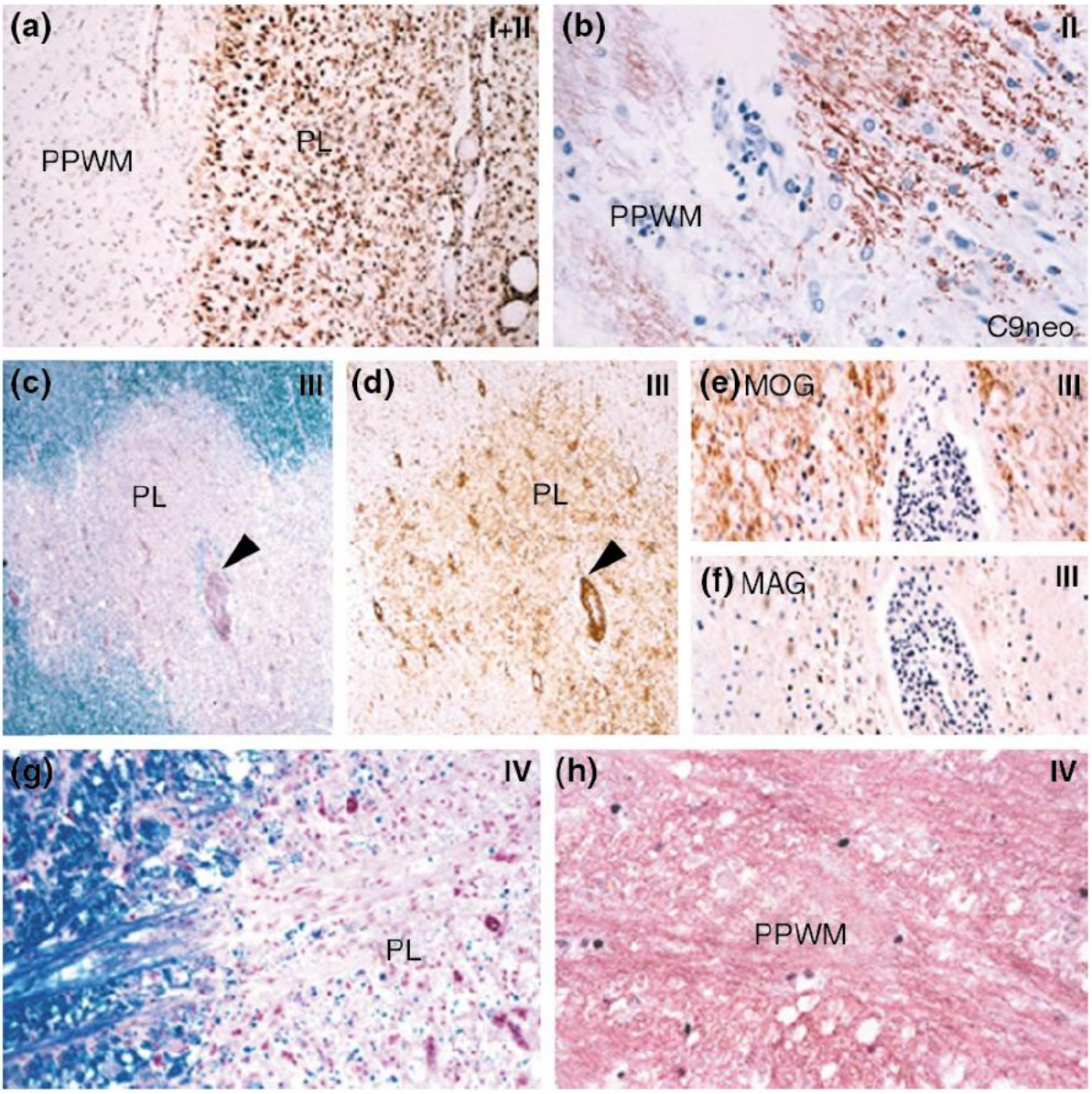

Figure 3. Histopathology of different patterns of demyelination in multiple sclerosis. (a) Active lesion (pattern I and II). (b) Active lesion of pattern II. (c, d) Active lesion of pattern III. (e-f) Higher magnifications of arrows in panels (c) and (d). (g) Active lesion of pattern IV. (h) Periplaque white matter (PPWM) lesion in panel (g). Abbreviations: PL, plaque; PPWM (periplaque white matter). Images from Lassman *et al*., 2001.

1.5 Clinical course

In 1996, the National MS Society Advisory Committee on Clinical Trials in MS first defined the clinical courses of MS. The terminology adopted to define the MS clinical course included the classification in: relapsing-remitting (RRMS), primary progressive (PPMS), secondary progressive (SPMS) and progressive relapsing (PRMS) phenotypes, in accordance to the appearance and type of MS lesions. Since then, an increased understanding of MS and its pathological mechanisms encouraged to consider a new grouping of the clinical phenotypes. In 2013, it has been reported the inclusion of a new phenotype named clinically isolated syndrome (CIS), while the progressive relapsing (PRMS) form has been excluded from the medical definitions of MS (Figure 4).

Neurologists currently define the CIS as the first clinical presentation of neurological symptoms which results characterized by inflammation and demyelination in the CNS. When the CIS produces detectable lesions in MRI scans, the probability of a second attack increases. The remaining forms of MS, relapsing-remitting (RRMS), primary progressive (PPMS) and secondary progressive (SPMS), are further subdivided into two new subcategories: active and non-active. Active MS phenotype is defined by the occurrence of clinical relapse, associated with focal areas of demyelination and inflammation, or in presence of new MRI visible lesions lasting one year from the previous event. Moreover, patients with progressive MS are distinguished into two new groups according the presence or not of signs of disability progression over a given time (Lublin *et al.*, 2014)(Figure 4).

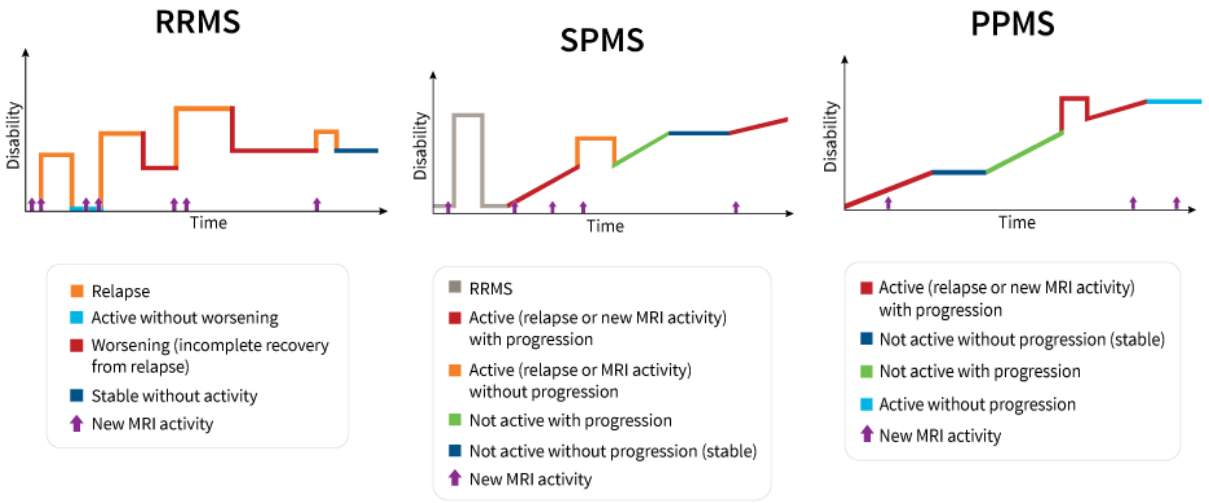

Figure 4. Clinical course of multiple scleorosis. Images obtained from the National Multiple Sclerosis Society web site (www.nationalmssociety.org), data source: Lublin *et al.*, 2014.

1.5.1 Clinical symptoms

MS patients show an individual and unpredictable variability in symptoms. The most frequent symptoms are fatigue and difficulty of walking. The disability in performing daily skills make this disease highly debilitating and decrease the quality of the life, resulting in high cost for the society. About the 80% of patients with MS accuses a state of chronic debilitating fatigue that interfere with daily life.

The walking impairments are due to the onset of various conditions such as numbness of limbs, involuntary muscle spasms and weakness, resulting in an imbalanced gait often cause of accidental falls injuries. Numbness can also affect the rest of the body and the face. Some patients present vision problems (i.e. diplopia) related to unclear vision and pain on ocular movement. Patients also experience with neurological disorders such as speech disorders, dizziness and vertigo, tremor, cognitive deficits leading to memory and in problem-solving skills impairment, lost in attention and altered perception of environment, acute or chronic pain. Patients with MS usually have intact intellectual faculties during the onset, but progressive and severe subcortical white matter lesions might produce a clinical outcome of dementia. Also the emotional sphere appears compromised, since patient can undergo in rapid mood changes and depression.

1.5.2 Diagnosis

The diagnosis of MS is confirmed when physicians exclude other neuropathological disorders causing similar neurological symptoms. This process could be rapid for some individuals but longer for others. A powerful test that can itself be used for the diagnosis is not yet available. Physicians use some criteria to diagnose MS such as: 1) the finding of at least two separated damaged areas in the CNS, including brain, spinal cord and optic nerve; 2) evidence that the damage occurred during the course of time; 3) the exclusion of other neurological diseases.

Hence, the diagnosis of MS can be assessed by the examination of the patient's anamnesis, the appearance of neurological symptoms and the presence of lesions or plaques, separated "in space and in time".

The exact diagnosis of MS requires the presence of clinical findings evaluated through the following three major techniques:

- Magnetic Resonance Imaging (MRI)
- Lumbar puncture
- Evoked potential testing

Magnetic resonance imaging (MRI) is the most sensitive diagnostic test for MS. MRI uses magnetic fields and radio waves to measure the relative water/lipid content in tissues. Two specific types of MRI scans that are used called T1-weighted and T2-weighted MRI that measure the relaxation time of hydrogen protons when a strong magnetic field is applied. Then a software convert data into body sections scans. Since the myelin is highly enriched in lipids, in case of its damage, the nervous tissue hold more water, thus resulting in spotted area in the scans.

The appearance of demyelinating lesions in the brain and spinal cord can be monitored over space and time by MRI scans. In the 90% of MS patients several abnormalities are found with MRI scans. Lesions, depicted as "patchy" or multifocal areas in the white matter, can be efficiently observed by using T2-weighted MRI images. Lesions are usually placed in the paraventricular area, cerebellum and brain stem, but also in cervical and/or thoracic spinal cord.

The McDonald criteria and their new versions introduced in 2001, 2005 and 2010 (McDonald *et al.*, 2001; Polman *et al.*, 2005; Polman *et al.*, 2011) are used in research studies to discriminate the MS clinical outcome from other MS-like neurological disorders.

In fact, several demyelinating and non-demyelinating disease should be taken in account before confirm the diagnosis of MS since it is important to distinguish MS lesions from those produced by some multiple emboli and disseminated vasculitis as they could also result as small infarcts injuring the white matter at MRI scans.

According to the 2010 McDonald criteria, the disease dissemination in time (DTI) can be evaluated by the presence of at least one new T2-weighted or gadolinium enhancing lesion on the next follow-up MRI or the simultaneous presence of asymptomatic gadolinium-enhanced and non-enhanced lesions at any time (Filippi *et al.*, 2016).

The chemical compound gadolinium, also known as "contrast", is injected in patients to evaluate the presence of active lesions. In fact, gadolinium normally does not pass through the blood-brain barrier. In some pathologies, such as MS, in which the BBB is disrupted, the gadolinium enter the brain and spinal cord, allowing the visualization of MS lesions lighting them up on MRI scans as brighter spots.

MRI is commonly considered an easy and not invasive exam to acclaim the appearance and evolution of plaques, although in some patients is required more in depth analysis of the clinical picture obtained by analysis of cerebrospinal fluid (CSF) and neurophysiological tests.

Lumbar puncture, also known as spinal tap, is an invasive test used to evaluate the content of cells and molecules in the CSF. Most patients affected by MS present abnormal results in this test (Stangel *et al.*, 2013). CSF analysis is used to exclude other diseases in the differential diagnosis of MS. In the CSF of MS patients are commonly found elevated levels of IgG antibodies, as well as specific protein called oligoclonal bands, and sometimes certain products of the breakdown of myelin (McDonald *et al.*, 2001; Link and Huang, 2006). The presence of all these markers suggest an abnormal response mediated by immune system against self-component of the myelin. Only 5-10% of MS patients does not present CSF abnormalities. For this reason, more in detail clinical analysis should be considered. A more in depth description of CSF content abnormalities in MS is given in the next paragraph 1.6.

The Evoked potential (EP) test can reveal damaged areas in the brain, spinal cord and in the optic nerve that other tests, i.e. the neurological test, may not detect. The EP test measures the electrical activity of the brain in response to stimulation of specific sensory nerve pathways. Specific types of sensory input are sounds, light or sensations. The decrease in electrical fiber conduction relies to the demyelinating event in course. Since the diagnosis of MS requires evidence of demyelination in two distinct areas of the CNS, EP test could be useful to recognize a second demyelinating event that remains undiagnosed with other tests. To induce the evoked potentials, a wire is placed on the scalp in proximity of the brain areas that are supposed to be stimulated. Specific sensory inputs are given and recorded by the software.

1.6 Biomarkers

In the last two decades the interest in finding and validate a predictable biomarker of MS increased. The biomarker search currently requires the collection of blood and CFS from MS patients. Blood is easy to collect and a few amount is sufficient to evaluate many different markers. The only disadvantage of hematic analyses is the huge daily variability of biomarkers searched (for a review see Eikelenboom *et al.*, 2011; Teunissen *et al.*, 2015). On the other hand, the CSF biochemical examination is well recognized as loyal test reflecting the inflammatory condition, being the CSF in tight proximity of CNS. The CSF withdrawal prevents the variability due to hepatic and renal excretion and, even with invasive lumbar puncture procedure, remains one of the most important clinical analysis for MS diagnosis. Current diagnosis of MS relies on the assessment of the presence in CSF of oligoclonal band(s) of IgG (OCBs) (Dobson *et al.*, 2013; Petzold *et al.*, 2013). Several studies correlated for diagnosis also the importance of detection of IgG directed against neurotropic viruses, rubella and varicella zoster defined as MRZ-specific IgGs (Sawcer *et al.*, 2014.), κ free light

chains (FLC) secreted by plasmacytes (Senel *et al.*, 2014; Presslauer *et al.*, 2014.) and inflammatory and immunological biomarkers such as the B-cell-attracting C–X–C motif chemokine 13 (CXCL13), reported to be expressed in actively demyelinating MS lesions, but not in chronic inactive lesions of patients with CIS, RRMS, SPMS or PPMS (Kademi *et al.*, 2014). The chitinase-3-like protein 1 (CHI3L1) levels, which has been reported to have both inflammatory and tissue remodelling functions, was found higher in CIS converters than in CIS non-converters (Hinsinger *et al.*, 2015).

Recently, small non-coding microRNAs (miRNAs) have been proposed as potential new biomarkers, due to the evidence of the role of miR-20a-5p in T-cell regulation, and the upregulated miR-22-5p in the blood and brain lesions in patients with MS (Cox *et al.*, 2010; Keller *et al.*, 2014). Neurofilaments light chains (NfL) levels in CIS patients have been reported to increase as consequence to axonal damage (Kuhle *et al.*, 2013; Khalil *et al.*, 2013; Fialová *et al.*, 2013).

1.7 Therapy

There is no yet cure treatment available for MS. The clinical treatments for MS are based on different medical strategies: 1) a rapid intervention immediately after attacks appearing; 2) the attempt to slowdown the progression of the disease; 3) the managing of symptoms (for a review see Goldberg *et al*., 2012).

Treatment of MS attacks includes corticosteroids and plasmapheresis. Corticoids (i.e. prednisone in oral administration and methylprednisolone i.v.) act counteracting the nerve inflammation. In alternative, plasmapheresis, is employed in case of severe new symptoms and in case of inefficient steroids cure.

Treatments to slow progression. Nowadays, there are no available therapies to slow the progression of PPMS. By contrast, several pharmacological treatments are available for the relapsing-remitting MS phenotype. Since many of these treatments have serious side effects, their clinical administration requires the awareness on their negative effects and the size and severity of the disease. In women, great care is taken in account also in prevision of future pregnancy. The current approved drugs for the RRMS are the immunomodulators, immunosuppressants and monoclonal antibodies.

- ***Immunomodulators***, such as the Beta Interferon 1a and 1b (Avonex®, Rebif22®, Rebif44®, Betaferon®, Extavia®, Plegridy), regulate or modulate the immune response. Injected under skin or intra-muscle, it is the most prescribed drug capable to reduce the frequency and the severity of relapses. Side effects include flu-like symptoms and local

reaction in the site of injection. Applied to the first CIS, it is able to counteract the appearance of a new attack. Another drug approved by the The Food and Drug Administration (FDA) is the synthetic form of myelin basic protein MBP (i.e. copolymer I or Copaxone).

- ***Immunosuppressants*** decrease the activation of the immune system. The most effective drug against severe forms of MS is the Mitoxantrone, which use is under the stringent prescription from a neurologist only in case of severe form of MS, since this drug is highly associated with cardiac complications and blood cancers development. The FDA also approved the Teriflunomide, Dimethyl Fumarate and Fingolimod.
- Specific ***monoclonal antibodies***, i.e. Natalizumab and Alemtuzumab selectively target cells or molecules, thus modifying the immune response. Even so greatly specific, monoclonal antibodies have a black side of the medallion. In fact, the use of Natalizumab is associated with the risk of a viral infection of the brain called progressive multifocal leukoencephalopathy while the administration of Alemtuzumab is associated with the risk of infections and other autoimmune disorders.

Treatment for symptoms. The purpose of symptomatic treatment is to relieve symptoms of the disease, in order to achieve an improved quality of life for people with MS. For some symptoms such as fatigue, cognitive disorders, neuropathic pain, there is still much to invest and do, although several molecules are avalaible. Uncontrollable spasms symptoms are cured with muscle relaxants.

Not less important is the research devoted to rehabilitation. In recent years several reports demonstrate the effectiveness of the rehabilitation treatment in MS patients. A typical physical therapy session may include stretching and strengthening exercises, useful to reduce weakness and other gait problems often associated with MS.

2. OLIGODENDROCYTES

Oligodendrocytes (from G*reek:* cells with few branches), also named oligodendroglia, are non-neuronal cells which take part of glial cells, known also with the term "neuroglia" or simply "glia". The term neuroglia appeared for the first time in 1846 by Virchow whom described the presence of cells different from neurons in the connective tissue of brain with the term *nervenkitt* (*German*: nerve glue) or neuroglia (Virchow, 1846). Subsequently, Ramon y Cajal and his student Rio Hortega improved the technique of metallic impregnation and started to classify the major glial cell types. Ramon y Cajal classified the astrocytes by using gold impregnation, while Rio Hortega described the oligodendrocytes and microglia by using silver carbonate impregnation technique.

Glial cells constitute the majority of the existing cells in the nervous system. They maintain homeostasis, produce the multilayer lipid fiber-insulator myelin, and provide protection and support to the CNS and PNS. In the CNS glial cells are classified in "Macroglia" (astrocytes, oligodendrocytes and ependymal cells) and "Microglia". In the PNS glial cells include Schwann cells and satellite cells.

In the last decades was commonly believed that the role of glial cells was marginal, simply confined to the stage of development and support ("glue") to neurons during the course of individual life. In the last few years, the scenario changed and the discoveries of numerous receptors and neurotransmitters in glial cells suggested a possible interaction of these cells in the glial-neuronal network, perhaps taking part in CNS physiological processes which dysfunction relies to several neurological conditions.

2.1 Morphology

In comparison to astrocytes, oligodendrocytes extent a restrict number of branches (ramifications or processes) from the soma, showing a lower size, higher density in cytoplasm, clumping of nuclear chromatin and a great number of microtubules in the cytoplasm (Lunn *et al.*,1997).

Oligodendrocytes are classified as *myelinating oligodendrocytes*, *satellite oligodendrocyte*, and *oligodendrocyte precursor cells (OPCs).*

Myelinating oligodendrocytes extend their processes to the axons which continuous envelopment and condensation produces the multi-spiral membrane called myelin. The number of connections with axonal segments depends on CNS areas and on the different species. In fact, oligodendrocytes may contact up to 40 segments in the CNS (Peters *et al.*, 1991).

Rio Hortega classified oligodendrocytes in four major categories, types I to IV, in relation to the number of processes, morphology, size and thickness of myelin sheath formed (Pérez-Cerdá *et al.*, 2015).

Electronic microscopy studies still evidenced a huge heterogeneity in oligodendrocytes morphology having differences in cytoplasms and nuclear chromatin densities. In fact, Mori and Leblond (1970) tried to define this finding defining the oligodendrocytes as "light", "medium" and "dark" (Mori and Leblond, 1970). Labeling with tritiated thymidine evidenced light oligodendrocytes in proliferation phase, then they become darker when maturation occurred.

During their maturation, myelinating oligodendrocytes pass through many different stages of development, from the proliferating stage of OPCs to the mature myelinating phenotype. Oligodendrocyte diversity is well appreciated by the specific expression patterns of antigenic markers (Figure 5).

Oligodendrocyte processes myelinate axonal segments. Each of this myelinated segment is several hundred micrometers long and is also termed "internode". Segments are interrupted by structures known as node of Ranvier which spans for less than 1 micron. At the node, as compared to the internodal region, the axon is not enwrapped by myelin. The end of internodal segment contains more cytoplasm and forms so called paranodal loop creating septate-like junctions with the axon. In addition, astrocyte processes contact the axonal membrane at the nodal region. Oligodendrocytes, similarly to astrocytes, are also connected by specific cell type gap junctions formed by connexins which alteration causes myelin disfunctions (Cotrina and Nedergaard, 2012).

In the gray matter it has been described an oligodendrocyte type not directly connected to myelin sheath formation. These *satellite oligodendrocytes* are perineuronal oligodendrocytes that maintain the ionic homeostasis and regulate the microenvironment around neurons (Baumann and Pham-Dinh, 2001).

A considerable number of *Oligodendrocyte Precursor cells (OPCs)* do persist in the adult brain at the pre-oligodendrocyte stage, and may provide a source of new oligodendrocyte, protoplasmic astrocytes, and neurons (Annunziato *et al.*, 2013; Boscia *et al.*, 2016) Because of their apparent stem-cell-like characteristics, adult OPCs have recently gained much attention for their potential reservoir of cells capable of self-renewal, differentiation, and remyelination after CNS injury.

2.2 Origin

The exact site of origin of oligodendrocytes was greatly debated in the last years. Many researchers proposed a unique origin in the ventral neuronal tube, while others approached the hypothesis of multiple origin (Spassky *et al.*, 2000; de Castro *et al.*, 2013).

Initially, OPCs were thought to exclusively originate in the ventral ventricular zone in response to the morphogen sonic hedgehog (Shh) (Noll and Miller, 1993; Warf *et al.*,1991). Then, the dorsal origin has been described occurring from the spinal cord to the telencephalon in an Shh-independent manner (Cai *et al.*, 2005; Fogarty *et al.*, 2005; Kessaris *et al.*, 2006; Vallstedt *et al.*, 2005)

During the half of fetal life OPCs first arise in a restricted germinal area in the ventral spinal cord, then a dorsal-lateral migration occurs. Oligodendrogenesis follows a double direction from ventral to dorsal and from caudal to rostral (de Castro and Zalc, 2013).

Some interesting findings show that radial glia, which is widespread throughout the CNS in development, may differentiate in oligodendrocytes after neurogenic phase (Choi *et al.*, 1983; Choi and Kim, 1985; Hirano and Goldman, 1988).

More recently, an important source of oligodendrocytes has also been found in ventral and dorsal forebrain. (Richardson *et al.*, 2006; Bradl and Lassmann, 2010). A first wave of OPCs is generated in the medial ganglionic eminence and anterior entopeduncular area of the ventral forebrain. OPCs populate the entire embryonic telencephalon including the cerebral cortex. Finally, a second wave of OPCs derived from the lateral and/or caudal ganglionic eminences and a third wave of OPCs arises within the postnatal cortex (Kessaris *et al.*, 2006).

From their sites of origins, OPCs migrate for long distances to colonize both gray and white matter (Thomas *et al.*, 2000; Qi *et al.*, 2002).

In late gestational and early postnatal mammalian brain, oligodendrocytes originate mainly from subventricular zone (SVZ) (Baumann *et al*., 2001; Menn, 2006; Perez, 2014).

OPC population is widely distributed in the adult brain. This functional redundancy represents as reservoir for oligodendrocyte replacement and myelin plasticity/remodelling. (Ettle *et* al., 2016) but may also serve as a promising source for neuronal replacement (Crawford *et al.*, 2014; Nishiyama *et al.*, 2009).

Some speculations about the oligodendrocyte population competition to the final place migration is well review by Richardson 2006 who elegantly examined the "Oligodendrocyte wars". In brief, ventrally-generated OPCs seems to predominate in the spinal cord, while the dorsal origin has been found in the ventricular telencephalon (Richardson *et al.*, 2006). Some OPCs persist inside the adulthood, ready to migrate within the brain at certain stimuli,

maturates and eventually differentiate in myelinating oligodendrocytes (Franklin and Ffrench-Constant, 2008).

2.3 Function

The major function of oligodendrocytes is the formation of myelin (for a review see Baumann and Pham-Dinh, 2001; Kettenmann and Verkhratsky, 2011; Ettle *et al.*, 2016). Oligodendrocyte processes surround the axons of nerve fibers generating a complex concentric wrapping multilayer membrane, called myelin. In PNS the same function in covered by the Schwann cells. Myelin works as electrical insulation that separates the nerve fiber from the environment.

Oligodendrocytes display also an homeostatic role in buffering extracellular potassium arises following the neuronal excitation (Menichella *et al.*, 2006).

Increasing the resistance and lowering the capacitance of axonal membranes is not the only function of oligodendrocytes and Schwann cells. Myelinating glia communicate lifelong with axons, and is required for the long-term integrity and survival of axons (Beirowski, 2013; Lee *et al.*, 2012). Oligodendrocytes secretes glial- and brain-derived neurotrophic factors (GDNF and BDNF) which supports either the axonal functionality and outgrowth, playing an important role in preserving neuronal circuitries (Wilkins *et al.*, 2003; Du and Dreyfus, 2002; Dai et *al.*, 2003).

2.4 Oligodendrocyte lineage

Oligodendrocytes are end product of a cell lineage which requires a complex and precisely timed program of proliferation, migration, differentiation, and myelination to finally generate the insulating sheath. OPCs pass through four major steps of differentiation that can be easily identified by the cellular ability to migrate, the acquisition of an elaborate morphology and expression of a specific antigenic pattern (Barateiro and Fernandez, 2014) (Figure 5).

Schematically, oligodendrocytes lineage steps can be classified as:

- precursor cells (OPC)
- pre-oligodendrocytes (or late OPC)
- immature oligodendrocyte (or pre-myelinating oligodendrocyte)
- mature oligodendrocyte (or myelinating oligodendrocyte).

OPCs appear in successive waves in prenatal life (Kessaris *et al.*, 2006; Richardson *et al.*, 2006) and continue proliferating even in the adulthood. OPCs shows a bipolar or multipolar morphology with tree-like fine processes (Huges *et al.*, 2013) and a surprising migratory capability to reach each part of the brain. OPCs specifically express antigenic markers such as the platelet-derived growth factor receptor α (PDGF-αR), the ganglioside A2B5, the proteoglycan NG2 (Nishiyama *et al.*, 1996; Pringle *et al.*, 1992), the polysialic acid-neural cell adhesion molecule (Grinspan and Franceschini, 1995) and fatty-acid-binding protein (FABP) (Sharifi *et al.*, 2013).

Given the appropriate environmental signals, OPCs exit the cell cycle and differentiate into pre- oligodendrocytes (Emery, 2010a, b; Zuchero and Barres, 2013).

Pre-oligodendrocytes extend multipolar short processes and expresse as markers the sulfatide recognized by the O4 antibody (Sommer and Schachner, 1981) and the GPR17 protein (Boda *et al.*, 2011), which may be found also in immature oligodendrocytes.

Immature oligodendrocytes do not express A2B5 and NG2 markers while continue to express O4 and a new marker called galactocerebroside C (GalC) (Yu *et al.*, 1994).

During this differentiation phase, oligodendrocytes stop proliferating and extent long ramified branches (Armstrong *et al.*, 1992, Gard and Pfeiffer, 1989).

Mature oligodendrocytes extend membranes that form compact enwrapping sheaths around the axons and express myelin proteins such as myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG) (Reynolds and Wilkin 1988; Scolding *et al.*, 1989; Zhang, 2001), MRF/Gm98 (Koenning *et al.*, 2012), zinc finger protein 488 (Wang *et al.*, 2006) and FABP5 (Sharifi *et al.*, 2013).

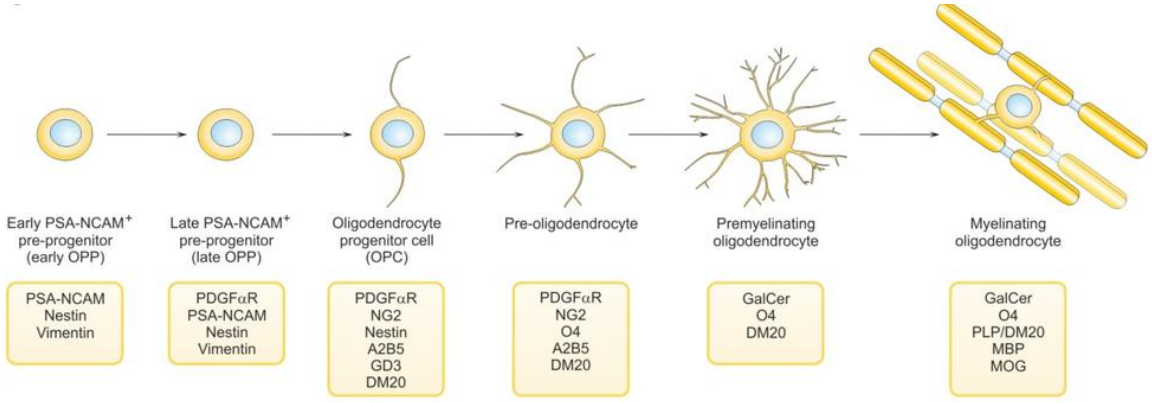

Figure 5. Oligodendrocyte maturation and differentiation. From Podbielska *et al.*, (2013).

3. MYELIN

The term myelin coined by Rudolf Virchow derives from the greek word "myelós" (marrow or core). In 18th century, myelin was thought to be within the inner nerve fiber and incorrectly linked the substance to bone marrow (Virchow, 1854). Myelin is a complicated structure produced by the enrolling of membrane layers around nerve fibers by oligodendrocyte and Schwann cells, respectively in the CNS and PNS.

Myelin presents a unique architecture with a characteristic composition in protein and lipids.

The most important function of myelin is the electric insulation of nerve fibers resulting in a rapid and efficient transmission of action potential signals, well known as saltatory conduction. During saltatory conduction, action potentials "jump" among specialized gaps in the myelin sheath, called nodes of Ranvier where sodium channels are clustered (Baumann and Pham-Dinh, 2001; Jessen, 2004, Freeman *et al*., 2016). The high specificity of myelin in increasing the electrical impulses rate along axons is made possible by the decrease in capacitance accompanied with increase in electrical resistance across the axolemma. Myelin permits rapid and efficient signal transmission for long distances, thus suggesting an evolutionary advantage for organism with larger body size (Hartline, 2008).

The production of the myelin sheath is referred with the name of *myelination* or *myelinogenesis*. Electron microscopy studies on mouse brain showed that myelinogenesis begins after birth, reaching a pick at P20 and being almost concluded at P60 (Baumann and Pham-Dinh, 2001; Vincze *et al.,* 2008). In humans, the myelinating process begins early in the 3rd trimester proceeding after birth through the early adult life. The threshold for axon diameter myelination ranges from 0.2 µm in CNS to 1 µm in the peripheral nervous system (PNS) (Waxman and Bennet, 1972; Voyvodic, 1989). Myelination is a highly demanding process requiring energy to synthetize all protein lipids membrane and all the other proteic components.

3.1 Myelin composition

Myelin *in situ* has a water content of about 40% both in CNS and PNS. The dry mass of myelin is composed by a high amount of lipid (80%) and a low quantity of protein (20%), thus having a ratio index opposite to the rest of biological body membranes. The high ***lipidic composition*** of myelin gives a white appearance to the nervous system, hence the coined name "white matter". Lipids are not exclusively present in the myelin, but the same molecules are found in membranes spread out in the brain. What is typical of myelin is the specific

composition in membranes which is almost composed of *cerebrosides*, also known as well as *galactosylceramides*. With the only exception of the developmental stage, cerebrosides can be referred proportional to the amount of total myelin.

The *galactolipids*, mostly represented by galactosylceramides (GalC) and their sulfated derivatives, named sulfatides, are around the fifth of lipidic composition of myelin. In the last decades, the sulfatides group were considered essential for oligodendrocytes differentiation, since the evidence of a great quantity of sulfatides in oligodendrocytes and myelin. Conversely, studies with knock-out mice for the UDP-galactose-ceramide galactosyltransferase showed that, also in absence of this enzyme, the biosynthesis of myelin is not altered and just few difference in myelin structure and in axonal signal conductivity were observed (Coetzee *et al.,* 1996).

In addition to cerebrosides and galactolipids, myelin contains high levels in *cholesterol* and *ethanolamine-containing plasmalogens*. Among this last group of lipids, lecithin is the majior component while sphingomyelin represent just a minority.

The abundance in cholesterol and the relative presence of lipid microdomains influences also biophysical properties of membranes such as fluidity, curving, budding and fission (Huttner and Zimmerberg 2001). A high cholesterol amount associates with great myelin membrane growth (Saher *et al.*, 2005). Cholesterol is important for partitioning, trafficking, and other metabolic properties of myelin such as the formation of membrane subdomains, called lipid rafts, (Ikonen and Jansen, 2008) that in last analysis could act as transport unit that deliver myelin proteins and lipids newly synthetized to the developing sheaths (de Vries and Hoekstra, 2000; Kramer *et al.*, 2001).

Minor components of myelin include at least three fatty acid esters of cerebroside and two glycerol-based lipids, diacylglyceryl-galactoside and monoalkylmonoacylglycerylgalactoside, collectively called galactosyldiglyceride.

The distinctive myelin composition in lipids increases the compaction grade of lateral chains of fatty acids, thus facilitating the insulator properties of myelin.

CNS and PNS have a similar myelin composition. There are few quantitative differences, since PNS myelin has less cerebroside and sulfatide and a little more sphingomyelin than CNS.

Proteins and glicoproteins in myelin are the same of the other biological membranes, but in lower amount. Polyacrilamide gel electrophoresis technique clarifies that myelin protein content has a predominance of Myelin Basic Protein (MBP) and Proteolipid Protein (PLP/DM20) proteins that are widely used to classify the late stage of maturation of

oligodendrocytes. Another group of myelin proteins, insoluble after solubilization of purified myelin in chloroform-methanol 2:1, have been designated as the Wolfgram proteins, which include the CNP and other proteins (Wolfgram, 1966). Several glycoproteins are present in myelin, among which are the myelin associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG) (Quarles, 1997).

MBP is one of the most abundant protein in myelin. The MBP was sequenced in 1971. (Eylar *et al.*, 1971). Mice deficient for the MBP gene presented decreased amount of myelin in the CNS accompanied by several neurologic disorders such as epilepsy, seizures, tremor.

The human gene is located to the chromosome 18 with a complex gene structure that is highly conserved during evolution (Saxe *et al.*, 1985). As strongly positively charged protein, MBP may bind with charged lipid head-groups via electrostatic interactions. The MBP isoforms are the result of alternative splicing of a gene also called Golli MBP (=gene of oligodendrocyte lineage). mRNA transcripts for MBP have been found not only in the brain but also in bone marrow and the immune system. The three major isoforms of MBP have a molecular weight of 21.5, 18.5, 17, and 14 kDa in mice and 21.5, 20.2, 18.5, and 17.2 kDa in humans (Compagnoni, 1988). A great number of post-translational modifications of the protein, which include phosphorylation, methylation, deamination, and citrullination are described (Wood *et al.*, 1999; Kim *et al.*, 2003). In mature myelinating oligodendrocytes, MBP redistributes from the soma and primary processes into the myelin sheaths, reflecting a change in the site of MBP mRNA expression (Barbarese *et al.*, 1999).

MBP plays a major role in myelin compaction as revealed by studies on the *shiverer* mutant mouse, where a large deletion of the MBP gene results in severe perturbation of myelin compaction (Privat *et al.*, 1979). In fact, MBP is thought to be required for facilitating the approach of apposed inner leaflets of the plasma membrane, structurally characterized by the intraperiod line.

Proteolipid proteins (PLP) were discovered in 1951 by Folch and Lees by using organic solvent extraction technique. Initially, the name “proteolipid” was assumed to distinguish these proteins, apparently composed of lipid-protein complexes, from other hydro-soluble lipoproteins (Folch and Lees, 1951). The PLP protein has a molecular mass of 25 kDa, whereas its most common isoform, the PLP/DM20 has a mass of 20 kDa. Usually, PLP migrates higher than its molecular mass due the covalent binding/ligation of acylated to cysteine residues at the intracytoplasmatic side or glycosilated domains at the extracytoplasmatic side. In humans, the gene coding for PLP is placed at the region position Xq22 of the X chromosome, having seven exons which alternative splicing forms the PLP full

length protein and its isoform DM20 that lacks the a.a. 116-150 of PLP sequence, thus having a deletion of 35 aminoacids (Nave *et al.*, 1987). The human sequence for PLP is identical to that one found in mouse. The integral-membrane protein PLP as well as its isoform DM20 is transported to the final location in myelin by vesicular transport (Colman et *et al.*, 1982). On its way to the plasma membrane, PLP/DM20 associates with cholesterol and galactosylceramide in the Golgi complex, which might assist the targeting of PLP/DM20 to myelin (Simons *et al.*, 2000). The PLP spans the membrane four times with its 4 alpha-helix hydrophobic structure, forming two extracellular and three intracellular domains. As a structural protein, PLP plays a major role in assembly and stabilization of the myelin, driving the most stable structure formation after membrane layers compaction (Rosenbluth *et al.*, 2006).

Experiments performed in mouse oligodendrocytes in absence of PLP gene, demonstrated that they are still able to myelinate. Despite this findings, ultrastructural studies revealed a condensation in the intraperiodic segments, as also seen in PLP mutants, which reduces stability of myelin sheaths. Hence, the integral-membrane PLP could act as biological "zipper" that stabilizes the myelin membrane layers (Boison *et al*., 1995; Klugmann *et al*., 1997).

2',3'-Cyclic nucleotide-3-phosphodiesterase (CNPase) isoforms, CNP1 and CNP2, representing 4% of the total of CNS myelin proteins (Pfeiffer *et al.*, 1993), having a molecular mass ranging from 48 to 55 kDa (Baumann and Pham-Dinh, 2001). CNP is mostly expressed in the CNS myelin and oligodendrocytes and only partially expressed at the early myelination stages in Schwann cells, whereas it concentrates in the cytoplasm of uncompacted myelin, inner and outer tongue processes and lateral loops (Siegel *et al.*, 1999; Trapp *et al.*, 1988). The CNPase gene is located on the murine chromosome 11 (Bernier *et al.*, 1988) and on human chromosome 17q21 (Douglas *et al.*, 1992). The gene consists of 4 exons spanning 7 kb which alternative splicing produce the two CNP isoforms, CNP1 and CNP2 (Kurihara *et al.*, 1990). The protein receives posttranslational modifications such as acylation and phosphorylation, especially the larger isoform (Vogel and Thompson, 1988). Cnp null mice present normal myelin development and structure (Baumann and Pham-Dinh, 2001; Lappe-Siefke *et al.*, 2003), whilst present increased inflammation response and progressive structural deficits which induce premature mice death. (Edgar *et al.*, 2009; Lappe-Siefke *et al.*, 2003; Rasband *et al.*, 2005). Cnp overexpression in transgenic mice perturbs normal myelin formation generating anomalous oligodendrocyte membrane expansion (Lappe-Siefke *et al.*, 2003).

The CNPase structure and function in vertebratae were recently reviewed by Myllykoski *et al.*, (2016). The CNPase catalyze the hydrolysis of 2',3'-cyclic nucleotides (Verrier *et al.*, 2012). CNP display a role in cytoskeletal network of myelin through binding of F-actin and tubulin, and overexpression of CNP stimulates process outgrowth (Quarles *et al.*, 2006; Siegel *et al.*, 1999). A recent study evidenced the fundamental role of CNPase and MBP interaction to regulate the time of myelin compaction during development (Snaidero *et al.*, 2014).

Myelin-associated glycoprotein (MAG) is a heavily glycosylated protein belonging to the family of sialic acid binding lectins, the sialoadhesins, present as minor constituent of myelin both in CNS (1%) and PNS (0,1%) (Baumann and Pham-Dinh, 2001). MAG gene includes 13 exons which alternative splicing generate the 2 isoforms S- and L-MAG which differs for the cytoplasmatic domain (S=short; L=long) (Pfeiffer *et al.*,1993; Siegel *et al.*, 1999). MAG glycoprotein play an important role in the axon-myelin interactions by functioning as recognition molecule (Pfeiffer *et al.*, 1993) helping oligodendrocyte to recognize myelinated from unmyelinated axons (Li *et al.*, 1994; Montag *et al.*, 1994). Another important role of MAG could be the inhibition of neurite outgrowth (for a review see McKerracher and Rosen, 2015). MAG knockout mice display a normal myelination process, while exhibiting subtle periaxonal structural abnormalities (Li *et al.*, 1994; Montag *et al.*, 1994; Siegel *et al.*, 1999) and developing a dying-back oligodendropathy which affects both myelin and axons (Lassmann *et al.*, 1997; Siegel *et al.*, 1999).

Myelin Oligodendrocyte Glycoprotein (MOG) is a minor glycoprotein of CNS myelin (Lebar *et al.*, 1986), exclusively expressed in mammalians with high homology of sequences among species (Birling *et al.*, 1993). MOG have a molecular mass spanning from 25-28 kDa which eventually forms dimers (Amiguet *et al.*, 1992; Birling *et al.*, 1993). The mouse and the human MOG gene present a total length of 12.5 and 19 kb long, respectively, composed by 8 exons. In humans different alternative variants are described (Pham-Dinh *et al.*, 1995). The MOG protein presents a structure with N-terminal domain at the extracellular side displaying typical characteristics of an immunoglobulin variable domains (Gardinier *et al.*, 1992; Pham-Dinh *et al*., 1993). MOG is located on the plasma membrane of the oligodendrocyte mainly at processes side and on the external lamellae of myelin (Brunner *et al.*, 1979). MOG function could refer to the maintaining structural integrity of the myelin sheath as well as it could interact with proteins of the immune system. Indeed, MOG located at the cell surface, is the unique CNS component able to induce an antibody-mediated response and a T-cell mediated immune reaction in the animal model for MS, the experimental autoimmune encephalomyelitis (EAE) (Linington *et al.*, 1988).

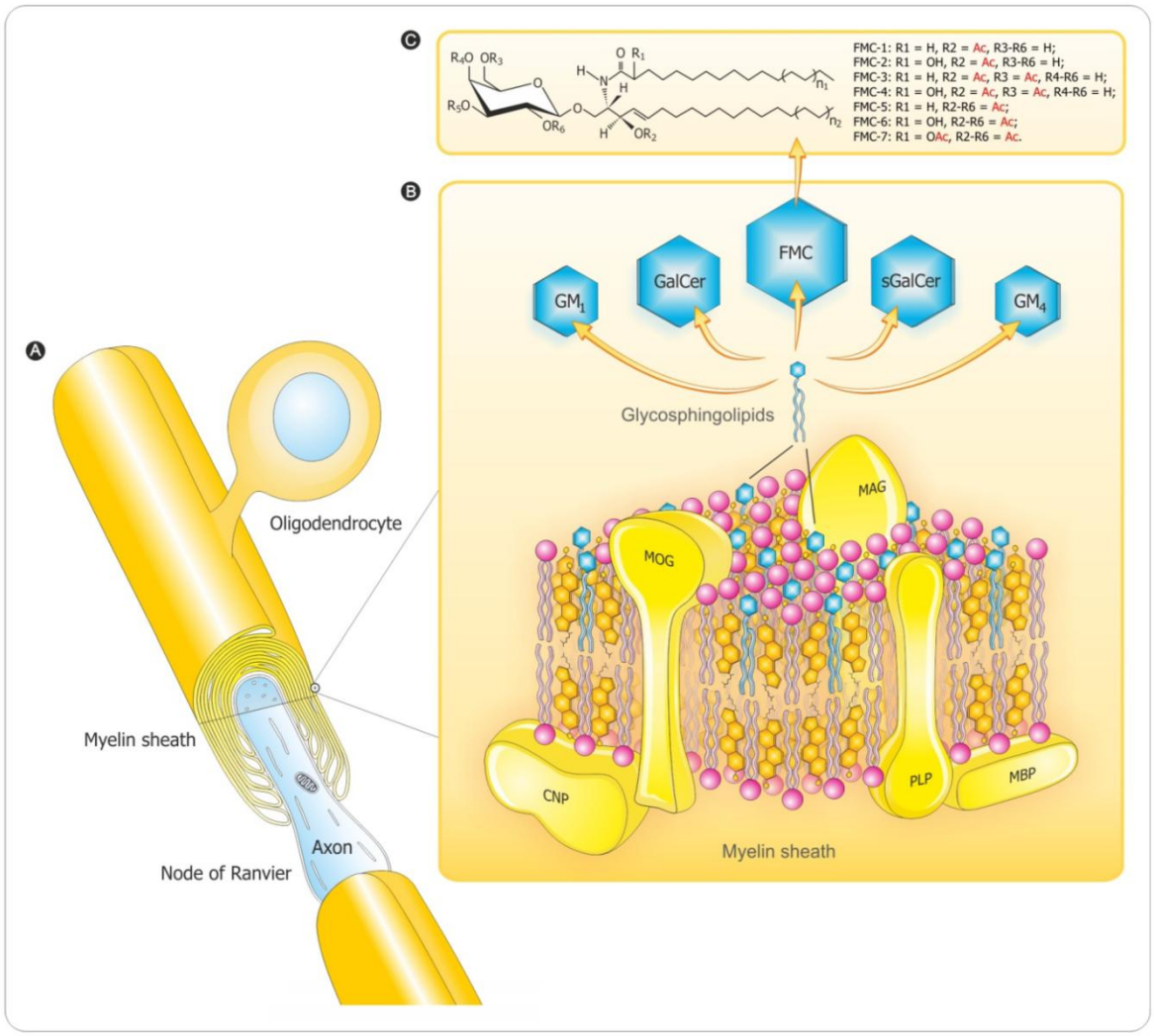

Figure 6. Structural features of CNS myelin. (**A**) Architecture; (**B**) 3D-molecular composition and conformation-based assembly and (**C**) sphingosine 3-*O*-acetylated galactosylceramide series (from Podbielska *et al.*, 2013).

3.2 Myelination

Myelination is defined as the process of synthesis mediated by oligodendrocytes and Schwann cells with formation of myelin around axons. Myelin is the results of a complex biosynthetic machinery in which newly synthetized molecules are transported to the final destination in membranes with a complex trafficking which guarantees a fixed composition in lipids and proteins. Myelination is characterized by several steps progression:

- OPC migration to the axon and proliferation
- Adhesion to axons
- Wrapping
- Compaction

At first, *OPCs migrates* in proximity to the axons to myelinate, while *maintaining mitotic divisions*. OPCs start to express new markers as immature oligodendrocytes. After the *adhesion* to the axon by extending its processes specifically mediated by integrins and its derivates laminins, oligodendrocytes start to form the myelin sheath by spiral *wrapping* of its membranes around axon, which finally undergo to *compaction* phase (Baumann and Pham-Dinh, 2001).

During myelination oligodendrocytes change in shape are drived by the reorganization of the actin cytoskeleton (Novak *et al.*, 2011). In 2014, Snaidero *et al.* (2014) described the mechanisms underlying the assembly of these multi-layered myelin sheaths.

The entire myelination process is tightly regulated (for a review see Tauheed *et al.*, 2016). The number of wrappings and thickness of myelin is determined by the axonal properties of diameter and brain area interested (Friede, 1972). Friede (1972) described the relation between the diameter of the axon and the thickness of myelin with the term "*g-ratio*", being calculated as the inner and outer perimeter of the myelin sheaths (Das *et al.*, 2011). It was reported that the optimal g-ratio value for CNS is approximately 0.77, while in the PNS is approximately 0.6 (Chomiak and Hu, 2009).

Myelination start early in life during developmental stage. In mice, myelination starts at birth in the spinal cord and is almost completed at postnatal day 60 (P60) (Baumann and Pham-Dinh, 2001). In humans, the peak of myelination is registered during the first year, while it precedes till the young adult life, as revealed in some cortical areas of the brain (Fields, 2008). Myelination direction has been described occurring caudo-rostral in the brain and rostro-caudal in the spinal cord (Baumann and Pham-Dinh, 2001). During the adult life myelination still provides the correct axons electric insulation, whilst OPCs in the adult still persist ready to myelinate.

3.3 Remyelination

Remyelination is an important physiological process in which oligodendrocyte repair demyelinated axons, restoring the saltatory conduction and reverting functional deficits (Liebetanz and Merkler 2006; Jeffery *et al.*, 1999; Smith *et al.*, 1979) through generation of new mature oligodendrocytes. In the adult healthy brain resides a population of adult stem precursor cells spread out in the entire CNS called adult oligodendrocyte precursor or NG2-positive cells. Adult OPC can be induced to proliferate and migrate *in vitro* after stimulation with growth factors such as PDGF and FGF (Wolswijk and Noble 1992, Hinks and Franklin 1999). After a demyelinating insult, *in vivo* adult OPCs nearby lesion pass from the quiescent

state to a regenerative phenotype, well distinguishable with both morphological changes and upregulation of genes, including the oligodendrocytes transcription factors Olig2, Nkx2.2, MyT1 and Sox2 (Watanabe *et al.*, 2004; Fancy *et al.*, 2004; Shen *et al.*, 2008). The most rapid OPCs response is observed in presence of acute injury-activated microglia and astrocytes releasing factors (Glezer *et al.*, 2006; Rhodes *et al.*, 2006).

After the *recruitment* of adult OPCs through active *migration* and *proliferation*, OPCs *differentiate* into mature *myelinating oligodendrocytes*. Similarly, to the myelination phase, oligodendrocytes increase myelin genes expression and *wrap* axons with their newly formed membranes. Finally, they compact the myelin sheaths. However, this processes often fails after successive multiple demyelination insults as occurr in the most advanced stages of MS (Franklin and ffrench-Constant, 2008).

As reviewed by Rodgers *et al.* (2013), the therapeutic strategies approached to counteract the MS digression include the combination of three major cardinal strategies, which include (Figure 7):

- stopping the immune attack against self myelin components through immunomodulatory drugs administration;
- protect the oligodendrocyte from injury;
- enhance remyelination process both by transplantation of exogenous cells or by stimulating the spontaneous repair via endogenous OPCs that regenerate the myelin sheets and prevent the further axonal loss.

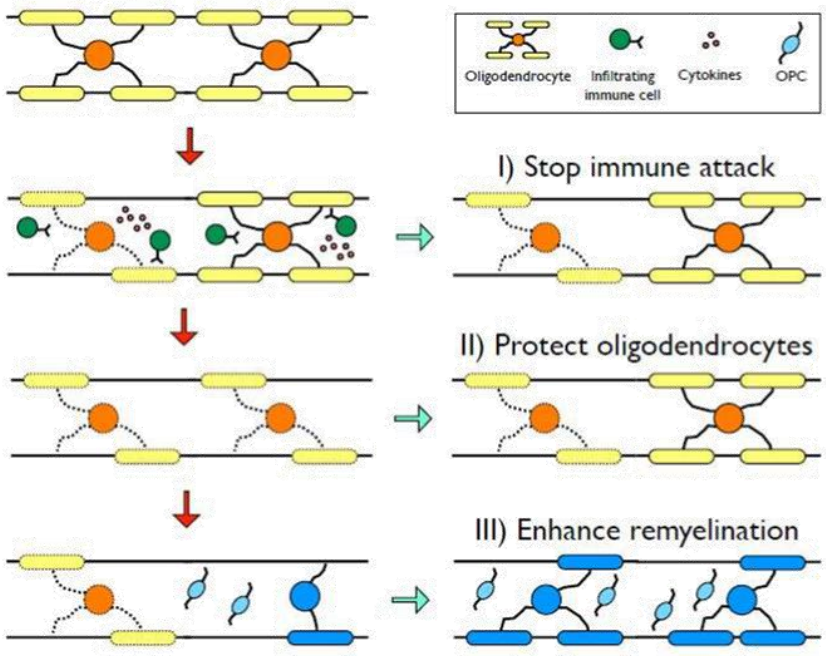

Figure 7. Combinatorial treatment strategies for multiple sclerosis (from Rodgers *et al.*, 2013).

4. IN VIVO MODELS TO STUDY DEMYELINATION AND REMYELINATION

Several models of demyelination were proposed along decades as primary research effort in understanding the cellular and molecular mechanisms basis of demyelinating diseases such as the MS (De Vries and Boullerne, 2010; Van der Star, 2012).

Animal models for MS are significantly used in research since they mimic the complex immune-pathological mechanisms of the disease as well. *In vivo* models are widely advantageous for testing novel therapeutic and reparative trials. A single model cannot resume all the clinical, radiological, pathological and genetic features of MS. The most common *in vivo* models of demyelinating disease include the experimental autoimmune encephalomyelitis (EAE), toxic demyelination, viral infections and transgenic mouse models presenting genetic mutations in myelin genes.

4.1 EAE

The EAE animal model was discovered by Rivers in 1930s as attempt to elucidate the strange cases of paralyses consequential to anti-rabies vaccine immunization. (Rivers, 1933). EAE is one of the most important animal model for MS, as reproduces many symptoms and characteristic of the disease similar to those observed in human MS patients, such as the mononuclear cell infiltration, the brain inflammation, the disruption of the BBB and demyelination of axonal segments. EAE is a demyelinating autoimmune model disease characterized by CD4+ T cell infiltration in the CNS. This model reproduces most of the features of MS through the immunization against self-component of myelin by injecting an emulsion under skin containing synthetic peptides, that replicate the myelin components such as the MOG, MBP or PLP, and an adjuvant to better configure the immunologic reaction. As the immunization begins, cells of the immune system activate and proliferate thus increasing the number of peripheral antigen-specific CD4+ T cells. T cells enter the CNS, bind specifically the myelin antigenic components destroying the integrity of sheaths.

The effects of EAE model are subjected to enormous variability, thus requiring the consideration of parameter such as the animal strain, age and treatment duration (Matsushima *et al.*, 2001; Sachs *et al.*, 2014).

4.2 Viral infections

Several of epidemiological studies have been reported that early viral infections in childhood might mediate the immune-attack against nervous tissue then visible in adulthood, with the rise of demyelinating diseases, such as the MS. A genetic susceptibility could have some

relevance as critical factor that rely the viral infection to the uprising of MS (Asherio and Munger, 2007). To date, there is no specific virus identified as the responsible agent for the onset of MS. Epstein-Barr Viruses (EBV) are currently the most accredited viruses group having some evidence with the susceptibility to develop the disease (Asherio, 2001; Salvetti *et al.*, 2009). Other virus showing a link with the immune attack rise against myelin are the influenza virus type A (Markovic-Plese *et al.*, 2005), human papilloma virus (HPV) (Ruiz *et al.*, 1999) and human herpes virus type 6 (HHV-6) (Tait and Straus, 2008; Kimberlin and Whitley, 1998) which is well known to express some role in mimicking myelin basic protein (MBP) sequence.

Oligodendrocyte may be infected by using several viral models (Atkins 2000; Amor 2010; Virtanen and Jacobson 2012). The two most known models include the attack on neurons secondarily affecting the myelinating oligodendrocytes, the "inside out model", and the direct attack of myelin, the "outside in model".

4.3 Toxic models

The toxic models are widely diffused in remyelination studies. The initial demyelinating insult is delivered through focal or systemic administration of a toxin. Focal lesions are induced via injection of lysophosphatidylcholine, known as lysolecithin or LPC, or ethidium bromide (EB) (Blakemore and Franklin, 2008), antibodies directed against oligodendrocyte antigens (Morris *et al.*, 2002; Rosenbluth *et al.*, 2003; Rosenbluth and Schiff, 2009), bacterial endotoxins (Felts *et al.*, 2005), 6-aminonicotinamide (Blakemore, 1968), electrolytes (Rojiani *et al.*, 1994) or antibodies conjugated to complement.

The systemic administration of the copper-chelating agent, cuprizone, induces more heterogeneous lesion in the CNS (Kipp *et al.*, 2009).

The ***copper chelator cuprizone*** [oxalic acid bis (cyclohexylidene hydrazide)] is a selective and sensitive toxin model for demyelinating diseases. The cuprizone, added as a supplement to rodent chow, exert its effect specifically on oligodendrocyte energetic balance, by interfering with the copper-dependent mitochondrial enzymes cytochrome oxidase and monoamine oxidase function. The energetic metabolism failure induces oligodendrocytes apoptotic death, hence resulting in an extensive demyelination in the CNS.

Interestingly, a spontaneous remyelination occurs already 4 days after withdrawal of cuprizone. The subsequent reversibility of the model makes the cuprizone an excellent model for studying factors which can prevent demyelination and stimulate remyelination.

Even the cuprizone is a copper chelator, its demyelinating effect may not be antidoted by administration of exogenous copper.

The cuprizone model has attracted increasing interest during the last decade since, contrary to other models of MS, this model provides a highly reproducible system of primary oligodendrocytes apoptosis and secondary demyelination.

The dosage necessary to induce appreciable demyelination is strain- and age-dependent.

A common protocol used in the last years includes as strain the C57BL⁄6 mice, preferentially males, of 8 weeks of age fed with 0.2% cuprizone (w⁄w) for 5-6 weeks, named "acute demyelination" protocol. The administration of cuprizone for a period superior to 6 weeks induces a "chronic demyelination" with detrimental effects on myelin that difficulty will be restored (Matsushima and Morell, 2001; Torkildsen *et al.*, 2008)(Figure 8).

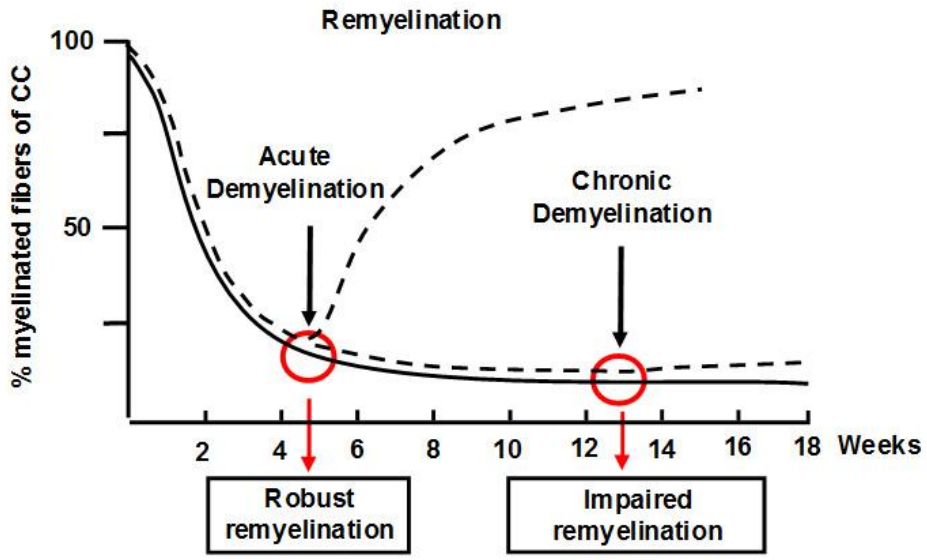

Figure 8 Characteristics of the cuprizone model. Image obtained from the web http://www.promyelo.de/multiple-sclerosis-models/remyelination-models/.

A higher degree of demyelination can be achieved by increasing the dosage of the toxin in the rodent chow up to 0.3% (w⁄w) (Lindner *et al.*, 2008).

Demyelinating lesions have been believed to exist exclusively in the caudal regions of the *corpus callosum* (CC) and in the superior cerebellar peduncles (Matsushima and Morell, 2001), but more recent studies attest the presence of lesions also in other nervous regions such as hippocampus, putamen, cerebellum and even distinct grey matter areas in the cortex (Kipp *et al.*, 2009; Nathoo *et al.*, 2014).

The primary loss in oligodendrocyte reflects the characterization of pattern III and IV lesions described by Lucchinetti *et al.* (2000), as in the pattern III lesions oligodendrocyte function is disturbed and in the patter IV lesions the demyelination secondarily occurs (Lucchinetti *et al.*, 2000).

In the cuprizone model, a specific time activation of microglia, macrophages and astrocytes occurs, while the BBB, that is defecting and destroyed in other models like the EAE, is maintained intact (Torkildsen *et al.*, 2008).

In C57BL6/J mice the cuprizone induces several behavioral responses spanning from the weight loss to the motor-behavioral disabilities. From the third week of cuprizone administration mice may present hyperactivity and decreased anxiety behavior. Around the fifth week the motor dysfunction becomes more evident and persists many weeks even after withdrawal of the cuprizone administration. Also social interaction and impaired bilateral sensorimotor coordination are impaired after six weeks of cuprizone intoxication (Franco-Pons *et al.*, 2007; Hibbits *et al.*, 2009; Liebetanz and Merkler, 2006; Makinodan *et al.*, 2009). Stopping the cuprizone administration, the mice weight becomes to the normality.

After 5 weeks of cuprizone administration the CC is almost completely demyelinated (Figure 9). The effect of this acute demyelination is still reversible. Conversely, chronic demyelination lesions are not completely restored (Komoly, 2005; Stidworthy *et al.*, 2003).

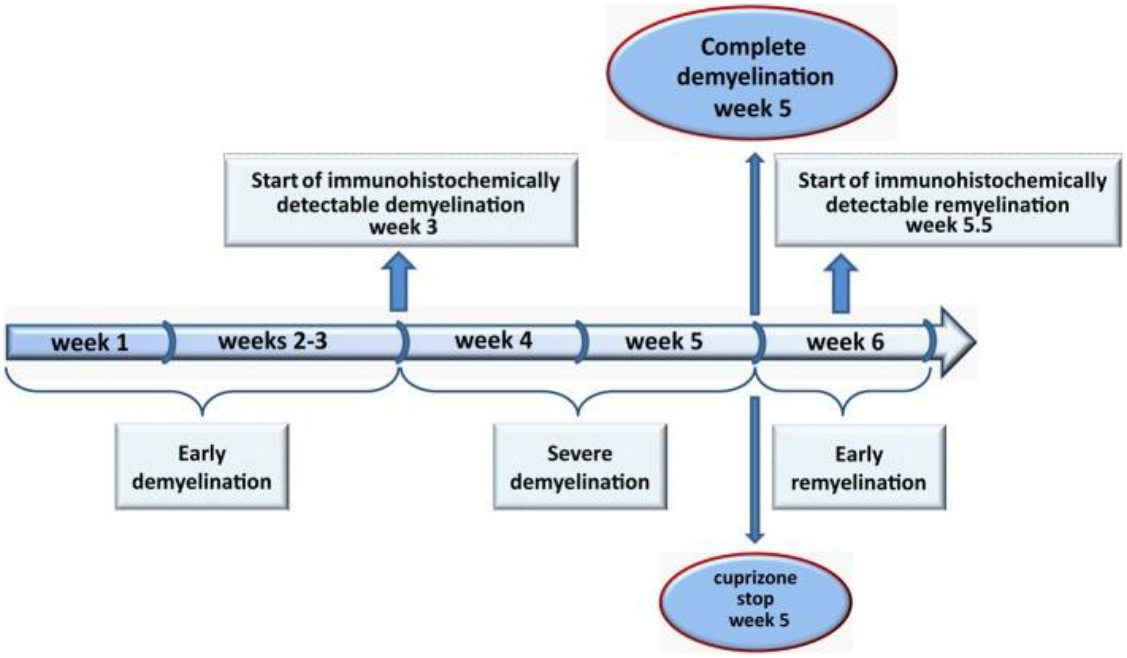

Figure 9. The course of demyelination and remyelination in the medial corpus callosum. Image obtained from Gudi *et al.* (2014).

Demyelination and remyelination processes are mostly investigated in the CC through different techniques including: 1) the myelin-specific histochemical stainings, such as the Luxol Fast Blue combined with the periodic acid Schiff (LFB-PAS) (Matsushima and Morell, 2001); 2) immunohystochemistry (IHC) by using specific antibodies, such as MBP and PLP, binding antigens present in the mature myelin; 3) ultrastructural morphological observations of myelin rings with electron microscopy analyses.

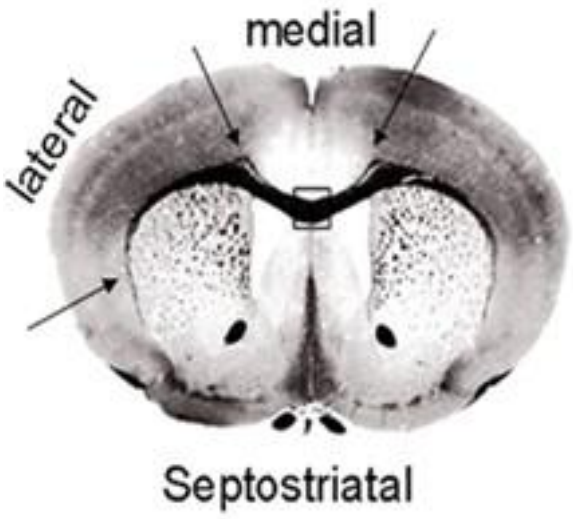

Figure 10. Corpus callosum septostriatal section. Image obtained from Kim *et al*., (2011).

In rodent experimental models, both MRI analysis and behavioral tests, as not invasive techniques, identified the CC as the region mainly involved in the evolution of demyelinating lesions in the cuprizone mouse model (Matsushima and Morell, 2001; Armstrong *et al.*, 2002; Nathoo *et al.*, 2014; Adamo *et al.*, 2006; Armstrong *et al.*, 2006; Lindner *et al.*, 2008; Thiessen *et al.*, 2013)

The CC function is associated to the integration of informations between the two hemispheres. The callosal agenesis in human brains does not impair superior functions despite some defectiveness in speech and motor skills (Paul *et al.*, 2007; Devinsky and Laff, 2003). As reported by Paul *et al.* (2007), the language disability often is linked to the lack of social interaction and other autistic symptoms (Paul et al. 2007). The different CC structure also associates with motor skills impairment such as the bimanual finger co-ordination.

In rodents, CC alterations impair motor coordination and balance (Schalomon and Wahlsten, 2002), thus suggesting that association of both imaging techniques with behavioral tests could be an easy and not invasive investigative strategies for longitudinal studies.

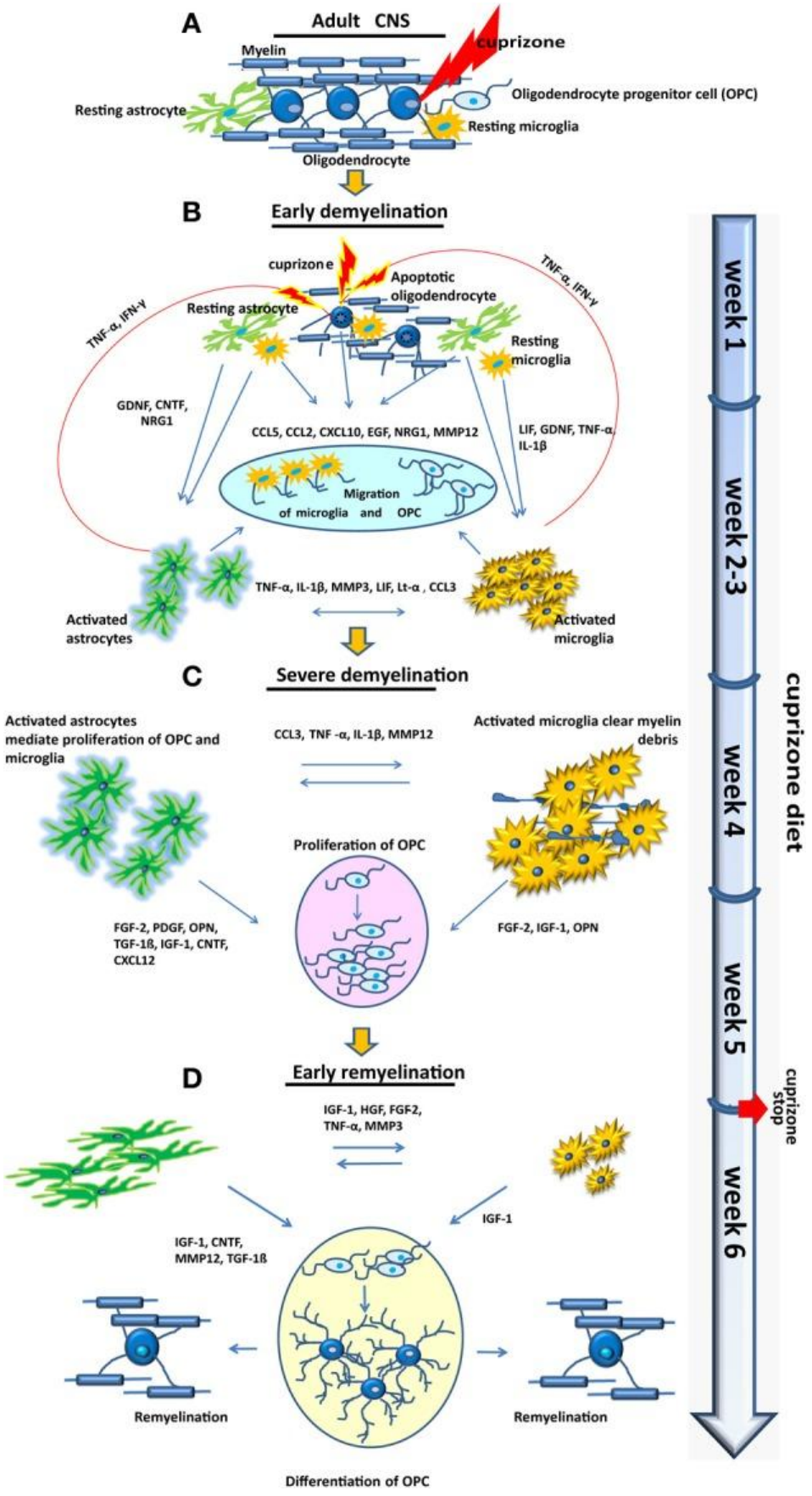

Figure 11. Cellular and molecular response in the medial corpus callosum during cuprizone-induced demyelination and remyelination. Image obtained from Gudi *et al*. (2014).

5. REGULATION OF OLIGODENDROCYTE DEVELOPMENT

Each step of the oligondedrocyte lineage differentiation is tightly time-space controlled by both intrinsic and extracellular factors (Emery, 2010a; Zuchero and Barres, 2013). First studies started on OPCs derived from the postnatal CNS (Temple and Raff, 1986; Gao and Raff, 1997; Gao *et al.,* 1998). Raff and colleagues proposed that there is an internal clock that determined the number of divisions of cultured OPCs in the presence of growth factors (Raff, 2006, 2007; Wang *et al.*, 2001). Then, many researchers started to enlighten the complex regulatory mechanisms of OPC differentiation which are well appreciated in a tight temporo-spatially manner.

Recently, great interest has been given to the transcriptional events behind the differentiation of oligodendrocytes (Nicolay, 2007; Wegner, 2008; Li et al., 2009; Emery, 2010b). Specific environmental and intrinsic regulatory factors orchestry the complex oligodendrocytes lineage through various stages of myelination (Emery, 2010a; He and Lu, 2013; Zuchero and Barres, 2013).

Here, I summarize some of the most important extrinsic and intrinsic cues controlling oligodendrocyte maturation and differentiation.

5.1 Sonic hedgehog, bone morphogenetic proteins and Wnt signaling

Oligodendrogenesis is committed to the activation of specific signaling patterns such as the Shh, BMP and Wnt/β-catenin.

In the developing spinal cord telencephalon OPC maturation is tightly regulated by the morphogenic *Sonic hedgehog* (Shh) which is secreted from the notochord and floor-plate at the ventral mid-line and by neuroepithelial cells in the preoptic area, respectively (Nicolay, 2007). Shh signaling pattern induces several transcription factors including Nkx2.2, Nkx6.1, Nkx6.2, Olig1 and Olig2 (Richardson *et al.*, 1988; Rowitch, 2004). The transcription factor *Nkx2.2* is not sufficient *per se* for OPC formation, but is required for the progression of oligodendrocyte differentiation and maturation. Nkx6.1 and Nkx6.2 induce the expression of Olig2, which indeed is strongly reduced in the spinal cord of Nkx6.1 knockout mice (Vallstedt *et al.,* 2005).

The *bone morphogenetic proteins* (BMPs) belong to the superfamily of transforming growth factor (TGF-β) and regulates cell proliferation and differentiation of several cellular types (Chen *et al.*, 2004) including astrocytes and neural progenitor oligodendrocytes (Hu *et al.*, 2010). BMP signaling antagonizes the effect of Shh-activated specification. In fact, it has

been demonstrated that the inhibition of BMP signaling induces OPC formation both *in vivo* and *in vitro* (Vallstedt *et al.*, 2005; Mekki-Dauriac *et al.*, 2002).

Sustained BMP signaling activation is necessary for OPC maintenance through upregulation of negative regulators of OPC differentiation, such as the inhibitor of DNA binding 2/4 (Id2/4) (Samanta and Kessler, 2004).

Interestingly, the morphogen *Wnt signaling* acts similarly to BMP. By antagonizing dorsal OPC specification promotes astrocyte development (Shimizu *et al.*, 2005). Wnt pathway acts to stabilize β-catenin protein, which then translocates to the nucleus and forms a complex with T-cell factor/lymphoid enhancer factor (Tcf/LEF) to regulate the expression of target genes (MacDonald *et al.*, 2009). Even Wnt signaling was considered as a negative regulator of the OPC differentiation, a recent study indicates that baseline activation of the Wnt pathway is important for OPC differentiation (Fancy *et al.*, 2014). However, unrestricted pathological high-activity of Wnt signaling impedes differentiation of oligodendrocytes (Fancy *et al.*, 2014).

5.2 Transcription factors

Besides these environmental cues, which are important to define the domains that will generate oligodendrocytes in CNS, cell commitment toward the OLG lineage is regulated by the dynamic interaction between transcription factors and also epigenetic factors, including *microRNA* (miRNA) (Liu and Casaccia, 2010).

In spinal cord and forebrain embryonic neuronal stem cells, the binding of *Shh* to its receptors initiates intracellular signalling cascades that activate or repress transcription factors whith visible effect on the oligodendrocyte differentiation. The morphogen sonic hedgehog (Shh) secreted in the neural tube (Pringle *et al.*, 1996; Orentas *et al.*, 1999) induces several transcription factors including Olig1 and Olig2, Nkx2.2, Nkx6.1, Nkx6.2, (Richardson *et al.*, 1988; Rowitch, 2004).

In fact, the first transcription factors that appears in differentiating OPCs, often used as markers for early OPCs, are Olig1 and Olig2 (Nikolay *et al.*, 2007). *Olig1* and *Olig2* are transcription factors belonging to the basic helix–loop–helix (bHLH) family expressed during fetal development and postnatal brains. Even sharing a similar structure, Olig2 seems to be more related to the generation of OPCs in association to Nkx2.2, while Olig1 function is linked to OPCs survival and maturation in spinal cord even remains necessary for the induction of cortical OPCs (Nikolay *et al.*, 2007). Overexpression of both Olig1 and Olig2 in

combination with blocking BMP has recently been shown to enhance oligodendrocyte differentiation of adult NSCs (Cheng, 2009).

Furthermore, two members of the zinc-finger superfamily of transcription factors, *Myt1* and *YinYang1* (YY1), have been recently investigated as potent regulators of OPC cell cycle exit towards differentiation phase (Sher *et al.*, 2008).

Also the *SOX* family of transcription factors are crucially involved in OPC progression lineage (Wegner and Stolt, 2005; Stolt *et al.*, 2006). Sox9 is involved in oligodendrocyte specification while Sox10 is required for OPC differentiation (Stolt *et al.*, 2004). Sox5 and Sox6 control OPC migration in spinal cord and forebrain (Baroti *et al.*, 2016).

5.3 Growth factors signaling

The OPCs development from the ventral neural tube results from the interaction of all the extrinsic factors cited and the release of specific growth factors, such as PDGF, FGF2, IGF1 and neurotrophins (NGF, BDNF, NT-3, NT-4/5) Kessaris *et al.*, 2004; Lachapelle *et al.*, 2002; Hsieh *et al.*, 2004)

The *platelet-derived growth factor (PDGF)* is synthesized during development from both astrocytes and neurons. It was described as the first discovered potent mitogen for O-2A progenitor cells *in vitro* (Pringle *et al.*, 1989). PDGF-AA allows signals of survival and proliferation to the OPCs niches. As the PDGF-α receptor (PDGF-αR) is one of the first to be induced upon Olig2 expression, it can be used as a marker for early OPCs. In PDGF-αR null mice, the number of OPCs is dramatically reduced in developing spinal cord, whereas its overexpression induces ectopic overproduction of OPC (Calver *et al.*, 1998).

Interestingly, it has been demonstrated that PDGF-AA acts as survival factor controlling oligodendrocyte progenitor proliferation also during the early phase of remyelination both in the LPC and cuprizone models (Woodruff *et al.*, 2004; Murtie *et al.*, 2005). PDGF also acts as potent chemoattractant for OPC *in vitro.*

PDGF-AA ligand binding to the PDGF-αR results in a series of intracellular signaling cascade events, including the activation of phospholipase C-γ (PLCγ), phosphoinositide-3 kinase (PI_3K), the Src family of tyrosine kinases, and the mitogen activated kinases (MAPK) (Heldin and Westermark, 1999).

Fibroblast Growth Factor (FGF) family, including the *FGF2*, sinergically acts with the PDGF-AA as crucial mitogen for OPCs survival and proliferation. FGF upregulates the expression of PDGF-αR thus long-lasting the proliferating phase during which OPCs or pre-oligodendrocytes are able to respond to PDGF (McKinnon *et al.*, 1990). FGF family factors

are described to stimulate rapid OPC division while show an inhibitory effect to the final oligodendrocyte maturation (Goddard *et al.*, 2001).

Similarly to PDGF-AA and FGF2, the *insulin-like growth factor 1 (IGF1)* promotes both OPC expansion and differentiation (Ye *et al.*, 2002).

Oligodendrocyte maturation is facilitated by both IGF1 and *thyroid hormone (T3)* receptor-dependent pathways (Barres *et al.*, 1994,1993). IGF-1 has been shown to induce myelination *in vitro* (Mozell and McMorris, 1991) and *in vivo* (D'Ercole *et al.*, 1996; Werther *et al.*, 1998). Furthermore, IGF-1 promotes the long-term survival of mature oligodendrocytes in culture (Barres *et al.*, 1993) and inhibits mature oligodendrocyte apoptosis *in vitro* (Cho *et al.*, 1997; Ye and D'Ercole, 1999).

It has long been appreciated that these different growth factors interact with each other to a certain extent. PDGF, FGF and IGF-1 have been shown to cooperate to promote OPC expansion (Goddard *et al.*, 1999; Baron *et al.*, 2000; Jiang *et al.*, 2001). Once they bind their receptors, they activate the Erk1/2 or PI3K/Akt signaling pathways to trigger downstream effectors (Baron *et al.*, 2000; Frederick *et al.*, 2007). However, their oligodendrocyte specific downstream targets are largely unknown.

NGF, BDNF, NT-3, NT-4/5 are a family of small neurotrophins secreted in the nervous system having a crucial role in the regulation of differentiation, migration, proliferation and cellular maturation. BDNF is notoriously linked to proliferation, axonal outgrowth and myelination *in vivo.* Neurotrophin-3 (NT-3) has been shown to stimulate OPC proliferation and survival via the activation of its receptor trkC (Rosenberg, 2006).

5.4 Ionic signaling through voltage-dependent ion channels

OPCs express *voltage-dependent sodium channels* with the classic rapid kinetics in both activation and deactivation, and high sensitivity blocked with TTX. Sodium currents are required for the early stages of OPCs maturation but not in the subsequent lineage steps (Paez *et al*., 2009a, De Biase *et al.*, 2010, Káradóttir *et al*, 2008).

Oligodendrocytes express a wide range of *potassium channels* during the entire developmental stage. OPCs express both voltage- and time-dependent potassium channels. Delayed rectifying channels have been found in OPC either in culture (Sontheimer *et al.*, 1989) and in brain slices (Berger *et al.*, 1991; Chvátal *et al.*, 1997). In cultured cells some of the outward rectifying potassium channels are activated by calcium (Sontheimer *et al.*, 1989). According to the transcriptome studies of Cahoy *et al.* (2008), OPCs express high levels of members of a newly-identified potassium channel two-pore domain (K2P) family, named

TASK-1, TREK-1 and TWIK-1 (Cahoy *et al.,* 2008). K(V)7/KCNQ channels are functionally expressed in oligodendrocyte progenitor cells (Wang *et al.*, 2011). OPCs express inwardly rectifying potassium channels, including Kir4.1 (Olsen and Sontheimer, 2008) and ATP-sensitive channels (Butt and Kalsi, 2006). Actually, there is no disease related to potassium channel dysfunction in oligodendrocyte lineage cells even in some pathological conditions is described for downregulated levels of Kir4.1 channel expression in astrocytes (Olsen and Sontheimer, 2008).

The voltage-gated calcium channels (VDCCs) existence in OPCs remained for many years problematic, since the great number of adversal studies. Initially, OPCs and oligodendrocytes were thought to be lacking this channels (Southeimer *et al.,* 1989; Barres *et al.,* 1990). More recently, the types R- and L-VDCCs were found to be expressed in precursor and immature oligodendrotcytes (Chen *et al.*, 2000; Paez *et al.,* 2007; Paez *et al.,* 2009b), probably having a role in axon-glial signaling (Chen *et al.,* 2000), cell migration and processes elongation (Paez *et al.*, 2009b).

Recent studies confirmed the presence of the calcium permable *Acid-Sensing Ion Channels* (*ASICs)* both in cultured oligodendrocytes and in brain slices (Feldman *et al.*, 2008).

5.5 Glutamate receptors signaling

Oligodendrocytes express different receptors including those of ATP, GABA, Glutamate, Serotonin, Acetylcolin and Glycine (Karadiottir and Attwel, 2007). Here, I will focus on the role of glutamate receptors in oligodenrocytes due to their relevance in the present study.

Recently, great evidence on the role of *glutamate signaling* in oligodendrocyte comes from the intimate relation between neurons and oligodendrocytes. *Glutamate* is the most abundant excitatory non-aminoacidic neurotransmitter spread out in the central and enteric nervous system of mammals (Dingeldine *et al.,* 1999; Kirchgessner, 2001). The almost totality of neurons (90%) forming synapses in the CNS are glutamatergic synapses (Schmidt and Pierce, 2010).

Oligodendrocyte expresses all the type of glutamate receptors including ionotropic (iGluRs) and metabotropic (mGluRs) during all the lineage progression from OPC to mature myelinating oligodendrocytes enabling these cells to sense and respond to the neuronal activity (Verkhratsky and Kirchhoff, 2007). The iGluRs are ligand-gated ion channels divided into 3 distinct subtypes according to their prototypic agonists: AMPA (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate, Kainate (KA) and NMDA (N-methyl-D-aspartate) receptors.

The iGluR receptors are composed by heterogenic assembling of 4-5 subunits, which determines the specificity for each receptor.

AMPA receptors are composed of four major subunits GluR1 to 4 encoded by separate genes and assembled in tetramers with different stoichiometry (Sommer *et al.* 1991, Hollmann and Heinemann, 1994)(Figure 12). AMPA are usually permeable to Na^+, K^+ and Ca^{2+} when lacking the GluR2 subunit, whereas the presence of this subunit make the AMPA receptor impermeable to Ca^{2+} (Hsu *et al.*, 2010). The RNA editing of the GluR2 sequence in the pore-forming site with a change of Q (Glutamine) to R (Arginine) may possible explain the exhibition of specific properties such as outwardly rectifying currents and minimal Ca^{2+} permeability. By contrast, the not edited GluR2 presence associates with inwardly rectifying currents and permeability to Ca^{2+} (Verdoorn *et al.*, 1991; Burnashev *et al.*, 1995).

Oligodendrocytes express GluR2, 3, 4, 6, 7 AMPA subunits (Patneau *et al.*, 1994; Matute *et al.*, 1997; Itoh *et al.*, 2002) which generate a glutamate-evoked inward currents in oligodendrocytes both in culture (Barres *et al.*, 1990; Patneau *et al.*, 1994; Gallo *et al.*, 1996) and in brain slices (Berger *et al.*, 1992b).

Although it was initially reported that functional AMPA receptors lack GluR2 subunit in oligodendrocytes (Matute *et al.*, 1997), more recently changes in GluR2 subunits levels during the different developmental stages have been described. In fact, mature oligodendrocytes express GluR2 subunit, while it has not been found in OPCs and immature oligodendrocytes (Itoh *et al.*, 2002). This finding may explain the higher susceptibility of mature oligodendrocytes to excitotoxic death (Back *et al.*, 2002; Deng *et al.*, 2003).

Transcriptosome studies performed by Cahoy and co-workers (2008) demonstrated a downregulation of AMPA receptor expression during development (Cahoy *et al.*, 2008), whilst some functional studies produced controversial results. In fact, either in culture or in brain slices, the AMPA receptor response to glutamate was larger in OPCs and immature rather than in mature oligodendrocytes (Itoh *et al.*, 2002; Deng *et al.*, 2003). By contrast, studies performed in brain slices documented a comparable response at different stages of development (Berger *et al.*, 1992b).

The AMPA receptors have been proposed to play an important role in oligodendrocyte development. It has been reported that, by mediating an intracellular Na^+ influx which blocks delayed rectifier potassium channels, AMPA receptor activation leads to the block of cell proliferation and differentiation but not of migration (Gallo *et al.*, 1996). Conversely, other studies reported that the increased cell migration upon AMPA receptor activation correlated with $[Ca^{2+}]i$ changes and mGluR-indipendent G_i-coupled proteins activation (Wang *et al.*,

1996; Gudz *et al.*, 2006). Recently, AMPA receptor has been detected at synapse connecting OPCs to neuronal axons of both white (Kukley *et al.*, 2007; Ziskin *et al.*, 2007) and grey matter (Bergles *et al.*, 2000). Unmyelinated axons extent glutamatergic synapses to near OPCs which activate in return through binding on their AMPA/kainate (KA) receptors. As major effect, the OPCs stop to proliferate and initiate the maturation program towards the mature myelinating oligodendrocytes which extent their processes to wrap the axon and enrolling it with the concentric myelin sheaths (Kukley *et al.*, 2007; Ziskin *et al.*, 2007).

The number of AMPA receptors at synapses is reduced when OPCs differentiate into mature oligodendrocytes (De Biase *et al.*, 2010; Kukley *et al.*, 2010). In mature myelinating oligodendrocytes the expression of AMPA receptors is lower and mainly confined at the soma (Salter and Fern, 2005; Káradóttir and Attwell, 2007).

NMDA receptor subunits are encoded by several genes belonging to 3 major superfamilies (Paoletti *et al.*, 2013; Vyklicky *et al.*, 2014), named GluN1-3 or NR1-3 (Collingridge *et al.*, 2009) (Figure 12). Specifically, GluN1 subunits binds glycine, GluN2 subunits (GluN2A, 2B, 2C and 2D) binds glutamate and GluN3 subunits (GluN3A and 3B) binds glycine (Collingridge *et al.*, 2009). NMDA receptors (NMDARs) display an heterotetrameric conformation in which GluN1, GluN2 and GluN3 subunits assemble with differ stoichiometry. Typically, the receptor is formed by the assembly of at least two glycine-binding subunits GluN1 and two glutamate binding subunits (GluN2A, 2B, 2C and 2D subunits), other receptors may be composed by GluN1 and GluN3 subunits, or may contain all subunits, GluN1 GluN2 GluN3 (Cull-Candy and Leszkiewicz, 2004).

NMDA receptors are voltage-gated receptors permeable to Na^+, K^+ and Ca^{2+}. Their activation need a membrane potential depolarization which remove the Mg^{2+} from the pore site, and require also the binding of either extracellular glutamate and a co-agonist (Glycine or D-serine) (Dingledine *et al.*, 1999; Wollmuth and Sobolevsky, 2004; Papouin *et al.*, 2012).

NMDARs are widely distributed through in the CNS with a specific expression pattern depending on the cerebral area and the developmental stage (Sanz-Clemente *et al.*, 2013), GluN1-GluN2B-GluN3A and GluN1-GluN2B-GluN2D during development, while GluN1-GluN2A-GluN2B and GluN1-GluN2A-GluN2C in the adult life (Al-Hallaq *et al.*, 2007; Brothwell *et al.*, 2008).

Initially, the role of NMDA receptors in oligodendrocytes was considered controversial, since some studies rejected the presence of NMDA receptors in oligodendrocytes. More recently, several works underlined the importance of NMDA receptors in mediating $[Ca^{2+}]_i$ influx

changes during oligodendrocytes differentiation and early myelination (Káradóttir *et al.*, 2005; Salter and Fern, 2005; Micu *et al.*, 2006).

A recent paper of Saab *et al.* (2016) showed the importance of NMDA receptors in coordinating the energetic metabolism required during the initial steps of myelination (Saab *et al.*, 2016). In particular, an NMDA-dependent mechanism is required to regulate glucose uptake in response to axonal glutamate release, thus making possible a metabolic communication between oligodendrocytes and axons (Saab *et al.*, 2016).

NMDA receptors expressed in oligodendrocytes display a characteristic weak sensitivity to Mg^{2+} which may be due to the specific receptor composition, mainly formed by NR1, NR2C and NR3 subunits which allow NMDA receptors to function even at the normal resting potential of oligodendrocytes (Káradóttir *et al.*, 2005; Salter and Fern, 2005; Micu *et al.*, 2006).

The physiological role of NMDA receptors in oligodendrocytes needs further investigations. In fact, it has been speculated the existence of an extrasynaptic location of receptors in the OPCs similarly to the neuronal extrasynaptic sites of NMDA receptors (Kukley *et al.*, 2007; Ziskin *et al.*, 2007). NMDA receptors, in fact, have been preferentially detected at myelinating oligodendrocytes processes (Káradóttir *et al.*, 2005; Salter and Fern, 2005; Micu *et al.*, 2006).

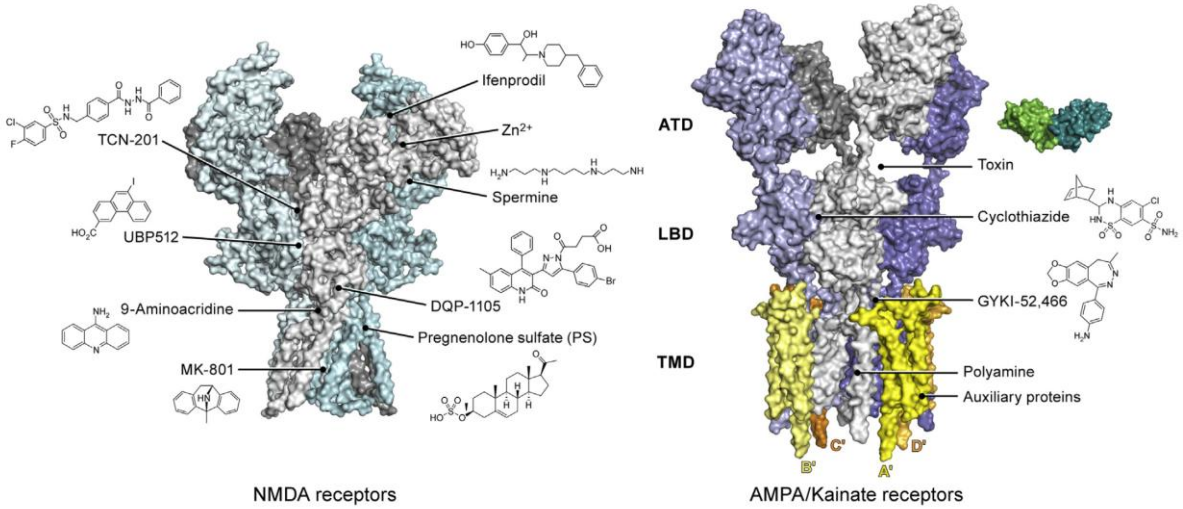

Figure 12. Pharmacological properties of iGluRs. Left panel: NMDA receptors have binding pockets on ATD, LBD and TMD layers. Right panel: AMPA/kainate receptors have binding cavities for both small molecules and proteins between the ATD-LBD layer, within the LBD dimer interface, and within the TMDs. Abbreviations: Amino Terminal Domain (ATD), Ligand-Binding Domain (LBD), Transmembrane Domain (TMD). Image obtained from Zhu and Gouaux (2017).

The ***metabotropic glutamate receptors (mGluRs)*** are seven transmembrane (7TM) receptor belonging to the family of G proteins-coupled protein (GPCRs). The 8 types of metabotropic receptors (mGluR1-8) are subdivided into 3 major groups in according to their sequence homology, different coupling to intracellular transduction mechanisms and pharmacological properties. In particular, mGluRs of Group I (mGluR1 and mGluR5, with their splice variants) are G_q-receptors coupled to the activation of phospholipase C (PLC) that hydrolyses some membrane phosphoinositides producing IP3 and dyacylglicerol and consequently activating the protein kinase C (PKC). Group II (mGluR2 and mGluR3) and Group III (GluR4, mGluR6, mGluR7 and mGluR8) are G_i-protein coupled receptors which activation has an inhibitory effect on the adenylate cyclase (AC) function, thus resulting in a decrease in cyclic AMP levels and blockade of the subsequent pathways (Niswender and Conn, 2010).

Metabotropic glutamate receptors exert modulator effects on other receptor, such as the NMDARs (Skeberdis *et al.*, 2001; Lea *et al.*, 2002).

OPC cultures express both transcripts and protein levels of mGluRs, preferentially with mGluR3 and mGluR5 subunits (Luyt *et al.*, 2003; Deng *et al.*, 2004; Luyt *et al.*, 2006; Cahoy *et al.*, 2008). With the only exception of the mGluR3 receptor, the expression level of metabotropic receptors decreases during maturation, probably due to a prior role in the differentiation stage of oligodendrocytes (Deng *et al.*, 2004; Cahoy *et al.*, 2008; Luyt *et al.*, 2006). Luyt and coworkers described an $[Ca^{2+}]_i$ oscillatory pattern mediated by the activation of mGluRs, while the only activation of mGluR3 reduces the intracellular levels of cAMP. (Luyt *et al.*, 2003; Luyt *et al.*, 2006). Both mechanisms are needed for the motility of OPCs (Othman *et al.*, 2003; Gudz *et al.*, 2006), due to a null response occuring in case of individual mechanisms activation (Luyt *et al.*, 2006).

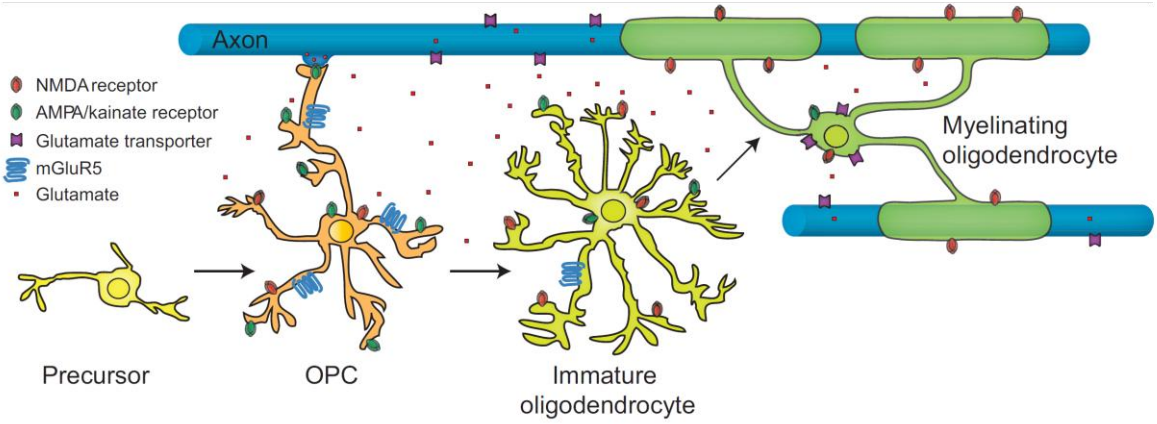

Figure 13. Glutamate receptor expression on oligodendrocyte lineage cells. OPCs are in tight connection to the unmyelinated axons. Either OPCs, immature oligodendrocytes, and mature oligodendrocytes express glutamate receptors. Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, NMDA, *N*-methyl *D*-aspartate; mGluR, metabotropic glutamate receptor. Image obtained from Kolodziejczyk *et al*. (2010).

5.6 Glutamatergic input role on oligodendrocyte lineage

Several recent studies evidence the importance of glutamate signaling as important effector of the intricate myelin plasticity phenomenon.

Káradóttir and its group elegantly described the importance of glutamate signaling in all the oligodendrocyte lineage stages and how it may influence oligodendrocyte migration, proliferation, differentiation, myelination and other cellular properties such as cell death and regeneration properties (Spitzer *et al.*, 2016).

Disagreeing to the simple-minded vision which consider neurotransmitters just released at synapses, glutamate may be release in many different ways. Glutamate may be released either in synaptic and not-synaptic sites. Remarkably, in the synaptic signaling pathway, glutamate may be released in a "canonical" vescicular manner when mediated by the complex SNARE protein machinery activation. Thus, at synapses, presynaptic neurons releases glutamate in activity- and calcium-dependent manner well distinguishable from the other vesicular glutamate release which spontaneously and asynchronously occurs in activity-, calcium- and SNARE-independent manner (Keaser and Regehr, 2014; Kavalali, 2015). In not-synaptic sites, glutamate may be released either in vesicular and not vesicular way occurring in activity-dependent or independent manner (Ziv and Garner, 2004). Even few is known about non-synaptic release, their role is associated with increased receptor expression in early developmental synaptogenesis steps (Furuta *et al.*, 1997; Demarque *et al.*, 2002; Ziv and Garner, 2004; Andreae and Burrone, 2015).

The different way of glutamate release may have some importance in oligodendrocyte lineage progression in which a different timing in location and expression of glutamate receptors have been described (Káradóttir *et al*., 2005, 2008; Bergles *et al*., 2000; Ziskin *et al*., 2007; Kukley *et al*., 2007).

Oligodendrocytes receive glutamatergic inputs from unmyelinated and myelinated axons (Kukley *et al.*, 2007; Tomassy *et al.* 2014; Ziskin *et al.*, 2007) both in the white (Káradóttir *et al.*, 2005, 2008; Kukley *et al.*, 2007; Ziskin *et al.*, 2007) and grey matter (Bergles *et al.*, 2000; Jabs *et al.*, 2005; Lin and Bergles, 2004).

5.6.1 OPC migration

OPCs migrates and populates the entire CNS. The growth and extension of filopodia are necessary for OPCs to sense the environment (Hughes *et al.*, 2013).

Motility seems to be influenced by the presence of both AMPA/Kainate and NMDA receptors which modulate OPCs leading processes. Studies focused on their role in the modulation of

the adhesion protein PSA-NCAM (Wang *et al.*, 1996), the $\alpha_v$integrin-PLP complex formation (Gudz *et al.*, 2006) and the Tiam1 signaling (Xiao *et al.*, 2013). In particular, the $\alpha_v$integrin-PLP complex seems to bind the GluR2 subunit of AMPA receptors making the channels not permeable to calcium (Gudz *et al.*, 2006; Harlow *et al.*, 2015). The lowering in GluR2 levels at the membrane increases the AMPA-mediated Ca^{2+}conductance. The slight OPCs interaction to the extracellular matrix facilitates the progenitors motility.

Controversial studies argued to the different effect of glutamate on OPC migration *in vitro* (neonatal) and *in vivo* (postnatal OPCs), mainly due to the respective different glutamate receptor pattern expression (Ziskin *et al.*, 2007; Káradóttir and Attwell, 2007). Likely, the OPCs might be initially responsive to non-synaptic signaling, such as consequence of glutamate transporter reverse operation mode (Káradóttir and Attwell, 2007), whereas acting as long-range glutamate signals drive the OPCs migration, by influencing the NMDARs-dependent dendritic arborization (Andreae and Burrone, 2015).

5.6.2 OPC proliferation

OPCs continuously proliferate maintaining the endogenous pool of precursors. The role of glutamate signaling in proliferating OPCs is found controversial, apparently due to the different OPC origin. In fact, *in vitro* studies performed on OPC cultures (Gallo *et al.*, 1996) and in cultured slices (Fannon *et al.*, 2015; Yuan *et al.*, 1998), glutamate signaling blocked OPCs proliferation through the activation of AMPA and Kainate receptors, while it has a stimulatory effect on striatal- or SVZ-derived OPCs (Brazel *et al.*, 2005; Redondo *et al.*, 2007).

In vitro, the proliferating effect of the PDGF-αR is antagonized via sodium influx through AMPA receptor activation. It is now retained that glutamate may work as signal that temporally pauses the proliferation thereby allowing the OPCs to reach the axon for the correct axon-OPC synapse formation (Borges *et al.*, 1994; Chittajallu *et al.*, 2005; Hossain *et al.*, 2014; Larson *et al.*, 2015).

In vivo, the understanding of glutamate input implication on OPCs proliferation is less defined. Proliferation of OPCs is equally increased either as in the case of lost of glutamate synaptic input (Mangin *et al.*, 2012) and in case of increased neuronal activity (Gibson *et al.*, 2014; McKenzie *et al.*, 2014). Curiously, there is also the case of a study in which no correlation evidence between glutamate input and proliferation occurs (Hines *et al.*, 2015; Mensch *et al.*, 2015). The most interesting explanation of this apparently discrepancy is described by Hughes *et al.* (2013) that suggest a sort of "sensing *quorum*" case. Indeed, neural

activity stimulate OPCs differentiation, but a secondary OPC proliferation response may occur to substitute OPC underwent differentiation, in order to replace the lacking cells in the progenitors pool (Hughes *et al.*, 2013).

5.6.3 OPC differentiation and myelination

The role of glutamate in OPCs differentiation is still not completely understood. Initially, Duncan and colleagues correlated the myelin thickness to the axonal diameter (Duncan *et al.*, 1934). By using more modern techniques such as nanofibers and OPCs co-cultures, several group joined to the point of an existing axonal activity-independent myelination drived by axonal diameter which not necessitate of glutamate signaling (Bechler *et al.*, 2015; Lee *et al.*, 2012; Li *et al.*, 2014).

Recently, Lundgaad *et al.* (2013) described a double mechanism in which differentiation may occur in both axonal dependent- and independent- activity manner (Lundgaard *et al.*, 2013). The differentiating mechanism which depends from the axonal activity seems to date possibly being modulated by glutamate, even the real contribute of glutamate is not entirely elucidated. Some findings suggest a more complex intervention of both ionotropic AMPA/Kainate and NMDARs and metabotropic glutamate receptors (mGluRs) in the OPC differentiation and in the general oligodendrocytes homeostasis. (Lundgaard *et al.*, 2013).

Recent studies evidenced the role of metabotropic glutamate receptors (mGluRs) as initial maturation and differentiation step promoters. In fact, mGluRs trigger the upregulation of the AMPA receptors expression (Spampinato *et al.*, 2014; Wake *et al.*, 2011; Zonouzi *et al.*, 2011), thereby mediating a higher calcium influx via ionotropic glutamate receptors iGlurRs. The higher calcium influx acts in return with stimulation of early genes explicating their role principally in cell growth and differentiation processes (Gallo *et al.*, 1996; Lundgaard *et al.*, 2013). The latter phase with increase in myelin proteins production and covering of axonal segments is tightly controlled by NMDARs activation via Akt/mTOR pathway. Hence, the glutamate input derived from neuronal activity start the myelination program via NMDARs activation. (Li *et al.*, 2013; Lundgaard *et al.*, 2013, Káradóttir *et al.*, 2005; Salter and Fern, 2005; Micu *et al.*, 2006). In mature oligodendrocytes, the roles exerted by glutamate receptor is slighter, being both necessary for the myelin maintenance, plasticity and architecture, including several structural adjustments such as myelin thickness and Ranvier's nodes conformation (Pajevic *et al.*, 2014; Ford *et al.*, 2015).

AMPA Receptor Subunits			
GluA1	RNA	OPC	Low [1][4][5][6][28]/Yes [25]
		Oligo.	Low [4][5][6]/Yes [25] /No [3]
	Protein	OPC	Yes [18][20][28][30] /No/Low [4][24]
		Oligo.	No [2][3][4][20] /Yes [22]
GluA2	RNA	OPC	Yes/High [1][4][5][6][25][28]
		Oligo.	Yes/High [4][5][6][25] /No [3]
	Protein	OPC	Yes [4][18][20][23][24][30][33]
		Oligo.	Yes [4][22] /No [2] /Low [20]
GluA3	RNA	OPC	Yes/High [1][4][5][6][25][28]
		Oligo.	Yes/High [3][4][5][6][25]
	Protein	OPC	Yes [4][24][30]
		Oligo.	Yes [2][4]
GluA4	RNA	OPC	High [1][4][5][6][28][29] /No [25]
		Oligo.	High [3][4][5][6] /No [25]
	Protein	OPC	Yes [4][17][18][20][23][24][28][30][31][33]
		Oligo.	Yes [2][3][4][7][15][26] /Low [20]

Kainate Receptor Subunits			
GluK1	RNA	OPC	Yes [5][6][25] /No [1][28]
		Oligo.	Yes/Low [5][6][25] /No [3]
	Protein	OPC	No [14]
		Oligo.	N/A
GluK2	RNA	OPC	Yes [1][5][6][25][28]
		Oligo.	Yes [3][5][6][25]
	Protein	OPC	N/A
		Oligo.	N/A
GluK3	RNA	OPC	Yes [1][6][25][28]
		Oligo.	Yes [3][6][25]
	Protein	OPC	N/A
		Oligo.	N/A
GluK4	RNA	OPC	Yes [1][5][6][25][28]
		Oligo.	Yes/Low [3][5][6][25]
	Protein	OPC	N/A
		Oligo.	Yes [27]
GluK5	RNA	OPC	Yes/High [1][5][6][25][28]
		Oligo.	Yes/High [3][5][6][25]
	Protein	OPC	Yes [23]
		Oligo.	Yes [3][8][15][26]

NMDA Receptor Subunits			
GluN1	RNA	OPC	Yes [5][6][16]
		Oligo.	Low [5][6][19]
	Protein	OPC	Yes [10][16][32][34]
		Oligo.	Yes [9][10][11][13][15][21][32] /Low [19]
GluN2A	RNA	OPC	Low [5] /No [6][16]
		Oligo.	Low [5] /No [6]
	Protein	OPC	Yes [32] /No [12]
		Oligo.	Yes [19][32] /No [12]
GluN2B	RNA	OPC	Low [5] /No [6][16]
		Oligo.	Low [5] /No [6]
	Protein	OPC	Yes [32]
		Oligo.	Yes [32]
GluN2C	RNA	OPC	Low [6] /No [5][16]
		Oligo.	Low/No [5][6]
	Protein	OPC	N/A
		Oligo.	Yes [10][11]
GluN2D	RNA	OPC	Yes [5] /Low [6] /No [16]
		Oligo.	Low [5][6]
	Protein	OPC	No [12]
		Oligo.	No [12]
GluN3A	RNA	OPC	Yes [6]
		Oligo.	Low [6]
	Protein	OPC	Yes [12]
		Oligo.	Yes [11]
GluN3B	RNA	OPC	Low/No [5][6]
		Oligo.	Low/No [5][6]
	Protein	OPC	N/A
		Oligo.	N/A

Figure 14. Ionotropic glutamate receptor expression in oligodendrocytes and OPCs. Image obtained from Spitzer *et al.* (2016).

5.7 The Na^+/Ca^{2+} exchanger

The SLC8A gene family encoding Na^+/Ca^{2+} exchanger (NCX) family belongs to the superfamily membrane proteins of Calcium-Cation-Antiporters (CaCA). The NCX exchanger is usually found in the plasmatic, mitochondrial and endoplasmic reticulum membranes of excitable cells (Kiedrowski *et al.*, 1994; Patterson *et al.*, 2007). NCX binds Ca^{2+} ions with low affinity even displaying a high capacity of transport reaching up to five thousand Ca^{2+} ions per second (Carofoli *et al.*, 2001). In order to be activated, NCX requires great changes in intracellular calcium amount, as occurs after the firing of action potentials in neurons (Annunziato *et al.*, 2004)(Figure 15).

5.7.1 Structure and function

The Na^+/Ca^{2+} exchanger is composed of a ten transmembrane segments (TSM1-10) spanning in the plasmatic membranes. The first six segments (TSM1-6) are considered N-terminus hydrophobic and the remaining TSM7-10 the C-terminus hydrophobic domains. The sequence comprised between TSM 2-3 and TSM 7-8 are named α1- and α2- repeats,

respectively at the extracellular and intracellular side where they participate to the ion translocation portion architecture. (Nicoll *et al.*, 1996; Ottolia *et al.*, 2005).

The N- and C-terminus hydrophobic domain are spatially separated by a 550 a.a. long sequence, well known as "f-loop" (Nicoll *et al.*, 1999), which role is not associated to the ions translocations, whilst it could be the side for the regulatory effects mediated by cytoplasmic messengers and transductional events. In fact, the f-loop includes CD1 and CD2 domains (Ca^{2+}-binding domains) with different sensitivity threshold to Ca^{2+} ion, thus allowing the exchanger a more various functioning, whilst CBD1 detect slight increases in cytosolic Ca^{2+} and CBD2 binds Ca^{2+} upon Ca^{2+} concentration increases (Annunziato *et al.*, 2004; Gomez-Villafuertes *et al.*, 2007). A particular autoinhibitory sequence in the N-terminus domain of the f-loop, called XIP (exchanger inhibitory peptide) (Maack *et al.*, 2005) was thought being activated by calmodulin (Matsuoka *et al.*, 1997). Recently, the crystal structure of the bacterial NCX_Mj (*Methanococcus jannaschii*) has been described having 10 transmembrane domains and four sites for the binding of Na^{+} and Ca^{2+} ions (Liao *et al.*, 2012).

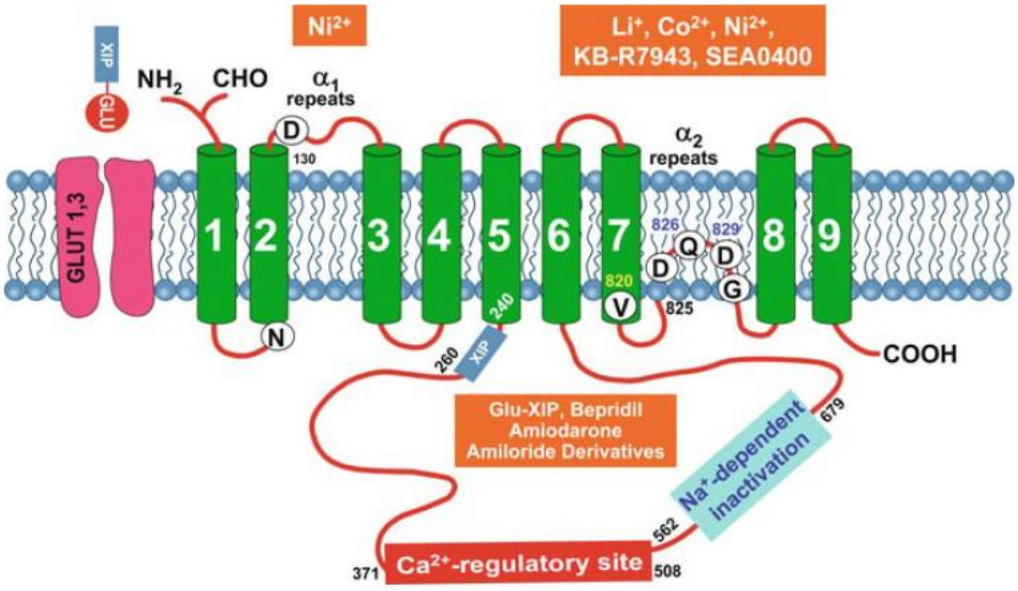

Figure 15. **Structural architecture of NCX**, from Annunziato *et al*. (2004)

5.7.2 Bidirectional operation mode

The NCX exchanger may work efficiently in a bidirectional manner described as forward and reverse operation mode (Annunziato *et al.*, 2004). When intracellular levels of Ca^{2+} increase, the NCX exchanger mediated the influx of three Na^{+} for each Ca^{2+} ion exchanged by using the electrical gradient of Na^{+} (forward mode). By contrast, when intracellular Na^{+} levels increase, as in case of depolarization or in some pathological state, the NCX revert its mode of operation by mediating the efflux of Na^{+}, restoring the membrane potential.

5.7.3 Role of NCX in oligodendrocytes

Quednau *et al*. (1997) demonstrated the presence of NCX isoforms (NCX1, NCX2 and NCX3) transcripts in oligodendrocytes. Since there their role in oligodendrocyte maturation and differentiation remained unexplored. More recently, our research group evidenced the important role of NCX3 in oligodendrocyte lineage progression from OPCs to mature myelinating oligodendrocyte (Boscia *et al.*, 2012). In particular, an upregulation of NCX3 during the differentiation of both oligodendrocytes MO3.13 progenitor and primary OPCs occurred when cells were differentiated into mature myelinating oligodendrocyte with phorbol-myristate-acetate (PMA) or with thyroid hormones T3 and T4, respectively. Specific expression analyses revealed the NCX1 and NCX3 protein and transcripts levels, but not those of NCX2, were differentially modulated during OPC differentiation. In fact, NCX1 was downregulated and NCX3 was intensely upregulated during OPC maturation. In the same study, the importance of NCX3 in oligodendrocytes was evidenced by silencing and knocking out studies of NCX3 gene. In fact, NCX3 silencing via siRNA technique prevented myelin markers expression upregulation during OPC differentiation. By contrast, NCX3 overexpression upregulated the myelin marker synthesis. The role of NCX3 role in oligodendrocyte was demonstrated by the observation that NCX3 knock out mice show a decreased spinal cord size and reduced expression of MBP protein levels (Boscia *et al.*, 2012). More recently, the importance of NCX3 in oligodendrocyte has been investigated by our research group in an animal model of multiple sclerosis, the MOG-induced experimental autoimmune encephalomyelitis (EAE) (Casamassa *et al.*, 2016). NCX3 protein levels were found significantly upregulated during the chronic EAE stage in wild type ncx3+/+ mice, whilst ncx3-/- mice displayed an impaired response of OPC and a worsening of EAE symptoms. These effects were accompanied by a reduced diameter of axons and a dramatic decrease in OPC and premyelinating cells in the white matter of spinal cord in ncx3-/- mice if compaired to ncx3+/+ (Casamassa *et al.*, 2016).

7. D-AMINOACIDS

In nature, biological molecules, such as sugars and aminoacids, may present a double stereo-chemical configuration, named chirality, which occurs in a L- and D- form. The D- and L-form are mirror images of same compound also named enantiomers or optical isomers.

D-sugars and L-Aminoacids are the most common forms of structure founded or incorporated in organic molecules.

L-form aminoacids are predominantly present in biological systems as free or aminoacids participating to the protein elongation process, while its enantiomer, the optically inversed D-forms, are found in lower concentrations in mammalians. The recent progress with more sensitive analytical technologies, revealed the presence of various D-Aminoacids in mammalian tissues including humans (for a review see Fujii *et al.,* 2011).

D-Aminoacids found in proteins can be the end products of a spontaneous mechanism of aminoacidic racemization that occurrs with age (Fuji 2002, 2005)

D-Aspartate (D-Asp), D-Serine (D-Ser) and D-Alanine (D-Ala) are the only free D-enantiomers occurring in substantial levels in mammalian tissues. These D-Aminoacids are found in both animals and humans in considerable concentrations in the nervous and endocrine systems (Hashimoto and Oka, 1997)

Emerging evidence support a role for some D-Aminoacids as neurotransmitter or neuromodulators (Ota and Sweedler, 2012). More recently, a possible role of D-Aminoacids as "gliotransmitters" involved in several physiological processes, including dendritic morphology, synaptic plasticity and cognition has been proposed (Billard, 2012; Wolosker *et al.,* 2008).

Alterations in the concentrations of D-Aminoacids might occur in some neurodegenerative and neuropsychiatric disorders (Fuchs *et al.,* 2005).

Initially, the presence of D-Aminoacids found in mammalian tissues were supposed to be introduced exclusively through alimentation, since only plants and bacteria are able to synthesize D-Aminoacids in free state or incorporated in peptides and protein linkages (Corrigan, 1969; Meister *et al.,* 1965).

7.1 D-Aspartic acid

In 1992, Nishikawa's group settled the high performance liquid chromatography (HPLC) system to separate isomers in biological samples. Nishikawa and coworkers confirmed the presence of free D-Ser and D-Asp in the rodent brains. Afterwards, they discovered the enzyme serine and aspartate racemase that synthesizes the D-Ser and D-Asp, respectively.

The metabolism of D-Asp is regulated by the enzymes aspartate racemase and D-Asp oxidase. Temporal reduction of D-Asp levels depends on the postnatal onset of D-Asp oxidase (DDO) activity, the only enzyme able to selectively degrade this D-amino acid (Errico *et al.*, 2012). Notably, previous studies debated for decades the existence of the enzyme related to the endogenous synthesis of D-aminoacid Oxidase and D-Asp oxidase in mammalians. (Mothet and Snyder, 2012). Free D-Asp is inserted in protein chains during the early phases of development and the days after birth (Errico *et al.*, 2012).

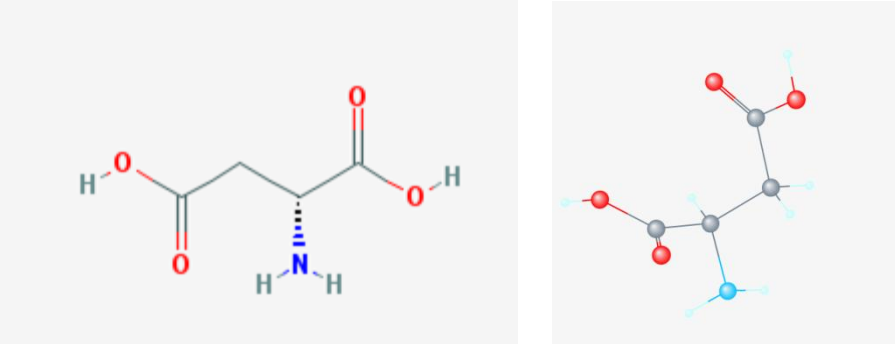

Figure 16. D-Aspartic acid 2D and 3D structure. Images obtained from PubChem web site (https://pubchem.ncbi.nlm.nih.gov).

7.1.1 Distribution

D-Aspartic Acid (D-Asp) is an endogenous aminoacid found in both invertebratae and vertebratae (for review see Ota *et al.*, 2012). D-Asp was first found in the nervous system of marine mollusks and subsequently in nervous and endocrine tissues of many mammals, including humans (Fisher 1991, Hamase 1997, Morikawa, 2007).

The distinct heterogenous localization in nervous and reproductive tissues suggests a specific physiological role. In fact, several studies focused on its function in nervous system development and sexual hormones regulation. (Furuchi and Homma, 2005; D'Aniello, 2007; Errico *et al.*, 2012;).

D-Asp levels transiently increase during the development of nervous system but decrease after birth at least in the rat (Dunlop 1986, Hashimoto 1993), chicken (Neidle and Dunlop 1990) as well as in humans (Hashimoto 1993).

Regional changes in D-Asp levels in the rat nervous system were characterized by Sakai in the late '90 by using immunohistochemistry with specific antibody recognition of D-Asp. The major findings of this work was the demonstration of the appearance of D-Asp in the hindbrain during the embryonic development of the brain, then spreading out to the forebrain area (Sakai, 1998). Initially, D-Asp was found in the cellular soma of neuron at the external

layer of the neuro-epithelium and in the axons, suggesting a role of D-Asp in neuronal differentiation. Studies in invertebrate animals demonstrated the presence of the D-Asp both in the cell soma as well as in neuronal processes. The location of D-Asp in both synaptic terminals and in vesicle released from synaptosomes further corroborated the hypothesis of a potential role of D-Asp in neuronal and neuroendocrine tissues (Spinelli, 2006; D'Aniello *et al.*, 2011).

7.1.2 Biosynthesis

D-Asp could be uptaken by dietary alimentation, since it has been demonstrated that both plants and bacteria are able to synthetize it through the bactivation of enzymatic pathways including racemase and transaminase enzymes (Lamont, 1972; Gosling and Fottrell 1978; Ogawa 1973).

Studies with the rat pheocromocytome PC12 cells demonstrated how these cells did not spontaneosly uptake exogenous D-Asp, indeed they convert the L-Aspartate (Long 1998, 2000, 2002). Other groups hypothesize the intracellular synthesis pathway by the interaction of racemase and transaminase enzymes acting on the aspartyl residues of existing peptides (Homma 2007, Katane and Homma 2011). In this regard, other groups studied the synthesis of D-Asp from the L-Aspartate by using radiolabeling techniques (Wolosker, 2000).

D'Aniello *et al*. (2011) suggested that the D-Asp synthesis may occur in the soma being lower D-Asp levels in the synaptosomes and synaptic vesicles if compared to the whole brain (D'Aniello *et al.*, 2011).

In the last years, other possible biosynthetic pathway of D-Asp were proposed in mammalians, being present mitochondrial aminoacid transaminases in chicken and *E.Coli* (Kochhar and Christen, 1992; Vacca *et al.*, 1997). The same mechanism was reported in plants (Funakoshi, 2008). Fujii's group demonstrated the spontaneous isomerization of L-Aspartic acid residues in proteins resulting in the release of free D-Asp (Fujii, 2002).

7.1.3 Uptake, transport, degradation and release

Uptake and accumulation of extracellular D-Asp was followed in experiments by using [3H] radiolabeled D-Asp. D-Asp is distributed both in neurons (Scanlan *et al.*, 2010) and in glia (Kimmich *et al.*, 2001; Gadea *et al.*, 2004; Lau *et al.*, 2010). D-Asp uptake was supposed to be carried in by the L-glutamate transporter following the Na^+ gradient. The L-Glu transporter showed a similar affinity for L-Asp and L-Glu, thus suggesting a competition for the same transport (Kanai and Hediger, 1992).

The removal of D-Asp is mediated by the enzyme D-Asp Oxidase (DAspO) that degrades the dicarboxylic D-Aminoacids via oxidation such as in the case of the D-Asp, D-Glu and NMDA. For the rest of D-Amino enantiomers, the degradation is settled by the D-Amino Acid Oxidase DAAO (D'Aniello 1993a and c). The DAspO gene has been found activated in several higher mammals such as human brains (Setoyama and Miura 1997; Katane *et al.*, 2007a; Simonic *et al.*, 1997). DAspO was found in peroxisome of both humans and rat liver (Vanveldhoven *et al*. 1991; Katane *et al*. 2010).

Several studies demonstrated the release of D-Asp upon electrical and chemical stimulation in a Ca^{2+}-dependent manner (Scanlan *et al.*, 2010; Wolosker, 2000). Savage and colleagues investigated the D-Aspartate and L-Glutamate release with radiolabeling in hippocampal slices (Savage, 2001). D-Asp release was blocked by treating synaptic vesicles with toxin or magnesium, and just moderately decreased by using antagonists for the voltage-gated Ca^{2+} channels. Spontaneous release of D-Asp occurred in PC12 cells (Long, 1998) either in the intracellular and in the extracellular environment, in absence of external stimulations.

7.1.4 Function

The D-Asp received great attention since it was supposed to function as neurotransmitter and neuromodulator (Spinelli *et al.*, 2006; Brown *et al*., 2007; D'Aniello 2007; Fieber *et al.*, 2010; Scanlan *et al.*, 2010; D'Aniello *et al*., 2011), being synthesized and delivered upon stimulation by synaptic terminals and by modulating the depolarization state of post-synaptic membrane in presence of other neurotransmitters (Kandel *et al.*, 2000; Ota *et al.*, 2012). Even recent findings about D-Asp, the exact receptor binding the D-Aminoacid remains still unknown. D-Asp may activate the NMDA receptors (Verdoorn and Dingledine, 1988). D-Asp is able to activate the L-Glu receptors in the same manner of the L-Glutamate. It may increase the second messenger cAMP levels both in rat and *L.Vulgaris,* suggesting a possible role in intracellular signaling pathways (D'Aniello *et al.*, 2011).

Moreover, even its role as neurotransmitter acting in short cell-to-cell signaling, D-Asp was supposed to have also a hormone-like role in the neuroendocrine system, since it may regulate steroid hormone synthesis in rat pituitary glands and testis, increases the LH and GH hormones and prolactin release (D'Aniello 2000a and 2000b, Nagata 1999) by acting in both cAMP/cGMP dependent (Topa 2009) and independent mechanisms (Nagata 1999a and 1999b).

D-Asp may serve as precursor of endogenous synthesis of NMDA via activation of D-Asp methyltransferase enzyme, also namend NMDA synthetase, that converts D-Asp in NMDA through transfer of a methyl residue (D'Aniello 2000a and 2000b)

D-Asp Oxidase may oxidate and degrade also NMDA (D'Aniello 1993a and 1993c).
Nuclear function for D-Asp were hypotized as it was discovered in nucleoli (Wang 2002) and it was associated to changes in supercoiled DNA (Bharathi *et al.,* 2003).
D-Asp acts as an endogenous agonist of NMDA receptors and its effect on this receptor has been associated with cognitive processes like learning and memory. D-Asp activate the NMDA receptor after binding of the L-Glutamate site (Fagg and Matus, 1984). D-Asp enhances LTP in mouse hippocampus (Errico *et al.*, 2008a) and ameliorates rat spatial learning and memory skills in rats (Topo *et al*., 2010). Remarkably, reduced levels of D-Asp were found in Alzheimer's brains (Fisher 1991, D'Aniello 1998).
D-Asp may also act on AMPA receptors, although both stimulatory and blocking effects have been reported (Gong *et al.,* 2005; Errico *et al.*, 2012; Krashia *et al.*, 2017).
Interestingly, recent works speculate a possible correlation between altered levels of D-Asp and several mental disorders such as Alzheimer's disease and schizophrenia (D'Aniello 2007; Katane and Homma 2011).

AIM OF THE STUDY

Previous studies demonstrated that the impaired myelin sheath regeneration in demyelinating conditions as occurs in multiple sclerosis (MS) is significantly associated with the failure of oligodendrocyte precursors cells (OPC) differentiation. A significant current drive in new MS therapeutics is to identify pharmacological compounds that promote remyelination by boosting OPC to form new myelin before axons become irreversibly damaged (Crawford *et al.*, 2014; Franklin *et al.*, 2012; Hauser *et al.*, 2013).

Electrical activity induced by glutamate signaling has been shown to regulate OPC proliferation, differentiation, and myelination (Wake *et al.*, 2011). In particular, mounting evidence suggests that $[Ca^{2+}]_i$ signaling through glutamate receptors and the Na^+/Ca^{2+} exchanger NCX3 may influence oligodendrocyte development, myelin synthesis and remyelination processes (Gautier *et al.*, 2015; Friess *et al.*, 2016; Boscia *et al.*, 2012; Casamassa *et al.*, 2016).

Recently, D-Aminoacids are emerging as important molecules with several important physiological roles in glial cells. Among them, D-Asp, plays an important role during nervous system development and in the neuroendocrine system as it is currently commercially available as supplement for fertility and cognition. The observation that D-Asp has been detected in considerable levels in the human white matter (Man *et al.*, 1987) and it may influence NMDA receptor signaling in oligodendrocytes led us to investigate the effects of D-Asp treatment on oligodendrocytes both *in vitro,* during OPC differentiation, and *in vivo,* in mice fed with the copper chelator cuprizone, an animal model to study demyelination and remyelination processes.

The overall objective of this study was to investigate the effect of D-Asp exposure on OPC differentiation and during demyelination and remyelination processes in an animal model of multiple sclerosis, the cuprizone model.

In details, the specific aims of our study have been:

1) to determine whether OPC differentiation may be influenced by D-Asp exposure in human oligodendrocyte MO3.13 progenitors. To this aim, we performed quantitative RT-PCR and Western Blot experimentes for myelin markers in presence or in absence of D-Asp exposure.
2) to investigate by means of RT-PCR analyses, the effects of NMDA and NCX3 blockers on D-Asp-induced myelin transcripts increase.

3) to investigate, by using single-cell Fura2-AM video imaging the intracellular $[Ca^{2+}]_i$ levels after D-Asp exposure. The contribution of NMDA and AMPA receptors, and the NCX3 exchanger to D-Asp-induced $[Ca^{2+}]_i$ increase have been studied both in MO3.13 cells and primary rat OPC.
4) to investigate, by using behavioral testing, Western Blot, Confocal and Electron Microscopy, the effects of D-Asp treatment in the cuprizone model of demyelination and remyelination, during cuprizone feeding (demyelination) and after cuprizone withdrawal (remyelination).

CHAPTER II

MATERIALS AND METHODS

II. MATERIALS AND METHODS

1. Animals

Male C57BL/6 mice aged 2 months were housed in a temperature and humidity controlled room under diurnal lighting conditions. Animal handling and care was in accordance with the International Guidelines for Animal Research and the experimental protocol was approved by the Animal Care and Use Committee of "Federico II" University of Naples. Wild-type *ncx3+/+* and knockout *ncx3−/−* mice (Sokolow *et al.*, 2014) were provided by Dr. S Sokolow (UCLA School of Nursing, Los Angeles, CA, USA) and Dr. A Herchuelz (Faculté de Médecine, Université Libre de Bruxelles, Brussels, Belgium).

2. Cell Cultures

The human oligodendrocyte MO3.13 cell line and D-Asp exposure

The MO3.13 cell line is an immortalized human clonal model that expresses the phenotypic characteristics of OPCs (McLaurin *et al.*, 1995; Boscia *et al.*, 2012). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 10 μg/ml streptomycin, and 2 mmol/l –glutamine (Normal Medium). To induce an oligodendrocyte phenotype, human MO3.13 cells were cultured in a serum-free chemically defined medium composed of DMEM supplemented with 500 μg/l insulin, 100 μg/ml human transferrin, 0.52 μg/l sodium selenite, 0.63 μg/ml progesterone, 16.2 μg/ml putrescin, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (OPC medium), and containing 100 nM Phorbol-12-Myristate-13-Acetate (PMA) or D-Asp for 3 days *in vitro* (DIV). 10-200 μm D-Asp was added in absence of PMA every day, by replacing the medium, and for 3 days.

3. Rat and mouse primary OPC cultures and D-Asp exposure

Purified OPC cultures were prepared as previously described (Boscia et al., 2012). In brief, primary mixed glial cell cultures were isolated from the cerebral cortex of postnatal day 1 Wistar rats or C57BL/6 mice, dissociated into single cells, and cultured into poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA)-coated tissue culture flasks in Normal medium at 37 °C in a humidified, 5% CO_2 incubator (Boscia *et al*., 2009). Once confluent (after 7–9 days), the microglia were separated by mechanical shaking of flasks on a rotary shaker for 60 min at 180 r.p.m. and removed. The cultures were then subjected to an additional 16 h of shaking at 180 r.p.m. To minimize contamination by microglial cells, the suspension of detached cells was

incubated twice for 40 min at room temperature (RT). The non-adhering OPCs were plated into 10µg/ml polyD-lysine-coated plates in Normal medium and maintained at 37 °C in a humidified, CO_2 atmosphere. This procedure yields 98% A2B5-positive cells. Six hours after plating, the culture medium was replaced with OPC medium supplemented with 10 ng/ml PDGF-AA and 10 ng/ml bFGF each day for 3 days to maintain the undifferentiated state and support OPC survival. Then, PDGF and bFGF were withdrawn from the OPC medium and were exposed to D-Asp for 3 days. For electrophysiological and microfluorimetric experiments, cells were seeded on glass coverslips coated with 10 ng/ml poly-L-lysine.

4. Quantitative Real-Time PCR

Total RNA was extracted from human MO3.13 cells using Trizol (Invitrogen, Milan, Italy). After DNase-I treatment (1 U/ml; Sigma-Aldrich) for 15 min at RT, the first-strand cDNA was synthesized using 5 mg of the total RNA and 500 ng of random primers by using the SuperScript (high capacity cDNA RT kit; Applied Biosystems, Monza, Italy). Using 1/10 of the cDNAs as a template, quantitative real-time PCR was performed in a 7500 fast real-time PCR system (Applied Biosystems) by using the Fast SYBR Green Master Mix (cod. 4385610; Applied Biosystems). Samples were amplified simultaneously in triplicate in one assay run for 40 cycles with a single fluorescence measurement. PCR data were then collected by using the ABI Prism 7000 SDS software (Applied Biosystems). Afterwards, the products were electrophoretically separated on 3% agarose gels and bands were visualized with ethidium bromide and documented by using the Gel Doc Imaging System (Bio-Rad, Hercules, CA, USA). Normalization of data was performed by using ribosomal protein β-Actin as an internal control; differences in mRNA content between groups were calculated as normalized values by using the 2_DDC t formula and the results were tested for significance by using the Relative Expression Software Tool (REST). The oligonucleotide sequences were as follows: NCX1: 5'-CTGGAGCGCGAGGAAATGTTA-3' and 5'-GACGGGGTTCTCCAATCT-3' ; NCX3: 5'-GGCTGCACCATTGGTCTCA-3' and 5'-GACGGGGTTCTCCAATCT-3'; β-Actin 5'-TGCTGTCCCTGTATGCCTCT-3' and 5'GATGTCACGCACGATTT-3'.

5. Western blotting

Protein samples were separated on 8-15% polyacrylamide gel and electrophoretically transferred onto nitrocellulose membranes 0,22 µm filter pore. Filters were probed using the indicated primary antibodies: polyclonal anti-CNPase (1:400; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), polyclonal anti-MBP (1:1000; Millipore), polyclonal anti-GFAP

(1:1000; Sigma), polyclonal anti-NF-200 (1:1000; Sigma), monoclonal anti-α-tubulin (1:1000; Sigma), polyclonal anti-Amyloid precursor protein (APP) (1:1000, Abcam), monoclonal mouse anti-Iba1 (1:2000, Dako). Proteins were visualized with peroxidase-conjugated secondary antibodies, using the enhanced chemiluminescence system (Amersham-Pharmacia Biosciences LTD, Uppsala, Sweden).

6. Microfluorimetric $[Ca^{2+}]_i$ measurement

Intracellular changes in $[Ca^{2+}]_i$ were measured by single-cell FURA-2AM video-imaging technique, as previously described (Secondo *et al.*, 2005). Oligodendrocyte MO3.13 progenitors were plated on 10μg/ml poly-L-lysine glass coverslip, and after 24 hours of incubation in OPC medium were loaded with 6 μM FURA-2 AM for 30 minutes at 37 °C in normal Krebs solution containing 5.5 mM KCl, 160 mM NaCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES-NaOH (pH 7.4). Then, coverslips were placed into a perfusion chamber (Medical System Co., Greenvale, NY, USA) and mounted onto the stage of an inverted Zeiss Axiovert 200 microscope (Carl Zeiss, Milan, Italy). Images were acquired with a FLUAR x40 oil objective, whereas cells were alternatively illuminated at wavelengths of 340 and 380 nm by a Xenon lamp.

$[Ca^{2+}]_i$ oscillations were identified and quantified using a computer program written in Java computer language as reported in Secondo *et al.*, (2005). Briefly, for each single cell, the software calculated the $[Ca^{2+}]_i$ mean ± SD during the baseline recording interval before drug addition; these values were used to define a cutoff to identify $[Ca^{2+}]_i$ oscillation, which was set at mean $[Ca^{2+}]_i$ ± 1 SD. Subsequently, the software identified as a single $[Ca^{2+}]_i$ oscillation each group of consecutive $[Ca^{2+}]_i$ values higher than this cutoff point. To quantify the effect of specific pharmacological treatments on the occurrence of $[Ca^{2+}]_i$ oscillations, the oscillation frequency was used to define the number of peaks divided by the duration of observation. In control experiments, no significant changes in the frequency occurred after the addition of drug vehicle.

The initial calcium peak was quantified and expressed as Δ% of $[Ca^{2+}]_i$ peak *versus* basal values of calcium. The NMDA receptor antagonist MK-801 and the AMPA receptor blacker DNQX were incubated 10 minute before microfluorimetric Ca^{2+} oscillations recordings.

7. Electrophysiology

AMPA currents were recorded from human oligodencrocyte MO3.13 precursors by using patch-clamp technique in whole-cell configuration using a commercially available amplifier

Axopatch200B (Molecular Devices, CA, USA) and data were acquired with a Digidata1322A acquisition system (Molecular Devices, CA, USA) and pCLAMP 10 software (Molecular Devices, CA, USA). Patch borosilicate glass pipettes were prepared with a puller (Narishige, PC-10, Tokyo, Japan). The resistance of the pipette was 4–5 MΩ. The dialyzing pipette solution contained the following (in mM): 100 Cs-gluconate, 10 tetraethylammonium (TEA), 20 NaCl, 1 Mg-ATP, 0.1 $CaCl_2$, 2 $MgCl_2$, 0.75 EGTA, and 10 HEPES, adjusted to pH 7.2 with CsOH. The cells were perfused with external Ringer's solution containing the following (in mM): 126 NaCl, 1.2 $NaHPO_4$, 2.4 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, and 18 $NaHCO_3$, pH 7.4. The holding potential was maintained at -70 mV in order to record AMPA (Sekiguchi *et al*., 2002; Brown *et al*., 2007; Schurmann *et al.,* 1997; Kung *et al.,* 2013). Signals were low-pass filtered at 5 kHz, sampled at 10 kHz. Drugs were applied using a hand-held pipette and used at the following concentrations: 1-100 µM AMPA, 100 µM D-Asp and 10 µM DNQX.

8. Silencing

Silencing of NCX3 in MO3.13 cells was performed by using the HiPerFect Transfection kit (Qiagen, Milan, Italy), by using two different FlexiTube siRNAs for NCX3, (#7) Hs_SLC8A3_7 (5'-ACCATTGGtCTCAAAGATTCA-3') and (#8) Hs_Slc8A3_8 (5'-CACCACGCTCTTGCTTCCTAA-3'), and a validated irrelevant AllStars siRNA as a negative control (siCtl). Cells were incubated with OptiMEM (Invitrogen) supplemented with the RNAiFect Transfection Reagent (Qiagen) and 20 nM of each siRNA duplex for 15 h. Then, cells were incubated in culture medium for an additional 48–96 h. The construct expressing NCX3–Flag (GeneCopeia, Rockville, MD, USA) was transiently transfected with a standard procedure using Lipofectamine 2000 (Invitrogen).

9. Cuprizone-induced demyelination/remyelination and D-Asp treatment protocols

Experimental toxic demyelination was induced by feeding 8-week-old male C57BL/6 mice a diet with 0.2% (w/w) cuprizone [oxalic bis-(cyclohexylidenehydrazide); Sigma–Aldrich] mixed into milled chow pellets (Harlan Laboratories) Food containing cuprizone was changed every 2 days for 5 weeks (Torkildsen *et al.*, 2008; Sachs *et al.*, 2014).

The effects of D-Asp administration were investigated during cuprizone-induced demyelination and during remyelination, after cuprizone withdrawal (n=6-8 animals per group).

To analyze the effects of D-Asp on acute demyelination, animals were divided into 4 groups: (1) control mice, which were fed with normal chow for all the time of experiments (5 weeks); (2) control D-Asp mice, which received 20mM D-Asp in drinking solution for 5 weeks; (3) cuprizone mice, which were fed with 0.2% cuprizone for 5 weeks; (4) cuprizone plus D-Asp mice, which received 20mM Dasp in drinking solution and were fed with 0.2% cuprizone for 5 weeks.

To analyze the effects on remyelination, animals were divided into five groups: (1) control mice, which were fed with normal chow for all the time of experiments (7 weeks); (2) control D-Asp mice, which received 20mM D-Asp in drinking solution during remyelination, for 2 weeks after cuprizone withdrawal; (3) Cuprizone mice, which were fed with 0.2% cuprizone for 5 weeks, and maintained with normal chow feeding for 2 weeks after cuprizone withdrawal; (4) cuprizone plus D-Aspartate mice (REM 4-7), which received cuprizone for 5 weeks, and 20mM D-Asp in drinking solution one week before cuprizone withdrawal, and maintained with normal chow feeding for 2 weeks; (5) cuprizone plus D-Asp mice (5-7), which received cuprizone for 5 weeks, and 20mM D-Asp in drinking solution after cuprizone withdrawal (REM 5-7) (Table 1).

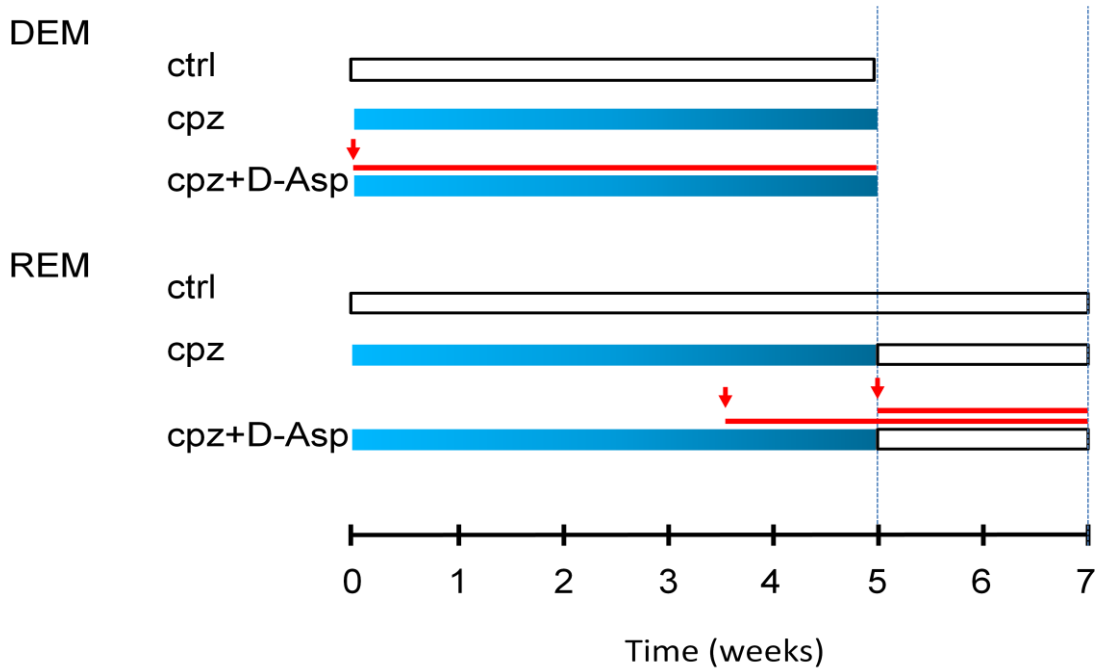

Table 1. Demyelination (DEM) and remyelination (REM) protocols adopted in the present study.

10. Behavioral testing

10.1 Beam Balance. Sensory-motor co-ordination was evaluated by beam balance test. Balance beam apparatus consist in 1 cm wide and 50 cm long beam, elevated 50 cm from the work-surface with a 10 degree inclination. In order to prevent mice injury, a soft sawdust-pad attenuates the mice foot-slips. During the training session the mice were individually placed at the start of the beam and allowed to freely transverse till the end of the runway where a resting box was located. After training mice returned to their home cages. The test consisted of three trials spaced out at least 5 minutes. The experiment was repeated for 3 consecutive days. During the session test the latency to transverse the beam has been timed by the operator.

10.2 Rotarod. Rotarod performance is a widely used motor test to evaluate motor coordination and balance in rodents. The rotaraod apparatus consists in a rotating rod with forced motor activity applied (Panlab Harvard Apparatus, LE8200 device) with variable speeds determined empirically. Rotarod test provides an easy solution to study the effect of drugs, brain damage, motor diseases in rodents (Franco-Pons *et al.*, 2007). In this study two different rotarod protocols were performed. In the first protocol, the apparatus rotation was set at a constant rate of 16 rpm and the number of falls recorded during 60 seconds. In the second protocol, the rod is accelerated (20 rpm/min, from 0 to 16 rpm in 48 seconds) then maintained at constant rate of 16 rpm rotation. The time latency to fall from the rod was automatically recorded (seconds) for each animal. When the animal safely drops into a plastic lane below, the test is considered concluded and the final time recorded (maximum 3 minutes). The experiment consisted in three consecutive sessions conducted with an inter-trial interval of 10 min. The experiment was repeated for 3 consecutive days.

11. Confocal microscopy

Mice were deeply anesthetized with Zoletil 100 (zolazepam/tiletamine, 1:1, 10 mg/kg, Laboratoire Virbac) and Xilor (xilazine 2%, 0.06 ml/kg, Bio98), and transcardially perfused with 4% (wt/vol) paraformaldehyde in 0.1M phosphate buffer (McCarthy and de Vellis 1980; Boscia *et al.*, 2012). Brains were cryoprotected in 30 % sucrose and sectioned coronally at 40 µm on a cryostat. After being blocked with Rodent M Block (Biocare Medical, Concord, CA, USA) for 60 minutes to block non-specific binding, sections were incubated with primary antibodies for 48 hours. The primary antibodies used were the following: polyclonal anti-NF200 (1:1000, Sigma, Milan, Italy); monoclonal anti-MBP (1:400, Covance). Subsequently, sections were incubated with corresponding fluorescence-labeled secondary antibodies

(Alexa-488- or Alexa-594-conjugated anti-mouse or anti-rabbit IgGs). Hoechst-33258 was used to stain nuclei.

12. Quantitative data analysis

All stainings and morphological analyses were blindly conducted (Boscia *et al*., 2008; Boscia *et al*., 2012). Images were observed using a Zeiss LSM 700 laser (Carl Zeiss) scanning confocal microscope. Single images were taken with an optical thickness of 0.5 µm and a resolution of 1024x1024. The MBP fluorescence intensity on coronal tissue sections of mice *corpus callosum* was quantified in terms of pixel intensity value by using the NIH image software ImageJ boundled to JAVA 'co-localization highlighter' (NIH, Bethesda, MA, USA). Digital images were taken with 60× objective and identical light intensity and exposure times were applied to all the photographs from each experimental set. Images from the same areas of the middle septostriatal *corpus callosum* were compared. Before analyses, colocalization threshold settings for each image were determined, and quantification was achieved by counting the number of MBP and NF200 colocalized points (white) per microscope field. Percentage of colocalization among MBP and NF200 signals was achieved by mathematical analysis according to the software features and represented as white dots.

13. Electron microscopy

Brains were fixed 4% PAF and 2.5 % glutaraldehyde in 0.1M phosphate buffer. Then, small blocks of tissue were post-fixed in uranyl acetate and in OsO4. After dehydration through a graded series of ethanol, the tissue samples were cleared in propylene oxide, embedded in the Epoxy resin (Epon 812) and polymerized at 60°C for 72 h. From each sample, thin sections were cut with a Leica EM UC7 ultramicrotome. Thin sections were further investigated using a FEI Tecnai-12 (FEI, Eindhoven, The Netherlands) electron microscope equipped with a Veletta CCD camera for digital image acquisition.

14. G-ratio

The G-ratio value were obtained by dividing the diameter of each axon by the combined diameter of axon and myelin. Assuming that every axon was perfectly circular, the diameter was calculated with the formula: diameter = C/ π (C=Circumference or axonal/myelin perimeter; π= Pi). Selected areas in the middle *corpus callosum* were imaged on the electron microscope at a fixed magnification (20X). Images were semiautomatically analyzed by using g-ratio calculator Image J tool (National institute of health, Bethesda, MD, USA). At least 100 axons were measured per group, and at least 3 animals were analysed per condition (n=3).

15. Hormonal dosage

Cells were collected and centrifuged at 3.000 rpm for 5-10 min. Pellets were homogenised with 200 µl of 80% methanol and centrifuged at 13.000 rpm for 2 minutes. The supernatants were transferred in 1.5 ml epperdorf tube and dried by evaporation at 40-45°C under the hood aspiration. Then, the residue of each tube was dissolved in 100 µl of Tris-HCl 0.05 M pH 7.5 containing 2 mg/ml bovine serum albumin (BSA) and testosterone, progesterone and 17β-estradiol levels were determined using the ELISA method with the corresponding reagent kits from DIAMEDRA s.r.l., Segrate (MI), Italy.

16. Statistical analysis

The data are expressed as the mean ± S.E.M. of the values obtained from individual experiments. Statistical comparisons between controls and treated groups were performed by ANOVA one-way analysis of variance followed by Tuckey's *Post Hoc Test.* P value was significant for values $P<0.05$.

CHAPTER III

RESULTS

III. RESULTS

1. D-Aspartate exposure stimulates oligodendrocyte differentiation and upregulates hormone levels in OPC cells

Quantitative RT-PCR experiments revealed that, when MO3.13 cells were exposed to 10-200 μM D-Asp or PMA for 3 days, a significant dose-dependent increase in CNPase and MBP transcripts was observed (Figure 17A-B). In accordance, Western blotting experiments revelaed that 100μM D-Asp exposure upregulated MBP protein levels, as shown in Figure 17C. In accordance with the well-known role of D-Asp on steroidogenesis (D'Aniello, 2007), when MO3.13 cells were exposed to D-Asp for 3 days, a significant upregulation of both progesterone and testosterone levels was recorded in MO3.13 oligodendrocytes if compared to untreated cells (Figure 17D).

2. Blocking the NMDA receptor and the Na^{+}/Ca^{2+} calcium exchanger NCX3 prevented D-Aspartate-induced myelin markers expression and intracellular calcium oscillations in oligodendrocyte precursors

The documented ability of D-Asp to activate NMDA receptors (Errico *et al*., 2012) and the significant role played by the activation of these ionotropic glutamate receptors (Cavaliere *et al*., 2012, Káradóttir *et al*., 2005) and the sodium calcium exchanger NCX3 during oligodendrocyte maturation (Boscia *et al*., 2012, 2015; Casamassa *et al*., 2016; Friess *et al*., 2016) led us to investigate whether the boosting actions of D-Asp on OPC differentiation might involve NMDA receptors and NCX3 activation. To this aim we first analyze the effect of selective NMDA and NCX3 blockers on D-Asp-induced myelin markers increase. When oligodendrocyte MO3.13 progenitors were exposed for 3 days to 200μM D-Asp in presence of 10 μM MK-801 or the NCX3 blockers, 30nM YM-244769 or 100nM BED (Secondo *et al*., 2015), the upregulation of both CNPase and MBP mRNA expressions induced by D-Asp incubation was significantly prevented (Figure 18A-B). Interestingly, exposure of MO3.13 progenitors to 10-200μM D-Asp significantly and dose-dependently upregulated NCX3 transcripts (Figure 18C), and this upregulation was significantly prevented by the NMDA antagonist MK-801 (Figure 18D). By contrast, D-Asp incubation, either in presence or in absence of 10μM MK-801, did not influence NCX1 transcripts levels (Figure 18E).

Then, we analyze whether the activation of NMDA receptors and NCX3 exchangers might contributed to the changes of intracellular calcium levels following D-Asp treatment (Figures 19 and 20). Acute 100 μM D-Asp application induced an initial calcium peak of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) followed by an oscillatory $[Ca^{2+}]_i$ pattern both in oligodendrocyte MO3.13 progenitors (~90%) and in the majority of primary OPC (~70%), as recorded by Fura-2 video-imaging (Figure 19). Only a small percentage of primary OPCs (~20%) underwent a single rise in $[Ca^{2+}]_i$ (data not shown). The selective NMDA antagonist MK-801 (10μM) completely suppressed D-Asp induced $[Ca^{2+}]_i$ oscillations both in MO3.13 cells and primary OPC (Figure 19A-B), but only partially affected the first $[Ca^{2+}]_i$ peak (see insert of Figures 19A-B). The $[Ca^{2+}]_i$ oscillation frequency in MO3.13 progenitors or primary OPC after D-Asp stimulation in presence of MK-801 was 0.05 ± 0.007 Hz and 0.1 ± 0.003 Hz, respectively, if compared to 0.2 ± 0.023 Hz (MO3.13) and 0.4 ± 0.005 Hz (primary OPC) calculated in absence of MK-801 (for both $p < 0.01$). Similarly, pharmacological blockade of NCX3 exchanger with either YM-244769, or the most recently developed compound BED (Secondo *et al.,* 2015), completely suppressed D-Asp-induced $[Ca^{2+}]_i$ oscillations both in MO3.13 cells and in primary OPC (Figure 20A-B). The oscillation frequency in oligodendrocyte MO3.13 progenitors or primary OPC after D-Asp stimulation pretreated with BED was 0.013 ± 0.001 Hz and 0.2 ± 0.002 Hz, respectively, if compared to 0.2 ± 0.023 Hz (MO3.13) and 0.4 ± 0.005 Hz (primary OPC) calculated in absence of BED (for both $p < 0.01$). The oscillation frequency in oligodendrocyte MO3.13 progenitors or primary OPC pretreated with YM-244769, was 0.004 ± 0.001 Hz (MO3.13) and 0.1 ± 0.002 Hz (primary OPC), respectively, if compared to 0.2 ± 0.023 Hz (MO3.13) or 0.4 ± 0.005 Hz (primary OPC) calculated in absence of YM-244769 (for both $p < 0.01$). Nevertheless, both YM-244769 or BED only partially prevented the initial rise of $[Ca^{2+}]_i$ observed following D-Asp application (see insert of Figures 20A-B).

To further investigate the contribution of NCX3 exchanger to D-Asp induced $[Ca^{2+}]_i$ oscillations we recorded calcium response in MO3.13 progenitors previously silenced for *ncx3* gene or in mouse primary OPC obtained from *ncx3*+/+ and *ncx3*-/- mice. As shown in Figure 20C-D, quantitative analysis of the oscillatory index revealed that silencing or knocking out *ncx3* gene considerably and significantly suppressed the $[Ca^{2+}]_i$ oscillatory pattern following D-Asp exposure. Similarly to what we observed by using pharmacological approach, blocking NCX3 activity by using silencing or transgenic approaches only partially affected the the initial $[Ca^{2+}]_i$ peak following D-Asp application (see insert of Figure 20C-D).

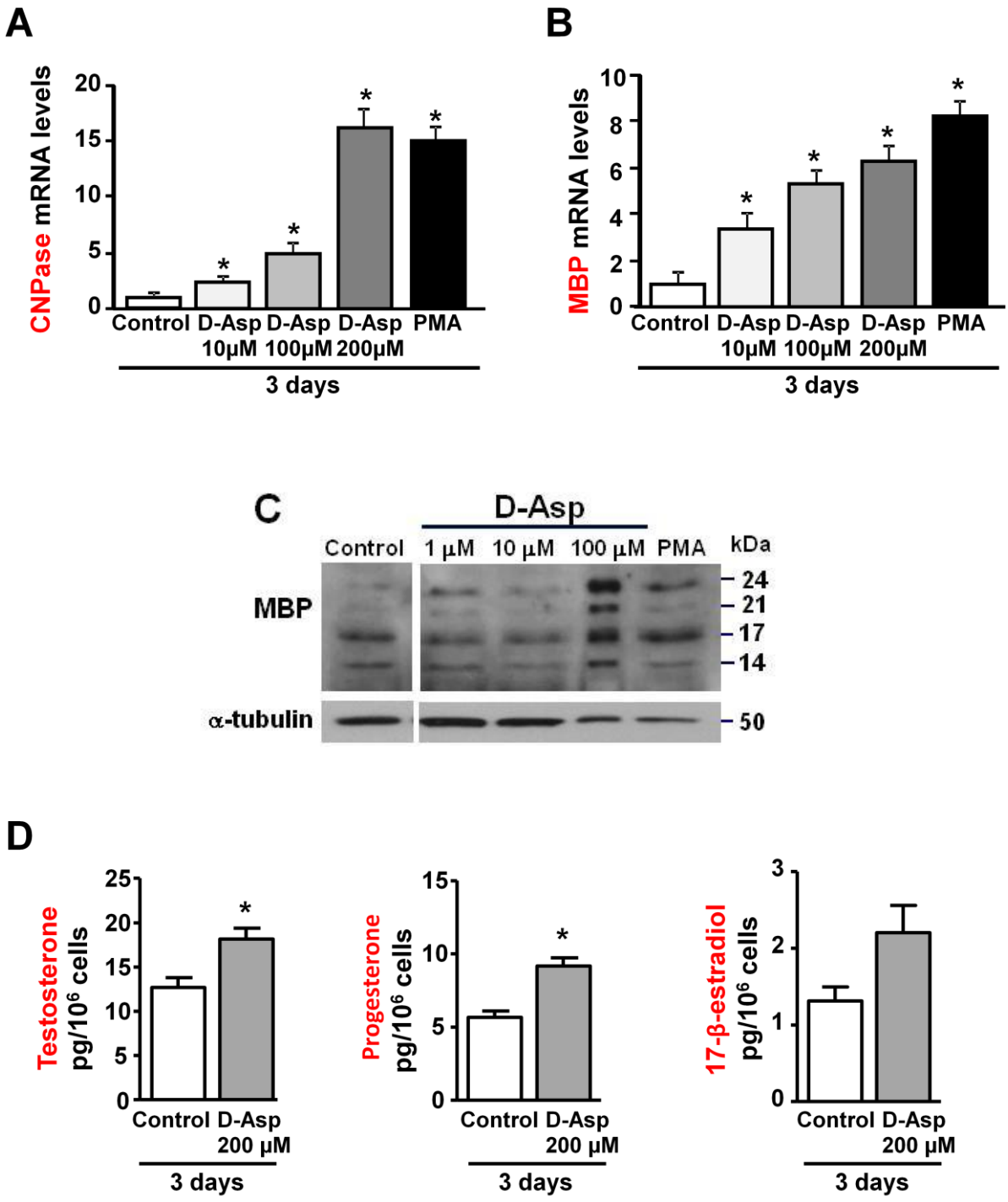

Figure 17. Effects of D-Asp exposure on OPC differentiation and hormone levels. A-B, Real-time PCR of CNPase and MBP mRNA expression in MO3.13 cells under control conditions and following D-Asp exposure (10mM, 100mM and 200mM) for 3 days. A positive control of differentiation was obtained by PMA stimulation. **C**, Western Blot analysis of MBP protein levels in MO3.13 cells under control conditions and following D-Asp exposure (1mM, 100mM and 100mM) for 3 days. A positive control of differentiation was obtained by PMA stimulation. **D,** Elisa assay of testosterone, progesterone and 17-β-estradiol hormone levels.in progenitor MO3.13 oligodenderocytes exposed to D-Asp 100 mM for 3 days The values represent the means ± S.E.M. (n=3). *P< 0.05 *versus* control.

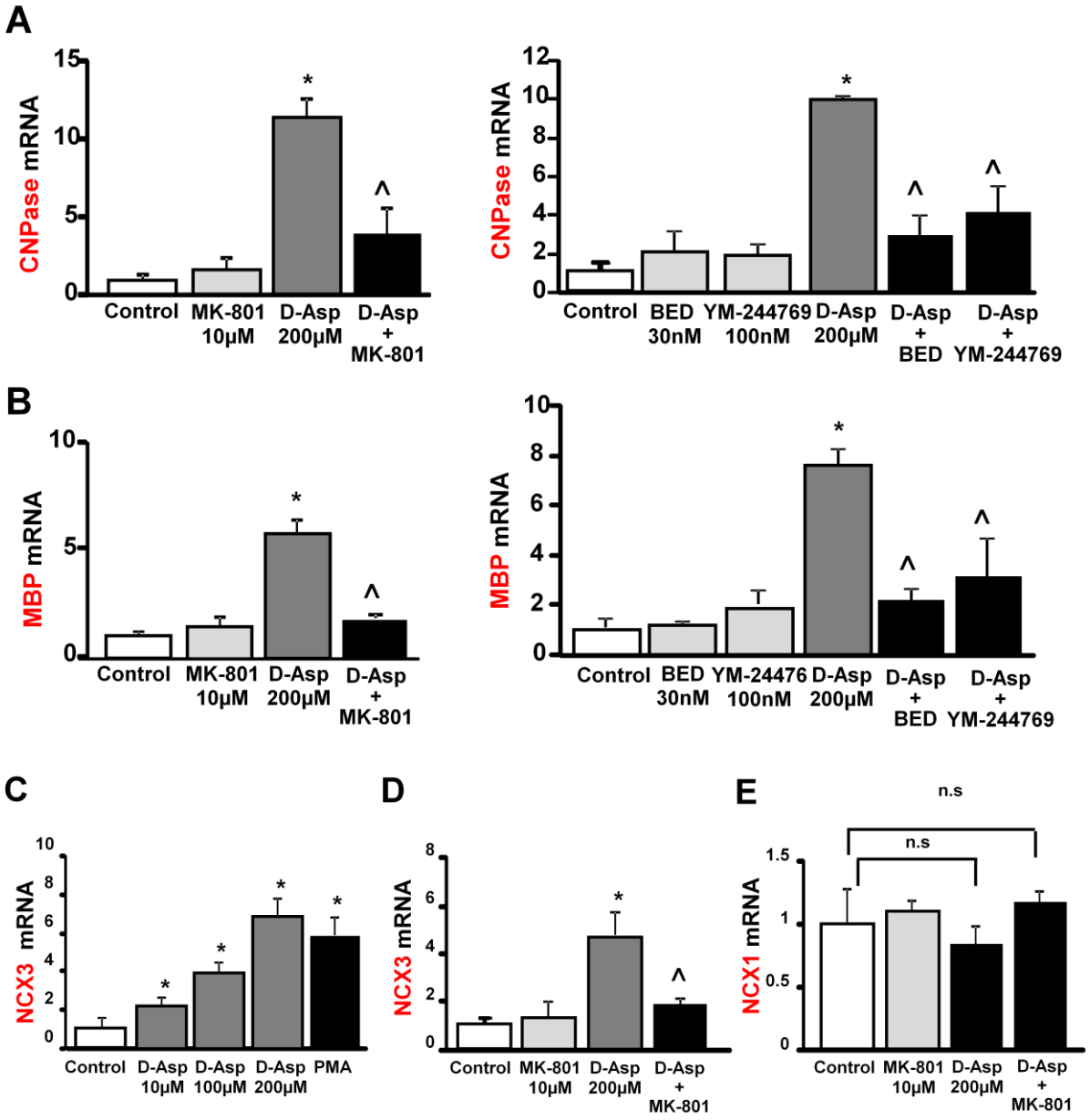

Figure 18. Effects of the NMDA receptor antagonist and NCX3 blockers on D-Asp-induced myelin marker expression. A, Real-time PCR of CNPase mRNA expression in MO3.13 cells under control conditions and following D-Asp exposure for 3 days, in absence or in presence of 10µM MK-801 (left panel), or in absence or presence of 30nM YM-244769 or 100nM BED (right panel). **B**, Real-time PCR of MBP mRNA expression in MO3.13 cells under control conditions and following D-Asp exposure for 3 days, in absence or in presence of 10µM MK-801 (left panel), or in absence or presence of 30nM YM-244769 or 100nM BED (right panel). **C**, Real-time PCR of NCX3 mRNA expression under control conditions and following 10-200µM D-Asp exposure or 100nM PMA for 3 days; **D**, Real-time PCR of NCX3 mRNA expression following 200µM D-Asp exposure, in absence or in presence of 10µM MK-801; **E,** Real-time PCR of NCX1 mRNA expression following 200µM D-Asp exposure, in absence or in presence of 10µM MK-801. The data were normalized on the basis of the β-actin levels and expressed as percentage of controls. The values represent the means ± S.E.M. (n=4). *P< 0.05 *versus* control. ^ P< 0.05 *versus* D-Asp.

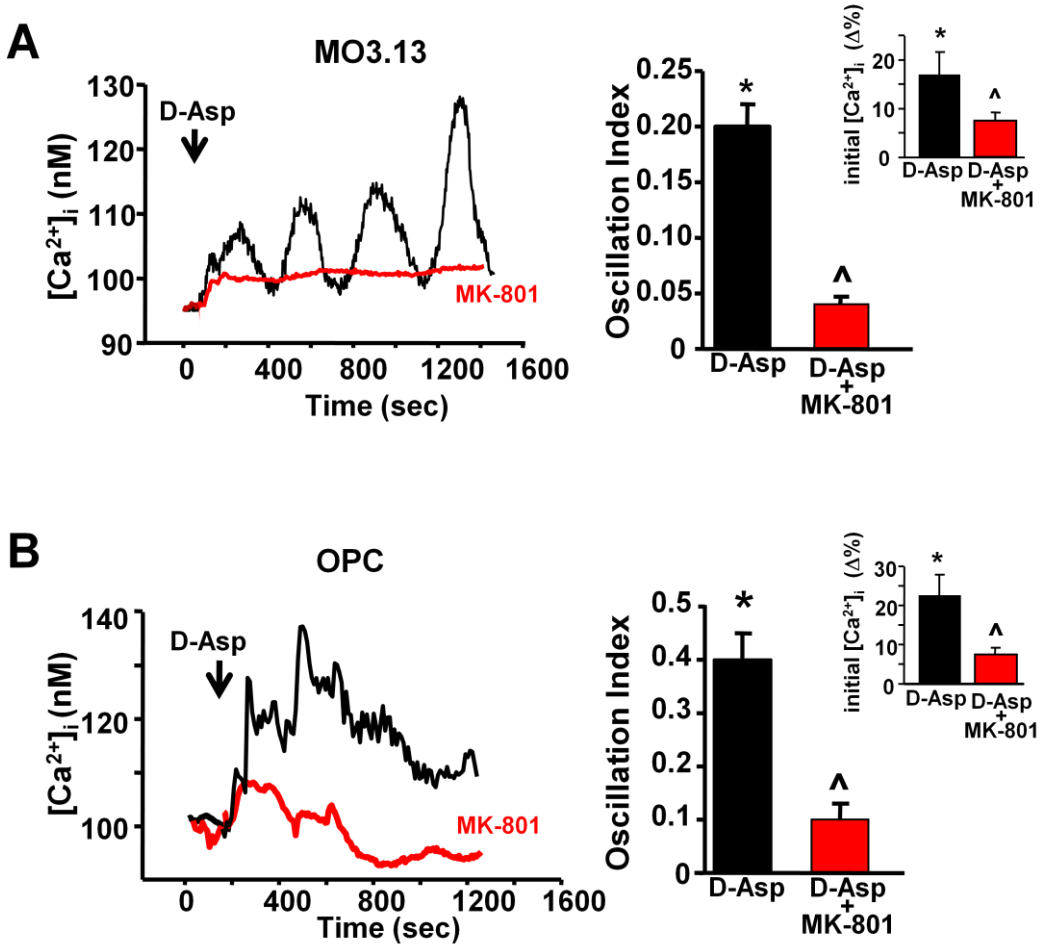

Figure 19. Contribution of NMDA receptor on D-Asp-induced intracellular $[Ca^{2+}]_i$ levels in oligodendrocyte precursors. A-B, left panels; superimposed single-cell traces representative of the effect of 100μM D-Asp on $[Ca^{2+}]_i$ detected in MO3.13 cells (**A**) and primary OPC (**B**) in absence or in presence of the NMDA receptor antagonist, 10μM MK-801. **A-B**, right panels; quantification of the oscillation index in MO3.13 cells (**A**) and primary OPC (**B**) in absence or in presence of the NMDA receptor antagonist, 10μM MK-801. The panel inserted on the right top indicated the quantification of the initial $[Ca^{2+}]_i$ increase elicited by D-Asp measured as Δ% of peak *versus* basal values in absence or in presence of 10μM MK-801, both in M03.13 cells (**A**) and primary OPC (**B**). MK-801 was preincubated 10 minutes before registration. Data are reported as mean of n=30 cells recorded in 3 different experimental conditions. *P< 0.05 *versus* control (basal value). ^ P< 0.05 *versus* D-Asp.

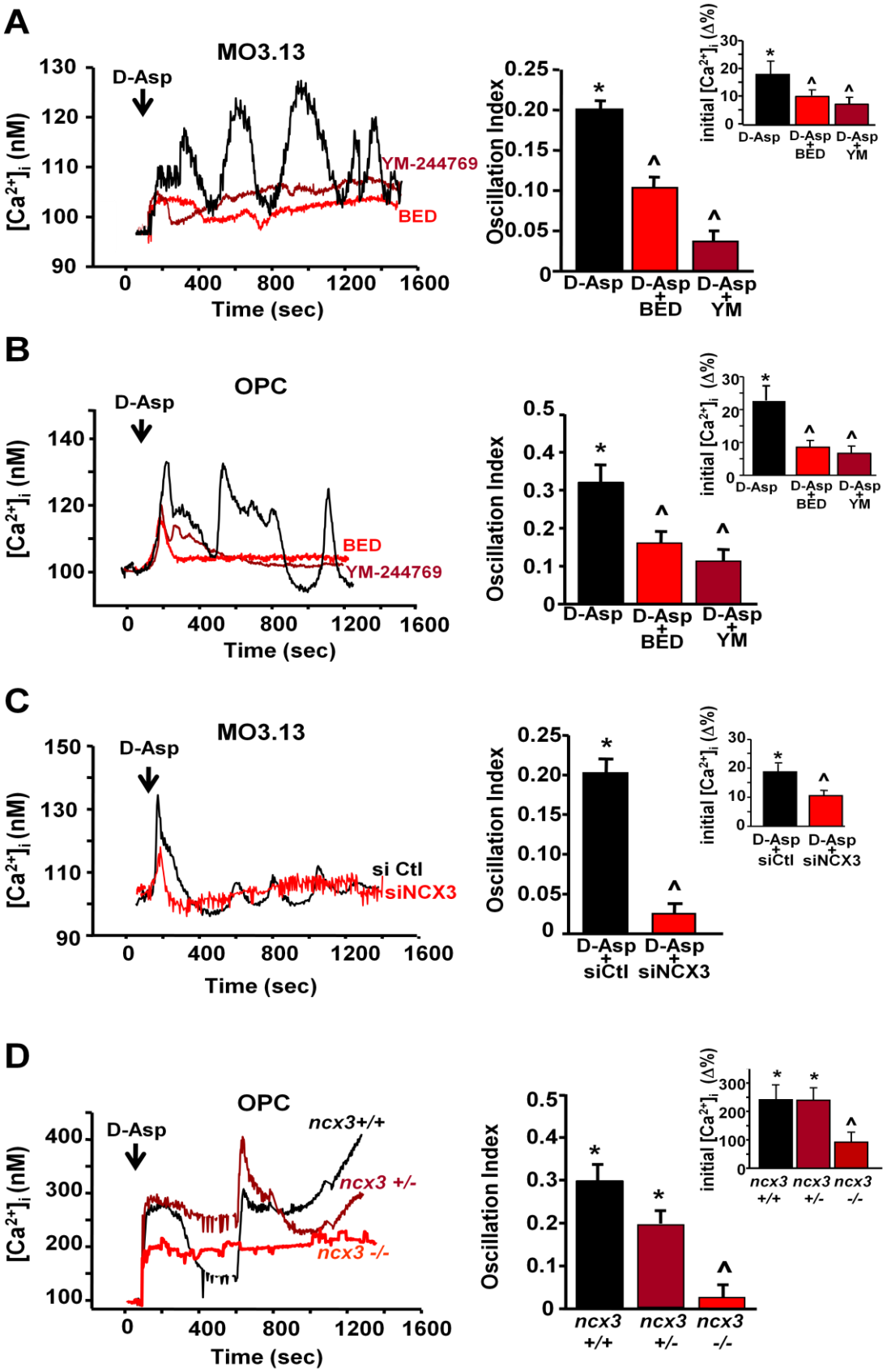

Figure 20. Contribution of NCX3 exchanger activities on D-Asp-induced intracellular $[Ca^{2+}]_i$ levels in oligodendrocyte precursors. A-B, left panels; superimposed single-cell traces representative of the effect of 100μM D-Asp on $[Ca^{2+}]_i$ detected in MO3.13 cells (**A**) and primary OPC (**B**) in absence or in presence of the

NCX3 blockers, 30nM YM-244769 or 100nM BED. **A-B**, right panels; quantification of the oscillation index in MO3.13 cells (**A**) and primary OPC (**B**) in absence or in presence of 30nM YM-244769 or 100nM BED. The panel inserted on the right top indicated the quantification of the initial $[Ca^{2+}]_i$ increase elicited by D-Asp measured as Δ% of peak *versus* basal values in absence or in presence of 30nM YM-244769 or 100nM BED, both in M03.13 cells (**A**) and primary OPC (**B**). YM244769 or BED were preincubated 10 minutes before registration. Data are reported as mean of n= 30 cells recorded in three different experimental conditions. *P< 0.05 *versus* control (basal value). ^ P< 0.05 *versus* D-Asp. **C,** left panel; superimposed single-cell traces representative of the effect of 100μM D-Asp on $[Ca^{2+}]_i$ detected in MO3.13 cells in presence of sictl or ncx3 silencing. **C**, right panel; quantification of the oscillation index in MO3.13 cells in absence or in presence of sincx3. The panel inserted on the right top indicated the quantification of the initial $[Ca^{2+}]_i$ increase elicited by D-Asp and measured as Δ% of peak *versus* basal values in absence or in presence of sincx3. Data are reported as mean of n= 30 cells recorded in three different experimental conditions. *P< 0.05 *versus* sictl (basal value). ^ P< 0.05 *versus* D-Asp + sictl. **D,** left panel, superimposed single-cell traces representative of the effect of 100μM D-Asp on $[Ca^{2+}]_i$ detected in primary OPC obtained from wild-type *ncx3*+/+, heterozygous *ncx3* +/-, and knock-out *ncx3*-/- mice. **D**, right panel; quantification of the oscillation index elicited by D-Asp in primary mouse OPC obtained from *ncx3*+/+, *ncx3* +/-, and *ncx3*-/- mice. The panel inserted on the right top indicated the quantification of the initial $[Ca^{2+}]_i$ increase measured as Δ% of peak *versus* basal values registrations. Data are reported as mean of n=30 cells recorded in 3 different experimental conditions. *P< 0.05 *versus* basal value. ^ P< 0.05 *versus ncx3*+/+.

3. AMPA receptors blockade prevented D-Asp-elicited currents in MO3.13 oligodendrocyte precursors and the initial $[Ca^{2+}]_i$ rise after D-Asp stimulation

Based on the emerging role of glutamate AMPA receptors during OPC development (Fannon *et al.*, 2015; Gautier *et al.*, 2015), we investigated whether the boosting effects of D-Asp on oligodendrocyte maturation may also involve AMPA receptor activation.

Electrophysiological experiments performed by patch-clamp in whole cell configuration showed that when MO3.13 cells were clumped at -70 mV, 1-100 μM AMPA elicited a concentration-dependent inward current which was prevented by the AMPA receptor antagonist 10μM DNQX (Figure 21A-B). Similarly, in the same experimental conditions, 100μM D-Asp elicited an inward current that was completely prevented by 10μM DNQX (Figure 21C-D). Both D-Asp and AMPA were able to elicit comparable inward currents after two consecutive applications (I and II, Figure 21B and D), thus excluding the possibility that the desensitization of AMPA receptors might contribute to the inhibitory effects observed with DNQX. Moreover, the inhibition exerted by DNQX was reversible, since both AMPA and D-Asp were able to trigger again the inward currents after the washout (Figure 21B and 21D).

Based on these results, we aimed to characterize whether the activation of AMPA receptor might be involved in the initial $[Ca^{2+}]_i$ peak elicited by 100μM D-Asp stimulation in oligodendrocyte precursors. As shown in Figure 22, 10μM AMPA perfusion triggered $[Ca^{2+}]i$ oscillations in oligodendrocyte MO3.13 progenitors. The selective and competitive AMPA receptor antagonist DNQX (10μM) was preincubated with 10μM AMPA for 10 minutes to block the channel before AMPA stimulation. Under these experimental conditions, no significant changes in $[Ca^{2+}]_i$ were recorded after 10μM AMPA addition (Figure 22A). Interestingly, in oligodendrocyte MO3.13 progenitors pretreated with DNQX+AMPA, D-Asp failed to induce the first $[Ca^{2+}]_i$ peak as well as the $[Ca^{2+}]_i$ oscillatory pattern (Figure 22B).

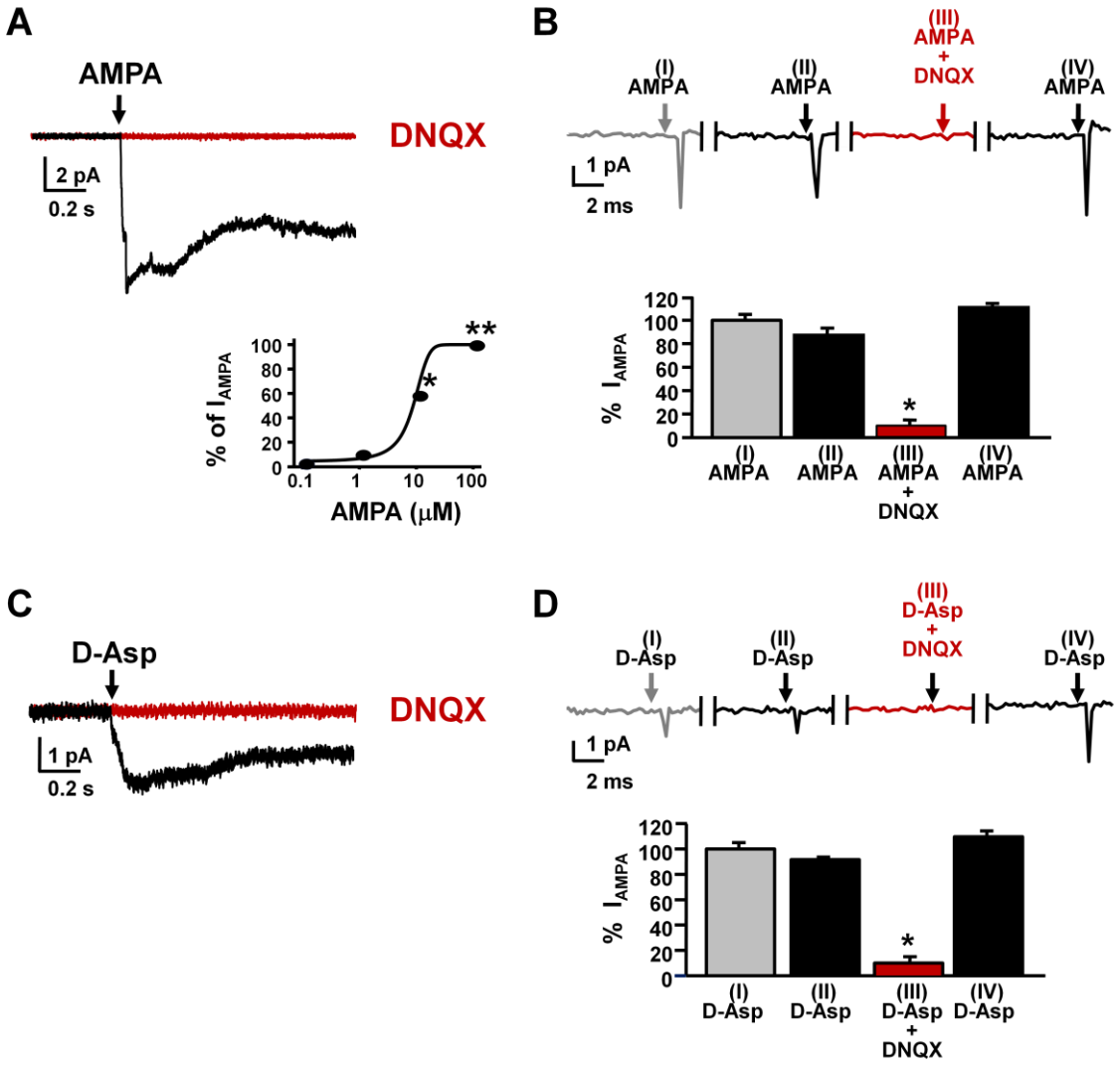

Figure 21. Effects of AMPA receptor blockade on D-Asp-induced inward currents in oligodendrocyte precursors. A, representative inward current traces elicited by 10μM AMPA application in human oligodendrocyte MO3.13 progenitor cells in absence or in presence of the AMPA antagonist, 10μM DNQX. The quantification of the concentration-dependent effect of AMPA (1-100 μM) on inward currents is showed at the bottom of panel A. **B**, representative traces of the inward current observed after 4 consecutive AMPA (10 μM) applications in human oligodendrocyte MO3.13 progenitors: (I), the first application with 10 μM AMPA alone; (II), the second application with 10μM AMPA alone; (III) the third application with 10μM AMPA in presence of 10μM DNQX; (IV) the fourth application with 10μM AMPA alone. The quantification of the inward currents represented at the bottom of panel B. The values are expressed as mean ± SEM of current densities of 3 independent experimental sessions and expressed as % of control (n=9 cells for each group). *P< 0.05 *versus* AMPA (I). **C**, representative inward current traces elicited by 100μM D-Asp application in MO3.13 cells in absence or in presence of the AMPA antagonist, 10μM DNQX. **D**, representative traces of the inward current observed after 4 consecutive D-Asp (100 μM) applications in human oligodendrocyte MO3.13 progenitors: (I), the first application with 100 μM D-Asp alone; (II), the second application with 100μM D-Asp alone; (III) the third application with 100μM D-Asp in presence of 10μM DNQX; (IV) the fourth application with 100μM D-Asp alone. The quantification of the inward currents represented at the bottom of panel D. The values are expressed as mean ± SEM of current densities of 3 independent experimental sessions and expressed as % of control (n=9 cells for each group). *P< 0.05 *versus* D-Asp (I).

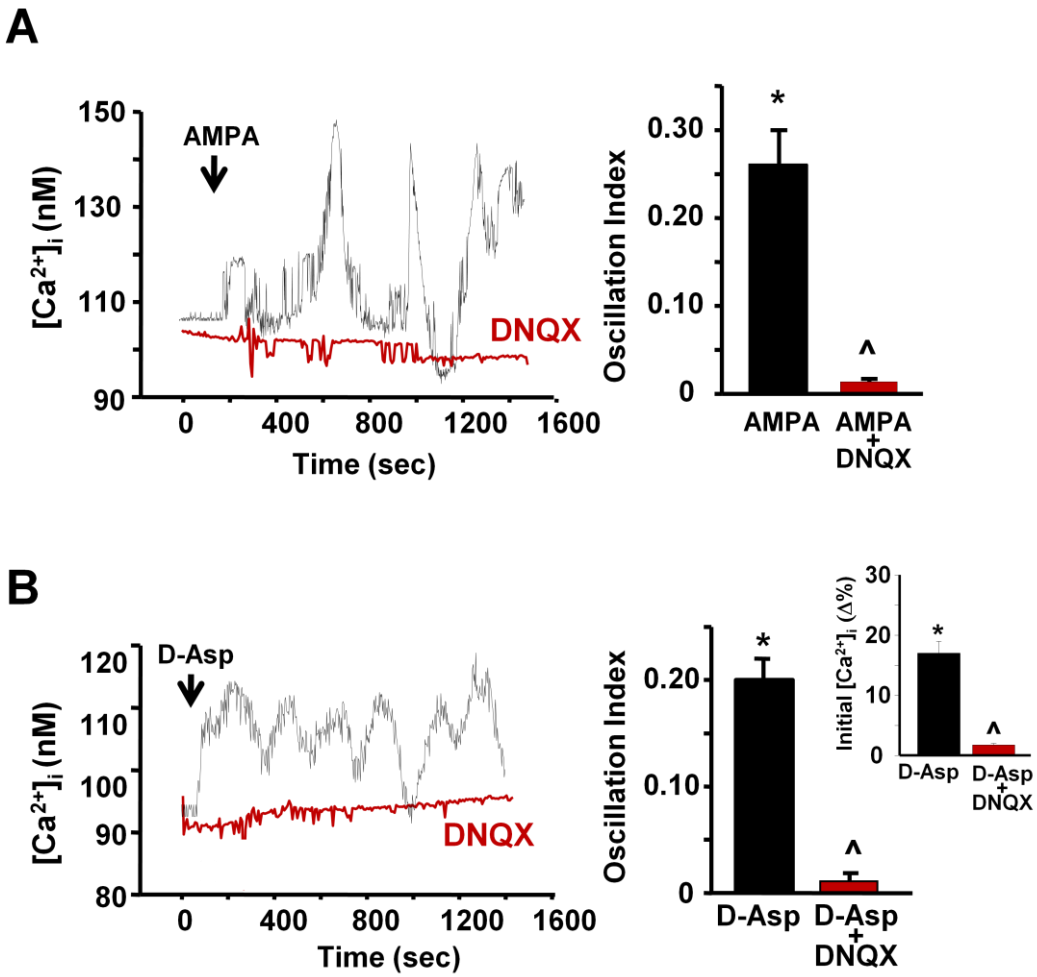

Figure 22. Effects of AMPA receptor blockade on D-Asp-induced initial $[Ca^{2+}]_i$ increase in oligodendrocyte precursors. **A**, left panel; superimposed single-cell traces representative of the effect of 10μM AMPA on $[Ca^{2+}]_i$ detected in MO3.13 cells in absence or in presence of 10μM DNQX. **A**, right panel; quantification of the oscillation index in MO3.13 cells after 10μM AMPA exposure in absence or in presence of 10μM DNQX. DNQX was preincubated 10 minutes before registration. Data are reported as mean of n= 30 cells recorded in 3 different experimental conditions. *P< 0.05 *versus* control (basal value). ^ P< 0.05 *versus* AMPA. **B**, left panel; superimposed single-cell traces representative of the effect of 100μM D-Asp on $[Ca^{2+}]_i$ detected in MO3.13 cells in absence or in presence of 10μM DNQX. B, right panel; quantification of the oscillation index in MO3.13 progenitors after 100μM D-Asp exposure in absence or in presence of 10μM DNQX. The panel inserted on the right top of panel B indicate the quantification of the initial $[Ca^{2+}]_i$ increase elicited by D-Asp in M03.13 cells measured as Δ% of peak *versus* basal values in absence or in presence of 10μM DNQX. Data are reported as mean of n=30 cells recorded in 3 different experimental conditions. *P< 0.05 *versus* control (basal value), ^ P< 0.05 *versus* D-Asp.

4. D-Aspartate treatment improves motor performance in the cuprizone mouse model of demyelination and remyelination

To explore whether the positive effects of D-Asp on oligodendrocytes may also be beneficial during acute demyelination and remyelination, we analyzed the effect of D-Asp treatment *in vivo*, in mice fed with the cupper chelator cuprizone. To analyze the effects on acute demyelination, D-Asp was given to the mice in drinking solution for 5 weeks during all the cuprizone treatment (DEM). To explore the effects on remyelination, D-Asp treatment was initiated one week before cuprizone withdrawal (REM 4-7) or immediately after cuprizone withdrawal (REM 5-7) and, in both cases, maintained for additional 14 days (Table 1).

The effects of D-Asp treatment in DEM and REM were assessed on motor coordination performance in beam balance and both fixed-speed and accelerating rotarod test. Mice completed 3 trials for each task during 1, 3 and 5 weeks of demyelination and after 2 weeks of remyelination. The number of falls and the latencies across daily trials and their average was calculated and showed in Figures 23, 24, 25. One week after cuprizone feeding no significant changes in motor performance among animal groups were assessed with both beam balance and rotarod tests (data not shown). By contrast, after 3 and more so after 5 weeks, cuprizone mice displayed a significant increase in latency to transverse the beam and number of falls from the rotarod, and a shorter latency to fall off the accelereted rotarod if compared to control mice (Figures 23, 24, 25). Interestingly, cuprizone mice treated with D-Asp performed significantly better and the performance often improved over trials when compared to cuprizone group during 3 and 5 weeks of demyelination. In particular, D-Asp mice showed significantly shorter latency to walk the beam, reduced number of falls from the rotararod and increased latencies to fall off the accelereted rotarod, when compared to cuprizone group (Figures 23, 24, 25).

Assessment of motor performance during REM revealed that mice treated with D-Asp one week before cuprizone withdrawal and for two weeks after cuprizone withdrawal presented improved motor performance when compared to cuprizone mice (REM 4-7). By contrast, no significant improvement of motor skills was observed when D-Asp was given immediately after cuprizone withdrawal (REM 5-7). No difference in averall performance accross days were observed between control groups on beam balance and rotarod tests (Figures 23, 24, 25).

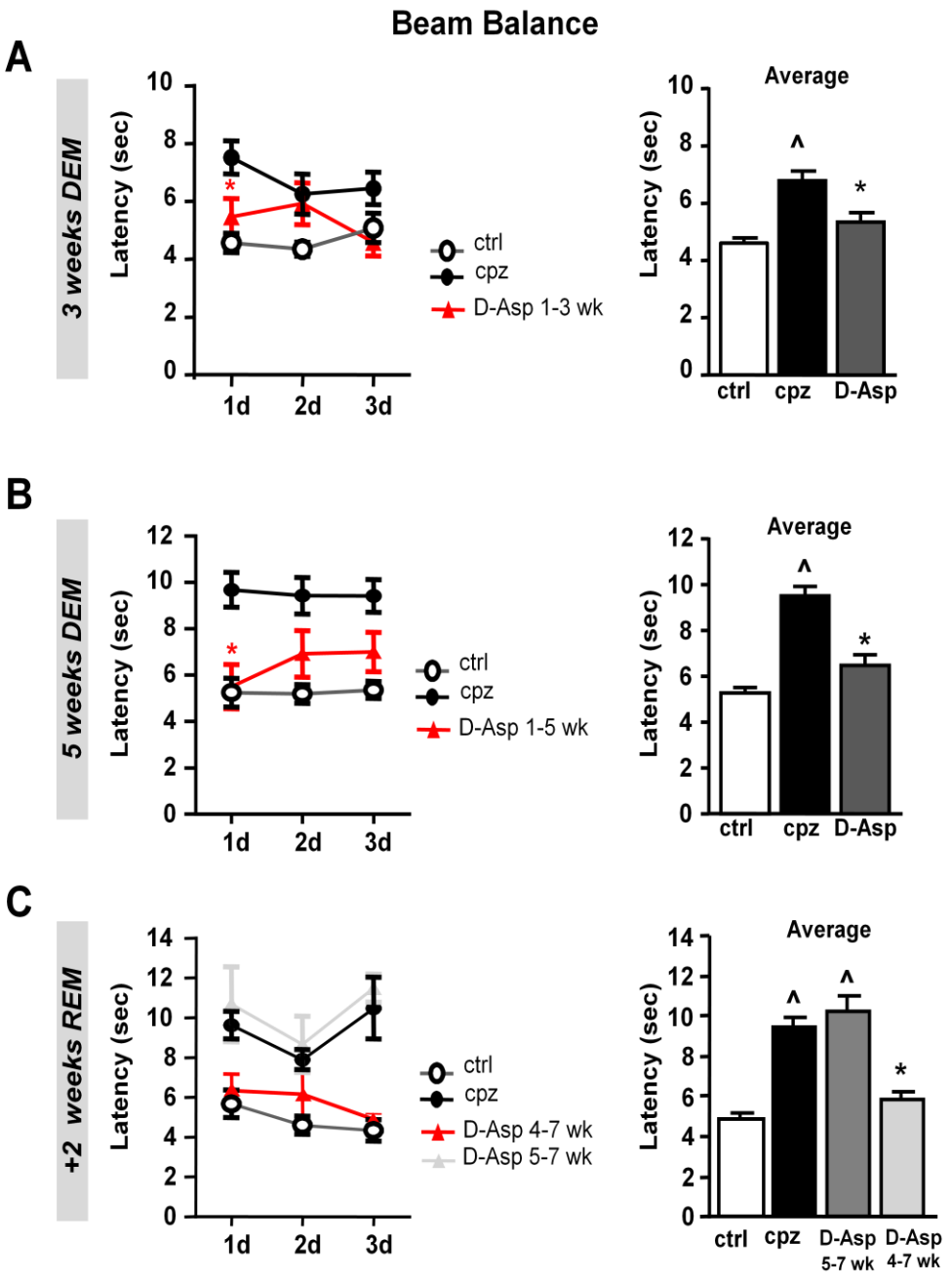

Figure 23. Effects of D-Asp treatment on motor coordination performance in beam balance test. A-B, beam crossing latency during daily training (left panel, averaged across 3 trials per day) and average latency to cross the beam over 3 consecutive days (right panel) recorded during demyelination (DEM) in control (open circles), cuprizone- (filled black circles) and in D-Asp- treated mice (filled red triangles) at 3 weeks (**A**), and 5 weeks (**B**). **C,** beam crossing latency during daily training (left panel, averaged across three trials per day) and average latency to cross the beam over 3 consecutive days (right panel) recorded after 2 weeks of remyelination (REM) in control (open circles), cuprizone- (filled black circles) and in D-Asp- treated mice. D-Asp 4-7 weeks (filled red triangles) refers to the group of mice which received D-Asp during the last week of cuprizone feeding and 2 additional weeks after cuprizone withdrawal; D-Asp 5-7 weeks (filled grey triangles) refers to the group of mice which received D-Asp only for 2 weeks after cuprizone withdrawal. The values represent the means ± S.E.M (n=6-8 mice for each group). ^P<0.05 *versus* control; *P<0.05 *versus* cpz.

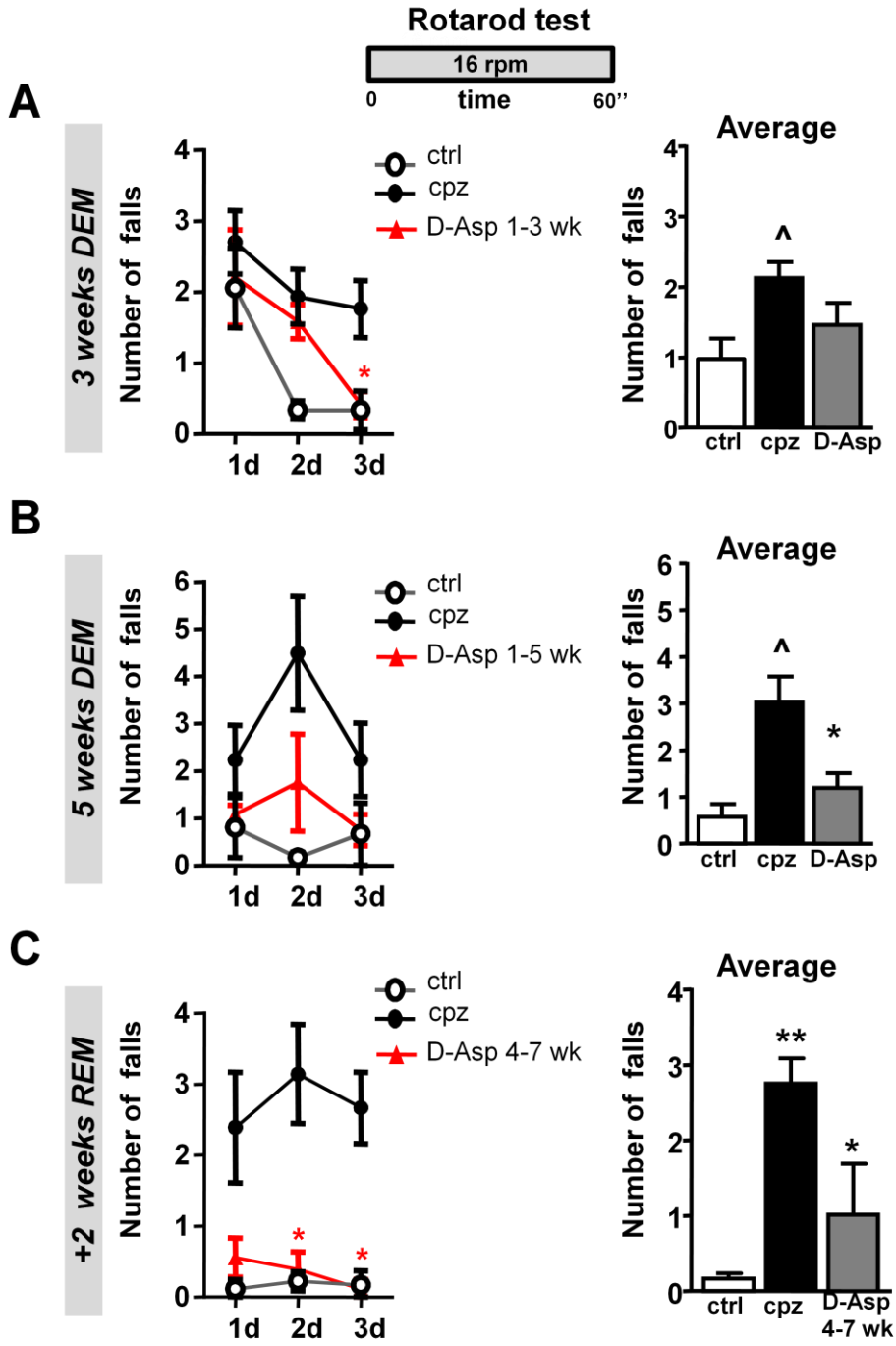

Figure 24. Effects of D-Asp treatment on motor coordination performance in fixed-speed rotarod test. A-B, number of falls during daily training in fixed-speed rotarod test (left panel, averaged across 3 trials per day) andaverage of falls over 3 consecutive days (right panel) recorded during demyelination (DEM) in control (open circles), cuprizone- (filled black circles) and cuprizone plus 20mM D-Asp- treated (filled red triangles) mice at 3 weeks (**A**), and 5 weeks (**B**). **C**, number of falls during daily training in fixed-speed rotarod test (left panel, averaged across 3 trials per day) and average of falls over 3 consecutive days (right panel) recorded after 2 weeks of remyelination (REM) in control, cuprizone-, and in D-Asp- treated mice. D-Asp 4-7 weeks refers to the group of mice which received D-Asp during the last week of cuprizone feeding and 2 additional weeks after cuprizone withdrawal. The values represent the means ± S.E.M (n=6-8 mice for each group). ^P<0.05 *versus* control; *P<0.05 *versus* cpz. **P<0.05 *versus* control and D-Asp.

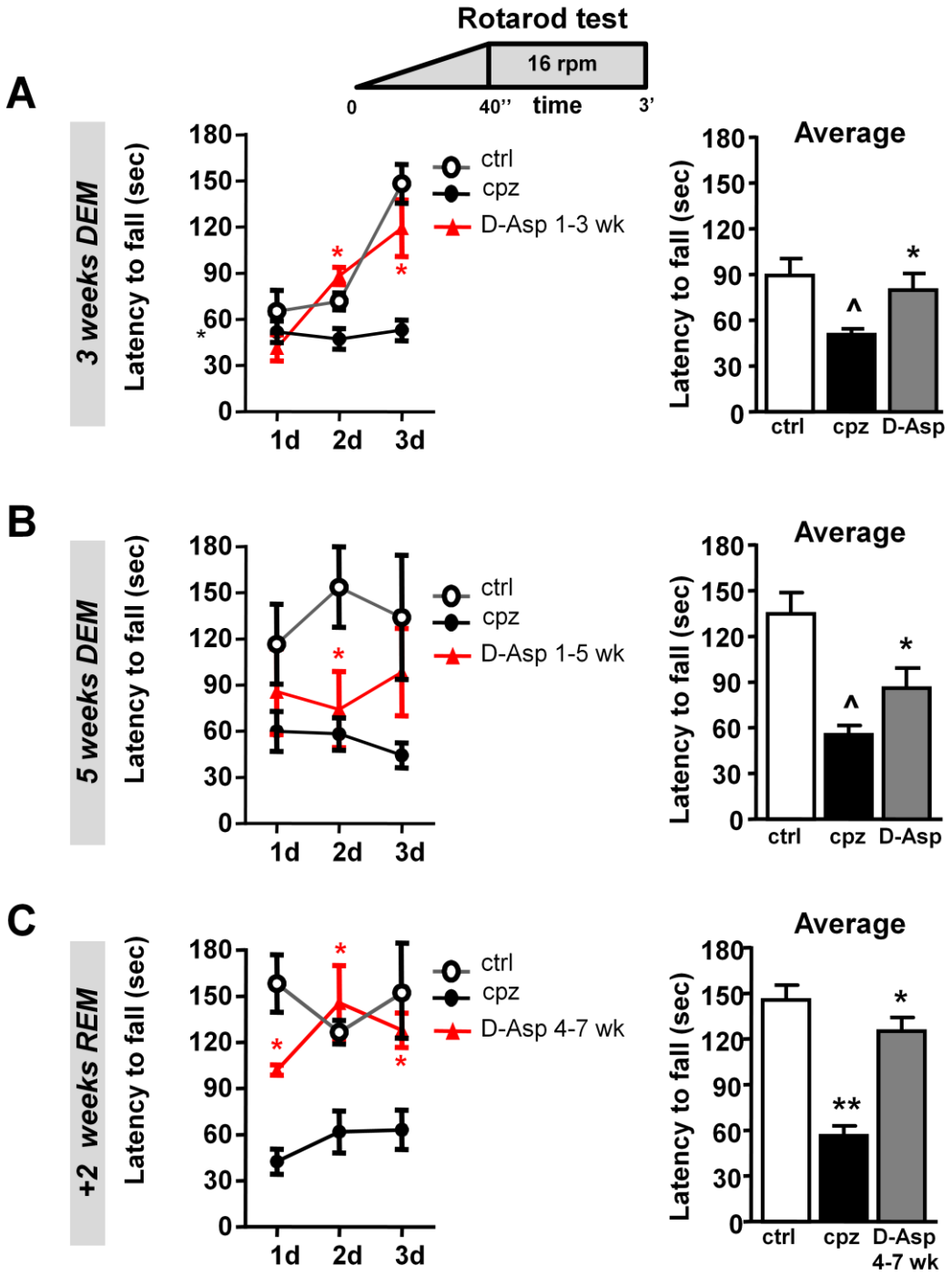

Figure 25. Effects of D-Asp treatment on motor coordination performance in accelerating rotarod test. A-B, latency to fall during daily training in accelerating rotarod test (left panel, averaged across 3 trials per day) and average of falls over 3 consecutive days (right panel) recorded during demyelination (DEM) in control, cuprizone- and cuprizone plus 20mM D-Asp- treated mice at 3 weeks (**A**), and 5 weeks (**B**). **C**, number of falls during daily training in accelerating rotarod test (left panel, averaged across 3 trials per day) and average of falls over 3 consecutive days (right panel) recorded after 2 weeks of remyelination (REM) in control, cuprizone- and in D-Asp- treated mice. D-Asp 4-7 weeks refers to the group of mice which received D-Asp during the last week of cuprizone feeding and 2 additional weeks after cuprizone withdrawal. The values represent the means ± S.E.M (n=6-8 mice for each group). ^P<0.05 *versus* control; *P<0.05 *versus* cpz. **P<0.05 *versus* control and D-Asp.

5. D-Aspartate treatment prevents demyelination and accelerates remyelination in the cuprizone mouse model

Immunoblot analysis performed on *corpus callosum* lysates after cuprizone treatment for 5 weeks revealed a significant reduction of MBP protein levels if compared to controls. This reduction was significantly prevented by D-Asp treatment, as revealed the quantitative analysis (Figure 26A). By contrast no significant alterations were observed in protein levels of the axonal marker APP, both in absence or in presence of D-Asp (Figure 26A). Quantitative immunofluorescence analysis performed in the middle *corpus callosum* showed a significant reduction in MBP fluorescence intensity in cuprizone mice when compared with control mice (Figure 26B). Furthermore, quantitative double immunofluorescence analysis performed with anti-MBP and anti-NF200 antibodies in the *corpus callosum* revealed that the percentage of colocalization between MBP and NF200, used as myelination index, was intensely reduced after cuprizone treatment, but partially preserved by D-Asp treatment (Figure 26B). D-Asp treatment during DEM also prevented demyelination-associated inflammation 5 weeks after cuprizone treatment, as revealed by the less immunoreactivity intensity of the bands corresponding to the microglial marker Iba1 and the astrocytic marker GFAP in *corpus callosum* lysates if compared to cuprizone treatment (Figure 26C). To analyze the effects of D-Asp during remyelination we performed electron microscopy studies both in absence or in presence of D-Asp. As shown in Figure 27, quantitative analysis of myelinated axons revealad that mice feeded with cuprizone for 5 weeks and 2 additional weeks with normal chow in presence of D-Asp (4-7) displayed a significant higher number of myelinated axons in the middle *corpus callosum* when compared to those oberved in absence of D-Asp treatment.

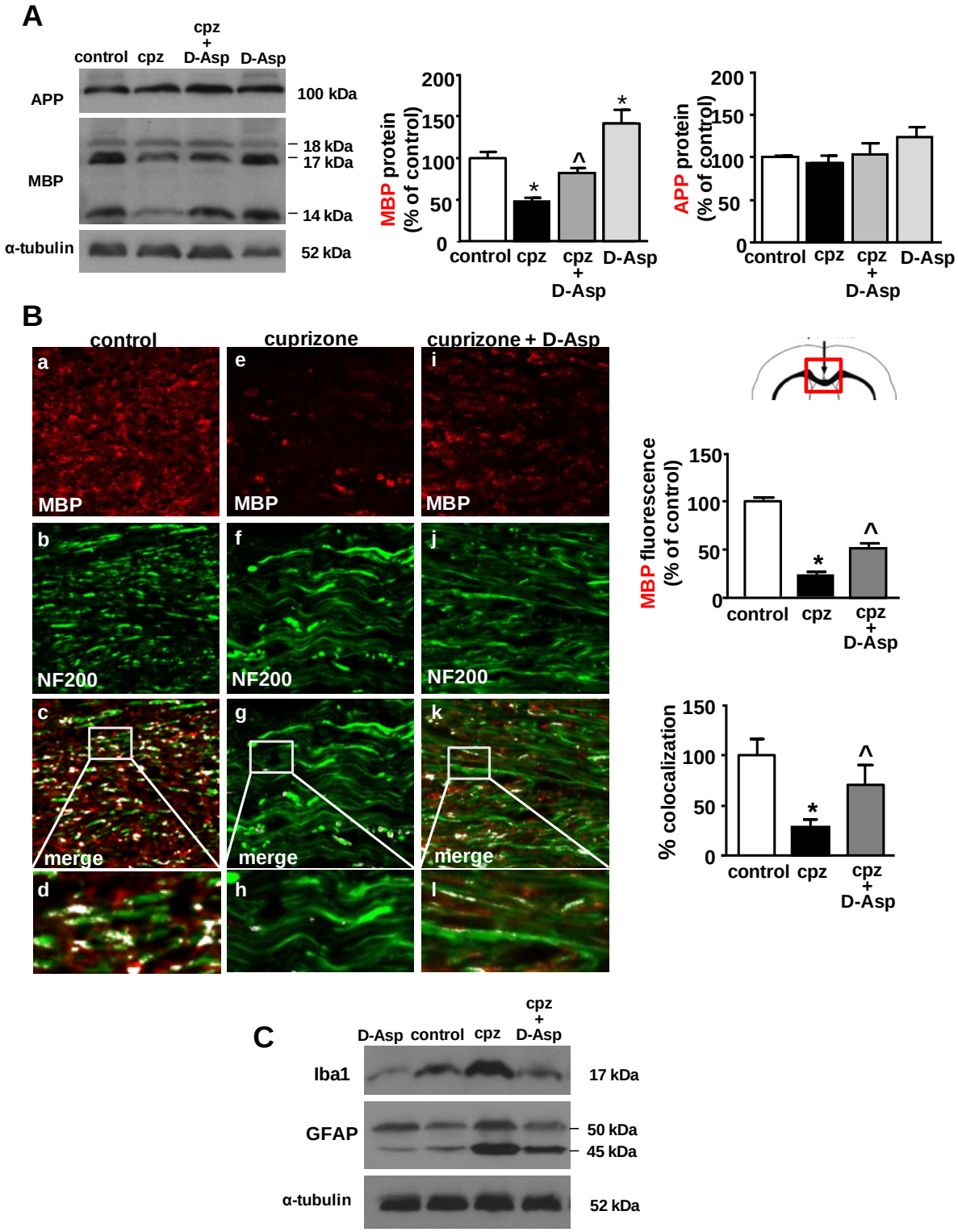

Figure 26. Effect of D-Asp treatment on *corpus callosum* demyelination. A, Western Blot analysis of MBP and APP protein levels in *corpus callosum* lysates under control conditions and following D-Asp exposure (100 μM) during 5 weeks of cuprizone feeding. **B**, left; Quantitative immunofluorescence analysis performed with anti-MBP and anti-NF200 antibodies in the middle *corpus callosum*. **B,** right; MBP immunofluorescence and percentage of colocalization between MBP and NF200, used as myelination index, upon D-Asp (20mM) stimulation during 5 weeks of cuprizone treatment. The values represent the means ± S.E.M (n=6-8 mice for each group). *P<0.05 *versus* control; ^P<0.05 *versus* cpz.

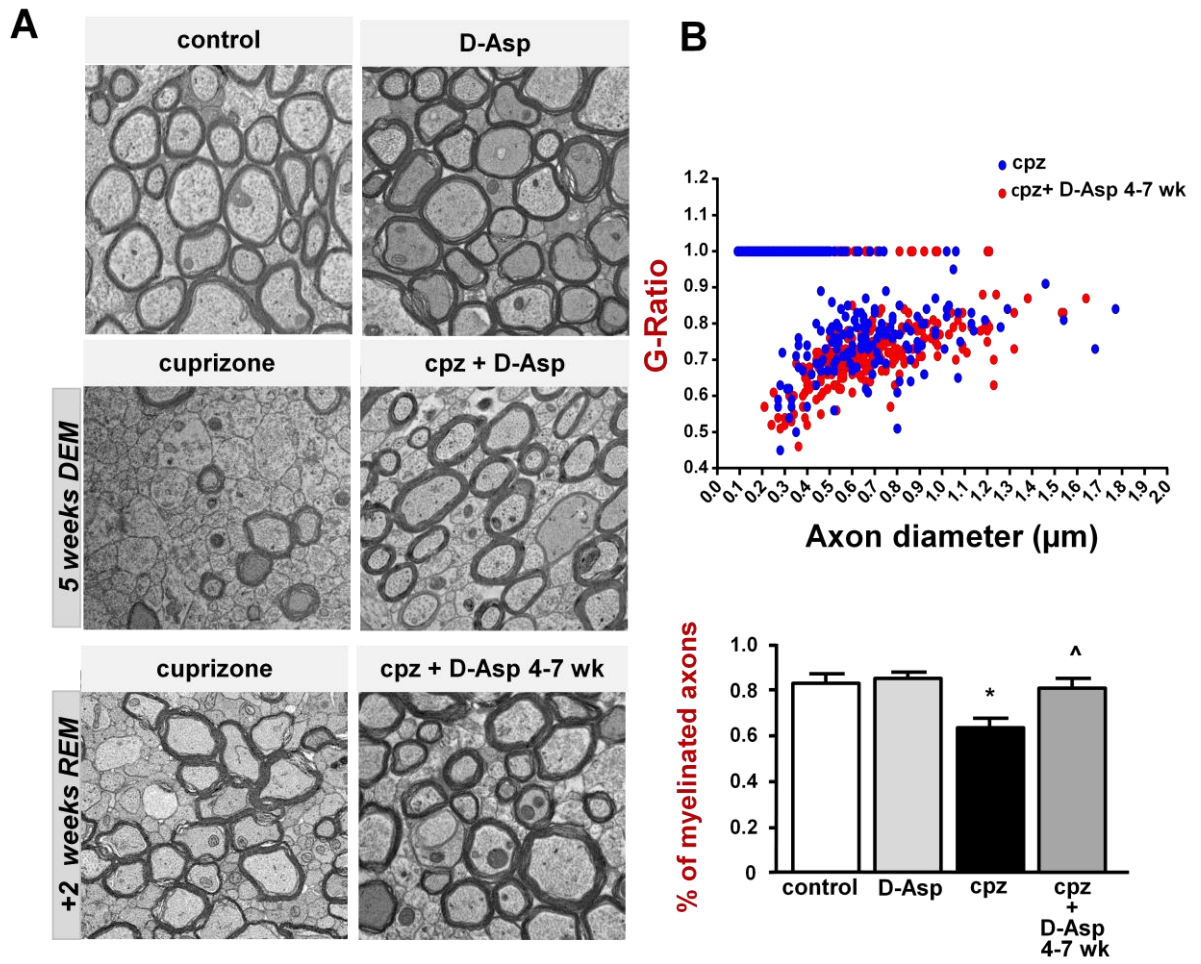

Figure 27. Effect of D-Asp treatment on *corpus callosum* remyelination. A, representative electron miscroscopy images of middle *corpus callosum* sections (Magnification 20X). **B,** upper panel; axonal G-Ratio value dispersion (y-axis: G-Ratio; x-axis: axonal diameter in µm). **B,** lower panel; quantitative analysis of myelinated axons in presence and in absence of D-Asp exposure after 5 weeks of demyelination and 2 weeks (D-Asp 4-7) of remyelination in the middle *corpus callosum* . The values represent the means ± S.E.M (n=at least one hundred axons for each group). *P<0.05 *versus* control; ^P<0.05 *versus* cpz.

CHAPTER IV

DISCUSSION

IV. DISCUSSION

Myelination is a physiological mechanism occurring when, during the early stages of development, OPCs contact target axons and generate the myelin sheets which protect axons, sustaining and guaranteeing the electrical signaling conduction in the CNS.

During normal adulthood, mature oligodendrocytes are actively replaced by OPCs, a widely expressed glial population spanning from 5% and 8% according to the specific region taken into account (Dawson *et al.*, 2000; Young *et al.*, 2013). In some pathological conditions, such as demyelinating insults (multiple sclerosis, leukodystrophies), traumatic and vascular injuries, neurodegenerative diseases and schizofrenia (Edgar and Sibille, 2012; Goldman *et al.*, 2012), the death of injured oligodendrocytes affects the total number of mature myelinating cells and latterly the myelin amount and its function. Multiple sclerosis is one of the more common demyelinating diseases as it is the more frequent neurological disease affecting young adults (Prineas and Parratt, 2012; Cui *et al.*, 2013; Noseworthy *et al.*, 2000; Compston and Coles, 2008; Henderson *et al.*, 2009). In the early phases of MS, a spontaneous program of remyelination may occur. However, at later stages, this process for some reasons fails (Franklin and Ffrench-Constant, 2008).

Recent studies evidenced the importance of D-Aminoacids in the nervous system playing important roles in neurotransmission and neuroendocrine regulation (Billard 2012; Wolosker *et al.*, 2008). In particular, we focused our study on the effects exerted by D-Aspartate, which is also considered an NMDA receptor agonist, on oligodendrocytes during differentiation and remyelination processes, with particular attention to its effects on intracellular $[Ca^{2+}]_i$ levels. We first determined whether D-Aspartate could effectively stimulate *in vitro* the differentiation of oligodendrocytes. Quantitative RT-PCR analyses revealed a significant dose-dependent increase in myelin CNPase and MBP transcripts 3 days after D-Asp exposure, thus suggesting that D-Asp may stimulate oligodendrocyte maturation. In accordance with our results, Martinez-Lozada *et al.* (2014) found that D-Asp exposure stimulated oligodendrocyte maturation, as revealed by the increased network area of oligodendrocyte arborization. In accordance with the boosting effects of D-Asp on OPC maturation, we found that D-Asp exposure also upregulated the transcripts of NCX3 exchanger, but not those of NCX1. This result is in line with a previous study from our research group demonstrating that the progression of OPCs into mature myelinating oligodendrocytes involves changes in $[Ca^{2+}]_i$ levels through the Na^+/Ca^{2+} exchanger NCX3 (Boscia *et al.*, 2012). Indeed, Boscia *et al.* (2012) found that while NCX3 was intensely upregulated during OPC development, NCX1

was instead downregulated. The importance of NCX3 exchanger was further demonstrated by the results showing that NCX3 silencing prevented myelin markers upregulation during oligodendrocyte differentiation, while its overexpression induces myelin markers increase (Boscia *et al.*, 2012). More recently, the crucial role of NCX3 was further highlighted by the observation that *ncx*3-/- knockout mice display increased susceptibility to EAE insult (Casamassa *et al.*, 2016), and an impairment of OPCs response to demyelination in the spinal cord of EAE mice.

In addition, we found that the increase in myelin CNPase and MBP transcripts elicited by D-Asp exposure in oligodendrocyte MO3.13 precursors was significantly prevented by the selective NMDA receptor blocker, MK-801, and the two NCX3 blockers, YM-244769 and BED (Secondo *et al.*, 2015). Collectively, our findings further suggest that both the glutamate NMDA receptor and the Na^+/Ca^{2+} exchanger NCX3 are involved in the mechanism leading to oligodendrocytes differentiation (Li *et al.*, 2013; Lundgaard *et al.*, 2013; Martinez-Lozada *et al.*, 2014; Boscia et al., 2012). Next, we explored the functional contribution of the NMDA receptor and NCX3 activities on D-Asp-induced intracellular $[Ca^{2+}]_i$ levels in oligodendrocytes. Microfluorimetric analyses show that the pharmacological blocking of both NMDA receptors and NCX3 completely prevented D-Asp–induced $[Ca^{2+}]_i$ oscillations, but only partially affected the initial $[Ca^{2+}]_i$ rise both in oligodendrocyte MO3.13 precursors and primary rat OPCs. To further investigate the contribution of NCX3 exchanger to D-Asp induced $[Ca^{2+}]_i$ oscillations we recorded calcium response in oligodendrocyte MO3.13 progenitors previously silenced for *ncx3* gene or in mouse primary OPC obtained from *ncx3*+/+ or *ncx3*-/- mice. Indeed, by using both silencing and transgenic approaches we found that D-Asp-induced $[Ca^{2+}]_i$ oscillations in OPC lacking NCX3 were significantly prevented.

Our functional data highlight the crucial role of NMDA receptor and NCX3 in mediating D-Asp-induced $[Ca^{2+}]_i$ levels changes during OPC differentiation. In line, several data support the importance of intracellular $[Ca^{2+}]_i$ changes during OPCs maturation and differentiation, as well as the intracellular $[Ca^{2+}]_i$ oscillatory pattern during oligodendrocyte development (Barres *et al.*, 1990; Kettenmann *et al.*, 1994; Soliven *et al.*, 2001; Boscia *et al.*, 2012; Paez *et al.*, 2012). In fact, intracellular $[Ca^{2+}]_i$ oscillations are considered as important signals during OPCs proliferation, survival, growth and differentiation (Paez *et al.*, 2009). Similarly, a large number of studies emerging in the last years point to glutamate signaling-mediated $[Ca^{2+}]_i$ changes as crucial regulator of various aspects of the biology of oligodendrocytes, including oligodendrocyte lineage progression, proliferation, survival, growth, migration and differentiation (Bergles *et al.*, 2000; Gudz *et al.*, 2006; Paez *et al.*, 2009; Cavaliere *et al.*,

2012; de Castro *et al.*, 2013; Martinez-Lozada *et al.*, 2014). Recently, great importance was given to the intracellular $[Ca^{2+}]_i$ changes in OPCs after release of glutamate at NG2-neuron synapse (Kolodziejczyk *et al.*, 2010). In addition, oligodendrocyte differentiation and myelination has also been demonstrated to be promoted by the NMDA receptor-mediated increase in cytosolic calcium levels, as revealed by microfluorimetric analysis of intracellular $[Ca^{2+}]_i$ changes in cocultures models in response to glutamate (Cavaliere *et al.*, 2012). In this regard, it is now clear that neuronal activity influences oligodendrocyte development and myelin formation (Demerens *et al.*, 1996; Gibson *et al.*, 2014; Stevens *et al.*, 2002; Wake *et al.*, 2011, Fannon *et al.*, 2015). Neuronal activity promotes myelination via AMPA and NMDA glutamate receptors activation on OPC surface (Gautier *et al.*, 2015; Fannon *et al.*, 2015). In fact, OPCs express functional glutamate AMPA and NMDA receptors which enable these cells to sense axonal action potential activity (Bakiri *et al.*, 2009; Gallo *et al.*, 2008). The intracellular $[Ca^{2+}]_i$ changes mediated by the activation of AMPA and NMDA receptors during the OPCs lineage progression make these cells able to sense the neuronal activity (Gautier *et al.*, 2015; Fannon *et al.*, 2015). As reviewed in Spitzer *et al.* (2016), the complex temporal intervention of both receptors orchestrate important Ca^{2+} influxes necessaries to boost and regulate morphological development of OPCs. Most recent studies indicated that the glutamate released from demyelinated axons may first preferentially bind the AMPA receptor on OPC membrane, with consequent inhibition of OPC proliferation and consequently start of the myelination program (Kukley *et al.*, 2007; Ziskin *et al.*, 2007).

It has been reported that D-Asp might also interact with AMPA receptors, although both stimulatory and blocking effects have been described (Gong *et al.*, 2005). Although it is not clear whether these opposite roles exerted by D-Asp maybe ascribed to the different D-Asp concentrations used or different experimental models employed in these studies, we investigated whether, in our experimental model, D-Asp might also exert a role on AMPA receptors. This hypothesis arise from the observation that, in our experiments both in oligodendrocytes MO3.13 and primary rat OPC, the NMDA receptor antagonist MK-801completely prevented $[Ca^{2+}]_i$ oscillations but only partially affected the initial $[Ca^{2+}]_i$ peak. Thus, in order to further characterize the functional contribution of AMPA receptors to the inward currents elicited by D-Asp stimulation in oligodendrocyte differentiation, we performed electrophysiological recordings in single-cell oligodendrocytes M03.13, by using the patch-clamp technique. First, we verified the existence of the dose-response of oligodendrocytes MO3.13 progenitors elicited by AMPA stimulation.

Interestingly, AMPA induced a first $[Ca^{2+}]_i$ rise followed by an oscillatory pattern in oligodendrocyte MO3.13 progenitors, and both effects were completely prevented by the AMPA antagonist, DNQX. Remarkably, in oligodendrocyte MO3.13 progenitors pretreated with DNQX, D-Asp failed to induce the first $[Ca^{2+}]_i$ peak as well as the $[Ca^{2+}]_i$ oscillatory pattern, suggesting a crucial role for AMPA receptor in D-Asp-induced inward currents.

Collectively, our *in vitro* results suggest that D-Asp may promote oligodendrocyte differentiation via a mechanism involving changes in intracellular $[Ca^{2+}]_i$ levels through both glutamate AMPA and NMDA receptors, and consequently the activation of the Na^+/Ca^{2+} exchanger NCX3.

These *in vitro* findings led us to investigate whether D-Asp administration *in vivo* might have a beneficial role on oligodendrocytes in the cuprizone mouse model of demyelination and remyelination. Cuprizone feeding in 8-old weeks C57BL/6J mice induces selective oligodendrocyte death with decrease in myelin markers protein expression (Hiremath *et al.*, 1998; Mason *et al.*, 2000a; Morell *et al.*, 1998). The cuprizone model has been well characterized over time and specific regional damage in the brain of treated animals are described (Van der Star *et al.*, 2012). The most affected brain regions in animals which received 0,2% (w/w) copper chelator cuprizone are those enriched in white matter such as the *corpus callosum*, but also the hippocampus and cerebral cortex (Gudi *et al.*, 2014). The c*orpus callosum* is an important brain region which integrates information coming from both hemispheres, regulating important functions such as balance and motor coordination that can be assessed by monitoring performance in mice with two behavioral tests, the beam balance and rotarod (Hagemeyer *et al.*, 2012; Franco-Pons *et al.*, 2007). Indeed, we investigated the effect of D-Asp on motor performance in beam balance and rotarod test. These behavioral tests have been largely characterized in the cuprizone model by Franco-Pons *et al.* (2007). When animals received D-Asp, we observed an improvement in motor performance either when was given during demyelination and during remyelination. D-Asp mice showed significantly shorter latency to walk the beam, reduced number of falls from the rotarod and increased latencies to fall off the accelerated rotarod, when compared to cuprizone group. Interestingly, the effect of D-Asp during the remyelination phase was more evident in mice treated with D-Asp one week before the cuprizone withdrawal. This effect may be possible ascribed to the fact that, as it was given by oral administration, longer time might be required to reach significant levels in the brain.

Our behavioral findings are in accordance with our results obtained in biochemical and morphological studies. In fact, immunoblot analysis performed after cuprizone treatment for 5

weeks revealed a significant reduction of MBP protein levels in the *corpus callosum* if compared to controls. Interestingly, this reduction was significantly prevented by D-Asp treatment. We did not observe significant alterations in APP protein levels both in presence or in absence of D-Asp, thus suggesting that no axonal damage occurred in both animal groups (Anthony *et al.*, 2000; Dutta and Trapp, 2011). Moreover, quantitative immunofluorescence analysis showed a significant reduction in MBP fluorescence intensity in cuprizone mice when compared to control or D-Asp mice 5 weeks after cuprizone treatment. Consistently with these results, quantitative double immunofluorescence analysis performed with anti-MBP and anti-NF200 antibodies revealed that the percentage of colocalization between MBP and NF200, used as myelination index, was intensely reduced after cuprizone treatment, but partially preserved by D-Asp treatment.

D-Asp treatment also prevented demyelination-associated inflammation following cuprizone treatment, as revealed by the lower immunoreactivity intensity corresponding to the bands detected with the microglial marker Iba1 and the astrocytic marker GFAP if compared to cuprizone alone treatment.

Collectively, our *in vivo* results show that D-Asp treatment partially prevented the cuprizone-induced demyelinating and inflammation.

In accordance with the beneficial effect of exogenous D-Asp treatment, it has been demonstrated that D-Asp increases levels of sexual hormones, such as testosterone and progesterone. In this regard, a large number of studies demonstrated that sexual hormones testosterone and estrogen have been inferred in some neuroprotective mechanisms in myelin via their immunomodulatory and neuroprotective properties (for a review see Gold and Voskuhl, 2009).

In conclusion, our findings shed light on the mechanisms promoting oligodendrocyte differentiation. The beneficial role of D-Asp in preventing demyelination and enhancing remyelination could be important in the next future to design a possible translational therapy for human demyelinating disease such as Multiple Sclerosis.

BIBLIOGRAPHY

Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ (2010). Structure and function of the blood-brain barrier *Neurobiol Dis 37*, 13-25.

Adamo AM, Paez PM, Escobar Cabrera OE, Wolfson M, Franco PG, Pasquini JM, Soto EF (2006) Remyelination after cuprizone-induced demyelination in the rat is stimulated by apotransferrin. Exp Neurol 198:519–529.

Agholme L, Lindström T, Kågedal K, Marcusson J, Hallbeck M (2010) An *in vitro* model for neuroscience: differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons *J Alzheimers Dis 20*, 1069-1082

Agrawal SK, Nashmi R and Fehlings MG (2000). Role of L- and N-type calcium channels in the pathophysiology of traumatic spinal cord white matter injury. *Neuroscience* 99(1), 179-188.

Al-Hallaq RA, Conrads TP, Veenstra TD, Wenthold RJ (2007). NMDA di-heteromeric receptor populations and associated proteins in rat hippocampus. *J Neurosci* 27(31):8334–8343.

Amiguet P, Gardinier MV, Zanetta JP, Matthieu JM(1992). Purification and partial structural and functional characterization of mouse myelin oligodendrocyte glycoprotein (MOG). *J Neurochem* 58: 1676-1682.

Amor S, Puentes F, Baker D, van der Valk P (2010). Inflammation in Neurodegenerative Diseases. *Immunology 129*, 154-169.

Annunziato L, Pignataro G, and Di Renzo GF (2004). Pharmacology of brain Na+/Ca2+ exchanger: from molecular biology to therapeutic perspectives. *Pharmacol Rev* 56: 633–654.

Annunziato L, Boscia F, Pignataro G (2013). Ionic transporter activity in astrocytes, microglia, and oligodendrocytes during brain ischemia. *J Cereb Blood Flow Metab* 33:969-982.

Anthony DC, Hughes P, Perry VH (2000). [The evidence for primary axonal loss in multiple sclerosis]. *Rev Neurol* 30:1203-1208.

Armstrong RC, Dorn HH, Kufta CV, Friedman E, Dubois-Dalcq ME(1992). Preoligodendrocytes from adult human CNS. *J Neurosci* 12 1538–1547.

Armstrong RC, Le TQ, Flint NC, Vana AC, Zhou YX (2006) Endogenous cell repair of chronic demyelination. *J Neuropathol Exp Neurol* 65:245–256.

Armstrong RC, Le TQ, Frost EE, Borke RC, Vana AC (2002) Absence of fibroblast growth factor 2 promotes oligodendroglial repopulation of demyelinated white matter. J Neurosci 22:8574–8585.

Ascherio A, Munger KL (2007). Environmental risk factors for multiple sclerosis. Part I: the role of infection. *Ann Neurol* 61:288–299

Ascherio A, Munger KL, Lennette ET, Spiegelman D, Hernan MA, Olek MJ, Hankinson SE, Hunter DJ (2001). Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. *JAMA* 286:3083–3088.

Ascherio A, Zhang SM, Hernán MA, Olek MJ, Coplan PM, Brodovicz K, Walker AM (2001). Hepatitis B vaccination and the risk of multiple sclerosis. *N Engl J Med* 1;344(5):327-332.

Ascherio A, Munger KL. (2007). Environmental risk factors for multiple sclerosis. Part I: the role of infection. *Ann Neurol* 61(4):288-299.

Assisi L, Botte V, D'Aniello A, Di Fiore MM (2001). Enhancement of aromatase activity by D-aspartic acid in the ovary of the lizard Podarcis s. sicula. *Reproduction* 121(5):803–808.

Atkins GJ, McQuaid S, Morris-Downes MM, Galbraith SE, Amor, S, Cosby SL, Sheahan BJ (2000). Transient virus infection and multiple sclerosis. *Rev Med Virol 10*, 291-303.

Atlas of MS 2013: Mapping Multiple Sclerosis Around the World. London: *Multiple Sclerosis International Federation 2013.*

Back SA, Han BH, Luo NL, Chricton CA, Xanthoudakis S, Tam J, Arvin KL, Holtzman DM (2002). Selective vulnerability of late oligodendrocyte progenitors to hypoxia-ischemia. *J Neurosci* 22(2), 455-463.

Baker PF, Blaustein MP, Hodgkin AL, and Steinhardt RA (1969). The influence of calcium on sodium efflux in squid axons. *J Physiol* 200:431– 458.

Bakiri Y, Burzomato V, Frugier G, Hamilton NB, Káradóttir R, Attwell D (2009). Glutamatergic signaling in the brain's white matter. *Neuroscience* 158:266-274.

Barbarese E, Brumwell C, Kwon S, Cui H, Carson JH (1999). RNA on the road to myelin. *J Neurocytol* 28: 263-270.

Barkovich AJ, Deon S (2016). Hypomyelinating disorders: An MRI approach. *Neurobiol Dis* 87:50-58.

Barres BA, Jacobson MD, Schmid R, Sendtner M, Raff MC (1993). Does oligodendrocyte survival depend on axons? *Curr Biol* 3(8):489-497.

Barres BA, Koroshetz WJ, Swartz KJ, Chun LL, Corey DP (1990). Ion channel expression by white matter glia: the O-2A glial progenitor cell. *Neuron* 4:507-524.

Barres BA, Lazar MA, Raff MC (1994). A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. *Development* 120(5):1097-108.

Baryshnikov SG, Pulina MV, Zulian A, Linde CI, Golovina VA (2009). Orai1, a critical component of store-operated Ca2+ entry, is functionally associated with Na+/Ca2+ exchanger and plasma membrane Ca2+ pump in proliferating human arterial myocytes. *Am J Physiol Cell Physiol* 297, C1103–C1112.

Baumann N, Pham-Dinh D (2001). Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol Rev 81*, 871-927.

Beirowski B (2013). Concepts for regulation of axon integrity by enwrapping glia. *Front Cell Neurosci* 7:256.

Berger T, Schnitzer J, Kettenmann H (1991). Developmental changes in the membrane current pattern, K+ buffer capacity, and morphology of glial cells in the corpus callosum slice. *J Neurosci* 11(10), 3008-3024.

Berger T, Schnitzer J, Orkand PM, Kettenmann H (1992a). Sodium and Calcium Currents in Glial Cells of the Mouse Corpus Callosum Slice. *Eur J Neurosci* 4(12), 1271-1284.

Berger T, Walz W, Schnitzer J, Kettenmann H (1992b). GABA- and glutamateactivated currents in glial cells of the mouse corpus callosum slice. *J Neurosci Res* 31(1), 21-27.

Berger UV, Luthi-Carter R, Passani LA, Elkabes S, Black I, Konradi C, Coyle JT (1999). Glutamate carboxypeptidase II is expressed by astrocytes in the adult rat nervous system. *J Comp Neurol*, 415(1), 52-64.

Bernier L, Colman DL, D'Eustachio P (1988). Chromosomal locations of genes encoding 2',3'-cyclic nucleotide -3'-phosphodiesterase and glial fibrillary acidic protein in the mouse. *J Neurosci Res* 20: 497–504.

Bers DM (2002). Cardiac excitation–contraction coupling. *Nature* 415, 198–205.

Billard JM (2012). D-Amino acids in brain neurotransmission and synaptic plasticity. *Amino Acids* 43(5):1851-1860.

Birling MC, Roussel G, Nussbaum F, Nussbaum JL (1993). Biochemical and immunohistochemical studies with specific polyclonal antibodies directed against bovine myelin/oligodendrocyte glycoprotein. *Neurochem* Res 18: 937-945.

Blakemore WF (1978). Lesions in the cat spinal cord following local injections of 6-aminonicotinamide. *Res Vet Sci 24*, 390-391.

Blakemore WF and Franklin RJ (2008). Remyelination in experimental models of toxin-induced demyelination. Curr Top Microbiol Immunol 318:193–212.

Blaustein MP (2010). Knockout of Na+/Ca2+ exchanger in smooth muscle attenuates vasoconstriction and L-type Ca2+ channel current and lowers blood pressure. *Am J Physiol Heart Circ Physiol* 298, H1472–H1483.

Bo L, Mork S, Kong PA, Nyland H, Pardo CA, Trapp BD (1994) Detection of MHC class II-antigens on macrophages and microglia, but not on astrocytes and endothelia in active multiple sclerosis lesions. *J Neuroimmunol* 51:135–146.

Boda E, Viganò F, Rosa P, Fumagalli M, Labat-Gest V, Tempia F, Abbracchio MP, Dimou L, Buffo A (2011). The GPR17 receptor in NG2 expressing cells: focus on in vivo cell maturation and participation in acute trauma and chronic damage. *Glia* 59(12):1958-1973.

Boison D, Bussow H, D'urso D, Muller HW, Stoffel W (1995). Adhesive properties of proteolipid protein are responsible for the compaction of CNS myelin sheaths. *J Neurosci* 15: 5502–5513.

Boscia F, D'Avanzo C, Pannaccione A, Secondo A, Casamassa A, Formisano L, Guida N, Sokolow S, Herchulelz A, Annunziato L (2012). Silencing or knocking out the Na^{+}/Ca^{2+} exchanger-3 (NCX3) impairs oligodendrocyte differentiation. *Cell Death and Differentiation* 19(4):562-572.

Boyman L, Hiller R, Lederer WJ, Khananshvili D (2008). Direct loading of the purified endogenous inhibitor into the cytoplasm of patched cardiomyocytes blocks the ion currents and calcium transport through the NCX1 protein. *Biochemistry* 47, 6602–6611.

Bradl M, Lassmann H (2010). Oligodendrocytes: biology and pathology. *Acta Neuropathologica* 119(1):37-53.

Brothwell SL, Barber JL, Monaghan DT, Jane DE, Gibb AJ, Jones S (2008). NR2B- and NR2Dcontaining synaptic NMDA receptors in developing rat substantia nigra pars compacta dopaminergic neurones. *J Physiol* 586(3):739–750.

Brown ER, Piscopo S, Chun JT, Francone M, Mirabile I, D'Aniello A (2007). Modulation of an AMPA-like glutamate receptor (SqGluR) gating by L- and D-aspartic acids. Amino *Acid* 32(1):53–57.

Browne P, Chandraratna D, Angood C, Tremlett H, Baker C, Taylor BV, Thompson AJ (2014). Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity. *Neurology* 83(11):1022-1024.

Brunner C, Lassmann H, Waehneldt TV, Matthieu JM, Linington C (1989).Differential ultrastructural localization of myelin basic protein, myelin-oligodendroglial glycoprotein, and 2',39-cyclic nucleotide 3'-phosphodiesterase in the CNS of adult rats. *J Neurochem* 52:296–304.

Bunge MB, Bunge RP, Ris H (1961). Ultrastructural study of remyelination in an experimental lesion in adult cat spinal cord. *J Biophys Biochem Cytol* 10: 67–94.

Buntinx M, Vanderlocht J, Hellings N, Vandenabeele F, Lambrichts I, Raus J, Ameloot M, Stinissen P, Steels P (2003) Characterization of three human oligodendroglial cell lines as anmodel to study oligodendrocyte injury: morphology andnoligodendrocyte-specific gene expression *J Neurocytol 32,b*25-38

Burnashev N, Zhou Z, Neher E, Sakmann B (1995). Fractional calcium currents through recombinant GluR channels of the NMDA, AMPA and kainate receptor subtypes. *J Physiol* 485: 403-418.

Butt AM and Kalsi A (2006). Inwardly rectifying potassium channels (Kir) in central nervous system glia: a special role for Kir4.1 in glial functions. *J Cell Mol Med* 10(1), 33-44.

Buttery PC, ffrench-Constant C (1999). Laminin-2/integrin interactions enhance myelin membrane formation by oligodendrocytes. *Mol Cell Neurosci* 14(3):199-212.

Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, Lubischer JL, Krieg PA, Krupenko SA, Thompson WJ, Barres BA (2008). A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J Neurosci* 28(1), 264-278.

Cai J, Qi Y, Hu X, Tan M, Liu Z, Zhang J, Li Q, Sander M, Qiu M (2005). Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling. *Neuron* 45:41–53

Campagnoni AT, Macklin WB (1988). Cellular and molecular aspects of myelin protein gene expression. *Mol Neurobiol* 2: 41–89.

Canitano A, Papa M, Boscia F, Castaldo P, Sellitti S, Taglialatela M, and Annunziato L. B (2002). Brain distribution of the Na+/Ca2+ exchanger-encoding genes NCX1, NCX2, and NCX3 and their related proteins in the central nervous system. Ann N Y Acad Sci 976: 394–404.

Carafoli E, Santella L, Branca D, Brini M (2001). Generation, control, and processing of cellular calcium signals. *Critical Reviews in Biochemistry and Molecular Biology* 36 (2): 107–260.

Casamassa A, La Rocca C, Sokolow S, Herchuelz A, Matarese G, Annunziato L, Boscia F (2016). Ncx3 gene ablation impairs oligodendrocyte precursor response and increases susceptibility to experimental autoimmune encephalomyelitis. Glia 64(7):1124-1137.

Cavaliere F, Benito-Muñoz M, Panicker M, Matute C (2013). NMDA modulates oligodendrocyte differentiation of subventricular zone cells through PKC activation. *Frontiers in Cellular Neuroscience* 7:261.

Cavaliere F, Urra O, Alberdi E, Matute C (2012). Oligodendrocyte differentiation from adult multipotent stem cells is modulated by glutamate. Cell Death Dis 2;3:e268.

Charcot JM (1868). Histologie de la scle´rose en plaques. *Gaz Des hospitaux* 41:554–555, 557–558, 566.

Chen K, Hughes SM, Connor B (2007) Neural progenitor cells derived from the adult rat subventricular zone: characterization and transplantation *Cell Transplant 16,* 799-810.

Chen S, Ren YQ, Bing R and Hillman DE (2000). Alpha 1E subunit of the R-type calcium channel is associated with myelinogenesis. *J Neurocytol* 29(10), 719-728.

Choi BH, Kim RC(1985). Expression of glial fibrillary acidic protein by immature oligodendroglia and its implications. *J Neuroimmunol* 8, 215–235.

Choi BH, Kim RC, Lapham LW (1983). Do radial glia give rise to both astroglial and oligodendroglial cells? *Dev. Brain Res.* 8, 119–130.

Chomiak T, Hu B (2009). What is the optimal value of the g-ratio for myelinated fibers in the rat CNS? A theoretical approach. *PLoS ONE* 4, e7754.

Chvátal A, Berger T, Vorísek I, Orkand RK, Kettenmann H, Syková E (1997).Changes in glial K+ currents with decreased extracellular volume in developing rat white matter. *J Neurosci Res* 49(1), 98-106.

Clanet M (2008). Jean-Martin Charcot. 1825 to 1893.*Int MS J* 15(2):59-61.

Coetzee T, Fujita N, Dupree J, Shi R, Blight A, Suzuki K, Suzuki K, Popko B (1996). Myelination in the absence of galactocerebroside and sulfatide: Normal structure with abnormal function and regional instability. *Cell* 86:209–219.

Collingridge GL, Olsen RW, Peters J and Spedding M (2009). A nomenclature for ligand-gated ion channels. *Neuropharmacology* 56, 2–5.

Collingridge GL, Olsen RW, Peters J, Spedding M (2009). A nomenclature for ligand-gated ion channels. *Neuropharmacology* 56(1):2-5.

Colman DR, Kreibich G, Frey AB, Sabatini DD (1995). Synthesis and incorporation of myelin polypeptides into CNS myelin. J Cell Biol 95(2 Pt 1):598-608.

Compston A, Coles A (2002). Multiple sclerosis. *Lancet* 359 (9313): 1221–1231.

Compston A, Coles A (2008). *The Lancet* Volume 372, Issue 9648, 1502 – 1517.

Cotrina ML, Nedergaard M (2012) Brain Connexins in Demyelinating Diseases: Therapeutic Potential of Glial Targets. *Brain research*. 1487:61-68.

Cox MB, Cairns MJ, Gandhi KS, Carroll AP, Moscovis S, Stewart GJ, Broadley S, Scott RJ, Booth DR, Lechner-Scott J (2010). MicroRNAs miR-17 and miR-20a inhibit T cell activation genes and are under-expressed in MS whole blood. PLoS One 5(8):e12132.

Crawford AH, Stockley JH, Tripathi RB, Richardson WD, Franklin RJ (2014). Oligodendrocyte progenitors: adult stem cells of the central nervous system? *Exp Neurol* 260:50–55.

Cucullo L, Marchi N, Hossain M, Janigro DA (2011). Dynamic *in vitro* BBB model for the study of immune cell trafficking into the central nervous system *J Cereb Blood Flow Metab 31*, 767-777.

Cui QL, Kuhlmann T, Miron VE, Leong SY, Fang J, Gris P, Kennedy TE, Almazan G, Antel J (2013). Oligodendrocyte progenitor cell susceptibility to injury in multiple sclerosis. *Am J Pathol* 183, 516–525.

Cull-Candy SG, Leszkiewicz DN (2004). Role of distinct NMDA receptor subtypes at central synapses. *Sci STKE* 255, 16.

D'Aniello A, Donofrio G, Pischetola M, Daniello G, Vetere A, Petrucelli L, Fisher GH (1993a). Biological role of D-amino acid oxidase and D-aspartate oxidase. Effects of D-amino acids. *J Biol Chem* 268 (36):26941–26949.

D'Aniello A, Vetere A, Petrucelli L (1993c). Further study on the specificity of D-amino acid oxidase and Daspartate oxidase and time course for complete oxidation of D-amino acid. *Comp Biochem Physiol B Biochem Mol Biol* 105(3-4):731–734.

D'Aniello S and Garcia-Fernandez J (2007). D-Aspartic acid and L-amino acids in the neural system of the amphioxus Branchiostoma lanceolatum. *Amino Acids* 32(1):21–26.

D'Aniello S, Somorjai I, Garcia-Fernandez J, Topo E, D'Aniello A (2011). D-Aspartic acid is a novel endogenous neurotransmitter. *FASEB J* 25(3):1014–1027.

Dai X, Lercher LD, Clinton PM, Du Y, Livingston DL, Vieira C, Yang L, Shen MM, et al. (2003).The trophic role of oligodendrocytes in the basal forebrain. *J Neurosci Off J Soc Neurosci* 23(13):5846–5853.

D'Aniello A (2007). D-Aspartic acid: An endogenous amino acid with an important neuroendocrine role *Brain Res Rev* 53(2):215-234.

D'Aniello S, Somorjai I, Garcia-Fernàndez J, Topo E, D'Aniello A (2011). D-Aspartic acid is a novel endogenous neurotransmitter. *FASEB J* 25(3):1014-1027.

Das K, Majumder A, Siegenthaler M. et al. (2011). Automated cell classification and visualization for analyzing remyelination therapy *Vis Comput* 27: 1055.

Dawson MR, Levine JM, Reynolds R (2000). NG2-expressing cells in the central nervous system: are they oligodendroglial progenitors? *J Neurosci* Res. 61, 471–479.

De Biase LM, Nishiyama A and Bergles DE (2010). Excitability and synaptic communication within the oligodendrocyte lineage. *J Neurosci* 30(10), 3600-3611.

de Castro F, Zalc B (2013) Migration of myelin-forming cells in the CNS. In: Cellular migration and formation of neuronal connections: comprehensive developmental neuroscience. Elsevier, Amsterdam.

de Vries GH, Boullerne AI (2010). Glial cell lines: an overview *Neurochem Res 35*, 1978-2000.

de Vries H, Hoekstra D (2000). On the biogenesis of the myelin sheath: cognate polarizedtrafficking pathways in oligodendrocytes. *Glycoconj J* 17: 181-190.

Deconinck N, Dan B (2007). Pathophysiology of duchenne muscular dystrophy: current hypotheses. *Pediatr Neurol* 36, 1–7.

Defaux A, Zurich MG, Honegger P, Monnet-Tschudi F (2011) Minocycline promotes remyelination in aggregating rat brain cell cultures after interferon-gamma plus lipopolysaccharide-induced demyelination *Neuroscience 187*, 84-92.

Demerens C, Stankoff B, Logak M, Anglade P, Allinquant B, Couraud F, Zalc B, Lubetzki C (1996). Induction of myelination in the central nervous system by electrical activity. *Proc Natl Acad Sci USA* 93:9887-9892.

Deng W, Rosenberg PA, Volpe JJ, Jensen FE (2003). Calcium-permeable AMPA/kainate receptors mediate toxicity and preconditioning by oxygen-glucose deprivation in oligodendrocyte precursors. *Proc Natl Acad Sci USA*, 100(11),6801-6806.

Deng W, Wang H, Rosenberg PA, Volpe JJ, Jensen F.E (2004). Role of metabotropic glutamate receptors in oligodendrocyte excitotoxicity and oxidative stress. *Proc Natl Acad Sci USA* 101(20), 7751-7756.

Devinsky O, Laff R (2003) Callosal lesions and behavior: history and modern concepts. *Epilepsy Behav* 4:607–617.

Devon RM (1987). Comparison of oligodendrocytes grown in neocortex and spinal cord aggregate cultures *Brain Res 429*, 289-294.

Di Fiore MM, Assisi L, Botte V, D'Aniello A (1998). D-Aspartic acid is implicated in the control of testosterone production by the vertebrate gonad. Studies on the female green frog, Rana esculenta. *J Endocrinol* 157(2):199–207.

Diemel LT, Wolswijk G, Jackson SJ, Cuzner ML (2004). Remyelination of cytokine- or antibody-demyelinated CNS aggregate cultures is inhibited by macrophage supplementation *Glia 45*, 278-286.

Dingledine R, Borges K, Bowie D, Traynelis SF (1999). The glutamate receptor ion channels. *Pharmacol Rev* 51:7-61.

Dingledine R, Borges K, Bowie D, Traynelis SF (1999). The glutamate receptor ion channels. *Pharmacol Rev* 51(1), 7-61.

DiPolo R and Beaugé L (2006). Sodium/calcium exchanger: influence of metabolic regulation on ion carrier interactions. *Physiological Reviews*. 86 (1): 155–203.

Dobson R, Ramagopalan S, Davis A, Giovannoni G (2013). Cerebrospinal fluid oligoclonal bands in multiple sclerosis and clinically isolated syndromes: a meta-analysis of prevalence, prognosis and effect of latitude. *J Neurol Neurosurg Psychiatry* 84(8):909-914.

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J Neurosci* 17:5046–5061, 1997.

Douglas AJ, Fox MF, Abbott CM, Hinks LJ, Sharpe G, Povey S, Thompson RJ (1992). Structure and chromosomal localization 2',3'-cyclic nucleotide -3'-phosphodiesterase gene. *Ann Hum Genet* 56:243–254.

Drago I, Pizzo P, Pozzan T (2011). After half a century mitochondrial calcium in- and efflux machineries reveal themselves. *EMBO J* 30,4119–4425.

Du Y, Dreyfus CF (2002). Oligodendrocytes as providers of growth factors. *J Neurosci Res* 68(6):647–654.

Dunlop DS, Neidle A, McHale D, Dunlop DM, Lajtha A (1986). The presence of free D-aspartic acid in rodents and man. *Biochem Biophys Res Commun* 141(1):27–32.

Dunn J, Elias CL, Le HD, Omelchenko A, Hryshko LV, Lytton J (2002). The molecular determinants of ionic regulatory differences between brain and kidney Na+/Ca2+ exchanger (NCX1) isoforms. *J Biol Chem* 277, 33957–33962.

Dutta M, Hanna E, Das P, Steinhubl SR (2006). Incidence and prevention of ischemic stroke following myocardial infarction: review of current literature.*Cerebrovasc Dis* 22, 331–339.

Dutta R, Trapp BD (2011). Mechanisms of Neuronal Dysfunction and Degeneration in Multiple Sclerosis. *Progress in neurobiology*. 93(1):1-12.

Edgar JM, McLaughlin M, Werner HB, McCulloch MC, Barrie JA, Brown A, Faichney AB, Snaidero N, Nave KA, Griffiths IR (2009). Early ultrastructural defects of axons and axon–glia junctions in mice lacking expression of *Cnp1*. *Glia* 57, 1815–1824.

Edgar JM, McLaughlin M, Werner HB, McCulloch MC, Barrie JA, Brown A, Faichney AB, Snaidero N, Nave KA, Griffiths IR (2009). Early ultrastructural defects of axons and axon-glia junctions in mice lacking expression of Cnp1. *Glia* 57, 1815–1824.

Edgar JM, McLaughlin M, Yool D, Zhang SC, Fowler JH, Montague P, Barrie JA, McCulloch MC, Duncan ID, Garbern J, Nave KA, Griffiths IR (2004). Oligodendroglial modulation of fast axonal transport in a mouse model of hereditary spastic paraplegia. *J. Cell. Biol.* 166, 121–131.

Edgar N, Sibille E (2012). A putative functional role for oligodendrocytes in mood regulation. *Transl Psychiatry* 2, e109.

Eikelenboom MJ, Uitdehaag BM, Petzold A (2011). Blood and CSF Biomarker Dynamics in Multiple Sclerosis: Implications for Data Interpretation. *Mult Scler Int* 823176.

Emery B (2010a). Regulation of oligodendrocyte differentiation and myelination. *Science* 330, 779-782.

Emery B (2010b) Transcriptional and post-transcriptional control of CNS myelination. *Current opinion in neurobiology* 20,601-607.

Emery B, Agalliu D, Cahoy JD, Watkins TA, Dugas JC, Mulinyawe SB, Ibrahim A, Ligon KL, Rowitch DH, Barres BA (2009) Myelin gene regulatory factor is a critical transcriptional regulator required for CNS myelination. *Cell* 138,172-185.

Errico F, D'Argenio V, Sforazzini F, Iasevoli F, Squillace M, Guerri G, Napolitano F, Angrisano T, Di Maio A, Keller S, Vitucci D, Galbusera A, Chiariotti L, Bertolino A, de Bartolomeis A, Salvatore F, Gozzi A, Usiello A (2015). A role for D-aspartate oxidase in schizophrenia and in schizophrenia-related symptoms induced by phencyclidine in mice. *Translational Psychiatry* 5(2):e512.

Errico F, Nisticò R, Palma G, Federici M, Affuso A, Brilli E, Topo E, Centonze D, Bernardi G, Bozzi Y, D'Aniello A, Di Lauro R, Mercuri NB, Usiello A (2008a). Increased levels of Daspartate in the hippocampus enhance LTP but do not facilitate cognitive flexibility. *Molecular and Cellular Neuroscience* 37, 236-246.

Errico F, Rossi S, Napolitano F, Catuogno V, Topo E, Fisone G, D'Aniello A, Centonze D, Usiello A (2008b). D-Aspartate prevents corticostriatal long-term depression and attenuates schizophrenialike symptoms induced by amphetamine and MK-801. *Journal of Neuroscience* 28,10404-10414.

Errico F, Napolitano F, Nisticò R, Usiello A (2012). New insights on the role of free D-aspartate in the mammalian brain. *Amino Acids* 43(5):1861-1871.

Ettle B, Schlachetzki JCM, Winkler J (2016). Oligodendroglia and Myelin in Neurodegenerative Diseases: More Than Just Bystanders? *Molecular Neurobiology* 53:3046-3062.

Eylar EH, Brostoff S, Hashim G, Caccam J, Burnett P (1971). Basic A1 protein of the myelin membrane.The complete amino acid sequence". *J Biol Chem* 246 (18): 5770–5784.

Fancy SPJ, Baranzini SE, Zhao C, Yuk DI, Irvine KA, Kaing S, Sanai N, Franklin RJM, Rowitch DH (2009). Dysregulation of the Wnt pathway inhibits timely myelination and remyelination in the mammalian CNS. *Genes Dev* 23:1571–1585.

Fancy SPJ, Chan JR, Baranzini SE, Franklin RJM, Rowitch DH (2011a) Myelin regeneration: a recapitulation of development? *Annu Rev Neurosci* 34:19–41.

Fancy SPJ, Harrington EP, Yuen TJ, Silbereis JC, Zhao C, Baranzini SE, Bruce CC, Otero JJ,Huang EJ, Nusse R, Franklin RJM, Rowitch DH (2011b) Axin2 as regulatory and therapeutic target in newborn brain injury and remyelination. *Nat Neurosci* 14(8):1009–1016.

Fancy SPJ, Kotter MR, Harrington EP, Huang JK, Zhao C, Rowitch DH, Franklin RJM (2010). Overcoming remyelination failure in multiple sclerosis and other myelin disorders. *Exp Neurol* 225:18–23.

Fancy SPJ, Zhao C, Franklin RJM (2004). Increased expression of Nkx2.2 and Olig2 identifies reactive oligodendrocyte progenitor cells responding to demyelination in the adult CNS. *Mol Cell Neurosci* 27:247–254.

Fannon J, Tarmier W, Fulton D (2015). Neuronal activity and AMPA-type glutamate receptor activation regulates the morphological development of oligodendrocyte precursor cells. *Glia* 63(6):1021-1035.

Feldman DH, Horiuchi M, Keachie K, Mccauley E, Bannerman P, Itoh A, Itoh T, Pleasure D (2008). Characterization of acid-sensing ion channel expression in oligodendrocyte-lineage cells. *Glia* 56(11), 1238-1249.

Felts PA, Woolston AM, Fernando HB, Asquith S, Gregson NA, Mizzi OJ, Smith KJ (2005). Inflammation and primary demyelination induced by the intraspinal injection of lipopolysaccharide. *Brain 128*, 1649-1666.

Fialová L, Bartos A, Švarcová J, Zimova D, Kotoucova J (2013). Serum and cerebrospinal fluid heavy neurofilaments and antibodies against them in early multiple sclerosis. *J Neuroimmunol* 15;259(1-2):81-87.

Fieber LA, Carlson SL, Capo TR, Schmale MC (2010). Changes in D-aspartate ion currents in the Aplysia nervous system with aging. *Brain Res* 1343:28–36.

Fields RD (2008). White matter in learning, cognition and psychiatric disorders. *Trends Neurosci* 31, 361–370.

Filippi M, Rocca MA, Ciccarelli O, De Stefano N, Evangelou N, Kappos L, Rovira A, Sastre-Garriga J, Tintorè M, Frederiksen JL, Gasperini C, Palace J, Reich DS, Banwell B, Montalban X, Barkhof F (2016). MRI criteria for the diagnosis of multiple sclerosis: MAGNIMS consensus guidelines. *The Lancet Neurology* Volume15, Issue 3, 292 - 303

Fisher GH, Daniello A, Vetere A, Padula L, Cusano GP, Man EH (1991). Free D-aspartate and D-alanine in normal and Alzheimer brain. *Brain Res Bull* 26(6):983–985.

Fogarty M, Richardson WD, Kessaris N (2005) A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord. *Development* 132:1951–1959

Folch J, Lees MB (1951). Proteolipids, a new type of tissue lipoproteins, their isolation from brain. *J Biol Chem* 191: 807–817.

Ford AL, Goodsall AL, Hickey WF, Sedgwick JD (1995) Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared *J Immunol 154*, 4309-4321

Forsby A, Bal-Price AK, Camins A, Coecke S, Fabre N, Gustafsson H, Honegger P, Kinsner-Ovaskainen A, Pallas M, Rimbau V, Rodriguez-Farre E, Sunol C, Vericat JA, Zurich MG (2009) Neuronal *in vitro* models for the estimation of acute systemic toxicity *Toxicol In Vitro 23*, 1564-1569

Franco-Pons N, Torrente M, Colomina MT, Vilella E (2007). Behavioral deficits in the cuprizone-induced murine model of demyelination/remyelination. *Toxicology Letters* 169:205–213.

Franklin RJM, ffrench-Constant C (2008). Remyelination in the CNS: from biology to therapy. *Nature Reviews Neuroscience* 9(11), 839-855.

Freeman SA, Desmazières A, Fricker D, Lubetzki C, Sol-Foulon N (2016). Mechanisms of sodium channel clustering and its influence on axonal impulse conduction. *Cellular and Molecular Life Sciences* 73:723-735.

Friede RL, Miyagishi T, Johnstone M (1972). Adjustment of the myelin sheath to changes in axon caliber. *Anat Rec* 172: 1–13.

Friese MA, Craner MJ, Etzensperger R, Vergo S, Wemmie JA, Welsh MJ, Vincent A, Fugger L (2007). Acid-sensing ion channel-1 contributes to axonal degeneration in autoimmune inflammation of the central nervous system. *Nat Med* 13(12), 1483-1489.

Fujii N (2002) D-Amino acids in living higher organisms. Orig Life Evol Biosph 32:103–127.

Fujii N (2005) D-Amino acid in elderly tissues. Biol Pharm Bull 28:1585–1589.

Fujii N, Kaji Y, Fujii N (2011) D-Amino acids in aged proteins: analysis and biological relevance. J Chromatogr B 879:3141–3147.

Funakoshi M, Sekine M, Katane M, Furuchi T, Yohda M, Yoshikawa T, Homma H (2008). Cloning and functional characterization of Arabidopsis thaliana D-amino acid aminotransferase - D-aspartate behavior during germination. *FEBS J* 275(6):1188–1200.

Gadea A, Lopez E, Lopez-Colome AM (2004). Glutamate-induced inhibition of D-aspartate uptake in Muller glia from the retina. *Neurochem Res* 29(1):295–304.

Gallo V, Mangin JM, Kukley M, Dietrich D (2008). Synapses on NG2-expressing progenitors in the brain: Multiple functions? *J Physiol* 586:3767-3781.

Gallo V, Zhou JM, McBain CJ, Wright P, Knutson PL, Armstrong RC (1996). Oligodendrocyte progenitor cell proliferation and lineage progression are regulated by glutamate receptor-mediated K+ channel block. *J Neurosci* 16(9), 2659-2670.

Ganter S, Northoff H, Mannel D, Gebicke-Harter PJ (1992) Growth control of cultured microglia *J Neurosci Res 33*, 218-230

Garberg P, Ball M, Borg N, Cecchelli R, Fenart L, Hurst RD, Lindmark T, Mabondzo A, Nilsson JE, Raub TJ, Stanimirovic D, Terasaki T, Oberg JO, Osterberg T (2005). *In vitro* models for the blood-brain barrier *Toxicol In Vitro 19*, 299-334.

Gard AL, Pfeiffer SE (1989). Oligodendrocyte progenitors isolated directly from developing telencephalon at a specific phenotypic stage: myelinogenic potential in a defined environment. *Development* 106,119–132.

Gardinier MV, Amiguet P, Linington C, Matthieu JM (1992). Myelinoligodendrocyte glycoprotein is a unique member of the immunoglobulin superfamily. *J Neurosci Res* 33: 177–187.

Gautier HO, Evans KA, Volbracht K, James R, Sitnikov S, Lundgaard I, James F, Lao-Peregrin C, Reynolds R, Franklin RJ, Káradóttir RT (2015). Neuronal activity regulates remyelination via glutamate signalling to oligodendrocyte progenitors. *Nat Commun* 6;6:8518.

Gibson EM, Purger D, Mount CW, Goldstein AK, Lin GL, Wood LS, Inema I, Miller SE, Bieri G, Zuchero JB, Barres BA, Woo PJ, Vogel H, Monje M (2014). Neuronal activity promotes oligodendrogenesis and adaptive myelination in the mammalian brain. *Science* (New York, NY) 344:1252304.

Giulian D, Baker TJ (1986) Characterization of ameboid microglia isolated from developing mammalian brain *J Neurosci 6*,2163-2178

Glezer I, Lapointe A, Rivest S (2006) Innate immunity triggers oligodendrocyte progenitor reactivity and confines damages to brain injuries. *FASEB J* 20:750–752.

Gold SM, Voskuhl RR (2009). Estrogen and Testosterone Therapies in Multiple Sclerosis. *Progress in brain research* 175:239-251.

Goldenberg MM (2012). Multiple Sclerosis Review. *P T* 37(3): 175–184.

Goldman SA, Nedergaard M, Windrem MS (2012). Glial progenitor cellbased treatment and modeling of neurological disease. *Science* 338, 491–495.

Gomez-Villafuertes R, Mellström B, Naranjo JR (2007). Searching for a role of NCX/NCKX exchangers in neurodegeneration. *Mol Neurobiol* 35(2):195-202.

Gonzalez-Perez O (2014). The ventricular-subventricular zone: a source of oligodendrocytes in the adult brain. *Front Cell Neurosci* 16;8:137.

Gosling JP, Fottrell PF (1978). Purification and characterization of D-amino acid aminotransferase from Rhizobium japonicum. *Biochim Biophys Acta, Enzymol* 522:84–95.

Goverman JM (2011). Immune Tolerance in Multiple Sclerosis. *Immunological reviews* 241(1):228-240.

Gravel M, Peterson J, Yong VW, Kottis V, Trapp B, Braun PE (1996). Overexpression of 2'3'-cyclic nucleotide-3'-phosphodiesterase in transgenic mice alters oligodendrocyte development and produces aberrant myelination. *Mol Cell Neurosci* 6: 453–466.

Griffiths I, Klugmann M, Anderson T, Yool D, Thomson C, Schwab MH, Schneider A, Zimmermann F, McCulloch M, Nadon N, Nave KA (1998). Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. *Science* 280, 1610–1613.

Grinspan JB, Franceschini B (1995). Platelet-derived growth factor is a survival factor for PSA-NCAM+ oligodendrocyte pre-progenitor cells. *J Neurosci Res* 41(4):540-551.

Gudz TI, Komuro H, Macklin WB (2006). Glutamate stimulates oligodendrocyte progenitor migration mediated via an alphav integrin/myelin proteolipid protein complex. *J Neurosci* 26(9), 2458-2466.

Hagemeyer N, Boretius S, Ott C, von Streitberg A, Welpinghus H, Sperling S, Frahm J, Simons M, Ghezzi P, Ehrenreich H (2012). Erythropoietin Attenuates Neurological and Histological Consequences of Toxic Demyelination in Mice. *Mol Med* 18(1):628–635.

Hamase K, Homma H, Takigawa Y, Imai K (1999). Alteration in the D-amino acid content of the rat pineal gland under anesthesia. *Amino Acids* 17(3):277–283.

Hao C, Richardson A, Fedoroff S (1991) Macrophage-like cells originate from neuroepithelium in culture: characterization and properties of the macrophage-like cells *Int J Dev Neurosci 9,* 1-14

Hartline DK(2008). What is myelin? *Neuron Glia Biology*. 4: 153–163.

Hashimoto A, Kumashiro S, Nishikawa T, Oka T, Takahashi K, Mito T, Takashima S, Doi N, Mizutani Y, Yamazaki T, Kaneko T, Ootomo E (1993). Embryonic development and postnatal changes in free D-aspartate and D-serine in the human prefrontal cortex. *J Neurochem* 61(1):348–351.

Hashimoto A, Oka T (1997). Free D-aspartate and D-serine in the mammalian brain and periphery. *Prog Neurobiol* 52(4):325-353.

Hayes GM, Fox RM, Cuzner ML, Griffin GE (2000) Human rotation-mediated fetal mixed brain cell aggregate culture: characterization and N-methyl-D-aspartate toxicity *Neurosci Lett 287*, 146-150.

He L, Lu QR (2013) Coordinated control of oligodendrocyte development by extrinsic and intrinsic signaling cues. Neuroscience bulletin 29:129-143

Heise N, Shumilina E, Nurbaeva MK, Schmid E, Szteyn K, Yang W, Xuan NT, Wang K, Zemtsova IM, Duszenko M, Lang F (2011). Effect of dexamethasone on Na+/Ca2+ exchanger in dendritic cells. *Am J Physiol Cell Physiol* 300, C1306–C1313.

Henderson AP, Barnett MH, Parratt JD, Prineas JW (2009). Multiple sclerosis: distribution of inflammatory cells in newlyforming lesions. *Ann Neurol* 66, 739–753.

Herchuelz A, Diaz-Horta O, van Eylen F (2002). Na/Ca exchange and Ca2+ homeostasis in the pancreatic beta-cell. *Diabetes Metab* 28, 3S54-60; discussion 53S108-112.

Herchuelz A, Kamagate A, Ximenes H, Van Eylen F (2007). Role of Na/Ca exchange and the plasma membrane Ca2+-ATPase in beta cell function and death. *Ann NY Acad Sci* 1099, 456–467.

Hibbits N, Pannu R, Wu TJ and Armstong RC (2009) Cuprizone demyelination of the corpus callosum in mice correlates with altered social interaction and impaired bilateral sensorimotor coordination. ASN NEURO 1(3):art:e00013.doi:10.1042/AN20090032

Hilgemann DW (1990). Regulation and deregulation of cardiac Na^{+}–Ca^{2+} exchange in giant excised sarcolemmal membrane patches. *Nature* 344, 242–245.

Hilgemann DW, Collins A, Matsuoka S (1992a). Steady-state and dynamic properties of cardiac Na/Ca. Secondary modulation by cytoplasmic calcium and ATP. *J Gen Physiol* 100, 933–961.

Hilgemann DW, Matsuoka S, Nagel GA, Collins A (1992b). Steady-state and dynamic properties of cardiac sodium–calcium exchange, sodium-dependent inactivation. *J Gen Physiol* 100, 905–932.

Hinks GL, Franklin RJM (1999) Distinctive patterns of PDGF-A, FGF-2, IGF-I and TGF-beta1 gene expression during remyelination of experimentally-induced spinal cord demyelination. *Mol Cell Neurosci* 14:153–168.

Hinsinger G, Galéotti N, Nabholz N, Urbach S, Rigau V, Demattei C, Lehmann S, Camu W, Labauge P, Castelnovo G, Brassat D, Loussouarn D, Salou M, Laplaud D, Casez O, Bockaert J, Marin P, Thouvenot E (2015). Chitinase 3-like proteins as diagnostic and prognostic biomarkers of multiple sclerosis. Mult Scler 21(10):1251-1256.

Hirano M, Goldman JE (1988). Gliogenesis in the rat spinal cord: evidence for origin of astrocytes and oligodendrocytes from radial precursors. *J. Neurosci. Res.* 21, 155–167.

Hollmann M and Heinemann S (1994). Cloned glutamate receptors. *Annu Rev Neurosci* 17: 31-108.

Homma H (2007). Biochemistry of D-aspartate in mammalian cells. *Amino Acids* 32(1):3–11.

Hsu CI, Wang TC, Hou SYT, Chin TY, Chang YC (2010). Quantitative study of the developmental changes in calcium-permeable AMPA receptor-expressing neurons in the rat somatosensory cortex. *J Comp Neurol* 518:75-91.

Hu BY, Du ZW, Zhang SC (2009) Differentiation of human oligodendrocytes from pluripotent stem cells *Nat Protoc 4,*1614-1622.

Hughes EG, Kang SH, Fukaya M, Bergles DE (2013). "Oligodendrocyte progenitors balance growth with self-repulsion to achieve homeostasis in the adult brain". *Nature Neuroscience. 16(6): 668–676.*

Huttner WB, Zimmerberg J. 2001. Implications of lipid microdomains for membrane curvature, budding and fission. *Curr Opin Cell Biol* 13: 478-484.

Ikonen E, Jansen M. 2008. Cellular sterol trafficking and metabolism: spotlight on structure. *Curr Opin Cell Biol* 20: 371-377.

Itoh T, Beesley J, Itoh A, Cohen AS, Kavanaugh B, Coulter DA, Grinspan JB, Pleasure D (2002). AMPA glutamate receptor-mediated calcium signaling is transiently enhanced during development of oligodendrocytes. *J Neurochem* 81(2), 390-402.

Iwamoto T, Kita S, Zhang J, Blaustein MP, Arai Y, Yoshida S, Wakimoto K, Komuro I, Katsuragi T (2004). Salt-sensitive hypertension is triggered by Ca2+ entry via Na+/Ca2+ exchanger type-1 in vascular smooth muscle. *Nat Med* 10, 1193–1199.

Jeffery ND, Crang AJ, O'Leary MT, Hodge SJ, Blakemore WF (1999) Behavioural consequences of oligodendrocyte progenitor cell transplantation into demyelinating lesions in rat spinal cord. *Eur J Neurosci* 11:1508–1514.

Jensen AB, Raff MC (1997). Continuous observation of multipotential retinal progenitor cells in clonal density culture. *Dev Biol* 188:267–279.

Jeon D, Yang YM, Jeong MJ, Philipson KD, Rhim H, Shin HS (2003). Enhanced learning and memory in mice lacking Na+/Ca2+ exchanger 2. *Neuron* 38, 965–976.

Jessen K (2004). Glial cells.*The International Journal of Biochemistry & Cell Biology* 36:10, 1861–1867.

Juhaszova M, Shimizu H, Borin ML, Yip RK, Santiago EM, Lindenmayer GE, Blaustein MP (1996). Localization of the Na+-Ca2+ exchanger in vascular smooth muscle, and in neurons and astrocytes. Ann N Y Acad Sci 779: 318–335.

Kanai Y, Hediger MA (1992). Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature* 360(6403):467–471.

Kandel ER, Schwartz JH, Jessell TM. Principles of Neural Science (2000). McGraw-Hill, New York.

Káradóttir R, Attwell D (2006). Combining patch-clamping of cells in brain slices with immunocytochemical labeling to define cell type and developmental stage. *Nat Protoc* 1(4), 1977-1986.

Káradóttir R, Attwell D (2007). Neurotransmitter receptors in the life and death of oligodendrocytes. *Neuroscience* 145(4), 1426-1438.

Káradóttir R, Cavelier P, Bergersen LH, Attwell D (2005). NMDA receptors are expressed in oligodendrocytes and activated in ischaemia. *Nature* 438(7071), 1162-1166.

Káradóttir R, Cavelier P, Bergersen LH, Attwell D (2005). NMDA receptors are expressed in oligodendrocytes and activated in ischaemia. *Nature* 438(7071), 1162-1166.

Káradóttir R, Hamilton NB, Bakiri Y, Attwell D (2008). Spiking and nonspiking classes of oligodendrocyte precursor glia in CNS white matter. *Nat Neurosci* 11(4), 450-456.

Káradóttir R, Hamilton NB, Bakiri Y, Attwell D (2008). Spiking and nonspiking classes of oligodendrocyte precursor glia in CNS white matter. *Nat Neurosci* 11(4), 450-456.

Katane M, Furuchi T, Sekine M, Homma H (2007a). Molecular cloning of a cDNA encoding mouse D-Aspartate oxidase and functional characterization of its recombinant proteins by site-directed mutagenesis. *Amino Acids* 32(1):69–78.

Katane M, Homma H (2007). D-Aspartate-An important bioactive substance in mammals: A review from an analytical and biological point of view. *J Chromatogr B Analyt Technol Biomed Life Sci* 879(29):3108–3121.

Katane M, Saitoh Y, Seida Y, Sekine M, Furuchi T, Homma H (2010). Comparative characterization of three D-aspartate oxidases and one D-amino acid oxidase from Caenorhabditis elegans. *Chem Biodivers* 7(6):1424–1434.

Kelland EE, Toms NJ (2001). Group I metabotropic glutamate receptors limit AMPA receptor-mediated oligodendrocyte progenitor cell death. *Eur J Pharmacol* 424(3), 3-4.

Keller A, Leidinger P, Steinmeyer F, Stähler C, Franke A, Hemmrich-Stanisak G, Kappel A, Wright I, Dörr J, Paul F, Diem R, Tocariu-Krick B, Meder B, Backes C, Meese E, Ruprecht K (2014). Comprehensive analysis of microRNA profiles in multiple sclerosis including next-generation sequencing. *Mult Scler* 20(3):295-303.

Kessaris N, Fogarty M, Iannarelli P, Grist M, Wegner M, Richardson WD (2006) Competing waves ofl oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. *Nat Neurosci* 9:173–179.

Kettenmann H, Hanisch UK, Noda M, Verkhratsky A (2011). Physiology of microglia. *Physiol Rev* 9, 461–553.

Kettenmann H, Verkhratsky A (2011) Neuroglia - Living Nerve Glue, *Fortschritte der Neurologie und Psychiatrie* 79: 588-597.

Khademi M, Kockum I, Andersson ML, Iacobaeus E, Brundin L, Sellebjerg F, Hillert J, Piehl F, Olsson T (2011). Cerebrospinal fluid CXCL13 in multiple sclerosis: a suggestive prognostic marker for the disease course. *Mult Scler* 17(3):335-343.

Khalil M, Enzinger C, Langkammer C, Ropele S, Mader A, Trentini A, Vane ML,Wallner-Blazek M, Bachmaier G, Archelos JJ, Koel-Simmelink MJ, Blankenstein MA, Fuchs S, Fazekas F, Teunissen CE (2013). CSF neurofilament and N-acetylaspartate related brain changes in clinically isolated syndrome. *Mult Scler* 19(4):436-442.

Khananshvili D (2013). The SLC8 gene family of sodium-calcium exchangers (NCX) - structure, function, and regulation in health and disease. *Mol Aspects Med* 34(2-3):220-235.

Kiedrowski L, Brooker G, Costa E, Wroblewski JT (1994). Glutamate impairs neuronal calcium extrusion while reducing sodium gradient. *Neuron*. 12 (2): 295–300.

Kiedrowski L, Brooker G, Costa E, Wroblewski JT (Feb 1994). Glutamate impairs neuronal calcium extrusion while reducing sodium gradient *Neuron* 12 (2): 295–300.

Kim B, Takeuchi A, Koga O, Hikida M, Matsuoka S (2012). Pivotal role of mitochondrial Na+–Ca2+ exchange in antigen receptor mediated Ca2+ signaling in DT40 and A20 B lymphocytes. *J Physiol* 590, 459–474.

Kim HJ, Miron VE, Dukala D, Proia RL, Ludwin SK, Traka M, Antel JP, Soliven B(2011). Neurobiological effects of sphingosine 1-phosphate receptor modulation in the cuprizone model. *FASEB J* 25,1509-1518.

Kim JK, Mastronardi FG, Wood DD, Lubman DM, Zand R, Moscarello MA (2003). Multiple sclerosis: an important role for post-translational modifications of myelin basic protein in pathogenesis. *Mol Cell Proteomics* 2(7):453-462.

Kim S, Steelman AJ, Koito H, Li J (2011) Astrocytes promote TNFmediated toxicity to oligodendrocyte precursors *J Neurochem 116,* 53-66

Kimberlin DW, Whitley RJ (1998). HHV-6: neurological implications of a newly described viral pathogen. *J Neurovir* 4: 474-485.

Kimmich GA, Roussie J, Manglapus M, Randles J (2001). Characterization of Na+-coupled Glutamate/Aspartate transport by a rat brain astrocyte line expressing GLAST and EAAC1. *J Membr Biol* 182(1):17–30.

Kipp M, Clarner T, Dang J, Copray, Beyer C (2009). The cuprizone animal model: new insights into an old story. *Acta Neuropathol* 118:723–736.

Kiskin NI, Krishtal OA, Tsyndrenko AY (1990). Cross-desensitization reveals pharmacological specificity of excitatory amino-acid receptors in isolated hippocampal neurons. *Eur J Neurosci* 2, 461-470.

Klugmann M, Schwab MH, Pulhofer A, Schneider A, Zimmermann F, Griffiths IR, Nave KA (1997). Assembly of CNS myelin in the absence of proteolipid protein. *Neuron* 18: 59–70.

Kochhar S, Christen P (1992). Mechanism of racemization of amino acids by aspartate aminotransferase. *Eur J Biochem* 203(3):563–569.

Koenning M, Jackson S, Hay CM, Faux C, Kilpatrick TJ, Willingham M, Emery B (2012). Myelin gene regulatory factor is required for maintenance of myelin and mature oligodendrocyte identity in the adult CNS. *J Neurosci* 32, 12528–12542.

Kolodziejczyk K, Saab AS, Nave KA, Attwell D (2010). Why do oligodendrocyte lineage cells express glutamate receptors*?* F1000 Biol Rep 2:57.

Komoly S (2005). Experimental demyelination caused by primary oligodendrocyte dystrophy. Regional distribution of the lesions in the nervous system of mice [corrected]. *Ideggyogy Sz* 58:40–43.

Kramer EM, Schardt A, Nave KA (2001). Membrane traffic in myelinating oligodendrocytes. *Microsc Res Tech* 52: 656-671.

Krämer-Albers EM, Gehrig-Burger K, Thiele C, Trotter J, Nave KA (2006). Perturbed interactions of mutant proteolipid protein/DM20 with cholesterol and lipid rafts in oligodendroglia: implications for dysmyelination in spastic paraplegia. *J Neurosci* 26 (45): 11743–11752.

Krashia P, Martini A, Nobili A, Aversa D, D'Amelio M, Berretta N, Guatteo E, Mercuri NB (2017), On the properties of identified dopaminergic neurons in the mouse substantia nigra and ventral tegmental area. *Eur J Neurosci* 45: 92–105.

Kuhle J, Plattner K, Bestwick JP, Lindberg RL, Ramagopalan SV, Norgren N, Nissim A, Malaspina A, Leppert D, Giovannoni G, Kappos L (2013). A comparative study of CSF neurofilament light and heavy chain protein in MS. *Mult Scler* 19(12):1597-1603.

Kukley M, Capetillo-Zarate E, Dietrich D (2007). Vesicular glutamate release fromaxons in whitematter. *Nat Neurosci* 10:311-320.

Kumar Ss, Bacci A, Kharazia V, Huguenard JR (2002). A developmental switch of AMPA receptor subunits in neocortical pyramidal neurons. *J Neurosci* 22: 3005-3015.

Kurihara T, Monoh K, Sakimura K, Takahashi Y (1990). Alternative splicing of mouse brain 2',3' cyclic nucleotide-3'-phosphodiesterase mRNA. *Biochem Biophys Res Commun* 152: 837–842.

Kurtzke JF (1980). Epidemiologic contributions to multiple sclerosis: an overview. *Neurology* 30:61–79.

Lamont HC, Staudenbauer WL, Strominger JL (1972). Partial purification and characterization of an aspartate racemase from Streptococcus faecalis. *J Biol Chem* 247:5103–5106.

Landtblom AM, Fazio P, Fredrikson S, Granieri E (2010). The first case history of multiple sclerosis: Augustus d'Este (1794–1848). *Neurol Sci Italy* 31 (1): 29–33.

Lappe-Siefke C, Goebbels S, Gravel M, Nicksch E, Lee J, Braun PE, Griffiths IR, Nave KA (2003). Disruption of *Cnp1* uncouples oligodendroglial functions in axonal support and myelination. *Nature Genet.* 33, 366–374.

Lassmann H, Brück W, Lucchinetti C (2001). Heterogeneity of multiple sclerosis pathogenesis: implications for diagnosis and therapy. *Trends Mol Med* 7(3):115-121.

Lau CL, Beart PM, O'Shea RD (2010). Transportable and Non-transportable Inhibitors of L-glutamate Uptake Produce Astrocytic Stellation and Increase EAAT2 Cell Surface Expression. *Neurochem Res* 35(5):735–742.

Lea PM, Custer SJ, Vicini S, Faden AI (2002).Neuronal and glial mGluR5 modulation prevents stretch-induced enhancement of NMDA receptor current. *Pharmacology, Biochemistry, and Behavior* 73 (2): 287–298.

Lebar R, Boutry JM, Vincent C, Robineaux R, Voisin GA(1976). Studies on autoimmune encephalomyelitis in the guinea pig. II. An in vitro investigation on the nature, properties, and specificity of the serum-demyelinating factor. *J Immunol* 116: 1439–1446.

Lebar R, Lubetzki C, Vincent C, Lombrail C, AND Boutry JM (1986). The M2 antigen of central nervous system myelin, a glycoprotein present in oligodendrocyte membrane. *Clin Exp Immunol* 66: 423–443.

Lee SL, Yu AS, Lytton J (1994) Tissue-specific expression of Na (+)–Ca2+ exchanger isoforms. *J Biol Chem* 269:14849–14852.

Lee Y, Morrison BM, Li Y, Lengacher S, Farah MH, Hoffman PN, Liu Y, Tsingalia A, et al. (2012). Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* 487(7408):443–448.

Lerma J (2003). Roles and rules of kainate receptors in synaptic transmission. *Nature* 4:481-495.

Levison SW, Goldman JE (1993). Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat brain. *Neuron* 10: 201–212.

Li C, Tropak MB, Gerlai R, Clapoff S, Abramow-Newerly W, Trapp B, Peterson A, Roder J (1994). Myelination in the absence of myelin-associated glycoprotein. *Nature* 30;369(6483):747-750.

Li Z, Matsuoka S, Hryshko LV, Nicoll DA, Bersohn MM, Burke EP, Lifton RP, Philipson KD (1994). Cloning of the NCX2 isoform of the plasma membrane Na^{+}-Ca^{2+} exchanger. *J Biol Chem* 269: 17434–17439.

Liao J, Li H, Zeng W, Sauer DB, Belmares R, Jiang Y (2012). Structural insight into the ion-exchange mechanism of the sodium/calcium exchanger. *Science* 335, 686–690.

Liebetanz D, Merkler D (2006). Effects of commissural de- and remyelination on motor skill behaviour in the cuprizone mouse model of multiple sclerosis. *Experimental Neurology* 202 :217–224.

Lilliu V, Pernas-Alonso R, Trelles RD, Di Porzio U, Zuddas A, Perrone-Capano C (2001). Ontogeny of AMPA receptor gene expression in the developing rat midbrain and striatum. *Mol Brain Res* 96: 133-141.

Lindner M, Heine S, Haastert K, Garde N, Fokuhl J, Linsmeier F, Grothe C, Baumgärtner W, Stangel M (2008) Sequential myelin protein expression during remyelination reveals fast and efficient repair after central nervous system demyelination. *Neuropathol Appl Neurobiol* 34:105–114.

Link H, Huang YM (2006). Oligoclonal bands in multiple sclerosis cerebrospinal fluid: an update on methodology and clinical usefulness. *J Neuroimmunol* 180 (1–2): 17–28.

Linnington C, Webb M, Woodhams PL (1984). A novel myelin-associated glycoprotein defined by a mouse monoclonal antibody. *J Neuroimmunol* 6:387-396.

Long Z, Lee JA, Okamoto T, Nimura N, Imai K, Homma H (2000). D-aspartate in a prolactin-secreting clonal strain of rat pituitary tumor cells (GH(3)). *Biochem Biophys Res Commun* 276(3):1143– 1147.

Long ZQ, Homma H, Lee JA, Fukushima T, Santa T, Iwatsubo T, Yamada RH, Imai K (1998). Biosynthesis of D-aspartate in mammalian cells. *FEBS Lett* 434(3):231–235.

Long ZQ, Sekine M, Adachi M, Furuchi T, Imai K, Nimura N, Homma H (2002). Cell density inversely regulates D- and L-aspartate levels in rat pheochromocytoma MPT1 cells. *Arch Biochem Biophys* 404(1):92–97.

Love S (2006). Demyelinating diseases. *Journal of Clinical Pathology* 59(11):1151-1159.

Lublin FD, Reingold SC, Cohen JA, Cutter GR, Sørensen PS, Thompson AJ,Wolinsky JS, Balcer LJ, Banwell B, Barkhof F, Bebo B Jr, Calabresi PA, Clanet M, Comi G, Fox RJ, Freedman MS, Goodman AD, Inglese M, Kappos L, Kieseier BC, Lincoln JA, Lubetzki C, Miller AE, Montalban X, O'Connor PW, Petkau J, Pozzilli C, Rudick RA, Sormani MP, Stüve O, Waubant E, Polman CH (2014). Defining the clinical course of multiple sclerosis: The 2013 revisions. *Neurology* 83(3):278-286.

Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H (2000). Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* 47:707–717.

Lunn KF, Baas PW, Duncan ID (1997). Microtubule organization and stability in the oligodendrocyte. *J Neurosci* 17: 4921–4932.

Luyt K, Váradi A, Durant CF, Molnár E (2006). Oligodendroglial metabotropic glutamate receptors are developmentally regulated and involved in the prevention of apoptosis. *J Neurochem* 99(2), 641-656

Luyt K, Varadi A, Molnar E (2003). Functional metabotropic glutamate receptors are expressed in oligodendrocyte progenitor cells. *J Neurochem* 84(6), 1452-1464.

Maack C, Ganesan A, Sidor A, O'Rourke B (2005). Cardiac sodium-calcium exchanger is regulated by allosteric calcium and exchanger inhibitory peptide at distinct sites. *Circ Res* 96: 91–99.

Makinodan M, Yamauchi T, Tatsumi K, Okuda H, Takeda T, Kiuchi K, Sadamatsu M, Wanaka A, Kishimoto T. (2009). Demyelination in the juvenile period, but not in adulthood, leads to long-lasting cognitive impairment and deficient social interaction in mice. *Prog Neuropsychopharmacol Biol Psychiatry* 33(6):978-985.

Mammucari C, Patron M, Granatiero V, Rizzuto R (2011). Molecules and roles of mitochondrial calcium signaling. *Biofactors* 37, 219–227.

Man EH, Sandhouse ME, Burg J, Fisher GH (1983). Accumulation of D-Aspartic acid with age in human brain. Science 220,1407-1408.

Marin-Husstege M, Muggironi M, Raban D, Skoff R, P, Casaccia-Bonnefil P (2004). Oligodendrocyte Progenitor Proliferation and Maturation Is Differentially Regulated by Male and Female Sex Steroid Hormones. Dev Neurosci 26:245-254.

Marin-Husstege M, Muggironi M, Raban D, Skoff R, P, Casaccia-Bonnefil P (2004). Oligodendrocyte Progenitor Proliferation and Maturation Is Differentially Regulated by Male and Female Sex Steroid Hormones. *Dev Neurosci* 26:245-254.

Markovic-Plese S, Hemmer B, Zhao Y, Simon R, Pinilla C, Martin R (2005). High level of cross-reactivity in influenza virus hemagglutinin-specific CD4+ T-cell response: implications for the initiation of autoimmune response in multiple sclerosis. *J Neuroimmunol* 169(1-2): 31-38.

Mason JL, Ye P, Suzuki K, D'Ercole AJ, Matsushima GK (2000). Insulin-like growth factor-1 inhibits mature oligodendrocyte apoptosis during primary demyelination. *J Neurosci* 20(15):5703-5708.

Matalon R, Michals-Matalon K, Surendran S, Tyring SK (2006). Canavan disease: studies on the knockout mouse. Adv Exp Med Biol Advances in Experimental Medicine and Biology 576: 77–93; discussion 361–363.

Matsuoka S, Nicoll DA, He Z, Philipson KD (1997) Regulation of cardiac Na(+)–Ca2+ exchanger by the endogenous XIP region. *J Gen Physiol* 109:273–286.

Matsushima GK, Morell P (2001). The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. *Brain Pathol* 11:107–116.

Matute C (2008). P2X7 receptors in oligodendrocytes: a novel target for neuroprotection. *Mol Neurobiol* 38(2), 123-128.

Matute C (2011). Glutamate and ATP signalling in white matter pathology. J Anat 219:53–64.

Matute C, Sánchez-Gómez MV, Martínez-Millán L, Miledi R (1997). Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes. *Proc Natl Acad Sci USA*, 94(16), 8830-8835.

Matute C, Torre I, Pérez-Cerdá F, Pérez-Samartín A, Alberdi E, Etxebarria E, Arranz AM, Ravid R, Rodríguez-Antigüedad A, Sánchez-Gómez M, Domercq M (2007). P2X(7) receptor blockade prevents ATP excitotoxicity in oligodendrocytes and ameliorates experimental autoimmune encephalomyelitis. *J Neurosci* 27(35), 9525-9533.

Mayer ML (2005). Glutamate receptor ion channels. *Curr Opin Neurobiol* 15:282–288.

McCarthy DP, Richards MH, Miller SD (2012). Mouse Models of Multiple Sclerosis: Experimental Autoimmune Encephalomyelitis and Theiler's Virus-Induced Demyelinating Disease. *Methods Mol Biol* 900: 381–401.

McCarthy KD, de Vellis J (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue *J Cell Biol 85*, 890-902

McKerracher L, Rosen KM (2015). MAG, myelin and overcoming growth inhibition in the CNS. *Front Mol Neurosci* 7;8:51.

Menichella DM, Majdan M, Awatramani R, Goodenough DA, Sirkowski E, Scherer SS, Paul DL (2006). Genetic and physiological evidence that oligodendrocyte gap junctions contribute to spatial buffering of potassium released during neuronal activity. J Neurosci Off J Soc Neurosci 26(43):10984–10991.

Micu I, Jiang Q, Coderre E, Ridsdale A, Zhang L, Woulfe J, Yin X, Trapp BD, McRory JE, Rehak R, Zamponi GW, Wang W, Stys PK (2006). NMDA receptors mediate calcium accumulation in myelin during chemical ischaemia. *Nature* 439(7079), 988-992.

Molinaro P, Cuomo O, Pignataro G, Boscia F, Sirabella R, Pannaccione A, Secondo A, Scorziello A, Adornetto A, Gala R, Viggiano D, Sokolow S, Herchuelz A, Schurmans S, Di Renzo G, Annunziato L (2008). Targeted disruption of Na+/Ca2+ exchanger 3 (NCX3) gene leads to a worsening of ischemic brain damage. *J Neurosci* 28, 1179–1184.

Molinaro P, Viggiano D, Nisticò R, Sirabella R, Secondo A, Boscia F, Pannaccione A, Scorziello A, Mehdawy B, Sokolow S, Herchuelz A, Di Renzo GF, Annunziato L (2011). Na+–Ca2+ exchanger (NCX3) knock-out mice display an impairment in hippocampal long-term potentiation and spatial learning and memory. *J Neurosci* 31, 7312–7321.

Montag D, Giese KP, Bartsch U, Martini R, Lang Y, Blüthmann H, Karthigasan J, Kirschner DA, Wintergerst ES, Nave KA, et al. (1994). Mice deficient for the myelin-associated glycoprotein show subtle abnormalities in myelin. *Neuron* 13(1):229-246.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12: 529-540.

Morell P, Barrett CV, Mason JL, Toews AD, Hostettler JD, Knapp GW, Matsushima GK (1998) Gene expression in the brain during cuprizone-induced demyelination and remyelination. *Mol Cell Neurosci* 12:220-227.

Mori S, Leblond CP (1970). Electron microscopic identification of three classes of oligodendrocytes and a preliminary study of their proliferative activity in the corpus callosum of young rats. *J Comp Neurol* 139(1):1–28.

Morikawa A, Hamase K, Inoue T, Konno R, Zaitsu K (2007). Alterations in D-amino acid levels in the brains of mice and rats after the administration of D-amino acids. *Amino Acids* 32(1):13–20.

Morris-Downes MM, McCormack K, Baker D, Sivaprasad D, Natkunarajah J, Amor S (2002). Encephalitogenic and immunogenic potential of myelin-associated glycoprotein (MAG),oligodendrocyte-specific glycoprotein (OSP) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) in ABH and SJL mice. *J Neuroimmunol 122*, 20-33.

Mothet JP, Snyder SH. (2012). Brain D-amino acids: a novel class of neuromodulators. *Amino Acids* 43(5):1809-1810.

Myllykoski M, Seidel L, Muruganandam G, Raasakka A, Torda AE, Kursula P (2016). Structural and functional evolution of 2',3'-cyclic nucleotide 3'-phosphodiesterase. Brain Res 1641(Pt A):64-78.

Naik P, Cucullo L (2012). *In vitro* blood-brain barrier models: Current and perspective technologies *J Pharm Sci 101,* 1337- 1354.

Nathoo N, Yong VW, Dunn JF (2014). Understanding disease processes in multiple sclerosis through magnetic resonance imaging studies in animal models. *Neuroimage Clin* 4:743-756.

National Multiple Sclerosis Society web site: http://www.nationalmssociety.org

Nave KA, Lai C, Bloom FE, Milner RJ (1987): Splice site selection in the proteolipid protein (PLP) gene transcript and primary structure of the dm-20 protein of central nervous system myelin. *Proc Natl Acad Sci USA* 84: 5665-5669.

Neidle A, Dunlop DS (1990). Developmental changes in free D-aspartic acid in the chicken embryo and in the neonatal rat. *Life Sci* 46(21):1517–1522.

Nicolay DJ, Doucette JR, Nazarali AJ (2007). Transcriptional control of oligodendrogenesis. *Glia*, 55: 1287–1299.

Nicoll DA, Longoni S, Philipson KD (1990). Molecular cloning and functional expression of the cardiac sarcolemmal Na^{+}-Ca^{2+} exchanger. *Science* 250: 562–565.

Nicoll DA, Ottolia M, Lu L, Lu Y, Philipson KD (1999). A new topological model of the cardiac sarcolemmal Na+-Ca2+ exchanger. *J Biol Chem* 274: 910–917.

Nicoll DA, Quednau BD, Qui Z, Xia YR, Lusis AJ, Philipson KD (1996). Cloning of a third mammalian Na^{+}-Ca^{2+} exchanger, NCX3. *J Biol Chem* 271: 24914–24921.

Nicoll DA,Hryshko LV,Matsuoka S, Frank JS, Philipson KD (1996).Mutation of amino acid residues in the putative transmembrane segments of the cardiac sarcolemmal Na+-Ca2+ exchanger. *J Biol Chem* 271:13385–13391.

Nishiyama A, Komitova M, Suzuki R, Zhu X (2009). Polydendrocytes (NG2 cells): Multifunctional cells with lineage plasticity. *Nature Reviews Neuroscience* 10(1): 9–22.

Nishiyama A, Lin XH, Giese N, Heldin CH, Stallcup WB (1996). Interaction between NG2 proteoglycan and PDGF alpha-receptor on O2A progenitor cells is required for optimal response to PDGF. *J Neurosci Res* 43(3):315-330.

Niswender CM, Conn PJ (2010). Metabotropic Glutamate Receptors: Physiology, Pharmacology, and Disease. *Annual review of pharmacology and toxicology* 50:295-322.

Noll E, Miller RH (1993) Oligodendrocyte precursors originate at the ventral ventricular zone dorsal to the ventral midline region in the embryonic rat spinal cord. *Development* 118:563–573

Noseworthy JH, Lucchinetti C, Rodríguez M, Weinshenker BG (2000). Multiple sclerosis. *N Engl J Med.* 343, 938–952.

Noseworthy JH, Lucchinetti C, Rodríguez M, Weinshenker BG (2000). Multiple sclerosis. *N Engl J Med.* 343, 938–952.

Novak N, Bar V, Sabanay H, Frechter S, Jaegle M, Snapper SB, Meijer D, Peles E (2011). N-WASP is required for membrane wrapping and myelination by Schwann cells. *J Cell Biol* 192(2):243-250.

Ogawa T, Fukuda M, Sasaoka K (1973). Occurrence of D-amino acid aminotransferase in pea seedlings. *Biochem Biophys Res Commun* 52:998–1002.

Olah M, Raj D, Brouwer N, De Haas AH, Eggen BJ, Den Dunnen WF, Biber KP, Boddeke HW (2012) An optimized protocol for the acute isolation of human microglia from autopsy brain samples *Glia 60,* 96-111

Olsen ML, Sontheimer H (2008). Functional implications for Kir4.1 channels in glial biology: from K+ buffering to cell differentiation. *J Neurochem* 107(3), 589-601.

Ota N, Shi T, Sweedler JV (2012). D-aspartate acts as a signaling molecule in nervous and neuroendocrine systems. *Amino acids*.;43(5):1873-1886.

Ota N, Shi T, Sweedler JV (2012). D-Aspartate acts as a signaling molecule in nervous and neuroendocrine systems. *Amino Acids* 43, 1873-1886.

Othman T, Yan H, Rivkees SA (2003). Oligodendrocytes express functional A1 adenosine receptors that stimulate cellular migration. *Glia* 44(2),166-172.

Ottolia M, Nicoll DA, Philipson KD (2005). Mutational analysis of the alpha-1 repeat of the cardiac Na+-Ca2+ exchanger. *J Biol Chem* 280:1061–1069.

Paez PM, Cheli VT, Ghiani CA, Spreuer V, Handley VW, Campagnoni AT (2012). Golli myelin basic proteins stimulate oligodendrocyte progenitor cell proliferation and differentiation in remyelinating adult mouse brain. *Glia* 60(7):1078-1093.

Paez PM, Fulton D, Colwell CS, Campagnoni AT (2009a). Voltage-operated $Ca^{(2+)}$ and $Na^{(+)}$ channels in the oligodendrocyte lineage. *J Neurosci Res* 87(15), 3259-3266.

Paez PM, Fulton DJ, Spreuer V, Handley V, Campagnoni CW, Macklin WB, Colwell C, Campagnoni AT (2009b). Golli myelin basic proteins regulate oligodendroglial progenitor cell migration through voltage-gated Ca^{2+} influx. *J Neurosci* 29(20), 6663-6676.

Paez PM., Spreuer V, Handley V, Feng JM, Campagnoni C, Campagnoni AT (2007). Increased expression of golli myelin basic proteins enhances calcium influx into oligodendroglial cells. *J Neurosci* 27(46), 12690-12699.

Paoletti P, Bellone C, Zhou Q (2013). NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat Rev Neurosc* 14: 383-400.

Paoletti P, Bellone C, Zhou Q (2013). NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat Rev Neurosci* 14(6):383-400.

Papouin T, Ladepeche L, Ruel J, Sacchi S, Labasque M, Hanini M, Groc L, Pollegioni L, Mothet JP, Oliet SH (2012). Synaptic and extrasynaptic NMDA receptors are gated by different endogenous coagonists. *Cell* 150: 633-646.

Patneau DK, Wright PW, Winters C, Mayer ML, Gallo V (1994). Glial cells of the oligodendrocyte lineage express both kainate- and AMPA-preferring subtypes of glutamate receptor. *Neuron* 12:357-371.

Patterson M, Sneyd J, Friel DD (2007). Depolarization-induced calcium responses in sympathetic neurons: relative contributions from Ca2+ entry, extrusion, ER/mitochondrial Ca2+ uptake and release, and Ca2+ buffering. *The Journal of General Physiology* 129 (1): 29–56.

Paul LK, Brown WS, Adolphs R, Tyszka JM, Richards LJ, Mukherjee P, Sherr EH (2007) Agenesis of the corpus callosum: genetic, developmental and functional aspects of connectivity. *Nat Rev Neurosci* 8:287–299.

Pérez-Cerdá F, Sánchez-Gómez MV, Matute C (2015). Pío del Río Hortega and the discovery of the oligodendrocytes. *Frontiers in Neuroanatomy* 9:92.

Peters A, Palay S, Webster H DE F (1991). The Fine Structure of the Nervous System: the Neuron and the Supporting Cells. *Oxford, UK: Oxford Univ. Pres.*

Petzold A (2013). Intrathecal oligoclonal IgG synthesis in multiple sclerosis. *J Neuroimmunol* 262, 1–10.

Pham-Dinh D, Della Gaspera B, Kerlero DE, Rosbo N, Dautigny A (1995). Human myelin/oligodendrocyte glycoprotein gene: alternative splicing generates multiple isoforms. *Genomics* 29: 345–352.

Pham-Dinh D, Mattei MG, Nussbaum JL, Roussel G, Pontarotti P, Roeckel N, Mather IH, Artzt K, Lindahl KF, Dautigny A (1993). Myelin/oligodendrocyte glycoprotein is a member of a subset of the immunoglobulin superfamily encoded within the major histocompatibility complex. *Proc Natl Acad Sci U S A.* 190(17):7990-7994.

Philipson KD, Longoni S, Ward R (1988). Purification of the cardiac Na^{+}-Ca^{2+} exchange protein. *Biochim. Biophys. Acta* 945, 298–306.

Philipson KD, Nicoll DA (2000). Sodium-calcium exchange: a molecular perspective. *Annu Rev Physiol* 62: 111–133.

Philipson KD, Nicoll DA, Ottolia M, Quednau BD, Reuter H, John S, and Qiu Z (2002). The Na+/Ca2+ exchange molecule: an overview. *Ann N Y Acad Sci* 976: 1–10.

Piaton G, Aigrot MS, Williams A, Moyon S, Tepavcevic V, Moutkine I, Gras J, Matho KS, Schmitt A, Soellner H, Huber AB, Ravassard P, Lubetzki C (2011) Class 3 semaphorins influence oligodendrocyte precursor recruitment and remyelination in adult central nervous system. *Brain* 134:1156–1167.

Pignataro G, Sirabella R, Anzilotti S, Di Renzo G, Annunziato L (2014). Does Na^{+}/Ca^{2+} exchanger, NCX, represent a new druggable target in stroke intervention? *Transl Stroke Res* 5(1):145-155.

Pleasure SJ, Page C, Lee VM (1992) Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons *J Neurosci 12*, 1802-1815

Podbielska M, Banik NL, Kurowska E, Hogan EL (2013). Myelin Recovery in Multiple Sclerosis: The Challenge of Remyelination Brain Sci 3(3), 1282-1324.

Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, Fujihara K, Havrdova E, Hutchinson M, Kappos L, Lublin FD, Montalban X, O'Connor P, Sandberg-Wollheim M, Thompson AJ, Waubant E, Weinshenker B, Wolinsky JS (2011). Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* 69(2):292-302.

Popescu BF, Lucchinetti CF (2012). Pathology of demyelinating diseases. *Annu Rev Pathol* 7:185-217.

Popescu BFG, Pirko I, Lucchinetti CF (2013). Pathology of Multiple Sclerosis: Where Do We Stand? *Continuum : Lifelong Learning in Neurology* 19(4 Multiple Sclerosis):901-921.

Poser CM (1986). Pathogenesis of multiple sclerosis. A critical reappraisal. *Acta Neuropathol* 71:1–10.

Presslauer S, Milosavljevic D, Huebl W, Parigger S, Schneider-Koch G, Bruecke T(2014). Kappa free light chains: diagnostic and prognostic relevance in MS and CIS. *PLoS ONE* 9, e89945.

Prineas JW, Parratt JD (2012). Oligodendrocytes and the early multiple sclerosis lesion. *Ann Neurol* 72, 18–31.

Pringle NP, Mudhar HS, Collarini EJ, Richardson WD (1992). PDGF receptors in the rat CNS: during late neurogenesis, PDGF alpha-receptor expression appears to be restricted to glial cells of the oligodendrocyte lineage. *Development* 115(2):535-551.

Privat A, Jacque C, Bourre JM, Dupouey P, Baumann N (1979). Absence of the major dense line in myelin of the mutant mouse ‘shivere’. *Neurosci Lett* 12: 107-112.

Pulliam L, Stubblebine M, Hyun W (1998) Quantification of neurotoxicity and identification of cellular subsets in a threedimensional brain model *Cytometry 32*, 66-69

Qi Y, Stapp D, Qiu M (2002) Origin and molecular specification of oligodendrocytes in the telencephalon. *Trends Neurosci* 25:223–225.

Quarles RH (1997). Glycoprotein of myelin sheaths. *J Mol Neurosci* 8:1–12.

Quednau BD, Nicoll DA, Philipson KD (1997). Tissue specificity and alternative splicing of the Na+/Ca2+ exchanger isoforms NCX1, NCX2, and NCX3 in rat. *Am J Physiol* 272(4 Pt 1):C1250-261.

Raasakka A, Kursula P (2014).Themyelinmembrane-associated enzyme 2’3’-cyclic nucleotide-3’-phosphodiesterase:on a high way to structure and function.*Neurosci Bull* 30,956–966.

Raff MC, Williams BP, Miller RH (1984) The *in vitro* differentiation of a bipotential glial progenitor cell *EMBO J 3*, 1857-1864.

Ramon Y Cajal (1913). Contribucion al conocimiento de la neuroglia del cerebro humano. *Trab Lab Invest Biol (Madrid)* 11: 255–315.

Rasband MN, Tayler J, Kaga Y, Yang Y, Lappe-Siefke C, Nave KA, Bansal R (2005). CNP is required for maintenance of axon–glia interactions at nodes of Ranvier in the CNS. *Glia* 50, 86–90.

Raucci F, Di Fiore MM (2010). The maturation of oocyte follicular epithelium of Podarcis s. sicula is promoted by D-aspartic acid. *J Histochem Cytochem* 58(2):157–171.

Reuter H, Seitz N (1968). The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *J Physiol* 195:451– 470.

Reynolds KD (2004). 'D'Este, Sir Augustus Frederick (1794–1848)', *Oxford Dictionary of National Biography*, Oxford University Press.

Reynolds R, Wilkin GP (1988). Development of macroglial cells in rat cerebellum. II. An in situ immunohistochemical study of oligodendroglial lineage from precursor to mature myelinating cell. *Development* 102,409–425.

Rhodes KE, Raivich G, Fawcett JW (2006) The injury response of oligodendrocyte precursor cells is induced by platelets, macrophages and inflammation-associated cytokines. *Neuroscience* 140:87–100.

Richardson WD, Kessaris N, Pringle N (2006). Oligodendrocyte wars. *Nat Rev Neurosci* 7(1):11–18.

Rio Hortega DP (1921). Histogenesis y evolucion normal; exodo y distribucion regional de la microglia. *Memor Real Soc Esp Hist Nat* 11: 213–268.

Rio Hortega DP (1928). Tercera aportacion al conocimiento morfologico e interpretacion funcional de la oligodendroglia. *Memor Real Soc. Esp Hist Nat* 14: 5–122.

Rivers T, Sprunt DH, Berry GP (1933). Observations on attempts to produce acute disseminated encephalomyelitis in monkeys. J Exp Med 58:39–53.

Rogers JM, Robinson AP, Miller SD (2013). Strategies for Protecting Oligodendrocytes and Enhancing Remyelination in Multiple Sclerosis. *Discov Med* 16(86):53-63.

Rojiani AM, Prineas JW, Cho ES (1994). Electrolyte-induced demyelination in rats. Role of the blood-brain barrier and edema. *Acta Neuropathol 88,* 287-292.

Rosenbluth J and Schiff R (2009). Spinal cord dysmyelination caused by an antiproteolipid protein IgM antibody: implications for the mechanism of central nervous system myelin formation. *J Neurosci Res 87,* 956-963.

Rosenbluth J, Nave KA, Mierzwa A and Schiff R (2006). Subtle myelin defects in PLPnull mice. *Glia* 54: 172-182.

Rosenbluth J, Schiff R, Liang WL, Dou W (2003). Antibodymediated CNS demyelination II. Focal spinal cord lesions induced by implantation of an IgM antisulfatide-secreting hybridoma. *J Neurocytol 32,* 265-276.

Rowitch DH (2004) Glial specification in the vertebrate neural tube. *Nat Rev Neurosci* 5:409–419

Ruiz PJ, Garren H, Hirschberg DL (1999). Microbial epitopes act as altered peptide ligands to prevent experimental autoimmune encephalomyelitis. *J Exp Med* 189(8): 1275-1284.

Saab AS, Tzvetavona ID, Trevisiol A, Baltan S, Dibaj P, Kusch K, Möbius W, Goetze B, Jahn HM, Huang W, Steffens H, Schomburg ED, Pérez-Samartín A, Pérez-Cerdá F, Bakhtiari D, Matute C, Löwel S, Griesinger C, Hirrlinger J, Kirchhoff F, Nave KA (2016). Oligodendroglial NMDA Receptors Regulate Glucose Import and Axonal Energy Metabolism. *Neuron* 6;91(1):119-132.

Sachs HH, Bercury KK, Popescu DC, Narayanan SP, and Macklin WB. (2014). A New Model of Cuprizone-Mediated Demyelination/Remyelination. *ASN Neuro 6*(5).

Saher G, Brügger B, Lappe-Siefke C, Möbius W, Tozaw Ra, Wehr MC, Wieland F, Ishibashi S, Nave KA (2005). High cholesterol level is essential for myelin membrane growth *Nature Neuroscience* 8, 468-475.

Sakai K, Homma H, Lee JA, Fukushima T, Santa T, Tashiro K, Iwatsubo T, Imai K (1998). Emergence of Daspartic acid in the differentiating neurons of the rat central nervous system. *Brain Res* 808(1):65–71.

Salter MG, Fern R (2005). NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury. *Nature* 438(7071), 1167-1171.

Salvetti M, Giovannoni G, and Aloisi F (2009). Epstein-Barr virus and multiple sclerosis. *Curr Opin Neurol* 22(3): 201-206.

Sanz-Clemente A, Nicoll RA, Roche KW (2013). Diversity in NMDA receptor composition: many regulators, many consequences. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* 19(1):62-75.

Sarbu N, Shih RY, Jones RV et-al. (2016). White Matter Diseases with Radiologic-Pathologic Correlation. *Radiographics* 36 (5):1426-1447.

Savage DD, Galindo R, Queen SA, Paxton LL, Allan AM (2001). Characterization of electrically evoked H-3-D-aspartate release from hippocampal slices. *Neurochem Int* 38(3):255–267.

Sawcer S, Franklin RJ, Ban M (2014). Multiple sclerosis genetics. *Lancet Neurol* 13, 700–709.

Saxe DF, Takahashi N, Hood L, Simon MI (1985). Localization of the human myelin basic protein gene (MBP) to region 18q22----qter by in situ hybridization. *Cytogenet. Cell Genet*. 39 (4): 246–249.

Scanlan C, Shi T, Hatcher NG, Rubakhin SS, Sweedler JV (2010). Synthesis, accumulation, and release of Daspartate in the Aplysia californica CNS. *J Neurochem* 115(5):1234–1244.

Schalomon PM and Wahlsten D (2002). Wheel running behavior is impaired by both surgical section and genetic absence of the mouse corpus callosum. *Brain Res Bull* 57, 27–33.

Schwab ME, Schnell L (1989). Region-specific appearance of myelin constituents in the developing rat spinal cord. *J Neurocytol* 18:183–190.

Scolding NJ, Frith S, Linington C, Morgan BP, Campbell AK, Compston DA (1989). Myelin-oligodendrocyte glycoprotein (MOG) is a surfacemarker of oligodendrocyte maturation, *J. Neuroimmunol* 22,169–176.

Senel M, Tumani H, Lauda F, Presslauer S, Mojib-Yezdani R, Otto M, Brettschneider J (2014). Cerebrospinal fluid immunoglobulin kappa light chain in clinically isolated syndrome and multiple sclerosis. *PLoS ONE* 9, e88680.

Setoyama C and Miura R (1997). Structural and functional characterization of the human brain D-aspartate oxidase. *J Biochem* 121 (4):798–803.

Sharifi K, Ebrahimi M, Kagawa Y, Islam A, Tuerxun T, Yasumoto Y, Hara T, YamamotoY, Miyazaki H, Tokuda N, Yoshikawa T, Owada Y (2013). Differential expression and regulatory roles of FABP5 and FABP7 in oligodendrocyte lineage cells. *Cell Tissue Res* 354 683–695.

Sharp J, Hatch M, Nistor G, Keirstead H (2011) Derivation of oligodendrocyte progenitor cells from human embryonic stem cells *Methods Mol Biol 767*, 399-409.

Shen S, Sandoval J, Swiss V, Li J, Dupree J, Franklin RJM, Casaccia-Bonnefil P (2008) Age dependent epigenetic control of differentiation inhibitors: a critical determinant of remyelination efficiency. *Nat Neurosci* 11:1024–1034.

Sherwin AL (1957). Multiple sclerosis in historical perspective. *McGill Med J* 26:39–48.

Simonic T, Duga S, Negri A, Tedeschi G, Malcovati M, Tenchini ML, Ronchi S (1997). cDNA cloning and expression of the flavoprotein D-aspartate oxidase from bovine kidney cortex. *Biochem J* 322:729–735.

Simons M, Krämer EM, Thiele C, Stoffel W, Trotter J (2000). Assembly of myelin by association of proteolipid protein with cholesterol- and galactosylceramide-rich membrane domains. *J Cell Biol* 151(1):143-154.

Sipido KR, Varro A, Eisner D (2006). Sodium calcium exchange as a target for antiarrhythmic therapy. *Handb Exp Pharmacol* 159–199.

Sipido KR, Volders PG, Vos MA, Verdonck F (2002). Altered Na+/Ca2+ exchange activity in cardiac hypertrophy and heart failure: a new target for therapy? *Cardiovasc Res* 53, 782–805.

Skeberdis VA, Lan J, Opitz T, Zheng X, Bennett MV, Zukin RS (2001). mGluR1-mediated potentiation of NMDA receptors involves a rise in intracellular calcium and activation of protein kinase C. *Neuropharmacology* 40 (7): 856–865.

Smith KJ, Blakemore WF, McDonald WI (1979) Central remyelination restores secure conduction. *Nature* 280:395–396.

Snaidero N, Möbius W, Czopka T, Hekking LH, Mathisen C, Verkleij D, Goebbels S, Edgar J, Merkler D, Lyons DA, Nave KA, Simons M (2014). Myelin membrane wrapping of CNS axons by PI(3,4,5)P3-dependent polarized growth at the inner tongue. *Cell* 156(1-2):277-290.

Sokolow S, Manto M, Gailly P, Molgó J, Vandebrouck C, Vanderwinden JM, Herchuelz A, Schurmans S (2004). Impaired neuromuscular transmission and skeletal muscle fiber necrosis in mice lacking Na/Ca exchanger 3. *J Clin Invest* 113, 265–273.

Sommer B, Kohler M, Sprengel R, Seeburg PH (1991). RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell* 67: 11-19.

Sommer I, Schachner M (1981). Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. *Dev Biol* 30;83(2):311-327.

Sontheimer H, Trotter J, Schachner M and Kettenmann H (1989). Channel expression correlates with differentiation stage during the development of oligodendrocytes from their precursor cells in culture. *Neuron* 2(2), 1135-1145.

Sontheimer H, Trotter J, Schachner M, Kettenmann H (1989). Channel expression correlates with differentiation stage during the development of oligodendrocytes from their precursor cells in culture. *Neuron* 2(2),1135-1145.

Spassky N, Olivier C, Perez-Villegas E, Goujet-Zalc C, Martinez S, Thomas Jl, Zalc, B (2000). Single or multiple oligodendroglial lineages: a controversy. *Glia* 29, 143–148 (2000).

Spinelli P, Brown ER, Ferrandino G, Branno M, Montarolo PG, D'Aniello E, Rastogi RK, D'Aniello B, Baccari GC, Fisher G, D'Aniello A. D-aspartic acid in the nervous system of Aplysia limacina: Possible role in neurotransmission (2006). *J Cell Physiol* 206(3):672–681.

Spinelli P, Brown ER, Ferrandino G, Branno M, Montarolo PG, D'Aniello E, Rastogi RK, D'Aniello B, Baccari GC, Fisher G, D'Aniello A (2006). D-aspartic acid in the nervous system of Aplysia limacina: Possible role in neurotransmission. *J Cell Physiol* 206(3):672–681.

Spitzer S, Volbracht K, Lundgaard I, Káradóttir RT (2016). Glutamate signalling: A multifaceted modulator of oligodendrocyte lineage cells in health and disease. *Neuropharmacology* 110(Pt B):574-585.

Stadelmann C, Wegner C, Brück W (2011). Inflammation, demyelination, and degeneration — Recent insights from MS pathology. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* Volume 1812, Issue 2, 275-282.

Stangel M, Fredrikson S, Meinl E, Petzold A, Stüve O, Tumani H (2013). The utility of cerebrospinal fluid analysis in patients with multiple sclerosis. *Nat Rev Neurol* 9(5):267-276.

Steinman L, Zamvil S (2003). Transcriptional analysis of targets in multiple sclerosis. *Nature Reviews Immunology* 3, 483-492.

Stevens B, Porta S, Haak LL, Gallo V, Fields RD (2002). Adenosine: A neuronglial transmitter promoting myelination in the CNS in response to action potentials. *Neuron* 36:855-868.

Stidworthy MF, Genoud S, Suter U, Mantei N, Franklin RJ (2003). Quantifying the early stages of remyelination following cuprizone-induced demyelination. *Brain Pathol* 13:329–339.

Stolt CC, Schlierf A, Lommes P, et al. SoxD proteins influence multiple stages of oligodendrocyte development and modulate SoxE protein function. Dev Cell. 2006; 11: 697–709.

Strober W (2001). Trypan blue exclusion test of cell viability. *Curr Protoc Immunol* Appendix 3:Appendix 3B.

Sundberg M, Skottman H, Suuronen R, Narkilahti S (2010) Production and isolation of NG2+ oligodendrocyte precursors from human embryonic stem cells in defined serum-free medium *Stem Cell Res 5*, 91-103.

Tait AR and Straus SK (2008). Phosphorylation of U24 from Human Herpes Virus type 6 (HHV-6) and its potential role in mimicking myelin basic protein (MBP) in multiple sclerosis. *FEBS Lett* 582; 582(18): 2685-2688.

Tauheed AM, Ayo JO, Kawu MU (2016). Regulation of oligodendrocyte differentiation: Insights and approaches for the management of neurodegenerative disease.*Pathophysiology* 23(3):203-210.

Teunissen CE, Malekzadeh A, Leurs C, Bride Cl and Killestein J (2015). Body fluid biomarkers for multiple sclerosis —the long road to clinical application. *Nat Rev Neurol* 11, 585–596.

Teunissen CE, Malekzadeh A, Leurs C, Bride Cl and Killestein J (2015). Body fluid biomarkers for multiple sclerosis -the long road to clinical application. *Nat Rev Neurol* 11, 585–596.

Thiessen JD, Zhang Y, Zhang H, Wang L, Buist R, Del Bigio MR, Kong J, Li XM and Martin M(2013).. Quantitative MRI and ultrastructural examination of the cuprizone mouse model of demyelination. *NMR Biomed* 26:1562–1581.

Thomas JL, Spassky N, Perez Villegas EM, Olivier C, Cobos I, Goujet-Zalc C, Martinez S, Zalc B (2000) Spatiotemporal development of oligodendrocytes in the embryonic brain. *J Neurosci Res* 59:471– 476.

Tkachev D, Mimmack ML, Huffaker SJ, Ryan M, Bahn S (2007). Further evidence for altered myelin biosynthesis and glutamatergic dysfunction in schizophrenia. *Int J Neuropsychopharmacol* 10 (4): 557–563.

Torkildsen O, Brunborg LA, Myhr KM, Bø L (2008), The cuprizone model for demyelination. *Acta Neurol Scand Suppl* 188:72-76.

Tracy ET, Zhang CY, Gentry T, Shoulars KW, Kurtzberg J (2011) Isolation and expansion of oligodendrocyte progenitor cells from cryopreserved human umbilical cord blood *Cytotherapy 13*, 722-729.

Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mörk S, Bö L (1998). Axonal transection in the lesions of multiple sclerosis. *N Engl J Med* 338:278-285.

Trotter J (1993) The development of myelin-forming glia: studies with primary cell cultures and immortalized cell lines *Perspect Dev Neurobiol 1*, 149-154.

Vacca RA, Giannattasio S, Graber R, Sandmeier E, Marra E, Christen P (1997). Active-site Arg->Lys substitutions alter reaction and substrate specificity of aspartate aminotransferase. *J Biol Chem* 272(35):21932–21937.

Vallstedt A, Klos JM, Ericson J (2005) Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain. *Neuron* 45:55–67

van der Valk P, De Groot CJ (2000). Staging of multiple sclerosis (MS) lesions: pathology of the time frame of MS. *Neuropathol Appl Neurobiol* 26:2-10.

Van Eylen F, Svoboda M, Herchuelz A (1997). Identification, expression pattern and potential activity of Na/Ca exchanger isoforms in rat pancreatic b-cells. *Cell Calcium* 21, 185–193.

Van Eylen F, Svoboda M, Herchuelz A (1997). Identification, expression pattern and potential activity of Na/Ca exchanger isoforms in rat pancreatic b-cells. *Cell Calcium* 21, 185–193.

Vanveldhoven PP, Brees C, Mannaerts GP (1991). D-aspartate oxidase, a peroxisomal enzyme in liver of rat and man. *Biochim Biophys Acta* 1073(1):203–208.

Verdoorn TA, Burnashev N, Monyer H, Seeburg PH, Sakmann B (1991). Structural determinants of ion flow through recombinant glutamate receptor channels. *Science* 252: 1715-1718.

Verdoorn TA, Dingledine R (1988). Excitatory amino acid receptors expressed in Xenopus oocytes: agonist pharmacology. *Mol Pharmacol* 34, 298-307.

Vereyken EJ, Fluitsma DM, Bolijn MJ, Dijkstra CD, Teunissen CE (2009) An *in vitro* model for de- and remyelination using lysophosphatidyl choline in rodent whole brain spheroid cultures *Glia 57*, 1326-1340.

Verkhratsky A, Kirchhoff F (2007). Glutamate-mediated neuronal–glial transmission. *Journal of Anatomy* 210(6):651-660.

Vincze A, Mazlo M, Seress L, Komoly S, Abraham H (2008). A correlative light and electron microscopic study of postnatal myelination in the murine corpus callosum. *Int J Dev Neurosci 26*, 575-584.

Virchow R (1846). Ueber das granulirte Aussehen der Wandungen der Gehirnventrikel. *Allg Z Psychiat* 3: 242–250.

Virchow R (1854). Über das ausgebreitete Vorkommen einer dem Nervenmark analogen Substanz in den tierischen Geweben. *Archiv für pathologische Anatomie und Physiologie und für klinische Medizin* (in German) 6 (4): 562–572.

Virtanen JO, Jacobson S (2012). Viruses and multiple sclerosis. *CNS Neurol Disord Drug Targets 11*(5)

Vogel US, Thompson RJ (1988). Molecular structure, localization and possible functions of the myelin-associated enzyme 2'3'-cyclic nucleotide-3'-phosphodiesterase. *J Neurochem* 50: 1667–1677.

Voyvodic JT (1989).Target size regulates calibre and myelination of sympathetic axons. *Nature* 1989: 342:430-433.

Vyklicky V, Korinek M, Smejkalova T, Balik A, Krausova B, Kaniakova M, Lichnerova K, Cerny J, Krusek J, Dittert I, Horak M, Vyklicky L (2014). Structure, function, and pharmacology of NMDA receptor channels. *Physiol Res* 63 Suppl 1:S191-203.

Wake H, Lee PR, Fields RD (2011). Control of local protein synthesis and initial events in myelination by action potentials. *Science* (New York, NY) 333(6049):1647-1651.

Wang C, Pralong WF, Schulz MF, Rougon G, Aubry JM, Pagliusi S, Robert A, Kiss JZ (1996). Functional N-methyl-D-aspartate receptors in O-2A glial precursor cells: a critical role in regulating polysialic acid-neural cell adhesion molecule expression and cell migration. *J Cell Biol* 135(6), 1565-1581.

Wang H, Kunkel DD, Martin TM, Schwartzkroin PA, Tempel BL (1993). Heteromultimeric K+ channels in terminal and juxtaparanodal regions of neurons. *Nature*, 365(6441), 75-79.

Wang H, Yan Y, Kintner DB, Lytle C, Sun D (2003). GABA-mediated trophic effect on oligodendrocytes requires Na-K-2Cl cotransport activity. *J Neurophysiol* 90(2), 1257-1265.

Wang S, Sdrulla AD, diSibio G, Bush G, Nofziger D, Hicks C, Weinmaster G, Barres BA (1998). Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* 21(1), 63-75.

Wang SZ, Dulin J, Wu, Hurlock E, Lee SE, Jansson K, Lu QR (2006). An oligodendrocyte-specific zinc-finger transcription regulator cooperates with Olig2 to promote oligodendrocyte differentiation. *Development* 133, 3389–3398.

Wang W, Gao XF, Xiao L, Xiang ZH, He C (2011). K(V)7/KCNQ channels are functionally expressed in oligodendrocyte progenitor cells. *PLoS One* 6(7):e21792.

Wang X, Arcuino G, Takano T, Lin J, Peng WG, Wan P, Li P, Xu Q, Liu QS, Goldman SA, Nedergaard M (2004). P2X7 receptor inhibition improves recovery after spinal cord injury. *Nat Med* 10(8), 821-827.

Warf BC, Fok-Seang J, Miller RH (1991) Evidence for the ventral origin of oligodendrocyte precursors in the rat spinal cord. *J Neurosci* 11:2477–2488

Watanabe M, Hadzic T, Nishiyama A (2004) Transient upregulation of Nkx2.2 expression in oligodendrocyte lineage cells during remyelination. *Glia* 46:311–322.

Waxman SG, Bennet MV (1972). Relative conduction velocities of small myelinated and non-myelinated fibres in the central nervous system. *Nat New Biol* 1972: 238:217-219.

Wegner M, Stolt CC (2005). From stem cells to neurons and glia: a Soxist's view of neural development. *Trends Neurosci* 28: 583–588.

Wenzel A, Fritschy Jm, Mohler H, Benke D (1997). NMDA receptor heterogeneity during postnatal development of the rat brain: differential expression of the NR2A, NR2B, and NR2C subunit proteins. *J Neurochem* 68:469-478.

Wilhelm I, Fazakas C, Krizbai IA (2011). *In vitro* models of the bloodbrain barrier *Acta Neurobiol Exp (Wars) 71*, 113-128.

Wilkins A, Majed H, Layfield R, Compston A, Chandran S (2003). Oligodendrocytes promote neuronal survival and axonal length by distinct intracellular mechanisms: a novel role for oligodendrocyte-derived glial cell line-derived neurotrophic factor. *J Neurosci Off J Soc Neurosci* 23(12):4967–4974.

William D. Richardson, Nicoletta Kessaris, Nigel Pringle (2006) Oligodendrocyte wars *Nature Reviews Neuroscience* 7, 11-18.

Wolfgram F (1966). A new proteolipid fraction of the nervous system. Isolation and amino acid analysis. *J Neurochem* 13: 461–470.

Wollmuth LP, Sobolevsky AI (2004). Structure and gating of the glutamate receptor ion channel. *Trends Neurosci* 27(6), 321-328.

Wolosker H, D'Aniello A, Snyder SH (2000). D-aspartate disposition in neuronal and endocrine tissues: Ontogeny, biosynthesis and release. *Neuroscience* 100(1):183–189.

Wolosker H, Dumin E, Balan L, Foltyn VN (2008). D-amino acids in the brain: D-serine in neurotransmission and neurodegeneration. *FEBS J* 275(14):3514-3526.

Wolswijk G, Noble M (1989) Identification of an adult-specific glial progenitor cell. *Development* 105:387–400.

Wolswijk G, Noble M (1992) Cooperation between PDGF and FGF converts slowly dividing O-2Aadult progenitors to rapidly dividing cells with characteristics of O-2Aperinatal progenitor cells. *J Cell Biol* 118:889–900.

Wood DD, Moscarello MA (1989). The isolation, characterization and lipid-aggregating properties of a citrulline containing myelin basic protein. *J Biol Chem* 264: 5121–5127.

Young KM, Psachoulia K, Tripathi RB, Dunn SJ, Cossell L, Attwell D, Tohyama K,Richardson WD (2013). Oligodendrocyte dynamics in the healthy adult CNS: evidence for myelin remodeling. *Neuron* 77, 873–885.

Yu WP, Collarini EJ, Pringle NP, Richardson WD (1994). Embryonic expression of myelin genes: evidence for a focal source of oligodendrocyte precursors in the ventricular zone of the neural tube. *Neuron 12*(6):1353-1362.

Zhang J, Ren C, Chen L, Navedo MF, Antos LK, Kinsey SP, Iwamoto T, Philipson KD, Kotlikoff MI, Santana LF, Wier WG, Matteson DR, Blaustein MP (2010). Knockout of Na+/Ca2+ exchanger in smooth muscle attenuates vasoconstriction and L-type Ca2+ channel current and lowers blood pressure. *Am J Physiol Heart Circ Physiol* 298, H1472–H1483.

Zhang SC(2001). Defining glial cells during CNS development. *Nat Rev Neurosci* 2,840–843.

Zhang Z, Drzewiecki GJ, Hom JT, May PC, Hyslop PA (1994) Human cortical neuronal (HCN) cell lines: a model for amyloid beta neurotoxicity *Neurosci Lett 177*, 162-164

Zhu S and Gouaux E (2017). Structure and symmetry inform gating principles of ionotropic glutamate receptors *Neuropharmacology* Volume 112, Part A, pages 11–15.

Ziskin JL, Nishiyama A, Rubio M, Fukaya M, Bergles DE (2007). Vesicular release of glutamate from unmyelinated axons in white matter. *Nat Neurosci* 10:321-330.

Zuchero JB, Barres BA (2013) Intrinsic and extrinsic control of oligodendrocyte development. Current opinion in neurobiology 23:914-920.

Zulian A, Baryshnikov SG, Linde CI, Hamlyn JM, Ferrari P, Golovina VA (2010). Upregulation of Na+/Ca2+ exchanger and TRPC6 contributes to abnormal Ca2+ homeostasis in arterial smooth muscle cells from Milan hypertensive rats. *Am J Physiol Heart Circ Physiol* 299, H624–H633.