Redox signal transduction in oligodendrocytes differentiation:
implications for Multiple Sclerosis pathogenesis

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In memory of My Mother

"Because I am part of You"
INDEX

ABSTRACT .................................................................................................................. 1

1. INTRODUCTION

1.1. Multiple Sclerosis ............................................................................................. 2
    1.1.1. Histopathology .......................................................................................... 2
    1.1.2. Epidemiology ............................................................................................ 5
    1.1.3. Pathogenesis ............................................................................................. 6
    1.1.4. Clinical manifestations .............................................................................. 10
    1.1.5. Diagnosis ................................................................................................ 12

1.2. Oligodendrocytes ............................................................................................. 14
    1.2.1. Function of Oligodendrocytes .................................................................. 16
    1.2.2. Myelin ...................................................................................................... 16
    1.2.3. Myelination ............................................................................................... 17
    1.2.4. Remyelination ........................................................................................... 18
    1.2.5. Oligodendrocyte proliferation and differentiation .................................... 19
    1.2.6. Factors influencing oligodendrocyte maturation and survival. ............... 21

1.3. Signaling pathways influencing growth and differentiation in
    several cell types .................................................................................................. 24
    1.3.1. Harvey-Ras/ Extracellular Signal-Regulated Kinases 1/2,
        H-RAS/ ERK 1/2 .......................................................................................... 24
    1.3.2. Cyclic AMP Response Element Binding Protein,
        CREB protein .............................................................................................. 26

1.4. Markers of oligodendrocyte differentiation .................................................. 29
1.4.1. Oligodendrocyte Transcription Factor 2, Olig-2 ...................... 29
1.4.2. Myelin Basic Protein, MBP .............................................. 30
1.4.3. α-Smooth Muscle Actin, α-SMA ....................................... 31
1.5. Reactive Oxygen Species (ROS) and Oxidative stress .............. 32
  1.5.1. The NOX NADPH oxidases family ................................... 34
  1.5.2. Regulation of NADPH oxidase ....................................... 36
1.6. Relationship between oxidative stress and Multiple Sclerosis .... 36
  1.6.1. Oxidative stress in oligodendrocytes ................................ 38

2. OBJECTIVES ............................................................................. 40

3. MATERIALS AND METHODS

  3.1. Cell cultures ......................................................................... 43
  3.2. Flow cytometric assay of cell viability ................................... 44
  3.3. H₂O₂ treatments ................................................................... 44
  3.4. Inhibitors treatment .............................................................. 45
  3.5. Patients ................................................................................ 45
  3.6. Cerebrospinal fluid treatment ................................................ 46
  3.7. Purification of Immunoglobulins ........................................... 46
  3.8. Immunoglobulins treatment .................................................. 47
  3.9. Protein extraction ................................................................. 47
  3.10. Protein determination .......................................................... 48
  3.11. Western-blotting analysis ..................................................... 49
  3.12. Flow cytometric analysis of Mielin Basic Protein ................. 49
3.13. Immunofluorescence confocal microscopy .................................. 50
3.14. Real Time PCR analysis .......................................................... 51
3.15. Statistical analysis .................................................................... 52

4. RESULTS
   4.1. Effects of PMA on oligodendrocyte differentiation ...................... 53
   4.2. Effects of oxidative stress on growth and differentiation of oligodendrocytes ................................................................. 57
      4.2.1. Study of pathways involved in oxidative stress and oligodendrocytes differentiation ......................................................... 65
   4.3. Stimulation of cells with biological samples ............................... 69
      4.3.1. Effect of CSF from MS patients on the molecular mechanism of differentiation in oligodendrocytes ........................................ 70
      4.3.2. Effect of IgG from MS patients on oligodendrocytes differentiation ....................................................................................... 74

5. DISCUSSION AND CONCLUSIONS .............................................. 80
   5.1. Future perspectives ................................................................. 84

ACKNOWLEDGEMENTS ................................................................... 86
REFERENCES ................................................................................... 88
APPENDIX .......................................................................................... 104
CONGRESS PUBLICATIONS .............................................................. 105
ABSTRACT

MS is a chronic demyelinating disease affecting OLs, responsible for axon myelination in the CNS. Remyelination, in MS lesions, is dependent on the recruitment and differentiation of OPCs.

During inflammation high levels of ROS can be achieved within MS lesions changing the local environment where OPCs differentiation occurs. Moreover, in chronic MS lesions OPCs accumulate with loss of mature OLs, suggesting the existence of a differentiation block of OPCs.

We investigated the effects of low and high ROS levels, on signaling pathways involved and the role of the main source of ROS, NADPH-oxidase (NOX) enzymes, in OPCs differentiation.

We also tested the hypothesis of the presence of autoantibodies impairing OPCs differentiation in CSF or in serum of MS patients.

Our results demonstrated that OPCs, exposed to mild oxidative-stress, increase expression of OLs differentiation markers; thus ROS mediate the signals leading to OPCs differentiation.

Fine tuning of the type and the levels of ROS generated by NOX–PKC signals may have profound effects on OPCs differentiation. Thus, large amounts of ROS induce death of OPCs.

This finding is relevant for the pathogenesis of MS lesions: whereas low ROS in limited inflammation may represent a positive re-myelination stimulus, excess of ROS produced by extensive inflammation may reduce the pool the OLs precursors and worsen MS lesions.

In addiction, our data indicate that CSF and autoantibodies present in the IgG fraction from serum of MS patients inhibit OLs differentiation thus impairing myelination in CNS.
CHAPTER I

INTRODUCTION

1.1. *Multiple Sclerosis*

Multiple sclerosis (MS) is characterized by a chronic demyelinating inflammation, damaging the central nervous system (CNS) (1, 2). MS is the most common cause of disability in young adults. As in other chronic inflammatory diseases, the manifestations of MS change from a benign to a rapidly progressive and disabling form. Some indirect data suggest an autoimmune etiology for MS, perhaps triggered by a viral infection, in genetically susceptible individuals (3). Despite the number of studies on the disease and a multidisciplinary approach to the problem, the pathogenesis of MS is still obscure and the etiology is unknown.

1.1.1. *Histopathology*

The term MS derives from multiple sclerotic areas visible in macroscopic examination of the brain. These lesions, called *plaques*, are easily distinguishable from the surrounding white matter. The plaques vary in size from 1-2 mm to several centimetres. The acute lesion of MS, which is rarely found in a post mortem examination, is characterized by infiltration of mononuclear cells (mainly T lymphocytes and macrophages) and loss of myelin (*demyelination*). Myelin (Figure 1), formed by glial cells named *oligodendrocytes* (OLs), constitutes the sheath that insulates nerve cell extensions, allowing the rapid and integral transmission of nerve impulses.
Figure 1. Cross section of white-matter lesions targeting the myelin sheath and oligodendrocytes.

Panel A - Light microscopy reveals myelin sheaths (dark blue rings) around axons in a cross section of myelinated white matter (toluidine blue). Two darker staining OLs, the cells that make and maintain myelin, lie to the right of center (arrow).

Panel B - An electron micrograph reveals the myelin sheath in cross section to be a spirally wrapped membrane beginning in the lower left as an outer (oligodendroglial) “tongue” of cytoplasm and spiraling counterclockwise to terminate at an inner tongue inside the myelin sheath to the right of the axon. Microtubules and neurofilaments can be seen cut in cross section within the axoplasm.


With the progression of the disease the destruction of myelin sheaths causes blocking or slowing of nerve impulses. The inflammatory infiltrates appear to mediate the loss of the myelin sheath surrounding the axon cylinder. With the progression of the lesion, a large number of macrophages and microglial cells (phagocytes specialize in the CNS that derive from bone marrow) digests myelin fragments and astrocytes proliferate (gliosis). In chronic lesions are present a complete or nearly complete demyelination and dense gliosis (Figure 2) (4).
Figure 2. Chronic lesions of multiple sclerosis in Humans.

Panel A - An electron micrograph of a chronic active lesion shows a myelinated fiber undergoing demyelination. The arrow shows myelin droplets on the macrophage surface being internalized by the cell. The fiber is invested by a microglial cell, which is engaged in the phagocytosis of myelin droplets as they are divested from the myelin sheath. The end product of this process is shown in Panel B (toluidine blue stain). Panel B - An area from a chronic silent gliotic lesion is made up of astroglial scar tissue, in which intact demyelinated axons (light profiles) are embedded; mitochondria can be seen within the axons; the smaller nuclei belong to microglial cells, but no oligodendrocytes are present. Panel C - An electron micrograph with a field similar to that in Panel B shows large-diameter demyelinated axons (A) within the glial scar; an astroglial-cell body is at the upper right. Panel D - (toluidine blue stain) A biopsy specimen from a patient with secondary progressive multiple sclerosis shows an area of remyelination (shadow plaque) in which the myelin sheaths of many axons are disproportionately thin and OLs are overabundant. These cells are probably oligodendroglial precursor cells recently recruited into the lesion. Panel E - An electron micrograph shows remyelination; the myelin sheaths are thin in comparison to the diameters of the axons, and two OLs are evident (OL). Panel F - (Luxol fast blue and periodic acid–Schiff) There is an abrupt transition at the edge of the chronic MS lesion. The myelin internodes (blue) terminate sharply at the demyelinated plaque. OLs are present (arrows) up to the edge of the lesion, but not within the lesion. Rod cells (microglia) are lined up along the boundary. A denotes axon, and As astrocyte.

1.1.2. Epidemiology

MS is the most common neurological disease in young adults and, as shown by the descriptive studies, the geographical distribution of the disease is non homogeneous (5). It is frequent in the countries in central and northern part of Europe and in those non-European regions of northern European ancestry. MS is typically a disease of temperate climates; in both hemispheres its prevalence decreases with decreasing latitude (Figure 3).

![Figure 3. Prevalence of MS related to latitude.](image)

MS is typically a disease of temperate climates; in both hemispheres its prevalence decreases with decreasing latitude.

The comparison between the populations of North America and Europe indicates similar rates of prevalence and similar north-south gradient. Some areas of the world represent real focus of the disease (in Italy, Sardinia) (6, 7), suggesting that in MS there are environmental factors. Italy is one of the countries with a higher risk of developing the disease (Figure 4). The number of affected subjects in Italy is approximately 50,000 and more than 1.1
million worldwide. In Caucasians, the average rates of total prevalence vary between 30 and 180 cases/100,000 inhabitants (5) and the incidence is 10-20 new cases/100,000 inhabitants per year. It is most frequently diagnosed between 20 and 40 years, rarely affects children and the elderly. MS is about twice as common in women than in men (8, 9).

Figure 4. Prevalence of MS per 100,000 inhabitants.
Globally, the median estimated prevalence of MS is 30 per 100,000 (with a range of 5–80). Regionally, the median estimated prevalence of MS is greatest in Europe (80 per 100,000), followed by the Eastern Mediterranean (14.9), the Americas (8.3), the Western Pacific (5), South-East Asia (2.8) and Africa (0.3). (The Atlas of MS 2008).

1.1.3. Pathogenesis

MS can be considered the result of complex multifactorial interactions between genetic and environmental factors. Several studies suggest that MS is an immune-mediated disease related to T lymphocytes action and induced by external and unknown agents, such as viruses and bacteria, in selected
subjects. Most researchers seem to agree that the demyelination process includes at least three main factors:

- a particular immunogenetic pattern;
- an immunopathologic mechanism;
- environmental factors.

**IMMUNOGENETIC FACTORS**

The evidence related to the influence of genetic factors in the pathogenesis of MS derives from studies of families and twins (10-13). These studies have shown that the disease risk is higher in the biologically related family members with MS patients, compared to the general population (14). These findings also suggest a common sharing of exposure to environmental risk factors during critical periods of risk (childhood and adolescence). However, Ebers et al. (1995) have shown that the disease risk in non-consanguineous first degree relatives, adopted individuals, living with MS patients is similar to the expected value from the general population. In Caucasian patients with MS, population genetic studies have demonstrated an association with major histocompatibility complex (MHC) class II alleles (DRB1*1501, DRB5*0101 and DQB1*0602) (15). These alleles, in humans, are contained in HLA-DR2 haplotype, that is the most frequently associated with the disease in Caucasians. The genotypic and phenotypic analysis shows that the susceptibility is probably oligogenic or multigenic type and mediated by a set of genes that interact in a epistatic way (16, 17). In this interaction a specific genetic locus may influence the phenotypic expression of the another locus (18).

**IMMUNOPATHOLOGICAL MECHANISM**

MS seems to be a disease with autoimmune pathogenesis and it is mediated, at least in part, by T lymphocytes. In the autoimmune diseases there
is an alteration of the immune system, autoreactive to endogenous self-peptides. In MS the myelin is not more recognized as self-peptide (19-21). Normally, in our immune system the T cells play a key role in defence against foreign pathogens, such as viruses, bacteria and allergens. In MS, the T cells attack the myelin of the CNS (22-25).

Previous studies have showed that peptide sequences of very common viral agents, such as Epstein Barr virus (EBV) (26, 27), influenza virus type A (28), human papilloma virus (HPV) (29) and human herpes virus type 6 (HHV-6) (30, 31) are very similar to Myelin Basic Protein (MBP). Another virus, John Cunningham Virus (JCV), is a type of human polyomavirus (formerly known as papovavirus) and is genetically similar to BK virus and SV40. It was discovered in 1971 and named with the two initials of a patient (JC) with progressive multifocal leukoencephalopathy. This virus is able to infect OLs and has been shown to be reactivated in MS patients treated with interferon (32). The inflammatory reaction is associated with up-regulation of several Th1 cytokines, including interleukin 2 (IL-2), interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) that were found in the cerebrospinal fluid (CSF) from patients with the disease (33, 34). The endothelial cells of the brain and spinal cord, triggered by demyelinating lesions, express adhesion molecules, fibronectin, the receptor for urokinase plasminogen activator (uPAR), the MHC class II molecules, chemokines and stress proteins (35). Magnetic resonance imaging (MRI) studies in the early stages of disease suggest that most of the lesions is preceded by focal destruction of blood brain barrier (BBB); therefore, this condition would facilitate entry of autoreactive T cells and antibodies inside the CNS (36). Recently, in experimental models of encephalomyelitis (EAE) it has been shown that the breaking of the BBB is initiated by memory T cells expressing CCR-6, the receptor for the chemoattractant molecule CCL-20. These cells escape from choroid plexus vessels, penetrate into stroma and cross the
epithelial-choroid plexus barrier (37). The histopathological effect of this inflammatory mechanisms is the demyelination, that is the formation of a plaque, indicating damage to the myelin sheath. The neuron, therefore, devoid of the myelin sheath, loses the capability to transmit nerve signals leading to the appearance of a sign or a symptom. Neuropathological studies of MS lesions have clearly demonstrated a *remyelination process*, but this process is incomplete in chronic lesions and usually limited to the edges of demyelinating plaques.

**ENVIRONMENTAL FACTORS**

Several epidemiological data suggest the intervention of environmental factors in the genesis of MS. Environmental factors proposed as possible risk factors of MS are:

- specific or common bacteria or virus,
- heavy metal poisoning,
- industrial pollution,
- hygiene,
- diet,
- climate.

Individuals emigrated from high risk areas of disease in low risk areas, such as South Africa (38) or Hawaii (39), maintained the risk level of their country of origin. However, this trend was valid only for individuals emigrated after the age of 15 years (38). The individuals emigrated during childhood or before adolescence acquired the same risk of host countries. These data suggest that environmental factors influence the individual just before puberty (about 15 years) (40).

The prevalence and the incidence of MS show large differences within high risk areas with stable and ethnically homogeneous populations (5). Therefore, the MS could be a disease with a non homogeneous distribution both in space and time. Thus, it is possible that the etiologic agent responsible
for the disease spreads from area to area, from a population to other and it must be able to change its concentration over time. These qualities seem typical of an infectious agents. One of the most important epidemiological studies on MS describes the dramatic increase in the incidence of the disease in the Faroe Islands (North Atlantic, west of Norway), after the military occupation by 1500-2000 soldiers of the British troops between 1941 and 1944 (41). This event shows that the environmental factor is not only endemic, but may be transported from one area to another. From these observations and from the association between the upper airways viral infections and relapsing MS and T cell cross-reactivity between proteins of the myelin sheath and viral antigens, the viruses have attracted the attention of researchers (42, 43). The viruses mostly investigated are: HHV-6, for its neurotrophism (30, 31), Epstein-Barr virus according to an action characterized by “molecular mimicry” (26, 27) and LM7 retrovirus, know as “multiple sclerosis associated retrovirus” (MSRV) (44, 45). Nevertheless, no viral agent has been conclusively linked to MS. It is likely that various infectious agents, also non-specific, induce, under certain circumstances, an immune response against self-antigens (46). Recent studies have shown that heavy metal poisoning (47), industrial pollution and hygiene can contribute to onset of the disease. Finally, an environmental factor is currently, attracting great interest: nutrition (48). The different dietary habits, in fact, seem to provide satisfactory explanations for the non homogeneous distribution of MS in different geographical areas.

1.1.4. Clinical manifestations

The symptoms of MS are extremely various and not specific to the disease. In fact, many diseases such as cancer, stroke, systemic lupus erythematosus (SLE), vasculitis and other less severe diseases have, at least in part, common signs and/or symptoms with MS (49). The appearance of the
disease can be sudden or slow. The symptoms at onset can be severe or so slight not to require medical attention even after months from the onset. The nature of the symptoms depends on the lesion location (or plaque) in CNS. The most common symptoms at onset include weakness in one or more limbs, blurred vision, due to optic neuritis, abnormal sensitivity, diplopia and ataxia. In order to standardize the terminology to describe the clinical course and the subtypes of the disease, it has been created a task force of 215 experts, members of the international MS scientific community (50), which identified four types of disease:

**Relapsing-remitting MS:** is the most common form of MS in the people under 40 years and it represents the 45-50% of people with MS. The subjects are struck by acute attacks, also named exacerbations, followed by periods of remission, during which the patients fully or partially recover. This clinical variant evolves in 80% of cases in the secondary progressive form.

**Secondary progressive MS:** characterized by a continuous disease progression after a period of time attributable to the relapsing-remitting type, with or without relapses or remissions. The recovery after exacerbations is incomplete, resulting in a progressive deterioration of physical conditions over time. It affects approximately 25-30% of patients with MS.

**Primary progressive MS:** gradual progression of the disease from its onset. The signs and symptoms accumulate gradually over time without the appearance of a real attack and without remissions, but rarely causing permanent disability, because the course is very slow. This form is more common in subjects presenting their first symptoms after age 40 (approximately 10-15% of patients with MS).

**Progressive relapsing MS:** the patients with a primary progressive MS may have relapses. The intervals between relapses are characterized by a continuous progression of the disease, unlike relapsing-remitting MS. It affects 2-5% of subjects with MS.
A BENIGN DISEASE indicates the lack of detection of neurological deficit 15 years from onset with complete remission (10% of patients). On the contrary, MALIGNANT DISEASE is characterized by a rapid and progressive course that causes multiple neurological deficits or death in a short period of time (5% of patients).

1.1.5. Diagnosis

No definitive diagnostic tests for MS is currently available. Therefore, to reach a definitive diagnosis it is necessary to use different tools derived from clinical analysis (51), laboratory (52) and instrumental tests (53, 54).

1) CLINICAL DIAGNOSIS analyzes:

- Patient medical history;
- Evidence of altered sensibility, impaired strength and vision disturbances;
- Symptoms/signs attributable to white matter lesions are not justified by other diseases;
- Spatial dissemination of lesions with clinical signs referable to 2 or more lesions;
- Symptoms/signs attributable to the temporal dissemination of the lesions: two or more relapses;

2) LABORATORY DIAGNOSIS is based on CSF investigations (inflammatory and autoimmune disorders), assaying the intrathecal synthesis of Immunoglobulins G (IgG) and the presence of oligoclonal Ig bands.

3) INSTRUMENTAL DIAGNOSIS:

- Magnetic Resonance Imaging (MRI) detects pathological foci in the brain stem, cerebellum and spinal cord and the presence of
lesions in the corpus callosum and the ventricles. In addition, through the use of contrast medium it is possible highlight local impairments in BBB preceding the signs of exacerbation and possible injury to the optic nerve.

- Computerized Axial Tomography (CAT) shows less dense areas around the ventricles corresponding to the plaques where the myelin is lost.
- Testing of Evoked Potentials (EP) measures the transmission time of sensory messages that travel through the nerves.

In MS the diagnostic procedure is rather long and tortuous and at present the only diagnostic support provided by the laboratory is based on analysis of intrathecal IgG synthesis and research of oligoclonal Ig bands.

The CSF constitutes the extra-cellular component of the CNS and it is separated from the systemic circulation only through the BBB. The IgG dosage in CSF and serum appears of same clinical interest. Numerous formulas have been used to distinguish the IgG synthesized locally by those in serum, which may enter the CNS passively through an altered BBB. An useful formula expresses the relationship between IgG and CSF albumin and IgG and serum albumin ("index LCS-IgG"). This quantitative analysis is simple and rapid, but not specific. Therefore, a more specific test is based on the research of oligoclonal Ig bands.

The presence of oligoclonal Ig bands in the CSF is carried out through the lumbar puncture (colloquially know as a spinal tap). The spinal needle inserted between the lumbar vertebrae L3/L4 or L4/L5. The γ-globulin region in CSF appears perfectly homogeneous in the serum. The intrathecal IgG synthesis in CSF is represented by the appearance of small discrete bands in the γ-globulin region that becomes non homogeneous. Oligoclonal bands are thin strips composed of IgG antibodies that migrate on an appropriate agarose or polyacrylamide gel, according to their isoelectric point and constant pH.
The test provides an initial separation of the proteins by their isoelectric point, they are transferred to a nitrocellulose membrane and then they are immunodetected with anti-IgG conjugated to peroxidase. The presence of one band (a monoclonal band) is not considered significant. The presence of two or more bands appears specific. This test is laborious, requires a fair experience of the operator and gives exclusively qualitative results (mostly based on a subjective estimate). Regarding the sensitivity and the accuracy of the test to identify MS patients, the presence of oligoclonal IgG bands has a 75% sensitivity and 85% specificity. This means that 1/4 of MS patients are negative (false negatives) and 1/6 of healthy patients are positive in this test (false positives). Another problem of the analysis of oligoclonal bands is the low reproducibility. This is due to a subjective estimate of the final result, misinterpretation of experimental artefacts, lack of a solid technical experience (55).

1.2. Oligodendrocytes

Glial cells, commonly called neuroglia or simply glia, are non-neuronal cells that maintain homeostasis, form myelin, and provide support and protection for the brain's neurons. It is divided in microglia and macroglia (composed by astrocytes, OLs, ependymal cells and radial glia in CNS).

The most abundant type of macroglia cell, astrocytes (also called astroglia) have numerous projections that anchor neurons to their blood supply. They regulate the external chemical environment of neurons by removing excess ions, notably potassium, and recycling neurotransmitters released during synaptic transmission.

Ependymal cells, also named ependymocytes, line the cavities of the CNS and make up the walls of the ventricles. These cells create and secrete CSF and beat their cilia to help circulate CSF.
OLs are cells that coat axons in the CNS with their cell membrane forming a specialized membrane differentiation called myelin, producing the so-called myelin sheath. The myelin sheath provides insulation to the axon that allows electrical signals to propagate more efficiently (Figure 5).

![Figure 5. Glia cells.](image)

A oligodendrocyte simultaneously wrapping multiple axons with a myelin sheath. Also shown are nodes of Ranvier, which are small unmyelinated axonal regions. (*Silverthorn Dee U.*).

Oligodendroglia derive during development from oligodendrocyte precursor cells (OPCs), which can be identified by their expression of a number of antigens, including the ganglioside GD3 (56, 57), the NG2 chondroitin sulfate proteoglycan, and the platelet-derived growth factor-alpha receptor subunit (PDGF-αR). In the rat forebrain, the majority of oligodendroglial progenitors arise during late embryogenesis and early postnatal development from cells of the sub-ventricular zones (SVZ) of the
lateral ventricles. SVZ cells migrate away from germinal zones to populate both developing white and gray matter, where they differentiate and mature into myelin-forming oligodendroglia (57, 58). However, it is not clear whether all oligodendroglial progenitors undergo this sequence of events. It has been suggested that some undergo apoptosis and others fail to differentiate into mature oligodendroglia but persist as adult oligodendroglial progenitors (59).

1.2.1. Function of Oligodendrocytes

As part of the nervous system, OLs are closely related to nerve cells, and, like all other glial cells, OLs provide a supporting role for neurons. In addition, the nervous system of mammals depends crucially on myelin sheaths, which reduce ion leakage and decrease the capacitance of the cell membrane. Myelin also increases impulse speed, as saltatory propagation of action potentials occurs at the nodes of Ranvier in between Schwann cells (of the peripheral nervous systems -PNS) and OLs (of the CNS). OLs provide the same functionality as the insulation on a household electrical wire (with the rather large difference that, while household electrical wires are in a non-conducting medium - air - the axons run in a solution of water and ions, which conducts electrical current well). Furthermore, impulse speed of myelinated axons increases linearly with the axon diameter, whereas the impulse speed of unmyelinated cells increases only with the square root of the diameter. In contrast, satellite OLs are functionally distinct from most OLs. They are not attached to neurons and, therefore, do not serve an insulating role. They remain opposed to neurons and regulate the extracellular fluid (60).

1.2.2. Myelin

The myelin sheath around most axons constitutes the most abundant membrane structure in the vertebrate nervous system. Its unique composition (richness in lipids and low water content allowing the electrical insulation of
axons) and its unique segmental structure responsible for the saltatory conduction of nerve impulses allow the myelin sheath to support the fast nerve conduction in the thin fibers in the vertebrate system. High-speed conduction, fidelity of transfer signaling on long distances, and space economy are the three major advantages conferred to the vertebrate nervous system by the myelin sheath, in contrast to the invertebrate nervous system where rapid conduction is accompanied by increased axonal calibers.

Myelin proteins, which comprise 30% dry weight of myelin, are for most of the known ones, specific components of myelin and OLs.

The major CNS myelin proteins, MBP and Proteolipidic Protein (PLP) (and isoform DM-20), are low-molecular-weight proteins and constitute 80% of the total proteins. Another group of myelin proteins, insoluble after solubilization of purified myelin in chloroform-methanol 2:1, have been designated as the Wolfgram proteins, since their existence was suspected already in 1966 by Wolfgram. These proteins comprise the 2’,3’-cyclic nucleotide-3’-phosphohydrolase (CNP) and other proteins (60).

1.2.3. Myelination

Myelination consists of the formation of a membrane with a fixed composition and specific lipid-protein interactions allowing membrane compaction and the formation of the dense and intraperiodic lines of myelin. Therefore, myelination also needs activation of numerous enzymes of lipid metabolism necessary for the synthesis of myelin lipids, of synthesis and transport of specific protein components of myelin or their mRNAs to the OLs processes.

These are the sequential steps governing myelination:

1. the migration of OLs to axons that are to be myelinated, and the fact that axons and not dendrites are recognized;
2. the adhesion of the OL process to the axon;
3. the spiraling of the process around the axon, with a predetermined number of myelin sheaths and the recognition of the space not to be myelinated, i.e., the nodes of Ranvier (60).

In the first step, the pre-oligodendroglial multiprocessed cells settle along the fiber tracts of the future white matter, maintaining the ability to divide. Indeed, mitoses are present in the interfascicular longitudinal glial rows (61). Second, these pre-OLs become immature OLs, characterized by the acquisition of specific markers and ready for myelination.

1.2.4. Remyelination

The OLs and the myelin sheath are the main targets of the pathological process in MS. The loss of OLs involves the demyelination, and thus a considerable loss of efficiency of axons to conduct impulses (1, 2). After the demyelination a spontaneous regenerative or healing process by which new myelin layers are formed around demyelinated axons, is taking place. This process is named remyelination, which allows axons to restore efficient conduction of nerve impulses. In the first stage of the disease, when the axonal degeneration is not significant, the demyelinating injury is compensated by remyelination (62).

The remyelination is mediated by a population of stem cells abundantly distributed in the adult CNS. These cells are the OPCs, and the inflammation is vital to stimulate, inside of the lesion, the production of factors that promote the recruitment of these precursors (63). Soon the demyelination area is filled by the OPCs that have the ability to proliferate and differentiate into mature OLs, producing new myelin sheath around the demyelinated axons.
The continuous maturation of these cells ensures a continuous process of myelin formation. This correcting mechanism is not perfect and limited to the early stages of the disease.

In MS it is not known whether the remyelination is slowed or blocked. The failed remyelination in MS has been associated with limited availability, migratory capability or myelination of the OPCs. Previous studies have shown that in chronic demyelinating lesions in MS patients there is a small number of pro-OLs and an increased number of OPCs, suggesting the presence of defective mechanism in the OPCs maturation (66, 67).

1.2.5. Oligodendrocyte proliferation and differentiation

The growth and the development of neuronal cells is controlled by a set of different factors: NGF, IGF II, laminin, fibronectin, collagen and adhesion molecules such as N-CAM and cadherins (68). Moreover, in the adult brain the turnover and the replacement of OLs, as well as the remyelination process, are phenomena that occur continuously. The brain contain endogenous OPCs with the ability to proliferate and differentiate into mature OLs, through the stages of Pro-OL and immature OL, producing new myelin sheath around the demyelinated axons (Figure 6) (69, 70). The oligodendrocyte precursors are present during the development of the nervous system, but some of them remain in the fully developed brain. They constitute the largest group of cells subjected to mitosis in the adult brain.
Figure 6. Immunophenotype and morphology of perinatal oligodendrocyte lineage cells in culture.
The name of the cell type is listed along the top, with the most immature stage, the pre-O-2A or pre-progenitor, at the left and the most differentiated stage, the mature oligodendrocyte, at the right (71).

The generation of OPCs by neural stem cells is the result of interaction of local extrinsic induction factors (SHH, FGF2, PDGF, IGF1, neurotrophins) (72-74) with proteins of the intrinsic transcription machinery of CNS (75).

The neurotrophins (NGF, BDNF, NT-3, NT-4/5) are small secreted proteins in the nervous system and are important in the differentiation, migration, proliferation and activation of cells of the CNS. In fact, BDNF and NT3 are required for survival and myelination (76). The first transcription factors in the differentiating of OPCs in spinal cord and forebrain (therefore used as markers for early OPCs) are Olig-1 and Olig-2, members of the basic helix-loop-helix (bHLH) family (75). Olig-2 leads to the generation of OPCs, while Olig1 seems involved in the survival and maturation of OPCs.

A second group of transcription factors crucially involved in OPC differentiation includes members of the Sox family. In particular, Sox8, Sox 9 and Sox10 are expressed in OPCs directly after induction of OL lineage and, therefore, are also frequently used as markers for early OPCs. Sox5 and Sox6 are involved in the progression of OPCs differentiation towards more mature stages (77, 78). In addition, three members of the homeodomain transcription factor family, Nkx2.2, Nkx6.1 and Nkx6.2 (74), along with two members of the Zinc-finger superfamily of transcription factors: Myt1 and YinYang1, seem
to play a role in the differentiation of the oligodendrocyte progenitors (79, 80).

1.2.6. *Factors influencing oligodendrocyte maturation and survival*

Many growth factors have been found to be involved in the proliferation, differentiation, and maturation of the oligodendrocyte lineage.

- **PDGF**. Platelet Derived Growth Factor (PDGF) is synthesized during development by both astrocytes and neurons. In vitro, PDGF, a survival factor for oligodendrocyte precursors, is a potent mitogen for OPCs, although it triggers only a limited number of cell divisions. PDGF is also a survival factor for oligodendrocyte progenitors, as recently demonstrated by the impaired OL development in the PDGF-α deficient mice. In these mice, there are profound reductions in the numbers of PDGFR-α progenitors and OLs in the spinal cord and cerebellum, but less severe reductions of both cell types in the medulla. Infusion of PDGF into the developing optic nerve in vivo greatly reduces apoptotic cell death (81). PDGF also stimulates motility of oligodendrocyte progenitors in vitro and is chemoattractive. 

- **Basic FGF**. Basic Fibroblast Growth Factor (bFGF) (also called FGF 2) is also a mitogen for neonatal oligodendrocyte progenitors. It upregulates the expression of PDGFR-a and therefore increases the developmental period during which oligodendrocyte progenitors or pre-OLs are able to respond to PDGF (82). Pre-OLs can even revert to the oligodendrocyte progenitor stage when cultured with both PDGF and bFGF. This inhibition of oligodendrocyte differentiation can be overridden by the presence of astrocytes. bFGF is present in the developing nervous system in vivo. The levels of expression of mRNA for the high-affinity bFGF receptors-1, -2, and -3
are differentially regulated during lineage progression (83); this pattern of expression could provide a molecular basis for the varying response of cells to a common ligand that is seen during development.

- **IGF-I.** Insulin-like growth factor I (IGF-I) stimulates proliferation of both oligodendrocyte progenitors and pre-OL O41 positive cells, and IGF receptors have been shown to be present on cells of the oligodendrocyte lineage (84). IGF-I is also a potent survival factor for both oligodendrocyte progenitors and OLs in vitro. The morphology of myelinated axons and the expression of myelin specific protein genes have been examined in transgenic mice that overexpress IGF-I and in those that ectopically express IGF binding protein-1 (IGFBP-1), a protein that inhibits IGF-I action when present in excess. The percentage of myelinated axons and the thickness of the myelin sheaths are significantly increased in IGF-I transgenics. An alteration in the number of OLs is seen but cannot completely account for the changes in the increase in myelin gene expression. IGFBP-1 transgenic mice have a decreased number of myelinated axons and thickness of the myelin sheaths. IGF-I could be involved in both the increase in OLs number and in the amount of myelin produced by each OL (85).

- **NT-3.** Neurotrophin-3 (NT-3) is a mitogen for optic nerve oligodendroglial precursors only when added with high levels of insulin, with PDGF, or with their combination. Astrocytes express NT-3 in optic nerve. NT-3 promotes also OL survival in vitro (86). The TrkC tyrosine kinase or TrkC receptor for NT-3 is expressed in OLs. Mice lacking NT-3 or its receptor TrkC exhibit profound deficiencies in CNS glial cells, particularly in oligodendrocyte progenitors; there is an important reduction in the spinal cord diameter, thereby suggesting that cell populations other than neurons are affected (87).
It was recently shown that NT-3 in combination with brain-derived neurotrophic factor (BDNF) is able to induce proliferation of endogenous oligodendrocyte progenitors and the subsequent myelination of regenerating axons in a model of contused adult rat spinal cord (88).

- **GGF.** The glial growth factor (GGF), a member of the neuregulin family of growth factors generated by alternative splicing, including Neu, heregulin, and the acetylcholine receptor-inducing activity (ARIA), is a neuronal factor, mitogenic on oligodendrocyte precursors; it is also a survival factor for these cells. It delays differentiation into mature OLs (89). In mice lacking the family of ligands termed neuregulins, OLs in spinal cord failed to develop (90). This failure can be rescued in vitro by the addition of recombinant neuregulin to explants of spinal cord. In the embryonic mouse spinal cord, neuregulin expression by motoneurons and the ventral ventricular zone is likely to exert an influence on early OPCs. Neuregulin is a strong candidate for an axon-derived promoter of myelinating cell development.

- **CNTF.** The ciliary neurotrophic factor (CNTF) can also act as comitogen with PDGF. Animals deficient in CNTF have a reduced number of mitotic glial progenitors. CNTF also promotes OL survival in vivo (81).

- **IL-6.** Interleukin (IL-6) may also act on OL survival as well as leukemia inhibitory factor (LIF) and a related molecule (91).

- **TGF-β.** In vitro, transforming growth factor (TGF-β) inhibits PDGF-driven proliferation and promotes differentiation of oligodendrocyte progenitors (92).
1.3. **Signaling pathways influencing growth and differentiation in several cell types**

1.3.1. **Harvey-Ras/ Extracellular Signal-Regulated Kinases 1/2, H-RAS/ ERK 1/2**

The ERK 1/2 pathway is a signal transduction pathway that couples intracellular responses to the binding of growth factors to cell surface receptors. This pathway is very complex and includes many protein components. The pathway, described below, includes the major components of the basic pathway (Figure 7). In many cell types, activation of this pathway promotes cell division.

![Figure 7. The organization and function of the Ras–Raf–MEK–ERK pathway.](image)

Activated Ras activates the protein kinase RAF kinase; RAF kinase phosphorylates and activates MEK; MEK phosphorylates and activates the Extracellular-Regulated Kinase (ERK 1/2).

RAF, MEK, and ERK 1/2 are all serine/threonine-selective protein kinases.
As discussed below, many additional targets for phosphorylation by ERK 1/2 were later found, and the protein was re-named "Mitogen-Activated Protein Kinase" (MAPK). The series of kinases from RAF to MEK to MAPK is an example of a protein kinase cascade. Such series of kinases provide opportunities for feedback regulation and signal amplification.

_H-Ras_ is localized at the cytoplasmic surface of the plasma membrane. It is a target of post-translational modification via attachment of farnesyl or methyl lipid moieties catalyzed by Farnesyltransferase (FTase) and Methyltransferase (ICMT), respectively. These post-translational modifications affect localization and biological activity of _H-Ras_ (93).

Like other G-proteins, _H-Ras_ is found in two interconvertible forms, GDP-bound inactive and GTP-bound active. Conversion from GDP-bound form to GTP-bound is catalyzed by guanine nucleotide exchange factor (GEF). Activity of GEF is regulated by the upstream signals. GEFs that activate _H-Ras_ are Son of Sevenless (SOS), PDZ-GEF1, CALDAG-GEF II and CALDAG-GEF III, RASGRF1, RASGRF2, and RasGRP4.

GEF first interacts with the GDP-bound form and releases bound GDP. As a result, a binary complex of the small G protein and GEF is formed. Then GEF in this complex is replaced by GTP resulting in formation of the GTP-bound small G protein (94).

Conversion of GTP-bound form to GDP-bound form is a result of slow intrinsic GTPase activity of _H-Ras_.

Proteins known as GTPase activated proteins (GAP) have been shown to stimulate this reaction. GAPs that inactivate _H-Ras_ are p120GAP and RASA3.

The activity of GEFs and GAPs is induced by a large variety of extracellular signals, most notably by those that activate receptors with intrinsic or associated tyrosine kinase activity.
The phosphotyrosines of the receptors, such as platelet-derived growth factor receptor beta (PDGF-R-β), serve as docking sites for the adaptor proteins, such as Src homology 2 domain containing transforming protein (Shc). Shc forms an adaptor protein complex with Growth factor receptor bound 2 (GRB2). This protein complex recruits SOS, the most characterized H-Ras-GEF, from the cytosol to produce a receptor-adaptor-GEF complex.

G-protein-coupled receptors (GPCRs) can also activate H-Ras signaling. The β-1 adrenergic receptor binds to the PDZ-GEF1-leading to H-Ras activation.

Other receptors, RET proto-oncogene (RET) and TEK tyrosine kinase endothelial (TIE2), can directly activate Docking proteins 1 and 2 (DOK1 and DOK2). DOK1 and DOK2 in turn stimulate the GAP activity of p120GAP that down-regulate H-Ras signaling (95). In addition, cytoplasmic Ca\(^{2+}\) and second messenger 1,2-diacyl-glycerol (DAG) can activate calcium and DAG-regulated GEFs (CALDAG-GEF II and CALDAG-GEF III).

Major effectors of H-Ras protein are protein kinase v-Raf-1 murine leukemia viral oncogene homolog 1 (c-Raf-1) and Phosphatidylinositol 3-kinase (PI3K cat class 1A).

Small G-proteins are also known to cross-talk with each other. H-Ras activates guanine nucleotide exchange factors RalRGL and Tiam 1 that in turn activate small GTPases RalA and Rac1, respectively.

### 1.3.2. Cyclic AMP Response Element Binding Protein, CREB protein

CREB is a transcription factor; it binds to certain DNA sequences called cAMP response elements (CRE) and, thereby, increases or decreases the transcription of the downstream genes. CREB was first described in 1987 as a cAMP-responsive transcription factor regulating the somatostatin gene (96).

Genes whose transcription is regulated by CREB include: c-fos, the neurotrophin BDNF, tyrosine hydroxylase, and many neuropeptides (such as
somatostatin, enkephalin, VGF, and corticotropin-releasing hormone). \textit{CREB} is closely related in structure and function to CREM (cAMP response element modulator) and ATF-1 (activating transcription factor-1) proteins. \textit{CREB} proteins are expressed in many animals, including humans. \textit{CREB} has a well-documented role in neuronal plasticity and long-term memory formation in the brain (97).

A typical (albeit somewhat simplified) sequence of events is as follows: a signal arrives at the cell surface, activates the corresponding receptor, which leads to the production of a second messenger, such as cAMP or Ca\textsuperscript{2+}, which in turn activates a protein kinase. This protein kinase translocates to the cell nucleus, where it activates a \textit{CREB} protein. The activated \textit{CREB} protein then binds to a CRE region, and is then bound to by a CREB-Binding Protein (CBP), which coactivates it, allowing it to switch certain genes on or off. The DNA binding of \textit{CREB} is mediated via its basic leucine zipper domain (bZIP domain).

\textit{CREB} can also be phosphorylated by a variety of kinases in response to mitogen, calcium and stress dependent signals. For instance, upon stimulation of cellular G-protein coupled receptors and growth factor receptors, adenylate cyclase is activated, leading to increases in cAMP. This in turn activates PKA by dissociating the regulatory from the catalytic subunits. Catalytic subunits can be translocated into the nucleus, where \textit{CREB} is phosphorylated. The crucial event in the activation of \textit{CREB} is the phosphorylation of Ser133 in kinase-inducible domain (KID). This domain includes several consensus phosphorylation sites for a variety of kinases [e.g. protein kinase A (PKA), protein kinase C (PKC), casein kinases, calmodulin kinases (CaMKs), glycogen synthase kinase-3, p34cdc2, p70s6k] that can either increase or decrease the activity of \textit{CREB}. Phosphorylation on Ser133 promotes \textit{CREB} to recruit transcriptional co-activators that induce transcription of a variety of intermediate early response genes. Dephosphorylation of Ser133 is important
for the inactivation of CREB. Both protein phosphatase 1 (PP-1) and PP-2A may be involved in the dephosphorylation of CREB. Based on the extent of homology at this region, members of the CREB family can be divided into the CREB, CREM, and activating transcription factor (ATF) groups (Figure 8).

![Figure 8. CREB Activation Pathways.](image)

CREB has many functions in many different organs, however most of its functions have been studied in relation to the brain. CREB proteins in neurons are thought to be involved in the formation of long-term memories; this has been shown in the marine snail Aplysia, the fruit fly Drosophila melanogaster, and in rats. CREB is necessary for the late stage of long-term potentiation. CREB also has an important role in the development of drug addiction (98). There are activator and repressor forms of CREB. Flies genetically engineered to overexpress the inactive form of CREB lose their ability to retain long-term memory. CREB is also important for the survival of neurons, as shown in genetically engineered mice, where CREB and CREM
were deleted in the brain. If CREB is lost in the whole developing mouse embryo, the mice die immediately after birth, again highlighting the critical role of CREB in promoting survival.

Disturbance of CREB function in brain can contribute to the development and progression of Huntington's Disease. Abnormalities of a protein that interacts with the KID domain of CREB, the CBP is associated with Rubinstein-Taybi syndrome. CREB is also thought to be involved in the growth of some types of cancer.

1.4. Markers of oligodendrocyte differentiation

1.4.1. Oligodendrocyte Transcription Factor 2, Olig-2

Oligodendrocyte transcription factor 2 (Olig-2) is a protein that in humans is encoded by the olig-2 gene.

Olig-2 is a basic helix-loop-helix transcription factor. Its expression is predominantly restricted to the CNS. The protein is an essential regulator of ventral neuroectodermal progenitor cell fate and is required for OLs and motor neuron development.

The olig-2 gene was originally discovered as a chromosomal translocation t(14;21)(q11.2;q22) associated with T-cell acute lymphoblastic leukemia (99). Its chromosomal location is within a region of chromosome 21 which has been suggested to play a role in learning deficits associated with Down syndrome.

Olig-2 is a universal marker of diffuse gliomas (oligodendroglioma, astrocytoma, glioblastoma, and mixed glioma). The expression in diffuse gliomas is distinct from other types of brain tumors and therefore is clinically useful as a pathologic marker for distinguishing these cancers.
1.4.2. Myelin Basic Protein, MBP

The presence of the myelin sheath, a tightly packed multilamellar membrane, is crucial to the functioning of the vertebrate nervous system. Myelin is formed by specialized glial cells in both the CNS and PNS, and mutations in myelin components or autoimmune attack towards them leads to severe neurological defects. Many of the defects observed in dys- or demyelination can be attributed to potential disruption of the intimate interactions between myelin proteins and the myelin lipid bilayer (100).

Interest in MBP has centered on its role in demyelinating diseases, in particular MS; several studies have shown a role for antibodies against MBP in the pathogenesis of MS which is characterized by the active degradation of the myelin sheath (101). Some studies have linked a genetic predisposition to MS to the MBP gene, though a majority have not.

MBP is one of the most abundant proteins in myelin, and present at high concentration in both the CNS and PNS myelin (102, 103). MBP is a protein believed to be important in the process of myelination of nerves in the CNS.

MBP is a peripheral membrane protein, which is reminiscent of intrinsically disordered proteins, when put into aqueous solution (104-106). Several lines of evidence, however, point towards a scenario, where interactions with ligands trigger secondary structure formation and some degree of folding into a more compact structure in MBP (107).

MBP was initially sequenced in 1971 after isolation from myelin membranes (108). Since that time, knockout mice deficient in MBP that showed decreased amounts of CNS myelination and a progressive disorder characterized by tremors, seizures, and early death have been developed. The gene for MBP is on chromosome 18q22 (109) the protein localizes to the CNS and to various cells of the hematopoietic system.
The pool of MBP in the CNS is very diverse, with several splice variants being expressed and a large number of post-translational modifications on the protein, which include phosphorylation, methylation, deamidation, and citrullination. In melanocytic cell types, MBP gene expression may be regulated by MITF (110).

1.4.3. α-Smooth Muscle Actin, α-SMA

Actin is one of the most abundant and conserved protein in many eukaryotic cell types. It polymerizes forming microfilaments that have an array of functions including regulating contractility, motility, cytokinesis, phagocytosis, adhesion, cell morphology, and providing structural support.

Six distinct actin isotypes, falling into three categories, have been identified in mammalian cells. The three types, separated based on isoelectric point, include α (α-Skeletal, α-Cardiac, α-Smooth Muscle), β (β-Non-muscle) and γ (γ-Smooth Muscle, γ-Non-Muscle) isoforms (111, 112). The actin isoforms are highly conserved at the amino acid level and only differ at their N-termini (112,113).

Each is encoded by a separated gene and is expressed in a developmentally regulated and tissue-specific manner, α and β cytoplasmic actins are expressed in a wide variety of cells; whereas, expression of α-skeletal, α-cardiac, α-vascular, and γ-enteric actins are more restricted to specialized muscle cell type. Expression of α-SMA is regulated by hormones, cell proliferation, and altered by pathological conditions including oncogenic transformation and atherosclerosis.

The specific NH₂-terminal sequence Ac-EEED of α-Smooth Muscle Actin (α-SMA or Acta2) plays an important role in the regulation of polymerization in vitro and in vivo (114).
1.5. **Reactive Oxygen Species (ROS) and Oxidative stress**

For most living organisms, oxygen is an essential molecule for survival. Oxygen, in fact, is used as a terminal electron acceptor in the mitochondrial respiratory chain, leading to the synthesis of ATP needed for cellular metabolism. Almost all the oxygen used by mitochondria is reduced in the \( \text{H}_2\text{O} \) molecule after accepting four electrons. The oxygen accepts one electron at a time, when it accepts the first electron, decreases superoxide radical (\( \text{O}_2^- \)), the term indicates a radical molecular species with an unpaired electron in the last orbit and therefore extremely reactive. The superoxide radical can accept a second electron reducing further in hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) and molecular oxygen. This reaction is catalyzed by superoxide dismutase (SOD):

\[
2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

Hydrogen peroxide in the presence of transition metal ions (i.e. \( \text{Fe}^{2+/3+} \), \( \text{Cu}^{+/2+} \)), that are ions able to accept or donate electrons, changing their oxidation number, can be transformed into hydroxyl radical (\( \cdot\text{OH} \)) and hydroxyl ion (\( \cdot\text{OH}^- \)) according to the Fenton reaction:

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+}
\]

These partially reduced forms of oxygen are called reactive oxygen species (ROS).

The variety of ROS derived by both normal metabolic activities, both from the stresses generated by various mechanisms.

These short-lived ROS can play physiological roles in signal transduction, but in excess can contribute to the mechanisms of disease by dysregulation of signal transduction and/or by oxidative damage to cellular macromolecules (lipids, proteins, DNA, RNA, carbohydrates) that exceeds the cellular capacity for regeneration or repair. As elaborated later under signal transduction, dysregulation of signal transduction and/or
macromolecular lesions can adversely alter cellular function or trigger apoptotic or necrotic cellular death.

ROS are involved in many phenomena of damage to cell membranes, DNA and proteins with pathophysiological consequences of atherosclerosis, aging, and other degenerative disorders, but it is also true that free radicals are species that are produced by cells and as a result of the operation of the mitochondrial respiratory chain, both for the development of specific metabolic pathways necessary for the physiology of the cell, tissue and organism. ROS are generated by multiple pathways (Figure 9). The primary training site is the chain of mitochondrial electron transport, in fact, it is known that in this cellular compartment, 1,2% of electrons escape the chain of cytochromes and can go to reduce the oxygen superoxide (115). Due to the high concentration of SOD in the mitochondria, superoxide is converted into hydrogen peroxide. The latter is able to permeate the mitochondrial membrane (116) and reach the cytoplasm.

Another source of intracellular ROS is the cytochrome P450 in the endoplasmic reticulum, in fact, it is known that cytochrome generates ROS during the oxidation of unsaturated fatty acids (117-119). ROS are also generated by the system hypoxanthine/xanthine oxidase, lipoxygenase and cyclooxygenase, in fact, use these free radicals to form prostaglandins, thromboxanes and leukotrienes. These factors regulate the formation of the clot and the recall of leukocytes at the site of inflammation. In the system hypoxanthine/xanthine oxidase, free radicals are used for the formation of uric acid during degradation of purines. Finally, the major source of ROS is Nicotinamide Adenine Dinucleotide Phosphate (NADPH)-oxidase. The NADPH oxidase is a multiprotein enzyme complex that catalyzes the production of $O_2^-$ from oxygen molecule:

$$O_2+NADPH\rightarrow O_2^-+NADP^++H^+$$
1.5.1. The NOX NADPH oxidases family

The NOX-NADPH oxidases comprise a family of electron-transporting membrane enzymes family, whose primary function is the generation of ROS. ROS produced by NOX enzymes show a variety of biologic functions, such as microbial killing, blood pressure regulation, and otoconia formation. Strong evidence suggests that NOX enzymes are major contributors to oxidative damage in pathologic conditions.

Whereas NOX2 (also known as gp91phox), the phagocyte oxidase, has been known for several decades as the enzyme responsible for the oxidative burst and associated microbicidal activity, the other members of the gene family have been identified only recently. The NOX family now consists of seven members (NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2), each with a distinct tissue distribution. Since the discovery that NOX enzymes are not limited to white blood cells, an exponential increase in scientific reports describe how NOX enzymes are responsible for increased
ROS generation in numerous pathologic conditions, such as hypertension, ischemia/reperfusion, diabetes, cardiovascular diseases, and neurodegeneration. The elevated ROS production has been linked to the pathobiology of many of these conditions (120). The core catalytic domains of all seven NOX isoforms share similar structures, and their only known biochemical function is the generation of ROS. The basic catalytic subunit of NOX contains a C-terminal dehydrogenase domain featuring a binding site for NADPH and a bound flavin adenine nucleotide (FAD), as well as an N-terminal domain consisting of six transmembrane alpha helices that bind two heme groups. On activation, cytosolic NADPH transfers its electrons to the FAD, which in turn passes electrons sequentially to the two hemes and ultimately to molecular oxygen on the opposing side of the membrane, to form the superoxide anion (O$_2^-$) (121).

Although all seven NOX isoforms catalyze the reduction of molecular oxygen, they differ in their tissue distribution, their subunit requirements, domain structure, and the mechanism by which they are activated. In the case of NOX2, the activation mechanism is well described: on activation, the regulatory subunit p47$^\text{phox}$ is phosphorylated and translocates to the membrane, in the form of a complex that also contains p67$^\text{phox}$ and p40$^\text{phox}$. Once at the membrane, the cytosolic complex binds to the transmembrane cytochrome unit comprising both NOX2 and the closely associated p22$^\text{phox}$. Independently, the GTP-binding protein Rac also moves to the membrane, and the combination of regulatory subunits induces activation. Like NOX1, NOX2 and NOX3 require p22$^\text{phox}$, as well as association with cytosolic regulatory components (p47$^\text{phox}$/p67$^\text{phox}$ or their homologues NOXO1, NOXA1) p40phox and Rac. NOX4 requires p22$^\text{phox}$, but not the cytosolic regulatory factors (122). NOX5 and the DUOXes are activated by elevation of intracellular Ca$^{2+}$, which binds directly to N-terminal EF-hand domains (123).
1.5.2. Regulation of NADPH oxidase

Several lines of evidence support a role of PKC in NADPH oxidase activation. PMA, an activator of PKC, is a strong stimulus of $O_2^-$ production in whole cells. Purified p47$^{phox}$ is a good substrate for PKC in vitro. Staurosporine, a powerful inhibitor of PKC, inhibits superoxide production and p47$^{phox}$ phosphorylation. The data presented here provide clear evidence that, in addition to p47$^{phox}$, p67$^{phox}$ itself could play a role in the regulation of NADPH oxidase by phosphorylation/dephosphorylation reactions and that the phosphorylation events involve a PKC-dependent pathway. Little is known of the possible role of other protein kinases in the regulation of NADPH oxidase. It has been suggested that cyclic AMP-dependent protein kinase, MAPK, and p21-activated kinase could regulate NADPH oxidase by phosphorylating p47$^{phox}$ and that protein kinases other than PKC may participate in p67$^{phox}$ phosphorylation (124).

1.6. Relationship between oxidative stress and Multiple Sclerosis

Diseases that result in injury to the oligodendroglial cells include demyelinating diseases such as MS and leukodystrophies in the CNS and peripheral neuropathies in the PNS. Cerebral palsy (periventricular leukomalacia) is caused by damage to developing OLs in the brain areas around the cerebral ventricles. Spinal cord injury also causes damage to OLs (125). In cerebral palsy, spinal cord injury, stroke and possibly MS, OLs are thought to be damaged by excessive release of the neurotransmitter glutamate. OLs dysfunction may also be implicated in the pathophysiology of schizophrenia and bipolar disorder (126). Oligodendroglia are also susceptible to infection by the JC virus (Human polyomavirus), which causes progressive multifocal leukoencephalopathy (PML), a condition that specifically affects
white matter, typically in immunocompromised patients. Tumors of oligodendroglia are called oligodendrogliomas.

Recently, it has been shown that the production of ROS can be involved in the pathogenesis of several diseases affecting the CNS (127, 128). Our research group for several years have been studying the cellular and molecular mechanisms of redox signal transduction in several conditions (129, 130), such as systemic sclerosis (scleroderma), neuronal degeneration, and ischemia/cerebral and renal reperfusion. Specifically, we have found that in systemic sclerosis redox signalling is hypertrophic in immune cells and fibroblasts (131, 132). We wished to apply some of the findings in systemic sclerosis to MS and specifically, the molecular mechanism linked to oxidative stress in MS. The OLs and their precursors are very sensitive to oxidative stress, because they use large amounts of oxygen. They also have a reduced antioxidant defence system and a high intracellular concentration of Fe ions (133). This sensitivity is inversely correlated to the maturation level, in fact the OLs precursors show an increased in susceptibility to oxidative stress, compared to mature cells (134).

MS is a chronic demyelinating disease of CNS in which inflammatory processes and neurodegeneration contribute to demyelination and axonal damage. There are important experiments about the involvement of oxidative stress in the pathogenesis of this disease. The OLs and their precursors are, in fact, highly sensitive to oxidative stress, because of the large amounts of oxygen consumed, low levels of cellular antioxidant defense systems and high intracellular iron content (133).

In MS it is not known whether the remyelination is slowed or blocked. Failure in remyelination in MS has been associated with limited availability or migratory capacity of OPCs. In fact, in chronic demyelinating lesions it was observed a small number of mature cells and an increased number of
precursors, suggesting the presence of a block of the maturation process of OPCs. The intracellular redox state, regulated by extracellular signal molecules, is able to modulate the balance between self-renewal and differentiation in dividing cells (135). Therefore, alterations of cellular redox state in MS may represent a pro-apoptotic stimulus to OLs, but also can affect differentiation processes of OPCs resulting in demyelination.

1.6.1. *Oxidative stress in oligodendrocytes*

   Cellular proliferation and differentiation in the nervous system are closely linked processes, controlled by a number of growth factors and depend on environmental conditions. In tissue culture, serum addition provides neural cells with necessary extrinsic growth factor supplements that promote cell growth and enable survival.

   Serum deprivation, on the other hand, causes an arrest in cell division and may induce cellular differentiation.

   Extended periods of serum deprivation may eventually lead to cell death due to the lack of growth factors or increased oxidative stress.

   Cells in the nervous system are highly sensitive to oxidative stress because they utilize high levels of oxygen for normal function, and the brain has relatively poorly developed antioxidant mechanisms (136-138).

   OLs and their precursor cells exhibit a high sensitivity to oxidative stress. Oligodendrocyte precursors, for example, are easily damaged by hypoxic events because of low antioxidant levels and high iron content. The cytotoxic potential of oxygen radical-generating systems on bovine OLs prepared from adult brains was described. Maturation-dependent differences in the vulnerability of OLs to oxidative stress are observable, specifically oligodendrocyte precursors show a higher sensitivity to oxidative stress-induced death caused by glutathione depletion. There is little information at the present time on the effects of toxic radicals on the molecular mechanisms
regulating cell survival and death in proliferating compared to differentiating oligodendroglia cells (139).

The molecular mechanisms underlying H$_2$O$_2$-induced toxicity were characterized in rat oligodendrocyte cultures. While progenitor cells were more sensitive than mature OLs to H$_2$O$_2$, the antioxidant, N-acetyl-L-cysteine (NAC), blocked toxicity at both stages of development. As free radicals have been considered to serve as second messengers, was examined the effect of H$_2$O$_2$ on activation of the MAPK, ERK1/2 and p38. H$_2$O$_2$ caused a time and concentration-dependent increase in MAPK phosphorylation, an effect that was totally blocked by NAC. Further exploration of potential mechanisms involved in oligodendrocyte cell death showed that H$_2$O$_2$ treatment caused DNA condensation and fragmentation at both stages of development, whereas caspase 3 activation and poly (ADP-ribose) polymerase cleavage were significantly increased only in oligodendrocyte progenitors. The pan-caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, blocked DNA fragmentation in progenitors and produced a small but significant level of protection from H$_2$O$_2$ toxicity in progenitors and mature OLs. In contrast, inhibitors of both p38 and MEK reduced H$_2$O$_2$-induced death most significantly in OLs. The poly (ADPribose) polymerase inhibitor, PJ34, reduced H$_2$O$_2$-induced toxicity on its own but was most effective when combined with benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone or PD169316. The finding that molecular mechanisms conferring resistance to reactive oxygen species toxicity are regulated during oligodendrocyte differentiation may be of importance in designing therapies for certain neurological diseases affecting white matter (134).
CHAPTER II

OBJECTIVES

The balance between cell proliferation and differentiation is controlled by opposing cellular signals that modulate important cell functions as development, tissue repair, and tissue homeostasis; yet relatively little is known about physiological mechanisms central to such modulation.

It has been shown that redox state of the OLs is modulated by extracellular signaling molecules that alter the balance between self-renewal and differentiation: growth factors that promote self-renewal cause progenitors to become more reduced, while signaling molecules that promote differentiation cause progenitors to become more oxidized (135).

The correlation of cellular redox state with differentiation of OPCs is of great interest; however, it is not known whether this correlation is coincidental or whether it provides an important cue to understanding the molecular mechanisms inducing OLs to differentiate in mature myelinating cells.

The objectives of present thesis project are:

- the evaluation of ROS importance as OLs differentiation mediators.

To this end we used M03-13 cells, an immortal human cell line that expresses phenotypic characteristics of primary OLs. Under appropriate conditions, these cells are able to differentiate in mature OLs.

We first will consider the effects of a differentiation stimulus, Phorbol-12-Myristate-13-Acetate (PMA, an activator of PKC), in medium deprived of serum, on the appearance of early and late differentiation markers such as ERK1/2, P-CREB, MBP, α-SMA and Olig-2. Secondly, we will expose the cells to low doses of H₂O₂ to test if ROS modify differentiation, by
measuring the differentiation markers levels modulated by the PMA stimulus.

- the analysis of signal transduction pathways involved in the modulation of differentiation, mediated by PMA and/or H$_2$O$_2$.

Our group, for several years, has been studying signal transduction pathways linking membrane receptors to various transducers, including p21Ras and cAMP. We have shown that Ras isoforms, *Ha-Ras* and *Ki-Ras*, have opposing functions in regulation of redox signals. *Ha-Ras*-expressing cells produce high levels of ROS by inducing the NADPH-oxidase system. *Ki-Ras*, on the other hand, stimulates the scavenging of ROS by activating post-transcriptionally the mitochondrial antioxidant enzyme, Mn-superoxide dismutase (Mn-SOD), via an *ERK1/2*-dependent pathway (58). Ras proteins are exquisite redox sensors, that adapt the cell to various metabolic needs following receptor stimulation. We have shown that the levels of Ras proteins in human primary fibroblasts are regulated by cytokines, such as PDGF. PDGF stabilizes *Ha-Ras* by stimulating ROS and *ERK1/2*. Activation of *ERK1/2* and high ROS levels stabilize *Ha-Ras* protein, by inhibiting proteasomal degradation. We found a remarkable example *in vivo* of amplification of this circuit in fibroblasts derived from systemic sclerosis (scleroderma) lesions, producing vast excess of ROS and undergoing rapid senescence. High ROS, *Ha-Ras*, and active *ERK1/2* stimulated collagen synthesis, DNA damage, and accelerated senescence. Conversely ROS or Ras inhibition interrupted the signaling cascade and restored the normal phenotype. In primary cells, stabilization of Ras protein by ROS and *ERK1/2* amplifies the response of the cells to growth factors. In some autoimmune diseases, such as scleroderma, this represents a critical factor in the onset and progression of the disease (131). In this disease there are stimulatory autoantibodies against PDGF-R, which appear to be a specific hallmark of
scleroderma. Their biologic activity on fibroblasts strongly suggests that they have a causal role in the pathogenesis of the disease (132).

Starting from these findings, and since the cause and definitive therapy of the MS are still unknown, we wish to explore the possibility that such a circuit exists also in MS. We wish to use the OLs as tester cell system to explore the presence of molecules that interfere with their differentiation.

So we will study in OLs, in presence of the differentiation stimulus, the effects of stimulation with CSF or Ig, extracted from the serum of patients affected by MS, on cellular redox state and maturation processes (differentiation block).
CHAPTER III
MATERIALS AND METHODS

3.1. Cell cultures

**M03-13 cells** - The M03-13 cells are an immortal human-human hybrid cell line with the phenotypic characteristics of primary OLs, derived from the fusion of a 6-thioguanine-resistant mutant of a human rhabdomyosarcoma with OLs obtained from adult human brain. They were grown in Dulbecco’s Modified Eagles Medium (DMEM; GIBCO Invitrogen), containing 4.5g/L glucose (GIBCO, Auckland, New Zealand), supplemented with 10% Foetal Bovine Serum (FBS; Sigma S. Louis, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were kept in a 5% CO₂ and 95% air atmosphere at 37° C.

The cells were differentiated in FBS-free DMEM, supplementing with 100 nM of Phorbol-12-Myristate-13-Aacetate (PMA; Sigma-Aldrich). The cells were kept in a 5% CO₂ and 95% air atmosphere at 37° C for 30 minutes, or 1day (pro-differentiation conditions) or 4 days (mature OLs). The differentiated cells express markers of mature OLs such as MBP, Proteolipidic Protein (PLP) and 2’,3’-cyclic nucleoside 3’-phosphodiesterase (CNPasi). These cells represent an excellent model to study the maturation and differentiation process of oligodendrocyte precursors.

**HEK293 cells** - HEK293 is a cell line derived from human embryonic kidney cells, of a healthy, aborted fetus, grown in tissue culture. This particular line was initiated by the transformation and culturing of normal HEK cells with sheared adenovirus 5 DNA. The transformation resulted in the incorporation of approximately 4.5 kilobases from the viral
genome into human chromosome 19 of the HEK cells. Cells were grown in Dulbecco’s Modified Eagles Medium (DMEM; GIBCO Invitrogen), containing 4.5g/L glucose (GIBCO, Auckland, New Zealand), supplemented with 10% FBS (Sigma S. Louis, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were kept in a 5% CO₂ and 95% air atmosphere at 37°C.

3.2. Flow cytometric assay of cell viability

The toxicity of H₂O₂ treatment was tested by cytofluorimetric analysis of Propidium Iodide (PI) staining of treated and control M03-13 cells differentiated with PMA (100nM) for 4 days. These cells, plated in 35mm Petri dishes and grown to semi confluence, were treated with increasing doses of H₂O₂ (200, 400, 600, 800, 1000μM) for 18 hours in medium without serum. After trypsinization and wash in PBS, the cells were resuspended in 500μl of PBS and 1μg/ml of PI was added before the flow cytometric analysis of PI-positive cells performed with a FCSscan apparatus (Becton-Dickinson). Data were analyzed using WinMDI 2.8 software.

3.3. H₂O₂ treatments

M03-13 cells, grown to semi confluence in 60mm dishes in FBS-free DMEM 4.5g/L glucose, supplementing with 100nM of PMA for 4 days, were stimulated for 30minutes with decreasing concentrations (starting from non toxic dose of 200μM, 50 and10 μM) of H₂O₂. Afterwards, it was used a H₂O₂ concentration that in the cell viability assay didn’t show a significant stimulation of apoptosis and at the same time induced modulation, analyzed by Western-blot, of differentiation markers.

M03-13 cells, grown to semi confluence in 60mm dishes in DMEM 4.5g/L glucose, were stimulated with H₂O₂ (200μM). The cells were kept in a
5% CO₂ and 95% air atmosphere at 37° C for 1 day or 4 days and after the differentiation markers were analyzed by Western-blot, confocal microscopy, or by flow cytometric analysis.

3.4. **Inhibitors treatment**

**APOCYNIN**: 1-(4-Hydroxy-3-methoxyphenyl)ethanone, also known as apocynin or acetovanillone, is a natural organic compound structurally related to vanillin.

M03-13 cells, preincubated for 1 hour with Apocynin (50μM, Sigma-Aldrich) were stimulated for 4 days with PMA (100 nM) or with H₂O₂ (200μM).

**BISINDOLYLMALEIMIDE**: Bisindolylmaleimide VIII acetate is a selective inhibitor of PKC.

M03-13 cells, preincubated for 30 minutes with Bisindolylmaleimide (BIM, 100μM, Sigma-Aldrich) were stimulated for 4 days with PMA (100 nM) or with H₂O₂ (200μM).

**PD98059**: 2’-Amino-3’-methoxyflavone is a selective, reversible, and cell-permeable inhibitor of MEK, that acts by inhibiting the activation of MAP kinase and subsequent phosphorylation of MAP kinase substrates.

M03-13 cells, preincubated for 30 minutes with PD98059 (PD, 40μM, Calbiochem) were stimulated for 4 days with PMA (100 nM) or with H₂O₂ (200μM).

3.5. **Patients**

In the study were included men and women between 15 and 50 years of age who meet all the following criteria:

- diagnosis of relapsing/remitting MS, according to McDonald criteria;
- an Expanded Disability Scale Score (EDSS) between 0 and 5.0;
• lesions detected by MRI compatible with the diagnosis of MS;
• at least one acute episode in the last 12 months.

The control subjects are patients with neurological diseases that need differential diagnosis with MS (cerebral cancers, stroke, vasculitis, etc.) selected by sex and age similar to MS patients.

All the patients were subjected to CSF collection by lumbar puncture to execute the routine laboratory analysis in the hospital where they were hospitalized. A quantity of 1-2ml of CSF was sent to our laboratories to carry out the investigations of our interest. At the same time a blood sample, from each patients, was collected to purify the IgG fractions from blood serum.

The hospitals that have collaborated to our project are:
• Cardarelli Hospital, Naples - Department of Neurology- Dr. C. Florio; - Department of Neurophysiopathology- Dr. F. Habetswallner;
• Polyclinic Federico II University, Naples - Department of Neurology- Prof. Orefice.

3.6. Cerebrospinal fluid treatment

To analyze the cell maturation processes in response to CSF stimulation the M03-13 cells, grown in FBS-free DMEM 4.5g/L glucose, supplementing with 100nM of PMA, were stimulated with CSF (30% v/v) from MS patients and control group for 1 or 4 days. The differentiation markers were analyzed by Western-blot or by confocal microscopy.

3.7. Purification of Immunoglobulins

The purification of IgG fractions from serum of MS and control subjects will be carried out by affinity chromatography on A/G Sepharose columns (Pierce, Rockford, IL). The protein concentration of immunoglobulin fractions thus prepared will be assessed spectrophotometrically and used in oligodendrocyte differentiation cell models.
3.8. Immunoglobulins treatment

To check the effect of IgG fractions stimulation on the molecular mechanisms related to oxidative stress in OLs and on the cell maturation processes, the M03-13 cells, grown in FBS-free DMEM 4.5g/L glucose, supplementing with 100nM of PMA, were stimulated with 200µg/ml of serum IgG from MS patients and control group for 1 or 4 days. The differentiation markers were analyzed by Western-blot or by confocal microscopy.

3.9. Protein extraction

The RIPA buffer was used to extract the proteins from mammalian cells. A cocktail of protease inhibitors (Roche, USA) was added to the buffer to prevent the protein degradation during the extraction procedure.

RIPA buffer:

- 50 mM Tris-HCl, pH 7.5
- 150 mM NaCl
- 1% NP-40
- 0.5% Deoxycholic acid (DOC)
- 0.1% Sodium Dodecyl Sulfate (SDS)
- 2.5mM Sodium Pyrophosphate (SPP)
- 1mM β-Glycerophosphate
- 1mM Sodium Orthovanadate (NaVO₄)
- 1mM Sodium Fluoride (NaF)
- Protease inhibitor (1X)
- 0.5mM Phenyl-methane-sulfonyl-fluoride (PMSF)

The cells were detached from the dishes with a scraper using the RIPA buffer in appropriate doses depending on the number of cells (100µl RIPA
buffer per $5 \cdot 10^5$ cells), on ice. Then, to achieve a complete lysis, cells were kept for 15 min at 4°C and disrupted by repeated aspiration (for approximately 10 times) through a 21-gauge needle. The extract preparations were centrifuged at 13000 rpm for 15 minutes at 4°C and the pellets were discarded. The Lowry protein assay was used to determine the protein concentration.

3.10. Protein determination

The Lowry protein assay was used for determining the total level of protein in a solution. The total protein concentration is exhibited by a color change of the sample solution in proportion to protein concentration, which can then be measured using colorimetric techniques. It is named for the biochemist Oliver H. Lowry who developed the technique in the 1940s. His 1951 paper describing the technique is among the most-highly cited papers in biology. The method combines the reactions of cupric ions with the peptide bonds under alkaline conditions (the Biuret test) with the oxidation of aromatic protein residues. The Lowry method is best used with protein concentrations of 0.01-1.0 mg/mL and is based on the reaction of Cu$^+$, produced by the oxidation of peptide bonds, with Folin-Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin-Ciocalteu reaction). The reaction mechanism involves reduction of the Folin reagent and oxidation of aromatic residues (mainly tryptophan, also tyrosine). The concentration of the reduced Folin reagent is measured by absorbance at 660 nm. As a result, the total concentration of protein in the sample can be deduced from the concentration of Trp and Tyr residues that reduce the Folin reagent. A reference standard was performed on four samples with increasing concentrations of Bovine Serum Albumin (BSA).
3.11. Western-blotting analysis

50 μg of total cells lysates were subjected to SDS-PAGE under reducing conditions using precast gels 4-12% gradient (Invitrogen). The protein samples were heated at 70º C and loaded on the gel. After electrophoresis, the proteins were transferred onto a PDVF filter membrane (Invitrogen) with a Trans-Blot Cell (Invitrogen) and transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol. The transfer was carried out at 4°C for 75 minutes at 40V. Membranes were placed in Blocking buffer (Invitrogen) at 4°C over-night to block the nonspecific binding sites. Filters were incubated with specific antibodies before being washed two times in water. Then, the filter was washed with wash buffer 3 times for 5 minutes and incubated with a peroxidase-conjugated secondary antibody (Invitrogen). After washing with wash buffer, peroxidase activity was detected with the ECL (Enhanced ChemiLuminescence) system (Invitrogen).

The filters were also probed with an anti α-Tubulin antibody (Sigma, USA). Protein bands were revealed by ECL and, when specified, quantified by densitometry using ScionImage software. Densitometric values were normalized to α-Tubulin.

3.12. Flow cytometric analysis of Mielin Basic Protein

Cells were grown to semiconfluency in 60-mm culture dishes. After trypsin detachment, 5 · 10⁵ cells are suspended in 1 mL of phosphate buffered saline (PBS) and fixed overnight with 1% formaldehyde at room temperature. Next, cells were permeabilized with 0.1% Triton X-100 for 40 min at 4°C, washed 4x with PBS containing 2% FBS, 0.01% NaN₃, 0.1% Triton X-100 (buffer A), and incubated for 45 min at 4°C with 1:50 dilution of rabbit polyclonal anti-human MBP Ig. The cells were then washed twice with the same buffer and incubated for 45 min at 4°C with Cy3-conjugated anti-(rabbit
IgG) Ig at 1:50 dilution. Control cells were incubated with Cy3-conjugated anti-(rabbit IgG) Ig alone. After two washes in buffer A, cells were resuspended in PBS and analyzed by flow cytometry using FACSCAN (BD, Heidelberg, Germany) and WINMDI software.

3.13. **Immunofluorescence confocal microscopy**

**MBP and α-SMA:** M03-13 cells were grown on glass coverslip under culture conditions described in the specific experiments. Then, the medium was removed and cells immediately fixed in 3.7% Paraformaldehyde in PBS with 2% Sucrose, pH 7.4, for 5 min at 22°C, and, after 2 washes in PBS with 2% Sucrose, permeabilized for 10 min at 4°C with 0.01% Saponin (Sigma-Aldrich, from quillaja bark) in PBS.

The cells, after blocking with 20% FBS in PBS with 0.01% Saponin for 30 minutes at 4°C, were labelled with primary rabbit-polyclonal anti human MBP antibody or with primary mouse-polyclonal anti human α-SMA antibody. The cells were washed and labelled with secondary Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch, USA) or with secondary Cy2-conjugated anti-mouse IgG (Jackson ImmunoResearch, USA). Controls were incubated with secondary antibodies alone. After treatment with nuclear marker, 4',6-diamidino-2-phenylindole (DAPI), the coverslips were briefly washed first, in PBS and then in distilled water, and finally mounted on glass slides for microscopy examination. Cells were analyzed with a Zeiss LSM 510 Meta laser scanning confocal microscope.

**OLIG-2:** M03-13 cells were grown on glass coverslip under culture conditions described in the specific experiments. Then, the medium was removed and cells immediately fixed in 3.7% Paraformaldehyde in PBS with 2% Sucrose, pH 7.4, for 5 min at 22°C, and, after 2 washes in PBS with 2%
Sucrose, permeabilized for 5 min at 4°C with 0.1% Triton X-100 in 20mM Hepes, 300mM Sucrose, 50mM NaCl, 3 mM MgCl₂.

The cells, after blocking with 20% FBS in PBS for 30 minutes at 4°C, were labelled with primary rabbit polyclonal anti human Olig-2 antibody. The cells were washed and labelled with secondary Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch, USA). Controls were incubated with secondary antibodies alone. After treatment with nuclear marker, 4',6-diamidino-2-phenylindole (DAPI), the coverslips were briefly washed first, in PBS and then in distilled water, and finally mounted on glass slides for microscopy examination. Cells were analyzed with a Zeiss LSM 510 Meta laser scanning confocal microscope.

Afterwards, images were analysed using the ImageJ software: we set the threshold on the maximum fluorescent value of the sample treated only with secondary antibody, and quantified 25 cells for each sample. Regions of interest (ROI) were defined to restrict the analysis to a spatially confined cellular area.

3.14. Real Time PCR analysis

RNA isolation and real-time PCR were performed as follow: total RNA was extracted using TRI-reagent according to the protocol provided by the manufacturer (Sigma, USA). Total RNA (4 µg) was reverse transcribed with Omniscript Reverse Transcriptase (Quiagen, USA) by oligo-dT primers for 60 min at 37°C in 40 µl reaction volumes. Real-time PCR was performed with an ABI 5700 or ABI PRISM-7900HT Sequence Detection System (Applied Biosystems Inc., USA). Reactions were carried out in 96-well optical reaction plates in a 50 µl final volume containing 25 µl of the SYBR-Green (Applied Biosystems Inc., USA) PCR master mix, 1,25 µl of each gene-specific primer, 40 ng of sample cDNA. Gene-specific primers were designed to selectively
amplify $\alpha$-SMA and relative expression values were normalized using glucose-6-phosphate dehydrogenase (G6PD). The SYBRGreen (Applied Biosystems Inc., USA) fluorescence was measured at each extension step. The threshold cycle (Ct) value reflects the cycle number at which the fluorescence measurement reached an arbitrary threshold. Melting curve analysis was performed to determine the specificity of the reaction. Real-time PCR was conducted in triplicate for each sample and the mean value was calculated.

Primers for human $\alpha$-SMA and G6PD used are the following:

- $\alpha$-SMA Forward: CTG TTC CAG CCA TCC TTC AT;
- $\alpha$-SMA Reverse: TCA TGA TGC TGT TGT AGG TGG T;
- Human G6PD Forward: ACA GAG TGA GCCC TTC TTC AA;
- Human G6PD Reverse: ATA GGA GTT GCG GGC AAA G

3.15. **Statistical analysis**

Statistical differences were evaluated using a Student’s $t$-test for unpaired samples.
CHAPTER IV
RESULTS

4.1. Effects of PMA on oligodendrocyte differentiation

M03-13 cells were exposed to PMA 100nM in medium without serum for 4 days. Figure 10, an optical microscope image, shows the morphological changes of the differentiated compared to control cells: the differentiating cells are no longer motile and extend multiple processes.

Figure 10. Optical microscope image.
M03-13 undifferentiated (Undiff. M03-13) and differentiated (Diff. M03-13) for 4 days with PMA 100nM in the absence of serum.

We first analyzed the effects of 30 minutes exposure to 100nM PMA, in cells pre-incubated for 16 hours in a medium containing 0.2% FBS, on signaling molecules (P-ERK1/2 and P-CREB) involved in OLs differentiation and also on a negative differentiation marker, α-SMA. In fact, FGF2-mediated OL induction is dependent on MAP kinase signalling and is inhibited by BMP4; E14 rat dorsal spinal cord cultures, treated by BMP4, show a dose-dependent reduction of OLs and astrocytes, and an induction of SMA. Therefore OLs differentiation is accompanied by significant reduction of SMA (140). Differentiation induced by PMA decreased α-SMA protein levels and, instead, increased P-ERK1/2 and P-CREB levels (Figure 11).
Figure 11. Modulation of \( \alpha\)-SMA, P-ERK1/2 and P-CREB levels in M03-13 cells stimulated with PMA for 30min.
The cells were incubated for 16 hours in medium containing 0.2% of FBS (Ctr) and then stimulated with 100nM PMA in medium without serum for 30min before harvesting them for Western blotting analysis of \( \alpha\)-SMA, P-ERK1/2 and P-CREB levels. The histogram shows the values (means ± SEM) relative to control obtained by densitometric analysis of protein bands normalized to \( \alpha\)-Tubulin in three independent experiments. *p<0.01 vs Ctr.

Moreover, we measured the expression of \( \alpha\)-SMA, P-ERK1/2 and P-CREB protein levels after 1 day of treatment with 100nM PMA in the absence of serum. As it is shown in Figure 12, differentiation of the cells is accompanied by a reduction of \( \alpha\)-SMA levels. On the contrary, P-ERK1/2 and P-CREB levels are induced by differentiation.

Figure 12. Modulation of \( \alpha\)-SMA, P-ERK1/2 and P-CREB levels in M03-13 cells stimulated with PMA for 1d.
Western blotting analysis of \( \alpha\)-SMA, P-ERK1/2 and P-CREB levels in M03-13 cells after 1 day of differentiation of M03-13 cells with 100nM PMA in medium without serum. N.D. indicates Not Differentiated cells, growing in complete medium. The histogram shows the values (means ± SEM) relative to control obtained by densitometric analysis of protein bands normalized to \( \alpha\)-Tubulin in three independent experiments. *p<0.01 vs N.D.
The levels of \textit{MBP} were, then, evaluated by flow cytometry at 1 and 4 days of differentiation with PMA. Figure 13 shows that PMA 100nM in medium without serum induces a time-dependent increase of \textit{MBP} protein levels.

\textbf{Figure 13. Increasing levels of \textit{MBP} protein at different days of differentiation of M03-13 cells with PMA.}

Immunoreactivity for \textit{MBP} was evidenced by indirect immunofluorescence and flow cytometry, using primary antibodies against \textit{MBP} and CY3-conjugated anti rabbit IgG as secondary antibodies. Control was treated with secondary antibodies alone. 10,000 cells were counted for each sample. N.D. indicates Not Differentiated cells, growing in complete medium. A sample of cells differentiated for 4 days with 100nM PMA in medium without serum is also shown (Diff.). The histogram shows the mean ± SEM in three independent experiments. *p<0.01 vs N.D.

Afterwards we considered the expression of \textit{\(\alpha\)-SMA}, \textit{P-ERK1/2} and \textit{P-CREB} in M03-13 cells after several days of 100nM PMA treatment in the absence of serum.

Figure 14 shows that \textit{P-CREB} levels are induced by differentiation, with a peak at 1 day; conversely, a progressive decrease of \textit{\(\alpha\)-SMA} levels is observed in differentiated cells.
Figure 14. \(P\)-CREB and \(\alpha\)-SMA levels in M03-13 cells during differentiation with PMA.

Western blotting analysis of \(P\)-CREB and \(\alpha\)-SMA levels in M03-13 cells after 1, 2 and 4 days of differentiation of M03-13 cells with 100nM PMA in medium without serum. N.D. indicates Not Differentiated cells, growing in complete medium.

The histogram shows the values (means ± SEM) relative to control obtained by densitometric analysis of protein bands normalized to \(\alpha\)-Tubulin in three independent experiments. *p<0.01 vs N.D.

Figure 15 shows that \(P\)-ERK1/2 levels are progressively induced by differentiation.

Figure 15. Western blotting analysis of \(P\)-ERK1/2 levels in M03-13 cells after different days of differentiation with 100nM PMA in a medium without serum.

Western blotting analysis of \(P\)-ERK1/2 levels in M03-13 cells after 1, 2 and 4 days of differentiation of M03-13 cells with 100nM PMA in medium without serum. N.D. indicates Not Differentiated cells, growing in complete medium.

The histogram shows the values (means ± SEM) relative to control obtained by densitometric analysis of protein bands normalized to \(\alpha\)-Tubulin in three independent experiments. *p<0.01 vs N.D.
4.2. Effects of oxidative stress on growth and differentiation of oligodendrocytes

To find a correlation between cellular redox state and differentiation of OPCs, we evaluated the effects of H$_2$O$_2$ on differentiation markers in M03-13 cells.

To monitor the effects of H$_2$O$_2$ on M03-13 cell survival, we induced differentiation in FBS-free DMEM, supplemented with 100 nM of PMA for 4 days and then treated the cells with increasing doses of H$_2$O$_2$ (200, 400, 800, 1000 μM) for 18 hours. Flow-cytometric analysis of PI-positive cells shown an increase of cell death by high H$_2$O$_2$ doses (from 400 μM to 1000 μM) compared to control cells, while M03-13 cells treated with a 200 μM H$_2$O$_2$ did not show significant cell death (Figure 16).

![Figure 16. Dose-dependent effect of H$_2$O$_2$ on cell viability.](image)

The MO3-13 cells were differentiated in FBS-free DMEM supplementing with 100 nm of PMA for 4 days; the cell viability after stimulation with increasing doses of H$_2$O$_2$ was measured by cytofluorimetry with Propidium Iodide (PI) staining.

We then evaluated the dose-response of stimulation at concentrations lower than 200 μM of H$_2$O$_2$ on the expression of OLs differentiation markers.
The cells were differentiated with 100nM PMA in medium without serum for 4 days and treated for 30 minutes with H$_2$O$_2$.

Figure 17 shows that H$_2$O$_2$ reduces of $\alpha$-SMA. On the contrary, differentiation of the cells is accompanied by an increase of P-CREB levels. Furthermore, H$_2$O$_2$ 200µM dose seems a very effective inducer of differentiation. Therefore, we used this dose of H$_2$O$_2$ for our experiments.

**Figure 17. Short-term effects of H$_2$O$_2$ on $\alpha$-SMA and P-CREB levels in M03-13 cells.** Cells were differentiated with 100nM PMA in medium without serum for 4 days, treated for 30min with 10, 50 or 200µM H$_2$O$_2$ and then $\alpha$-SMA and P-CREB levels were evaluated by Western blotting analysis. The histogram shows the values (means ± SEM) relative to controls obtained by densitometric analysis of protein bands normalized to $\alpha$-Tubulin in three independent experiments.

We also quantified the levels of MBP in M03-13 cells treated for 4 days with H$_2$O$_2$ 200 µM; flow cytometric analysis shown an increase of total MBP protein levels (Figure 18).
Figure 18. Effects of H$_2$O$_2$ on MBP levels in M03-13 cells (4d).
Increase of MBP protein levels in M03-13 cells treated for 4 days with 200µM H$_2$O$_2$. Immunoreactivity for MBP was evidenced by indirect immunofluorescence and flow cytometric analysis, using primary antibodies against MBP and CY3-conjugated anti rabbit IgG as secondary antibodies. Control was treated with secondary antibodies alone. 10,000 cells were counted for each sample.
N.D. indicates Not Differentiated cells, growing in complete medium. A sample of cells differentiated for 4 days with 100nM PMA in medium without serum is also shown (Diff.). The histogram shows the mean ± SEM in three independent experiments. *p<0.01 vs N.D.

On the contrary, differentiation of cells for 4 days with H$_2$O$_2$ was accompanied by a significant decrease of cytosolic MBP levels, measured by protein fractionation and Western blotting analysis. These apparently contradictory data can be explained by the fact that MBP translocates from cytosol to the detergent-resistant lipid rafts membrane microdomain following differentiation (Figure 19).
Figure 19. Effects of H$_2$O$_2$ on cytosolic MBP levels in M03-13 cells (4d). Western blotting analysis of cytosolic MBP levels in M03-13 cells treated for 4 days with 200µM H$_2$O$_2$.

N.D. indicates Not Differentiated cells, growing in complete medium. A sample of cells differentiated for 4 days with 100nM PMA in medium without serum is also shown (Diff.). The histogram shows the values (means ± SEM) relative to control obtained by densitometric analysis of MBP normalized to α-Tubulin in three independent experiments. *p< 0.01 vs N.D.

The effects of the PMA stimulus (100 nM) in serum-free medium or of H$_2$O$_2$ (200 µM) for 1 and 4 days on α-SMA and MBP levels were also evaluated by confocal microscopy analysis.

Figure 20 shows that the signal derived from antibodies directed against α-SMA decreases both in cells stimulated by PMA and with H$_2$O$_2$, compared to undifferentiated cells (Table 1).
Figure 20. Decrease of α-SMA levels in M03-13 cells after treatment with PMA or H2O2 (1d-4d).

α-SMA staining after 1 or 4 days of differentiation of M03-13 cells with 100nM PMA in medium without serum or with 200μM H2O2. Cells were stained with anti human α-SMA antibodies and CY2-conjugated anti mouse IgG as secondary antibodies and analyzed by confocal microscopy.

Ctr was treated with secondary antibodies and nuclear dye DAPI alone. N.D. indicates Not Differentiated cells, growing in complete medium.

<table>
<thead>
<tr>
<th></th>
<th>PMA 1d</th>
<th>PMA 4d</th>
<th>H2O2 1d</th>
<th>H2O2 4d</th>
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</thead>
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<tr>
<td>N.D.</td>
<td>104.83±8.5</td>
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<td>43.27±7</td>
<td>51.12±8.3</td>
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<td></td>
<td>38.01±7.4</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 1. Quantitative analysis of the Figure 20.

The values are means ± SD of 25 cells for each sample.

Besides, in M03-13 cells immunostained with antibodies directed against MBP, the signal increased in differentiated cells with PMA, compared with undifferentiated cells and accumulated in cellular processes; a similar increase of the fluorescent signal was observed in the cells treated with H2O2 (Figure 21 and Table 2).
Figure 21. Increase of MBP levels in M03-13 cells after treatment with PMA or H₂O₂ (1d-4d).

MBP staining after 1 or 4 days of differentiation of M03-13 cells with 100nM PMA in medium without serum or with 200µM H₂O₂. Cells were stained with anti human MBP antibodies and CY3-conjugated anti rabbit IgG as secondary antibodies and analyzed by confocal microscopy.

Ctr was treated with secondary antibodies and nuclear dye DAPI alone. N.D. indicates Not Differentiated cells, growing in complete medium.

<table>
<thead>
<tr>
<th></th>
<th>N.D.</th>
<th>PMA 1d</th>
<th>PMA 4d</th>
<th>H₂O₂ 1d</th>
<th>H₂O₂ 4d</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.D.</td>
<td>39.86±4.6</td>
<td>71.73±21.4</td>
<td>97.93±38.4</td>
<td>56.44±3.9</td>
<td>63.91±4.8</td>
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</tbody>
</table>

Table 2. Quantitative analysis of the Figure 21.

The values are means ± SD of 25 cells for each sample.

Moreover, we measured, by confocal fluorescence analysis, the effects of the same stimuli on the expression levels of Olig-2, a specific OLs differentiation marker, in PMA-differentiated cells, as well as in H₂O₂-stimulated cells. The signal progressively increased compared to undifferentiated growing cells (Figure 22 and Table 3). In the figure the DAPI (nuclei) and Olig-2 signals are split in different panels to better appreciate the nuclear staining of Olig-2.
Figure 22 Increase of *Olig*-2 levels in M03-13 cells after treatment with PMA or H$_2$O$_2$ (1d-4d).

*Olig*-2 staining after 1 or 4 days of differentiation of M03-13 cells with 100nM PMA in medium without serum or with 200µM H$_2$O$_2$. Cells were stained with anti human *Olig*-2 antibodies and CY3-conjugated anti rabbit IgG as secondary antibodies and analyzed by confocal microscopy.

For each image are shown three panels: on the left *Olig*-2 (red); on the center nuclei (blue); on the right the merged image. Ctr was treated with secondary antibodies and nuclear dye DAPI alone. N.D. indicates Not Differentiated cells, growing in complete medium.
<table>
<thead>
<tr>
<th>N.D.</th>
<th>PMA 1d</th>
<th>PMA 4d</th>
<th>H$_2$O$_2$ 1d</th>
<th>H$_2$O$_2$ 4d</th>
</tr>
</thead>
<tbody>
<tr>
<td>39.86±4.6</td>
<td>50.61±6.4</td>
<td>72.27±9.4</td>
<td>54.2±9.9</td>
<td>54.99±2.8</td>
</tr>
</tbody>
</table>

**Table 3. Quantitative analysis of the Figure 22.**

The values are means ± SD of 25 cells for each sample.

Afterwards, we found that chronic stimulation (4 days) of the cells with H$_2$O$_2$ (200 µM) induced $P$-$ERK1/2$, $P$-$CREB$, $Olig$-2 and decreased $\alpha$-$SMA$ levels similarly to PMA (100 nM) (Figure 23). Also, the same markers were evaluated in response to H$_2$O$_2$ in cells preincubated with N-acetyl-L-cysteine (NAC, 50 µM), a precursor of Glutathione, a natural antioxidant that protects cells, together with catalase and SOD, from ROS toxicity: H$_2$O$_2$ effects were reversed by treatment with NAC (data not shown).

Figure 23: Effects of H$_2$O$_2$ on differentiation markers in M03-13 cells (4d).

Western blotting analysis of $\alpha$-$SMA$, $P$-$ERK1/2$, $P$-$CREB$ and $Olig$-2 levels in M03-13 cells treated for 4 days with 200µM H$_2$O$_2$.

N.D. indicates Not Differentiated cells, growing in complete medium. A sample of cells differentiated for 4 days with 100nM PMA in medium without serum is also shown.

The histogram shows the values (means ± SEM) relative to control obtained by densitometric analysis of MBP normalized to $\alpha$-Tubulin in three independent experiments.

*p< 0.01 vs N.D.
Real-time PCR analysis evidenced a reduction of \( \alpha\text{-SMA} \) mRNA levels in M03-13 cells treated for 4 days with \( \text{H}_2\text{O}_2 \) relative to control cells (Figure 24), confirming the decrease of \( \alpha\text{-SMA} \) protein levels evidenced by Western blotting experiments.

![Graph](image.png)

**Figure 24:** \( \alpha\text{-SMA} \) m-RNA levels in M03-13 cells treated with PMA or \( \text{H}_2\text{O}_2 \) (4d).

RT-PCR analysis of \( \alpha\text{-SMA} \) m-RNA levels in M03-13 cells treated for 4 days with 100nM PMA in medium without serum or with 200µM \( \text{H}_2\text{O}_2 \). N.D. indicates Not Differentiated cells, growing in complete medium. Expression values were normalized using glucose-6-phosphate-dehydrogenase mRNA (G6PD). The histogram shows means ± SEM values relative to control in three independent experiments.

4.2.1. **Study of pathways involved in oxidative stress and oligodendrocytes differentiation**

To identify the signalling pathways involved in \( \text{H}_2\text{O}_2 \) and PMA-induced OLs differentiation, we used specific inhibitors of signaling molecules involved in oxidative stress and/or OLs differentiation.

We first evaluated the involvement of NADPH-ox-dependent ROS production in PMA-mediated differentiation using Apocynin, a specific NAPDH oxidase inhibitor.
To this aim the effects of stimulation with PMA (100 nM) in serum-free medium or with H₂O₂ (200 μM) for 4 days, were evaluated, on the expression levels of P-ERK1/2, P-CREB, Olig-2 and Ha-Ras, with or without Apocynin (50 μM), by Western-blotting. Incubation of cells with H₂O₂ and apocynin for 4 days, induced cell death.

Figure 25 shows an increase in protein levels seen following the stimulation with PMA or with H₂O₂; the effect of PMA is reversed by preincubation with Apocynin.

![Figure 25. Apocynin reverts PMA effect (4d).](image)

Western blotting analysis of p-ERK1/2, p-CREB, Olig-2 and Ha-Ras expression levels in M03-13 cells stimulated with H₂O₂ (200 μM) or PMA (100 nM) for 4 days in serum-free medium in the presence or absence of Apocynin (50 μM). N.D. indicates Not Differentiated cells, growing in complete medium.

The histogram shows the values (means ± SEM) relative to control obtained by densitometric analysis of protein bands normalized to total-ERK1/2, compared to undifferentiated control, in three independent experiments. * p< 0.01 vs N.D.

The same protocol was used to verify the action of Bisindolylmaleimide (BIM, 100 μM), a PKC inhibitor, on differentiation induced by H₂O₂ or by PMA; increased expression levels, verified by Western-blot, of proteins p-ERK1/2, p-CREB, Olig-2 and Ha-Ras, as a result of stimulation with PMA or H₂O₂, were reversed by the BIM (Figure 26).
Similarly MBP, using confocal microscopy, decreased in the samples treated with BIM and PMA (100 nM) or with H$_2$O$_2$ (200 μM) for 4 days compared with that cells stimulated with PMA or H$_2$O$_2$ alone (Figure 27 and Table 4).

**Figure 26. BIM reverts PMA and H$_2$O$_2$ effects (4d).**
Western blotting analysis of p-ERK1/2, p-CREB, Olig-2 and Ha-Ras expression levels in M03-13 cells stimulated with H$_2$O$_2$ (200 μM) or PMA (100 nM) for 4 days in serum-free medium in the presence or absence of BIM (100 μM). N.D. indicates Not Differentiated cells, growing in complete medium. The histogram shows the values (means ± SEM) relative to control obtained by densitometric analysis of protein bands normalized to total-ERK1/2 compared to undifferentiated control, in three independent experiments. * p< 0.01 vs N.D.
Figure 27. BIM reverts PMA and H$_2$O$_2$ effects (4d) on MBP levels in M03-13 cells. MBP staining after 1 or 4 days of differentiation of M03-13 cells with 100nM PMA in medium without serum or with 200µM H$_2$O$_2$ in the presence or absence of BIM (100 µM). Cells were stained with anti human MBP antibodies and CY3-conjugated anti rabbit IgG as secondary antibodies and analyzed by confocal microscopy. Ctr was treated with secondary antibodies and nuclear dye DAPI alone. N.D. indicates Not Differentiated cells, growing in complete medium.

<table>
<thead>
<tr>
<th></th>
<th>PMA 1d</th>
<th>PMA 4d</th>
<th>BIM+PMA 4d</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.D.</td>
<td>37.49±2.9</td>
<td>96.53±11.6</td>
<td>38.10±11.4</td>
</tr>
<tr>
<td>BIM 4d</td>
<td>50.71±7.9</td>
<td>51.54±2.7</td>
<td>35.51±4.1</td>
</tr>
<tr>
<td>H$_2$O$_2$ 1d</td>
<td>44.84±9.5</td>
<td>45.85±6.2</td>
<td>51.54±2.7</td>
</tr>
<tr>
<td>H$_2$O$_2$ 4d</td>
<td>45.85±6.2</td>
<td>51.54±2.7</td>
<td>35.51±4.1</td>
</tr>
</tbody>
</table>

Table 4. Quantitative analysis of the Figure 27.
The values are means ± SD of 25 cells for each sample.
The expression levels of \( \alpha\text{-SMA}, \) P-ERK1/2, P-CREB were also measured after 4 days of differentiation with PMA (100 nM) in the absence of serum or with H\(_2\)O\(_2\) in the presence or absence of MEK-ERK1/2 pathway inhibitor, PD98059 (PD, 40 \( \mu \)M). Incubation of cells with H\(_2\)O\(_2\) and PD, for 4 days, induced cell death. As shown in Figure 28, P-ERK1/2 and P-CREB were induced by differentiation; in contrast, levels of \( \alpha\text{-SMA} \) were reduced by differentiation. PD reversed the effect of PMA on the expression levels of these proteins.

**Figure 28. PD reverts PMA effect (4d).**
Western blotting analysis of \( \alpha\text{-SMA}, \) p-ERK1/2 and p-CREB expression levels in M03-13 cells stimulated with H\(_2\)O\(_2\) (200 \( \mu \)M) or PMA (100 nM) for 4 days in serum-free medium in the presence or absence of PD (40 \( \mu \)M). N.D. indicates Not Differentiated cells, growing in complete medium.
The histogram shows the values (means ± SEM) relative to control obtained by densitometric analysis of protein bands normalized to total-ERK1/2 compared to undifferentiated control, in three independent experiments. *p<0.01 vs N.D.

### 4.3. Stimulation of cells with biological samples

Immunocytochemistry and immunohistochemistry previous studies have shown that in chronic demyelinating lesions of MS patients is present a small number of pro-OL and an increased number of OPCs, suggesting an impairment in the OPCs maturation (66, 67). To assess the presence in
biological fluids (serum and CSF) of MS patients factors (such as Ig extracted from serum or CSF) that alter the redox state and differentiation of OPCs, we evaluated the effects of stimulation with these biological samples, in differentiated OLs, on expression levels of proteins involved in differentiation.

4.3.1. Effect of CSF from MS patients on the molecular mechanism of differentiation in oligodendrocytes

To evaluate whether in CSF from MS patients there are molecules that alter the OLs differentiation, we incubated M03-13 cells, grown in FBS-free DMEM supplemented with 100nM of PMA for 1or 4 days (pro-differentiation conditions), in the presence of CSF (30% v/v) of MS or control patients.

In these conditions \(\alpha\)-SMA, measured by confocal microscopy, which decreased as a result of differentiation for 4 days, in presence of CSF of MS patients displayed expression levels comparable to that of undifferentiated cells; this did not occur after treatment with CSF of control patients (Figure 29 and Table 5).
Figure 29. CSF from MS patients inhibits PMA differentiative effect on α-SMA levels (4d).

The cells were stained with anti-α-SMA and anti-mouse secondary antibody conjugated to CY2 and the nuclear dye DAPI, and analyzed by confocal microscopy. N.D. indicates Not Differentiated cells, growing in complete medium; Diff was treated only with PMA (100 nM) for 4 days in serum-free medium; CSF N indicates cells stimulated by CSF from control patients; CSF MS indicates cells stimulated with CSF of MS patients.

<table>
<thead>
<tr>
<th>N.D.</th>
<th>Diff.</th>
<th>CSF N1</th>
<th>CSF N2</th>
<th>CSF MS1</th>
<th>CSF MS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>98.21±4.1</td>
<td>38.15±2.1</td>
<td>36.18±1.7</td>
<td>58.16±6.3</td>
<td>123.02±30.7</td>
<td>101.38±8.7</td>
</tr>
</tbody>
</table>

Table 5. Quantitative analysis of the Figure 29.

The values are means ± SD of 25 cells for each sample.

Under the same conditions MBP, measured by confocal microscopy, which increased as a result of differentiation for 1 day, in the presence of CSF of MS patients displayed expression levels comparable to that of undifferentiated cells; this did not occur after treatment with CSF of control patients (Figure 30 and Table 6).
Figure 30. CSF from MS patients inhibits PMA differentiative effect on MBP levels (1d).
The cells were stained with anti-MBP and anti-rabbit secondary antibody conjugated to CY3 and the nuclear dye DAPI, and analyzed by confocal microscopy.
N.D. indicates Not Differentiated cells, growing in complete medium; Diff was treated only with PMA (100 nM) for 1 day in serum-free medium; CSF N indicates cells stimulated by CSF from control patients; CSF MS indicates cells stimulated with CSF of MS patients.

<table>
<thead>
<tr>
<th>N.D.</th>
<th>Diff.</th>
<th>CSF N1</th>
<th>CSF N2</th>
<th>CSF MS1</th>
<th>CSF MS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>96.87±4.2</td>
<td>127.15±8.6</td>
<td>100.44±5.5</td>
<td>121.79±12</td>
<td>89.26±5</td>
<td>86.66±4.6</td>
</tr>
</tbody>
</table>

Table 6. Quantitative analysis of the Figure 30.
The values are means ± SD of 25 cells for each sample.

Using the same protocol, we evaluated the effects of stimulation with CSF on the transcriptional factor *Olig-2*. In M03-13 cells, after 1 day of stimulation with PMA (100 nM) in the absence of serum, the specific *Olig-2* signal increased compared to growing cells (N.D.), and became evident in the cytosol, in cells treated for 1 day with PMA in the presence of CSF of MS patients, the signal derived from *Olig-2* was similar to that of undifferentiated cells (Figure 31 and Table 7).
Figure 31. CSF from MS patients inhibits PMA differentiative effect on Olig-2 levels (1d).

The cells were stained with anti-Olig-2 and anti-rabbit secondary antibody conjugated to CY3 and the nuclear dye DAPI, and analyzed by confocal microscopy.

For each image are shown three panels: on the left Olig-2 (red); on the center nuclei (blue); on the right the merged image.

N.D. indicates Not Differentiated cells, growing in complete medium; Diff. was treated only with PMA (100 nM) for 1 day in serum-free medium; CSF N indicates cells stimulated by CSF from control patients; CSF MS indicates cells stimulated with CSF of MS patients.
Table 7. Quantitative analysis of the Figure 31.
The values are means ± SD of 25 cells for each sample.

<table>
<thead>
<tr>
<th></th>
<th>N.D.</th>
<th>Diff.</th>
<th>CSF N1</th>
<th>CSF N2</th>
<th>CSF MS1</th>
<th>CSF MS2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95.21±7.6</td>
<td>114.56±5.1</td>
<td>93.35±5.4</td>
<td>103.12±3.4</td>
<td>84.77±8.5</td>
<td>93.57±2.6</td>
</tr>
</tbody>
</table>

4.3.2. Effect of IgG from MS patients on oligodendrocytes differentiation

Further experiments for the analysis of the effects of biological samples on cellular maturation processes of OLs, were performed by incubating M03-13 cells for 1 day with PMA (100 nM), in the absence of serum (pro-differentiative conditions), and in the presence of IgG (200 µg/ ml), purified by affinity chromatography, from the serum of MS and control patients.

In this case the expression levels of P-ERK1/2 and Olig-2 were assessed by Western-blot. Both differentiation markers increased as a result of differentiation; in cells incubated in the presence of IgG purified from serum of patients with MS, their levels were lower than that observed after treatment of cells with IgG from control patients (Figure 32).

**Figure 32.** IgG from MS patients inhibit PMA differentiative effect on p-ERK1/2 and Olig-2 expression levels (1d).

Western blotting analysis of p-ERK1/2 and Olig-2 expression levels in M03-13 cells stimulated with PMA (100 nM) for 1 day in serum-free medium in the presence of IgG (200 µg/ml) purified from the serum of patients with and not from MS. N.D. indicates Not Differentiated cells, growing in complete medium; Diff. was treated only with PMA (100 nM) for 1 day in serum-free medium; Ig N indicates cells stimulated by IgG of control patients; Ig MS indicates cells stimulated with IgG of patients with MS.

The histogram shows the values (means ± SEM) relative to control obtained by densitometric analysis of protein bands normalized to α-Tubulin, compared to cells stimulated by IgG control patients, in three independent experiments. *p<0.01 vs Ig Ctr.
Using the same protocol and confocal microscopy, we monitored the effects on \( \alpha\text{-SMA} \) after 4 days, and on \( \text{MBP} \) and on \( \text{Olig-2} \) after 1 day.

In M03-13 cells incubated with anti-\( \alpha\text{-SMA} \) antibodies, after 4 days of stimulation with PMA (100 nM) in the absence of serum, the signal decreased compared to growing cells (N.D.); in cells treated for 4 days with PMA, in the presence of IgG from MS patients, the signal arising from \( \alpha\text{-SMA} \) was increased (Figure 33 and Table 8).

![Figure 33. IgG from MS patients inhibit PMA differentiative effect on \( \alpha\text{-SMA} \) levels (4d).](image)

The cells were stained with anti-\( \alpha\text{-SMA} \) and anti-mouse secondary antibody conjugated to CY2 and the nuclear dye DAPI, and analyzed by confocal microscopy.

N.D. indicates Not Differentiated cells, growing in complete medium; Diff. was treated only with PMA (100 nM) for 4 days in serum-free medium; Ig N indicates cells stimulated by IgG of control patients; Ig MS indicates cells stimulated with IgG of MS patients.

<table>
<thead>
<tr>
<th></th>
<th>N.D.</th>
<th>Diff.</th>
<th>Ig N1</th>
<th>Ig N2</th>
<th>Ig MS1</th>
<th>Ig MS2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>125.02±11.1</td>
<td>36.15±4.8</td>
<td>40.17±1.8</td>
<td>37.44±3.5</td>
<td>69.74±12.9</td>
<td>81.85±4.4</td>
</tr>
</tbody>
</table>

**Table 8. Quantitative analysis of the Figure 33.**

The values are means ± SD of 25 cells for each sample.
In M03-13 cells incubated with anti-MBP antibodies, after 1 day of stimulation with PMA (100 nM) in the absence of serum, the signal increased compared to growing cells (N.D.); in cells treated for 1 day with PMA, in the presence of IgG from MS patients, the signal arising from MBP was decreased (Figure 34 and Table 9).

![Image of microscopy images](image)

**Figure 34.** IgG from MS patients inhibit PMA differentiative effect on MBP levels (1d).
The cells were stained with anti-MBP and anti-rabbit secondary antibody conjugated to CY3 and the nuclear dye DAPI, and analyzed by confocal microscopy.
N.D. indicates Not Differentiated cells, growing in complete medium; Diff. was treated only with PMA (100 nM) for 1 day in serum-free medium; Ig N indicates cells stimulated by IgG of control patients; Ig MS indicates cells stimulated with IgG of MS patients.

<table>
<thead>
<tr>
<th></th>
<th>N.D.</th>
<th>Diff.</th>
<th>Ig N1</th>
<th>Ig N2</th>
<th>Ig MS1</th>
<th>Ig MS2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>97.1±3.5</td>
<td>150.55±4.4</td>
<td>112.20±13.9</td>
<td>144.18±12.3</td>
<td>88.84±4.1</td>
<td>86.39±5.9</td>
</tr>
</tbody>
</table>

**Table 9. Quantitative analysis of the Figure 34.**
The values are means ± SD of 25 cells for each sample.
Similar results were obtained in M03-13 cells incubated with anti-
*Olig-2* antibodies, after 1 day of stimulation with PMA (100 nM) in the absence of
serum: the signal increased compared to growing cells (N.D.), and was
evident in the cytosol; in cells treated for 1 day with PMA in the presence of
IgG from MS patients, the signal derived from *Olig-2* was decreased (Figure
35 and Table 10).
Figure 35. IgG from MS patients inhibit PMA differentiative effect on Olig-2 levels (1d).
The cells were stained with anti-Olig-2 and anti-rabbit secondary antibody conjugated to CY3 and the nuclear dye DAPI, and analyzed by confocal microscopy.
For each image are shown three panels: on the left Olig-2 (red); on the center nuclei (blue); on the right the merged image.
N.D. indicates Not Differentiated cells, growing in complete medium; Diff. was treated only with PMA (100 nM) for 1 day in serum-free medium; Ig N indicates cells stimulated by IgG of control patients; Ig MS indicates cells stimulated with IgG of MS patients.
Table 10. Quantitative analysis of the Figure 35.
The values are means ± SD of 25 cells for each sample.

![Table 10. Quantitative analysis of the Figure 35.](image)

Finally, to assay the tissue-specificity of the IgG effect on the expression levels of *P-ERK1/2*, we applied the same protocol on another cell line, the HEK293. Also in these cells, PMA (100 nM) treatment for 1 day, stimulated *P-ERK1/2*, but this effect was not modified by the presence of IgG from MS patients (Figure 36).

![Figure 36. Inhibition of PMA differentiative effect by Ig from MS patients is tissue specific.](image)

Western blotting analysis of *p-ERK1/2* expression levels in M03-13 and HEK293 cells stimulated with PMA (100 nM) for 1 day in serum-free medium in the presence of IgG (200 µg/ml) purified from the serum of patients with and not from MS. N.D. indicates Not Differentiated cells, growing in complete medium; Diff. was treated only with PMA (100 nM) for 1 day in serum-free medium; Ig N indicates cells stimulated by IgG of control patients; Ig MS indicates cells stimulated with IgG of MS patients.
The histogram shows the values (means ± SEM) relative to control obtained by densitometric analysis of protein bands normalized to α-Tubulin, compared to cells stimulated by IgG control patients, in three independent experiments. *p<0.01 vs Ig Ctr.
CHAPTER V
DISCUSSION AND CONCLUSIONS

OLs, the myelin-forming cells of the CNS, are derived from bipotential precursor cells. In culture, these cells differentiate to astrocytes type II in the presence of serum, and proliferate or differentiate to mature OLs when certain growth factors are added (81). These cells respond to serum deprivation by a cessation of cell proliferation and by an increase in morphological differentiation (138).

In this study, M03-13 cells, a cell line with oligodendroglial properties, were used to search for a link between oxidative stress and differentiation.

We identified several molecular markers modulated by differentiation stimuli such as PMA (PKC activator). We identified the effective concentrations of these substances and the incubation time required for the modulation of markers of differentiation, following the pattern of their expression over time up to 4 days of treatment.

We demonstrated a close link between ROS and differentiative processes of OLs. The chronic stimulation of the cells with low doses of H2O2 induces the differentiation of OPC in differentiated cells; H2O2 modulates, in fact, different markers of differentiation, in similar manner to chronic treatment with PMA 100nM in medium without serum.

Several studies have shown that H2O2 activates signaling pathways associated with protein tyrosine kinases and their downstream signaling components, such as MAPKs and transcription factors regulating cell survival (141); our results show that P-ERK1/2 and P-CREB, in fact, increase with differentiation.
MBP, protein associated with the process of myelinating OLs, and Olig-2, a specific OLs transcription factor, increase with differentiation and chronic stimulation of the cells with low doses of H$_2$O$_2$ exert similar effects.

Instead $\alpha$-SMA, a negative marker of differentiation, decreases with differentiation induced both by PMA and H$_2$O$_2$.

Important experimental evidence suggest the existence of a significant contribution of ROS in the formation and persistence of chronic demyelinating lesions in MS. This condition of oxidative stress is associated with epigenetic modulation of gene transcription and with a block of OLs maturation.

Therefore, we demonstrated that the differentiation of OLs can be monitored and manipulated in culture under very defined conditions. Moreover, we discovered that hydrogen peroxide at low dose stimulates differentiation, whereas at higher doses induces oxidative stress and compromises cell viability.

To study the signal transduction pathway involved in PMA and H$_2$O$_2$–induced OLs differentiation we treated the cells with differentiation stimuli in the presence of different inhibitors:

- Apocynin’s observed anti-inflammatory capabilities proved to be a result of its ability to selectively prevent the formation of free radicals, oxygen ions, and peroxides in the body.  
  **MODE OF ACTION:** NADPH oxidase is an enzyme that effectively reduces O$_2$ to superoxide (O$_2^-$), which can be used by the immune system to kill bacteria and fungi. Apocynin is a selective inhibitor of NADPH oxidase activity and thus is effective in preventing the production of the superoxide in human white blood cells or neutrophilic granulocytes (142). Apocynin was used to determine whether ionic activation due to proton flux across the membrane of renal medulla cells was
coupled to NADPH oxidase production of superoxide. Apocynin was introduced to the cells and completely blocked the production of superoxide, and was a key component in determining that the proton outflow was responsible for the activation of NADPH oxidase (143).

- Bisindolylmaleimide (BIM) is a selective inhibitor of PKC, that enhances Fas- and TRAIL-mediated apoptosis and inhibits T cell-mediated autoimmune diseases.
- PD 98059 is a selective, reversible, and cell-permeable inhibitor of MEK, that acts by inhibiting the activation of MAP kinase and subsequent phosphorylation of MAP kinase substrates.

We observed that these three inhibitors reverse PMA effects, and BIM reverts H₂O₂ effects too; we don’t yet know if Apocynin and PD reverse H₂O₂ effects, because used doses caused cell death.

On the basis of these results, we have hypothesized that stimuli of PMA and H₂O₂ may converge into one pathway: a seven transmembrane receptor activates phospholipase C (PLC); inositol triphosphate leads to an increase (IP₃), which, by interacting with its receptor (IP₃-R) in the endoplasmic reticulum (ER), causes an increase of intracellular Ca²⁺ concentration, which, together with diacylglycerol, activates PKC; PKC acts on the NADPH-oxidase, resulting in an increase of ROS, which activate the MAP kinase cascade, that self-amplifies the circuit (Figure 37).
Figure 37. Scheme of PKC/ROS signaling pathways involved in OLS differentiation. ROS are downstream of PMA and are generated by NADPH oxidase to induce differentiation of OLs precursors.

An important objective of the project is to verify the presence in biological fluids from MS patients of factors able to induce oxidative stress and/or to alter OLs differentiation. The CSF is in communication with the brain interstitial fluid and therefore substances or antibodies present in CSF can reach neurons and glial cells activating specific cellular responses. Moreover, in MS patients, it has been demonstrated a disruption of the BBB; therefore, it is possible to hypothesize that serum antibodies can reach the CNS, producing demyelination. Based on these assumptions, the effects of liquor and Ig from serum and liquor from MS patients were analyzed on the OLS differentiation through the analysis of the expression levels of differentiation markers.
We found that in the CSF from patients with MS there is a molecule(s) that blocks the OLs differentiation; this inhibition may alter the normal remyelination process, giving rise to permanent neurological damage.

Using the CSF or IgG from serum of MS patients to stimulate the cells, we found that \textit{P-ERK1/2, MBP} and \textit{Olig-2} expression levels are significantly reduced compared with control group, likewise to undifferentiated cells. Instead, \textit{\alpha-SMA} expression levels are decreased in cells stimulated by CSF or IgG from serum of MS patients, similarly to undifferentiated cells. Therefore, in biological fluids from MS patients, there are molecules that act on redox pathways and inhibits the OLs differentiation. Importantly, this effect is cell specific, since it is detectable only in OLs and the source of inhibition is in the serum, presumably an Ig. This opens a new scenario in the search of the pathogenic clue of MS.

Previous studies have shown that in chronic demyelinating lesions in MS patients there is a small number of pro-OL and an increased number of OPCs, suggesting an impairment in the OPCs differentiation (66, 67).

This data is actually based on immunocytochemistry and immunohistochemistry investigations of the brain tissue, while our results allow us to evaluate the differentiation mechanisms at a cellular level and not organ level.

5.1. Future perspectives

In the next future we wish to validate the effects of Ig derived from CSF on OLs precursors and differentiated cells and the effects of CSF and Ig derived from serum and CSF on OLs differentiated with H$_2$O$_2$.

Finally, the identification of specific markers of oxidative stress and/or cell differentiation, modulated in M03-13 cells exposed to biological fluids from MS patients, is relevant because it can represent an indirect measurement of a specific cell receptor activation, giving the opportunity to
use this assay as guide identify the receptor affected. The identification of a putative receptor target of molecules present in the biological fluids of MS patients will pave the way to dissect the primary cause of the disease. The identification of receptor will be performed through the use of specific inhibitors. Once identified, the receptor will be purified by chromatography and an in vitro binding assay the blood serum Ig from patients will be developed (131).

It is not yet clear whether the axonal damage, the main cause of disability in MS, is secondary to the myelin sheath damage. Therefore, we cannot exclude that neurons represent the direct target of auto-antibodies or substances present in body fluids of MS patients leading to deficiency of axon-glial signaling and demyelination. Consequently, primary cultures of mouse neurons will also be used in our study.

From these experiments we expect to get information on the nature and identity of factors that can affect the maturation processes of OLs and on the pathogenesis of the disease.
ACKNOWLEDGEMENTS

In these years I grew up, and with me my consciousness grew too: primarily, on the life’s precariousness, but especially on the fact that “some things” never let you down……the gestures, the words, the glances and smiles of the people, who, rather than simply to be near with you, accompany you, guide you in your path, do you support or at least try to do their best for you.

And it is from this consciousness that the desire to thank all these people was born; people that know, in one way or another one, to helping me!

First of all, I would like to thank Prof. Vittorio Enrico Avvedimento for being my scientific guidance, for having contributed to the development of my preparation during these years and for having conveyed to me the deep passion that he puts in the research.

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My thanks to Prof. Roberto Paternò, for his suggestions and for braving with me “the medical records”.

Futhermore, emphasizing his availability, I would like to express my sincere gratitude to Prof. Paolo Mondola, which allowed me to work in Physiology laboratories.

A special thanks also to all friends and colleagues (Simona Damiano, Valentina Ucci, Tiziana Petrozziello, Giusi Russo, Concetta Santarpia, Maria Rosaria De Mizio) for their continued support that allowed me to endure the stress and disheartening moments.
How can we forget the place where it all began???

Thanks friends, I have always felt to me!

Thanks Francesca, because in the same room, in the building next door or anywhere in the world, I know you're there with your valuable advice or (even better) with your reassuring hugs.

Thanks are also to do to all my friends, that even if they have worked with me have certainly enriched my life during the PhD.

Olga, you know to be near me, also from far away;

Paola, especially, thanks for having done just in time;

Ilaria… only we know what it means… “Hamster Sister”…

Special thanks, also, to Rosa… I think that “your name” is not only a coincidence.

Moreover, my hearty thanks to Roberto for being by my side in every moment, not only during this hard and long experience.

I wish to thank affectionately Francesco, “my half-sister”, for always managing to comfort me in all bad moments; and I want to remind my sisters, Maty and Manu, that “♪♫♪ we are very lucky girls……♫♪♫”!

Lastly, my biggest thanks goes always to You, Mom, my source of inspiration, my guide, my love. Wherever you are, I find you in my smiles, in my thoughts, in my words, in my days.

I hope You're proud of me!

Roberta
REFERENCES


54. Achten E, Deblaere K. Health technology assessment on the use of magnetic resonance imaging (MRI) and computed tomography (CT) in the


126. Tkachev D, Mimmack ML, Ryan MM, Wayland M, Freeman T, Jones PB, Starkey M, Webster MJ, Yolken RH and Bahn S. *Oligodendrocyte...*
dysfunction in schizophrenia and bipolar disorder. Lancet. 2003; 362: 798-805.


## APPENDIX

### ABBREVIATIONS USED

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Apo, Apocynin</td>
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<tr>
<td>BIM, Bisindolylmaleimide</td>
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<td>CSF, Cerebrospinal fluid</td>
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<td>CREB protein, Cyclic AMP Response Element Binding Protein</td>
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<td>ERK ½, Extracellular Signal-Regulated Kinases 1/2</td>
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<td>H-Ras, Harvey-Ras</td>
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<td>MAPK, Mitogen-Activated Protein Kinase</td>
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<td>MS, Multiple sclerosis</td>
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<td>MBP, Myelin Basic Protein</td>
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<td>NAC, N-acetyl-L-cysteine</td>
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<td>NADPH oxidase, Nicotinamide Adenine dinucleotide phosphate-oxidase</td>
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<td>OLs, Oligodendrocytes</td>
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<td>OPCs, Oligodendrocyte precursor cells</td>
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<td>Olig-2, Oligodendrocyte Transcription Factor 2</td>
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<td>PD, PD98059 (2’-Amino-3’-methoxyflavone)</td>
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<td>PMA, Phorbol-12-Myristate-13-Acetate</td>
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<td>PI3K, Phosphatidylinositol 3-kinase</td>
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<td>PLC, Phospholipase C</td>
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<td>PDGF, Platelet-derived growth factor</td>
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<td>PI, Propidium Iodide</td>
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<td>PKC, Protein kinase C</td>
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<td>ROS, Reactive Oxygen Species</td>
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<tr>
<td>α-SMA, α-Smooth Muscle Actin,</td>
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Abstracts of the 62nd National Congress of the Italian Physiological Society
25–27 September 2011, Sorrento, Italy
Reactive oxygen species generated by NADPH oxidase induce oligodendrocytes differentiation

ACCETTA R¹, DAMIANO S¹, UCCI V¹, MORANO A², PETROZZIELLO T¹, SANTARPIA C³, MONDOLA P¹, AVVEDIMENTO VE², PATERNO’ R³, SANTILLO M¹

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Reactive oxygen species (ROS) are signaling molecules involved in many physiological processes including cell differentiation. We investigated the role of redox signaling pathways on differentiation of oligodendrocytes (OL), the myelin forming cells in the CNS.

OL cell line MO3-13, with the phenotypic characteristics of oligodendrocyte precursors cells (OPCs), exposed for 4 days to mild oxidative stress (200μM H2O2) show increased expression of OL differentiation markers P-ERK1/2 (1.5+/−0.2), P-CREB (1.7+/−0.2), Olig-2 (2.5+/−0.3) and Myelin Basic Protein (MBP, 3.9+/−0.3) and reduced levels of the negative differentiation marker α-Smooth Muscle Actin (0.55+/−0.05) relative to unstimulated cells. Confocal analysis of MBP shows accumulation of the protein in the cell processes and membrane.

Cell differentiation by 100nM phorbol myristate acetate (PMA/no serum), is dependent on ROS generated by the membrane-bound superoxide generating NADPH oxidase (NOX) enzyme, since co-incubation of the cells for 4 days with differentiation stimulus and a specific NOX inhibitor, apocynin (50μM), inhibits cell differentiation. The Protein Kinase C (PKC) signaling pathway is involved in oxidative stress-induced differentiation since 2,3-butanedione 2-monoxime (100μM), a PKC inhibitor, reverted cell differentiation induced by oxidative stress or PMA/no serum. These data demonstrate that ROS generated by NOX enzyme induce OPCs differentiation through PKC signaling pathway.
Abstracts of papers presented
Molecular Mechanisms in Neuroscience
October 3-4 2011

Accademia
Nazionale dei Lincei
Immunoglobulin fraction purified from serum of multiple sclerosis patients inhibits oligodendrocyte differentiation
Dept. Clinical and Experimental Medicine, Dept. Neuroscience., Dept. of Cell Biol. and Pathol. L. Califano; Dept of Neurology - University Federico II; Santobono Children’s Hospital; Cardarelli Hospital, Naples, Italy.
Multiple sclerosis (MS) is a chronic demyelinating disease affecting oligodendrocytes (OL), responsible for axon myelination in the CNS. OL originate from progenitor cells (OPCs) with migratory and mitotic capacity, maturing in postmitotic myelin-producing cells. In chronic MS lesions OPCs accumulate with loss of mature myelinating cells suggesting the existence of a differentiation block of OPCs contributing to the reduction of OL and to the limited remyelination in MS. We tested the hypothesis of the presence in serum of MS patients of autoantibodies impairing OPC differentiation. We determined the biological effects of the serum immunoglobulin (IgG) fraction on oligodendrocyte differentiation using the human OL cell line MO3-13 cells. 72 MS and 64 control subjects were enrolled in the study. Controls were affected by neurological disorders. MO3-13 cells with the phenotypic characteristics of OPCs, were differentiated by growing them in medium without serum and in the presence of 100nM Phorbol Myristate Acetate (PMA). IgG fraction purification from serum was carried out by affinity chromatography on A/G Sepharose columns. The extent of OL differentiation was evaluated by measuring early and late differentiation markers by Western blotting (P-ERK1/2, Olig-2) and confocal microscopy (Olig-2 and myelin basic protein, MBP). Incubation of MO3-13 cells with the differentiation stimulus for 24h significantly increased P-ERK1/2 and Olig-2 protein levels 2.9 +/- 0.3 and 1.49 +/- 0.2 fold induction, respectively, relative to undifferentiated cells. Confocal analysis showed that, with cell differentiation, MBP and Olig-2 increased, MBP accumulated in the cell processes and membranes. In cells incubated with 200ug/ml of IgGs from MS subjects, in the presence of the differentiation stimulus, P-ERK1/2 and Olig-2 levels were significantly lower 0.52 +/- 0.06 and 0.64 +/- 0.09, fold decrease, respectively, relative to cells with IgGs from controls. Confocal analysis of MBP and Olig-2 in cells incubated with IgGs from MS subjects, in the presence of the differentiation stimulus, showed a decrease of both protein compared to cells treated with IgGs from controls. MBP signal appeared more diffuse with low accumulation of the protein in the cell processes and membrane. Data indicate that autoantibodies present in the IgG fraction from serum of MS patients inhibit OL differentiation thus impairing myelination in CNS. Funded by the academic spin off Prts of the Federico II

100
5th Joint Triennial Congress of the European and Americas Committees for Treatment and Research in Multiple Sclerosis

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spontaneous recovery. We were able to confirm these findings in an independent EAE model, induced in SJL/J mice by adoptive transfer of activated myelin proteolipid protein (PLP) p139-151-specific lymphocytes (passive EAE). Taken together, our data suggest that chronic inflammatory processes in the hippocampus lead to long-lasting pathological processes that affect hippocampal neurogenesis. Thus, together with recently described neurodegenerative changes in the hippocampus such as synaptic alterations, the failure of this stem cell niche to generate new neurons may contribute to cognitive dysfunction in MS.


P818

Reactive oxygen species modulate the differentiation of oligodendrocytes: the good and the bad of oxidation in multiple sclerosis lesions


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Background: Remyelination, that takes place in Multiple Sclerosis (MS) lesions, is dependent on the recruitment and differentiation of Oligodendrocyte Progenitor Cells (OPCs). During inflammation high levels of Reactive Oxygen Species (ROS) can be achieved within MS lesions changing the local environment where OPCs differentiation occurs.

Aims: To investigate the effects of low and high ROS levels, signaling pathways involved and the role of the main membrane source of ROS, NADPH oxidase (NOX) enzymes, in OPCs differentiation.

Methods: Oligodendrocyte (OL) cell line, MO3-13 cells, with the phenotypic characteristics of OPCs, differentiate in the absence of serum with 100mM phenol myristate acetate (PMA/no serum) for 4 days. A mild oxidative stress was induced by 200mM H2O2 for 1-4 days. OL differentiation markers were measured by Western blotting (WB), confocal microscopy or flow cytometry.

Results: OPCs exposed for 4 days to mild oxidative stress increased expression of OL differentiation markers P-ERK1/2 (1.5±0.2), P-CREB (1.7±0.2), Olig-2 (2.5±0.3) and Myelin Basic Protein (MBP, 3.9±0.3) and reduced levels of the negative differentiation marker a-Smooth Muscle Actin (α-SMA) (0.55±0.05) relative to unstimulated cells. Confocal analysis of MBP showed accumulation of the protein in the cell processes and membrane. Cell differentiation by PMA/no serum, is dependent on ROS generated by NOX, since co-incubation of the cells with differentiation stimulus and a specific NOX inhibitor, apocynin (50μM), inhibited cells differentiation evaluated after 4 days by WB analysis of P-ERK1/2, P-CREB and Olig-2. The Protein Kinase C (PKC) signaling pathway is involved in oxidative stress-induced differentiation since 2,3-Butanedione 2-Monoxime (100μM), a specific inhibitor of PKC, reverted cell differentiation induced by oxidative stress or PMA/no serum. OPCs exposed for 24h to H2O2 at doses higher than 500μM induced high rate of cell death measured by PI staining.

Conclusions: ROS mediate the signals leading to OPCs differentiation. Fine tuning of the type and levels of ROS generated by NOX-PKC signals may have profound effects on OPC differentiation. Thus, large amounts of ROS induce death of OPCs.

This finding is relevant for the pathogenesis of MS lesions: while ROS in limited inflammation may represent a positive re-myelinating stimulus, excess of ROS produced by extensive inflammation may reduce the pool of OL precursors and worsen MS lesions.

This project was funded by the academic spin off Firma of the University "Federico II" of Naples.

The authors have nothing to disclose.

P819

Plasma lipid profile and magnetic resonance imaging and spectroscopy in multiple sclerosis

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Background: Alterations of plasma lipid profile have been reported in multiple sclerosis (MS) patients. However, the relevance of these alterations for the pathophysiology of the disease is uncertain. In this study, we investigated whether the plasma lipid profile was related to the inflammatory and neurodegenerative processes of the disease as assessed by magnetic resonance imaging (MRI) and spectroscopy.

Methods: 44 patients (32 females and 12 males) with relapsing-remitting (RR) MS before the beginning of disease-modifying treatments (DMT) were studied. No patient was medicated with lipid lowering agents or steroids at the moment of analytical determinations. Plasma level of triglycerides (TG), total cholesterol (TC) and HDL-cholesterol (HDLc) were determined by enzymatic assays and LDL-cholesterol (LDLc) using the Friedewald formula. MRI was obtained on a 1.5 Tesla scanner. Hyperintense T2 lesions and hypointense T1 lesions were counted for each case, summed and averaged. Lesion Load was determined manually by ROI segmentation using Osiris 3.6.1 1H spin-echo single-voxel spectroscopy (MRS) was performed with long TE over the caloso-septal interface. For statistical analysis Pearson correlation coefficient and student’s T-Test were used.

Results: Females mean age was 39.6±10.1 years with a mean disease duration (DD) of 5.5±5.3 years. In males, the mean age was 32.2±8.7 years, while the mean DD was 4.3±6.7 years. In males, significant correlations were found between the TG levels and T2 lesion load (r=0.45, p=0.05), and between LDLc and NAA/Cho ratio (r=0.42, p<0.05). A trend to an association between TCho and NAA/Cho ratio was also observed (r=0.41, p=0.07). No correlations between lipid plasma levels and imaging data were found in female patients.

Conclusion: These data suggest a possible gender-related association of plasma lipid profile with the pathogenesis of RR-MS.

Supported by Bayer-Health Care, Merck-Serono and Sanofi-Aventis

P820

Retinoic acid induces the blood-brain barrier during human brain development