UNIVERSITY OF NAPLES

"FEDERICO II"

Doctorate Program in Molecular Pathology and Physiopathology

Doctorate School in Molecular Medicine

XXV cycle

5-HT2a receptor pathway in oligodendrocyte differentiation: its involvement in Multiple Sclerosis pathogenesis

Coordinator

Prof. Vittorio Enrico Avvedimento

Candidate

Dr. Ucci Valentina

Tutor

Prof. Mariarosaria Santillo

ACADEMIC YEAR 2012-2013

INDEX

ABSTRACT

Multiple sclerosis (MS) is a chronic demyelinating disease affecting the neurons of brain and spinal cord. In chronic MS lesions oligodendrocytes (the myelin synthesizing cells) precursor cells (OPCs) accumulate with loss of mature OLs, suggesting a differentiation block of OPCs. In MS patients during the active phase of the disease (*poussè*) the disruption of the blood brain barrier can allow serum antibodies and other molecules to reach the CNS, producing demyelination.

Here we demonstrate that IgGs, isolated from MS patients during the acute phase, inhibit PMA-induced differentiation of OPCs by reducing the expression of the OLs differentiation markers in the human OL cell line, MO3-13. In a more specific setting, in which differentiation is driven by the action of serotonin (5-HT) on its specific receptor (5-HT2aR subtype), IgGs from MS patients inhibit 5-HT-induced signalling. We also demonstrated, by flow cytometry experiments, the binding of 5-HT2aR with serum-IgG from a MS patient.

In conclusion, these data demonstrate that MS-derived autoantibodies interfere and inhibit oligodendrocyte differentiation-induced by 5HT. The identification of a putative receptor targeted by IgGs present in the biological fluids of MS patients will pave the way to dissect the signalling pathway leading to demyelination in MS.

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.

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. 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) digest myelin fragments and astrocytes proliferate (gliosis). In chronic lesions are present a complete or nearly complete demyelination and dense gliosis (Figure1) (4).

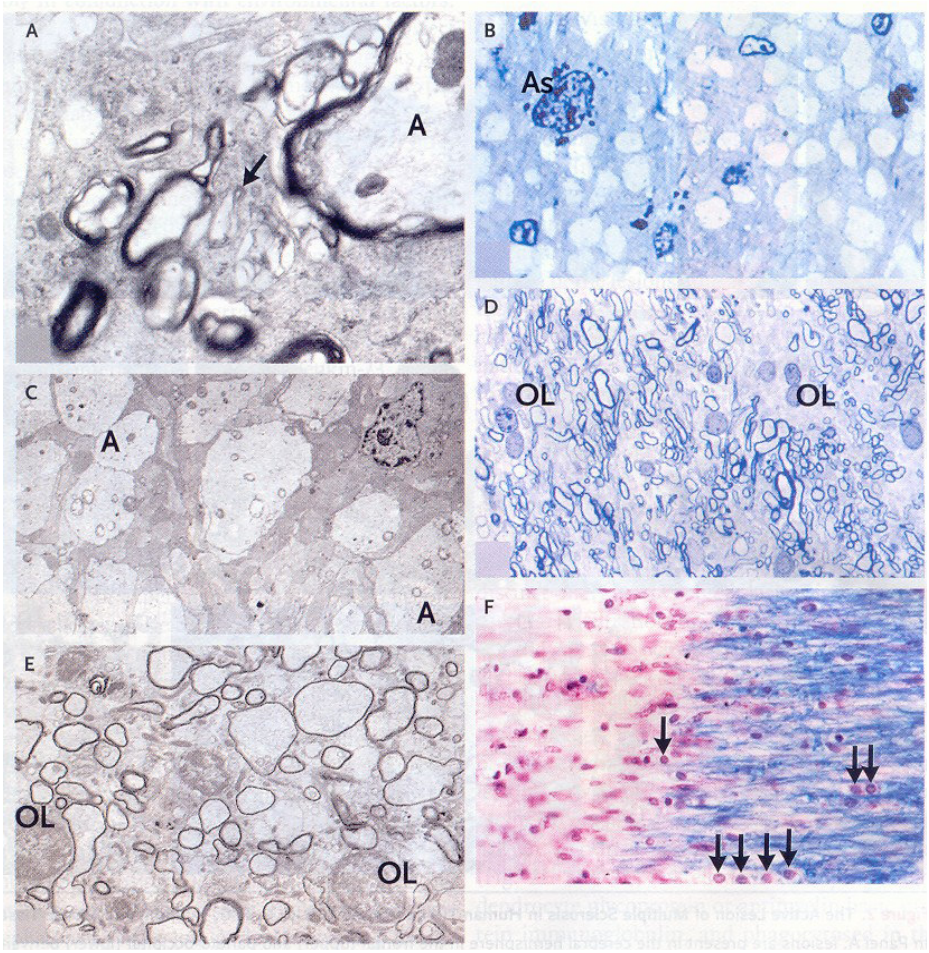


Figure 1. **Chronic lesions of multiple sclerosis in Humans.**
Panel A - An electron micrograph of a chronic active lesion shows a myelinated fiber undergoing demyelination. The arrow shows myelin droplets on the macrophage surface being internalized by the cell. The fiber is invested by a microglial 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; mitochrondria 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.
(Elliot M. Frohman et al. –Multiple Sclerosis-The plaque and its pathogenesis-N Engl J Med 2006).

1.1.1. *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 (5-8). These studies have shown that the disease risk is higher in the biologically related family members with MS patients, compared to the general population (9). These findings also suggest a common sharing of exposure to environmental risk factors during critical periods of risk (childhood and adolescence).

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 (10-12). 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 (13-16).

Previous studies have showed that peptide sequences of very common viral agents, such as Epstein Barr virus (EBV) (17, 18), influenza virus type

A (19), human papilloma virus (HPV) (20) and human herpes virus type 6 (HHV-6) (21, 22) 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 (23). The inflammatory reaction is associated with up-regulation of several Th1 cytokines, including interleukin 2 (IL-2), interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) that were found in the cerebrospinal fluid (CSF) from patients with the disease (24, 25). 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 (26). Magnetic resonance imaging (MRI) studies in the early stages of disease suggest that most of the lesions are preceded by focal destruction of blood brain barrier (BBB); therefore, this condition would facilitate entry of autoreactive T cells and antibodies inside the CNS (27). 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 chemo attractant molecule CCL-20. These cells escape from choroid plexus vessels, penetrate into stroma and cross the epithelial-choroid plexus barrier (28). The histopathological effect of these inflammatory mechanisms is the demyelination, which 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.

The viruses mostly investigated are: HHV-6, for its neurotrophism (21, 22), Epstein-Barr virus according to an action characterized by "molecular mimicry" (17, 18) and LM7 retrovirus, know as "multiple sclerosis associated retrovirus" (MSRV) (29, 30). 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 (31).

1.1.2. *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 (32). 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 (33), 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).

1.1.3. *Diagnosis*

No definitive diagnostic tests for MS are currently available. Therefore, to reach a definitive diagnosis it is necessary to use different tools derived from clinical analysis (34), laboratory (35) and instrumental tests (36, 37).

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.
    - 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.

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 2).

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 (38, 39), the NG2 chondroitin sulfate proteoglycan, and the platelet-derived growth factor-alpha receptor subunit (PDGF-αR).

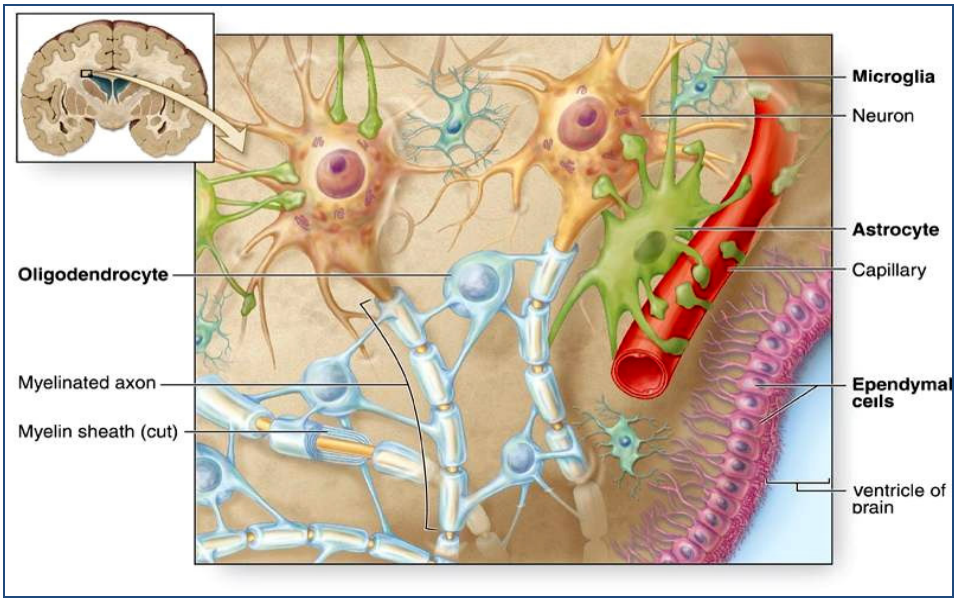


Figure 2. Glia cells. An oligodendrocyte simultaneously wrapping multiple axons with a myelin sheath. Also shown are nodes of Ranvier, which are small unmyelinated axonal regions.

1.2.1. *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 (40).

1.2.2. *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 (40).

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 (41). Second, these pre-OLs become immature OLs, characterized by the acquisition of specific markers and ready for myelination.

1.2.3. *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 remyelinization (42).

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 (43). 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 (44, 45). 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 (46, 47).

1.2.4. *Oligodendrocyte proliferation and differentiation*

The growth and the development of neuronal cells are controlled by a set of different factors: NGF, IGF II, laminin, fibronectin, collagen and adhesion molecules such as N-CAM and cadherins (48). 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 3) (49, 50). 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.

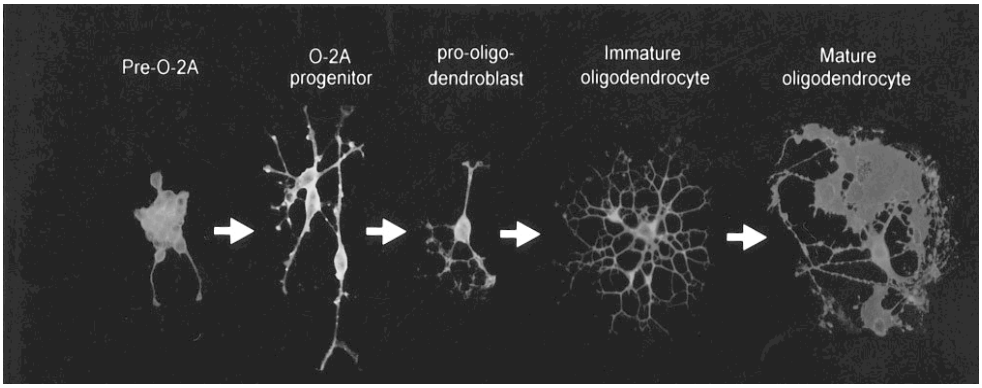


Figure 3. Immunophenotype and morphology of perinatal oligodendrocyte lineage cells in culture.
The name of the cell type is listed along the top, with the most immature stage, the pre-O-2A or pre-progenitor, at the left and the most differentiated stage, the mature oligodendrocyte, at the right.

The generation of OPCs by neural stem cells is the result of interaction of local extrinsic induction factors (SHH, FGF2, PDGF, IGF1, neurotrophins) (51-53) with proteins of the intrinsic transcription machinery of CNS (54).
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 (55). 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 (54). *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 (56,57). In addition, three members of the homeodomain transcription factor family, Nkx2.2, Nkx6.1 and Nkx6.2 (53),

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 (58, 59).

1.2.5. *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 (60). PDGF also stimulates motility of oligodendrocyte progenitors in vitro and is chemo attractive.

- *BASIC FGF*. Basic Fibroblast Growth Factor (bFGF) (also called FGF 2) is also a mitogen for neonatal oligodendrocyte progenitors. It up regulates the expression of PDGFR-a and therefore increases the developmental period during which oligodendrocyte progenitors or pre-OLs are able to respond to PDGF (61). 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 (62); 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-1*. 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 (63). 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 transgenic. 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 (64).

- *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 (65). 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 (66).
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 (67).

- *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 (68). In mice lacking the family of ligands termed neuregulins, OLs in spinal cord failed to develop (69). 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 (60).

- *IL-6*. Interleukin (IL-6) may also act on OL survival as well as leukemia inhibitory factor (LIF) and a related molecule (50).

- *TGF-β*. In vitro, transforming growth factor (TGF-β) inhibits PDGF-driven proliferation and promotes differentiation of oligodendrocyte progenitors (71).

1.3. *Serotonin*

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter. Biochemically derived from tryptophan, and it is primarily found in the gastrointestinal (GI) tract, platelets, and in the central nervous system (CNS) of animals including humans.

5-HT signaling pathways integrate not only basic physiology, but also essential brain functions, including sensory processing, cognitive control, emotion regulation, autonomic responses, and motor activity. It is a target of many physiologic regulators, including modulators of gene transcription, neurotrophic peptides, and steroids as well as psychotropic therapeutics, which impact the formation and activity of 5-HT subsystems.

Serotonin (5-HT) is synthesized from the amino acid l-tryptophan by the rate-limiting enzyme l-tryptophan-hydroxylase (TPH), resulting in the formation of 5-hydroxytryptophan (5-HTP). 5-HT is subsequently obtained by removal of a carboxyl group catalyzed by the enzyme 5-hydroxy-tryptophan-decarboxylase. 5-HT is, like other neurotransmitters, stored in storage and release vesicles via the vesicular monoamine transporter (VMAT). When released in the synapse, serotonin exerts its effects through 16 distinctive 5-HT receptors. Furthermore, 5-HT reuptake in the presynaptic neuron occurs via the serotonin transporter (5-HTT) (72).

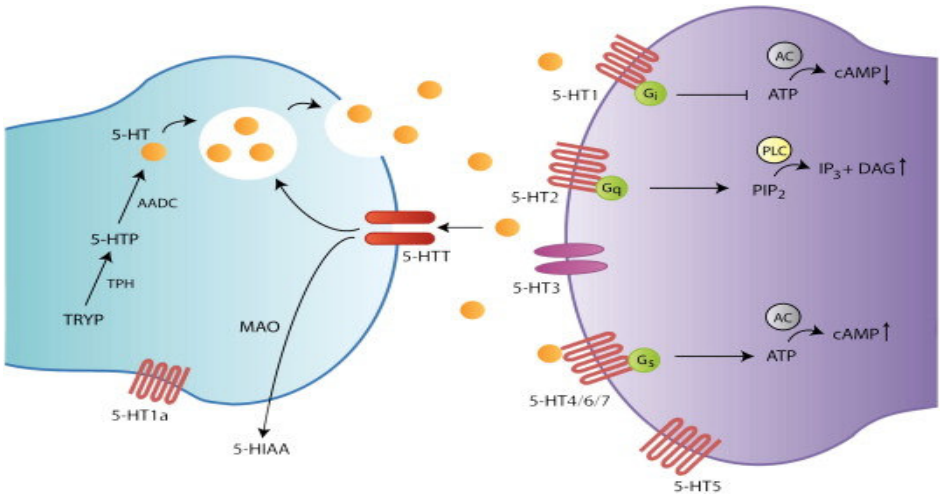


Figure 4. Serotonin main signaling pathways.

1.4. *Serotonin receptors*

Serotonin (5-hydroxytryptamine; 5-HT) receptors are a family of G protein coupled receptors (GPCRs) and ligand-gated ion channels (5-HT 3) found in the central and peripheral nervous systems where they mediate neurotransmission. 5-HT receptors influence various processes and are hence a primary target of several drugs, including many antidepressants, antipsychotics, anorectics, antiemetics, and hallucinogens.

GPCRs are characterized by seven membrane-spanning helices with an extracellular amino-terminus, an intracellular carboxy-terminus, and three intracellular and three extracellular loops connecting each of the transmembrane segments. Binding of an agonist to its GPCR leads to conformational changes in the receptor that induce the dissociation and activation of a receptor-specific heterotrimeric G protein into its α- and βγ-subunits.

The seven transmembrane domain (7TMD) serotonin receptors are coupled to different G proteins. The 5-HT1 receptors couple to $G\alpha_i$ /$G\alpha_o$ proteins; the 5-HT2 receptors couple to $G\alpha_q$ proteins; the 5-HT4 , 5-HT6

and 5-HT7 receptors couple to $G\alpha_s$ proteins, and the 5-HT5 receptors are related to $G\alpha_i$ /$G\alpha_o$ proteins.

Activation of $G\alpha_s$ coupled receptors leads to the stimulation of adenylyl cyclases elevating cyclic AMP (cAMP), which as a second messenger interacts with other proteins including ion channels and activating the protein kinase A (PKA). This phosphorylating enzyme also activates cAMP-responsive transcription factors like CREB modifying gene expression. The interaction with other exchange proteins directly activated by cAMP leads to alternative signaling cascades besides the classical PKA. The interaction with $G\alpha_i$ leads to inhibition of adenylyl cyclases, decreasing production of cAMP.

The activation of $G\alpha_{q/11}$ coupled receptors lead to the hydrolysis of membrane phosphoinositides resulting in the formation of diacyl glycerol (DAG) and inositol phosphates (IP3). IP3 can interact with the calcium reservoirs, elevating intracellular levels and activating protein kinase C. Serotonin receptors may also be coupled to $G\alpha_{12/13}$, mediating structural changes within the cell through activation of the Rho signaling pathway.

The G ßγ dimeric subunit can interact with a variety of enzymatic effectors within the cell, like their action on gated ion channels, regulation of particular isoforms of adenylyl cyclase and phospholipase C, and phosphoinositide-3-kinase isoforms (and ERK signaling) (73).

1.4.1. *5-HT2a receptor*

Serotonin 5-HT2a receptors are important for mediating a large number of physiologic processes both in the periphery and in the central nervous system. These processes include platelet aggregation, smooth muscle contraction, and the modulation of mood and perception. Most of these The 5-HT2 class that is divided into 5-HT2a, 5-HT2b, and 5-HT2c.

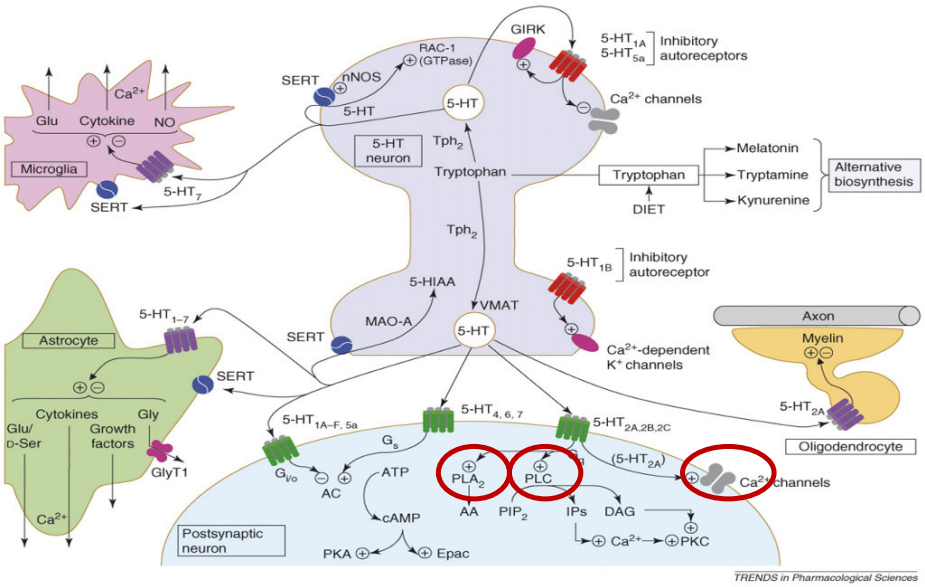


Figure 5. 5-HT2 receptors signaling.
Main pathways of intracellular signaling for these serotonin receptors subtype involve rupture of membrane phospholipids, particularly with phospholipase C (PLC) producing diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) from phosphatidylinositol 4,5-bisphosphate (PIP2). These second messengers activate protein kinase C (PKC) which in time may activate the extracellular signal-regulated kinases 1 and 2 (ERK1/2)

A large number of drugs mediate their actions, at least in part, by interactions with 5-HT2a receptors. These include hallucinogens, atypical antipsychotic drugs and antidepressants.

The 5-HT2a receptor activates PLC through G_q and leads to an accumulation of IP_3, di-acylglycerol (DAG) and activation of protein kinase C (PKC). Increase in cytoplasmic IP_3 causes a release of calcium from intracellular endoplasmic reticulum stores, a characteristic activation signature of many GPCRs. This cascade has been the most studied and is perhaps the most important signal transduction pathway regulated by this receptor.

In addition to initiating intracellular signal transduction cascades, agonist activation of GPCRs also triggers cellular and molecular mechanisms that lead to the attenuation of receptor signaling. Thus, GPCR responsiveness to agonist-induced stimulation wanes over time, a process termed

desensitization. Other regulatory phenomena include resensitization, a recovery of receptor responsiveness following desensitization, and down-regulation, a reduction in receptor number.

1.4.2. *5-HT2a receptor desensitization and the role of kinases*

Desensitization is an adaptive mechanism by which cells regulate receptor responsiveness to repetitive environmental stimuli. Two major patterns of rapid GPCR desensitization have been characterized, homologous or agonist-specific, and heterologous or agonist-nonspecific. Homologous desensitization refers to the attenuation of a cell's response to only that agonist. For example, a cell exposed to a 5-HT2a receptor agonist would, over time, become desensitized to repeated exposure to the same 5-HT2a receptor agonist. In contrast, heterologous desensitization is the attenuation of the response to multiple distinct agonists acting at different receptor types following stimulation by a single agonist. Thus, exposure of a cell to a 5-HT2a receptor agonist may result in the desensitization of the cells responsiveness to an agonist with activity at another GPCR. In both homologous and heterologous desensitization, phosphorylation of the intracellular domains of GPCRs is thought to be essential. G protein-coupled receptor kinases (GRKs) and arrestins are thought to be involved in mediating homologous desensitization while the second messenger-dependent kinases, protein kinase A (PKA) and protein kinase C (PKC), are typically involved in heterologous desensitization.

The mechanism of 5-HT2a receptor desensitization is incompletely understood. For some time though, it has been clear that 5-HT2a receptors may be desensitized following PKC activation, though cell-type specific effects have been noted. Infact it has been demonstrated that PKC activation is necessary for 5-HT-mediated 5-HT2a receptor internalization and is sufficient to bring about receptor endocytosis (74).

CHAPTER II
OBJECTIVES

In chronic demyelinating MS lesions it was found a significant reduction of mature differentiated cells and an increased number of precursors, suggesting the presence of a block of the maturation process of OPCs. However, a possible mechanism leading to this differentiation block has not been clarified.

Recent evidence indicates that higher or unbalanced ROS production can be involved in the pathogenesis of several diseases affecting the CNS, including MS. In fact, the OLs and their precursors are highly sensitive to oxidative stress, for the large amount of oxygen consumed, low levels of cellular antioxidant defence systems and high intracellular iron content. Therefore, an unbalance between ROS production and elimination and alteration of redox-sensitive signalling may be an important factor leading to the maturation block of OPCs observed in MS lesions (79).

Our research group for several years has been studying the cellular and molecular mechanisms of redox signal transduction in several conditions, such as systemic sclerosis (scleroderma), neuronal degeneration, and cerebral and renal ischemia reperfusion. In all these conditions, the unbalance between ROS production and scavenging was critical for the initiation and progression of the disease (77).

On the basis of this information , we wish to dissect the MS pathogenesis by analysing : 1. The impact of ROS signalling on the differentiation of Ols: 2. The presence in the serum of MS patients of molecules interfering with OLs differentiation

The specific aims of this study are the following:

- Molecular analysis of OLs differentiation induced by phorbol esters (PMA)
- Analysis of the biological effects of immunoglobulins (Igs) isolated from MS in the active phase (Poussè) and in quiescent phase (Not Poussè);

- Molecular analysis of 5-HT2a signalling in oligodendrocyte differentiation
- Interference between MS IgGs isolated from serum of MS patients and 5-HT2a induced differentiation of OLs.

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% CO_2 and 95% air atmosphere at 37° C.

The cells were differentiated in FBS-free DMEM, supplementing with 100 nM of Phorbol-12-Myristate-13-Acetate (PMA; Sigma-Aldrich). The cells were kept in a 5% CO_2 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% CO_2 and 95% air atmosphere at 37°C.

3.2 *5-HT and PMA treatments*

HEK-293 and M03-13 cells, grown to semi confluence in 60mm dishes in FBS-free DMEM 4.5g/L glucose, were stimulated for 15 minutes with 5μM of Serotonin (5-HT), and after the differentiation markers were analyzed by Western-blot or by flow cytometric analysis.

M03-13 cells, grown to semi confluence in 60mm dishes in DMEM 4.5g/L glucose, were stimulated with 100 nM of PMA. The cells were kept in a 5% CO_2 and 95% air atmosphere at 37° C for 1day or 4 days and after the differentiation markers were analyzed by Western-blotting or by flow cytometric analysis.

3.3 *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.

From each patient was collected a blood sample 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.

The MS patients were divided into two subsets according to whether or not the patient was in the active phase of the disease at the time of collection of CSF and blood.

The attribution to the subset of patients with Poussé is established on the presence at least one of the following three criteria:

- MRI brain and spinal cord in which there is evidence of at least one lesion absorbing contrast medium spread over a period ranging from 24 hours before to 24 hours after taking the CSF or blood
- Increase in 'index of albumin calculated at the time of sampling
- Symptoms with onset no later than 48 hours and no earlier than 24 hours prior to the withdrawal or SM type PP and SP.

3.4 *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.

3.5 *Immunoglobulins treatment*

To check the effect of IgG fractions stimulation on the molecular mechanisms related to 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 30 minutes. The differentiation markers were analyzed by Western-blot,

3.6 *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 Dodecil Sulfate (SDS)
- 2.5mM Sodium Pyrophosphate (SPP)
- 1mM β-Glycerophosphate
- 1mM Sodium Orthovanadate ($NaVO_4$)
- 1mM Sodium Fluoride (NaF)
- Protease inibitor (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 15min 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.

3.7 *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).

3.8 *Trasfection*

The cells were transfected with a plasmid containing the gene that expresses the 5-HT2A receptor conjugated to a green fluorescent protein, GFP, linked to the C-terminal of the receptor.

One day before transfection, 450.000 cells (HEK293) grown to semi confluence in 35mm dishes in growth medium so that cells will be 70-90% confluent at the time of transfection.

For each transfection sample, prepare complexes as follows:

- Dilute 1 γ/λ DNA (5-HT2a receptor coniugated to GFP) in 80µl of growth medium without serum and antibiotics. Mix gently.
- Dilute 4 µl of Lipofectamine™ 2000 in 80 µl of growth medium without serum and antibiotics.
- Combine the diluted DNA with diluted Lipofectamine™ 2000 (total volume = 160 µl). Mix gently and incubate for 45 minutes at room temperature.

Add the 160 µl of complexes to each dishes containing cells and medium.
Incubate cells at 37°C in a CO_2 incubator for 18-48 hours prior to testing for transgene expression. Medium may be changed after 4-6 hours.

3.9 *Flow cytometric assay for binding 5-HT2aR and IgG serum*

The HEK293 cells were plated in 100mm Petri dishes and grown to semi confluence. After trypsinization and wash in PBS, these cells were resuspended in 200µl PBS and then incubated with mouse serum for 30 min at 4 °C, to block non specific binding; then, were incubated for 30 min with 200µg of serum IgG (MS or neurological), and stained for 30 min with PE-conjugated goat anti human IgG. Cells were washed and resuspended in 200 µL of PBS for flow cytometric analysis of phycoerythrin positive cells with a FCSscan apparatus (Becton-Dickinson).
Data were analyzed using WinMDI 2.8 software.

3.10 *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 Chemiluminescent Western Blot Immunodetection Kit (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.

3.11 *RT-PCR analysis*

Total RNA was extracted from 10 mm petri dish cells by using Trizol solution (Invitrogen, Carlsbad, CA, USA). Total RNA was treated with DNase. RNA concentration and quality were determined by agarose gel electrophoresis and spectrophotometry. The RNA was re-suspended in 50 ml diethyl pyrocarbonate treated water, and stored at -80 °C until used. The synthesis of cDNA was performed by using a reverse transcription (RT) system (Promega). The primers used for the detection of human 5-HT2aR mRNA were: forward 5'-TCATCATGGCAGTGTCCCTA-3' and reverse 5'-TGAGGGAGGAAGCTGAAAGA-3'. To rule out the possibility of amplifying genomic DNA, one PCR was carried out prior RT of the RNA. As internal control for RT and reaction efficiency, amplification of a 597 bp fragment of Human β-actin mRNA was carried out in parallel in each sample, using the primer pair: forward 5'-TCACCCTGAAGTACCCCATC -3' and reverse 5'-GGCTGGAAGAGTGCCTCA-3'. As a negative control for all reactions, distilled water was used in place of cDNA. The PCR products were separated on a

2% agarose gel and visualized by ethidium bromide, using a 1 kb DNA ladder to estimate the band sizes. Finally, the bands were cut off from the gel, purified and sequenced by the Primm (Milan, Italy).

3.12 *Statistical analysis*

Statistical differences were evaluated using a Student's *t*-test for unpaired samples.

CHAPTER IV
RESULTS

4.1 *Molecular analysis of oligodendrocyte differentiation*

Immunocytochemistry and immunohistochemistry studies indicate that in chronic demyelinating lesions of MS patients the number of pro and mature oligodendrocytes (OL) is reduced and there is a significant fraction of oligodendrocyte precursors (OPCs), suggesting an impairment of OPCs differentiation in MS (66, 67). To assess the presence in the serum of MS patients of factors that alter the differentiation of OPCs, we isolated the immunoglobulin fraction (Ig) from serum of MS patients and exposed to these fractions growing or differentiating oligodendrocytes. The cell line we used was M03-13 cells, which display several features of OPCs and differentiate in OLs after stimulation with Phorbol-12-Myristate-13-Acetate (PMA, an activator of PKC).

We have, previously partly characterized PMA induced differentiation in M03-13 cells by assessing. 1. the levels and the localization of the transcription factor, Olig2 and the myelin basic protein, MBP; 2. the activation of ERK1/2 and CREB and; 3. the levels of α-SMA, which disappear when oligodendrocytes differentiate (86).

Figure 6 shows the morphological changes of the cells exposed to PMA 100nM: differentiating cells are characterized by inhibition of migration and the appearance of multiple cytoplasmic extensions.

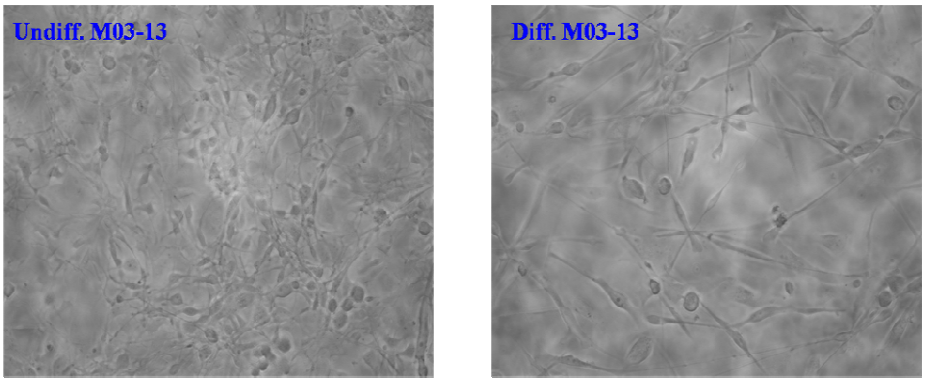


Figure 6. Optical microscope image. M03-13 undifferentiated (Undiff. M03-13) and differentiated (Diff. M03-13) for 4 days with PMA 100nM in the absence of serum.

The differentiation of the cells exposed to 100nM PMA for 1 day is marked by a significant reduction of α-SMA levels (a negative differentiation marker) and by the activation of ERK1/2 (P-ERK1/2) and the cAMP-transcription factor CREB (P-CREB) (positive differentiation markers) (Figure 7).

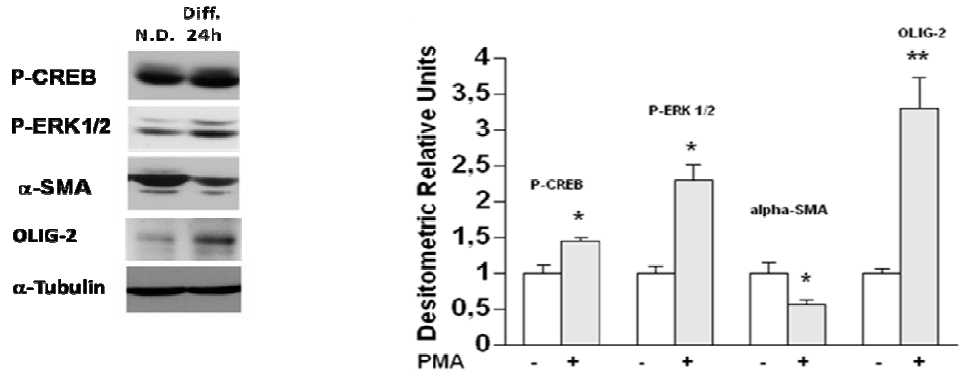


Figure 7. Modulation of differentiation markers in M03-13 cells stimulated with PMA for 1d.
Western blotting analysis of α-SMA, P-ERK1/2, P-CREB and Olig-2 levels in M03-13 cells after 1 day of differentiation with 100nM PMA in medium without serum. N.D., indicates Not Differentiated cells, growing in complete medium, Diff., indicates Differentiated cells.
The histograms show the values (means ± SEM) relative to N.D. obtained by densitometric analysis of protein bands normalized to α-Tubulin in three independent experiments. *p<0.05 vs N.D.; **p<0.01 vs N.D.
On the left a representative experiment is shown.

The levels of MBP, a specific marker of mature oligodendrocytes, assessed by flow cytometry, progressively increase 1 and 4 days of exposure to PMA (Figure 8).

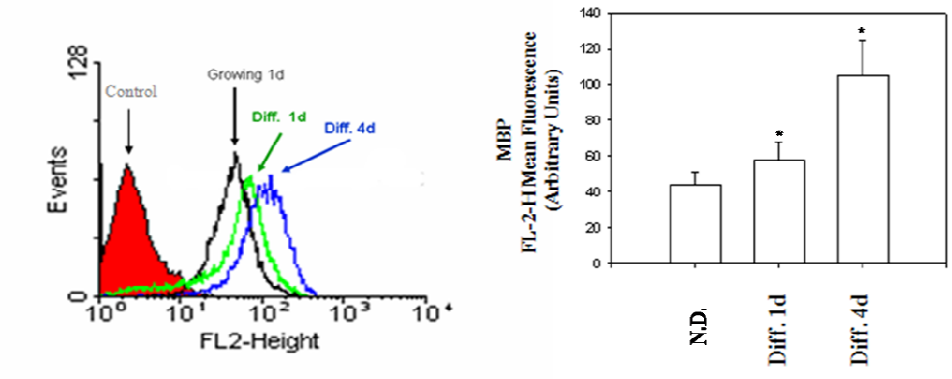


Figure 8. Increasing levels of MBP protein at different days of differentiation of M03-13 cells with PMA.
Immunoreactivity for MBP was evidenced by indirect immunofluorescence and flow cytometry, using primary antibodies against MBP and CY3-conjugated anti rabbit IgG as secondary antibodies. Control was treated with secondary antibodies alone. 10,000 cells were counted for each sample. N.D. indicates Not Differentiated cells, growing in complete medium, Diff., indicates Differentiated cells.
The histograms show the mean ± SEM of three independent experiments. *p<0.01 vs N.D.
On the left a representative is shown.

The levels of P-ERK1/2 and P-CREB in M03-13 cells were induced by differentiation, with a peak at 1 day; conversely, a progressive decrease of -SMA levels was observed during differentiation (Figure 9).

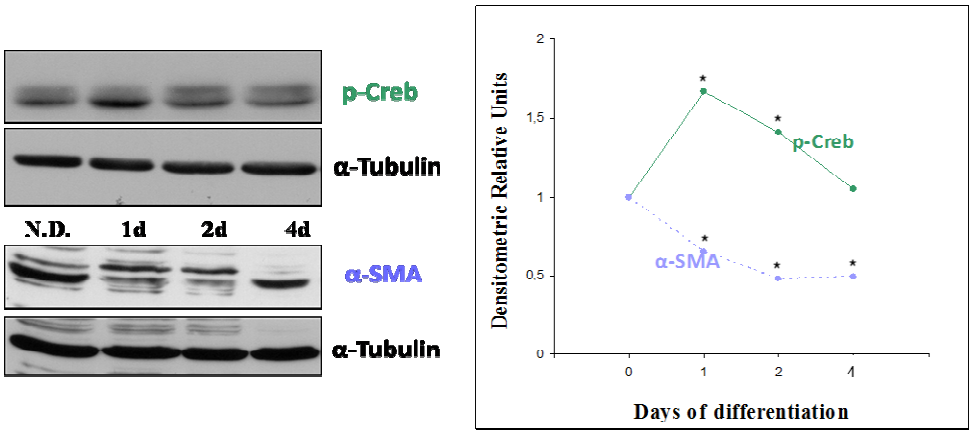


Figure 9. P-CREB and α-SMA levels in M03-13 cells during differentiation with PMA.
Western blotting analysis of P-CREB and α-SMA levels in M03-13 cells after 1, 2 and 4 days of differentiation of M03-13 cells with 100nM PMA in medium without serum. N.D. indicates Not Differentiated cells, growing in complete medium. The histogram shows the values (means ± SEM) relative to N.D. obtained by densitometric analysis of protein bands normalized to α-Tubulin of three independent experiments. *p<0.01 vs N.D.
On the left a representative experiment is shown.

Figure 10 shows that P-ERK1/2 levels are progressively induced by differentiation.

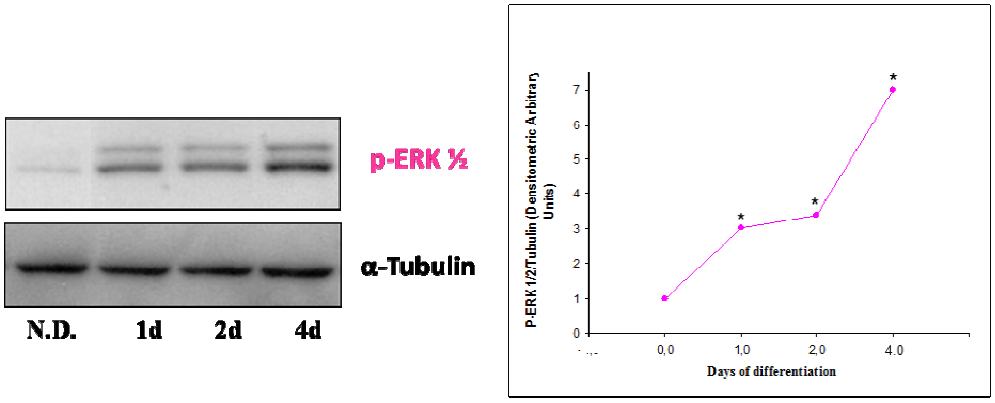


Figure 10. P-ERK1/2 levels in M03-13 cells during differentiation with PMA
Western blotting analysis of P-ERK1/2 levels in M03-13 cells after 1, 2 and 4 days of differentiation of M03-13 cells with 100nM PMA in medium without serum. N.D. indicates Not Differentiated cells, growing in complete medium.
The histogram shows the values (means ± SEM) relative to N.D. obtained by densitometric analysis of protein bands normalized to α-Tubulin of three independent experiments. *p<0.01 vs N.D.
On the left a representative experiment is shown

4.2 *The Ig fraction isolated from MS patients inhibits PMA-induced differentiation of oligodendrocytes*

We hypothesized that serum of MS patients may contain molecules that interfere with oligodendrocyte differentiation. To this end we incubated M03-13 cells for 30 minutes with PMA (100nM), in the absence of serum. Under these conditions oligodendrocyte exit the cell cycle and progressively differentiate (pro-differentiative conditions). We exposed differentiated oligodendrocytes to the IgG fraction (200 μg/ ml), purified by affinity chromatography from serum of MS and control patients. Controls patients were subjects with neurological diseases needing differential diagnosis with MS (cerebral cancers, stroke, vasculitis, etc.). The expression levels of P-ERK1/2 were assessed by Western-blotting.

The differentiation marker, p-ERK1/2, increased progressively with differentiation and was not modified by exposure of the cells to the to IgG derived from control patients. M03-13 cells incubated in the presence of IgG purified from serum of MS patients instead, showed a significant reduction of P-ERK1/2 levels (Figure 11).

It was observed production of intrathecal IgG presents in 90% of patients with MS and the presence of oligoclonal bands (OCB) ; studies in the early stages of disease suggest that in the acute phase of the disease (Poussè) 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 of exit of intratecal antibodies outside the CNS (36). For this reason, we, also, evaluated the different effects of IgG from MS in the active phase (Poussè) and in quiescent phase (Not Poussè); the P-ERK1/2 levels in cells incubated with IgG from MS patients in the active phase (Poussè) were significantly lower compared to cells stimulated with IgG of control patients; on the contrary, there was no significantly difference of P-ERK1/2

levels between Not Poussè and Differentiated or Differentiated with IgG Ctr groups.

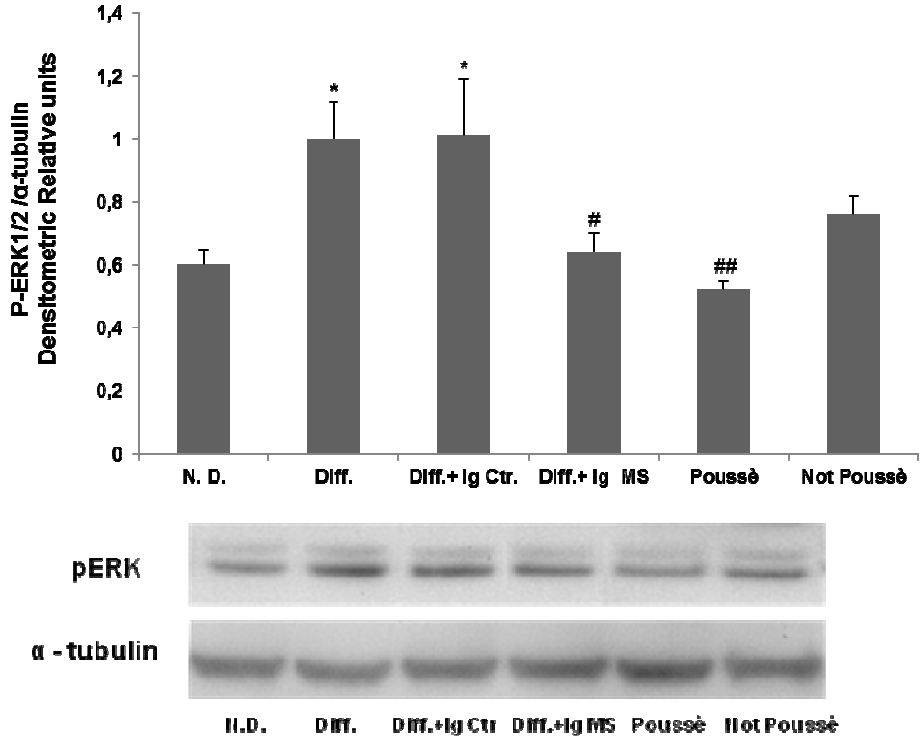


Figure 11. IgG from MS patients inhibit PMA effect on p-ERK1/2 levels.
Western blotting analysis of p-ERK1/2 levels in M03-13 cells stimulated with PMA (100 nM) for 30 minutes in serum-free medium in the presence of IgG (200 μg/ml) purified from the serum of patients.
N.D. indicates Not Differentiated cells, growing in complete medium; Diff. was treated only with PMA (100 nM) for 30 minutes in serum-free medium; Ig Ctr indicates cells stimulated with IgG of control patients (n=6); Ig MS indicates cells stimulated with IgG of patients with MS (n=10); Poussè and Not Poussè indicate cells stimulated with IgG from MS patients in the active (n=5) and quiescent phase of disease (n=5), respectively.
The histograms show the values (means ± SEM) relative to Diff. obtained by densitometric analysis of protein bands normalized to α-Tubulin of three independent experiments. *p<0.05 vs N.D; # p<0.05 vs Diff and vs Diff+IgG Ctr.; ## p<0.01 vs Diff. and vs Diff.+IgG Ctr.

Using the same protocol, we monitored the effects of IgG from MS patients on P-CREB levels.

In M03-13 cells, after 30 minutes of stimulation with PMA (100 nM) in the absence of serum, the P-CREB levels were increased compared to growing

cells (N.D.); in cells treated for 30 minutes with PMA, in the presence of IgG from MS patients, the P-CREB levels were decreased (Figure 12). Moreover, the P-CREB levels in cells incubated with IgG from MS patients in the active phase (Poussè) were significantly lower compared to IgG from control patients; on the contrary, not statistically significant differences were observed between IgG from MS patients in the silent phase (Not Poussè) and that from controls.

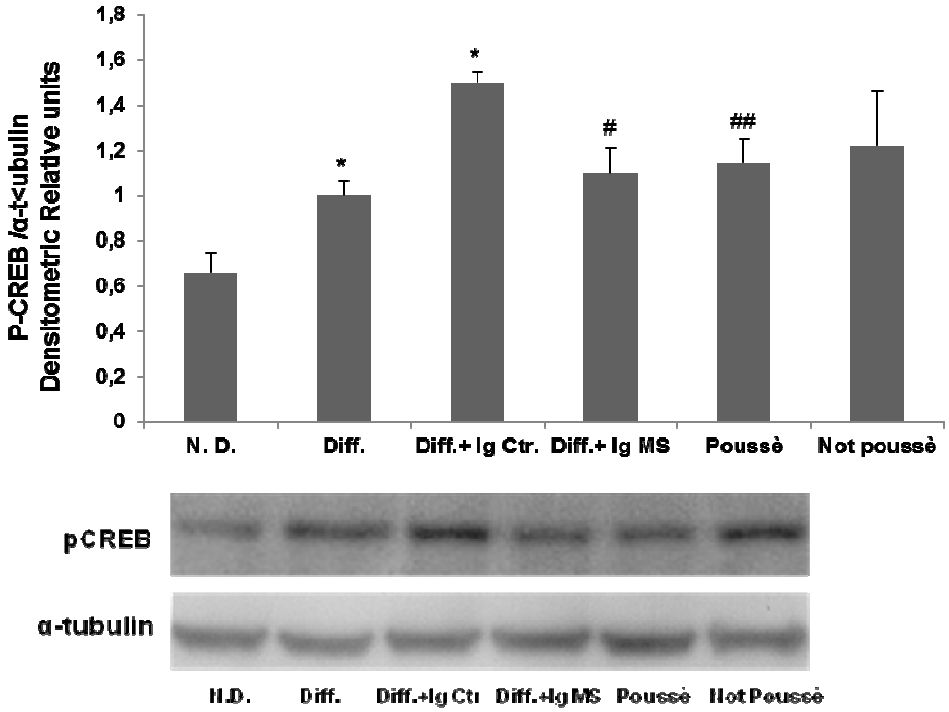


Figure 12. IgG from MS patients inhibit PMA effect on p-CREB levels.
Western blotting analysis of p-CREB levels in M03-13 cells stimulated with PMA (100 nM) for 30 minutes in serum-free medium in the presence of IgG (200 µg/ml) purified from the serum of patients.
N.D. indicates Not Differentiated cells, growing in complete medium; Diff. was treated only with PMA (100 nM) for 30 minutes in serum-free medium; Ig Ctr indicates cells stimulated with IgG of control patients (n=6); Ig MS indicates cells stimulated with IgG of patients with MS (n=10); Poussè and Not Poussè indicate cells stimulated with IgG from MS patients in the active (n=5) and quiescent phase of disease (n=5), respectively.
The histograms show the values (means ± SEM) relative to Diff. obtained by densitometric analysis of protein bands normalized to α-Tubulin of three independent experiments. *p<0.05 vs N.D, # p<0.05 vs IgGCtr

Further experiments for the analysis of late 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 oligodendrocyte transcription factor, Olig-2, were assessed by Western-blotting. Olig-2 increased as a result of differentiation; in cells incubated in the presence of IgG purified from serum of patients with MS, the levels were lower than that observed after treatment of cells with IgG from control patients (Figure 13).

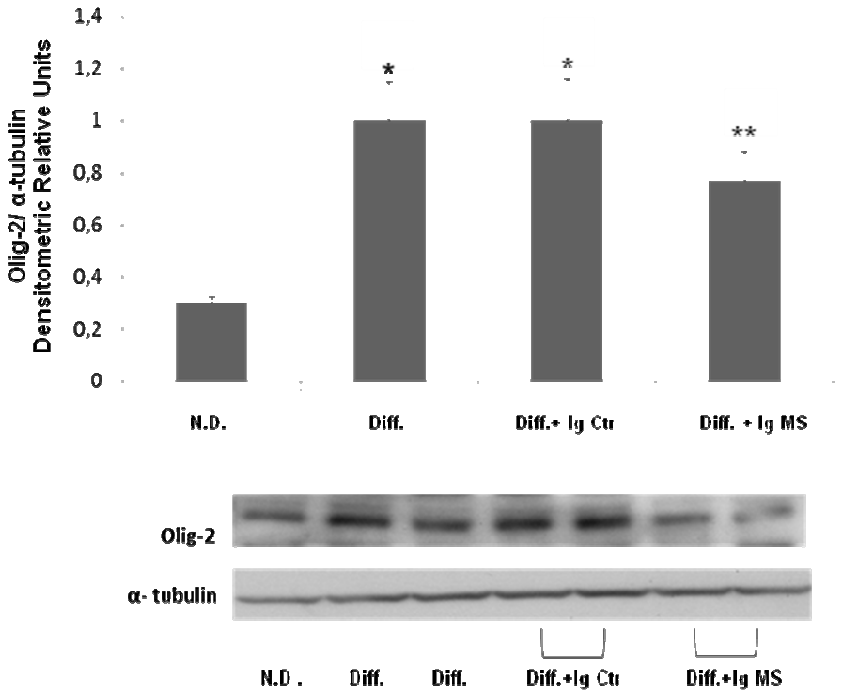


Figure 13 IgG from MS patients inhibit PMA effect on *Olig-2* levels
Western blotting analysis of *Olig-2* levels in M03-13 cells stimulated with PMA (100 nM) for 1 day in serum-free medium in the presence of IgG (200 μg/ml) purified from the serum of patients.
N.D. indicates Not Differentiated cells, growing in complete medium; Diff. was treated only with PMA (100 nM) for 1 day in serum-free medium; IgG Ctr indicates cells stimulated with IgG of control patients; Ig MS indicates cells stimulated with IgG of patients with MS.
The histograms show the values (means ± SEM) relative to Diff. obtained by densitometric analysis of protein bands normalized to α-Tubulin of three independent experiments.
* $p<0.01$ vs ND; ** $p< 0.05$ vs IgGCtr.

Subsequently, we evaluated the effects of IgG from MS patients in the acute and quiescent phase of disease on Olig-2 levels.

In particular, we evaluated the effect of IgG derived from serum of 11 control patients, 6 patients with MS during the active phase (Poussè) and 5 patients with MS quiescent (Not Poussè). Even in this case M03-13 cells were maintained in culture for 24h with PMA in the presence of IgG extracted from serum before the Western blotting analysis of Olig-2 levels.

As it is shown in Fig.14, the Olig-2 levels in cells incubated with IgG from MS patients in the active phase (Poussè) were significantly lower compared to IgG from controls patients; on the contrary, not statistically significant differences were observed between IgG from MS patients in the silent phase (Not Poussè) and that from controls.

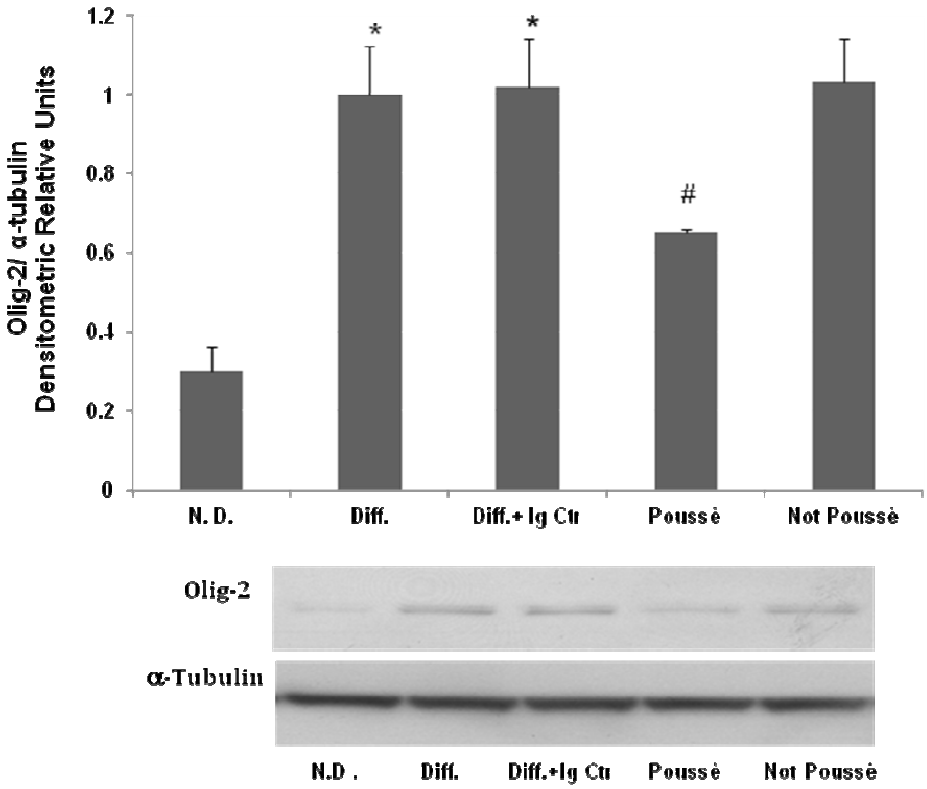


Figure 14 Modulation of *Olig-2* levels in M03-13 cells stimulated with IgG from MS patients during phase Poussè and Not Poussè.
Western blotting analysis of Olig-2 levels in M03-13 cells after 1 day of differentiation of M03-13 cells with 100nM PMA in medium without serum and stimulated with IgG (200 μg/ml) purified from the serum of patients
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; IgG Ctr indicates cells stimulated with IgG of control patients (n=6); Poussè and Not Poussè indicate cells stimulated with IgG from MS patients in the active (n=5) and quiescent phase (n=5) of disease, respectively.
The histograms show the values (means ± SEM) relative to Diff. obtained by densitometric analysis of protein bands normalized to α-Tubulin of three independent experiments. *p <0.01 vs ND; # p<0,05 vs Diff and vs Diff + Ig Ctr

4.3 *Serotonin (5-HT) induces differentiation of oligodendrocytes*

To dissect the signalling pathways targeted by these IgG from active MS patients, we investigated the activity of several G protein coupled and tyrosine signalling receptors in oligodendrocytes. We focused our attentention to the serotonin receptor 2A, because human embryonic oligodendrocyte progenitors express this receptor (85). We first, analysed the expression of the receptor in various tissues and cell lines. We first, analysed the expression of the receptor in various tissues and cell lines.

a. Expression of 5 HT receptor

M03-13 cells express the 5-HT2a receptor as protein (Fig. 15A) and as mRNA (Fig. 15B). Western Blotting analysis shows that receptor protein is also expressed in human, rat and mouse brain and in several neuronal cell lines, such as SK-N-BE, SH-SY5Y, U87 and Hela cells (Fig.15A). Conversely, the human embryonic kidney cell line , HEK293, did not express either 5-HT2aR both as protein (Figure 15A) and as mRNA (Fig. 15B).

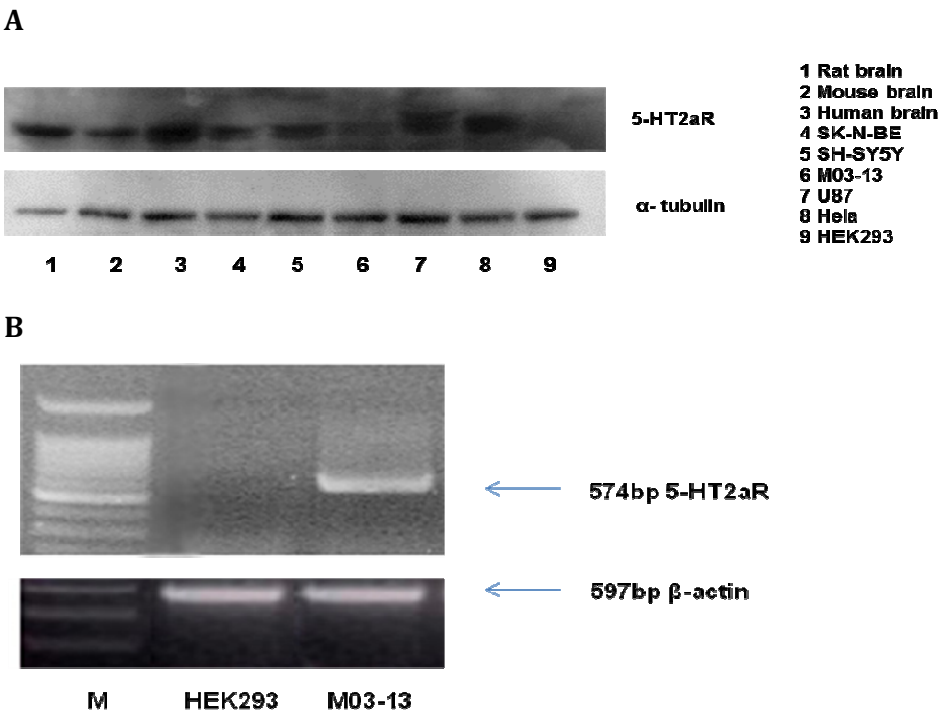


Figure 15 Expression of the 5-HT2aR in different cell lines and tissues.
The panel A shows the Western Blotting analysis of 5-HT2a receptor protein in different tissues and several cell lines; the panel B shows the RT-PCR analysis of the expression of 5-HT2a receptor mRNA in two different cell lines (HEK 293 and M03-13). As internal control for RT and reaction efficiency, amplification of fragment of Human β-actin mRNA was carried out in parallel in each sample.

b. HT stimulates the early oligodendrocytes differentiation markers

The M03-13 cells express the 5-HT2a receptor (Fig.11). To ascertain whether 5-HT induced oligodendrocyte differentiation, we exposed the oligodendrocyte cell line cells to 5μM 5-HT or to 100nM PMA s and examined the effects on P-ERK1/2 and Olig-2, as markers of OL differentiation.

Figure 16 shows that 5HT induced a sharp P-ERK1/2 peak at 15-30 min, which returned to the un-stimulated levels 2 h after the initial stimulation. Conversely, the P-ERK1/2 induction by PMA persisted at least 2h.

Using the same protocol, we monitored the effects on Olig-2 expression of 5μM of 5-HT or 100nM PMA. Olig-2 levels were induced after 15 min of stimulation with 5-HT and then decreased when the cells were exposed for longer period to 5 HT, returning to basal levels after 2h. On the contrary, PMA induced Olig-2 with a sharp peak at 15 min and after a transient drop at 1 h, stimulated Olig-2 levels for 24h (Fig. 14 and ref 15).

Figure 16 shows that 5HT induced a sharp P-ERK peak at 15-30 min, which returned to the un-stimulated levels 2 h after the initial stimulation. Conversely, the P-ERK1/2 induction by PMA persisted at least 2h.

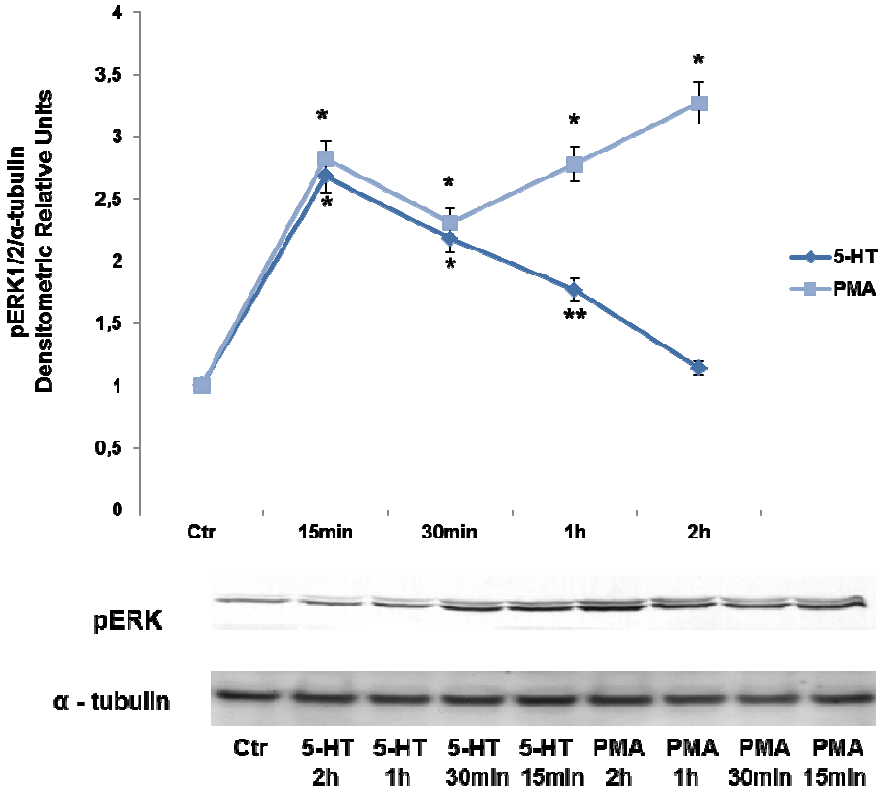


Figure 16. Modulation *of P-ERK1/2* levels in M03-13 cells stimulated with 5-HT and PMA. The cells were incubated for 16 hours in medium containing 0.2% of FBS (Ctr) and then stimulated with 5μM 5-HT or 100nM PMA in medium without serum for the times indicated before harversting them for Western blotting analysis of *P-ERK1/2* levels. The graph shows the values (means ± SEM) relative to control obtained by densitometric analysis of protein bands normalized to α-tubulin of three independent experiments. *p<0.01 vs Ctr, **p<0.05 vs Ctr.

Using the same protocol, we monitored the effects on Olig-2 expression of 5μM of 5-HT or 100nM PMA. Olig-2 levels were induced after 15 min of stimulation with 5-HT and then decreased when the cells were exposed for longer period to 5 HT, returning to basal levels after 2h. On the contrary, PMA induced Olig-2 with a sharp peak at 15 min and after a transient drop at 1 h, stimulated Olig-2 levels for 24 h (Fig. 13 and 14).

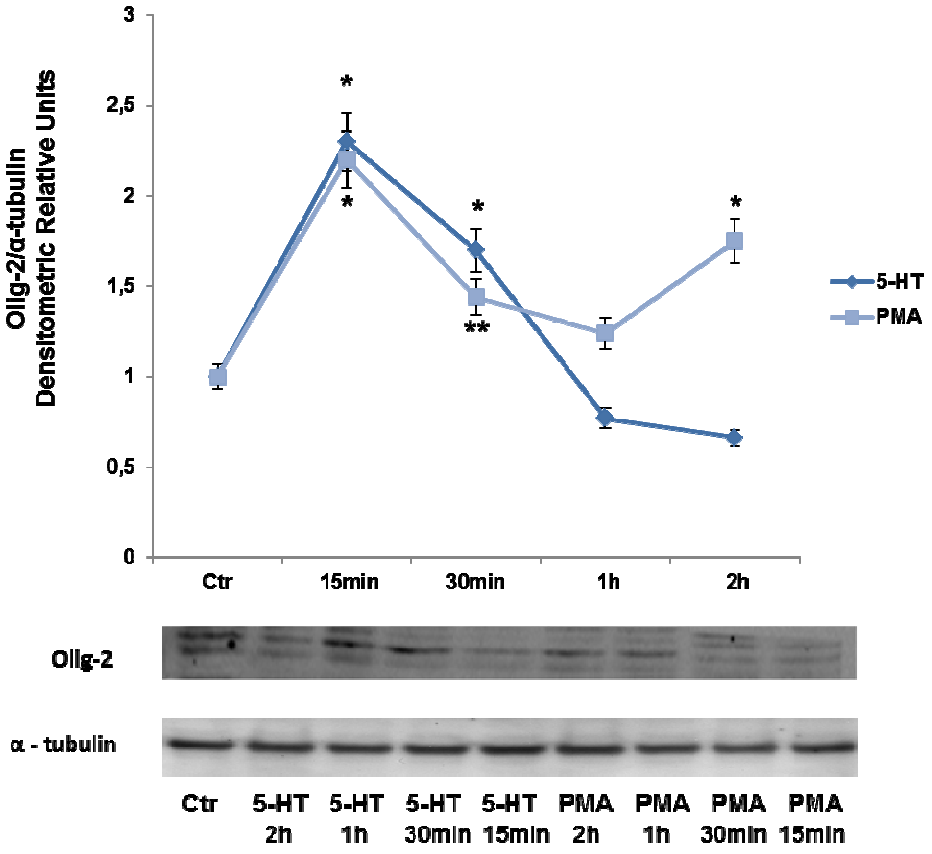


Figure 17. Modulation of Olig-2 levels in M03-13 cells stimulated with 5-HT or PMA.
The cells were incubated for 16 hours in medium containing 0.2% of FBS (Ctr) and then stimulated with 5μM 5-HT or 100nM PMA in medium without serum for the indicated times before harversting them for Western blotting analysis of Olig-2 levels. The graph shows the values (means ± SEM) relative to control obtained by densitometric analysis of protein bands normalized to α-tubulin of three independent experiments. *p<0.01 vs Ctr, **p<0.05 vs Ctr

c. Engineering heterologous cell lines expressing the 5HT receptor

To verify the ability of serum IgG from MS patients to interfere with 5-HT2a receptor signaling, we transfected with the recombinant 2aHt receptor the human embryonic kidney cells (HEK 293) , which do not express the endogenous receptor. The cells were transiently transfected with an expression vector encoding the 5-HT2a receptor cDNA fused to the green fluorescent protein, GFP. The fusion protein contained GFP linked to the C-terminal of the receptor, leaving the N-terminal portion available for ligand binding. The transfection efficiency (approximately 80% at 24 h) was assessed by cytofluorimetry.

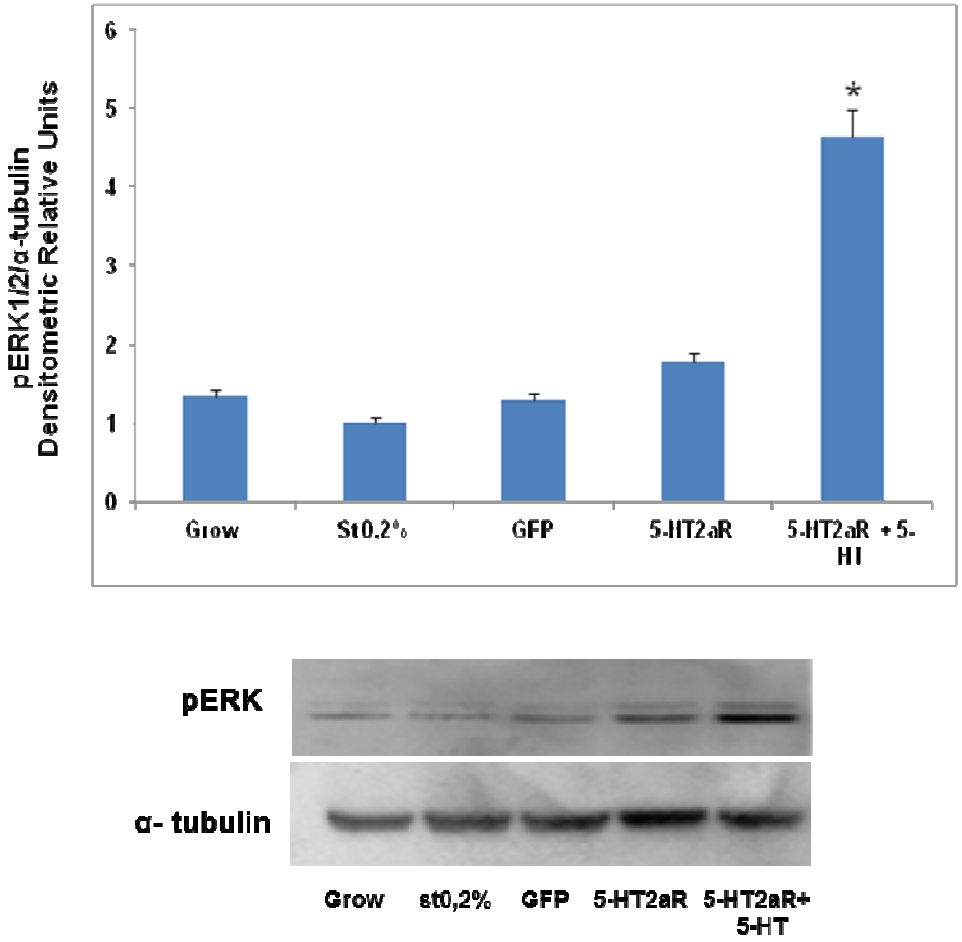


Figure 18. The serotonin induces levels of P-ERK1/2 levels in HEK293 cells transfected with 5-HT2A-GFP construct.
The cells were transfected and after 24h, incubated for 4h in medium containing 0.2% FBS and then stimulated with 5μM of 5-HT for 15 minutes, before harvesting them for Western blotting analysis of *P-ERK1/2* levels. Grow indicates cells growing in complete medium; st 0,2% indicates cells incubated with medium containing 0,2% FBS for 4 h The histograms show the values (means ± SEM) relative to GFP obtained by densitometric analysis of protein bands normalized to α-tubulin of three independent experiments. *$p < 0.01$ vs 5-HT2aR
In the lower part of the figure a representative experiment in shown

Subsequently, we tested whether the stimulation of cells with the receptor agonist, serotonin, for 15 minutes at a concentration of 5μM, induced P-ERK1/2 levels.

In transient transfected cells (5-HT2aR-GFP construct) exposed to serotonin robustly activated ERK1/2 (Fig.18), whereas control transfected cells (GFP construct) did not respond to 5HT (data not shown).

4.4 *IgGs from MS patients selectively inhibit ERK1/2 activation by serotonin in HEK293cells transiently or stably expressing the 5-HT2a receptor*

After testing the functionality of the system, we studied the effects of preincubation of the cells with human IgG derived from MS or neurological patients on 5-HT2aR signaling. HEK293 cells, transiently expressing the 5-HT2a receptor-GFP fusion protein, were exposed to MS IgG for 30 minutes and subsequently with 5Ht for 15 minutes. P-ERK1/2 levels in these cells was significantly inhibited by MS IgG. The same cells pre-incubated with IgG from control patients and 5Ht did not change the pERK1/2 levels. Inhibition of 5 HT-induced ERK1/2 by MS IgG was highly reproducible and well controlled, since the levels of pERK1/2 were normalized to total ERK1/2 and to 5-HT2AR expression in transfected cells. Also, not-transfected or control transfected cells did not respond to 5HT or MS IgG (Fig.18, legend).

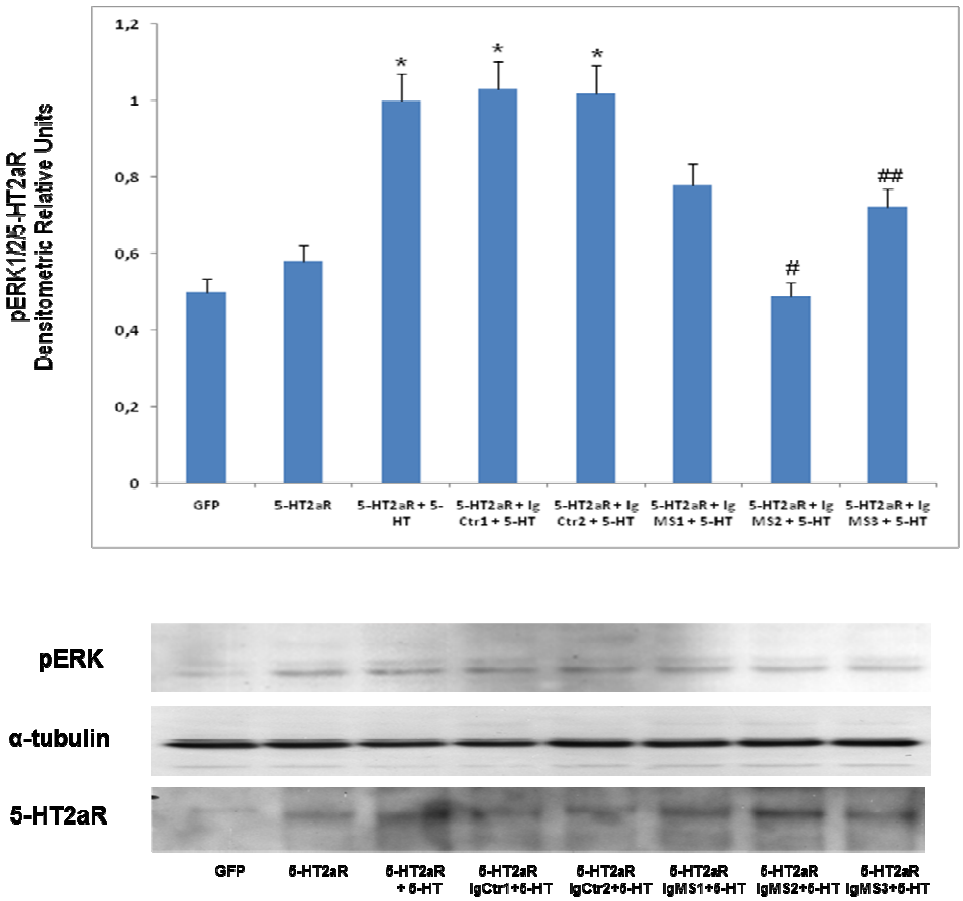


Figure 19. Effect of serum IgG from MS and neurological patients on P-ERK 1/2 levels in transient 5-HT2aR transfected cells

The HEK293 cells transfected in transient with 5-HT2aR-GFP were incubated for 4h in medium containing 0.2% FBS. Then the cells were pre-incubated for 30 minutes with serum IgG (3 MS and 2 neurological) and subsequently stimulated with 5μM of 5-HT for 15 minutes, before harversting them for Western blotting analysis of P-ERK1/2 levels. The histograms show the values (means ± SEM) relative to control (5-HT2aR + 5-HT) obtained by densitometric analysis of protein bands normalized to α-tubulin and 5-HT2a receptor of three independent experiments. *p < 0.01 vs 5-HT2aR; # p < 0.01 vs 5-HT2aR + 5-HT; ## p< 0.05 vs 5-HT2aR + 5-HT
In the lower part of the figure a representative experiment in shown.

The serotonin receptor 5-HT2a is not present in the HEK 293 cells, but the efficiency of transient transfection, although very high and reproducible, is a variable to be considered in every single experiment. To minimize this

variable, was created a cellular model of HEK293 stably transfected with the 5-HT2aR-GFP construct on which functional tests have been performed, analyzing the P-ERK1/2 levels after 5-HT stimulation.

As in previous experiments, the cells stably transfected with 5-HT2aR-GFP were stimulated for 30 minutes with serum-IgG of patients with MS and of patients with neurological diseases, and subsequently stimulated for 15 minutes with serotonin (5-HT).

Figure 20 shows that serotonin was able to induce pERK1/2 and that selectively, MS IgG inhibited this induction. Under the same conditions control cells did not respond to 5Ht or MS IgG**.**

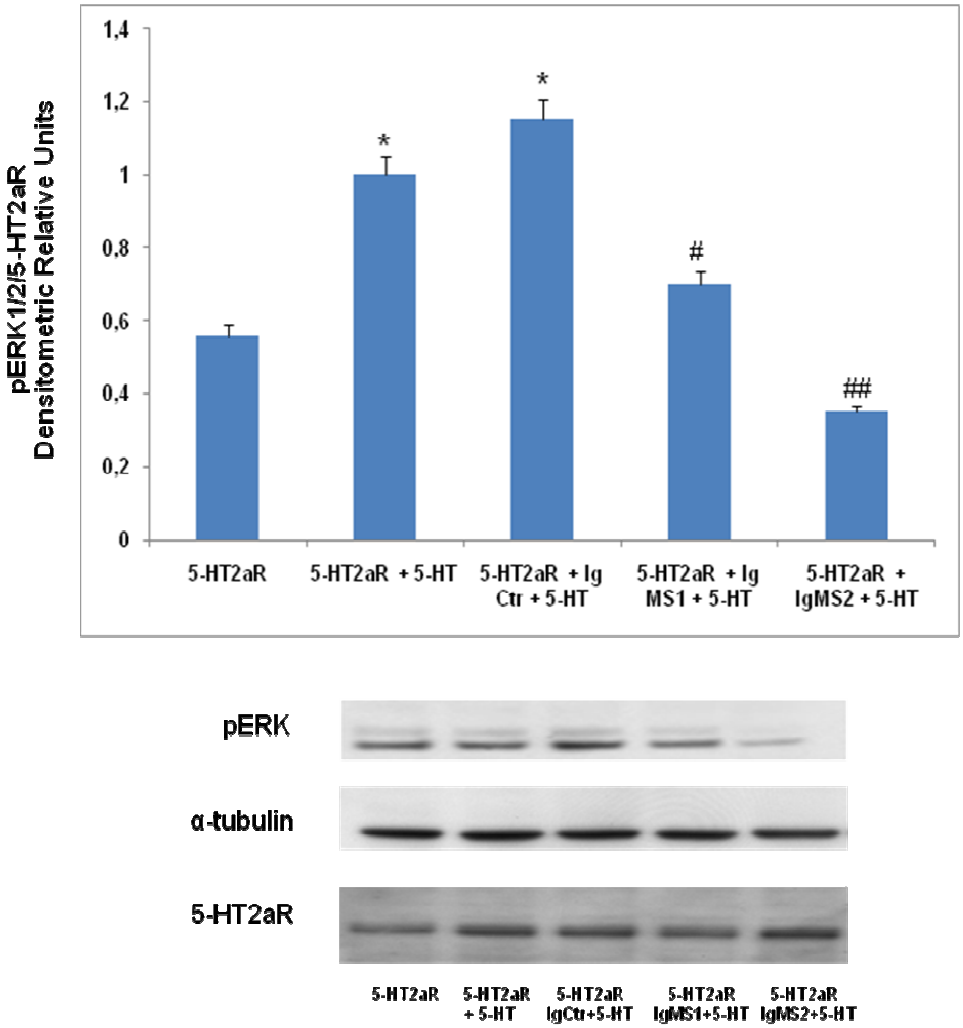


Figure 20 Effect of serum IgG from MS and neurological patients on P-ERK 1/2 levels in stably transfected cells.

The HEK293 cells stably transfected with 5-HT2aR-GFP were incubated for 4h in medium containing 0.2% FBS. Then the cells were pre-incubated for 30 minutes with serum Ig (2 MS and 1 neurological) and subsequently stimulated with 5μM of 5-HT for 15 minutes, before harversting them for Western blotting analysis of P-ERK1/2 levels. The histograms show the values (means ± SEM) relative to control (5-HT2aR) obtained by densitometric analysis of protein bands normalized to α-Tubulin and 5-HT2a receptor of three independent experiments. *$p < 0.05$ vs 5-HT2aR; # $p < 0.05$ vs 5-HT2aR + 5-HT; ## $p< 0.01$ vs 5-HT2aR + 5-HT.

In the lower part of the figure a representative experiment in shown

4.5 *Presence in MS serum of HT2a receptor binding antibodies*

The experiments shown above indicate that in the serum of MS patients are present IgG, able to interfere with oligodendrocyte differentiation, possibly through the 5Ht receptor. If this is the case, cells expressing the 5Ht receptor should be able to bind selectively serum IgG from MS not control patients, assayed by flow cytometry.

Cytofluorimetry was performed on HEK 293 cells transiently transfected with the 5-HT2aR-GFP.

The cells were incubated for 30 min with 200μg of serum IgG (MS or control) and subsequently challenged for 30 min with phycoerythrin-conjugated (PE) anti human IgG.

Figure 21 shows that PE fluorescence, which is a measure of IgG binding, marked a significant fraction of cells in the sample incubated with IgG from MS compared to the control samples (Figure 21 and Table 1). These data suggest that there is a specific binding between 5-HT2a receptor expressing cells and MS IgG and point furthermore to a possible specific assay to purify antibodies recognizing the 5-HT receptor.

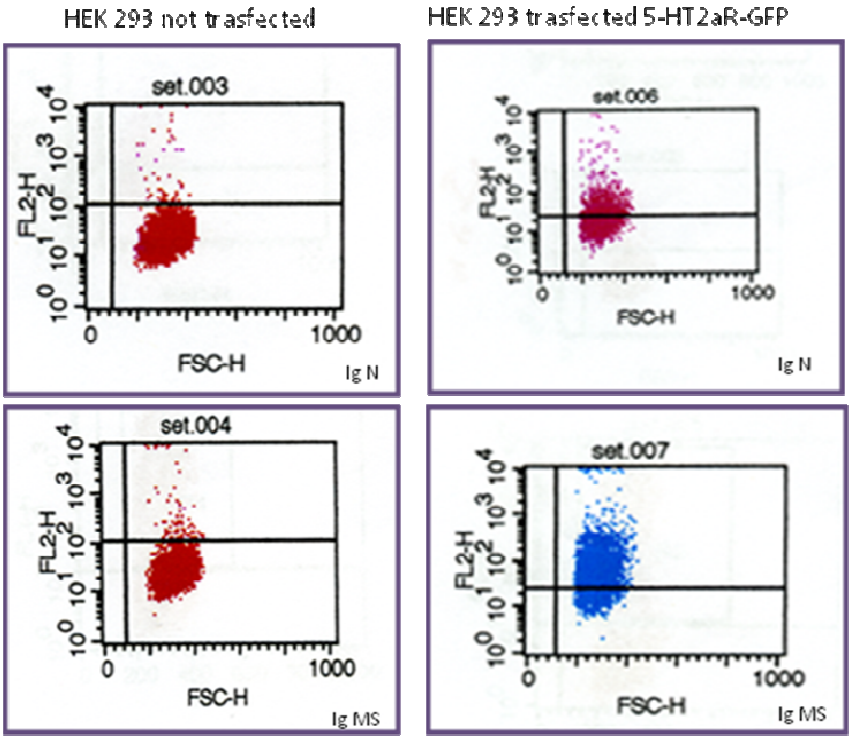


Figure 21. Binding between serum IgG from neurological or MS patients and 5-HT2a receptor. 5-HT2aR-GFP transfected cells were resuspended in 200µl PBS and then incubated with mouse serum for 30 min at 4 °C, to block non specific binding.
Then cells were incubated for 30 min with 200µg of serum IgG (MS or neurological), and stained for 30 min with PE-conjugated goat anti human IgG. Cells were washed and resuspended in 200 µL of PBS for flow cytometric analysis.

	% gated in HEK293 not trasfected	**% gated in HEK293 trasfected (5-HT2aR-GFP)**
IgG neurological	0,5%	**28%**
IgG MS	0,8%	**77%**

Table 1. Quantitative analysis of the Figure 21.

CHAPTER V
DISCUSSION AND CONCLUSIONS

Multiple sclerosis (MS) is a chronic demyelinating disease affecting oligodendrocytes (OLs), the cells responsible for axon myelination in the CNS. OLs 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, that may contribute to the reduction of OLs and to the limited remyelination in MS. These data is actually based on immunocytochemistry and immunohistochemistry investigations of the brain tissues, but the cellular mechanisms underlined to the failure of OLs maturation observed in MS has not been clarified.

In the first part of the project we verified the hypothesis of the presence, in serum of MS patients, of autoantibodies impairing the differentiation of OPCs. 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, through the analysis of the expression levels of differentiation markers, were analyzed the effects of serum IgG from MS patients on the OLs differentiation.

For the experiments we used the human oligodendrocyte cell line MO3-13. These cells were previously characterized in our laboratory. We identified several molecular markers modulated by differentiation stimuli such as PMA (PKC activator) identifying the effective concentrations and the incubation time required for the modulation of differentiation markers following the pattern of their expression over time up to 4 days of treatment (Figs 7-8-9-10).

Using IgG from serum of MS patients to stimulate the cells, we found that *P-ERK1/2*, *P-CREB* and *Olig-2* expression levels were significantly reduced compared with that of cells stimulated with IgG from control group (Figs 11-12-13-14).

The immune mechanisms leading to demyelination in MS are still unknown and the autoimmune hypothesis itself is not certain.

According to the hypothesis defined "outside-in", the onset of lesions are preceded by focal destruction of BBB that allows to autoreactive T cells and antibodies to reach CNS. This theory is opposed to the "inside-out" hypothesis. According to this hypothesis, an injury of the cells within CNS triggers an inflammatory response leading to pro-inflammatory lesions of the BBB that allows the entry of immune cells in CNS and their activation (80).

Studies of magnetic resonance imaging (MRI) in the early stages of the disease suggest that the focal destruction of the BBB is a hallmark of the acute phase of disease (Poussè) (81).

For this reason, we also compared the effects of IgG from MS patients in the active phase (Poussè) and in quiescent phase of disease on the OLs differentiation. We found that the P-ERK1/2, P-CREB and Olig-2 levels in cells incubated with IgG from MS patients during Poussè were reduced compared with that isolated during the quiescent phase of disease (Figs 11-12-14).

In the second part of the project we investigated the hypothesis of the presence, in MS patients, of autoantibodies directed concomitantly against a virus and a membrane receptor used by the virus to infect the OL, leading to differentiation failure.

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.

A candidate receptor expressed in oligodendrocytes is the 5-HT2aR subtype of the serotoninergic 5-HTR family. This receptor is used by human polyomavirus JC (JCV) to enter human embryonic stem cell-derived olygodendrocyte progenitor cells. JCV causes progressive multifocal

leukoencephalopathy (PML), a fatal demyelinating disease, in immunocompromised patients. The serotonergic receptor 5HT2A could act as the cellular receptor for JCV on human glial cells. In fact the administration of 5HT2A receptor antagonists inhibited JCV infection and monoclonal antibodies directed at 5HT2A receptors blocked infection of glial cells by JCV (82).

This virus may be involved in the disease. Two features of the biology of JCV make it a suitable candidate for MS: its neurotropic capability of targeting glial cells, and its latency and persistence. Recent studies have demonstrated that 70% of the human population worldwide is infected with JCV but the virus is absent from CSF of normal or neurological patients while is present in some MS subjects (83).

On the basis of these studies, we had to verify the involvement of 5-HT2a receptor signalling in OL differentiation and the direct interaction of 5-HT2a receptor with serum-Ig from MS patients, which might lead to an alteration of downstream oligodendrocytes intracellular signalling inducing differentiation block.

The $5\text{-}HT_{2A}$ receptor is a seven transmembrane receptor activating phospholipase C (PLC), through G_q, leading to accumulation of IP_3, di-acylglycerol (DAG) and activation of protein kinase C (PKC). Increase in cytoplasmic IP_3 causes a release of calcium from intracellular endoplasmic reticulum stores, and activaction of MAP kinase cascade (ERK1/2).

PKC activation is the target of PMA leading to differentiation in M03-13 cells, nevertheless, in our study we were not able to differentiate the cells with serotonin, probably, due to the rapid desensitization and internalization of the 5-HT2a receptor (84). However stimulation of M03-13 cells with serotonin for 15 min induced early differentiation markers such as P-ERK1/2 and Olig-2 (Figs 16-17).

Using IgGs from serum of MS patients to stimulate the cells, we found that 5-HT-mediated induction of *P-ERK1/2* levels, in HEK293 cells transfected with 5-HT2a receptor, was significantly reduced compared with that stimulated with IgG from control subjects (Figs 19-20).

Preliminary data also suggest a direct binding of IgG from MS patients with 5Ht2AR (Fig.21).

In conclusion, these data demonstrate that MS-derived autoantibodies interfere and inhibit oligodendrocyte differentiation-induced by 5HT opening new perspectives in the diagnosis and therapy for MS. Further studies will be focused on the purification of the whole receptor protein or peptides corresponding to the extracellular domains of the protein involved in the binding with the antibodies and/or JCV capsid protein.

These data give the opportunity to develop new diagnostic in vitro assay methods to execute a specific and early diagnosis of multiple sclerosis based on the measure of the binding activity between the purified receptor/peptides and the blood serum from patients.

REFERENCES

1. NOSEWORTHY JH, LUCCHINETTI C, RODRIGUEZ M, WEINSHENKER BG. *MULTIPLE SCLEROSIS*. N ENGL J MED 2000; 343: 938-52.
2. TRAPP BD, PETERSON J, RANSOHOFF RM, ET AL. *AXONAL TRANSECTION IN THE LESIONS OF MULTIPLE SCLEROSIS*. N ENGL J MED 1998; 338: 278-285.
3. MARRIE RA. *ENVIRONMENTAL RISK FACTORS IN MULTIPLE SCLEROSIS AETIOLOGY*. LANCET NEUROL 2004; 3: 709–718.
4. ELLIOT M, FROHMAN ET AL. *MULTIPLE SCLEROSIS-THE PLAQUE AND ITS PATHOGENESIS*. N ENGL J MED 2006.
5. COMPSTON A, SADOVNICK AD. *EPIDEMIOLOGY AND GENETICS OF MULTIPLE SCLEROSIS*. CURR OPIN NEUROL NEUROSURG 1992; 5: 175-181.
6. MCFARLAND HF. *TWINS STUDIES AND MULTIPLE SCLEROSIS*. ANN NEUROL 1992; 32: 722-723.
7. SADOVNICK AD, ARMSTRONG H, RICE GP, ET AL. *A POPULATION BASED OF MULTIPLE SCLEROSIS IN TWINS: UPDATE.* ANN NEUROL 1993; 33: 281-285.
8. EBERS GC, SADOVNICK AD. *THE ROLE OF GENETIC FACTORS IN MULTIPLE SCLEROSIS SUSCEPTIBILITY.* J NEUROIMMUNOL 1994; 54: 1-17.
9. SADOVNICK AD, DYMENT D, EBERS GC. *GENETIC EPIDEMIOLOGY OF MULTIPLE SCLEROSIS.* EPIDEMIOL REV 1997; 19: 99-104.
10. RAINE CS. THE DALE E. *MCFARLIN MEMORIAL LECTURE: THE IMMUNOLOGY OF THE MULTIPLE SCLEROSIS LESION.* ANN NEUROL 1994; 36 (SUPPL): S61-S72.
11. LASSMANN H, RAINE CS, ANTEL J, PRINEAS JW. *IMMUNOPATHOLOGY OF MULTIPLE SCLEROSIS: REPORT OF AN INTERNATIONAL MEETING HELD AT INSTITUTE OF NEUROLOGY OF THE UNIVERSITY OF VIENNA.* J NEUROIMMUNOL 1998; 86: 213-217.
12. KORNEK B, STORCH MK, WEISSERT R, ET AL. *MULTIPLE SCLEROSIS AND CHRONIC AUTOIMMUNE ENCEPHALOMYELITIS: A COMPARATIVE QUANTITATIVE STUDY OF AXONAL*

INJURY IN ACTIVE, INACTIVE, AND REMYELINATED LESIONS. AM J PATHOL 2000; 157: 267-76.

13. HUSEBY ES, LIGGITT D, BRABB T, SCHNABEL B, OHLEN C, GOVERMAN J. *A PATHOGENIC ROLE FOR MYELIN-SPECIFIC CD8(+) T CELLS IN A MODEL FOR MULTIPLE SCLEROSIS.* J EXP MED 2001; 194: 669-76.
14. CRAWFORD MP, YAN SX, ORTEGA SB, ET AL. *HIGH PREVALENCE OF AUTOREACTIVE, NEUROANTIGEN-SPECIFIC CD8+ T CELLS IN MULTIPLE SCLEROSIS REVEALED BY NOVEL FLOW CYTOMETRIC ASSAY.* BLOOD 2004; 55: 627-38.
15. ZANG YC, LI S, RIVERA VM, ET AL. *INCREASED CD8+ CYTOTOXIC T CELL RESPONSES TO MYELIN BASIC PROTEIN IN MULTIPLE SCLEROSIS.* J IMMUNOL 2004; 172: 5120-7.
16. BIELEKOVA B, GOODWIN B, RICHERT N, ET AL. *ENCEPHALITOGENIC POTENTIAL OF THE MYELIN BASIC PROTEIN PEPTIDE (AMINO ACIDS 83-99) IN MULTIPLE SCLEROSIS: RESULTS OF A PHASE II CLINICAL TRIAL WITH AN ALTERED PEPTIDE LIGAND.* NAT MED 2000; 6: 1167-75.
17. BRAY PF, LUKA J, ET AL. *ANTIBODIES AGAINST EPSTEIN- BARR NUCLEAR ANTIGEN (EBNA) IN MULTIPLE SCLEROSIS CSF AND TWO PENTAPEPTIDE SEQUENCE IDENTITIES BETWEEN EBNA AND MYELIN BASIC PROTEIN.* NEUROL 1992; 42: 1798-1804.
18. SALVETTI M, GIOVANNONI G, ALOISI F. *EPSTEIN-BARR VIRUS AND MULTIPLE SCLEROSIS.* CURR OPIN NEUROL. 2009 JUN; 22(3): 201-6.
19. MARKOVIC-PLESE S, HEMMER B, ZHAO Y, SIMON R, PINILLA C, MARTIN R. *HIGH LEVEL OF CROSS-REACTIVITY IN INFLUENZA VIRUS HEMAGGLUTININ-SPECIFIC CD4+ T-CELL RESPONSE: IMPLICATIONS FOR THE INITIATION OF AUTOIMMUNE RESPONSE IN MULTIPLE SCLEROSIS.* J NEUROIMMUNOL. 2005 DEC; 169(1-2): 31-8.
20. RUIZ PJ, GARREN H, HIRSCHBERG DL, ET AL. *MICROBIAL EPITOPES ACT AS ALTERED PEPTIDE LIGANDS TO PREVENT EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS.* J EXP MED. 1999 APR 19; 189(8): 1275-84.
21. TAIT AR, STRAUS SK. *PHOSPHORYLATION OF U24 FROM HUMAN HERPES VIRUS TYPE 6 (HHV-6) AND ITS POTENTIAL ROLE IN MIMICKING MYELIN BASIC PROTEIN (MBP) IN MULTIPLE SCLEROSIS.* FEBS LETT. 2008 AUG 6; 582; 582(18): 2685-8.

22. KIMBERLIN DW, WHITLEY RJ. *HHV-6: NEUROLOGICAL IMPLICATIONS OF A NEWLY DESCRIBED VIRAL PATHOGEN.* J NEUROVIR 1998; 4: 474-485.
23. O'HARA BA, ATWOOD WJ. *INTERFERON BETA1-A AND SELECTIVE ANTI-5HT(2A) RECEPTOR ANTAGONISTS INHIBIT INFECTION OF HUMAN GLIAL CELLS BY JC VIRUS.* VIRUS RES. 2008 MAR; 132(1-2): 97-103.
24. CANELLA B, RAINE CS. *THE ADESION MOLECULE AND CYTOKINE PROFILE OF MULTIPLE SCLEROSIS LESIONS.* ANN NEUROL 1995; 37: 424-435.
25. NAVIKAS V, LINK H. *CYTOKINES AND PATHOGENESIS OF MULTIPLE SCLEROSIS*. J NEUROSCI RES 1996; 45: 322-333.
26. LUCCHINETTI C, BRUCK W, RODRIGUEZ M, LASSMANN H. *MUTIPLE SCLEROSIS: LESSONS LEARNED FROM NEUROPATHOLOGY.* SEM NEUROL 1998; 18: 337-349.
27. KERMODE AG, THOMPSON AJ, TOFTS P, ET AL. *BREAKDOWN OF THE BLOOD-BRAIN BARRIER PRECEDES SYMPTOMS AND OTHER MRI SIGNS OF NEW LESIONS IN MULTIPLE SCLEROSIS.* BRAIN 1990; 113: 1477-1489.
28. RM RANSOHOFF. *IMMUNOLOGY: IN THE BEGINNING*. NATURE 2009 NOV; 462(7269): 41-2.
29. PERRON H, GARSON JA, BEDIN F. *MOLECULAR IDENTIFICATION OF A NOVEL RETROVIRUS REPEATEDLY ISOLATED FROM PATIENTS WITH MULTIPLE SCLEROSIS.* THE COLLABORATIVE RESEARCH GROUP ON MULTIPLE SCLEROSIS. PROC NATL ACAD SCI USA. 1997; 94: 7583-7588.
30. SOTGIU S, SERRA C, MAMELI G ET AL. *MULTIPLE SCLEROSIS-ASSOCIATED RETROVIRUS AND MS PROGNOSIS: AN OBSERVATIONAL STUDY.* NEUROL 2002; 59: 1071-1073.
31. JOHNSON RT. *VIRAL ASPECTS OF MULTIPLE SCLEROSIS.* HANDBOOK OF CLINICAL NEUROLOGY: DEMYELINATING DISORDERS, VOL. 3. AMSTERDAM/NEW YORK: ELSEVIER SCIENCE 1985; 259-287.
32. ITOVAMA Y. *MULTIPLE SCLEROSIS-DIAGNOSIS AND ITS PROBLEMS.* RINSHO SHINKEIGAKU. 1995 DEC; 35(12): 1496-7.

33. RUNMARKER B, ANDERSEN O. *PROGNOSTIC FACTORS IN A MULTIPLE SCLEROSIS INCIDENCE COHORT WITH TWENTY-FIVE YEARS OF FOLLOW-UP.* BRAIN 1993; 116: 117-134.
34. TROJANO M, PAOLICELLI D. *THE DIFFERENTIAL DIAGNOSIS OF MULTIPLE SCLEROSIS: CLASSIFICATION AND CLINICAL FEATURES OF RELAPSING AND PROGRESSIVE NEUROLOGICAL SYNDROME.* NEUROL SCI. 2001 NOV; 22 (SUPPL 2): S98-102.
35. LUQUE FA, JAFFE SL. *CEREBROSPINAL FLUID ANALYSIS IN MULTIPLE SCLEROSIS.* INT REV NEUROBIOL. 2007; 79: 341-56.
36. YOUNG IR, HALL AS, PALLIS CA, LEGG NJ, BYDDER GM, STEINER RE. *NUCLEAR MAGNETIC RESONANCE IMAGING OF THE BRAIN IN MULTIPLE SCLEROSIS.* LANCET. 1981 NOV 14; 2(8255): 1063-6.
37. ACHTEN E, DEBLAERE K. *HEALTH TECHNOLOGY ASSESSMENT ON THE USE OF MAGNETIC RESONANCE IMAGING (MRI) AND COMPUTED TOMOGRAPHY (CT) IN THE DIAGNOSIS OF MULTIPLE SCLEROSIS (MS) AND CLINICALLY ISOLATED SYNDROMES (CIS).* EUR J RADIOL. 2008 FEB; 65(2): 211-3.
38. CURTIS R, COHEN J, FOK-SEANG J, HANLEY MR, GREGSON NA, REYNOLDS R, AND WILKIN GP. *DEVELOPMENT OF MACROGLIAL CELLS IN RAT CEREBELLUM. I. USE OF ANTIBODIES TO FOLLOW EARLY IN VIVO DEVELOPMENT AND MIGRATION OF OLIGODENDROCYTES.* J NEUROCYTOL. 1988;17: 43-54.
39. HARDY R AND REYNOLDS R. *PROLIFERATION AND DIFFERENTIATION POTENTIAL OF RAT FOREBRAIN OLIGODENDROGLIAL PROGENITORS BOTH IN VITRO AND IN VIVO*. DEVELOPMENT. 1991;111: 1061-1080.
40. BAUMANN NICOLE AND DANIELLE PHAM-DINH. *BIOLOGY OF OLIGODENDROCYTE AND MYELININ THE MAMMALIAN CENTRAL NERVOUS SYSTEM*. PHYSIOLOGICAL REVIEWS. 2001 APRIL: VOL. 81, NO. 2. PRINTED IN U.S.A.
41. REYNOLDS R AND WILKIN GP. *OLIGODENDROGLIAL PROGENITOR CELLS BUT NOT OLIGODENDROGLIA DIVIDE DURING NORMAL DEVELOPMENT OF THE RAT*. J NEUROCYTOL. 1991; 20: 216-224.

42. PRINEAS JW, BARNARD RO, KWON EE, SHARER LR, CHO ES. *MULTIPLE SCLEROSIS: REMYELINATION OF NASCENT LESIONS.* ANN NEUROL. 1993; 33: 137-51.
43. SETZU A, LATHIA JD, ZHAO C, ET AL. *INFLAMMATION STIMULATES MYELINATION BY TRANSPLANTED OLIGODENDROCYTE PRECURSOR CELLS.* GLIA. 2006; 54: 297-303.
44. SCOLDING N, FRANKLIN R, STEVENS S, HELDIN CH, COMPSTON A, NEWCOMBE J. *OLIGODENDROCYTE PROGENITORS ARE PRESENT IN THE NORMAL ADULT HUMAN CNS AND IN THE LESIONS OF MULTIPLE SCLEROSIS.* BRAIN. 1998; 121: 2221-8.
45. WOLSWIJK G. *OLIGODENDROCYTE SURVIVAL, LOSS AND BIRTH IN LESIONS OF CHRONIC-STAGE MULTIPLE SCLEROSIS.* BRAIN. 2000; 123: 105-15.
46. CHANG A, TOURTELLOTTE WW, RUDICK R, TRAPP BD. *PREMYELINATING OLIGODENDROCYTES IN CHRONIC LESIONS OF MULTIPLE SCLEROSIS.* N ENGL J MED. 2002 JAN 17; 346(3): 165-73.
47. WOLSWIJK G. *CHRONIC STAGE MULTIPLE SCLEROSIS LESIONS CONTAIN A RELATIVELY QUIESCENT POPULATION OF OLIGODENDROCYTE PRECURSOR CELLS*. J NEUROSC. 1998 JAN 15; 18(2): 601-9.
48. HAGG T. *FROM NEUROTRANSMITTERS TO NEUROTROPHIC FACTORS TO NEUROGENESIS.* NEUROSCIENTIST. 2009 FEB; 15(1): 20-7.
49. ARMSTRONG RC, DORN HH, KUFTA CV, FRIEDMAN E, DUBOIS-DALCQ ME. *PRE-OLIGODENDROCYTES FROM ADULT HUMAN CNS.* J NEUROSCI. 1992 APR; 12(4): 1538-47.
50. ARMSTRONG RC, KIM JG, HUDSON LD. *EXPRESSION OF MYELIN TRANSCRIPTION FACTOR I (MYTI), A "ZINC-FINGER" DNA-BINDING PROTEIN, IN DEVELOPING OLIGODENDROCYTES.* GLIA. 1995 AUG; 14(4): 303-21.
51. KESSARIS N, JAMEN F, RUBIN LL, RICHARDSON WD. *COOPERATION BETWEEN SONIC HEDGEHOG ANS FIBROBLAST GROWTH FACTOR/MAPK SIGNALLING PATHWAYS IN NEOCORTICAL PRECURSORS*. DEVELOPMENT. 2004; 131: 1289-1298.
52. LACHAPELLE F ET AL. *FIBROBLAST GROWTH FACTOR-2 (FGF-2) AND PLATELET-DERIVED GROWTH FACTOR AB (PDGF AB) PROMOTE ADULT SVZ-DERIVED OLIGODENDROGENESIS IN VIVO*. MOL CELL NEUROSCI. 2002; 20: 390-403.

53. HSIEH J, AIMONE JB, KASPAR BK, ET AL. *IGF-I INSTRUCTS MULTIPOTENT ADULT NEURAL PROGENITOR CELLS TO BECOME OLIGODENDROCYTES.* J CELL BIOL 2004; 164: 111–122.
54. NICOLAY DJ, DOUCETTE JR, NAZARALI AJ. *TRANSCRIPTIONAL CONTROL OF OLIGODENDROGENESIS.* GLIA. 2007; 55: 1287–1299.
55. DE GROOT DM, COENEN AJ, VERHOFSTAD A, VAN HERP F, MARTENS GJ. *IN VIVO INDUCTION OF GLIAL CELL PROLIFERATION AND AXONAL OUTGROWTH AND MYELINATION BY BRAIN-DERIVED NEUROTROPHIC FACTOR.* MOL ENDOCRINOL. 2006 NOV; 20(11): 2987-98.
56. WEGNER M, STOLT CC. *FROM STEM CELLS TO NEURONS AND GLIA: A SOXIST'S VIEW OF NEURAL DEVELOPMENT.* TRENDS NEUROSCI. 2005; 28: 583–588.
57. STOLT CC, SCHLIERF A, LOMMES P, ET AL. *SOXD PROTEINS INFLUENCE MULTIPLE STAGES OF OLIGODENDROCYTE DEVELOPMENT AND MODULATE SOXE PROTEIN FUNCTION.* DEV CELL. 2006; 11: 697–709.
58. HE Y, DUPREE J, WANG J, ET AL. *THE TRANSCRIPTION FACTOR YIN YANG 1 IS ESSENTIAL FOR OLIGODENDROCYTE PROGENITOR DIFFERENTIATION.* NEURON. 2007; 55:217–230.
59. NIELSEN JA, BERNDT JA, HUDSON LD, ARMSTRONG RC. *MYELIN TRANSCRIPTION FACTOR 1 (MYT1) MODULATES THE PROLIFERATION AND DIFFERENTIATION OF OLIGODENDROCYTE LINEAGE CELLS.* MOL CELL NEUROSCI. 2004; 25: 111–123.
60. BARRES BA, SCHMID R, SENDTER M, AND RAFF MC. *MULTIPLE EXTRACELLULAR SIGNALS ARE REQUIRED FOR LONG-TERM OLIGODENDROCYTE SURVIVAL.* DEVELOPMENT. 1993; 118: 283–295.
61. MCKINNON RD, MATSUI T, DUBOIS-DALCQ M, AND ARONSON SA. *FGF MODULATES THE PDGF-DRIVEN PATHWAY OF OLIGODENDROCYTE DEVELOPMENT*. NEURON. 1990; 5: 603–614.
62. BANSAL R, KUMAR M, MURRAT K, MORRISON RS, AND PFEIFFER SE. *REGULATION OF FGF RECEPTORS IN THE OLIGODENDROCYTE LINEAGE*. MOL CELL NEUROSCI. 1996; 7: 263–275.

63. McMorris A and Dubois-Dalcq M. *Insulin-like growth factor 1 promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing in vitro.* J Neurosci. 1988; Res 21: 199–209.
64. Carson MJ, Behringer RR, Brinster RL, and McMorris FA. *Insulin-like growth factor 1 increases brain growth and central nervous system myelination in transgenic mice.* Neuron. 1993; 10: 729–740.
65. Barde YA. *A crucial role for neurotrophin-3 in oligodendrocyte development.* Nature. 1994; 367: 371–375.
66. Kahn MA, Kumar S, Liebl D, Chang R, Parada LF, and De Vellis J. *Mice lacking NT-3, and its receptor TrkC, exhibit profound deficiencies in CNS glial cells.* Glia. 1999; 26: 153–165.
67. McTigue DM, Horner PJ, Stokes BT, and Gage FH. *Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord*. J Neurosci. 1998; 18: 5354–5365.
68. Canoll PD, Kraemer R, Teng KK, Marchionni MA, and Salzer JL. *GGF/neuregulin induces a phenotypic reversion of oligodendrocytes*. Mol Cell Neurosci. 1999; 13: 79–94.
69. Vartanian T, Fischbach G, and Miller R. *Failure of spinal cord oligodendrocyte development in mice lacking neuregulin*. Proc Natl Acad Sci USA. 1999; 96: 731–735.
70. Gard AL, Burrell MR, Pfeiffer SE, Rudge JS, and Williams WC II. *Astroglial control of oligodendrocyte survival mediated by PDGF and leukemia inhibitory factor-like protein*. Development. 1995; 121: 2187–2197.
71. McMorris FA and McKinnon RD. *Regulation of oligodendrocyte development and CNS myelination by growth factors: prospects for therapy of demyelinating diseases*. Brain Pathol. 1996; 6: 313–329.

72. BERUMEN LC, *SEROTONIN RECEPTORS IN HIPPOCAMPUS*, THE SCIENTIFICWORLD JOURNAL, 2012.
73. NICHOLS DE AND NICHOLS CD*, SEROTONIN RECEPTORS, CHEM.* REV. 2008, 108, 1614–1641
74. GRAY JA, ROTH BL, *PARADOXICAL TRAFFICKING AND REGULATION OF 5-HT_{2A} RECEPTORS BY AGONISTS AND ANTAGONISTS*; BRAIN RES BULL, 2001 NOV 15; 56(5):441-51.
75. SANTILLO M, MONDOLA P, SERÙ R, ANNELLA T, CASSANO S, CIULLO I, TECCE MF, IACOMINO G, DAMIANO S, CUDA G, PATERNÒ R, MARTIGNETTI V, MELE E, FELICIELLO A, AVVEDIMENTO EV. *OPPOSING FUNCTIONS OF KI- AND 71 HA-RAS GENES IN THE REGULATION OF REDOX SIGNALS.* CURR BIOL. 2001 APR 17; 11(8): 614-9.
76. CUDA G, PATERNÒ R, CERAVOLO R, CANDIGLIOTA M, PERROTTI N, PERTICONE F, FANIELLO MC, SCHEPIS F, RUOCCO A, MELE E, CASSANO S, BIFULCO M, SANTILLO M, AVVEDIMENTO EV. *PROTECTION OF HUMAN ENDOTHELIAL CELLS FROM OXIDATIVE STRESS: ROLE OF RAS-ERK1/2 SIGNALING.* CIRCULATION. 2002 FEB 26; 105(8): 968-74.
77. SVEGLIATI S, CANCELLO R, SAMBO P, LUCHETTI M, PARONCINI P, ORLANDINI G, DISCEPOLI G, PATERNO R, SANTILLO M, CUOZZO C, CASSANO S, AVVEDIMENTO EV, GABRIELLI A. *PLATELET-DERIVED GROWTH FACTOR AND REACTIVE OXYGEN SPECIES (ROS) REGULATE RAS PROTEIN LEVELS IN PRIMARY HUMAN FIBROBLASTS VIA ERK1/2. AMPLIFICATION OF ROS AND RAS IN SYSTEMIC SCLEROSIS FIBROBLASTS.* J BIOL CHEM. 2005 OCT; 280(43): 36474-82.
78. BARONI SS, SANTILLO M, BEVILACQUA F, LUCHETTI M, SPADONI T, MANCINI M, FRATICELLI P, SAMBO P, FUNARO A, KAZLAUSKAS A, AVVEDIMENTO EV, GABRIELLI A. *STIMULATORY AUTOANTIBODIES TO THE PDGF RECEPTOR IN SYSTEMIC SCLEROSIS.* N ENGL J MED. 2006 JUN; 354(25): 2667-76.
79. DOWSON VL, DOWSON TM. *FREE RADICALS AND CELL DEATH*. CELL DEATH DIFFERENTIATION. 1996; 3: 71-78.

80. WOOTLA B, ERIGUCHI M, RODRIGUEZ M. *IS MULTIPLE SCLEROSIS AN AUTOIMMUNE DISEASE?,* AUTOIMMUNE DIS. 2012;2012:969657. EPUB 2012 MAY 16
81. KERMODE AG, THOMPSON AJ, TOFTS P, MACMANUS DG, KENDALL BE, KINGSLEY DP,MOSELEY IF, RUDGE P, MCDONALD WI. *BREAKDOWN OF THE BLOOD-BRAIN BARRIER PRECEDES SYMPTOMS AND OTHER MRI SIGNS OF NEW LESIONS IN MULTIPLE SCLEROSIS*. PATHOGENETIC AND CLINICAL IMPLICATIONS. BRAIN. 1990 OCT;113 (PT 5):1477-89
82. ALVAREZ-LAFUENTE R, GARCÍA-MONTOJO M, DE LAS HERAS V, BARTOLOMÉ M, ARROYO R*., JC VIRUS IN CEREBROSPINAL FLUID SAMPLES OF MULTIPLE SCLEROSIS PATIENTS AT THE FIRST DEMYELINATING EVENT,* MULTIPLE SCLEROSIS. 2007 JUN;13(5):590-5.
83. ELPHICK GF, QUERBES W, JORDAN JA, GEE GV, EASH S, MANLEY K, DUGAN A, STANIFER M, BHATNAGAR A, KROEZE WK, ROTH BL, ATWOOD WJ., *THE HUMAN POLYOMAVIRUS, JCV, USES SEROTONIN RECEPTORS TO INFECT CELLS*, SCIENCE, 2004 NOV 19;306(5700):1380-3.
84. BHATTACHARYYA S, PURI S, MILEDI R, PANICKER MM , *INTERNALIZATION AND RECYCLING OF 5-HT2A RECEPTORS ACTIVATED BY SEROTONIN AND PROTEIN KINASE C-MEDIATED MECHANISMS,* PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, 2002 OCT 29;99(22):14470-5
85. SCHAUMBURG C, O'HARA BA, LANE TE, ATWOOD WJ, *HUMAN EMBRYONIC STEM CELL-DERIVED OLIGODENDROCYTE PROGENITOR CELLS EXPRESS THE SEROTONIN RECEPTOR AND ARE SUSCEPTIBLE TO JC VIRUS INFECTION,* JOURNAL OF VIROLOGY, 2008 SEPT; 82(17): 8896–8899.
86. Siddharthan Chandrana, Hidemasa Kato, Dianne Gerreli, Alastair Compston, Clive N. Svendsen and Nicholas D. Allen. *FGF-DEPENDENT GENERATION OF OLIGODENDROCYTES BY A HEDGEHOG-INDEPENDENT PATHWAY*. Development 130, 6599.6609. Published by The company of Biologists, 2003.

PUBLICATIONS

Molecular and Cellular Neuroscience 52 (2013) 31–37

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Cu–Zn superoxide dismutase activates muscarinic acetylcholine M1 receptor pathway in neuroblastoma cells

Simona Damiano [a], Tiziana Petrozziello [a], Valentina Ucci [a], Stefano Amente [b], Mariarosaria Santillo [a], Paolo Mondola [a,*]

[a] Dipartimento di Neuroscienze, Unità di Fisiologia Umana, Università degli Studi di Napoli "Federico II", Napoli, Italy
[b] Dipartimento di Biologia Strutturale e Funzionale, Università degli Studi di Napoli "Federico II", Italy

ARTICLE INFO

Article history:
Received 4 April 2012
Revised 25 October 2012
Accepted 2 November 2012
Available online 10 November 2012

Keywords:
Cu–Zn superoxide dismutase (SOD1)
Neuroblastoma cells (SK-N-BE)
Muscarinic acetylcholine receptors (mAChRs)
ERK 1/2
Akt

ABSTRACT

Muscarinic receptors (mAChRs) control several neuronal functions and are widely expressed in the central nervous system (CNS): M1 subtype represents the predominant mAChR in the CNS.
Previously, we showed that antioxidant enzyme Cu–Zn superoxide dismutase (SOD1) is secreted by many cellular lines and specifically interacts with cell surface membrane of human neuroblastoma SK-N-BE cells thus activating phospholipase C (PLC) transduction pathway and increasing intracellular calcium concentration ($[Ca^{2+}]_i$). In addition, we demonstrated that a small amount of SOD1 is contained in large core dense vesicles and that it is secreted in response to depolarization induced by elevated extracellular K^+ concentration. In the present study, we investigated the involvement of muscarinic M1 receptors in SOD1-induced activation of PLC transduction pathway. We showed that, in SK-N-BE cells, SOD1 was able to activate muscarinic M1 receptor producing a phosphorylation of ERK 1/2 and Akt in dose- and time-dependent manner. Interestingly, in the presence of the M1 antagonist pirenzepine, ERK 1/2 and Akt phosphorylation induced by SOD1 was remarkably prevented. This effect was mimicked by knocking-down M1 receptor using two sequences of RNA silencing (siRNA). At functional level, siRNAs against M1 receptor were able to prevent the increase in $[Ca^{2+}]_i$ induced by SOD1. The same inhibitory effect on $[Ca^{2+}]_i$ changes was produced by the M1 antagonist pirenzepine. Collectively, the results of this study demonstrated that SOD1 could activate a transductional pathway through the involvement of M1 muscarinic receptor.

Introduction

Muscarinic acetylcholine receptors (mAChRs) are among the best-characterized of the seven transmembrane receptors. Five mAChR subtypes have been cloned and they are divided into two distinct classes based on signal transduction: M1, M3 and M5 mAChR subtypes, coupled to $G_{q/11}$ proteins activate phospholipase C and mobilize intracellular calcium, whereas M2 and M4 mAChRs signal through $G_{i/o}$ proteins thus reducing the intracellular concentration of cAMP (Caulfield and Birdsall, 1998). Moreover the M1 subtype also activates the extracellular signal-regulated kinase pathway (Berkeley et al., 2001).

Muscarinic receptors control several neuronal functions and are widely expressed in the central nervous system (CNS): M1 subtype represents the predominant mAChR in the CNS, which is located in the cortex, hippocampus, striatum and thalamus where it is found post-synaptically (Ellis, 2002). M2 mAChRs are located not only in the brainstem and thalamus, but also in the cortex, hippocampus and striatum at the level of cholinergic synaptic terminals (Rouse et al., 1997) where it controls ACh release (Raiteri et al., 1980). M3 and M5 mAChRs are expressed at much lower levels than M1 within the CNS. M4 mAChRs are most prominent in the striatum (Ellis, 2002), where they are thought to play a role in controlling dopamine release and locomotor activity. Given the wide and varied expression profile of the mAChRs in the CNS, it is not surprising that all the subtypes have been evaluated as potential drug targets in Alzheimer Disease (AD), schizophrenia, Parkinson's disease and drug dependence.

For AD, recent studies have provided strong evidence to suggest that the stimulation of M1 receptor attenuates the disease progression (Caccamo et al., 2006). Furthermore, genetic and biochemical studies have shown that mAChR density is reduced in the pre-frontal cortex, hippocampus and caudate putamen of schizophrenic subjects (Crook et al., 2000; Dean et al., 1996, 2002) in most of which a single-nucleotide polymorphism in the M1 mAChR gene (C267A) has been associated with prefrontal cortical dysfunction (Liao et al., 2003). It is predicted that selectively activating M1 mAChRs should lead to cognitive benefits in schizophrenia, whereas M4 mAChRs agonism may inhibit dopamine release to reduce positive symptoms associated with the disease. While the role of mAChRs in both AD, Parkinson's disease and, to a lesser extent, schizophrenia, is well established in

* Corresponding author at: Dept. of Neuroscience, Unit of Physiology, University of Naples "Federico II", Via S. Pansini, 5, 80131 Naples, Italy. Fax: +39 81 7463639.
E-mail address: mondola@unina.it (P. Mondola).

1044-7431/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.mcn.2012.11.001

the literature, more recent evidence has suggested that blockade of the M5 mAChRs may be a suitable approach in drug dependence and addiction.

Previously, we showed that antioxidant enzyme Cu–Zn superoxide dismutase (SOD1) is secreted by many cellular lines (Cimini et al., 2002; Mondola et al., 1996, 1998, 2003) and specifically interacts with cell surface membrane of human neuroblastoma SK-N-BE cells activating PLC transduction pathway that increases intracellular calcium concentration (Mondola et al., 2004).

Using rat pituitary GH3 cells, that express all the neuronal protein machinery involved in synaptic vesicle exocytosis, we demonstrated that small SOD1 amount is contained in large core dense vesicles. In addition we showed that this antioxidant molecule is secreted in response to depolarization induced by elevated extracellular K^+ concentration (Santillo et al., 2007).

Many studies have shown that the activation of muscarinic M1 receptor coupled to $G_{q/11}$ protein, can increase extracellular signal-regulated protein kinase (ERK 1/2) inducing long term depression (LTD) (Anagnostaras et al., 2003; Hamilton et al., 2001; McCoy and McMahon, 2007; Scheiderer et al., 2006).

In the present study, performed in SK-N-BE cells, we investigated whether the activation on PLC transduction mechanism induced by SOD1 may involve muscarinic M1 receptor and induce the downstream activation of ERK 1/2 and Akt signaling cascade.

Results

Effect of SOD1 on ERK 1/2 and Akt phosphorylation in SK-N-BE neuroblastoma cells

SOD1 induced an increase of ERK 1/2 phosphorylation (p-ERK 1/2) in SK-N-BE cells that was dose-dependent (200–400 ng/ml) (Fig. 1A); similarly, the enzyme produced Akt phosphorylation that increases from 200 to 400 ng/ml (Fig. 1B). These effects on both the kinases were time-dependent (Fig. 1C and D). At 400 ng/ml SOD1 induced ERK 1/2 and Akt phosphorylation already at 10 min of exposure and further increases at 30 min (Fig. 1C and D).

Moreover, we demonstrated that the effect of SOD1 on ERK 1/2 and Akt activation was not reactive oxygen species (ROS) dependent since stimulating the cells with 400 ng/ml of SOD1 in the absence and presence of 10 mM of the ROS scavenger N-acetylcysteine (NAC) the activation of the above transductional pathways was not reverted (data not shown). We have previously shown that SOD1 is able to trigger PLC/PKC transductional pathway thus increasing $[Ca^{2+}]_i$ (Mondola et al., 2004). In the light of this evidence, SK-N-BE cells were exposed to 400 ng/ml of SOD1 in the absence or in the presence of 100 nM of BIM, a PKC inhibitor (Fig. 2A and B) and in the absence or in the presence of 10 μM of intracellular Ca^{2+} chelator, BAPTA/AM (Fig. 2C and D). Both BIM and BAPTA/AM decrease mainly P-ERK levels but the effect is not

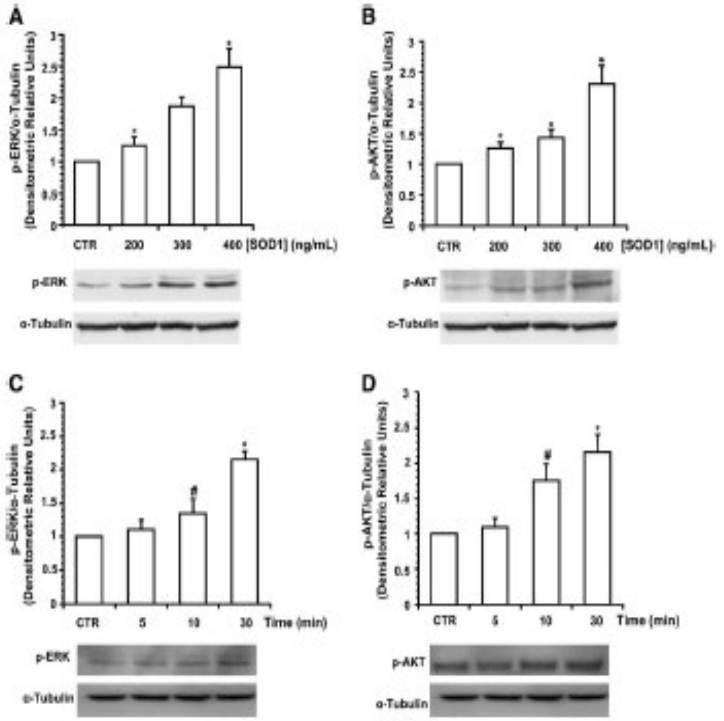


Fig. 1. Western blotting analysis of the effects of increasing concentration of SOD1 on p-ERK (panel A) and p-Akt (panel B) levels in SK-N-BE cells. Western blotting analysis of the effects of different times of incubation of SOD1 on p-ERK levels in cells is shown in panel C; the same time-course analysis of p-Akt is represented in panel D. The cells were incubated for 18 h in medium containing 0.2% FBS and then stimulated with indicated SOD1 concentration for the indicated time. The histograms show the mean values (± SE), obtained by densitometric analysis of three independent experiments. Panel A: * $p<0.05$ versus control and 200 ng/ml; Panel B: * $p<0.05$ versus control, 200 ng/ml and 300 ng/ml; Panel C: * $p<0.05$ versus control and 5 min, # $p<0.05$ versus control; Panel D: * $p<0.05$ versus control and 5 min; # $p<0.05$ versus control. Representative Western blotting is shown below the respective panels.

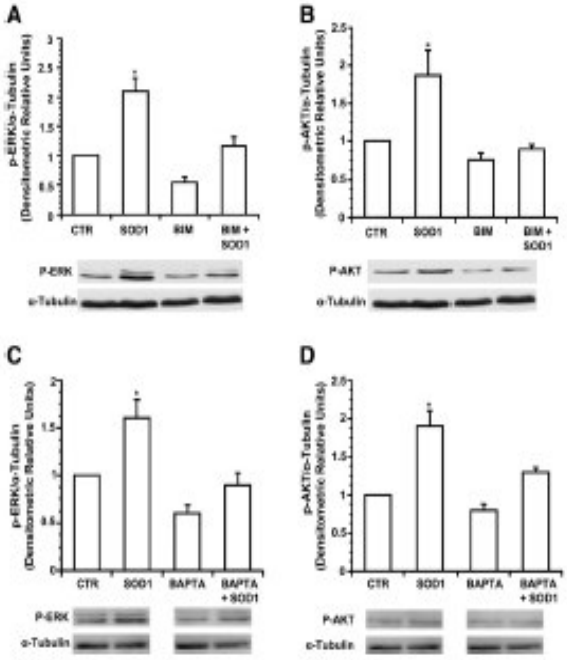


Fig. 2. Effect of SOD1 on ERK and Akt activation in the absence and presence of PKC inhibitor and calcium chelator. Western blotting analysis of p-ERK on SK-N-BE cells incubated with 400 ng/ml of SOD1 alone or in the presence of BIM (panel A); in panel B the same experiment related to p-Akt is illustrated. Western blotting experiments of p-ERK (panel C) and p-Akt (panel D) on cells incubated with 400 ng/ml of SOD1 in the presence and absence of calcium chelator BAPTA/AM. The cells were incubated for 18 h in medium containing 0.2% FBS, pre-incubated with 100 nM BIM for 30 min or with 10 μM BAPTA/AM for 5 min and then stimulated with 400 ng/ml of SOD1 for 10 min. The histograms show the mean values (± SE), evaluated by densitometric analysis of three independent experiments. Panel A: * $p < 0.05$ versus control, BIM and BIM + SOD1; Panel B: * $p < 0.05$ versus control, BIM and BIM + SOD1; Panel C: * $p < 0.05$ versus control, BAPTA and BAPTA + SOD1; Panel D: * $p < 0.05$ versus control, BAPTA and BAPTA + SOD1. Representative Western blotting is shown below the respective panels.

statistically significant. These two specific tools instead significantly prevented ERK 1/2 and Akt phosphorylation induced by SOD1 (Fig. 2). These data demonstrate the involvement of PKC/Ca^{2+}/ERK1/2/Akt pathway in the signaling activated by extracellular SOD1.

In order to study the involvement of muscarinic M1 receptor in the activation of p-ERK 1/2 and p-Akt, we evaluated the effects of the well known M1 antagonist pirenzepine and of the M1 agonist oxotremorine on the stimulatory action of SOD1. Neuroblastoma cells were therefore preincubated at 37 °C for 5 min with 10 μM pirenzepine or 15 μM oxotremorine, respectively. Fig. 3 shows that pirenzepine, by antagonizing M1 receptor function, inhibited the stimulatory effect of SOD1 (400 ng/ml) on p-ERK 1/2; similar results were obtained on p-Akt (Fig. 4B). In contrast, the well known M1 agonist oxotremorine produced per se a great stimulation of both ERK 1/2 and Akt phosphorylation when incubated at 15 μM (Fig. 3A, B).

Interestingly, SOD1 plus oxotremorine produced a phosphorylation of both ERK 1/2 and Akt at the same extent than when incubated alone (Fig. 3). We stimulated SK-N-BE cells with physiological M1 agonist Ach (10 μM acetylcholine) and we found, data not shown, that this increases P-ERK levels slightly more than SOD1; in addition this effect doesn't seem to be additive.

SOD1 induces activation of ERK 1/2 and Akt through M1 receptor in SK-N-BE neuroblastoma cells

To study more specifically the involvement of M1 receptor in the signaling of SOD1, this metabotropic receptor was knocked-down by two different sequences of siRNA. Western blot analysis revealed that siRNA # 37 and siRNA # 69 of M1 receptor were able to significantly and specifically reduce M1 protein expression in SK-N-BE cells (Fig. 4A). Furthermore siRNA # 37 and siRNA # 69 significantly prevented ERK 1/2 and Akt phosphorylation induced by SOD1 (400 ng/ml) (Fig. 4B and C). On the other hand, no effect was detected with their non targeting sequence on ERK 1/2 and Akt phosphorylation induced by SOD1 (Fig. 4B and C).

Hypothetically, the incubation of cells with SOD1 could alter the release of Ach; therefore, to demonstrate that the activation of M1 receptors with exogenous SOD1 is specific and not modulated by the release of Ach by SK-N-BE cholinergic cells, we demonstrated (data not shown) that neither in normal condition (control) nor after depolarization induced by 55 mM of extracellular K^+ we detected Ach release in culture medium. The levels of Ach in the culture medium were in all the above conditions less than 0.01 μg/l. The same result was obtained stimulating SK-N-BE cells with 400 ng/ml of SOD1.

Role of M1-receptor in SOD1-mediated $[Ca^{2+}]_i$ modulation in SK-N-BE cells

The involvement of $[Ca^{2+}]_i$ as an important player in the signaling of SOD1 has been highlighted by the inhibitory effect of BAPTA/AM on ERK 1/2 and Akt phosphorylation induced by SOD1. Furthermore, the role of M1 receptor in SOD1-mediated control of $[Ca^{2+}]_i$ homeostasis has been characterized in neuroblastoma cells by both pharmacological and biochemical approaches. In accordance to the greater

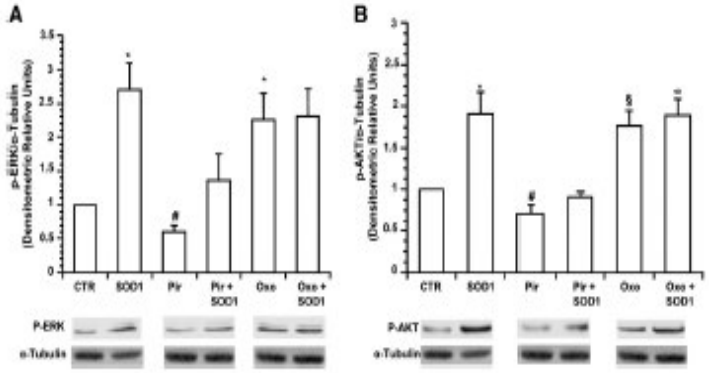


Fig. 3. Effect of SOD1 on ERK and Akt activation in the absence and presence of pirenzepine or oxotremorine. Effects of 400 ng/ml of SOD1 on p-ERK (panel A) and p-Akt (panel B) in the presence of M1 antagonist (pirenzepine) and M1 agonist (oxotremorine). The cells were incubated for 18 h in medium containing 0.2% FBS, pre-incubated with 15 μM pirenzepine or with 10 μM oxotremorine for 5 min and then stimulated with 400 ng/ml of SOD1 for 10 min. The histograms show the mean values (± SE), evaluated by densitometric analysis of three independent experiments. Panel A: * $p < 0.05$ versus control, pirenzepine and pirenzepine + SOD1; # $p < 0.05$ versus oxotremorine and oxotremorine + SOD1; Panel B: * $p < 0.05$ versus control, pirenzepine and pirenzepine + SOD1; # $p < 0.05$ versus oxotremorine and oxotremorine + SOD1; § $p < 0.05$ versus control and pirenzepine + SOD1; = $p < 0.05$ versus control and pirenzepine + SOD1. Representative Western blotting is shown below the respective panels.

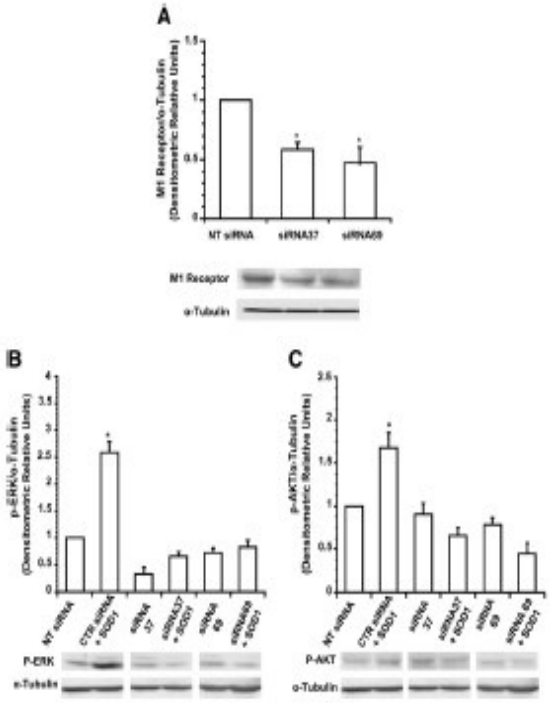


Fig. 4. Silencing of M1 muscarinic receptor gene. Western blotting analysis of M1 muscarinic receptor in control SK-N-BE cells (CTR) and in cells transfected by MicroPorator (MP-100) with two siRNA to M1 muscarinic receptor (# 37 and # 69) (panel A). Effects of SOD1 on p-ERK (panel B) and p-Akt (panel C) in control non targeting (NT) siRNA transfected neuroblastoma cells and in cells transfected with two siRNA to M1 muscarinic receptor (# 37 and # 69) stimulated in the absence or in the presence of 400 ng/ml of SOD1 for 10 min. The histograms show the mean values (±SE), evaluated by densitometric analysis of three independent experiments. Panel A: * $p < 0.05$ versus no targeting scramble; Panel B: * $p < 0.05$ versus all treatments; Panel C: * $p < 0.05$ versus all treatments. Representative Western blotting is shown below the respective panels.

expression of muscarinic (M) receptors, the non selective M receptor agonist carbachol and the selective M1 agonist oxotremorine were both able to induce $[Ca^{2+}]_i$ increase in these cells (data not shown). In addition, in SK-N-BE neuroblastoma cells, the M1 antagonist pirenzepine was able to inhibit $[Ca^{2+}]_i$ increase induced by carbachol in a dose-dependent way with an IC_{50} of about 5 μM (Fig. 5A). In the same model, SOD1 (400 ng/ml) produced a significant increase in $[Ca^{2+}]_i$ that was totally prevented by pirenzepine (15 μM) (Fig. 5B). More specifically, the effect of SOD1 on $[Ca^{2+}]_i$ was also prevented by the two specific siRNA # 37 and siRNA # 69 but not by their non targeting sequence (Fig. 5C).

Discussion

Many previous data show that SOD1 is secreted in the form of microvesicles in both normal and transformed cells, including neuroblastoma SK-N-BE cells, through an ATP dependent mechanism (Mondola et al., 1998, 2003, 2004). Export of SOD1 is linked to a brefeldin A-sensitive pathway suggesting the ER localization of this enzyme (Gomes et al., 2007; Mondola et al., 2003). Moreover, a recent study demonstrates that, besides the basal secretion of SOD1, an inducible release of this protein, mediated by depolarization, is also present in excitable cells like rat pituitary GH3 (Santillo et al., 2007). The SOD1 secretion in motor neurons from NSC-34 mice has been further confirmed by other studies; moreover, an impaired SOD1 secretion in familiar amyotrophic lateral sclerosis-linked SOD1 mutants has been observed (Atkin et al., 2006; Turner et al., 2005). This alteration of mutant SOD1 export could be attributed to reduction of cys-cys disulfide bound that facilitates the monomer aggregation (Furukawa et al., 2004). In addition it has been shown that a chronic intraspinal infusion of wild type SOD1 is able to delay the progression of disease in transgenic $SOD1^{G93A}$ mice, suggesting a therapeutic role of this enzyme (Turner et al., 2005). The secretion of wild type SOD1 in SK-N-BE cells suggests a neuroprotective antioxidant paracrine effect; however, it has been shown that SOD1 is able to activate PLC–PKC pathway in this cellular line (Mondola et al., 2004). Therefore, the release of inducible SOD1, in response to depolarization of excitable cells, was strongly inhibited after intoxication with botulin toxin (TB) and in the presence of calcium chelator EGTA (Santillo et al., 2007). This suggests that SOD1 may act as a putative extracellular messenger.

Previous cytometric analysis demonstrated that SOD1 specifically interacts with SK-N-BE cell membranes (Mondola et al., 2004). Some literature data demonstrate that muscarinic M1 receptor is well expressed in human brain and is able to activate the pathway PLC/PKC (Ellis, 2002). Of all other neurotransmitter systems, the muscarinic–cholinergic system carries out a key role in many important cerebral functions including learning, memory and synaptic plasticity (McCoy and McMahon, 2007; Scheideter et al., 2006, 2008). In order to characterize the specific receptor implicated in this interaction, by means of siRNA approach, we give strong evidence here that, in human neuroblastoma SK-N-BE cells, SOD1 causes an activation of a transductional mechanism that involves PLC and PKC and induces an activation dose- and time-dependent of the downstream signal molecules ERK 1/2 and Akt by modulating muscarinic M1 receptors.

The siRNA experiments cannot exclude that, in addition to a direct effect of SOD1 on M1 receptor, a constitutive activity of M1 receptor pathway could be necessary for ERK and AKT activation by SOD1 through an alternative pathway. Furthermore, the SOD1-dependent

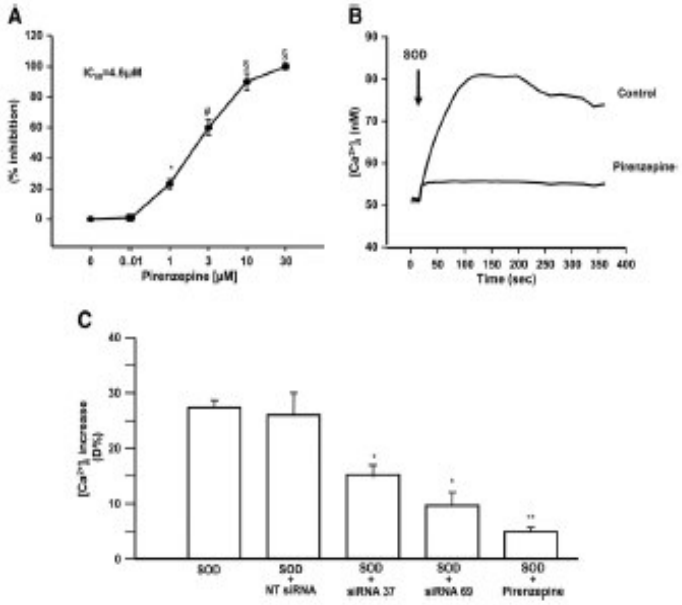


Fig. 5. Effect of siRNA of M1 receptor on SOD1-induced $[Ca^{2+}]_i$ increase in SK-N-BE neuroblastoma cells. Dose–response curve for the effect of pirenzepine on carbachol-induced $[Ca^{2+}]_i$ increase (panel A). To obtain the reported IC_{50} data were fitted using the equation: $a + b * \exp(-x/t)$. Each point represents the mean (± S.E.M.) of the data studied in three independent experimental sessions. *$p<0.05$ versus respective control; #$p<0.05$ versus its respective control and previous concentration; §$p<0.05$ versus all treatments. Superimposed single-cell traces representative of the effect of SOD1 on $[Ca^{2+}]_i$ in the presence or absence of pirenzepine. The arrow indicates the perfusion time (panel B). Bar graph represents the quantification of the effect of SOD1, SOD1 plus non targeting sequence, SOD1 plus siRNA # 37 and SOD1 plus siRNA # 69 on $[Ca^{2+}]_i$. The effects are reported as ΔX of increase over basal values (panel C). Each bar represents the mean ± SEM ($n = 50$ cells in 3 independent experimental sessions). * $p<0.05$ versus control (SOD1).

increase in $[Ca^{2+}]_i$ is prevented by pirenzepine at high concentration. This could be due to the relatively low affinity of the drug for the M1 receptors expressed in SK-N-BE cells as also demonstrated by the relatively high IC_{50} value of pirenzepine in the inhibition of carbachol-induced $[Ca^{2+}]_i$ increase. Interestingly, in the presence of the calcium chelator BAPTA/AM, SOD1-dependent activation of ERK 1/2 and Akt is remarkably reduced; nevertheless, because pirenzepine completely prevent the effect of SOD1 on intracellular Ca2+ while BAPTA/AM plus SOD1 still gives a slight increase of p-ERK 1/2 and p-Akt, we cannot completely exclude that SOD1 can partially activate ERK 1/2 and Akt through a Ca2+ independent pathway. In addition when PKC was inhibited the presence of M1 antagonist pirenzepine alone decreased P-ERK and P-AKT suggesting a possible constitutive M1 activity. Even if our data point out that SOD1 activates P-ERK and P-Akt transductional mechanisms through M1 receptor we cannot exclude that other concomitant pathways could be affected by SOD1 treatment.

Accordingly, our data on SK-N-BE neuroblastoma cells suggest that SOD1 induced the activation both of ERK 1/2 and Akt in a Ca^{2+}-dependent way. In addition, ERK1/2/Akt signaling down stream M1 receptor could be highly amplified; therefore M1 receptor knocking-down completely prevents ERK 1/2 and Akt phosphorylation. This strongly supports the relevance of the role played by M1 receptors in the SOD1 modulation of this transductional cascade, although the participation of other intermediate mediators cannot be excluded.

Collectively, the Ca^{2+}-dependent stimulatory effect of SOD1 on ERK 1/2 and Akt in SK-N-BE neuroblastoma cells, mimicked by the M1 agonist oxotremorine and prevented by the M1 antagonist pirenzepine and by M1 receptor knocking-down, suggests that SOD1 could carry out a neuromodulatory effect activating M1 muscarinic receptor signaling.

Experimental methods

Cell culture

Human neuroblastoma SK-N-BE cells (American Type Culture Collection, ATCC, USA) were grown in monolayer in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 μg/ml streptomycin and 50 IU/ml penicillin. The cells were kept in a 5% CO_2 and 95% air atmosphere at 37 °C.

Material

1,2-Bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA/AM), pirenzepine and oxotremorine were dissolved in distilled water. Bisindolylmaleimide (BIM) was dissolved in dimethyl sulfoxide (DMSO). SOD1 was purchased by Santa Cruz Biotechnology.

Western blotting analysis

SK-N-BE cells, grown to semi-confluence in 60-mm dishes, were washed twice with PBS and incubated for 18 h in medium containing 0.2% FBS before the experiments.

SK-N-BE cell lysates were obtained in RIPA buffer, containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 2.5 mM Na-pyrophosphate, 1 mM β-glycerophosphate, 1 mM $NaVO_4$, 1 mM NaF, 0.5 mM PMSF and a cocktail of protease inhibitors (Roche, USA). The cells were kept for 15 min at 4 °C and disrupted by repeated aspiration through a 21-gauge needle. Cell lysates were centrifuged for 15 min at 13,000 rpm and the pellets were discarded. Fifty micrograms of total proteins was subjected to SDS-PAGE under reducing conditions. After electrophoresis, the proteins were transferred onto a nitrocellulose filter membrane (GE-Healthcare, UK) with a Trans-Blot Cell (Bio-Rad Laboratories, UK) and transfer buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were placed in 5% non-fat milk in tris-buffered saline, 0.1% Tween 20 (TBST) at 4 °C for 2 h to block the nonspecific binding sites. Filters were incubated with specific antibodies before being washed three times in TBST and then incubated with a peroxidase-conjugated secondary antibody (GE-Healthcare, UK). After washing with TBST, peroxidase activity was detected with the ECL system (GE-Healthcare, UK).

SOD1 and M1 muscarinic receptor were detected with rabbit polyclonal antibodies; SOD1 antibodies were from Santa Cruz Biotechnology Inc. (USA), while M1 receptor antibodies were purchased by Sigma (USA); p-Akt and p-ERK 1/2 were purchased from Santa Cruz Biotechnology (USA). 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 antibodies. Each experiment was three times repeated.

RNA interference

Human M1 siRNAs from SK-N-BE cells were obtained from QIAGEN (Qiagen-Xeragon). Transfection of siRNAs was carried out by MicroPorator (MP-100) Digital Bio Technology, a pipette-type electroporation. Cells were dissociated by a brief treatment with trypsin–EDTA, and counted. Indicated plasmids, DNAs and siRNA were introduced into each 1×10^6 dissociated cells in 300 μl volume according to manufacturer's instructions. The experimental conditions were optimized for SK-N-BE cells: voltage 1200, width 20 ms, 3 pulse. Electroporated cells were then seeded into culture dishes containing pre-warmed culture medium without antibiotics. Then, after 5 h, the medium was changed and substituted with culture medium, supplemented with 10% FBS, 2 mM L-glutamine, 50 μg/ml streptomycin and 50 IU/ml penicillin. After 20 h, SK-N-BE cells were washed twice PBS and incubated for 18 h in medium containing 0.2% FBS before the experiments.

The siRNAs sequences utilized were:

siRNA6B : TGGCTCCGAAGTGGTGATCAA,
siRNA37 : CCGGCTTGGCTAGTCACATAT.

We transfected independently two different siRNAs against M1 receptor and obtained similar results. M1 receptor knockdown was tested by immunoblot. As controls, "non targeting" (NT) siRNAs were used. In all experiments, siRNAs were used at a final concentration of 100 nM and co-transfected with 2 μg of Green Fluorescent Protein (GFP) plasmid to evaluate transfection efficiency. Percent of GFP positive cells was evaluated after 48 h of transfection by flow cytometry. Transfection efficiency average was 70 ± 7%.

$[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was measured by single cell computer-assisted video imaging (Secondo et al., 1985). Briefly, SK-N-BE cells, grown on glass coverslips, were loaded with 10 μM Fura-2 acetoxymethyl ester (Fura-2AM) for 30 min at 37 °C in normal Krebs solution containing the following (in mM): 5.5 KCl, 160 NaCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, and 10 Hepes-NaOH, pH 7.4. At the end of the Fura-2AM loading period, the coverslips were placed into a perfusion chamber (Medical System, Co. Greenvale, NY, USA) mounted onto the stage of an inverted Zeiss Axiovert 200 microscope (Carl Zeiss, Germany) equipped with a FLUAR 40 × oil objective lens. The experiments were carried out with a digital imaging system composed of MicroMax 512BFT cooled CCD camera (Princeton Instruments, Trenton, NJ, USA), LAMBDA 10-2 filter wheeler (Sutter Instruments, Novato, CA, USA), and Meta-Morph/MetaFluor Imaging System software (Universal Imaging, West Chester, PA, USA). After loading, cells were alternatively illuminated at wavelengths of 340 nm and 380 nm by a Xenon lamp. The emitted light was passed through a 512 nm barrier filter. Fura-2 fluorescence intensity was measured every 3 s; the length of exposure/acquisition period for

FURA-2 fluorescence intensity was 500 ms. Forty to sixty-five individual cells were selected and monitored simultaneously from each cover slip. All the results are presented as cytosolic Ca^{2+} concentration. Assuming that the K_D for FURA-2 was 224 nM, the equation of Grynkiewicz et al. (1985) was used for calibration. Each experiment was three times repeated.

Statistical analysis

Statistical differences were evaluated using one-way ANOVA followed by a Bonferroni post-hoc test. A value of $p<0.05$ was considered statistically significant.

Funding declaration

This work was in part supported by the Italian Ministry of University and Research (PRIN 2004–2006 grants to PM and FB) that had no role in study design, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the paper for publication.

Acknowledgments

Special thanks to Dr. Agnese Secondo for her valuable help with $[Ca^{2+}]_i$ measurement experiments.

References

Anagnostaras, S.G., Murphy, G.G., Hamilton, S.E., Mitchell, S.L., Rahnama, N.P., Nathanson, N.M., Silva, A.J., 2003. Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. Nat. Neurosci. 6, 51–58.

Atkin, J.D., Berg, M.A., Turner, B.J., Tomas, D., Lysaght, J.A., Nunan, J., Rembach, A., Nagley, P., Beart, P.M., Cheema, S.S., Horne, M.K., 2006. Induction of the unfolded protein response in familial amyotrophic lateral sclerosis and association of protein-disulfide isomerase with superoxide dismutase 1. J. Biol. Chem. 281, 30152–30165.

Berkeley, J.L., Gomeza, J., Wess, J., Hamilton, S.E., Nathanson, N.M., Levey, A.I., 2001. M1 muscarinic acetylcholine receptors activate extracellular signal-regulated kinase in CA1 pyramidal neurons in mouse hippocampal slices. Mol. Cell. Neurosci. 18, 512–524.

Caccamo, A., Oddo, S., Billings, L.M., Green, K.N., Martinez-Coria, H., Fisher, A., 2006. M1 receptors play a central role in modulating AD like pathology in transgenic mice. Neuron 49, 671–682.

Caulfield, M.P., Birdsall, N.J., 1998. Classification of muscarinic acetylcholine receptors. Pharmacol. Rev. 50, 279–290.

Cirmi, V., Ruggieri, G., Buonomo, T., Serà, R., Sciorio, S., Zanzi, C., Santangelo, F., Mondola, P., 2002. Cu,Zn-superoxide dismutase in human thymus: immunocytochemical localization and secretion in thymus-derived epithelial and fibroblast cell lines. Histochem. Cell Biol. 118, 163–169.

Crook, J.M., Tomaskovic-Crook, E., Copolov, D.L., Dean, B., 2000. Decreased muscarinic receptor binding in subjects with schizophrenia: a study of the human hippocampal formation. Biol. Psychiatry 48, 381–388.

Dean, B., Crook, J.M., Opeskin, K., Hill, C., Keks, N., Copolov, D.L., 1996. The density of muscarinic M1 receptors is decreased in the caudate-putamen of subjects with schizophrenia. Mol. Psychiatry 1, 54–58.

Dean, B., McLeod, M., Keriakous, D., McKenzie, J., Scarr, E., 2002. Decreased muscarinic1 receptors in the dorsolateral prefrontal cortex of subjects with schizophrenia. Mol. Psychiatry 7, 1083–1091.

Ellis, J., 2002. Understanding G Protein-coupled Receptors and Their Role in the CNS: Muscarinic Receptors. Oxford University Press, p. 349.

Furukawa, Y., Torres, A.S., O'Halloran, T.V., 2004. Oxygen-induced maturation of SOD1: a key role for disulfide formation by the copper chaperone CCS. EMBO J. 23, 2872–2881.

Gomes, C., Keller, S., Altevogt, P., Costa, J., 2007. Evidence for secretion of Cu,Zn superoxide dismutase via exosomes from a cell model of amyotrophic lateral sclerosis. Neurosci. Lett. 428, 43–46.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Hamilton, S.E., Nardone, S.N., Anagnostaras, S.G., Murphy, G.G., Richmond, K.N., Silva, A.J., Beigi, E.Q., Nathanson, N.M., 2001. Alteration of cardiovascular and neuronal function in M1 knockout mice. Life Sci. 68, 2489–2493.

Liao, D.L., Hong, C.J., Chen, H.M., Chen, Y.E., Lee, S.M., Chang, C.Y., 2003. Association of muscarinic M1 receptor genetic polymorphisms with psychiatric symptoms and cognitive function in schizophrenic patients. Neuropsychobiology 48, 72–76.

McCoy, P.A., McMahon, L.L., 2007. Muscarinic receptor dependent long-term depression in rat visual cortex is PKC independent but requires ERK 1/2 activation and protein synthesis. J. Neurophysiol. 98, 1862–1870.

Mondola, P., Annella, T., Santillo, M., Santangelo, F., 1996. Evidence for secretion of cytosolic Cu,Zn superoxide dismutase by Hep G2 cells and human fibroblasts. J. Biochem. Cell Biol. 28, 677–681.

Mondola, P., Annella, T., Serà, R., Santangelo, F., Iossa, S., Ginelli, A., Santillo, M., 1998. Secretion and increase of intracellular Cu,Zn superoxide dismutase content in human neuroblastoma SK-N-BE cells subjected to oxidative stress. Brain Res. Bull. 45, 517–520.

Mondola, P., Ruggiero, G., Serà, R., Damiano, S., Grimaldi, S., Garbi, C., Monda, M., Greco, D., Santillo, M., 2003. The Cu, Zn superoxide dismutase in neuroblastoma SK-N-BE cells is exported by a microvesicles dependent pathway. Mol. Brain Res. 110, 45–61.

Mondola, P., Santillo, M., Serà, R., Damiano, S., Alvino, C., Ruggiero, G., Formisano, P., Terracciano, G., Secondo, A., Annunziato, L., 2004. Cu, Zn superoxide dismutase increases intracellular calcium levels via a phospholipase C-protein kinase C pathway in SK-N-BE neuroblastoma cells. Biochem. Biophys. Res. Commun. 324, 887–892.

Rainer, M., Marchi, M., Paradiso, P., 1990. Presynaptic muscarinic receptors in the central nervous system. Ann. N. Y. Acad. Sci. 604, 113–129.

Rouse, S.T., Thomas, T.M., Levey, A.I., 1997. Muscarinic acetylcholine receptor subtype M2: diverse functional implications of differential synaptic localization. Life Sci. 60, 1031–1038.

Santillo, M., Secondo, A., Serà, R., Damiano, S., Garbi, C., Taverna, E., Rosa, P., Glovedi, S., Benfenati, F., Mondola, P., 2007. Evidence of calcium- and SNARE-dependent release of Cu,Zn superoxide dismutase from rat pituitary GH3 cells and synaptosomes in response to depolarization. J. Neurochem. 102, 679–685.

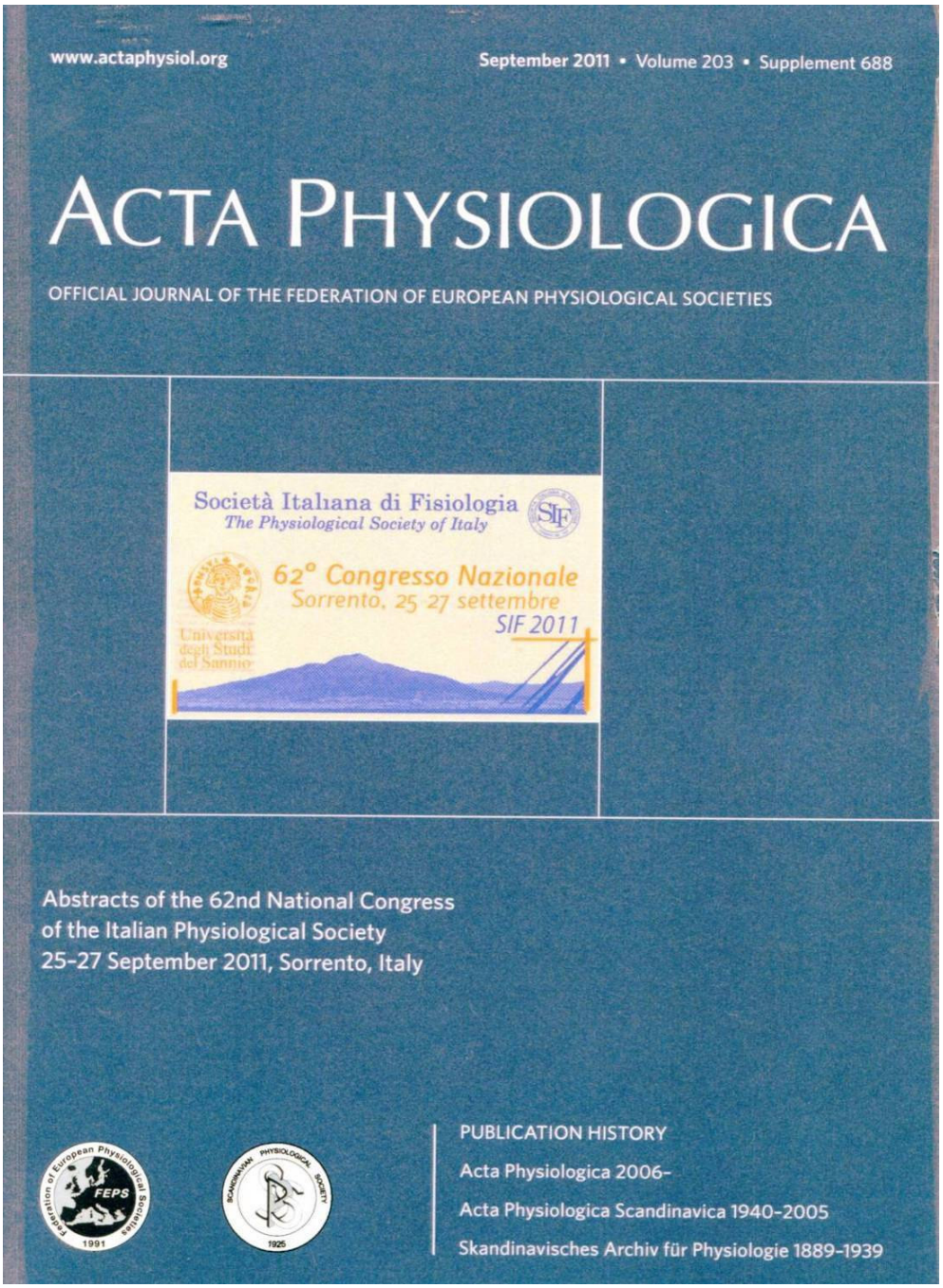
Scheiderer, C.L., McCutchen, E., Thacker, E.E., Kolasa, K., Ward, M.K., Pearson, D., Harrell, L.E., Dobrunz, L.E., McMahon, L.L., 2006. Sympathetic sprouting drives hippocampal cholinergic reinnervation that prevents loss of muscarinic receptor-dependent long-term depression at CA3–CA1 synapses. J. Neurosci. 26, 37–45–3756.

Scheiderer, C.L., Smith, C.C., McCutchen, E., McCoy, P.A., Thacker, E.E., Kolasa, K., Dobrunz, L.E., McMahon, L.L., 2008. Coactivation of M1 muscarinic and α1 adrenergic receptors stimulates extracellular signal-regulated protein kinase and induces long-term depression at CA3–CA1 synapses in rat hippocampus. J. Neurosci. 28, 5350–5358.

Secondo, A., Molinaro, P., Pannaccione, A., Esposito, A., Cantile, M., Lippiello, P., Sirabella, R., Iavarone, T., Di Renzo, G., Annunziato, L., 1985. Nitric oxide stimulates NCX1 and NCX2 but inhibits NCX3 isoform by three distinct molecular determinants. Mol. Pharmacol. 79, 558–568.

Turner, B.J., Atkin, J.D., Farg, M.A., Zang, D.W., Rembach, A., Lopes, E.C., Patch, J.D., Hill, A.F., Cheema, S.S., 2005. Impaired extracellular secretion of mutant superoxide dismutase 1 associates with neurotoxicity in familial amyotrophic lateral sclerosis. J. Neurosci. 25, 108–117.

CONGRESS PUBLICATIONS



P2.1

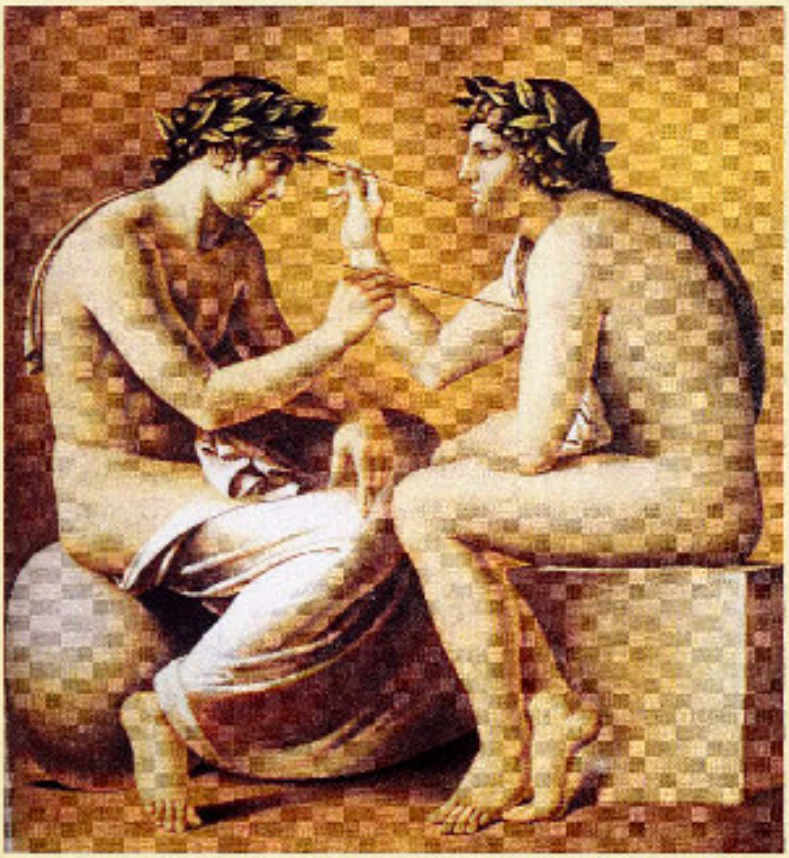
Reactive oxygen species generated by NADPH oxidase induce oligodendrocytes differentiation

ACCETTA R[1], DAMIANO S[1], UCCI V[1], MORANO A[2], PETROZZIELLO T[1], SANTARPIA C[3], MONDOLA P[1], AVVEDIMENTO VE[2], PATERNO' R[3], SANTILLO M[1]

[1]Dipartimento di Neuroscienze,Unità di Fisiologia Umana, Univ. "Federico II", Napoli, Italy
[2]Dipartimento di Biologia e Patologia Cellulare e Molecolare "L.Califano", Univ. "Federico II", Napoli, Italy
[3]Dipartimento di Medicina Clinica e Sperimentale , Univ. "Federico II", Napoli, Italy

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



(Carlo Maria Mariani, La mano ubbidisce all'intelletto)

Accademia
Nazionale dei Lincei

Immunoglobulin fraction purified from serum of multiple sclerosis patients inhibits oligodendrocyte differentiation

C. Santarpia, S. Damiano, R. Accetta, V. Ucci, M. Cicale, G. Cinalli, C. Florio, F. Habetswallner, G. Orefice, G. Vacca, V.E. Avvedimento, M. Santillo, R. Paternò.

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 Prius of the Federico II

100


VOLUME 17 | SUPPLEMENT 10 | OCTOBER 2011
ISSN 1352-4585 | http //msj.sagepub.com
MULTIPLE SCLEROSIS JOURNAL
Formerly Multiple Sclerosis
5TH JOINT TRIENNIAL CONGRESS OF THE EUROPEAN AND AMERICAS COMMITTEES FOR TREATMENT AND RESEARCH IN MULTIPLE SCLEROSIS
19 – 22 OCTOBER 2011
AMSTERDAM,
THE NETHERLANDS
ECTRIMS
actrims
L
R
L
R

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.

T.P., J.L., R.S.,P.H., P.K., A.L., S.W., A.K., H.-P.H., B.S., O.A. have nothing to disclose

P818

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

R. Accetta, S. Damiano, C. Santarpia, V. Ucci, A. Morano, P. Mondola, T. Petrozziello, A. Postiglione, E. Visciano, V.E. Avvedimento, R. Paternò, M. Santillo

University of Naples "Federico II" (Naples, IT)

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 100nM phorbol myristate acetate (PMA/no serum) for 4 days. A mild oxidative stress was induced by 200uM 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 (a-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 (50uM), 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 (100uM), 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 500uM 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 the 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 low ROS in limited inflammation may represent a positive re-myelination stimulus, excess of ROS produced by extensive inflammation may reduce the pool the OL precursors and worsen MS lesions.

This project was funded by the academic spin off Prius 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

C. Capela, P. Pereira, R. Manaças, R. Pedrosa, A. Sena

Hospital dos Capuchos (Lisbon, PT), Hospital dos Lusiadas (Lisbon, PT)

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 (TChol) 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 Osirix 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±4.6 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

M.R. Mizee, D. Wooldrik, K.A.M. Lakeman, J.A.R. Drexhage, B. van het Hof, D. Geerts, M. Bugiani, E. Aronica, R. Mebius, A. Prat, H.E. de Vries, A. Reijerkerk