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DYSREGULATION OF THE GPR17 RECEPTOR IN NEURO-INFLAMMATORY DISEASES: IMPLICATIONS FOR REMYELINATION IN MULTIPLE SCLEROSIS

Settore disciplinare BIO/14

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CONTENTS

ABSTRACT **5**

RIASSUNTO **6**

Chapter 1 ..... **9**

Multiple Sclerosis **9**

1.1 Multiple sclerosis: clinical features **9**

1.1.1 Definition 9

1.1.2 Symptoms and diagnosis 9

1.1.3 Clinical courses 12

1.2 Epidemiology and pathogenesis **14**

1.2.1 Prevalence and incidence 14

1.2.2 Immunopathophysiology 17

1.2.3 Pathological heterogeneity and staging of MS lesions 18

1.3 Therapy **19**

1.3.1 Approved treatments in MS 20

1.4 Future direction **23**

1.4.1 Personalized medicine for MS 23

1.4.2 Agents in trial 24

1.4.3 New strategies 26

1.5 Animal models of MS **29**

1.5.1 Experimental autoimmune encephalomyelitis 30

1.5.2 The cuprizone-induced experimental demyelination 33

Chapter 2 ..... **36**

Oligodendrocytes **36**

2.1 Oligodendrocytes: the myelin-forming cells in the CNS **36**

2.1.1 Embryonic and postnatal development of OLs 38

2.1.2 Oligodendrogliogenesis 41

2.1.3 Regulation of OL differentiation and myelination 42

2.1.4 Purinergic signaling and oligodendrogliogenesis 45

2.1.5 Structure and formation of myelin sheath 47

2.3 Demyelination and re-myelination **52**

2.3.1 Demyelination and re-myelination mechanisms 52

2.3.2 OPCs contribute to glial scar formation 56

2.3.3 OPCs as innate immune cells 57

Chapter 3 ..... **59**

The G protein coupled receptor GPR17 ..... **59**

3.1 Identification and gene cloning **59**

3.2 GPR17, a promiscous receptor: structure and pharmacological properties **59**

3.2.1 GPR17 agonists 60

3.2.2 GPR17 antagonists 62

3.3 GPR17 expression pattern in the CNS **63**

3.4 Transduction signaling pathways activated by GPR17 ..... **65**

3.5 GPR17 function **66**

3.5.1 Role of GPR17 in OL differentiation 67

3.5.2 GPR17 alterations in demyelinating diseases ... 70
3.6 GPR17 as a pharmacological target for MS ... 71
3.6.1 in silico identification of new GPR17 ligands ... 71
3.7 GPR17 fluorescent reporter mouse line: GPR17-iCreER^{T2} ... 73
Chapter 4 ... 75
Aim of the study ... 75
Chapter 5 ... 80
Materials and methods ... 80
5.1 EAE experiment ... 80
5.1.1 EAE induction ... 80
5.1.2 EAE in transgenic mice ... 80
5.1.3 Histology and immunofluorescence ... 80
5.1.4 In situ hybridization ... 81
5.1.5 Cell counts ... 82
5.2 In vitro experiments ... 83
5.2.1 Primary cultures, OPCs isolation and treatment ... 83
5.2.2 Astrocyte culture and generation of astrocyte-conditioned medium ... 84
5.2.2 Immunocytochemistry and cell counting ... 84
5.3 Total RNA extraction, retrotranscription and gene expression analysis ... 84
5.4 Analysis on human MS tissue ... 85
5.4.1 Tissue Source ... 85
5.4.2 Lesion Detection and Classification ... 85
5.4.3 Immunohistochemistry ... 85
5.5 Statistical analysis ... 86
Chapter 6 ... 88
Results ... 88
6.1 In mouse spinal cord, GPR17 is expressed in cells of the oligodendroglial lineage ... 88
6.2 Characterization of GPR17 expressing cells in EAE ... 88
6.3 Identification and fate of GPR17-expressing cells in inducible GFP-reporter mice ... 90
6.4 In the cuprizone-induced demyelination model GPR17 kinetics correlate with MBP expression pattern ... 93
6.5 *In vitro* exposure of OPCs to medium conditioned by activated astrocytes results in strong GPR17 stimulation and OPC maturation ... 94
6.6 *In vitro* exposure of OPCs to cytokines ... 95
6.7 GPR17 is a promiscuous receptor that can be also activated by CXCL12 ... 96
6.8 GPR17 is pathologically up-regulated in demyelinating lesions of human MS subjects ... 98
Chapter 7 ... 102
Discussion ... 102
Bibliography ... 108

ABSTRACT

Multiple Sclerosis (MS) is a chronic immune-mediated disease in which the immune system directs an abnormal response against myelin, an insulating lipidic structure produced by oligodendrocytes responsible of fast axonal electric transmission. During MS, demyelination disrupts neuronal conductance, leading to motor symptoms, and impairs oligodendroglial functions. Under these conditions, oligodendrocyte precursor cells (OPCs) are recruited at the injury site to re-myelinate damaged axons, but this process is often defective.

Many disease-modifying treatments (DMTs) are available but there are several unmet needs: delaying disease progression, providing neuroprotection and promoting re-myelination.

The aim of this thesis was to characterize GPR17 alterations both in murine MS models and in human MS lesions, to assess whether this receptor, a key actor of oligodendrogenesis, can be proposed as a pharmacological target in re-myelinating strategies.

GPR17 is a G protein-coupled receptor activated by both uracil nucleotides and cysteinyl-leukotrienes, mediators involved in inflammatory responses in the CNS. Under physiological conditions, GPR17 is expressed in OPCs, with maximal levels in immature oligodendrocytes and progressive downregulated in terminally differentiating cells. A marked GPR17 up-regulation was found in rodent models of cerebral trauma, ischemia and in lysolecithin-induced focal demyelination; suggesting that GPR17 takes part in the pathological mechanisms of demyelination either as a consequence of the disease or contributing to the lesion. In mice with Experimental Autoimmune Encephalomyelitis (EAE), we observed a marked and persistent upregulation of GPR17 in the OPCs accumulating at demyelinating lesions. Conversely, no GPR17 upregulation was found in a model characterized by a much lower degree of inflammation, i.e. cuprizone-induced demyelination. In a similar way to EAE, in autoptic samples from MS patients, many GPR17-positive activated cells accumulated at the border of active lesions. Furthermore, we demonstrated that the chemokine CXCL12 can also directly act as a promiscuous activator of GPR17, corroborating our hypothesis of a common pathophysiological role for GPR17 and chemokine receptors in leading the re-myelination processes. Characterizing the molecular defects of GPR17 in MS will help re-establishing its correct function in re-myelination and foster the identification of new pharmacological strategies to enhance OPCs reparative potential in MS.

RIASSUNTO

La sclerosi multipla (SM) è una patologia cronica mediata dal sistema immunitario il quale dirige una risposta anticorpale aberrante contro la mielina, la struttura lipidica prodotta dagli oligodendrociti (OL) che isola gli assoni favorendo la rapida trasmissione degli impulsi nervosi. Nella SM, in seguito alla demielinizzazione si verifica un'interruzione della comunicazione neuronale che porta a disabilità motorie e compromette le funzioni degli oligodendrociti. In queste condizioni, i precursori degli OL (OPC) vengono reclutati al sito di danno per rimielinizzare gli assoni danneggiati ma questo tentativo di riparo spesso non viene completato con successo.

Attualmente sono disponibili diverse terapie a base di farmaci in grado di modificare la malattia (DMT) ma rimangono dei bisogni clinici insoddisfatti: ritardare la progressione della malattia, fornire neuroprotezione e promuovere la rimielinizzazione.

Lo scopo di questa tesi è stato caratterizzare le alterazioni del recettore GPR17 sia in un modello murino di SM, sia nelle lesioni umane, per verificare se questo recettore, che ha un ruolo chiave nel differenziamento degli OL, possa essere proposto come bersaglio farmacologico per strategie terapeutiche rimielinizzanti.

GPR17 è un recettore accoppiato a proteina G che può essere attivato da diversi mediatori delle risposte infiammatorie nel sistema nervoso centrale quali ad esempio i leucotrieni cisteinici e i nucleotidi. In condizioni fisiologiche, GPR17 è espresso dagli OPC, raggiungendo livelli massimi di espressione allo stadio di OL immaturi e progressivamente spento durante la maturazione terminale. In modelli murini di trauma cerebrale, ischemia e demielinizzazione focale indotta con lisolecitina è stato riscontrato un marcato aumento dei livelli di GPR17 suggerendo che questo recettore è implicato nei meccanismi patologici di demielinizzazione e rimielinizzazione. In topi con encefalite sperimentale autoimmune (EAE), abbiamo osservato un marcato e persistente aumento di OPC esprimenti GPR17 nei siti di lesioni demielinizzanti. Al contrario, nessun aumento di GPR17 è stato riscontrato in un modello caratterizzato da una diversa tipologia di risposta infiammatoria quale il modello di demielinizzazione indotta da cuprizone. In un modo simile a quanto descritto nel modello di EAE, in campioni autoptici di pazienti umani con SM, abbiamo osservato la presenza di cellule GPR17-positive accumulate ai bordi delle lesioni attive. Inoltre, abbiamo dimostrato che anche la chemochina CXCL12 può attivare GPR17, confermando la nostra

ipotesi di un ruolo fisiopatologico comune per GPR17 e i recettori per le chemochine nel guidare i processi di rimielinizzazione. La caratterizzazione delle alterazioni di GPR17 in SM è quindi importante per ripristinarne le funzioni e promuovere la rimielinizzazione attraverso l'identificazione di nuove strategie farmacologiche volte a incrementare il potenziale riparativo endogeno degli OPC in corso di SM.

INTRODUCTION

Chapter 1

Multiple Sclerosis

1.1 Multiple sclerosis: clinical features

1.1.1 Definition

Multiple sclerosis (MS) is a human inflammatory demyelinating disease of the central nervous system (CNS) and the most common non-traumatic cause of neurological disability in young people in the western world (Coclitu et al., 2016).

MS is characterized by the development of multiple and diffuse foci (sclerosis) of demyelination in the brain and spinal cord as a result of an inflammatory process ongoing. Indeed, MS is a putatively autoimmune disease where inflammation is the cause for demyelination, axonal damage and neuronal loss but the exact antigen remains unknown, so for this reason MS is considered to be "immune-mediated" rather than "autoimmune". As in other chronic inflammatory diseases, the CNS of patients with MS shows infiltration of activated T cells and macrophages, dendritic cells, B cells and plasmacells. This implies potential roles for both cellular and humoral immune responses and the engagement of different immunopathological effector mechanisms in CNS tissue damage. (Coclitu et al., 2016; Peru et al., 2008; Keegan and Noseworthy, 2001).

1.1.2 Symptoms and diagnosis

MS symptoms are variable and unpredictable. Typical clinical signs include temporary loss of vision, sensory and motor problems but also fatigue, neurocognitive changes, and impairment of bladder-, bowel- and sexual functions (Keegan and Noseworthy, 2001).

Initially, most patients show subacutely “attacks” or “relapses” of neurological dysfunction over hours to days, then plateau and improve (sometimes incompletely) over days to weeks, either spontaneously or with corticosteroid treatment. The neurological symptoms reflect the location of the lesion within the CNS; for example, visual loss reflects a lesion of the optic nerve; hemi-, para-, or quadriparesis, with or without bowel/bladder dysfunction, reflects a lesion of the spinal cord; vertigo or diplopia, a lesion of the brain stem; and ataxia, a lesion of the cerebellum. However, many lesions are clinically silent. Other symptoms, such as debilitating fatigue, paresthesias on neck flexion (Lhermitte’s

symptom), and heat-exacerbated symptomatic worsening (e.g., Uhthoff's symptom), may be present. Occasionally, severe attacks occur with multifocal neurological involvement. Although MS is common, other causes for this symptom complex must be considered (Keegan and Noseworthy, 2001). Other conditions can damage myelin in the CNS, including viral infections, side effects from high exposure to certain toxic materials, severe vitamin B12 deficiency, autoimmune conditions that lead to inflammation of blood vessels (the "collagen-vascular diseases"), and some rare hereditary disorders (e.g., metachromatic leukodystrophy, - Poeppel et al. 2005). Demyelination of the peripheral nervous system (PNS) occurs also in Guillain-Barré Syndrome (Jasti et al., 2016).

Although it is facilitated by supportive laboratory and radiological investigations, MS continues to be diagnosed on a clinical basis. Careful and repetitive examinations may be needed to establish an exact diagnosis among the possible causes of neurologic symptoms. At this time, there are no symptoms, physical findings or laboratory tests that can univocally determine if a person has MS. Several strategies are used to determine if a person meets the long-established criteria for a diagnosis of MS, and to exclude other possible causes of symptoms that person is experiencing. These strategies include a careful medical history, a neurologic exam and various tests including magnetic resonance imaging (MRI), evoked potentials (EP) and spinal fluid analysis (McDonald WI, et al., 2001; Hessen C. et al., 2009).

The McDonald criteria for diagnosing MS were published in 2001 by a team led by Prof Ian McDonald, and were revised in 2005 and 2010. McDonald's criteria are the standard clinical case definition for MS and the 2010 version is regarded as the gold standard test for MS diagnosis.

The criteria specify that, in order to make a diagnosis of MS, the physician must:

- *Find evidence of damage in at least two separate areas of the CNS, which includes the brain, spinal cord and optic nerves AND*
- *Find evidence that the damage occurred at least one month apart AND*
- *exclude all other possible diagnoses*

The Revised McDonald Criteria, published by the International Panel on the Diagnosis of Multiple Sclerosis (Polman et.al, 2011), include specific guidelines for using MRI, visual evoked potentials (VEP) and cerebrospinal fluid analysis to speed the diagnostic process (Fig.1.1). These tests can be used to look for a second area of damage in a person who has

experienced only one attack (also called a relapse or an exacerbation) of MS-like symptoms — referred to as *clinically-isolated syndrome* (CIS).

Clinical Presentation	Additional Data Needed for MS Diagnosis
≥2 attacks[a]; objective clinical evidence of ≥2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack[b]	None[c]
≥2 attacks[a]; objective clinical evidence of 1 lesion	Dissemination in space, demonstrated by: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)[d]; or Await a further clinical attack[a] implicating a different CNS site
1 attack[a]; objective clinical evidence of ≥2 lesions	Dissemination in time, demonstrated by: Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack[a]
1 attack[a]; objective clinical evidence of 1 lesion (clinically isolated syndrome)	Dissemination in space and time, demonstrated by: For DIS: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)[d]; or Await a second clinical attack[a] implicating a different CNS site; and For DIT: Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack[a]
Insidious neurological progression suggestive of MS (PPMS)	1 year of disease progression (retrospectively or prospectively determined) plus 2 of 3 of the following criteria[d]: 1. Evidence for DIS in the brain based on ≥1 T2 lesions in the MS-characteristic (periventricular, juxtacortical, or infratentorial) regions 2. Evidence for DIS in the spinal cord based on ≥2 T2 lesions in the cord 3. Positive CSF (isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index)

If the Criteria are fulfilled and there is no better explanation for the clinical presentation, the diagnosis is "MS"; if suspicious, but the Criteria are not completely met, the diagnosis is "possible MS"; if another diagnosis arises during the evaluation that better explains the clinical presentation, then the diagnosis is "not MS."

[a]An attack (relapse; exacerbation) is defined as patient-reported or objectively observed events typical of an acute inflammatory demyelinating event in the CNS, current or historical, with duration of at least 24 hours, in the absence of fever or infection. It should be documented by contemporaneous neurological examination, but some historical events with symptoms and evolution characteristic for MS, but for which no objective neurological findings are documented, can provide reasonable evidence of a prior demyelinating event. Reports of paroxysmal symptoms (historical or current) should, however, consist of multiple episodes occurring over not less than 24 hours. Before a definite diagnosis of MS can be made, at least 1 attack must be corroborated by findings on neurological examination, visual evoked potential response in patients reporting prior visual disturbance, or MRI consistent with demyelination in the area of the CNS implicated in the historical report of neurological symptoms.

[b]Clinical diagnosis based on objective clinical findings for 2 attacks is most secure. Reasonable historical evidence for 1 past attack, in the absence of documented objective neurological findings, can include historical events with symptoms and evolution characteristics for a prior inflammatory demyelinating event; at least 1 attack, however, must be supported by objective findings.

[c]No additional tests are required. However, it is desirable that any diagnosis of MS be made with access to imaging based on these Criteria. If imaging or other tests (for instance, CSF) are undertaken and are negative, extreme caution needs to be taken before making a diagnosis of MS, and alternative diagnoses must be considered. There must be no better explanation for the clinical presentation, and objective evidence must be present to support a diagnosis of MS.

[d]Gadolinium-enhancing lesions are not required; symptomatic lesions are excluded from consideration in subjects with brainstem or spinal cord syndromes.

MS = multiple sclerosis; CNS = central nervous system; MRI = magnetic resonance imaging; DIS = dissemination in space; DIT = dissemination in time; PPMS = primary progressive multiple sclerosis; CSF = cerebrospinal fluid; IgG = immunoglobulin G.

Figure 1.1 - *The 2010 McDonald Criteria for Diagnosis of MS (modified from Polman et al., 2011)*

Furthermore, the use of brain MRI for evaluation of neurological disorders has increased in the past two decades, leading to an increased detection of incidental findings on brain MRI. The most common of these asymptomatic abnormalities are white matter lesions that are interpreted as demyelinating based on radiological criteria. However, in the absence of associated clinical symptoms suggestive of MS, a definite diagnosis cannot be made in patients with these incidental white matter lesions that are now diagnosed as *radiologically isolated syndrome* (RIS).

1.1.3 Clinical courses

Current diagnostic categories provide a clinical description of the disease course of MS. People with MS typically experience one of four disease courses (see table 1.1), which can be mild, moderate or severe and that will be described in this paragraph. But there are some exceptions represented by CIS, that may evolve in MS, and by the RIS, due to the easy and wide availability of brain MRI in the past two decades that has led to its increasing use in evaluation of a variety of neurological symptoms.

In most patients (85%), MS initially has a relapsing-remitting course (RRMS) which is characterized by discrete clinical "attacks" or "relapses" followed by periods of remission. RRMS is the most typical presentation in younger patients.

Many years after onset, in the majority of RRMS patients the relapsing course will be followed by a secondary progressive phase (SPMS). These patients will develop a slow, insidiously progressive, neurological deterioration over many years with or without clinical attacks superimposed.

A minority of patients (~15%) have primary progressive MS (PPMS) characterized by a progressive course from onset, an absence of clinically evident relapses, and less conspicuous inflammation on MRI. Progressive relapsing MS (PRMS) involves a progressive course from onset with occasional relapses later in the disease. This category may be of less prognostic significance, however, as occasional relapses occurring in the context of a predominantly progressive course do not appear to significantly alter long-term outcome (Compston et al., 2008).

Clinically isolated syndrome

CIS is now considered one of the MS disease courses. CIS refers to a first episode of neurologic symptoms that lasts at least 24 hours and is caused by inflammation or

demyelination in the CNS. Based upon clinical symptoms alone, CIS and MS may appear the same: like MS, CIS is two to three times more common in women than men; 70% of people diagnosed with CIS are between the ages of 20 and 40 years; a person with CIS has experienced more than one episode and may or may not evolve to MS. According to the 2010 revisions to the diagnostic criteria for MS, when CIS is accompanied by specific findings on MRI that demonstrate that another episode has occurred in the past, the diagnosis of MS can be made.

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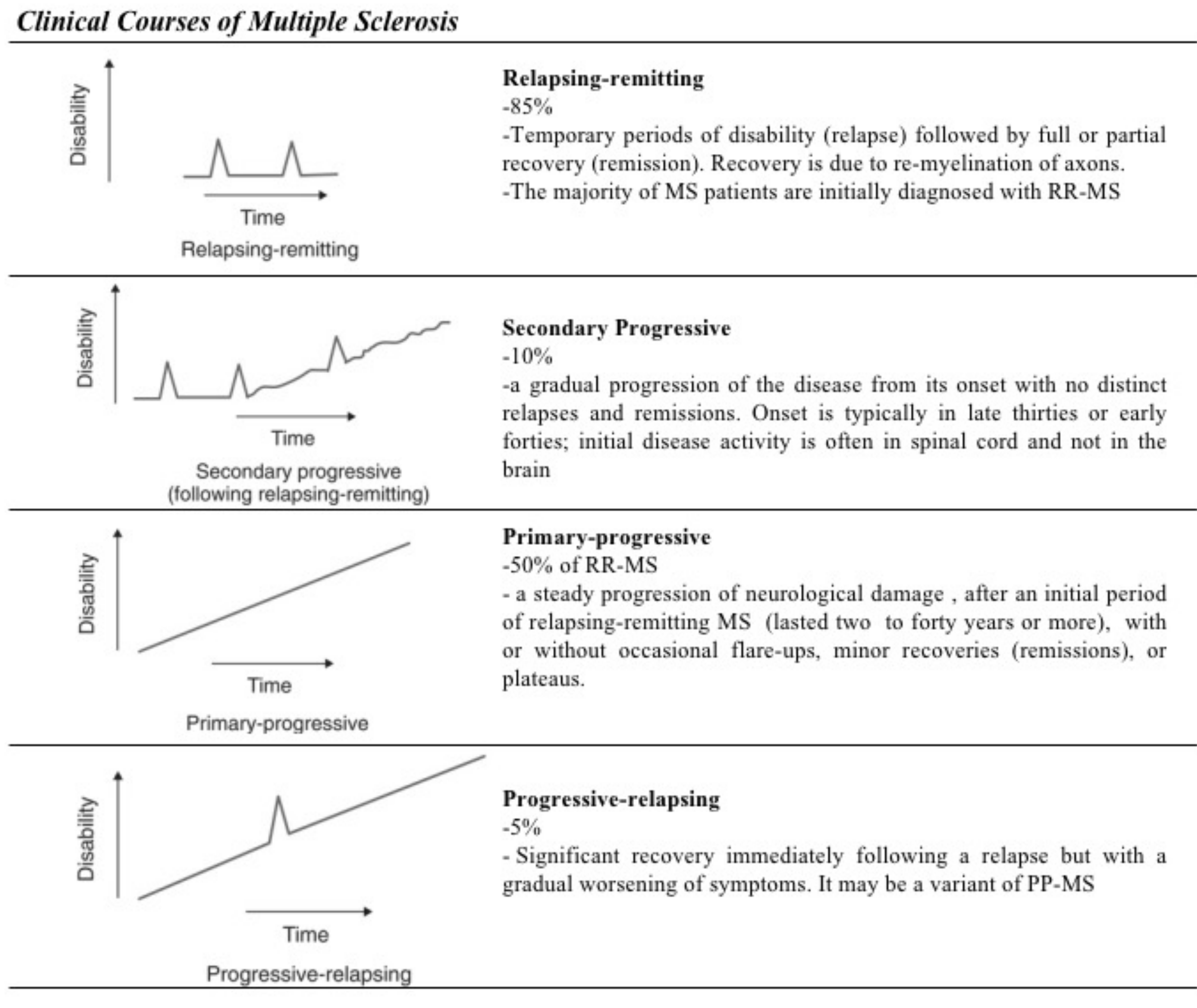

Clinical Courses of Multiple Sclerosis

Course	Description
Disability / Time / Relapsing-remitting	**Relapsing-remitting** -85% -Temporary periods of disability (relapse) followed by full or partial recovery (remission). Recovery is due to re-myelination of axons. -The majority of MS patients are initially diagnosed with RR-MS
Disability / Time / Secondary progressive (following relapsing-remitting)	**Secondary Progressive** -10% -a gradual progression of the disease from its onset with no distinct relapses and remissions. Onset is typically in late thirties or early forties; initial disease activity is often in spinal cord and not in the brain
Disability / Time / Primary-progressive	**Primary-progressive** -50% of RR-MS - a steady progression of neurological damage , after an initial period of relapsing-remitting MS (lasted two to forty years or more), with or without occasional flare-ups, minor recoveries (remissions), or plateaus.
Disability / Time / Progressive-relapsing	**Progressive-relapsing** -5% - Significant recovery immediately following a relapse but with a gradual worsening of symptoms. It may be a variant of PP-MS

Table 1.1 *- Main clinical courses of multiple sclerosis*

Radiologically isolated syndrome

Given to the widespread use of MRI, diagnosis of RIS often occurs during diagnosis of another unrelated condition (e.g. head trauma or migraine headache). The most common of these incidental abnormalities are white matter lesions that based on their appearance, location, and distribution are consistent with demyelination but are not associated with any clinical symptoms suggestive of MS. For this reason, the term radiologically isolated syndrome (RIS) has been proposed to describe this entity for the first time by Okuda in 2009 (Okuda et al., 2009).

The clinical significance and prognostic implication of subclinical lesions in patients with RIS remains controversial. There is some evidence suggesting that one-third of patients with RIS have an increased risk of developing MS in five years, implying that RIS, in some cases, constitutes a preclinical stage or subclinical form of MS. These results suggest that the McDonald criteria lack some degree of sensitivity in detecting MS in its earliest phases. Another issue relates to the treatment recommendations for patients with RIS (Leahy and Garg, 2013), but it will be discussed later.

1.2 Epidemiology and pathogenesis

The precise etiology of MS remains unknown. It likely results from complex interactions between environmental and genetic factors, which lead to an aberrant immune response and damage to the myelin sheath, oligodendrocytes, axons, and neurons. A number of factors appear to influence the risk of MS.

1.2.1 Prevalence and incidence

MS affects approximately 400,000 people in the United States (Evans et al., 2013) and 2.5 million worldwide. Europe is considered a high prevalence region for MS, containing more than half of the global population of people diagnosed with MS reported by the World Health Organization in 2008. MS typically begins between the ages of 20 and 40 years and it is the leading cause of non-traumatic disability in young adults. Initial symptoms rarely occur before age 10 years or after age 60 years. It is also clear that populations vary in their susceptibility to MS with a gender difference: women are affected approximately twice as often as men, except in individuals with the primary-progressive form of the disease, where there is no gender preponderance.

Thus, factors which influence MS incidence include population genetics, the interplay between genes and a geographically determined physical environment, and socioeconomic structure, including availability of medical facilities (Howard et al., 2016).
In the last years, many studies shed light on these components of MS and they are presented in the next paragraphs.

Geography and environmental risk factors

The prevalence rates of MS have been reported to vary by continent and geographical latitude. The condition is of high prevalence (>30 per 100,000) in northern parts of Europe and North America; medium prevalence (5-30 per 100,000) in southern Europe and southern United States; and Central and South America (10-20 per 100,000); and low prevalence (<5 per 100,000) in Asia and South America.

Koch-Henriksen and Sørensen conducted an extensive literature search and meta-regression analysis to evaluate the changes in MS incidence and prevalence worldwide. Their analysis indicated that the prevalence and incidence of MS are increasing over time. The increase in prevalence was presumed to be due to prolonged survival of patients with MS, while the increase in incidence was thought to be due to a number of factors. In particular, the ratio of disease in women to men has increased over time from less than 1.5 to greater than 2. This increase in MS among females appears to be driving the increase in incidence, and may be due to changes over time in occupation, cigarette smoking, obesity, birth control, and later childbirth. Furthermore, the previously proposed latitude-related differences in prevalence were dispelled in the northern hemisphere but supported in the southern hemisphere. MS is fairly common in Caucasians of northern European ancestry, but less common where non-Caucasians live, in low-income countries, and in tropical zones (Koch-Henriksen and Sørensen, 2010).

Other environmental factors which may relate to MS are sunlight and ultraviolet radiation exposure, vitamin D, Epstein-Barr virus (EBV) and other viruses, and other infective agents. The hypothesis that higher exposure to sunlight, and consequently ultraviolet radiation, is associated with a lower incidence of MS tends to conveniently fit with the latitude-based observations; however, exceptions exist. Israeli-born individuals of African descent have higher MS rates than their immigrant predecessors, although it is unlikely that their exposure to sunlight was different. For the same reasons, the relationship between vitamin D and MS is unclear. Although epidemiologic studies correlated increased vitamin D intake

with decreased MS incidence, exceptions among Israeli-born individuals do not support the association (Milo and Kahana 2010).

Genetics

Familial aggregation in MS has not been compelling. Evidence for a genetic predisposition includes a 20- to 40-fold increased risk of MS in first-degree relatives of patients with MS and a 25% to 30% concordance in monozygotic twins, compared with only 5% in dizygotic twins. However, monozygotic twins of afflicted individuals had a 30% risk of the disease, with a similar rate in dizygotic twins to other siblings (Ebers et al., 1986), demonstrating that environmental factors and other unknown influences most likely contribute to disease susceptibility.

Bashinskaya and colleagues provided an excellent review of genome-wide association studies (GWAS) in MS. The strongest known genetic factor affecting MS susceptibility is the HLA-DRB1*1501 haplotype. Because it is well known that the HLA locus is an essential component directing the immune response and immune developments, it is not surprising that the major histocompatibility complex (MHC) region still represents about one-half of the MS genetic risk.

However, it is not essential for the development of MS, as it only increases the risk by 2- to 4-fold and is present in approximately 20% to 30% of healthy individuals. Non-HLA genes associated with MS are associated with T-cell function and may indicate the leading role of T-cell immunity in MS development (Bashinskaya et al., 2015).

Exposure to infectious agents

Viral and other infectious exposures may predispose a host to an autoimmune attack.

Exposure to EBV at an early age in children has been linked to reduced incidence of MS, while exposure in the form of infectious mononucleosis later in life (late adolescence) is linked to an increased risk. EBV prevalence also appears to correlate with the observed differences in MS based on latitude and socioeconomic structure (Tzartos et al., 2012). It has also been postulated that the lower rate of herpes simplex virus (HSV) in patients with MS may suggest a protective effect of HSV, or an immunomodulatory effect on the outcome of EBV. Exposure to certain bacteria (i.e., Acinetobacter species, Chlamydia pneumonia, Pseudomonas aeruginosa), mycobacteria, or helminthes has also been linked to MS, although the data are not strongly associated (Tullman, 2013).

1.2.2 Immunopathophysiology

The primary trigger of immune response in MS is unknown but it is believed that early in the inflammatory cascade, a response is triggered against myelin antigens, such as myelin basic protein (MBP), proteolipid protein (PLP), myelin/oligodendrocyte glycoprotein, MAG, and gangliosides.

Studies in experimental allergic encephalomyelitis (EAE), histopathological studies of MS lesions, and immunologic markers in serum and cerebrospinal fluid of MS patients suggest that MS is an immune-mediated disease. A virus, bacterium, or other environmental toxin might induce an immune response in genetically susceptible persons (Frohman et al., 2006; Prineas and Parratt, 2012). Antigen-presenting cells (APCs) provide relevant antigens to $CD4^+$ T helper cells in the periphery, which lead to their activation and the subsequent generation of autoreactive pro-inflammatory T helper (Th) 1 and 17 subsets (Frohman et al., 2006). B-cells and monocytes are also activated. These autoreactive T-cells interact with adhesion molecules on the endothelial surface of CNS vessels and, with antibodies and monocytes, cross the disrupted blood-brain barrier with the aid of proteases (e.g., matrix metalloproteinases) and chemokines. Within the CNS, target antigens are recognized (putative antigens include myelin basic protein, myelin-associated glycoprotein, myelin-oligodendrocyte glycoprotein, proteolipid protein, alpha B-crystallin, phosphodiesterases, and S-100 protein), T-cells are reactivated, and the immune response is amplified. Pro-inflammatory Th cells proliferate and B cells continue their maturation to antibody-secreting plasma cells, while monocytes become activated macrophages (Wuest et al., 2011). Together, these immune cells produce inflammatory cytokines (e.g., IL-12, IL-23, interferon γ, tumor necrosis factor α), proteases, free radicals, antibodies, nitric oxide, glutamate, and other stressors that collectively lead to damage of myelin and oligodendrocytes. In the appropriate cytokine milieu, CD4+ Th2 cells proliferate and secrete anti-inflammatory cytokines (e.g. IL-4, Il-5, IL-13) and transforming growth factor β (TGFβ) that suppress the immune response. Depending on the location and extent of damage, demyelination may impair or block nerve conduction and result in neurologic symptoms (Bielekova et al., 2006; Kaur et al., 2012; Bielekova et al., 2000). With a loss of trophic support from oligodendrocytes, axons may degenerate to cause irreversible neurological deficits. Spontaneous improvement of symptoms is attributed to resolution of

inflammation, adaptive mechanisms (e.g. reorganization of sodium channels), or remyelination.

It had long been thought that Th1 and Th2 subsets arose from the terminal differentiation of the $CD4^{+}$ T cells. However, a third pathway has been identified, induced by IL-1, IL-6, and TGFβ, and then expanded and maintained by IL-23, which is secreted by APCs. This third subset, a pro-inflammatory T helper cell, is known as Th17 (because it produces IL-17). Th17 cells secrete a number of cytokines, including TNFα and GM-CSF, which are critical for the development of EAE. Patients with MS have monocyte-derived dendritic cells that secrete higher levels of IL-23 than healthy people. Higher levels of IL-17 mRNA-bearing mononuclear cells are found in the serum of patients with MS experiencing relapses than in those with MS in remission (Frohman et al., 2006; Wegner et al., 2010; Matusevicius et al., 1999).

Although MS is typically considered a T cell–mediated disease, a growing number of evidence supports a pathogenic role of B cells, including the frequent observation of intrathecal production of immunoglobulin in patients with MS, identification of antibodies that react to specific myelin antigens within MS lesions, a pathological pattern of MS characterized by antibody-associated demyelination, and the discovery of B cell follicles in the meninges of patients with secondary-progressive MS (pathological patterns are described in details in paragraph 1.2.3). Furthermore, B cells are efficient antigen-presenting cells, and B cell depletion is considered by many scientists a promising therapeutic approach in MS (Tullman, 2013).

1.2.3 Pathological heterogeneity and staging of MS lesions

Although plaques may occur throughout the CNS, they are most common in the optic nerves, cerebral periventricular white matter, brainstem, and spinal cord white-matter tracts. MS lesions are classified histologically as pre-active, acute, chronically active, and inactive (Van Der Valk and De Groot, 2000).

Preactive lesions, characterized by discrete abnormalities of WM, such as clusters of microglial cells, strongly HLA-DR-and CD45-positive, and few perivascular inflammatory cells, but no demyelination.

Acute MS lesions have indistinct margins, hypercellularity, intense perivascular infiltration by lymphocytes, parenchymal edema, loss of myelin and oligodendrocytes, widespread

axonal damage, plasma cells, myelin-laden macrophages, hypertrophic astrocytes, and little astroglial scarring.

Chronic MS lesions have sharp edges, with a perivascular cuff of infiltrating cells, lipid-laden and myelin-laden macrophages, hypertrophic astrocytes, some degenerating axons, dissolution of myelin into droplets which are phagocytized by macrophages, and demyelination associated with immunoglobulin deposition. In particular, *chronic active lesion* are defined with a hypocellular centre and a hypercellular rim; *chronic inactive*, defined as a hypocellular lesion.

Chronic lesions may also exhibit an increase in oligodendrocytes and remyelination.

Lucchinetti and colleagues identified 4 distinct pathological patterns in an immunohistopathological study of actively demyelinating MS lesions from 83 cases, which included 51 biopsies and 32 autopsies. All 4 patterns (see fig.1.2) contained an inflammatory infiltrate consisting of T lymphocytes and macrophages. The most common type, pattern II, was characterized by the deposition of immunoglobulin and complement. Pattern I was characterized by macrophage-associated demyelination. In patterns III and IV, demyelination was due to an oligodendrogliopathy. Pattern III was differentiated from pattern IV by a preferential loss of myelin-associated glycoprotein. The same lesion pattern was observed within each patient, but there was marked heterogeneity between patients, suggesting that MS might have multiple pathogenic mechanisms (Denic et al., 2011).

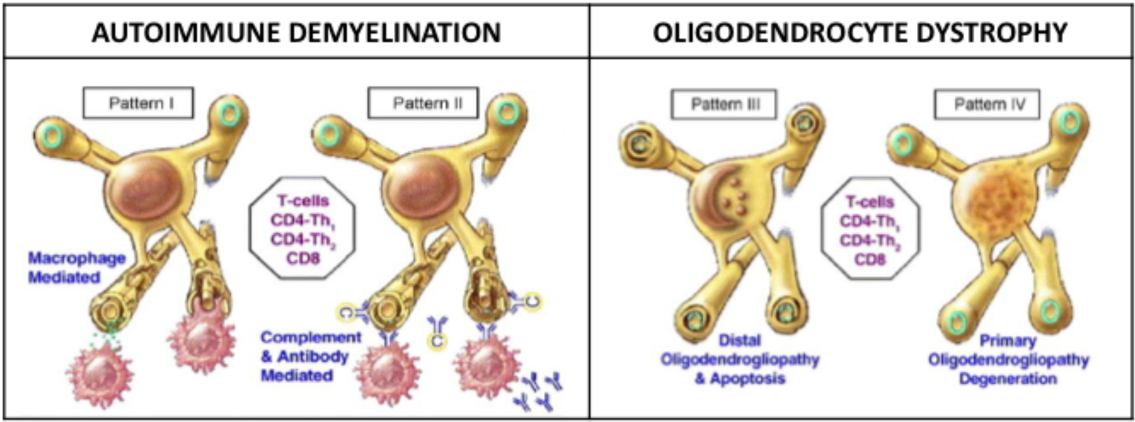

Figure 1.2 – *Pathological patterns of MS lesions (modified from Denic et al., 2011)*

1.3 Therapy

In MS, medications are used to modify the disease course (disease modifying therapies – DMTs), treat relapses and manage symptoms. Along with the other essential components of

comprehensive MS care, these medications help people manage their MS and enhance their comfort and quality of life.

1.3.1 Approved treatments in MS

Currently available DMTs, all of which have immunomodulatory or immunosuppressive properties, improve the course of RRMS (see table 1.2). However, current DMTs fail to benefit the later, in which neurodegenerative mechanisms assume overriding clinical importance, and a high proportion of patients, evolving to a progressive phase, are not responsive to any existing therapy (Wingerchuk and Weinshenker, 2016).

Such DMTs include injectable (interferon-beta and glatiramer acetate) and oral medications (fingolimod, teriflunomide, and dimethyl fumarate), monoclonal antibodies (natalizumab and alemtuzumab), and 1 chemotherapeutic agent (mitoxantrone). They are all indicated for patients with RRMS still in relapse, but, other than mitoxantrone, they do not have a role in the progressive phase of the illness. In patients with newly diagnosed MS and low disease activity, most authorities suggest starting treatment as soon as possible to influence the frequency of relapses, stabilize disease activity, and lessen long-term disability. The nonspecific immunosuppressants azathioprine and cyclophosphamide have been used frequently without clearly established efficacy. Cladribine, mitoxantrone, antilymphocyte globulins, cyclosporine, and tacrolimus are chemotherapeutic agents with use as semispecific suppressors of MS disease activity. In extremely severe cases, total lymphoid irradiation may modulate the immune system, potentially benefiting MS, though controlled trials are lacking. Several peptides are being explored that interfere with binding within the trimolecular complex (T-cell receptor, antigen, and MHC class II molecule), potentially leading to more specific agents decreasing the activity of the disease with minimal systemic immunosuppression (Vandenbark et al., 1996).

Acute exacerbations are often initially treated with corticosteroids that enhance the resolution of symptoms and signs, though do not significantly affect the long-term outcome of an exacerbation. There are no certain dosing guidelines, although solumedrol 1g for 5 days is an appropriate course of treatment. Pulse therapy with corticosteroids is associated with many temporary side effects such as insomnia, irritability, fluid retention, increased appetite, weight gain, hyperglycemia, hypertension, dyspepsia, depression, psychosis, bone fractures, and osteoporosis. In patients with poor venous access or otherwise intolerable

reactions to corticosteroids, adrenocorticotropic hormone may be used instead. Plasmapheresis is sometimes used in severe relapses that are refractory to corticosteroids.

Therapies focused on improving conduction include 4-aminopyridine (4-AP) and 3, 4-diaminopyridine (3, 4-DAP), both potassium channel blockers that amplify and prolong action potentials. Preliminary studies with 4-AP demonstrated improvement in may measures of neurologic function. However, when a large, multicenter, double- blind, placebo-controlled, study was performed, it failed to show an effect on the Expanded Disability Status Scale (Van Diemen et al., 1992). Unfortunately, higher levels of these medications can result in seizures and encephalopathy, potentially preventing sufficient dosage for demonstrable effect (Bever et al., 1994).

Symptomatic therapy for MS is an important aspect of management (Schapiro, 1994). Paresthesia may respond to antidepressants and anticonvulsants. Anticholinergic and β-blocker medications can improve bladder function, and fatigue can require amantadine and CNS stimulants. There are no medications currently available to treat muscle weakness, though physical therapy can optimize patient function. Spasticity, muscle cramps, and spasms respond to stretching and antispasticity medications, including baclofen, tizanidine, and benzodiazepines. If necessary, botulinum toxin can be introduced into specific muscles or, if generalized spasticity is refractory to other treatments, intrathecal baclofen administered by an implantable subcutaneous pump or dorsal root rhizotomy may be considered. Adaptive equipment includes ankle-foot orthoses for foot-drop dysfunction and canes, walkers, and wheelchairs for mobility. Tremor may respond to a variety of medications. Propranolol and primidone are often used initially, though isoniazid, buspirone, trazadone, baclofen, carbamazepine, gabapentin, benzodiazepines, and unilateral thalamotomy, can all be effective (Whittle and Haddow, 1995).

Approved disease modifying therapies for relapsing multiple sclerosis

Drug class	Approved drug(s)	Route of administration	Standard dose	Frequency	Key adverse effects	Monitoring requirements
Moderate efficacy or standard initial therapies						
Type I interferons	Interferon beta-1a and interferon beta-1b (4 drugs)	Subcutaneous or intramuscular	Variable	Every other day to weekly	Flu-like symptoms, ISRs, transaminase elevation	LFTs, CBC
	Pegylated interferon beta-1a	Subcutaneous		Every 2 weeks		
Random amino acid copolymer	Glatiramer acetate (2 drugs)	Subcutaneous	20 mg	Daily	ISRs, systemic syndrome (flushing, chest pain, anxiety)	None
			40 mg	Three times weekly		
Pyrimidine synthesis inhibitor	Teriflunomide	Oral	7 mg or 14 mg	Daily	Diarrhea, alopecia, hepatotoxicity, hypertension, peripheral neuropathy	Baseline tuberculosis test and pregnancy test; baseline and regular CBC, LFTs
Possible Nrf2 pathway activator and NFκB inhibitor	Dimethyl fumarate	Oral	240 mg	Twice daily	Flushing, gastrointestinal upset, lymphopenia, rare PML	Baseline and regular CBC with differential
Sphingosine-1-phosphate receptor modulator	Fingolimod	Oral	0.5 mg	Daily	First dose bradycardia, atrioventricular nodal blockade, VZV reactivation, macular edema, opportunistic infections, rare PML	Pre-treatment cardiac, VZV immunity, laboratory, and eye and skin examinations; on-treatment laboratory and ophthalmological assessment and skin examination
High efficacy or later line therapies						
Monoclonal antibody	Natalizumab	Intravenous	300 mg	Every 28 days	PML, infusion reactions	REMS program
Monoclonal antibody	Alemtuzumab	Intravenous	12 mg/m^2	5 infusions (year 1) and 3 infusions (year 2)	Autoimmune thyroid disease, ITP, Goodpasture's syndrome	REMS program; baseline and on-treatment monitoring of CBC, creatinine, and urinalysis (monthly) and thyroid function (quarterly); continue monitoring for 4 years after last infusion
Anthracenedione antineoplastic/general immunosuppressive	Mitoxantrone	Intravenous	12 mg/m^2	Every 3 months for maximum of 2 years	Cumulative dose dependent cardiomyopathy, acute leukemia (either may emerge post-treatment)	Regular echocardiography and CBC during and after treatment completion
Monoclonal antibody, anti-CD25 (anti-IL2-Rα)	Daclizumab	Subcutaneous	150 mg	Every 4 weeks	Transaminase elevation/ severe liver injury; cutaneous reactions (sometimes severe), non-infectious colitis, lymphadenopathy, infection risk, depression	REMS program; baseline and on-treatment monitoring of liver function monthly before each dose and for 6 months after final dose

CBC=complete blood count; ISR=injection site reaction; IL2-Rα=interleukin-2 receptor-alpha; ITP=idiopathic thrombocytopenic purpura; LFT=liver function test; PML=progressive multifocal leukoencephalopathy; REMS=required event monitoring system; VZV=varicella zoster virus.

Table 1.2 *- Approved disease modifying therapies for relapsing multiple sclerosis (modified from Wingerchuk et al., 2016)*

DMTs are often recommended for people diagnosed with a CIS that is considered more likely to progress to clinically definite MS (CDMS), with the goal of delaying a second attack.

At this time, it is difficult to predict the future course a person who is diagnosed with a CIS will experience. Many episodes of CIS are mild and resolve without treatment. In other cases, treatment with high dose oral or intravenous methylprednisolone (a steroid) is typically recommended. Several large-scale clinical trials have been conducted to determine whether early treatment following a CIS can delay the second clinical event, and therefore the diagnosis of CDMS. Based on the results of these studies, the U.S. Food & Drug Administration (FDA) has expanded the indication of several medications used to treat MS to include individuals who have experienced a first clinical episode and have MRI findings consistent with MS. The results of these trials, and the FDA's approval of expanded labeling for certain medications used to treat MS, support the earliest possible treatment for MS, which many believe may delay the development of permanent clinical disabilities.

Although some might support use of DMTs to delay the clinical or radiological progression similar to CIS patients, there are no studies to suggest this might be beneficial even for RIS

patients at higher risk of MS. Moreover, the risk factors are not always clear, and the other caveat may be misdiagnosis of RIS in some cases where other conditions may mimic MS radiologically. Given the uncertainty about the diagnosis and management of these patients, only a small proportion of RIS patients get treated with disease modifying therapy. There is currently no set protocol for managing patients with RIS. These patients are usually followed with surveillance MRIs every six months to a year or on as needed basis depending on the patient's wishes and the treating neurologist's preference. If clinical symptoms develop over time (conversion to CIS), most of these patients would be initiated on DMT. There is, however, a lack of evidence to support the use of DMT in RIS patients who show radiological progression on follow up imaging in absence of clinical progression (Leahy and Garg, 2013).

1.4 Future direction

1.4.1 Personalized medicine for MS

Although drug mechanisms can occasionally be personalized for cancer on the basis of genomic or tissue specific DNA mutation detection and pathobiology, this approach is not feasible for MS. Nevertheless, optimization of treatment for MS remains a highly individualized process. An intermediate approach between the escalation and induction strategies, in which risk for future disability, comorbidities, personal preferences, and risk mitigation strategies are considered to select a therapy, provides the best compromise between efficacy and potential risk.

The diverse efficacy and safety profiles of DMTs have resulted in greater emphasis on "personalized" approaches that tailor decisions about treatment to a patient's disease characteristics and preferences. And, because the medicines all are relatively expensive, better ways of making most effective use of them are needed. However, lack of prognostic and therapeutic biomarkers continues to hinder development of biologically based strategies.

Clarifying the monitoring strategies for adverse effects and breakthrough MS would be crucial. The development of radiological and biological biomarkers could allow in the future personalizing treatment and precision medicine in MS (Comabella et al., 2016).

Understanding the heterogeneity of the MS syndrome involves an active process of 'deconstruction' to define the biologically distinct diseases included within it and their interactions with individual, patient-specific factors. Coordinated collection and sharing of data (not just clinical data but also that from devices and patient-reported outcomes and behavioural, employment and life-style data), development of predictive models and their progressive evaluation in the care of individual patients will be an essential part of this. Treating the right patient with the right drug early in the disease course, before disability has been acquired, could yield long term benefits (Gafson et al., 2016).

1.4.2 Agents in trial

After Fingolimod, the first oral DMT approved in 2010, many other oral agents have been approved too or are currently in phase III trials or are going to be submitted to the regulatory agencies for approval (Thomas et al., 2015). Three monoclonal antibodies are now approved for MS treatment, and others are also in late stage development (breafly described below). And, despite a real breakthrough in treating MS, the available therapies are far from having sorted out the current unmet needs raised by the complexity of MS.
Three anti-CD20 agents (rituximab, ocrelizumab, and ofatumumab) that deplete pre-B cells and mature B cells without affecting plasma cells or progenitor cells in the bone marrow have been studied in MS (Singer et al., 2016).

- Rituximab is a human–mouse chimeric monoclonal antibody against CD20. Rituximab has been used off label for MS as well as neuromyelitis optica. Although rituximab rapidly and consistently decreases the numbers of peripheral $CD20^{+}$ and $CD19^{+}$ cells (Topping et al., 2016), a small phase II trial of intrathecal rituximab was terminated early because of low efficacy on the CSF biomarkers. No phase III trials of rituximab for MS have yet been performed.
- Ocrelizumab and ofatumumab are both humanized anti-CD20 monoclonal antibodies. Ocrelizumab was the first trialed drug to meet primary and key secondary efficacy outcomes in a phase III PPMS study. Ocrelizumab significantly reduced the relative risk of 12-week CDP by 24% and 24-week CDP by 25%, decreased the volume of T2 hyperintense lesions and reduced the whole brain volume loss compared with placebo. The results of all phase trials will be submitted for the approval of

ocrelizumab to the FDA (Coclitu et al., 2016).

- Ofatumumab is currently used for lymphocytic leukemia, which interacts with the early activation of the B lymphocyte and has lower potential for antigenicity. It was tested in a small phase II clinical trial with promising results, showing a 99% reduction of MRI activity, with no serious adverse events (Sorensen et al., 2014).

A part of the monoclonal antibodies that have been just described, other pharmacological agents are under clinical trials.

Cladribine is a cytotoxic drug, an adenosine deaminase-resistant purine nucleoside, used as a first-line chemotherapeutic agent in the treatment of hairy cell leukemia and other neoplasms, in its parenteral formulation (Huynh et al., 2009). It enters cells via purine nucleoside transporters (Liliemark, 1997). Cladribine works preferentially on lymphocytes and monocytes by disrupting cellular metabolism resulting in cell death (Beutler, 1992), being incorporated into the DNA of the dividing cells. The recently reported results of a 120-week extension demonstrated that in a majority of patients, the clinical benefits on relapses and disability as well as on MRI outcome measures of 3.5mg/kg cladribine given in the first two years of the trial can be maintained for at least 4 years (Coclitu et al., 2016). But, a recent analysis applied the 2010 McDonald criteria caused Cladribine fail to get regulatory approval by the EMA because of concerns over the risks of cancers in the CLARITY active arm (Coclitu et al., 2016). Now, PREMIERE (NCT01013350), an observational prospective study of patients who have participated in clinical trials with cladribine or other DMTs is ongoing Pakpoor et al., 2015).

Laquinimod is an orally available carboxamide derivative, derived from linomide, a drug that was proved to reduce activity in RRMS, but with the cost of severe adverse events (Brueck and Wegner, 2011). Laquinimod was or currently is tested for neurodegenerative disease such as Huntington’s disease and also for relapsing remitting and progressive MS (Kim et al., 2015).

Siponimod and ozanimod, two oral selective S1P receptor modulators, were recently successfully tested in a phase 2 trial with positive MRI outcomes (Cohen et al., 2016).

1.4.3 New strategies

Although several therapies for MS are already available, a large unmet clinical need for more effective immunomodulatory treatments still remains in this category of diseases, but also for interventions able to address their neurodegenerative component, which is currently untreated.

Preventing the entry of lymphocytes into the CNS and modifying the nature of the immune response are approaches that act on in the inflammatory component of MS, but have little or no effect on neurodegeneration.

Here, new strategies and potential approaches for the treatment of MS are briefly described.

Autologous Bone Marrow Transplantation

Hematopoietic stem cell transplantation (HSCT) has been used in many studies on animal models that showed a strong immunosuppression followed by syngeneic bone marrow transplantation can induce long term antigen-specific tolerance (Karussis et al., 1999).

There is evidence that high-dose immune ablation and autologous HSCT could renew the immune system repertoire and reinforce immune tolerance mechanisms thus having a clinical impact (Muraro and Douek, 2006). Although these results are promising, and progress has been made over the last decade in mitigating risks, there are many unknowns regarding the use of HSCT as a possible second-line therapy for refractory MS (Freedman and Atkins, 2016; Soelberg Sorensen, 2016).

However, the benefits of this procedure would only be seen with intense conditioning regimens which achieve a near-complete immune ablation (Freedman and Atkins, 2016). Considering HSCT benefit-risk profile and the availability of highly-effective treatments with monoclonal antibodies, which can achieve disease control in patients with active disease, intense immunosuppression with HSCT should remain a third-line therapy (Soelberg Sorensen, 2016). It is likely that the place of HSCT in MS will be re-evaluated over the next years, in light of the continuously-growing spectrum of available therapies and of new pragmatic, prospective, controlled multicentre trials.

Remyelination strategies

It is known that remyelination occurs initially in MS lesions but is inadequate, and the mechanism of repair in the CNS fails with time, especially in chronic disease stages (Franklin, 2002). The differentiation of oligodendrocyte precursor cells (OPC) into mature cells is essential and this process will be described in detail in Chapter 2. Remyelination develops in two steps: the colonization of the lesions by OPCs, and OPC differentiation into mature oligodendrocytes able to generate functional myelin sheath (Chari, 2007).

Most information on these processes comes from studies on animal models (EAE and the cuprizone model of MS) (Zendedel et al., 2013). Remyelination can be promoted either by intrinsic (altering intrinsic signaling pathways, for instance through the pharmacological modulation of a membrane receptor) or extrinsic (acting on lesion environment) repair mechanisms (Rodgers et al., 2013; Keough and Yong, 2013).

In this respect, GPR17 has been proposed as a new possible target for remyelinating strategies. GPR17 (that is the focus of this thesis and it will be introduced extensively in chapter 3) is a membrane receptor expressed by OPCs for a short-time window during their differentiation and it has been demonstrated to be essential for this process (Chen et al., 2009; Fumagalli et al., 2011). An upregulation of this receptor has been reported under many pathological conditions with demyelinating component, thus a pharmacological modulation aimed at restoring the receptor levels could be a promising strategy able to foster remyelination (Lecca et al., 2008; Boda et al., 2011; Ceruti et al., 2011).

Wang et al. differentiated OPCs from human induced pluripotent stem cells (iPS) and engrafted them in a myelin-deficient mouse model. The transplanted OPCs differentiated into astrocytes and oligodendrocytes, myelinated the brains of the animals, and increased their survival (Wang et al., 2013). However, since MS is a multifocal disease it would probably require repeated transplantation of the OPC in all the demyelinated regions (Munzel and Williams, 2013). These techniques are still under study, and their safety and efficacy are yet unknown.

It is not yet clear whether remyelination completely prevents neurodegeneration but it does appear to restore neuronal function and at least limit neuronal degeneration (Munzel and Williams, 2013), therefore remyelination strategies are likely to be part of the MS treatments in the future.

Mesenchymal stem cells

Mesenchymal stem cells (MSC) can be harvested from adult bone marrow and can be transplanted securely without the need for immunosuppression and with a low risk of aberrant proliferation.

Mechanisms of action would include immunoregulation and anti-inflammatory changes of the cellular environment. It is likely that the potential therapeutic efficacy of MSC could be based on systemic effects as recently shown (Abramowski et al., 2016).

A phase 1/2 open-safety clinical trial in patients with MS and with amyotrophic lateral sclerosis showed that intrathecal and intravenous administration of autologous MSCs is a clinically feasible and a relatively safe procedure which produced immediate immunomodulatory effects (Karussis et al., 2010).

T-cell directed strategies

It was suggested that the immune response in MS is directed at least in part against myelin proteins including MBP, myelin oligodendrocyte protein (MOG) and PLP (Amor et al., 1994; Johns et al., 1995). Although there are differences in the activation state or precursor frequencies of T cells from patients with MS and healthy subjects, people without MS also have immune responses against such antigens. However, it was suggested based on indirect evidence that molecular mimicry, epitope spreading and bystander activation are possible mechanisms to initiate and maintain disease activity. Consequently, an alternative treatment approach in MS could aim to selectively restore self-tolerance to auto-antigens via immunization (Fissolo et al., 2012) and epitope-specific induction of T cell tolerization (Billetta et al., 2012) or specifically by targeting regulatory T cells (Treg) signaling (Spence et al., 2015). A first phase I trial in humans published in 2013 showed that the antigen-coupled cell tolerization in MS is feasible and safe (Lutterotti et al., 2013).

Drug repurposing in MS

The failure to deliver successful neuroprotective therapies in MS has led to alternative strategies such as drug repurposing (Vesterinen et al., 2015). By exploiting existing trial and regulatory data on clinical safety and efficacy, “drug rescue” (evaluating drugs at advanced stage of development but abandoned before approval) and “repurposing” (evaluating drugs

already approved for other conditions), offer the potential to reduce both the cost and time to achieve licensed approval status.

Successful repurposing of drugs is not new; examples include dimethyl fumarate (Tecfidera)—originally marketed as a therapy for psoriasis, but later developed as a disease modifying therapy for relapsing-remitting MS (RRMS) (Gold R., et al, 2012). And recently, encouraging data suggest that phenytoin in acute optic neuritis (Raftopoulos et al., 2016) and amiloride in PPMS (Arun et al., 2013) could have a neuroprotective effect.

Thus, on this basis, it is probable that repurposing drug development will play a bigger role in the future despite the inherent pitfalls which impact its feasibility (Giovannoni et al., 2015).

1.5 Animal models of MS

As mentioned earlier, the mechanism of MS is largely unknown and despite serious efforts are made every day, there is no cure, but only symptomatological treatments. However, there are several well-characterized experimental animal models that provide a profound insight into the pathological processes that may cause or influence MS.

The autoimmune view of MS is strongly supported by the animal model experimental autoimmune encephalomyelitis (EAE), a group of disorders characterised by inflammation, myelin damage and neurodegeneration induced following immunisation with myein antigens (e.g., MOG or PLP).

For demyelinating disorders, where the aetiological agent is due to viral infection, several viral models to study MS have been developed. Theiler's murine encephalomyelitis (TMEV) model is based on virus-induced demyelination. Intracranial infection of susceptible mouse strains with TMEV results in biphasic disease of the CNS, consisting of early acute and late chronic demyelinating phase. The late chronic stage of demyelination in the TMEV infection makes this experimental model highly suitable for studying different aspects of the pathomechanism of MS (Denic et al., 2011).

Remyelination, however, is better studied in toxin models, where neurotoxic agents are used to induce the loss of myelin sheath in certain areas in the CNS. The most frequently used demyelinating agents are lysolecithin, ethidium bromide and the copper chelator cuprizone. Despite the extensive use of these models, the clinical course, immunology and

neuropathology reflect only part of the pathological spectrum of MS, indicating that responses to therapies in animal models often cannot predict efficacy in humans.
Below, a brief overview on the most used MS models.

1.5.1 Experimental autoimmune encephalomyelitis

Immunisation of susceptible animals with CNS antigens gives rise to a spectrum of inflammatory disorders collectively named EAE. Although the experimental disease in animals was originally termed experimental disseminated encephalomyelitis, the idea that the 'disease' was allergic gave rise to the name experimental allergic encephalomyelitis. More recently, allergic has been replaced by autoimmune. Despite differences in disease course and pathology, EAE is still the most intensely used experimental model of MS, that mimics MS pathology and allows a detailed insight into the immunological aspects of this disease. EAE has been extensively used to better understand immune-mediated mechanisms of demyelination and neurodegeneration in MS.
This experimental disease can be obtained in all mammals tested so far, including nonhuman primates, allowing very advanced preclinical studies. Its appropriate use has led to the development of the most recent treatments approved for MS (See table 1.3), also demonstrating its predictive value when properly handled (Kipp et al., 2012).

Treatment	"Proof on concept" in EAE	FDA approval	Approved indication
Glatiramer acetate	1971	1996	RR-MS
Mitoxantrone	1987	2000	SP-MS worsening RR-MS
Natalizumab	1992	2004 Withdrawn in 2005 Reinstated in 2006	RR-MS

Table 1.3 - *Therapeutics approved for preventive treatment of MS that have been developed in the EAE model (modified from Denic et al., 2011).*

This form of experimental encephalomyelitis displays an acute and/or chronic-relapsing, acquired, inflammatory demyelinating autoimmune disease. In active EAE, rodents (most commonly mice, rats or guinea pigs) or non-human primates are injected subcutaneously with a myelin related antigen/peptide (e.g. MOG, PLP, MBP) or spinal cord homogenate (SCH). The clinical, pathological, and immunological picture of autoimmune models of demyelination depends upon the mode of sensitization, the nature of the immunogen, and the genetic background of each species and strain (see Fig.1.3).

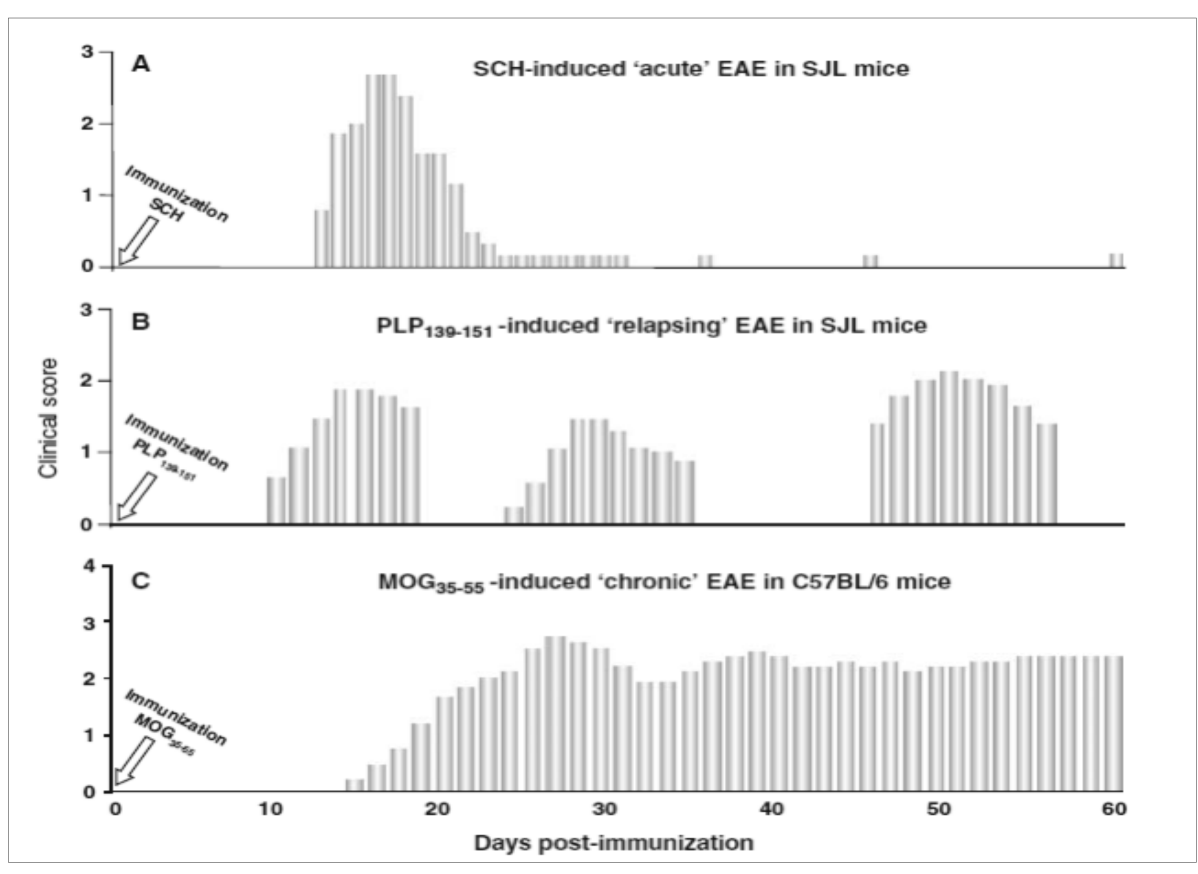

Figure 1.3 – *EAE clinical courses (modified from Furlan et al., 2009)*

Similar to MS, age, gender and environmental factors have a profound influence on disease susceptibility, severity and course of EAE. For example, young male SJL mice immunised with PLP are relatively resistant to EAE whereas older males and female SJL mice of any age are susceptible. Young C57BL/6 mice and Wistar rats develop acute EAE and remission whereas middle-age males developed severe chronic EAE (Ditamo et al., 2005; Matejuk et al., 2005). As in MS pregnant females show a reduced susceptibility to disease, probably as a result of immune suppression (Evron et al., 1984). These findings, along with the observation that older mice immunised in winter are more susceptible to EAE indicate an influence of genetics, gender and age in disease susceptibility.

In the classical picture of EAE, activated myelin-antigen specific $CD4^+$T-cell clones express adhesion molecules, enzymes, cytokines and chemokines and their receptors inducing the break-down of blood-brain barrier and migrating into the CNS. Antigenic determinants presented by antigen presenting cells (monocytes, macrophages, dendritic cells, B cells, microglia, astrocytes) then recognized by myelin-antigen specific T cells, which undergo continuous activation and exert cytotoxic effects in the CNS. A rather simplistic picture of

the EAE cytokine network would suggest that TNF-α, TNF-β, IFN-γ, and IL-12 (pro-inflammatory cytokines) have a disease-promoting role, while TGF-β, IL-10, and possibly IL-4 (anti-inflammatory cytokines) may protect from disease. A paralytic disease typically develops affecting predominantly the tail and hind limbs, but sometimes also the fore limbs (see below).

The initial phase of disease is usually termed the *acute phase*, and correlates with the mononuclear cell infiltrates in the CNS. In species and strains where the animals recover, this recovery period is referred to as *remission*. If animals do not recover the disease is referred to as *chronic EAE*.

A non-parametric scale with five steps is used for clinical evaluation (Fig. 1.4). Mice are taken from the cage by the tail and observed from the abdominal side: healthy mice extend their hind limbs very wide when held in this position. An asymmetric position, especially with one of the hind limbs retracted close to the abdomen, may represent an initial sign of disease. A weak tail and an unsteady gait are the signs at onset. To reveal an uncertain gait, it is useful to observe mice walking on a grid, like the cage cover. Healthy mice are quick in movements on a grid almost as on firm ground. Sick mice are unsure, move slowly, and often stumble. In the absence of other signs, we score the uncertain gait on the cage cover as 1.5. Another useful examination is that of the righting reflex. Trying to put healthy mice on its back results in such a quick flipping to the upright position that it is impossible to see the abdomen. A sick mice may have a slow, very slow, to complete impaired righting reflex. In the absence of other signs, we score any grade of impairment of the righting reflex as 2. Paraparesis (score 3) and involvement of fore limbs (score 4) are easily identified (Furlan et al., 2009).

EAE: Clinical score

0	Healthy mouse
1	Weak or flaccid tail.(When the mouse, put on a grid, has gait problems= **1.5**)
2	Unstaedy gait (ataxia), hind limb paresis, or slow righting reflex
3	Paraparesis, complete hind limb paralysis
4	Paraparesis with fore limb involvement
5	Moribund or death

Figure 1.4 – *EAE clinical evaluation (modified from Furlan et al., 2009)*

1.5.2 The cuprizone-induced experimental demyelination

The first experiments using cuprizone as a toxic compound were performed in the late 1960s (Carlton, 1969). Although cuprizone was used as a chelator for copper analysis, it was described that cuprizone administration induced microscopic lesions in the brain accompanied by edema, hydrocephalus, demyelination, astrogliosis and the effects of cuprizone were not antidoted by administration of copper (Carlton, 1967). Based on his findings, most laboratories use 6–9-week-old mice and feeding with a diet containing 0.2–0.3% cuprizone. Blakemore reported first that cuprizone causes OL degeneration (Blakemore, 1972) and the demyelination of the superior cerebellar peduncle (Blakemore, 1973). Primary OL degeneration and selective regional vulnerability of the cuprizone regimen were described by Komoly et al (Komoly et al., 1987; Komoly et al., 1992).

Cuprizone-induced demyelination model has attracted increasing interest during the last decade since contrary to other models of MS this one provides a highly reproducible system of primary OL apoptosis and secondary demyelination and where adaptive immune responses are not involved. The administration of the copper chelating agent cuprizone (bis-cyclohexanoneoxaldihydrazone) to mice induces spatially and temporally well-defined histopathological alterations in the CNS.

Furthermore, strain-dependent susceptibility to cuprizone has been reported. For example, SJL mice display a unique pattern of demyelination that does not follow the profile as seen in C57BL/6 mice. SJL mice do not readily demyelinate at the midline within the corpus callosum but show greater demyelination directly lateral to midline (Taylor et al., 2009).

The earliest event is the appearance of megamitochondria (Ludwin, 1978), followed by only specific mature OL apoptosis. Cuprizone is a copper chelator, which in turn leads to inhibition of the copper-dependent mitochondrial enzymes cytochrome oxidase and monoamine oxidase. Thus, a plausible hypothesis is that disturbance in energy metabolism leads to apoptosis in the oligodendrocytes, which causes demyelination (Matsushima et al., 2001). If the mice are exposed to a higher dose of cuprizone, this will lead to formation of megamitochondria in the liver, thus emphasizing the role of mitochondrial dysfunction in this model.

The peak of the apoptotic events is between the 3rd and 10th days (Matsushima et al., 2001) of the cuprizone challenge, but apoptotic OLs can be detected during the entire administration, and even during the recovery period 12 weeks post treatment (Lindner et al., 2009). The exact mechanism of OL apoptosis is not fully understood, and is often debated. However, it is generally accepted that cuprizone induces metabolic disturbances in OLs which leads to apoptosis involving a mitochondrial mechanism. A similar role of the mitochondria has been implicated in OL cell loss in MS as well (Kalman et al., 2007).

The massive OL apoptosis is followed by extensive demyelination. The loss of myelin is preceded and accompanied by a down-regulation of myelin-related proteins with varying kinetics. For example, down-regulation of MAG expression can be seen in a few days after the initiation of cuprizone administration, while complete demyelination of the corpus callosum is usually observed after six weeks of treatment (fig. 1.5). While demyelination was thought to affect only particular white matter tracts (i.e. corpus callosum, superior cerebellar peduncle) (Komoly, 2005), other studies reveal that other regions, including the hippocampus, putamen, cerebellum and even distinct gray matter areas in the cortex, also undergo demyelination (Kipp et al. 2009).

Another prominent pathological feature associated with OL apoptosis is the invasion of the demyelinated areas by activated microglial cells. These cells originate from residential microglia, but macrophages immigrating from the blood (Remington et al., 2007) also contribute to the marked numbers of phagocytic cells seen most abundantly around the third week of cuprizone treatment. If mice return to normal diet after five weeks of cuprizone exposure, demyelination is followed by a spontaneous and complete remyelination driven by the repopulation and maturation of OL progenitor cells (OPCs) (Lindner et al., 2008). If the cuprizone challenge is prolonged for 12 weeks, the degree of

remyelination may be limited or remyelination may even fail to occur (Lindner et al., 2009). If it occurs, this spontaneous but incomplete remyelination begins between the 4th and 6th weeks of the ongoing cuprizone administration (Armstrong, 2007).

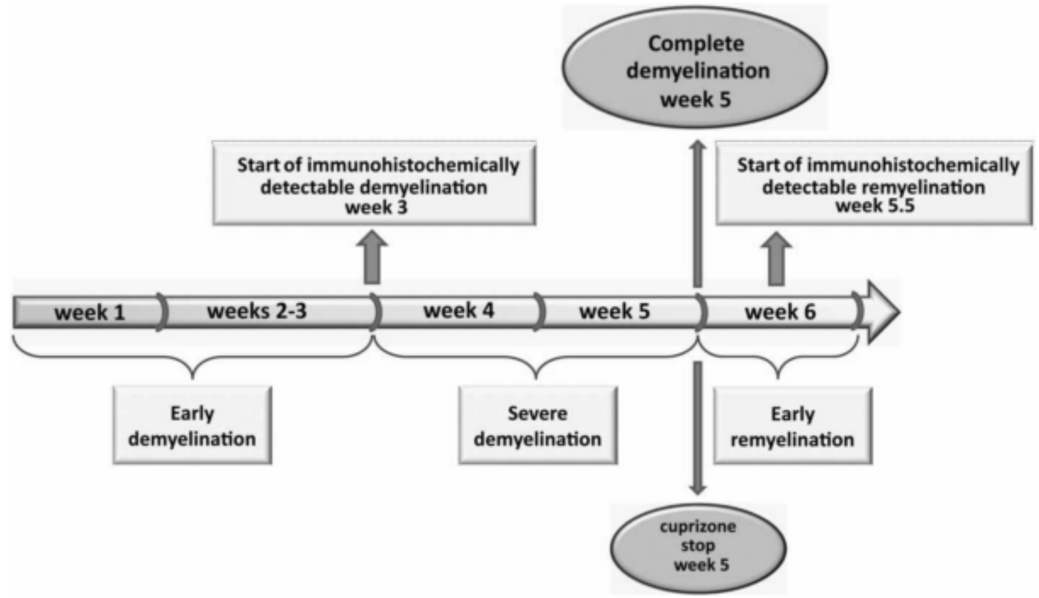

Figure 1.5 – *Course of de- and re-myelination in the medial corpus callosum (modified from Gudi et al., 2014)*

Histopathological features of the cuprizone-induced demyelination closely resemble those of the Lucchinetti et al. defined type III MS lesions (described before in paragraph 1.2.3) (Lucchinetti et al., 2000). The most significant similarities include a prominent OL apoptosis and microglial activation in the actively demyelinating lesions, the lesions are not perivascular and their borders are ill defined, and there is an early and profound downregulation of the MAG mRNA level (Morell et al., 1998). In addition, the cuprizone model shares common features with the earliest phases of MS lesion development as described by Barnett and Prineas (Barnett et al., 2004) and Henderson et al (Henderson et al., 2009), where first, apoptosis of OLs occurs in regions with intact myelin. As the pathology further evolves, early demyelinating lesions are invaded by scavenging macrophages (innate immune response), which phagocytose and clean up degraded myelin.

Considering the above histopathological features, the cuprizone model is highly suitable for studying basic mechanisms of acute and chronic demyelination and remyelination, exploring the pathophysiology of OL apoptosis, and testing preclinically new interventions for promoting remyelination and repair in MS lesions.

Chapter 2

Oligodendrocytes

2.1 Oligodendrocytes: the myelin-forming cells in the CNS

The CNS contains two major types of specialized cells, neurons and glial cells (Figure 2.1). Neurons are regarded as the elements mediating the electrical activity in the form of action potentials and are responsible for the relay of information throughout the nervous system. Neuroscience research of the past has mainly focused on this cell type, since until recently glial cells were believed to provide only structural support to neurons. However, it is now becoming increasingly clear that glial cells have active functions in the nervous system, as demonstrated by numerous experimental evidences showing that glial cells play a role in synaptic development and activity, provide guidance of neuronal migration and process outgrowth and influence the electrical activity of neurons. Thus, it is now recognized that different types of glial cells fulfil distinct tasks and are essential for the proper functioning of neural circuits.

Based on morphology, function and location in the nervous system, glial cells are classified in three main categories: astrocytes, microglia and oligodendrocytes (OLs) (Barres, 2008; Allen and Barres, 2009).

Astrocytes are star-shaped cells which extend many processes that contact both blood vessels and neurons; they provide leading structures during development and represent important elements for controlling the composition of the extracellular space mediating signals between the brain endothelium and the neuronal membrane.

Microglial cells are the resident immuno-competent cells of the nervous system. They have crucial functions in surveillance and homeostasis of CNS, reacting to damage and infection, by removing cellular debris and actively participating to neural tissue remodelling after injury.

OLs are the myelin-forming cells of the CNS that ensure the saltatory conduction of the nerve impulses in the WM. They are post-mitotic glial cells with small number of cytoplasmic processes, whose name comes from the Greek roots oligo, meaning ‘few’, dendro meaning ‘branch’, and kytos which denotes ‘cell’. This term was first introduced by

Rio Hortega to describe those neuroglial cells that show few processes in material stained by metallic impregnation techniques.

OLs are also involved in neuronal development and survival, in the regulation of extracellular ion concentrations, in the distribution of potassium channel along the axons and in the axonal transport. Moreover, some OLs form synapses with neurons and are actively involved in CNS functions (Barres, 2008; Emery, 2010).

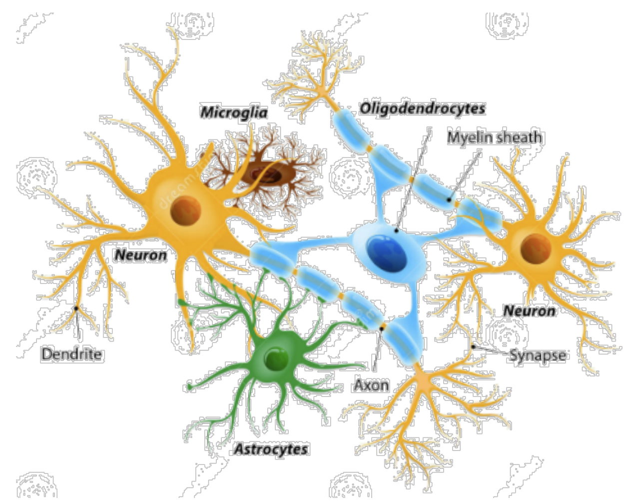

Figure 2.1 – *Glial populations within CNS*

In 1992, Rio Hortega classified OLs in four categories based on the characteristics of the number and orientation of their cellular processes, the shape and size of their somata, the size of the axons they were associated with, and their distributions within the CNS (Rio-Hortega, 1928). Type I and II OLs are indistinguishable and have four or more primary processes that branch repeatedly to myelinate 10-30 axons less than 2 μm in diameter. Type I OLs can be found in the forebrain, cerebellum and spinal cord, whereas type II OLs are observed only in WM. Type III OLs have large cell bodies, often applied directly to an axon, with one or more thick primary processes that rarely branch and myelinate a small number of axons, usually less than five, with external sheath diameters ranging 4 to 15 μm. They are localized in the cerebellar peduncles, the medulla oblongata and the spinal cord. Type IV OLs are similar to type III OLs but not have processes and form a single long myelin sheath over a large-diameter fiber; they are restricted to tracts containing the largest diameter fibers and occur near the entrance of nerve roots into the CNS (Baumann and Pham-Dinh, 2001).

This first section will be focused on this latter glial cell type. Specifically, the embryonic and postnatal development of OLs and their functional roles in physiological and pathological conditions will be described and discussed.

2.1.1 Embryonic and postnatal development of OLs

OLs are ubiquitous in both the WM and grey matter (GM) of brain and spinal cord and most of our knowledge about the biology of OLs and myelin derives from studies in rodents. These cells originate from pluripotent neuroepithelial cells of neural tube, that give rise to committed oligodendrocyte precursor cells (OPCs). These cells are able to divide and migrate throughout the CNS. In mouse, OPCs first appear in the embryo starting at about embryonic day (E) 12.5 in the ventral ventricular zones of the spinal cord, where they originate together with motoneurons from a common class of precursor cells (pMN, precursors motoneuron), defined by the expression of the transcription factor Olig2 (Lu et al., 2002; Takebayashi et al., 2002; Zhou et al., 2000). This OPC production is dependent on Sonic hedgehog (Shh) signaling and bone morphogenetic proteins. At about E15, generation of a secondary wave of precursors starts in more dorsal regions of spinal cord by trans-differentiation of radial glia (Lu et al., 2002) and this generation is independent on Shh pathway (Cai et al., 2005).

The origin of OPCs in the brain is instead more complex. In the embryonic telencephalon three different waves of OPC generations were observed, temporally progressing from ventral to dorsal regions (Kessaris et al., 2006). The first wave starts at E12.5 (overlapping with the appearance of OPCs in the spinal cord) in the medial ganglionic eminence (MGE) and in the anterior entopeduncular area (AEP). The second wave follows the first at E15.5, taking place in the lateral and caudal ganglionic eminence (LGE and the CGE) and final one arises at postnatal day 0 (P0) directly from the cortex (Kessaris et al., 2006).

These three distinct waves of OPCs originate from three distinct types of neural progenitors, expressing different transcription factors. OPCs from the first wave express platelet-derived growth factor receptor alpha (PDGFRα) reflecting their dependence on PDGF-AA for survival and proliferation (probably the most important molecule controlling the number of OLs in vivo); these cells successively migrate to the cortex (at E16) and, finally, disappear completely at P10. OPCs from second wave start at E15.5 and are generated from Gsh2-expressing progenitors and OPCs from third wave start around birth

(P0) and derived from Emx1-expressing progenitors (Figure 2.2). This fine embryonic regulation of OPC origin led once again to the idea of distinct subsets of OPCs, devoted to different functions. However, so far no study has correlated a distinct origin to a specificity in OPC behavior in the postnatal and adult CNS. Future research will clarify this aspect.

After the formation, OPCs migrate extensively from oligodendrogliogenic niches and colonise the entire CNS. The migration of these precursors is likely to be mediated by specific directional and substrate cues. Ventrally-generated OPCs predominate in the spinal cord, while the dorsally-generated ones prevail in the telencephalic vesicles; this distribution may reflect a compensatory redundancy to ensure rapid and efficient myelination throughout the entire CNS (Richardson, 2006). It is known that OPCs migrate along pre-existing axons. However, when these pre-existing axons are transected, the cell biology of OPCs does not change and they migrate properly, and even extensively, demonstrating that OPCs could respond to signals other than those expressed by viable axons (de Castro et al., 2005; Sugimoto et al., 2001; Ueda et al., 1999).

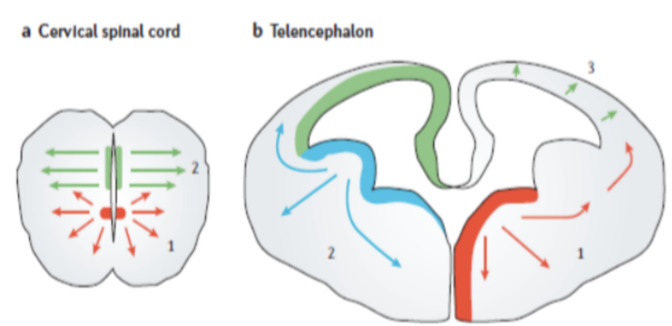

Figure 2.2 - ***Origins and migration of oligodendrocyte precursors in the rodent cervical spinal cord and telencephalon.*** *(a) In the mouse spinal cord,* OPCs *are generated from pMN in the ventral ventricular zones (1), starting at about embryonic day (E)12.5. At about E15, generation of a secondary wave of precursors starts in more dorsal regions by trans-differentiation of radial glia (2). (b) In the telencephalon, the ventral-most precursors in the medial ganglionic eminence are produced from about E12.5 (1), production of the lateral ganglionic eminence derived precursors starts a few days later (2), and production of the cortex-derived precursors occurs mainly after birth (3) (Richardson et al., 2006).*

To date, three different classes of secreted molecules seem to be involved in the migration of OPCs: *growth factors* - like PDGF-AA, FGF2 (Simpson and Armstrong, 1999; Bribian et al., 2006); *chemotropic molecules* - like netrins and secreted semaphorins (Spassky et al., 2002); and *the chemokine CXCL1* (Miller et al., 2002; De Castro et al., 2013). Although there is no doubt that these factors play a role in OPC migration, the exact mode of action of these factors is still a matter of controversy, in part due to differences in experimental models, culture systems, and time points studied. OPC migration is not only controlled by secreted molecules, but is also regulated by contact-mediated mechanisms involving many different extracellular matrix proteins and cell surface molecules, N-cadherins, and

possibly even additional, yet unidentified molecules. From all these different molecules, a common theme evolves, demonstrating contact-based migration of OPCs over extracellular matrices, axonal tracts, and astrocytic surfaces. Once located at their final destination, some OPCs persist into adulthood, while the vast majority differentiates to myelin-producing oligodendrocytes (Bradl and Lassmann, 2010).

Globally, the factors described above, highlight how complex and fine-tuning is OPC migration. The control of all these mechanisms ensures the proper migration of these precursors and allow them to migrate long distances and to populate the whole brain and spinal cord.

In humans, myelination is a largely postnatal process and it continues well into adulthood. Practicing a skill can increase the volume of WM regions employed in carrying out the task and, conversely, social isolation, with reduced external stimuli, leads to hypomyelination and impaired cognitive functions (Blumenfeld-Katzir et al., 2011; Gibson et al., 2014; Liu et al., 2012; Makinodan et al., 2012; Sampaio-Baptista et al., 2013). Thus, external stimuli may modulate myelination, which in turn could affect axonal transmission velocity and neural processing (Bergmann and Frise´ n, 2013; Fields, 2008, 2012). In a recent work, Yeung and colleagues demonstrates that the number of OLs in the corpus callosum is established in childhood and remains stable after that.

They found that the number of OPCs is highest in the youngest individuals and dropped during early childhood. Shortly after birth, there are very few mature OLs, but the number increased rapidly in the perinatal period and approach stable numbers at about 5 years of age (88% of the final number). The final number of OLs is reached at 9 years of age. After this age the number of OLs stays largely stable throughout the rest of the human lifespan. The OL population in human WM is remarkably static once the full complement is established, with only 1/300 OLs being exchanged annually, and OL generation cannot account for the increase in myelin volume in response to experience in humans. They conclude that myelin remodeling in WM is independent of cell turnover and mainly carried out by mature OLs in humans (Yeung et al., 2014).

2.1.2 Oligodendrogliogenesis

During migration, the precursors maintain the capability to proliferate but, then, when they reach their final destination, exit the cell cycle and start differentiating in order to acquire a mature phenotype for the formation of myelin sheaths (Greenwood and Butt, 2003).

OPC differentiation is a very complex process during which cells encounter their fate through a series of fine regulated maturation stages, characterized by changes in cell morphologies, cell cycle exit and by the expression of genes and proteins required for maturation and myelination (Fig. 2.3). At the beginning, OPCs have a bipolar morphology and express PDGFR-α, the proteoglycan NG2 (glial antigen 2), the isoform DM-20 of the proteolipidi protein (PLP) and the gangliosides A2B5 and GD3 (Baumann and Pham-Dinh, 2001). Other important markers of these cells are Olig1/Olig2, that are transcriptional factors present throughout all the development and the maturation process. Although these two transcriptional factors are structurally related, their biological functions are only partially redundant. Olig1 has a minor role in OPC specification and development, but it plays a pivotal role in terminal differentiation. By contrast, Olig2 functions at earlier developmental stages. Initially, Olig2 acts to oppose cell differentiation and sustains the replication competent state so as to expand the pool of progenitors. At later stages of development, Olig2 promotes the fate choice decision to form early oligodedrocyte progenitors (Meijer et al., 2012). However, it has been demonstrated that OPC terminal differentiation in the pMN domain requires the presence of both Olig1 and Olig2 and in some regions of CNS the role of ones prevail on the other (Wegner et al., 2008). Zhou and co-workers demonstrated that, during the last phase of OPC development, there is a reduction of Olig2 expression in parallel with an increase of Olig1, while Lu and its team claim that the expression of both genes remains throughout all the development in the adult CNS too (Wegner et al., 2001).

In the developmental brain, cells of the premyelinating stage assume a more complex morphology with many branching processes and are characterized by the expression of the sulfatide O4 (oligodendrocyte marker O4), GPR17, the tetraspanin protein CD9 and DM20. They start appearing in the corpus callosum and, then, they distribute in all the cortical parenchyma between P4 and P10; particularly at P7 all NG2-positive cells express O4 (Levine et al., 1993; Dawson et al., 2003). Subsequently, pre-oligodendrocytes become

immature OLs. In this stage, cells start expressing the galactosylceramide GalC, the myelin-associated enzyme 2', 3'-cyclic nucleotide 3'-phospho-diesterase (CNPase) and the ribosome inactivating protein (RIP), while they start losing the expression of NG2, A2B5, GD3 and GPR17. Pre-oligodendrocytes further progress to the mature stage during which they synthetize the myelin basic protein (MBP), the myelin associated glycoprotein (MAG) and the mature isoform of PLP1 (PLP). Maturing OLs transiently express the CC1 (APC immunogen, clone CC1 or "CC1") during normal oligodendroglial development and oligodendroglial regeneration (Lang et al., 2013). These cells are not able to form myelin yet, since the formation of myelin sheaths requires the contact with neuronal axons. After the establishment of this contact, cells become myelinating cells and start expressing the myelin oligodendrocyte glycoprotein (MOG) (Solly et al., 1996) and the pi-isoform of glutathione-S-transferase (GSTπ) (Tansey et al., 1991).

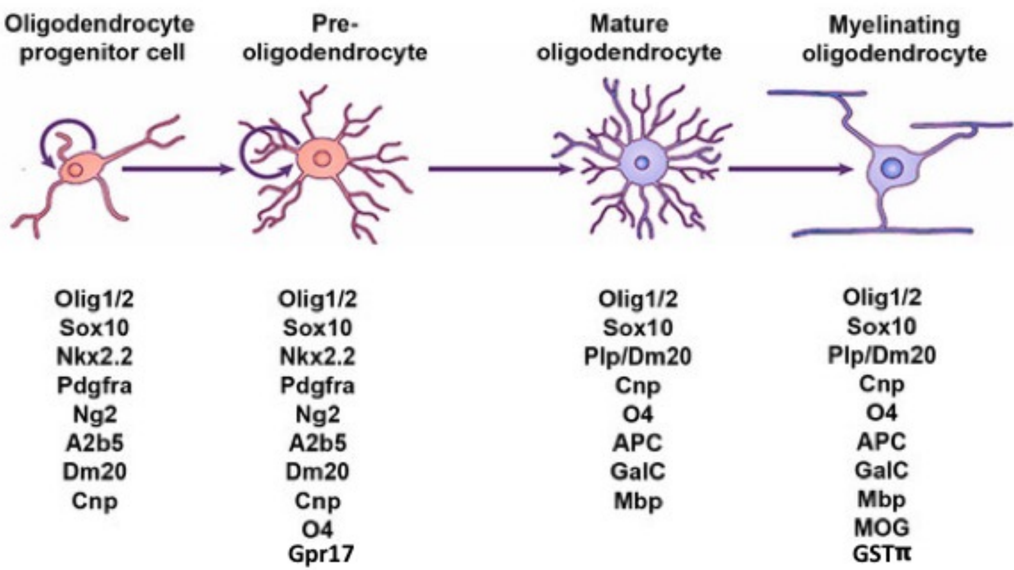

Figure 2.3 – OPC differentiation process. *Schematic representation of the developmental stages of the OL lineage (adapted from Traiffort et al., 2016).*

2.1.3 Regulation of OL differentiation and myelination

As described before, myelin may exhibit substantial plasticity throughout adult life. This has sparked renewed interest in the myelination process given that this plasticity may have profound implications for neural functioning. Every aspect of an OL's life is largely influenced by neuronal and astroglial signals which, in turn, regulate OL survival, proliferation, myelination rate and programmed cell death. An increasing number of studies highlighted the importance of one factor rather than others, in the control of OL behaviour.

It is extremely difficult to extrapolate to in vivo conditions, as multiple factors may act in concert to achieve the fine regulation of the complex process of OL development and myelination. Combinations of factors often produce effects that are significantly different from those seen with any one factor alone (Baumann and Pham-Dinh, 2001; Emery, 2010). Here it will be summarized extrinsic and intrinsic signalling mechanisms controlling OL differentiation and myelination (Fig. 2.4).

Extrinsic signaling mechanisms

- Extracellular ligands and secreted molecules. The simplest mechanism for determining whether an individual axon is myelinated would be the expression of inhibitory or permissive cues for myelination on the surface of the axon itself, with important benefit of allowing control of myelin at the subcellular level, explaining how individual axons proximal to an oligodendrocyte can be myelinated or not. For instance, Jagged, which signals via Notch in OPCs (Wang et al., 1998), PSA-NCAM (Charles et al., 2000), and LINGO-1 (Mi et al., 2005), are all axonal ligands that inhibit either OPC differentiation or myelination. On the contrary, the axonal expression of neuregulins is largely dispensable for myelination (Brinkmann et al., 2008).

  In addition to the above factors, it is almost certain that a number of extracellular ligands that modulate CNS myelination remain to be identified. For instance, GPR17 is transiently expressed during oligodendrocyte differentiation and orchestrates the transition between immature and myelinating oligodendrocytes (Chen et al., 2009). Indeed, GPR17 inhibition, by either antagonists or siRNAs, impaired the normal program of OPC differentiation (Fumagalli et al., 2011).

  The chemokine CXCL12 (also known as SDF-1) and its receptor CXCR4 have well known roles in the patterning and function of the immune and nervous systems where they localize various cell types to specific microenvironments (Klein et al., 2004). In the last years, several in vitro and in vivo studies demonstrated roles for CXCL12/CXCR4 in the migration and maturation of OPCs (Patel et al., 2010; Dziembowska et al., 2005).
- Neuronal activity. In addition to genetically programmed extracellular ligands, there is evidence that myelination is at least in part driven by the level of electrical

activity in the axons themselves. Neuronal activity may modulate the surface expression of the above mentioned axonal ligands or cytokines. Alternatively, release of adenosine by active axons may activate purinergic receptors on OPCs and promote their differentiation and myelination (Stevens et al., 2002). Because OPCs express ionotrophic glutamate receptors and voltage-gated ion channels (Barres et al., 1990), they can respond to this stimulation with a depolarization event not unlike the action potential of a neuron. This suggests an elegant mechanism in which activity of unmyelinated axons is associated with direct synaptic release from axo-glial synaptic junctions onto adjacent OPCs, which differentiate and myelinate the axon at a certain signal threshold.

Intrinsic signaling mechanisms

- Transcriptional regulation. The initial specification of the oligodendrocyte lineage is reliant on the transcription factor Olig2; ventrally derived OLs (and lower motor neurons) are derived from Olig2-expressing subventricular zone progenitors, and the oligodendrocyte lineage is absent in Olig2-null mice (Zhou et al., 2001; Lu et al., 2002). Subsequently, the downstream induction of a number of transcription factors, most notably Olig1, Ascl1, Nkx2.2, Sox10, YY1, and Tcf4, is required for the generation of mature, postmitotic oligodendrocytes (Wegner, 2008). All these factors are present in OPCs as well as in postmitotic OLs, with the exception of Tcf4, which is transiently expressed during differentiation (Fancy et al., 2009; Ye et al., 2009).
- Chromatin remodeling. OL differentiation is also regulated at the level of chromatin remodeling by histone deacetylases (HDACs), as pharmacological inhibition of HDAC activity in postnatal rats causes a delay in OL differentiation and myelination (36). Histone deacetylases likely promote oligodendrocyte differentiation by inhibiting the expression of pathways and genes that otherwise act to block differentiation.
- miRNAs. Post-transcriptional control of gene expression by microRNAs also plays a pivotal role in controlling CNS myelination. Use of microRNA profiling identified several microRNAs, most notably miR-219 and miR-338, that are induced concurrent with OL differentiation. These microRNAs target genes that usually act to maintain OPCs in the undifferentiated state, including PDGFRa, Sox6, and Hes5 (Dugas et al., 2010; Zhao et al., 2010). Recently, miR-125a-3p, a developmentally regulated miRNA, has been proposed as a new actor of oligodendroglial maturation. In cultured

OPCs, over-expression of miR-125a-3p by mimic treatment impairs while its inhibition with an antago-miR stimulates oligodendroglial maturation (Lecca et al., 2016).

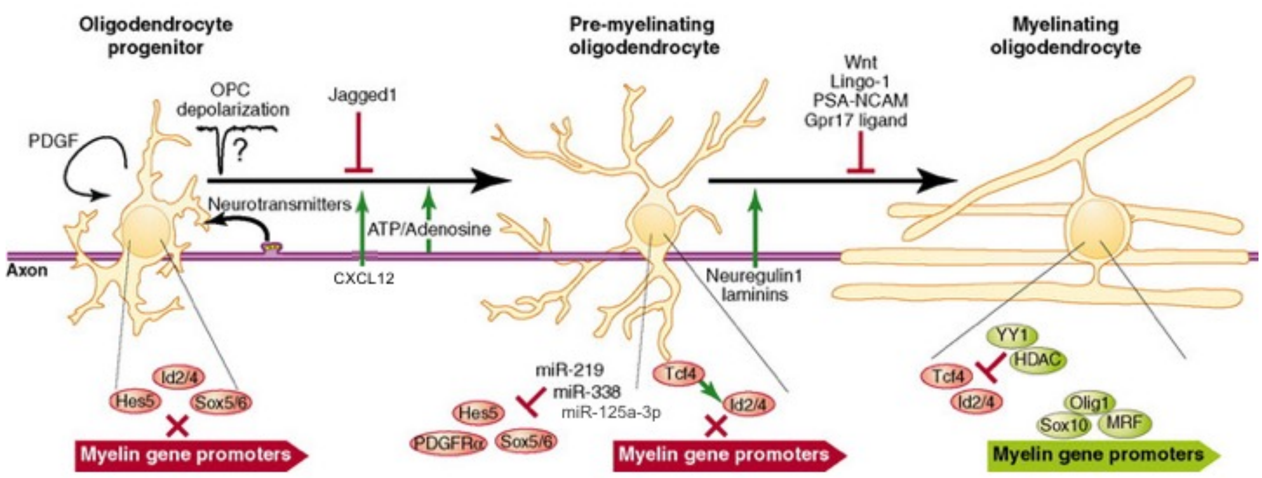

Figure 2.4 – *Schematic representation of the oligodendrocyte lineage showing some of the intrinsic and extrinsic factors that influence oligodendrocyte differentiation and the myelination of individual axons (adapted from Emery, 2010).*

2.1.4 Purinergic signaling and oligodendrogliogenesis

Many studies have revealed a role for purinergic signaling in OL development and death (Butt, 2006; Agresti et al., 2005; Fumagalli et al., 2011; Fumagalli et al., 2016). Since the early ‘90s, ATP has been identified as an important activity-dependent axonal signal that is able to activate purinergic receptors, increasing Ca^{2+} concentrations in OPCs and differentiated OLs. These cells express several P2X (P2X1, P2X2, P2X3, P2X4, P2X7) and P2Y (P2Y1, P2Y2, P2Y6, P2Y12, P2Y13) receptors. Among these, P2X7 and the ADP-sensitive P2Y1 have been identified as the main ionotropic and metabotropic P2 receptor active in OPCs (Agresti et al., 2005). In addition, OPCs have all four adenosine receptors (Fields and Burnstock, 2006).

Adenosine has been demonstrated to act as a potent neuron-glia transmitter, able to inhibit OPC proliferation, stimulate differentiation and promote the formation of myelin in both dissociated culture and cerebellar organotypic slice (Stevens et al., 2002). Indeed, upon application of adenosine, the incorporation of BrdU or 3H-thymidine in $NG2^{+}$-OPC nuclei decreases significantly, even in presence of potent mitogenic factors such as PDGF and bFGF. In parallel, OPCs start to show a more complex morphology and the expression of O4 and O1 (Stevens et al., 2002). Similarly, Agresti and colleagues (2005) demonstrated that ATP and ADP are able to inhibit OPC proliferation in vitro, both in purified cultures and in

cerebellar tissue slices. ATP and ADP, but not UTP have been shown also to induce OPC migration in vitro in dose-dependent manner. The effects of ATP and ADP on cell migration and proliferation are mediated by the ADP-sensitive P2Y1 receptor, as the P2Y1 antagonist MRS2179 completely prevents these changes (Agresti et al., 2005). Moreover, purinergic signaling has been found to stimulate myelination at later stages of OL development, through an indirect mechanism involving astrocyte release of promyelination factors (Ishibashi et al., 2006). In this respect, it has been demonstrated that upon treatment with ATP, 2-methylthio ATP and α,β-methylene ATP, astrocytes are induced to produce the leukemia inhibitory factor (LIF) (Yamakuni et al., 2002; Ishibashi et al., 2006), a cytokine belonging to the CNTF family, which has been shown to stimulate myelination in OPC/dorsal root ganglia (DRG) co- cultures (Ishibashi et al., 2006; Stankoff et al., 2002). Collectively, these findings revealed an important and positive role for purinergic signaling in OL development.

However, ATP signaling through P2X7 receptors has been demonstrated to mediate OL apoptosis (Matute et al., 2007; Neary and Zimmermann, 2009; Domercq et al., 2010). It has been shown that differentiated OLs of spinal cord and optic nerve have a robust expression of P2X7 (Matute et al., 2008). P2X7 channel opening causes rapid depolarization, Ca^{2+} influx and loss of Na^{+} and K^{+} gradients. Thus, its activation can amplify glutamate-receptor mediated excitoxicity. Matute and colleagues have demonstrated that ATP signaling can trigger OL excitoxicity through the activation of P2X7 receptors (Matute et al., 2007). Moreover, it has also been showed that the OL death induced by the activation of P2X7 receptor may have a role in the pathogenesis of diseases characterized by demyelination, such MS. Accordingly, sustained activation of P2X7 receptor in vivo has been shown to cause white matter lesions that are reminiscent of the major features of MS plaques, as characterized by OL death, intense microgliosis, demyelination and axonal damage. These features indicate that excess of extracellular ATP as a consequence of progressive tissue damage in MS may in turn aggravate the development of the pathology. Indeed, administration of Brilliant blue G, a P2X7 antagonist, in EAE mouse model inhibits demyelination and restores axonal conductance (Matute et al., 2007; 2008). This finding can be highly relevant for understanding the etiology of MS, since an increased P2X7 expression has been detected also in normal appearing axonal tracts in post-mortem tissues

from MS patients, suggesting that the elevated expression of this purinergic receptor may be a risk factor associated with early lesion formation in this disease (Matute et al., 2007).
Also the four adenosine receptors take part to OPC differentiation are involved in some OL diseases. For example, the deletion of A1 receptor induces a severe demyelination and causes a progressive form of EAE (Tsutsui et al., 2004). Similarly, the continuous activation of A1 receptor in the brain reduces the MBP expression and induces a damage in the white matter (Turner et al., 2003).
Besides the role of purines in OL development and differentiation, a number of very recent studies have demonstrated the existence of a pyrimidinergic signaling mediated by uracil nucleotides (i.e. UDP and UTP) and their sugar conjugates (i.e. UDP-glucose and UDP-galactose) which has been also reported to modulate OPC proliferation and differentiation (Lecca and Ceruti, 2008). In this respect, UDP, UDP-glucose and UDP-galactose have been shown to act as endogenous agonists at the P2Y-like receptor GPR17 (Ciana et al., 2006; Benned-Jensen and Rosenkilde, 2010), an important regulator of OL maturation (Lecca et al., 2008; Ceruti et al., 2011; Fumagalli et al., 2011). The role of this receptor in OL development will be described in detail in the paragraph 3.4.1. In conclusion, the current data suggest that ATP and other nucleotides, that are released in high concentrations during inflammatory events and after cell death, may take part to the reparative processes in the diseases characterized by demyelination (Lecca et al., 2012).

2.1.5 Structure and formation of myelin sheath

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 and insulation of nerve 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. The importance of myelin in human development is highlighted by its involvement in an array of different neurological diseases such as leukodystrophies and MS in the CNS and peripheral neuropathies in the peripheral nervous system (PNS). Moreover, in recent years, myelin has also generated new

interest for its involvement in normal cognitive function, learning and intelligence quotient and as an unexpected contributor to a wide range of psychiatric disorders, including depression and schizophrenia (Fields, 2008).

Myelin is a spiral structure composed of extensions of the OL plasma membrane. Myelinating OLs send out sail-like extensions of their cytoplasmic membrane, each of which forms a segment of sheathing around an axon, the myelin sheath. This myelin sheath is made up of two different layers: the major dense line (dark layer) formed by the cytoplasmic surfaces of the expanding myelinating processes of the OL and the double intraperiodic line constituted by the overlapping of the outer leaflets of OL membranes (Baumann and Pham- Dihn, 2001).

Myelin has a chemical composition very close to that of cellular plasmatic membranes but it has a peculiar lipid-to-protein ratio: it consists of 70% lipids and 30% proteins. The specific constituents of myelin, glycolipids and proteins are formed in the OL.

Concerning lipids, myelin contains cholesterol, phospholipids, and glycolipids in molar ratios ranging from 4:3:2 to 4:4:2. On the cytoplasmatic side of the membrane there are phospholipids and on the extracellular side there are glycolipids, cholesterol and among them long chain fatty acids. One of the major characteristics of the myelin lipids is their richness in glycosphingolipids, in particular galactocerebrosides (i.e. galactosylceramides, GalC) and their sulfated derivatives, sulfatides (i.e. sulfogalactosylceramides).

The major CNS myelin proteins MBP and PLP (and the immature isoform DM-20) are low-molecular-weight proteins and constitute 80% of the total proteins (fig. 2.5). MBP constitutes as much as 30% of the total proteins and is present in various isoforms of different molecular masses. The main role of MBP is to compact membrane during myelin maturation, whereas PLP and DM20 are mainly myelin constituents.

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 (Wolfgram, 1966). One of these proteins is the CNPase. This is an enzyme representing the 4% of total myelin proteins and known to hydrolyzes artificial substrates 2',3'-cyclic nucleotides into their 2'-derivatives.

Finally, several glycoproteins are present in myelin, such as MAG and MOG. MAG is quantitatively a minor constituent, representing 1% of the total protein found in myelin isolated from the CNS and 0.1% in the PNS. In the adult rat CNS, MAG is confined to the

periaxonal collar of the myelin sheath, whereas a larger distribution across different regions of PNS myelin has been reported.

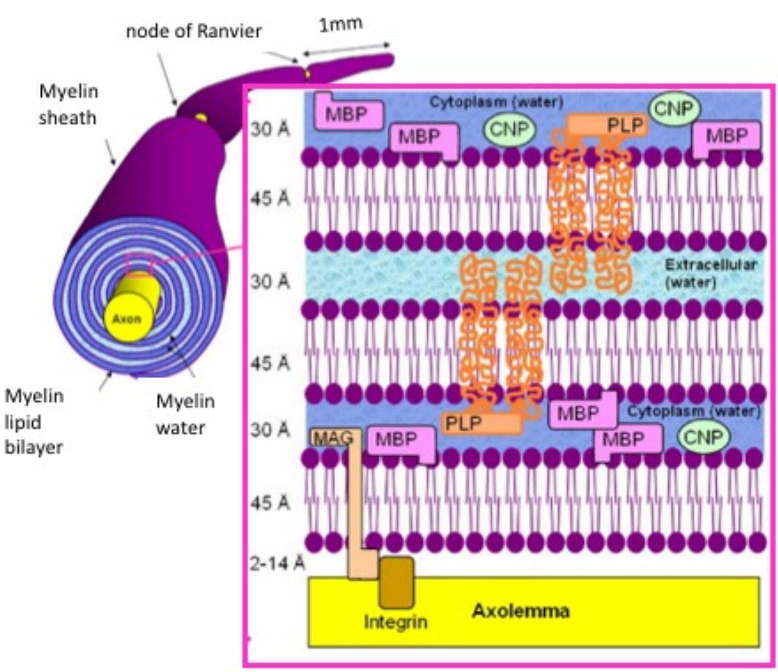

Figure 2.5 – *Schematic representation of myelin structure (modified from Laule et al., 2007)*

MOG was first identified by a polyclonal antibody directed against an antigen called M2 that induces EAE in the guinea pig. It was later identified as a minor glycoprotein specific for CNS myelin and further characterized by immunological methods, immunohistochemistry, and Western blot (WB), using a mouse monoclonal antibody against glycoproteins of rat cerebellum. MOG is only present in mammalian species and is highly conserved between species (Baumann and Pham-Dinh, 2001).

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 OL processes.

Individual OLs myelinate up to sixty axons by extending their cytoplasmic membrane and forming a compact wrapping in segments called internodes (Peters, 1964; Remahl and Hildebrand, 1990). Between two internodes there is a gap called the node of Ranvier. At

these nodes, voltage-gated sodium channels are clustered to conduct action potentials, resulting in a conduction phenomenon called *saltatory conduction*. Myelination permits nerves to conduct action potentials faster and for longer distances, since the insulation provided by the myelin sheaths reduces the current flow across the axonal membrane and increases its transverse resistance. In addition, the saltatory conduction of action potentials from node to node reduces the metabolic requirements of the neurons and increases conduction velocity (Poliak and Peles, 2003).

The development of OLs and myelination of individual axon is a highly regulated process controlled by a number of mechanisms that have been described in paragraph 2.1.3.

The following sequential steps characterize the myelination process: 1) the migration of OPCs to axons that are to be myelinated and their differentiation into highly ramified cells; 2) the adhesion of the OL processes to the axon, 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; 3) the compaction of the myelin layers resulting in the formation of the mature myelin sheath.

During the first step, the preoligodendroglial multiprocessed cells settle along the fiber tracts of the future white matter, maintaining the ability to divide. Then, these preoligodendrocytes become immature OLs, characterized by the acquisition of specific markers (see paragraph 2.1.2) and, then, ready for myelination. The exact mechanism through which axons are wrapped and myelinated by OLs has not been fully elucidated. Several theories have been put forward over the past few years to explain how myelination takes play. Two models have been proposed (Fig. 2.6). In the first, the leading edge of the OL spreads along the axon in a sheet like manner, concludes an initial wrap, and then moves underneath the growing sheet. In the second, the process remains compact and, only when a sufficient number of wraps have been generated by turns around the axon, extends laterally into overlapping sheets. These two theories are clearly not mutually exclusive, and intermediate mechanisms might exist.

The subsequent compaction phase is perhaps the most important part of the myelin sheath formation, because it involves the extrusion of the cytoplasm and both intracellular and extracellular sheet interconnection by specialized proteins, and is thus responsible for the highly specific insulating function of the sheath.

Both models indicate that the formation of myelin requires important morphological changes of OLs which are mediated by alteration of the cytoskeleton (Bauer et a., 2009). In fact, during the first stage of myelination, the extension and the branching of OL processes need the reorganization of the cytoskeleton mediated by extracellular signals, such as bFGF and PDGF secreted by neurons and astrocytes and extracellular matrix molecules such as fibronectin, vitronectin, collagen and laminin. Besides the pronounced cytoskeletal remodeling, the process outgrowth requires the extension of the membrane surface and some alterations in membrane composition. Among these, one prominent feature is the emergence of specialized microdomains in the sheets, termed lipid rafts. Oligodendroglial lipid rafts consist of a tightly packed array cholesterol and glycosphingolipids and phospholipids with saturated acyl chains. The role of lipid rafts is not so clear. Two lines of evidence suggest that one role of rafts must be to direct cytoskeletal assembly for myelination after axo-glial contact; the second ones is to recruit myelin protein. Finally, important morphological changes are also observed in the last stage of myelination, the compaction of the myelin sheath. This step requires retraction, disassembly or reorganization of the cytoskeleton and, concomitantly, relocation of bulky cellular organelles located in the peripheral processes (Bauer et al., 2009).

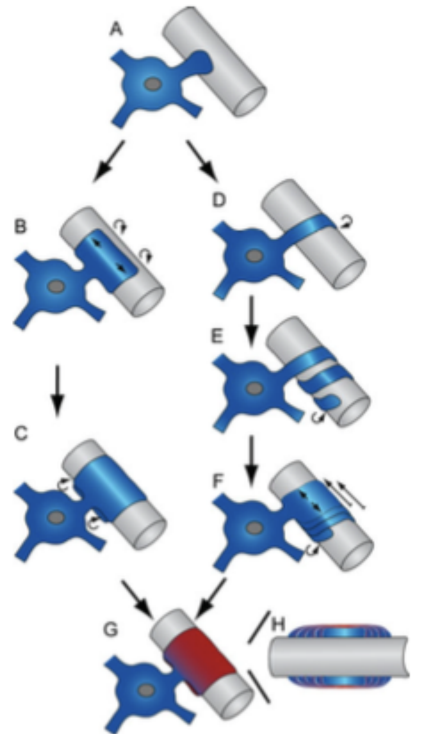

Figure 2.6 – Scheme representing the two models of myelination. *After contact formation of an OL process with an axon (A), the process could flatten into a broad sheet, wrap around the axon (B), and then move underneath itself (C) to form the multiple layers of the mature myelin sheath (G). Alternatively, the process could wrap around the axon-like twine around a post (D), and once the appropriate number of wraps has formed, flatten out and move laterally (E, F) to conclude sheath formation. From the images, it does become apparent that these models are not mutually exclusive, but that intermediate events might contribute to myelination. (H) depicts a longitudinal section through a myelinated axon, detailing the different layers of the compact myelin sheath, with cytoplasm-containing paranodal loops at either end (from Bauer et al., 2009).*

2.3 Demyelination and re-myelination

2.3.1 Demyelination and re-myelination mechanisms

As previously described, myelin integrity plays a fundamental role in the transmission of electrical impulses and it is the result of the proper ensheathment of axons by myelinating cells. OL degeneration causes a pathological process called demyelination. At present, too little is known about the etiology of many demyelinating disorders to enable a precise pathological classification. The alteration and the loss of axonal ensheathment may have different origin and may be caused by genetic defects, infectious agents, autoimmune reactions, chemical agents exposure or unknown factors.

Demyelination can be regarded as either primary or secondary. The primary form destroys or damages myelin or the myelin-forming cells, whereas the axons are relatively normal, at least in the early stages. On the other hand, secondary demyelination (also known as Wallerian degeneration) involves damages to neurons or axons, followed by breakdown of myelin.

From a clinical perspective, there are two major causes of primary demyelination in the CNS: genetic abnormalities that affect glia (e.g. leukodystrophies), and inflammatory damage to myelin and OLs (e.g. MS). Concerning the genetic diseases, they usually appear in childhood with generalized neurological symptoms, such as changes in gait, muscle tone and cognition, and can be subdivided into those that result from defects of lysosomal function (for example, metachromatic leukodystrophy and Krabbe's disease) or perioxsomal function (adrenoleukodystrophy), those that result from abnormal OL myelinogenesis, that is due to deficiencies in or misfolding of key myelin proteins (hypomyelinating leukodystrophies, such as Pelizaeus–Merzbacher disease) and those that result from defects in the astrocytes that provide trophic support for myelinating OLs (Alexander's disease and, probably, vanishing white matter disease).

As previously described, during the initial phase of pathological demyelination, neurons remain intact and they can respond to the loss of myelin with the redistribution and the insertion Na+ channels along the denuded axolemma, to allow non saltatory conduction along the demyelinated segment. This compensatory mechanism allows the transmission of electrical impulse, even though it is less efficient than normal transmission, but impairs Na^{+}- K^{+} activity. This enzyme is responsible for rapidly correcting Na^{+} and K^{+} levels following

an action potential but in these conditions, it does not work properly because of a pathological increase in intra-axonal Na^+ concentrations. As a consequence, there is the involvement of Na^+-Ca^{2+} pumps that, eliminating Na^+, increases intracellular Ca^{2+} levels which, in turn, causes severe damages (Franklin and Ffrench-Constant, 2008).
Besides the formation of myelin sheath, OLs have an important role in maintaining axonal integrity and in promoting neuronal growth by synthetizing neurotrophic factors such as CNTF, IGF and glial cell-derived neurotrophic factor (GNDF) (Dai et al., 2001; Du and Dreyfus, 2002).
Since it seems clear that a damage to myelin and OLs has direct consequences on neurons and, in general, on the entire CNS, it is important to understand the causes of demyelination.
One of the most frequent causes of OL death or dysfunction is oxidative injury. A toxic byproduct of ATP synthesis is hydrogen peroxide, which, if not metabolized, has been shown in vitro to cause DNA degradation and OL apoptosis. Cellular metabolism also creates reactive oxygen species, which are highly toxic and induce lipid peroxidation and DNA damage (McTigue and Tripathi, 2008). Moreover, paradoxically, OLs have low concentrations of glutathione (GSH), a robust antioxidative enzyme. This low glutathione concentration would allow intracellular iron levels rise. If these defenses are not able to keep reactive oxygen species (ROS) levels under the threshold of toxicity, there will be oxidative stress. The oxidative stress is a common feature of many pathological conditions as MS, Alzheimer's disease, spinal cord injury, CNS hypoxia and ischemia (Stankiewicz et al., 2007; McTigue and Tripathi, 2008).
Excitotoxicity is another important cause of OL death. It is the pathological process by which nerve cells are damaged and killed by excessive stimulation by neurotransmitters such as glutamate and similar substances. This occurs when receptors for the excitatory neurotransmitter glutamate (glutamate receptors) such as the NMDA receptor and AMPA receptor are over-activated by glutamatergic storm. Excitotoxins like NMDA and kainic acid, which bind to these receptors, as well as pathologically high levels of glutamate, can cause excitotoxicity by allowing high levels of Ca^{2+} ions to enter the cell. Ca^{2+} influx into cells activates a number of enzymes, including phospholipases, endonucleases, and proteases such as calpain. These enzymes go on to damage cell structures, degrade cytoskeletal proteins and cause mitochondrial disruption leading to reactive oxygen species production

and cytochrome c release. Glutamate release may be a consequence of a trauma or anoxia, which invert the direction of transporters or may be secreted by microglia and macrophages, exchanging glutamate with cysteine. It has also been demonstrated that immature OL can release glutamate via reverse glutamate transport, which can then feedback and damage the cells through calcium influx (McTigue and Tripathi, 2008).

The loss of OLs and of the myelin sheath has dramatic consequences in the CNS but in some particular conditions, our organism is able to face this pathological process and plays out a reparative response called *remyelination*. During this process, the entire myelin sheaths are restored to demyelinated axons, saltatory conduction is reinstated and functional deficits are resolved. Remyelination is a spontaneous process to the myelin damage but, the new myelin sheath, although functional, is shorter and thinner than the original one. For this reason, it is believed that the mechanism of remyelination is essentially different to that of myelination. One hypothesis of this difference is that, while the myelinating OL associates with a dynamically changing axon, that is yet to achieve its full length and diameter, the remyelinating OL engages an axon that is undergoing less change, having already reached its mature size. Therefore, the remyelinating OL is not subjected to the same degree of dynamic changes as the myelinating OL during development. Such changes may, by analogy with other cell types, regulate protein synthesis and a number of intracellular signaling pathways and make an essential contribution to the elaboration of the normal myelin sheath (Franklin and Ffrench-Constant, 2008).

Remyelination follows the steps listed below:

1. Generation of new OPCs
2. Migration of OPCs in the demyelinated area
3. Axon engagement and OPC differentiation
4. Formation of a new myelin sheath

All the steps listed before are in common to the myelination process, but the question where new OLs come from has been a central theme of remyelination research over several decades. Now, it is believed that in most cases remyelination is mediated by new OLs derived from a population of adult OPCs. These proliferating cells are also known as $NG2^{+}$-cells, based on the expression of the membrane chondroitin sulphate proteoglycan NG2 or polydendrocytes to reflect their multi-processed morphology and their lineal relationship to OLs. These cells represent 5-8% of the cell population and are dispersed in both white and

grey matter in various CNS regions and in the subventricular zone (SVZ). These precursors remain in the adult brain in a quiescent state and are able to proliferate and migrate, even if less rapidly, towards demyelinated areas (Franklin and Ffrench-Constant, 2008). Previous time-lapse imaging revealed how $NG2^+$ cells are recruited to sites of focal CNS injury and rapidly migrate and proliferate to restore their density (Hughes et al., 2013). Indeed, NG2 is a core organizer of Rho GTPase activity and localization in the cell, which controls OPC polarity and directional migration (Binamè et al., 2013).
Of note, during the first phases of demyelination, all the neural cells of the damaged area are involved in this process in particular microglia/macrophages and astrocytes which are immediately activated and start releasing different factors that are essential for the beginning of remyelination.
Among these, PDGF and FGF have been found over-expressed after demyelination (Hinks and Franklin, 1999) and some others are the same involved in the embryonic OPC development. For example, FGF and IGF1 have an important role in the inhibition of OLs differentiation but they are responsible of OPC recruitment during remyelination and regulate their transition towards a mature phenotype.
Following recruitment, the OPCs differentiate into remyelinating OLs. This differentiation phase encompasses three distinct steps: establishing contact with the axon that is to be remyelinated, expressing myelin genes and generating a myelin membrane, and finally wrapping and compacting the membrane to form the sheath. Interestingly, in recent years, several studies have provided compelling evidence for a key role of neuroinflammation in re-myelination. As previously mentioned, microglia and macrophages, as said before, are present within the sclerotic plaques typical of the demyelinating diseases and within the inflammatory lesions of neurodegenerative diseases. Even though it is believed that inflammatory factors, proteases and chemical mediators released from these cells contribute to worsen the primary lesions by determining the onset of a secondary neuronal damage, now scientific community has accepted that the microglia activation does not always produce a deleterious effect in CNS. In fact, their main function is to infiltrate the damaged area to phagocytize cellular debris, apoptotic cells and pathogens participating in this way to the reparative mechanisms. Furthermore, microglia and macrophages can counteract cerebral lesions by releasing inflammatory and neurotrophic cytokines and immunosuppressive factors which are able to promote neuronal tissue growth. However, re-

myelination does not always properly occur. The efficiency of re-myelination is affected by the non disease-related factors age, sex and genetic background. These generic factors will have a bearing on the efficiency of re-myelination regardless of the disease process that is involved (Franklin, 2002).

Like all regenerative processes, the efficiency of re-myelination decreases with age. This manifests as a decrease in the rate at which it occurs and is likely to have a profound influence on disease progression (which, in the case of MS, can occur over many decades). The consequences of slow re-myelination are compounded by an age-associated increase in the vulnerability of demyelinated axons to atrophy. The age-associated effects on remyelination are due to a decrease in the efficiency of both OPC recruitment and OPC differentiation. Moreover, during aging the production of cytokines, chemokines and growth factors diminishes (Franklin and Ffrench-Constant, 2008; Franklin and Kotter, 2008).

In addition to these generic factors, remyelination could also be incomplete or fail for disease specific reasons. This may be related to a primary deficiency in precursor cells or to a failure of precursor cell differentiation and maturation (Franklin and Ffrench-Constant, 2008).

It is clear that, in order to stimulate remyelination, it would be important to increase the knowledge on this issue and try to answer many of the still unresolved questions.

2.3.2 OPCs contribute to glial scar formation

After any kind of CNS injury, glial cells become activated and orchestrate the formation of the glial scar. The glial scar is characterized by a high content of extracellularmatrix (ECM) molecules forming an environment known to block axonal regeneration and remyelination (Cregg et al., 2014; Silver and Miller, 2004). While reactive astrocytes are the principal cells present within the glial scar, it is now clear that OPC are not only bystanders during scar formation. Following spinal cord injury, OPCs proliferate and upregulate NG2 expression, thereby inhibiting axonal regeneration (Tan et al., 2005). Rhodes et al. reported that treatment with antimitotic drugs, aimed at diminishing glial scar formation by eliminating OPC proliferation in knife wound injuries, results in a slight improvement in axonal regeneration (Rhodes et al., 2003). This result suggests that OPC proliferation and upregulation of NG2 can be detrimental to axon regrowth. Conversely, OPC expression of

NG2 has also been shown to support axon growth in vitro, even when NG2 was overexpressed in OPCs (Yang et al., 2006).
Chondroitin sulfate proteoglycans (CSPGs) are a key component of the inhibitory glial scar (Davies et al., 1997; Lau et al., 2012) and it have been show that OPCs also produce keratan sulfate proteoglycan after injury, and also neurocan and versican but CSPGs are known to impair CNS repair (Asher et al., 2000, 2002; Jones and Tuszynski, 2002). In conclusion, OPCs clearly participate in the formation of the glial scar and produce CSPGs that impair CNS repair and their differentiation into mature myelinating OL. This apparent duality in function clearly highlights the multi-functionality of OPC in the CNS (Fernandez-Castaneda et al., 2016).

2.3.3 OPCs as innate immune cells

Microglia and astrocytes are considered to be the classic innate immune cells of the CNS because of their response to pathogens or tissue damage and their ability to recruit peripheral immune cells (Ransohoff and Brown, 2012). However, the literature contains evidence that OPCs are not simple spectators of the CNS immune response. In a mouse model of cerebral prolonged hypoperfusion, OPCs are the initial producers of MMP9, an enzyme necessary for degradation of the extracellular matrix, prior to the appearance of white matter damage (Seo et al., 2013). Furthermore, authors demonstrated that OPC derived MMP9 could mediate the opening of the BBB and the infiltration of neutrophils that ultimately damage the myelin sheath in this model (Seo et al., 2013). Moyon et al. have recently demonstrated that OPCs isolated from the brain of mice undergoing cuprizone-induced demyelination express high levels of CCL-2 and IL-1ß (Moyon et al., 2015). CCL-2 has a critical role in recruiting monocytes (Deshmane et al., 2009), while IL-1ß is a powerful inflammatory cytokine involved in many aspects of the immune response (Sims and Smith, 2010). While authors did not explore the role of these mediators on immune cell recruitment/ activation, they discovered that CCL-2 promotes OPC migration in vivo and is also expressed by OPCs present in active multiple sclerosis (MS) lesions (Moyon et al., 2015). Recent work by Gadani et al. also highlights the role that myelinating glia play in repair after spinal cord injury. Oligodendrocytes release IL-33, a nuclear alarmin implicated in orchestrating the recruitment of peripheral immune cells necessary for CNS repair (Gadani et al., 2015). Although predominantly expressed by oligodendrocytes, OPCs also

express IL-33 and may participate in promoting CNS repair (Gadani et al., 2015b). Beyond producing inflammatory mediators, OPC can respond to cytokines and chemokines, such as TNF-α IL-1ß and IFN-γ (Moore et al., 2016; Arnett et al., 2001; Vela et al., 2002). The impact of these molecules on OPCs remains controversial, with reports suggesting both a beneficial outcome on myelination and induction of OPC death. For example, TNFα is highly expressed in demyelinating MS lesions and has been shown to potentiate IFN-γ-induced cell death in vitro (Andrews et al., 1998; Watzlawik et al., 2010). Arnett et al. showed a modest delay in cuprizone-induced oligodendrocyte death in TNF-α knockout mice, implicating it as a potentially harmful cytokine (Arnett et al., 2001). Unexpectedly, the study also showed that TNF-α knockout mice suffered from impaired remyelination (Arnett et al., 2001). Mice lacking TNF-α showed decreased decreased OPC proliferation and, as a result, less oligodendrocytes were ultimately generated, accounting for decreased remyelination (Arnett et al., 2001). This data implies that TNF-α has a dual role in both demyelination and remyelination.

A recent study with a TNF receptor 2 (TNFR2) conditional KO mice, with selective TNFR2 ablation in oligodendrocytes, provided an evidence for TNFR2 as an important signal for oligodendrocyte differentiation. They demonstrated that oligodendroglial TNFR2 is a key mediator of tmTNF-dependent protection in EAE. In fact, following activation by transmembrane TNF, TNFR2 initiates pathways that drive oligodendrocytes into a reparative mode contributing to remyelination following disease (Madesen et al., 2016).

Furthermore, another study demonstrated that OPCs respond to the T cell produced cytokine, IL-17, and actively participate in the amplification of pathology in EAE (Kang et al., 2013b). Finally, OPCs have been shown to be phagocytic in vitro, as isolated cultures of OPCs can efficiently engulf myelin debris (Gaultier et al., 2009). Further investigation should be performed in vivo to determine if OPC could participate in the clearance of cellular debris in vivo, during CNS diseases, especially considering their rapid recruitment at the injury site. In conclusion, OPCs also contribute to the immune response in the CNS by producing and responding to inflammatory mediators in a multitude of pathologies (Fernandez-Castaneda et al., 2016).

Chapter 3

The G protein coupled receptor GPR17

3.1 Identification and gene cloning

GPR17 is a G-protein coupled receptor (GPCR) that was first identified thanks to a screening of a human cDNA library for P2Y-receptor homologous sequences (Blasius et al, 1998). Two distinct transcripts of the human GPR17 receptor (hGPR17) were identified to be generated by alternative polyadenylation, resulting in the generation of two different isoforms lately named short (hGPR17-S) and long isoform (hGPR17-L) (Pugliese et al., 2009; Benned- Jensen and Rosenkilde, 2010). Subsequently also the rat and mouse GPR17 orthologues were identified and cloned, showing 80% homology in the amino acid sequence to the hGPR17 (Ciana et al., 2006; Lecca et al., 2008).

The expression pattern and functional characterization of GPR17 have been studied since few years, and our knowledge is therefore based on few in vitro studies on the hGPR17, and several in vitro and in vivo studies for the rGPR17 and mGPR17 (rat and mouse GPRI7) but there is an increasing interest for this peculiar receptor due to its strategic time-restricted expression during oligodendrogenesis.

Here, the main structural, pharmacological, functional characteristics and expression profiles of GPR17 receptor so far known are reported.

Although GPR17 amino acidic sequence is conserved among species, the gene structure is pretty different. The hGpr17 gene is indeed located on chromosome 2 and it is composed of four exons, of which only two contain coding sequences. The rat and mouse Gpr17 gene are both located on chromosome 18. rat Gpr17 (rGpr17) is composed only by one exon, whereas the mGpr17 (mouse Gprl7) has two different exons and only a small portion of the second exon encodes for the GPR17 protein.

3.2 GPR17, a promiscous receptor: structure and pharmacological properties

The receptor is named GPR17 because before its endogenous ligands were described, it was classified as an orphan GPCR, since it displayed the same structure of G protein coupled receptors, with 399 amino acids including 7 transmembrane domains, an N- and a C-terminus (Ciana et al., 2006; Lecca et al., 2008). As already mentioned, GPR17 structure is phylogenetically related to two classes of GPCR receptors: P2Y receptors and CysLT

receptors (Figure 3.1). The former class of receptors (P2Y receptors) includes P2Y1, 2, 4, 6, 11,12,13,14, that respond to adenine nucleotides (ATP adenosine-5 '-triphosphate and ADP adenosine monophoshate), uracil nucleotides (UTP uridine triphosphate and UDP uridine diphosphate) and sugar nucleotides (UDP-glucose and UDP-galactose). CysLT receptors, namely CysLT1 and CysLT2, are also a class of GPCRs responding solely to cystenil-leukotrienes such as LTD4, LTC4 and LTE4. Initially, GPR17 showed pharmacological responses to both classes of ligands, therefore it has been one of the first GPCRs to be classified as a "hybrid" or "dual" receptor (Ciana et al, 2006; Parravicini et al, 2008); recently, it has been demonstrated that GPR17 is a promiscous receptor and new ligands have been identified.

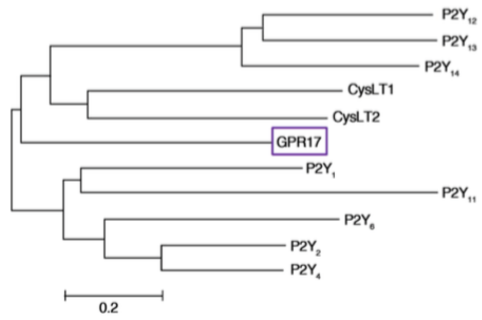

Figure 3.1 – Phylogenetic tree that represents the relationship between GPR17 and P2Y and CysLT receptors (from Ciana et al., 2006)

3.2.1 GPR17 agonists

In the last few years, many studies have been performed in order to clarify the pharmacological profile of this receptor. To find the natural ligands of GPR17, the cDNA of the short form of the human receptor was cloned in an expression vector pcDNA3.1, that was transfected in 1321N1 cells, COS-7 and HEK-293. $^{[35S]}$GTPγS binding studies showed that in 1321N1 cells, which do not express functional P2Y receptors (Communi et al., 1999), the expression of GPR17 induces concentration-dependent responses to LTC4 and LTD4 (with LTC4>>LTD4) and UDP, UDP-glucose and UDP-galactose (with UDP- galactose=UDP>UDP-glucose), whereas no responses were identified for ligands as ATP, ADP, 2-methyl-thio-ADP, UTP, α,β-methylene-ATP and guanosine (Ciana et al., 2006).

Moreover, additional studies conducted in 1321N1 cells expressing the GPR17 receptor demonstrated that prolonged exposure of these cells to agonists UDP-glucose and LTD4 determines the desensitization of the receptor, followed by a process of resensitization

consequent to the removal of agonists (Daniele et al., 2011). More recently, GPR17 has been reported to also respond to emergency signals signals like oxysterols, in a similar way to other related receptors involved in inflammatory responses (like EB2 and CXCR2) (Sensi et al., 2014) and to the chemokine SDF-1 (stromal derived factor-1 or CXCL12) that can specifically activate GPR17; it has been also demonstrated that cangrelor, a GPR17 orthosteric antagonist, can block the SDF-1-mediated activation of GPR17 in a concentration-dependent manner (Parravicini et al., 2016).

Pharmacological studies were conducted also on the rodent GPR17 receptor. The pharmacology of the rat GPR17 (rGPR17) is identical to that of human as regards the type of agonists, but shows variations in the order of the potency (LTD4>LTC4; UDP - glucose>UDP, and UDP-galactose has no effect). On the contrary, from the pharmacological studies conducted on the murine receptor emerges that the mouse GPR17 (mGPR17) has a pharmacological profile more similar to that of the human receptor (UDP>UDP - galactose>UDP-glucose; LTE4>LTD4>LTC4) (Lecca et al., 2008). Transfection of GPR17 in other cell lines such as COS-7, HEK-293 and CHO confirmed the results obtained in 1321N1 cells (Ciana et al., 2006).

It is interesting to emphasize that the EC50 values of the tested agonists are in agreement with the known characteristics of the P2Y receptors (micromolar, µM) and for cystenyl-leukotrienes; in fact, for the first, the EC50 is comprised in a range while for the cysteinyl-leukotrienes (nanomolar).

The long form of the human GPR17 receptor (hGPR17) was characterized pharmacologically too. Similarly to the short form, treatment with both uracil and cysteinyl- leukotrienes agonists induces the appearance of concentration-dependent responses to LTC4 and LTD4 (with LTC4 >LTD4) and also to UDP, UDP-glucose (UDP-glucose) and UDP- galactose (with UDP-galactose=UDP>UDP-glucose). However, while the values found for the cysteinyl-leukotrienes not show significant differences with those described for the short form, UDP-glucose appears to be slightly more potent on the long form (Pugliese et al., 2009). One explanation for these differences has been given by recent studies of molecular dynamics simulation on the short form of the receptor. These studies suggest that, in addition to an inner pocket for the binding of the nucleotides, GPR17 has an accessory site in a region formed by three extracellular domains (EL): EL2, EL3, and the N-terminal sequence. This binding site could drive small extracellular agonists towards the main inner binding site,

leading to receptor activation (Parravicini et al., 2008; 2010). Even though, modeling studies on the long form of the receptor have not yet been described, it is thought that the N-terminal sequence longer 28 amino acids than the long ones, might change the conformation of this accessory binding site, influencing the affinity of nucleotide agonists (Pugliese et al., 2009), thus explaining the observed differences between the two isoforms.
Similar to these studies, the group of Benned-Jensen has performed experiments in transfected HEK293 cells, demonstrating that the ligands UDP, UDP-glucose and UDP-galactose activate the two isoforms of the receptor GPR17, with more power on the long ones. Contrary to the above mentioned studies, they did not find receptor activation by cystenil- leukotrienes, LTD4 and LTE4 (Benned-Jensen and Rosenkilde, 2010; Nörregaard et al., 2011).
However, recent results in recombinant in vitro systems suggest that the GPR17 receptor may also act through a ligand-independent way. It appears that GPR17 behaves as a negative allosteric modulator of the CysLT1 receptor, that is able to respond to LTD4, through the formation of a dimer receptor-receptor. This action of GPR17 does not seem to be due to its activation by endogenous ligands (Maekawa et al., 2009).
In contrast with these results, the group of Qi demonstrated that, in C6 and CHO cells stabling expressing GPR17, neither UDP, UDP sugars, nor cysteinyl-leukotrienes activate GPR17 (Qi et al., 2013). The reasons for the discrepancies between the results obtained by different laboratories still remain to be clarified.

3.2.2 GPR17 antagonists

In the studies aimed at characterizing GPR17 pharmacological profile, the activity of some known purinergic and cysteinyl-leukotrienes antagonists was also assayed. As for agonists also for the antagonists the $^{[35S]}$GTPγS binding assay was performed in 1321N1 cells expressing the receptor (long or short isoform of the human receptor, mouse and rat receptor). As nucleotide antagonists both cangrelor (N(6)-(2-methyl-tioetil)-2-(3,3,3-trifluoropropiltio)- beta,gamma-diclorometilene-ATP), which is known to antagonize P2Y12 and P2Y13 (Ingall et al., 1999; Marteau et al., 2003; Fumagalli et al., 2004) and MRS2179 (2'-deoxy-N6-metiladenosina3',5'-bisphosphate) which antagonizes P2Y1 (Jacobson et al. 2002) were assayed; both molecules inhibit in a concentration-dependent the binding of the $^{[35S]}$GTPγS stimulated by UDP -glucose, with nanomolar IC50 values.

It was also demonstrated that a non-hydrolyzable analog of ATP, ATPßS, which does not act as an agonist neither on the short nor on the long form of the human receptor (Ciana et al., 2006), may act as an antagonist in blocking in a concentration-dependent manner the activation of GPR17 induced by UDP-glucose and LTD4. The fact that this compound behaves as an antagonist is in agreement with the capacity of cangrelor and MRS2179, two derivatives of ATP (Abbracchio et al., 2006), to act as antagonists of GPR17.
As cysteinyl-leukotriene antagonists, montelukast and pranlukast were assayed (Brink et al., 2003; Capra et al., 2006). Both these two compounds inhibit the activation induced by LTD4 of human, rat and mouse receptor, with values nanomolar IC50 typical of CysLT1.
Due to the dual nature of this receptor, it was therefore hypothesized the existence of two distinct binding sites on the receptor: one for nucleotides and one for the cysteinyl-leukotrienes.

3.3 GPR17 expression pattern in the CNS

So far the majority of the studies on GPR17 have been performed in rodent models (rats and mice). However, the receptor was first identified in a cDNA library from human hippocampus (Blasius et al, 1998). The expression of GPR17 in the human brain was lately confirmed by mRNA analysis from different human tissues (Ciana et al, 2006). hGPR17 is expressed at high levels in organs or tissues that can undergo ischemic damage, namely brain, heart and kidney (Ciana et al., 2006). The long form is, instead, expressed exclusively in the brain, especially in the cortex and striatum (Pugliese et al., 2009; Benned-Jensen and Rosenkilde, 2010).
Other studies showed GPR17 expression on human and mouse mastocysts and bone marrow derived monocytes (Maekawa et al, 2009; Maekawa et al, 2010), where the receptor seems to tightly co-localize with CysLTR1. Finally, and most interesting, a trascriptome analysis performed on human and mouse adult neural stem cells and fetal embryonic tissue revealed expression of the receptor specifically in adult neuroprogenitors (Maisel et al, 2007).
Concerning the GPR17 expression in the brain, the same pattern of expression observed in human has also been confirmed in rats and mice (Ciana et al, 2006; Lecca et al, 2008). In fact, immunohistochemistry experiments carried on in our laboratory demonstrated that, in rat and mouse brain cortex, GPR17 is expressed by two distinct cell types:

• neurons: where it co-localizes with typical neuronal proteins, SMI-311 (neurofilament marker), ß-tubulin (neuronal specific tubulin) and NeuN (neuronal nuclear protein)

• numerous cells dispersed in both grey and white matter, displaying small cell bodies with fine radiating processes and expressing typical OPC markers, such as NG2 and Olig2.

No co-localization of GPR17 was found with more mature myelinating oligodendroglial markers, such as CNPase, MAG and MBP, suggesting that GPR17 may be expressed at a specific stage of oligodendroglial differentiation.

In mice and rats, GPR17 was never found in astrocytes (no co-localization with GFAP, glial fibrillary acidic protein), and in non-activated microglia (no co-localization with Iba) assuming therefore that the receptor is not localized in these cell types (Lecca et al., 2008). GPR17 is instead co-expressed with the marker IB4 in activated microglia (Lecca et al., 2008).

Recently, the first characterization of GPR17 expression in neurosurgical and autoptic samples from patients with traumatic brain injury (TBI) has been published. In this study through immunohistochemistry analysis it has been demonstrated that GPR17 co-localizes with various specific markers of brain cells. Specifically, it has been shown that, in the damaged area, the receptor is expressed by injured neurons (NeuN^{+} in post-surgical samples; MAP2^{+} in autoptic samples), by a subpopulation of hypertrophic astrocytes (GFAP^{+}) with a stellate morphology, by infiltrating cells that participate in the processes of repair engulfing the dead cells, microglia/macrophages (IB4^{+} tissues in post-surgical samples, Iba1^{+} in autoptic samples), and OPCs (CNPase^{+} and O4^{+} in autoptic samples). Furthermore, through a spatio- temporal gradient analysis, it was shown that GPR17 expression is very strong in cells inside and at the borders of the necrotic core. These cells are activated microglia/macrophages; more externally GPR17 is expressed by activated astrocytes. Concerning neurons, a reduction of GPR17 expression has been observed as the distance from the injured area increased; finally, in the most distal areas, GPR17 is also found in ramified cells expressing O4 and CNPase, thus confirming that they are OPCs. These results confirm the findings from the studies in rodents concerning the distribution of the receptor in the damaged brain and its involvement in repair processes (Franke et al., 2013).

3.4 Transduction signaling pathways activated by GPR17

Studies on the system of signal transduction showed that, in cells transfected with GPR17, the receptor is mainly coupled to Gi protein that inhibits the activity of the enzyme adenylate cyclase and consequently the formation of cAMP (Ciana et al., 2006). It was also demonstrated the activation of Gq protein which, in turn, induce the activation of phospholipase C (PLC) and the increase of intracellular calcium levels. However, this transduction system does not seem to represent the main signaling pathway used by the receptor, as only 30% of the cells transfected with GPR17 is able to couple to PLC (Ciana et al., 2006).

Similarly, studies carry on in rat primary OPCs, which express physiologically GPR17 during a specific temporal window of their differentiation process (see paragraph 2.1.2), confirmed that this receptor is coupled to G_i and that its activation after the exposure to GPR17 agonists reduces cAMP levels (Fumagalli et al., 2011). Furthermore, the siRNA-induced GPR17 knock-down supports the hypothesis that the effects on cAMP levels observed after treatment with GPR17 agonists are specifically mediated by this receptor: in fact, they are completely obliterated in GPR17-silenced cells. No effect on the calcium intracellular levels was observed after GPR17 agonists exposure in primary OPCs, suggesting that, in this native system, GPR17 is not associated with protein-G_q (Fumagalli et al., 2011).

It has also been demonstrated by electrophysiological studies in 1321N1 cells stably expressing the hGPR17 that the activation of the receptor with micromolar concentrations of UDP, UDP-glucose and UDP-galactose and with nanomolar of LTD4 leads to an increase of the outward potassium currents while the treatment with MRS2179, specific antagonist for P2Y1, but also active on GPR17, blocks this effect (Pugliese et al., 2009). It seems that the effect observed is due to the activation of large-conductance, Ca^{2+}-activated BK (or maxi-K) potassium channels widely expressed in human glioma cells (Basrai et al., 2002). These ion channels react to increases in intracellular Ca^{2+} and membrane depolarization, which follow an acute ischemic stroke, by increasing K^+ efflux, rapidly hyperpolarizing the membrane and reducing further voltage-dependent Ca^{2+} influx (Gribkoff et al., 2001).

More recently, it has been demonstrated that, after the immature oligodendrocyte stage, to enable cells to complete maturation, GPR17 is physiologically down-regulated via phosphorylation/desensitization by G protein-coupled receptor kinases (GRKs); conversely,

GRKs are regulated by the "mammalian target of rapamycin" mTOR. However, how GRKs and mTOR are connected to each other in modulating GPR17 function and oligodendrogenesis has remained elusive. Fumalli and co-workers showed that, in maturing OPCs, both rapamycin and Nutlin-3, a small molecule inhibitor of Mdm2-p53 interactions, increased GRK2 sequestration by Mdm2, leading to impaired GPR17 down-regulation and OPC maturation block (Fumagalli et al., 2015).

3.5 GPR17 function

Studies aimed at understanding GPR17 function have been performed in two tissues: the CNS (brain and spinal cord) and the immune-system (Ceruti et al, 2011; Ciana et al, 2006; Lecca et al, 2008; Maekawa et al, 2010; Mao et al., 2013). Only one study has been published on the modulation of the immune response to allergenes by GPR17 activation (Maekawa et al, 2010). As previously mentioned GPR17 is co-expressed on dendritic cells together with CysLTR1. In case of forced allergene (dust) exposure, GPR17 seems to negatively modulate the response of CysLTR1-mediated inflammatory cell accumulation. This observation opens the possibility to use GPR17 ligands to positively modulate the immune reaction to allergenes.
On the other hand, more numerous are the studies aimed at understanding GPR17 function in the CNS. GPRl7 therapeutic potential was first assessed in a rat model, and subsequently in a mouse model, of ischemia (MCAo, medial cerebral artery occlusion) (Ciana et al, 2006; Lecca et al, 2008). rGPR17 was shown to be expressed in neurons, OPCs and in microglial cells upon pathological condition. The application of a P2Y antagonist and more specifically the use of anti-sense oligonucleotide targeting GPR17 are sufficient to reduce the penumbra area of the ischemic damage. It is not clear, however, if the ischemic damage reduction is due mainly to the effect on a specific cell type or if it was more general. Therefore, in this case GPR17 appears to be pro-inflammatory and its blockage, with the antagonist and anti-sense oligonucleotide, improves functional recovery (Ciana et al, 2006; Lecca et al, 2008).
Use of the anti-sense oligonucleotide, gave positive results in terms of damage reduction and functional recovery also in a model of spinal cord injury (SCI), again suggesting an active role of GPR17 in damage propagation (Ceruti et al, 2009). In this scenario the

function of GPR17 was described to be temporal and cellular specific; the first response to injury provokes neuronal and OL death through GPR17 activation; later, the recruitment of microglia and GPR17^{+}-macrophages seems to shed the first steps of tissue remodeling and repair, that was followed by the up-regulation of GFAP, as a marker of stem-cell like properties, on GPR17 positive ependymal cells. The multiplicity of roles exploited by GPR17 in the SCI model can be explained by the diverse actions that its endogenous ligands, abundantly released during injury, may play.

In a similar way, acute damage to myelin induced by lysolecithin injection in corpus callosum induced a strong over- expression of GPR17 at the lesion site 10 days after injury (Boda et al., 2011).

The fact that GPR17 may act as sensor for WM damage has also been confirmed in an animal model of periventricular leukomalacia (PVL), that is the most common ischemic brain injury in premature infants. In this work, it has been shown that GPR17^{+}-glial progenitor cells, within the white matter, together with the progenitors cells of the SVZ, are significantly increased after an ischemic event and migrate towards the lesion to support its repair. However, the endogenous self-repair capacity of these cells appear to be limited, since the more mature OLs do not completely recover from experimental ischemia. This limitation is likely associated with cerebral microenvironmental factors, such as ischemia-induced intracellular calcium overload, excitotoxicity caused by overstimulation or secondary effects from over-expression of certain immediate-early genes (Mao et al., 2013).

In line with these findings, treatment of highly proliferating neurospheres from murine oligodendroglioma cells with UDP-glucose, UDP or LTD4 reduced proliferation and expanded the pool of Olig2^{+} OLs, suggesting that GPR17 activation directs cells to differentiation (Dougherty et al., 2012).

Taken together the data described above suggest that GPR17 represents an interesting target to develop new approaches to foster tissue repair in different pathological conditions.

3.5.1 Role of GPR17 in OL differentiation

In 2009, Chen and colleagues demonstrated, through a chromatin immunoprecipitation (ChIP), that the transcription factor Olig1 can directly bind to the promoter of GPR17 and

regulates it negatively. Accordingly, GPR17 was also identified as one of the genes down-regulated in Olig1 mutant mice (Chen et al., 2009). In this work, GPR17 has been proposed as a negative regulator of the OPC differentiation. Both in vitro and in vivo (using a mouse model) it has been demonstrated that GPR17 over-expression blocks OPC maturation and, in some cases, induces death. In fact, although the precursors are properly generated, they are not able to proceed in the differentiation process and, as a consequence, there is a defective myelinogenesis and hypomyelination (Chen et al., 2009).

In vitro studies in rat postnatal OPC cultures, showed that GPR17 expression labels a specific temporal window of OL differentiation process. Specifically, during the early stages of differentiation, GPR17 receptor decorates two subsets of slowly proliferating cells. The former corresponds to early, morphologically immature slowly proliferating $NG2^+$ precursor cells that also express Olig2, PDGFRα, and the immature PLP isoform DM-20; the latter corresponds to more ramified, still immature pre-oligodendrocytes that are losing NG2 and PDGFRα immunoreactivity and already express O4, O1, and the two splicing variants of the myelin protein PLP. After this differentiation stage, GPR17 expression is progressively turned down, and the GPR17 protein is never found in fully mature MAG^+ or MBP^+ OLs (Fumagalli et al., 2011).

In this cells, it has been demonstrated that GPR17 receptor has a functional role during the differentiation process; in fact, its activation by UDP-glucose (100 μM) or LTD4 (100 nM) for 72 hours, induced a significant increase in the number of MBP^+-cells (Lecca et al., 2008; Fumagalli et al., 2011). Additional in vitro data also showed that, GPR17 antagonists (for example, cangrelor) or knock-down by siRNAs impaired the normal differentiation program of OPCs (Fumagalli et al., 2011). These data suggest that GPR17 may be involved in the control of the transition from OPCs to mature myelinating OLs enabling the repair or damaged myelin.

In support to these data, recent studies have also shown that GPR17 activation by UDP-glucose enhances delayed rectifier K^+ currents without affecting transient K^+ conductances. This effect was observed in a subpopulation of OPCs ($NG2^+$) and immature pre-OL ($O4^+$) whereas it was absent in mature OLs, in line with GPR17 expression, that peaks at intermediate phases of OL differentiation and is thereafter down-regulated to allow terminal maturation. The effect of UDP-glucose on K^+ currents is concentration-dependent, blocked by the GPR17 antagonists MRS2179 and cangrelor, and sensitive to the K^+ channel

blocker tetraethyl-ammonium (TEA), which also inhibits OL maturation. Moreover, the effect observed parallels the expression of distinct voltage-dependent currents during OPC differentiation. In fact, while in OPC progenitors most currents are represented by outward rectifying conductances (Ik), in mature OL inwardly rectifying currents (Kir) prevail. Altogether these data suggest the enhancement of IK currents after UDP-glucose exposure is responsible for the GPR17-mediated facilitating effect in OPC maturation (Coppi et al., 2013). It was also observed that the receptor has a different intracellular localization depending on the differentiation stage: during the first day in culture, it was present within the compartments of synthesis of OPCs, while in more advanced differentiation stages it was found on the cell surface and within the endosomal compartments. This observation suggests that the subcellular distribution of GPR17 is linked to the maturation of these cells. In this regard, an in vitro study in Oli-neu cells, an immortalized OPC cell line, showed that the exposure to UDP-glucose and LTD4 induces GPR17 internalization, which is delivered into early endosomes and sorted either to lysosomes for degradation or recycled to the cell surface via small G-protein Rab4-dependent pathway. GPR17 down-regulation is the results of these two processes and it seems to be a key event to allow OPCs to proceed to myelination (Fratangeli et al., 2013).

In astrocyte-OPC mixed cultures, it has been demonstrated that the expression of GPR17 receptor is markedly influenced by culturing conditions (Ceruti et al., 2011). In the presence of growth factors (GFs), no significant GPR17 expression is found. On the contrary, when cultures are shifted to a differentiating medium, a dramatic, time-dependent increase in the number of highly branched GPR17$^+$-cells is observed. Under these conditions, GPR17 is induced in the totality of O4$^+$-immature OLs. In the same work, it was also shown that the addition of ATP to the culture medium without GFs induces cell death, suggesting that GPR17 act as danger signal in the presence of high extracellular ATP concentrations. The influence of culturing conditions on GPR17 receptor was also demonstrated in Oli-neu cells line, in which the expression of the receptor is increased by factors released from neurons and astrocytes (Fratangeli et al, 2013).

An in vivo study has shown that, in cerebral cortex, during mouse brain development, GPR17 expression in the oligodendroglial lineage precedes the production of myelin. Immediately after birth, the receptor is not expressed, but it progressively appears in an increasing fraction of OPCs that stop proliferating and start differentiating up to covering

the majority of immature OLs (Boda et al., 2011). Similarly to what observed during the development in the rodent cerebral cortex, GPR17 expression begins to be detectable soon after birth within the spines and gradually increases until P14. Subsequently, it undergoes a strong decline, in parallel with the beginning of myelinogenesis. Finally, according to Chen and co-workers, GPR17 prevents OPC differentiation allowing the translocation into the nucleus of transcription factors that act as potent repressors OPC differentiation: ID2 and ID4. In line with these findings, in vitro studies showed an over- expression of ID2 in proliferating OPCs, in which the differentiation is slowed or stopped; the levels of this repressor are instead reduced during OPC maturation towards mature myelinating OLs. It is known that these repressors are able to translocate into the nucleus by passive diffusion thanks to their small size, but during OPC differentiation, they are internalized in this compartment through an active transport mechanism. Once inside the nucleus, they form a complex with Olig1/2 and E47, so that they prevent these factors to bind DNA, resulting in inhibition of OPC differentiation. On the contrary, during OPC differentiation, ID2 is found in the cytoplasm associated with Enigma Homolog (ENH), a cytoskeletal protein, thus allowing Olig1/2 and E47 to bind DNA and to induce the transcription of myelin genes. Specifically, Olig1 represses GPR17 thus promoting OPC maturation (Kondo and Raff, 2000; Wang et al., 2001; Chen et al., 2009; 2012). It has to be highlighted that, in the GPR17 over-expressing mice used in this work, GPR17 expression is under the control of the CNP1 promoter (Chen et al., 2009). As mentioned above, our studies suggest that, GPR17 levels are strongly reduced when CNPase starts appearing in immature OLs. Thus, GPR17 forced expression at this stage of differentiation, could seriously affect and compromise the oligodendrogliogenesis, making this animal model highly contestable. However, these data demonstrate a close relationship between GPR17 and the myelination process, suggesting a possible role of this receptor in demyelinating diseases.

3.5.2 GPR17 alterations in demyelinating diseases

GPR17 is abnormally upregulated in neurodegenerative conditions characterized by myelin disruption, independently of the original cause. In fact, it was observed that, both in patients with MS and in EAE mice, GPR17 levels are higher compared to controls, and this alteration may explained the failure of remyelination observed in this pathological conditions (Chen et al., 2009). Furthermore, as described in the previous paragraphs, after

a traumatic, ischemic or demyelinating injury GPR17^{+}-OPCs accumulate within the damaged area and take part to the reparative processes (Lecca et al., 2008; Ceruti et al., 2009; Boda et al., 2011). In contrast with the data described above, in a recent paper, Hennen and co-workers proposed GPR17 as a negative regulator of myelinogenesis (Hennen et al., 2013). In this paper this group claimed that the receptor should be block in order to promote OPC maturation. Specifically, they showed that in OPC cultures from heterozygous (GPR17$^{+/-}$), but not homozygous (GPR17$^{-/-}$) mice, MDL29,951 markedly attenuate the capacity of OLs to differentiate suggesting that the addition of GPR17 antagonists to anti-inflammatory drug cocktails that are already used in the management of MS may be an innovative strategy to promote remyelination (Hennen et al., 2013).

Despite the conflicting results obtained by different groups within the scientific community, GPR17 remains an attractive candidate for the developing of new pharmacological compounds. Further studies will clarify whether it will be more useful to obtain molecules enabling the activation or the block of this receptor.

3.6 GPR17 as a pharmacological target for MS

In the last years, the importance of GPR17 as a promising therapeutic target for the treatment of neurodegenerative diseases characterized by demyelination and other human diseases has been gradually consolidated. The need to have new molecules able to modulate the activity of this receptor is an important starting point for improving the atomic and molecular knowledge of GPR17. In this respect, the approach of the "drug discovery" is a good strategy.

3.6.1 in silico identification of new GPR17 ligands

In case of receptors such as GPR17 whose molecular structure is still unknown, the “homology modeling” is a powerful approach to study ligand-receptor interactions. Moreover, in several cases this technique allowed to successfully predict the ligand-receptor interaction.

Currently, two homology models of hGPR17 have been built: the first one is entirely based on the crystal structure of bovine rhodopsin (bRh), the only high-resolution crystal structure of a GPCR available when the model was published. This model allowed to successfully predict and characterize the binding site of endogenous ligands and various derivatives of GPR17 (Parravicini et al., 2008; 2010; Calleri et al., 2010). Few years later, thanks to

protein engineering, a chimeric structure of GPR17 was built (Fig. 3.2) (Eberini et al., 2011).

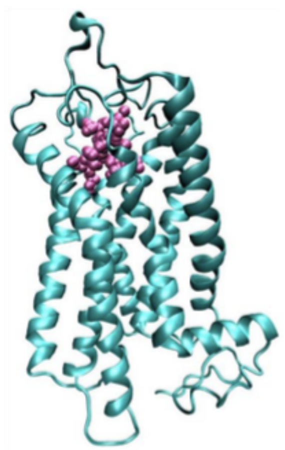

*Figure 3.2 -**Tridimensional model of human GPR17 receptor.** The color spheres represent the binding site predicted for extracellular nucleotides on the receptor.*

Merging the templates of the human adenosine A2A receptor (hA2AR), the human ß2-adrenergic receptor (hß2AR), the turkey ß1-adrenergic receptor (t ß1AR) and squid Rh, this model was more accurate, allowed the identification of five candidate agonist or partial agonist molecules, belonging to very different chemical classes, able to modulate GPR17 activity with higher potency and efficacy than the endogenous reference compounds. This represents the first step towards the rational identification of candidate molecules for the development of entirely novel drugs for demyelinating and ischemic diseases, for which no effective therapy is yet available (Eberini et al., 2011, see also below). Recently another molecule has been proposed as potential agonist of this receptor, the MDL29,951 (Hennen et al., 2013). Specifically, using different targeted pharmacological assays (measurement of cAMP accumulation, $^{[35S]}$GTPγS incorporation, inositol phosphate accumulation, Ca^{2+} release, label-free DMR and bioimpedance, and BRET assays), Hennen and co-workers demonstrated that in cell transfected with GPR17, stimulus with MDL29,951 is able to activate the entire set of intracellular second messengers and their reciprocal cooperation. Specifically, through cAMP assay, they showed a dual modulation of the adenylyl cyclase, suggesting an engagement of either the Gs and Gi mediated pathways. The coupling to G_i subunit after treatment with MDL29,951 was further demonstrated to block the inhibition of forskolin-induced cAMP increase with the pertussis toxin (PTX). Moreover, functional GPR17-mediated $G\alpha_q$ activity was confirmed by a specific inhibition of Ca^{2+} flux induced by

the agonist. The same effects/responses were not found in control cells, suggesting that the activation of the various intracellular cascades is a consequence of the modulation of GPR17 induced by the ligand MDL29,951 (Hennen et al., 2013).

3.7 GPR17 fluorescent reporter mouse line: GPR17-iCreER^{T2}

In collaboration with the University of Munich, we generated a novel BAC transgenic mouse line that expresses the improved Cre-recombinase (iCre) fused to the modified estrogen receptor (iCreER^{T2}, as described in Simon et al., 2012) under the control of the Gpr17 promoter, and the GFP (green fluorescent protein) under the control of the viral CAG promoters (Fig.3.3). This mouse line allowed us to specifically label and monitor the fate of GPR17-glia over time without affecting the physiological expression and function of GPR17. Indeed, upon tamoxifen administration, thanks to the activity of a Cre recombinase, cells expressing GPR17 and their progeny are labeled by GFP, costitutively expressed after recombination, thus allowing us to trace their destiny throughout animal’s life (Viganò et al., 2015).

In these mice, Viganò and co-workers found that, under physiological conditions, GPR17^{+}-cells did not differentiate within 3 months, a peculiarity that was overcome after cerebral damage induced by acute injury or ischemia. After these insults, GPR171 NG2-glia rapidly reacted to the damage and underwent maturation, suggesting that they represent a ‘reserve pool’ of adult progenitors maintained for repair purposes (Viganò et al. 2015).

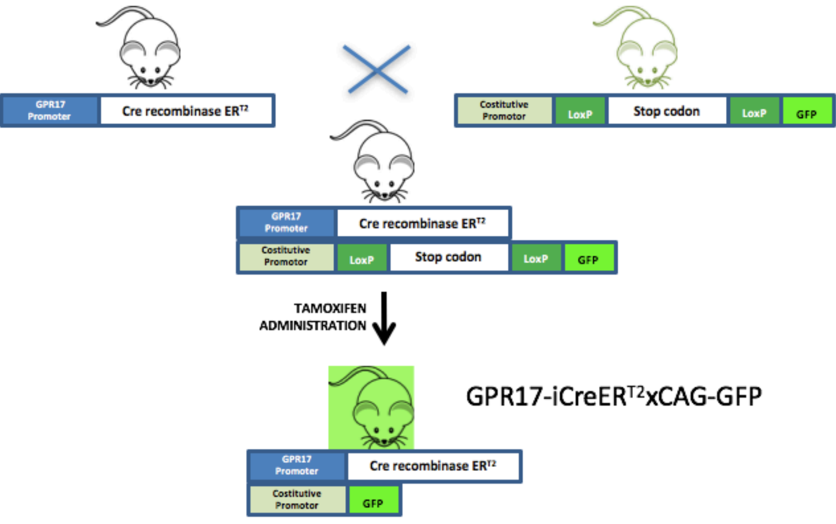

Figure 3.3 - Generation of the GPR17iCreER^{T2}-GFP reporter mouse line

AIM OF THE STUDY

Chapter 4

Aim of the study

MS is an immune-mediated demyelinating disease of the CNS and it is one of the principal causes of neurologic deficit and motor disability in young adults in western countries (Ellwardt & Zipp, 2014). MS is characterized by neuroinflammation and demyelinating plaques, where myelin is attacked by the immune system (Crawford et al., 2013). Myelin is a fundamental component of nerve transmission since it enables impulse saltatory conduction from a Ranvier node to another, making nerve transmission faster and more efficient compared to unmyelinated fibres.

Progressive myelin degeneration impairs transmission along nerve fibres in both brain and spinal cord (El Waly et al., 2014).

A number of DMTs are presently available for the treatment of MS and most of them are immune-modulatory treatments able to control the clinical course of the disease. Although they are proved to reduce the frequency of relapses in MS, they are only effective in about 50-60% of patients with the relapsing- remitting form of the disease (40-50% of the patients are "non-responders"). In a significant percentage of patients (with primary progressive and secondary progressive MS) currently available treatments are proved to be ineffective. Immune-modulatory treatments target only the immune mediated processes of MS, although it has been established that type III and type IV MS subtypes are characterized mostly by OL apoptosis and loss, resembling rather a neurodegenerative disease (Denic et al., 2011).

Treating neurodegeneration and halting the progression of disability still remain unmet needs. Therefore, there is a paramount need to discover new approaches of treatment for the non-immune mediated aspects of MS.

Two challenges confront us: (i) to develop cocktails of therapies able to shift the immune homeostasis of MS patients toward a healthy profile, and (ii) to identify and modulate the activity of targets within the neurodegenerative component of MS.

Re-myelination, the generation of new myelin in the adult nervous system, is an endogenous repair mechanism that restores function of denuded axons and delays their deterioration. Although re-myelination can be extensive in some patients, in the majority

of cases, repair is limited only to the acute phase of disease. A significant current drive in new MS therapeutics is to identify targets that can promote re-myelination by boosting endogenous OPCs to form new myelin, with the final aim to delay disease progression and recover lost neurological functions.

There are many potential strategies to directly promote myelin repair, and, in this respect, the GPCR superfamily is particularly interesting, since it represents the largest class of functionally selective drug targets for disease modulation and therapy. GPCRs have been studied in great detail in CNS neurons, but have been relatively understudied in glia. In recent years, however, exciting new roles for GPCRs in glial cell biology have emerged. Here, we focus on the key role of the GPR17 receptor as a regulator of myelinating glial cell development, the OPCs, and myelin repair in the most studied animal models of MS and in human MS lesions.

Originally identified as an incomplete sequence of a human GPCR many years ago (Blasius et al., 1998), GPR17, a G_i coupled receptor inhibiting cAMP formation, is mainly expressed in the CNS, in particular on OPCs (Lecca et al, 2008; Boda et al., 2011, Chen et al., 2009, Fumagalli et al., 2011). Since then, much interest on GPR17 has been aimed at understanding its function in CNS myelination.

In vitro experiments demonstrated that early GPR17 silencing profoundly affects OPCs ability to generate mature oligodendrocytes, suggesting that, in the absence of this receptor, cells are retained at a less differentiated stage and do not progress to maturation (Fumagalli et al., 2011). However, interferences with GPR17 physiological downregulation at late stages of OPC differentiation also maintained cells at a more immature phenotype (Fumagalli et al., 2015). In line with this result, CNP-GPR17 transgenic mice aberrantly and un-timely expressing GPR17 in late stage maturing OPCs showed defective myelinogenesis, motor disabilities, tremors and precocious death within the second week of life, likely due to a disrupted regulation of terminal maturation (Chen et al., 2009). Globally, these findings suggest that GPR17 exerts opposite stage-specific roles: a positive role for differentiation in early OPCs and a negative function for oligodendroglial maturation in late OPCs. They also suggest that, in late OPCs, physiological GPR17 silencing is needed to allow cells to complete their maturation program.

While physiologically GPR17 is mostly an oligodendroglial receptor, within 48 h after acute injury, GPR17 is induced, sequentially, in dying neurons inside and at the borders of the

ischemic/traumatic lesion, in infiltrating microglia/macrophages and in activated parenchymal OPCs in the lesion's surrounding areas, with similar expression patterns in different models of disease (Boda et al, 2011, Ceruti et al, 2009, Ciana et al, 2006, Lecca et al, 2008, and Zhao et al, 2012). Thus, GPR17 is abnormally upregulated in neurodegenerative conditions characterized by myelin disruption, independently of the original cause.

Based on current knowledge on GPR17, it could be hypothesized that, after damage, GPR17 is initially induced to promote re-myelination and repair; thus, pharmacological interventions targeting GPR17 may help to bypass this checkpoint, accomplishing terminal maturation and promoting neurorepair. In this respect, GPR17 is an ideal target, since it is a membrane receptor that, at variance from other intrinsic regulators of oligodendrogenesis, can be easily targeted and manipulated with pharmacological agents.

On this basis, in order to assess whether this receptor can be proposed as a pharmacological target in re-myelinating strategies, the aims of this thesis were:

1. Determining the physiological mechanisms that regulate GPR17 expression during oligodendrogenesis;
2. Identifying alterations in GPR17 in the most studied animal models of MS;
3. Testing new pharmacological/biotechnological strategies to correct GPR17 dysfunction in MS and resume myelination.

In particular, we investigated the role of GPR17 in MS pathophysiology through i) its analysis in accepted animal models of MS, i.e., EAE and cuprizone models, and ii) its characterization in human MS lesions.

The use of animal models has been necessary to characterize the role of GPR17 in MS pathophysiology. Animal models are indeed important to assess the predictivity and feasibility of therapeutic approaches directed to new targets, and their appropriate use has led to the development of the most recent treatments approved for MS (Kipp et al., 2012).

For this part of the study, we also took advantage of a new GPR17 transgenic reporter mouse line for fate-mapping studies, that allowed us to determine the final destiny of the GPR17^{+}-cell population during disease induction and at distinct time-points after the disease onset.

Regarding the point aim to unveil the mechanisms that could modulate GPR17 expression during oligodendrogenesis, for this thesis project, we focused on the effects of "inflammatory" stimuli.

It is known that cellular interactions between pro-inflammatory immune cells and CNS-resident cell promote CNS tissue injury. In contrast, "anti-inflammatory" immune cells/molecules can positively influence CNS-resident cells promoting tissue repair and/or neuroprotection (Bennet J.L., et al., 2009).

To get insight on the effects of immune cells and the other CNS cells on OPC differentiation and GPR17 expression we performed in vitro experiments, by exposing OPCs to either medium conditioned by reactive astrocytes or to different cocktails of cytokines typically released by polarized T-cells. Moreover, in light to the recent findings that GPR17 promiscuously bind CXCL12, we also evaluated if this chemokine, already demonstrated to have a pro-differentiating effect on OPCs, could exert its function, not only via its known receptor CXCR4, but also through a direct interaction with GPR17.

Finally, since there are no histological studies describing the pattern of GPR17^{+}-cells in human MS lesion, we studied the expression profile of GPR17 in human cerebral tissues from MS patients in respect to the lesion type. GPR17 characterization in human samples is important for future development of drugs against this target. Indeed, in our laboratory we are now testing new compounds, modulating GPR17, in the EAE model (according to the aim *"Testing new pharmacological/biotechnological strategies to correct GPR17 dysfunction in MS and resume myelination"*). The results of these experiments are not shown in this thesis because they are still ongoing.

MATERIALS AND METHODS

Chapter 5

Materials and methods

5.1 EAE experiment

5.1.1 EAE induction

EAE was induced in 8-week-old female wild-type C57Bl/6 mice (Charles River) by subcutaneous immunization in the flanks and in the tail base with 200µg of myelin oligodendrocyte glycoprotein (MOG_{35-55}, Espikem) per mouse in IFA (Sigma Aldrich) supplemented with 8 mg/ml of Mycobacterium tuberculosis (strain H37Ra, Difco). Mice immunized received 500 ng of pertussis toxin (PTX, Duotech) intravenously the day of the immunization and 48 h later. Animals were daily weighted and scored for clinical symptoms of EAE according the following scale: 0 = healthy, 1 = flaccid tail, 2 = ataxia and/or paresis of hindlimbs, 3 = paralysis of hindlimbs and/or paresis of forelimbs, 4= tetraparalysis, 5 =moribund or death. Non-EAE controls received PTX injections, as well as the initial injections of emulsion but without the encephalitogen, to ensure that observed effects are due to EAE and not to a non-specific reaction to the ancillary components that are used to facilitate disease induction. Animals were sacrificed 21 days post immunization (dpi) for histological and real-time PCR analysis.

5.1.2 EAE in transgenic mice

EAE was induced also in female adult mice (2- to 3-month-old) of the GPR17-iCreERT2xCAG-eGFP mouse line. Tamoxifen (40 mg/mL; Sigma Aldrich) was diluted in ethanol (final concentration 10%) and corn oil. Mice received for three times (every second day) 10 mg tamoxifen suspension by gavaging (for a total of 30 mg) 2 weeks before EAE induction and were sacrificed 21 dpi for histological and real-time PCR analysis.

5.1.3 Histology and immunofluorescence

Mice were anesthetized with chloral hydrate (400 mg/kg, Sigma Aldrich) and perfused transcardially with saline-0,1M EDTA (Sigma Aldrich) followed by 4% neutral buffered formalin (Sigma Aldrich) in deionized water. Spinal cords were collected and post-

fixed for 1h in the same solution at 4 °C, cryoprotected in 30% sucrose for 24h (until the tissue sinks to the bottom of the tube), embedded in OCT and then frozen at –80°C. Spinal cords were cut transversally into 20um-thick sections with a cryostat and processed for immunofluorescence.

Slides were incubated for 45 minutes at room temperature with a blocking solution composed by 10% normal goat serum and 0.1% triton-x 100 in PBS1x.

Then, the sections were incubated with primary antibodies (see table 5.1) overnight at 4°C in PBS1x with 5% goat normal serum and 0.1% Triton-X 100. The next day, the sections were exposed for 1 h at room temperature to secondary antibodies and Hoechst33528 was used to visualize cell nuclei. After processing, sections were mounted on microscope slides with fluorescent mounting medium (Dako).

GPR17 labeling was amplified with the high sensitivity tyramide signal amplification kit (Perkin Elmer) according to the manufacturer's instruction.

Name	**Company**	**Dilution**
GPR17	Home-made	1:10,000
Olig2	Millipore	1:500
NG2	Millipore	1:200
CC1	Calbiochem	1:50
GSTπ	MBL	1:500
GFAP	Cell Signalling technology	1:500
NeuN	Millipore	1:50
Iba1	Wako	1:1000
GFP	Aves Labs	1:1400

***Table 5.1** – List of all the primary antibodies used to perform immunohistochemistry.*

5.1.4 In situ hybridization

Slides were dried at RT for 5 minutes. After one wash in PBS1x-DEPC, slides were postfixed in 4% paraformaldehyde for 5 minutes, washed in PBS1x-DEPC, with SSC2x for 5 minutes each and then with Tris-Glycine 0.1M (pH7) for 20 minutes. Sections were permeabilzed in protease K (20 µg/ml) in PBS1x for 10 min at 37°C. The activity of protease K was stopped by fixation in 4% paraformaldehyde for 5 min, followed by 2x5 min washes in PBS1x to remove fixative from the sections.

Slides were incubated in 0.25% acetic diaminobenanhydride (Sigma-Aldrich) with 0.1 M triethanoloamine (pH 8.0) (Sigma-Aldrich) for 10 min at RT, followed by washing 2xSSC for 10 min. Digoxigenin-labeled cRNA (0.3µg/ml) of either antisense or sense probes were added to hybridization buffer containing 50% formamide, 10% dextran sulphate, 1x Denhardt's solution, 4xSSC, 250 µg/ml Salmon sperm, 10mM DTT and 250 µg/ml E. coli tRNA (RNase-free). Hybridization was carried out for 16 h at 55°C in a hybridization oven. The sections were washed in 2xSSC for 30 min at 52°C, 3x5min in 0.2xSSC at 52°C, 2x5min in 2xSSC at 52°C and then let to cold in PBS1x at RT. The following protocol was used to detect the hybridization signals. Briefly, the sections were first incubated in the blocking buffer containing 3% bovine serum albumin (Sigma-Aldrich) and 0.1% Triton X-100 in PBS at RT for 30 min, and then with anti-digoxigenin sheep IgG Fab fragments conjugated to alkaline phosphatase (Roche Boehringer Mannheim) diluted 1:500 in the blocking buffer o/n. Slides were washed with PBS1x for 3x5 min, followed by washing in Buffer 1 (0.1M Tris-HCl, pH 7.5, 0.1M NaCl), then equilibrated in Buffer 2 (0.1M Tris-HCl, pH 9.5, 0.1M NaCl, 0.05M MgCl). The colour development was performed with 400µg/ml nitro blue tetrazolium, 200µg/ml 5-bromo-4-chloro-3-indolyl phosphate and 100 µg/ml levamisole in buffer 2 in the dark at RT for 10 minutes. The sections were rinsed in distillate water to stop the colour development, then dried by soaking slides in successive baths of ethanol (50%, 75%, 95%, 100%) and xylene for 10 minutes. Slide were mounted with DPX (Sigma-Aldrich).
Mouse GPR17 cDNA sequences (sense: 5' GATGAACGGTCTGGAGGCAGCC3'; antisense: 5'CTCACAGCTCGGATCGGGCAC3') were inserted into a T-vector (pBlu2KSM-T). Digoxeginin-labeled RNA probes were synthesized following the manufacturer's instructions (Roche).

5.1.5 Cell counts

After immunofluorescence, one section from each level (cervical, thoracic, and lumbar-sacral) of the spinal cord was analysed for every animal. Seven arbitrary non-overlapping sample fields were counted separately in WM and six fields in GM of every section at 40x magnification (Fig. 5.1).

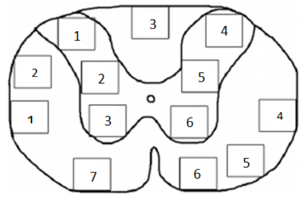

Figure 5.1 - *Schematic representation of a spinal cord transversal section indicating the locations of the sample boxes (area=0.035 mm^2) used for cell counts.*

5.2 In vitro experiments

5.2.1 Primary cultures, OPCs isolation and treatment

Mixed glial cultures were obtained from 12 postnatal day 2 (P2) Sprague-Dawley rat cerebral cortices pooled together. The shaking protocol (Chen et al., 2007) allowed the sequential isolation of OPCs, astrocytes and microglia from the same preparation.

OPCs were seeded onto 13 mm-glass coverlisps (15,000 cells/coverslip) coated with poly-D,L-ornithine-coated (final concentration 50 µg/ml; Sigma-Aldrich) coverslips for immunocytochemistry and poly-D,L-ornithine-coated 12-wells plates (30,000 cells/coverslip) for qRT-PCR assays. Cells were plated in Neurobasal medium supplemented with 2% B27 (Life Technologies), 2 mM L-glutamine, 10 ng/ml human platelet-derived growth factor BB (Sigma-Aldrich), and 10 ng/ml human basic fibroblast growth factor (Life Technologies) to promote proliferation (proliferating medium, PM). When OPCs reached a 60% confluency, cultures were switched to a Neurobasal medium lacking growth factors and containing triiodothyronine 15 nM (T3, Sigma-Aldrich) to allow differentiation (differentiating medium, DF).

To assay the effects of reactive astrocytes on oligodendrocyte differentiation, OPCs were incubated with supernatants derived from astrocytes activated by FBS starvation for 48 hours, thereafter cells were fixed and stained.

To determine the direct effects of cytokines on OPC differentiation, OPCs were treated for 48 hours with two different cocktails of cytokines mainly released by Th1- Th2- polarized T cells: TH1 (IL-1ß,30ng/ml; IL-12,10ng/ml; CXCL12, 1.2nM; IL-17, 25ng/ml) and TH2 (IL-4,10ng/ml; IL-6,10ng/ml; IL-10, 10ng/ml). Thereafter cells were fixed and stained.

To determine the effects of CXCL12 on OPC differentiation, 24h after being in DM, OPCs were treated with human beta CXCL12 (1.2 nmol/l, Sigma-Aldrich) or vehicle for 48 hours and then fixed at room temperature (RT). Cells were pre-incubated for 30 min with the

antagonists cangrelor (10 nM/l, The Medicines Company, Parsippany, NJ) or AMD3100 (1 μmol/l, Sigma-Aldrich), then treated with CXCL12.

5.2.2 Astrocyte culture and generation of astrocyte-conditioned medium

Astrocytes, obtained as described previously in 5.2.1., were plated in T75 flasks with DMEM containing 10% FBS (fetal bovine serum), penicillin/streptomycin, and glutamine. All cells were kept under sterile conditions in incubators at 37˚C. After 2 weeks, the medium was changed and replaced with a new one without FBS; astrocytes were cultured in this conditions for 10 days, after that the conditioned medium was collected, filtered and storage at -20°C.

5.2.2 Immunocytochemistry and cell counting

Cells were fixed in a 4% paraformaldehyde phosphate-buffered solution containing 4% sucrose. The following primary antibodies were used: rabbit anti-GPR17 (1:100; Cayman Chemical), rat anti-MBP (1:200; Merck Millipore). Incubation of primary antibodies were performed 2.5 hours at room temperature or over-night at 4°C. Cells were then incubated for 1 h at room temperature with secondary antibodies conjugated to either AlexaFluor 488 or AlexaFluor 555 (1:600; Life Technologies). All the antibodies were diluted in a phosphate-buffered blocking solution (pH 7.4) containing 0.3% Triton X-100. Nuclei were labeled with the UV fluorescent dye Hoechst 33258 (1:10,000; Life Technologies). Coverslips were then mounted in a fluorescent mounting medium (Dako). Positive cells for the selected markers were counted from 20 random fields for each coverslip (0.07 mm^2/field).

5.3 Total RNA extraction, retrotranscription and gene expression analysis

Total RNA was extracted from cells or tissues using Trizol reagent (Life Technologies).
For gene expression analysis, cDNA synthesis was performed starting from 800 ng of total RNA using SuperScript® II Reverse Transcriptase (Life Technologies). The expression of all genes was analysed using Sybr-green reagents (Bio-rad) and normalized to GAPDH expression using CFX96 real time PCR system (Bio-rad) following the manufacturer’s protocol. The Ct values were elaborated with the Comparative CT method (ΔΔCT) which allows the relative quantification of template comparing the expression levels of the interested gene with the ones of the housekeeping gene.

5.4 Analysis on human MS tissue

5.4.1 Tissue Source

The tissues supplied by the UK Multiple Sclerosis Tissue Bank at Imperial College, London, were collected postmortem with fully informed consent from both donors and close relatives. Procedures for retrieval, processing, and storage have gained ethical approval from all appropriate committees. The brain tissues analysed in this study were from 9 neuropathologically confirmed cases of MS (see Table 5.2). Analysis was performed also on samples from patients who died due to non-neurological diseases. Cerebral hemispheres were fixed with 4% paraformaldehyde for about 2 weeks, coronally sliced, and blocked. Individual blocks were cryoprotected in 30% sucrose for 1 week and frozen by immersion in isopentane precooled on a bed of dry ice. Frozen tissue blocks were stored at –80°C.

CASE	AGE (YEARS)	SEX	DTPI (h)	DISEASE COURSE	DISEASE PHASE	CLINICAL DIAGNOSIS
MS122	44	M	16	Secondary progressive	Progression.	Chronic multiple sclerosis.
MS200	44	F	20	Secondary progressive.	Stable.	Chronic multiple sclerosis.
MS234	39	F	15	Relapsing progressive.	Progression.	Chronic multiple sclerosis.
MS242	57	F	12	Secondary progressive.	Progressive.	Chronic multiple sclerosis.
MS297	58	F	8	Secondary progressive.	Progressive.	Pathological features of necrotising myelitis.
MS300	56	F	13	Secondary progressive.	Progressive.	Chronic multiple sclerosis.
MS325	51	M	13	Primary Progressive.	Progressive.	Chronic multiple sclerosis.
MS317	48	F	21	Secondary progressive.	Progressive.	Chronic multiple sclerosis.

Table 5.2 – Summary of patient details (DTPI, death-tissue preservation interval)

5.4.2 Lesion Detection and Classification

Cryostat sections (12 µm thick) were stained with Luxol fast blue, in order to detect WM lesions, or subjected to immunohistochemistry for MOG (1:200, Proteintech), in order to distinguish GM lesions. HLA (1:50, Dako) staining was performed to assess inflammation. Lesions were classified according to Van Der Valk and De Groot (2000).

5.4.3 Immunohistochemistry

After quenching endogenous peroxidase by a 10-min incubation with 1% H_2O_2 in methanol at -20°C, sections were incubated for 30 minutes in PBS 5% NGS at 4°C, with primary

antibodies as specified in 5.4.2 and in table 5.1. Sections were then incubated either with biotinylated secondary antibodies, followed by avidin-biotin-peroxidase reactions (Vectastain, ABC kit, Vector), using 3,3#-diaminobenzidine (Sigma-Aldrich) as a chromogen, or with fluorescent secondary antibodies (for immunofluorescence, as described in 5.1.3). The sections were rinsed in distillate water to stop the colour development, then dried by soaking slides in successive baths of ethanol (50%, 75%, 95%, 100%) and xylene for 2 minutes each. Slides were mounted with DPX (Sigma-Aldrich).

5.5 Statistical analysis

Data are presented as mean ± standard error (SEM) of replicates. GraphPad Prism 6.0 was used for statistical analysis. For all comparisons between two groups with a normal distribution, two-tailed unpaired t-test was performed. For multiple comparison testing, one-way analysis of variance (ANOVA) accompanied by Dunnett’s post-hoc test was used. P values < 0.05 were considered statistically significant.

RESULTS

Chapter 6

Results

6.1 In mouse spinal cord, GPR17 is expressed in cells of the oligodendroglial lineage

As a first step, we characterized the expression pattern of GPR17 in control mouse spinal cord. The GPR17 receptor protein was present in a relatively high number of cells throughout the whole spinal cord of adult mice (Fig. 6.1A), where it clearly decorates oligodendroglial cells, as demonstrated by co-localization with the typical oligodendroglial transcription factor Olig2 (Fig. 6.1 panel B, B'). In particular, it is expressed in early OPCs with a bipolar phenotype positive for NG2, but also in maturing oligodendrocyte precursors positive for CC1 (Fig. 6.1 panel C, D). No co-localization was found with the astroglial or microglial markers like GFAP and Iba1 and the neuronal marker MAP2 (Fig. 6.1 E-G). Thus, we confirmed that also in spinal cord, as previously shown for brain (Lecca et al., 2008; Boda et al., 2011; Ceruti et al., 2011), GPR17 is specifically expressed in a subset of oligodendrocyte precursors at an intermediate stage of differentiation.

6.2 Characterization of GPR17 expressing cells in EAE

The mRNA of GPR17 was previously found to be up-regulated in EAE, but the expression of the receptor protein was not investigated in detail. For this reason, we induced EAE, followed disease development for 21 days, sacrificed mice during disease acute phase, explanted the spinal cord and analysed GPR17 expression by qPCR and, in parallel, by immunohistochemistry.

As shown in Fig. 6.2 A, the disease evolved as expected with the first clinical symptoms appearing around day 10 after MOG immunization, followed by a strong and progressive neurological impairment peaking between day 16 and 21.

Real-time PCR analysis showed that GPR17 expression was almost doubled in EAE spinal cord compared to controls (Fig. 6.2 B), along with two inflammatory cytokines (i.e. IL-1ß and TGF-ß), that confirmed the presence of strong and acute inflammation (Fig. 6.2 C). The presence of increased receptor gene expression was also found by *in situ* hybridization showing GPR17-expressing cells accumulating at the borders of infiltration sites, where a large amount of blood borne cells are typically found (see Fig. 6.2 panel D). Accordingly, immunofluorescence analysis revealed GPR17-expressing cells accumulating at the lesions,

where both demyelination and inflammatory foci with infiltrating cells were found (Fig. 6.2 panel E).

In order to characterize whether the detected gene expression changes reflected alterations in the subpopulation of GPR17-positive oligodendrocytes, we evaluated by immunohistochemistry their co-localization rate with both NG2, a marker of early OPCs, and CC1, a marker of maturing oligodendrocytes. In line with the expected loss of oligodendrocytes in EAE, we found a reduction in the total number of $GPR17^+$ cells in WM, but, despite this trend, the number of $GPR17^+NG2^+$ double-positive cells was increased (Fig. 6.2 panel F), whereas no changes in the number of $GPR17^+CC1^+$ cells were found (Fig. 6.2 panel G). These data suggest that the up-regulation of GPR17 gene expression reflects the expansion of the early proliferating cells responding to the lesion and still expressing the receptor, whereas more mature cells do not react.

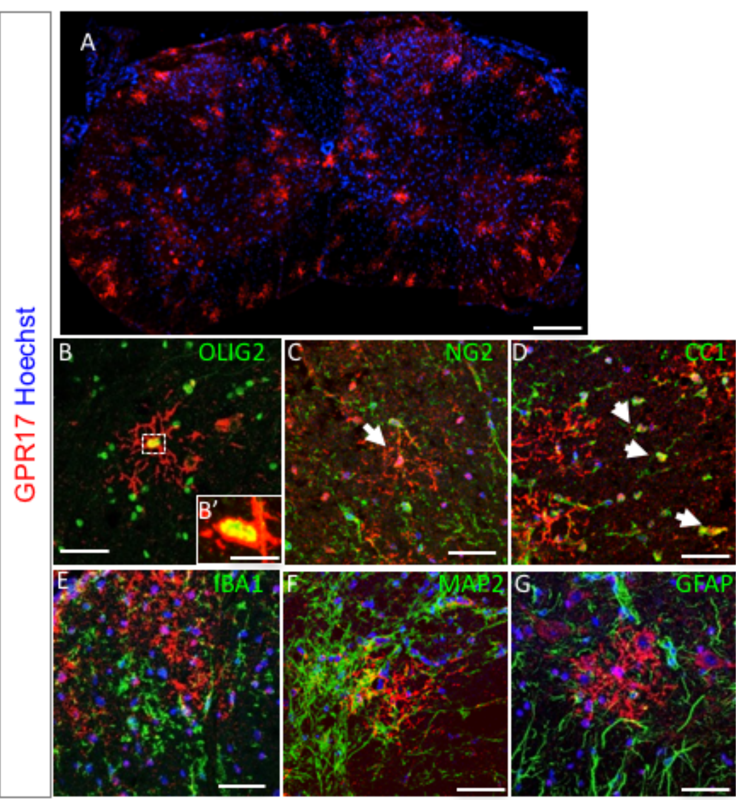

Figure 6.1 - Confocal images of control mice spinal cord immunolabeled for GPR17 and other markers. *(A) Distribution of $GPR17^+$-cells (in red) in the spinal cord of an adult mouse. Cell nuclei are labelled with Hoechst 33258 (in blue). $GPR17^+$-cells express the oligodendroglial marker Olig2 (B and inset B'). GPR17 presence was detected in cells showing co-localization of both the early OPC marker NG2 (C) and the more mature marker CC1 (D). No GPR17 positivity was found in microglia (Iba1), neurons (MAP2) and astrocytes (GFAP) (E, F, G). Images were taken at the confocal microscope (Zeiss LSM 510 Meta). Scale bars: 200µm (A), 50µm (B-G), 5µm (B').*

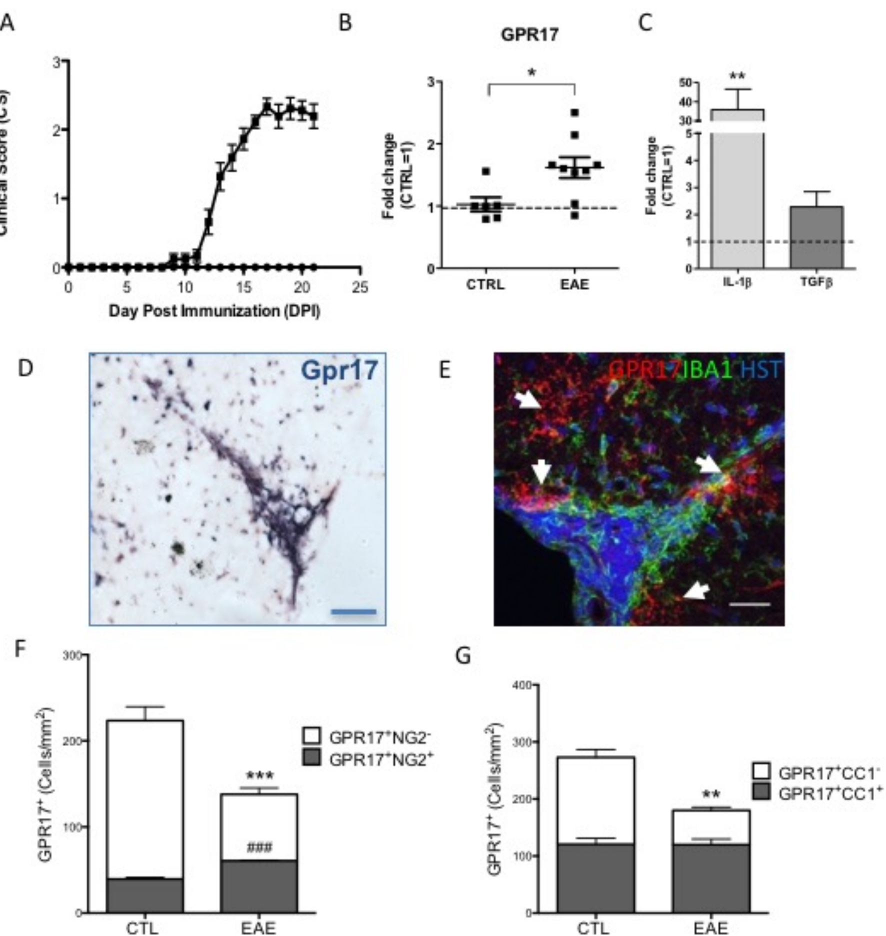

Figure 6.2 - Changes of GPR17 expression after acute EAE and alterations in the proportion of GPR17$^+$ oligodendrocytes pool in spinal cord of EAE mice. *Animals were analyzed on day 21 after immunization (acute EAE). The graph in A shows the clinical scores of mice during acute EAE ± SEM. By means of real-time PCR, a significant up-regulation of GPR17 was found in spinal cord of mice after acute EAE, compared to controls (B) and this correlated with the increased expression of inflammatory cytokines (C). Histograms show the fold change value ± SEM compared to control set to 1. Two-tailed Mann-Whitney t-student test, * p≤0.05, ** p< 0.01 from 3 independent experiments. GPR17 up-regulation was also confirmed by means of in situ hybridization at the lesion site (blue arrows indicate cells with increased levels of GPR17 mRNA (D). Scale bar 100µm. A local up-regulation of GPR17$^+$ cells (white arrows) was observed after EAE induction in the same area where inflammatory cells infiltrate the tissue (characterized by a high number of nuclei, in blue), bordered by Iba1$^+$ activated microglial cells (E). Scale bar 50µm. Proportion of GPR17$^+$-NG2$^+$ and GPR17$^+$-CC1$^+$ double-positive cells in white matter (F, G). Data are the mean±SEM of cervical, thoracic and lumbar sections; CTL n=3, EAE n=5). Unpaired two-tailed Student's t test; **, p< 0.01; ***, p< 0.001; ###, p< 0.001 compared to control from 2 independent experiments.*

6.3 Identification and fate of GPR17-expressing cells in inducible GFP-reporter mice

GPR17 typically decorates the 30-40% of OPCs (Boda et al., 2011; Viganò et al., 2015). We thus wondered whether this subset of GPR17$^+$ OPCs could be differentially recruited after damage compared to OPCs that do not express GPR17, and if they were able to undergo terminal differentiation at inflammatory sites during EAE.

To do so, we took advantage of the transgenic inducible reporter mouse line GPR17iCreER^{T2}:GFP (Fig. 6.3 panel A), in which, thanks to the activity of a Cre recombinase, upon tamoxifen administration, cells expressing GPR17 and their progeny are permanently labeled by the green fluorescent protein GFP (Viganò et al., 2015), thus allowing us to trace their destiny throughout animal's life.
First, to confirm successful transgene recombination and the identity of the recombined cells, we analyzed spinal cord sections in healthy mice. Importantly, we observed that virtually all the recombinant GFP^{+} cells remained within the oligodendrocyte lineage as nearly all of them were Olig2^{+} (Fig. 6.3 B-C). Many GFP^{+} cells were also positive for GPR17 (Fig. 6.3 panel D) and the NG2 marker (Fig. 6.3 panel E). Although they were not abundant, we also detected several GFP^{+}/GSTπ^{+} cells (Fig. 6.3 panel F), suggesting that the increase in the number of GFP^{+}-cells, observed in EAE, does not lead to augmented proportion of mature OLs. Vice versa, no co-localization of GFP^{+} cells was found with markers of microglia, neurons, astrocytes (Iba1, NeuN and GFAP, respectively) (Fig. 6.3 G-I).
Then, to determine the fate of GFP^{+} cells following EAE, we used the same protocol described above, after tamoxifen-induced recombination. To avoid any pharmacological interference with the onset of the disease, tamoxifen was administered to adult GPR17iCreER^{T2}:GFP transgenic mice 2 weeks before EAE induction (Fig. 6.4 panel A). After immunofluorescence, spinal cord sections were analyzed by counting cells at day 21 after immunization, when most animals reached the highest clinical score and clearly showed locomotor symptoms (Fig. 6.4 panel B). Interestingly, we observed a 3-fold increase in the number of GFP^{+} cells in WM, in particular in the regions of infiltration (Fig. 6.4 C-E), suggesting that cells expressing GPR17 responded to the insult with increased proliferation. Fate mapping analysis showed that most of the GFP^{+} cells were positive for NG2 (Fig. 6.4 F-H), suggesting that these cells are specifically proliferating in the lesion area. Although we found some differentiated GFP^{+} cells expressing the mature marker GSTπ in EAE mice, the number of these cells was not changed compared to control mice (Fig. 6.4 I-K), suggesting that their differentiative abilities are impaired, likely due either to demyelination itself, or to unfavourable local inflammatory environment. Globally these data suggest that the population of GFP^{+} cells is reacting mostly by expanding the pool of precursors, and that this is not followed by the maturation of these cells, at least at this disease stage.

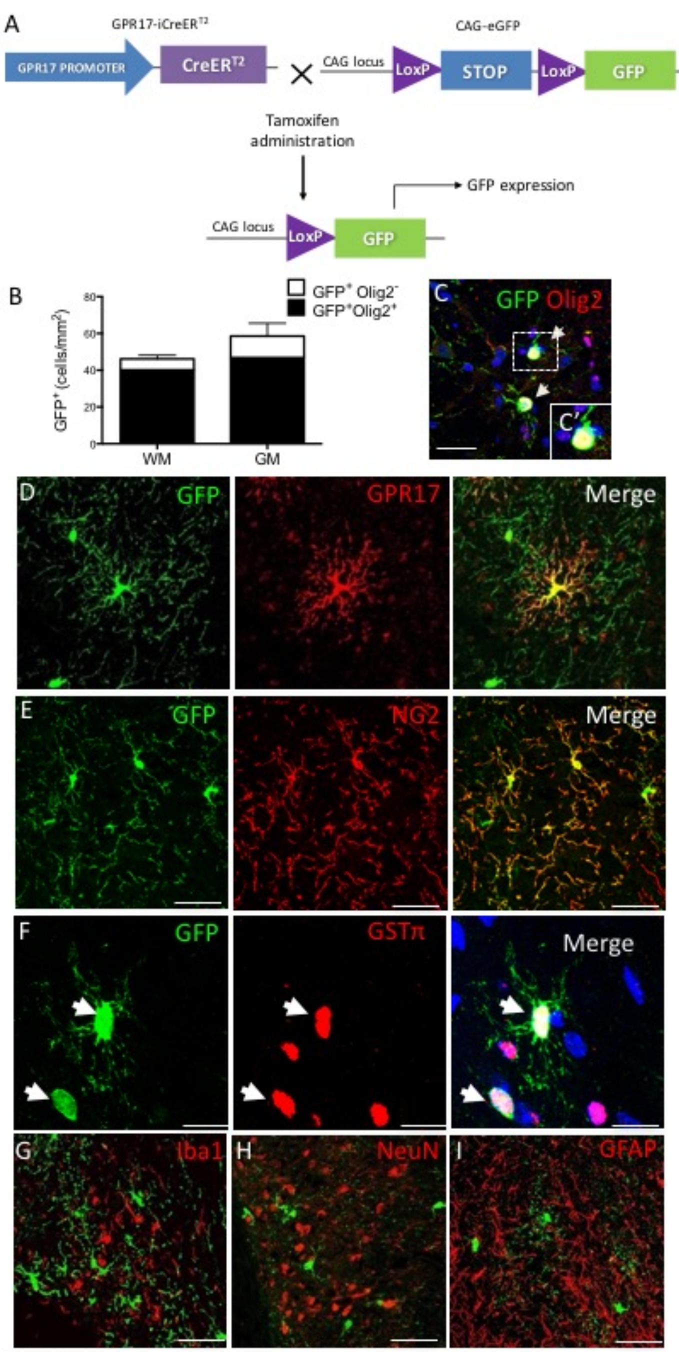

Figure 6.3 - Identity of recombinant cells in the adult spinal cord. *Schematic representation of the transgenic alleles in GPR17-iCreERT^2:CAG-GFP mice showing tamoxifen-responsive recombination of the CAG-GFP allele to induce GFP in cells expressing GPR17 (A). Quantification of Olig2$^+$ cells among GFP$^+$ cells in the spinal cord reveals that nearly all recombined cells belong to the oligodendroglial lineage (B, C). Many GFP$^+$ cells were also found positive for GPR17 (D) and the NG2 marker (E). Although they were not abundant, we detect the presence of some GFP$^+$-GST$^+$ cells (F). Vice versa, GFP$^+$ cells are not positive for the markers of microglia, Iba1 (G), neurons, NeuN (G) and astrocytes, GFAP (I). Images were taken at the confocal microscope (Zeiss LSM 510 Meta). Scale bar: 10µm (C, F), 5µm (C'), 50µm (D-I).*

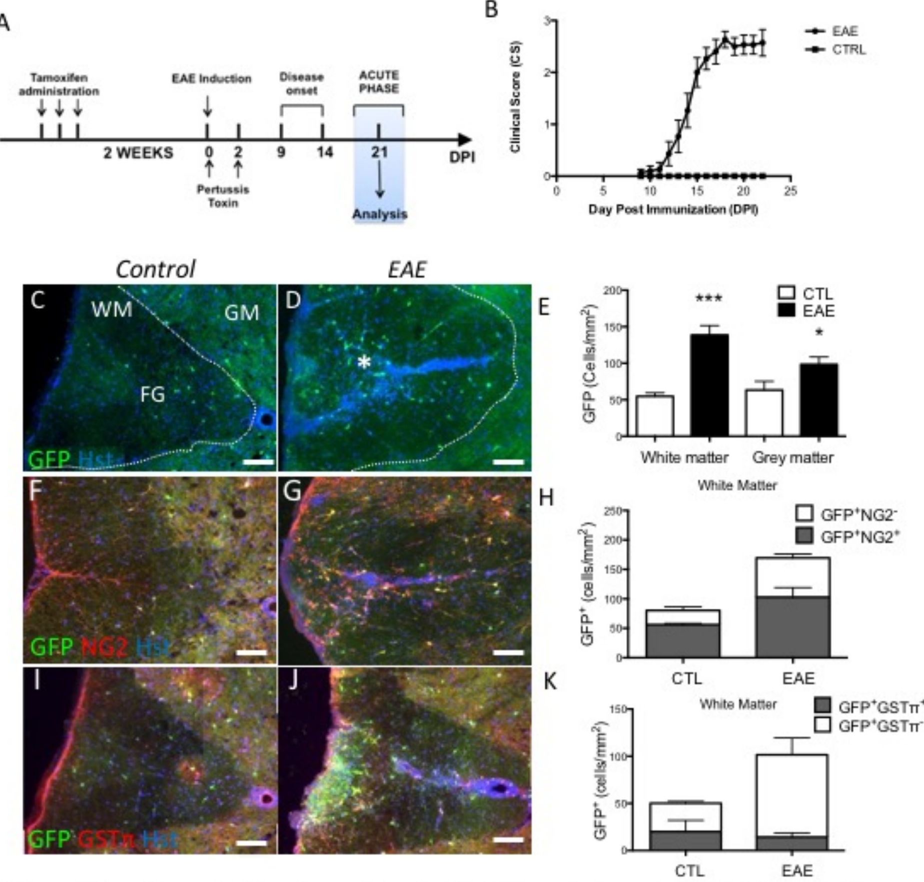

Figure 6.4 - Reaction and fate of recombined cells in the spinal cord of GPR17-iCreER^{T2} mice after acute EAE. *Mice received tamoxifen by oral gavage three times (once a day every second day for 3 d), starting 14 d before EAE induction. Animals were analyzed on day 21 after immunization (A). Clinical scores of mice during acute EAE. Results represent mean CS± SEM of 15 EAE mice (B). Representative images showing fasciculus gracilis (FG) of spinal cords of control and EAE mice. GFP^{+}-cells are visible under both conditions but, after EAE, their number significantly increased in the infiltration areas, where a lot of inflammatory cells were found (high number of nuclei, in blue, *) (C). Images were taken at 20X magnification and reconstructed. Quantification of the number of GFP^{+}-cells in EAE spinal cord (D). In H and K, characterization of the identity of the GFP^{+} cells in the white matter spinal cord of GPR17-iCreER^{T2} mice after EAE. Data are the mean±SEM of lumbar sections; CTL n=2, EAE n=5). Unpaired two-tailed Student's t test, * $p< 0.05$, *** $p< 0.001$ compared to control. Representative images showing of spinal cords of control and EAE mice. Scale bars: 100µm (C, D, F, G, I, J).*

6.4 In the cuprizone-induced demyelination model GPR17 kinetics correlate with MBP expression pattern

Next, we wondered if the local up-regulation of GPR17 at the lesion site was due to the inflammatory/immune response, or it was a specific feature of the demyelinating component of the disease. To address this issue, we took advantage of the cuprizone animal model, a highly reproducible system of primary OL apoptosis and secondary demyelination, which is characterized by much lower inflammation, and in which adaptive immune responses are not involved (Gudi et al., 2014). Wild-type mice received a cuprizone-supplemented diet for 5 weeks to cause demyelination and then switched to

normal diet up to 3 weeks to allow spontaneous re-myelination. As expected, the expression of MBP during the various disease stages followed a typical pattern (Tagge et al., 2016), with a strong down-regulation starting from the first week of treatment and a gradual increase during the re-myelination phase, reaching higher levels at weeks 7 and 8 (Fig. 6.5 panel A). Interestingly, also Gpr17 expression was significantly reduced in the early phases of demyelination and was then progressively restored to basal levels at the onset of the re-myelination phase, with no significant up-regulation compared to control mice and compared to the EAE model (Fig. 6.5 panel B).

We conclude that, at variance from the EAE model (see above), after toxic cuprizone-induced demyelination, there is no up-regulation of GPR17 levels. GPR17 changes during disease course instead follow the kinetics of other myelin genes.

We thus suggest that, in a more complex disease like EAE, further components, such as the adaptive immune system or inflammatory factors released by immune cells, are present at lesion sites and may be responsible for the detected up-regulation of Gpr17 expression levels under these conditions.

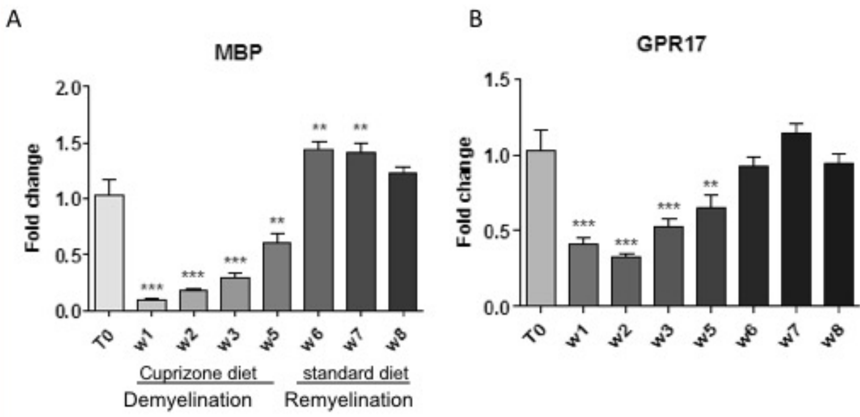

*Figure 6.5 - **Correlation of GPR17 expression with progression of re-myelination in the cuprizone-induced demyelinating model**. GPR17 expression detected by qRT-PCR using corpus callosum tissue from cuprizone treated wild-type animals (n=5) during 1, 2, 3, 4, 5 weeks of cuprizone treatment, and after animals were fed with standard diet for one (W6), two (W7) and three weeks (W8).*

6.5 *In vitro* exposure of OPCs to medium conditioned by activated astrocytes results in strong GPR17 stimulation and OPC maturation

To shed light on the mechanisms responsible for OPC activation after injury and to assess, in particular, the role of GPR17 in this activation, we focus our attention on astrocytes, another type of glia known to become “reactive” after damage (Kuhlmann et al., 2008).

Reactive astrogliosis is highly prevalent in inflammatory lesions and indeed represent one of the major pathological features of MS. Moreover, reactive astrocytes are known to release a variety of pro-inflammatory molecules, including cytokines, that could then play a role in GPR17 activation.

To investigate how astrocytes can directly impact on OPCs differentiation and GPR17 expression through the release of soluble products, rat primary OPCs were cultured with conditioned medium obtained from rat primary astrocytes activated by FBS starvation. After a 48h-exposure to astrocyte conditioned medium (ACM), we observed a strong positive effect on OPCs differentiation; in particular, the number of both GPR17$^+$ and MBP$^+$ cells was increased with respect to control condition (Fig. 6 panel A and B). Real-time PCR performed under the same conditions also confirmed up-regulation, with a 2-fold increase of GPR17 gene expression and an almost 10-fold increase of MBP expression by real-time PCR (Fig. 6 panel F). These data suggest that, as expected, astrocytes reacting to damage release soluble factors (including cytokines) that can then impact on OPC maturation. They also show that this likely occurs via GPR17 induction.

6.6 *In vitro* exposure of OPCs to cytokines

As a following step, we investigated the direct effect of cytokines typically released at inflammatory sites (both in EAE and MS lesions) on the expression of Gpr17. In attempt to model the contribution, to OPC differentiation, of typical cytokines released by both astrocytes and cells of adaptive immune system, primary rat OPCs were cultured with two different cocktails of cytokines mainly released by Th1- Th2- polarized T cells: TH1 (IL-1ß, IL-12, CXCL12, IL-17) and TH2 (IL-4, IL-6, IL-10) (Zeis et al., 2015). Cells were treated with TH1 and TH2 cocktails for 48h and were then fixed and stained to determine GPR17 and MBP. After exposure to TH1 cytokines, we observed an increase only in the number of GPR17$^+$ cells, without any pro-differentiating effect with respect to control (Fig. 6.6, panels C and E); instead, after exposure to TH2 cytokines, we observed an increase in the number of GPR17$^+$ cells associated with an increase in the number of MBP$^+$ mature OLs (Fig. 6.6 panels D and E). Real-time PCR revealed that TH1 cytokines induced a 2-fold increase of GPR17 expression that was not accompanied by an increase of MBP levels. No significant effects on GPR17 and MBP expression levels was observed after TH2 exposure (Fig. 6.6 panel F).

All together, these results suggest that impaired OPC maturation observed after the treatment with pro-inflammatory cytokines could be due to at least in part to GPR17 sustained up-regulation.

6.7 GPR17 is a promiscuous receptor that can be also activated by CXCL12

As already mentioned, after CNS injury, astrocytes secrete a variety of factors (including cytokines and chemokines like CXCL12) that can influence OPC survival, migration, differentiation, and subsequent myelination (Moore et al., 2011). This confirms that astrocytes can be considered important regulators of myelination and re-myelination in the CNS. Recently, we reported a promiscuous behavior for GPR17 and demonstrated that this receptor can indeed also bind to the chemokine CXCL12 (also known as SDF-1) both in silico and in vitro (Parravicini et al., 2016). Specifically, in primary OPC cultures, treatment with CXCL12 alone increases by approximately 30% the number of MBP-positive cells compared to control conditions, thus accelerating their differentiation toward a mature phenotype, in line with literature data (Li et al., 2012; Patel et al., 2010; Carbajal et al., 2010) (see Fig. 6.7, panel A, B and E). As also shown in Fig. 6.7, this increase is not observed when CXCL12 is added to cells either in combination with the GPR17 antagonist Cangrelor (panel C, E), or, as expected, with the CXCR4 antagonist Plerixafor (panel D, E). In both antagonist groups, the number of MBP-positive cells is comparable to, or slightly lower than, the one measured in the vehicle-treated cells.
Globally, these results demonstrate that CXCL12, at a concentration able to activate its cognate receptor CXCR4, can also directly act as a promiscuous activator of GPR17, corroborating our hypothesis of a common pathophysiological role for GPR17 and chemokine receptors in leading the re-myelination processes.

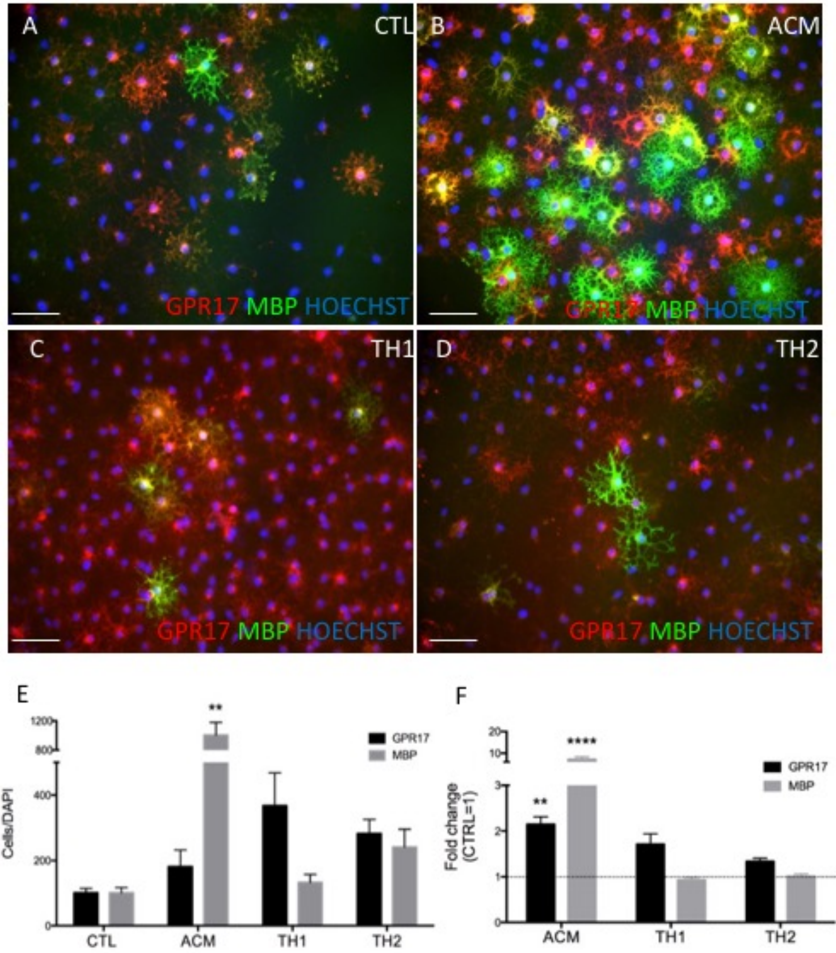

Figure 6.6 - Effects of reactive astrocyte conditioned medium and TH1- and TH2- cytokine cocktails on GPR17expression and OPC differentiation. Representative images of GPR17- (in red) and MBP-expressing cells (in green) treated for 48h with: vehicle (A) reactive astrocyte conditioned medium (ACM) (B), TH1 cytokine cocktail (IL-1b, IL-12, CXCL12, IL-17) (C) and TH2 cocktail (IL-4, IL-6, IL-10) (D). Cell nuclei are labelled with Hoechst 33258 (in blue). Exposure to ACM of rat OPCs increase the number of cells expressing both $GPR17^+$-OPCs and MBP^+-oligodendrocytes after 48 hours (B, E). Histograms show quantification of the percentage of GPR17- and MBP positive cells after 48 h of treatment with vehicle and with ACM, TH1 and TH2 cytokines cocktails. The number of positive cells was counted in 20 optical fields under a 40× magnification. Data are the mean ± SEM; from 2 independent experiments; one-way ANOVA Dunnett multiple-comparisons test $^{**}p<0.01$, compared to control. In F, histograms show quantification of Gpr17 and Mbp gene expression levels by real-time PCR after 48 h of treatment with vehicle and with ACM, TH1 and TH2 cytokines cocktails. Data are the mean ± SEM; from 2 independent experiments; one-way ANOVA Dunnett multiple-comparisons test$^{**}p<0.01$, $^{****}p<0.01$ compared to control=1.

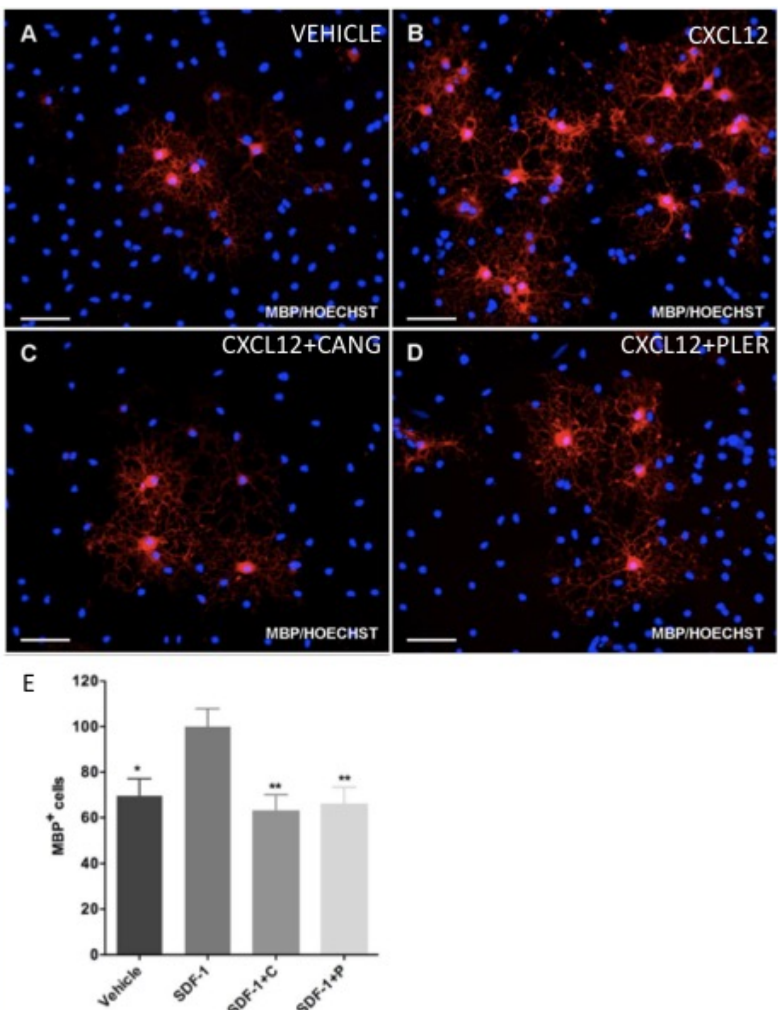

*Figure 6.7 – CXCL12-mediated activation of GPR17 in OPCs. Representative images of MBP expressing cells (in red) treated with vehicle (A) and CXCL12 (also known as SDF-1) alone (B), or in combination with the GPR17 antagonist cangrelor (C) and with the CXCR4 antagonist Plerixafor (D). Cell nuclei are labelled with Hoechst 33258 (in blue). (E) Histograms show quantification of the percentage of MBP positive cells after 48 h of treatment with vehicle and with CXCL12 alone (CXCL12-treated cells set to 100%), or in combination with cangrelor (C) and plerixafor (P). The number of positive cells was counted in 20 optical fields under a 40× magnification. Data are the mean ± SEM of at least three independent experiments. *, $p<0.05$; **, $p<0.01$ compared to CXCL12 treated cells; one-way ANOVA Dunnett's Multiple Comparison Test. Scale bar: 50 µm.*

6.8 GPR17 is pathologically up-regulated in demyelinating lesions of human MS subjects

Finally, we wondered whether GPR17 up-regulation was also associated with human MS demyelinating lesions like in EAE. To answer this question, we first analysed the presence of GPR17 in human cortex from autoptic specimens of control subjects died for non-neurological diseases (data not shown), and then we compared these data with GPR17 expression in a cohort of MS patients (see table 5.2).

As previously observed in mouse, GPR17$^+$ cells were homogeneously distributed throughout the normal appearing white matter (NAWM) (Fig. 8 panel A), co-expressing the marker

Olig2, confirming an abundant expression of the receptor also in human cells belonging to the oligodendroglial lineage (Fig. 8 panel B).

In the pathological specimens from MS patients, as observed in EAE, many GPR17^{+} cells were found at the border of active demyelinating MS lesions (Fig.8 panel C) also identified by means of the luxol-fast blue staining (Fig.8 panel D). However, at variance with the rodent tissues, these cells showed a completely different morphology, resembling that of reactive astrocytes (Fig.8 panel E). GPR17 clearly co-localized with both the human leukocyte antigen (HLA), which is typical of antigen presenting cells, microglia and astrocytes, and the markers Iba1 and GFAP (Fig.8 F). These data suggest that, in human cortex, GPR17 decorates different types of cells activated or recruited at the border of active MS lesion sites.

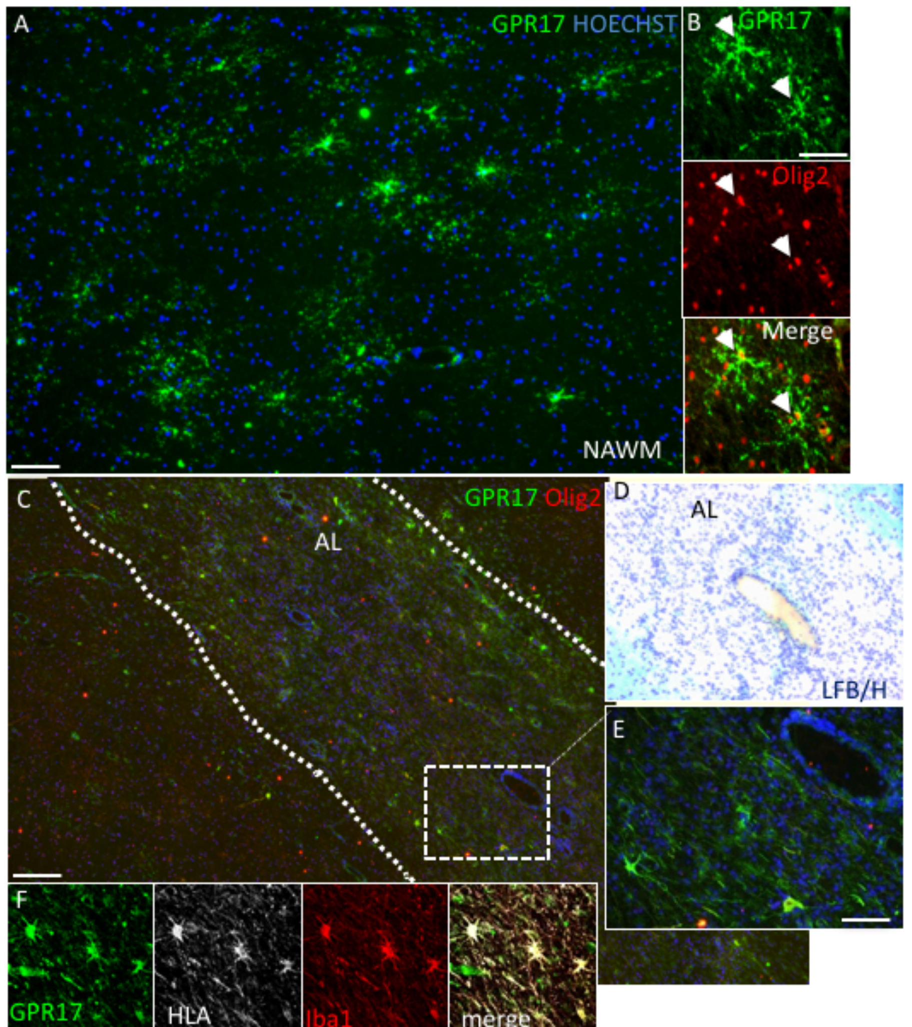

Figure 6.8 - Sections from autopsy frontal cortex of human MS patients. *GPR17 is present on oligodendrocytes in normal appearing white matter (NAWM) (A). Hoechst (blue) stains nuclei. Double immunofluorescence staining with GPR17 and Olig2 (B). In C, images of an active lesion (AL; demarcated with dashed lines) taken at 10X magnification and reconstructed. In this region, where demyelination occurs as shown by luxol fast blue/Hematossilin staining (LFB/H; panel D), many GPR17^{+} cells accumulate at the border but they have a different morphology compared to those in NAWM (E) and are GPR17^{+}HLA^{+}Iba1^{+}(F). Scale bar: 50µm (B, E, F) 100µm (A); 200µm (C).*

DISCUSSION

Chapter 7

Discussion

In recent years, GPR17 has emerged as an important key actor in oligodendrogenesis. Previous studies have shown that, in the brain, GPR17 indeed acts as an intrinsic regulator of this process, it is needed to start OPC differentiation but at a certain stage of differentiation, it has to be turned down to allow the terminal maturation of immature oligodendrocytes (Fumagalli et al., 2015). Here, we provide for the first time the characterization of GPR17 in the spinal cord and analyse its changes after induction of EAE in rodents. We also provide the first analysis of GPR17 in the CNS plaques of MS patients.

GPR17-positive OPCs react to EAE induction

The rodent studies have suggested that, under physiological conditions, GPR17 has a widespread expression on the OPCs in both brain and spinal cord, suggesting a role in the normal homeostasis and in the turnover of oligodendrocytes. Here, we confirm that GPR17 expressing cells are widespread throughout the whole spinal cord, both in grey and white matter (Figure 6.1 A). It was also demonstrated that, at least in the brain, under normal conditions, cells expressing GPR17 are relatively quiescent and they proceed in the differentiating process only after 11 weeks. However, these cells can very rapidly react to insults, thus representing a pool of cells able to react to emergency situations (Viganò et al., 2015). Thus, it seems that the presence of the receptor enables OPCs and immature oligodendrocytes to react to injury through its activation, which is also in line with our previous data suggesting GPR17 as a “sensor of damage” (Lecca et al., 2008; Ceruti et al., 2009; Buffo et al., 2011). Therefore, the presence of GPR17 in adult spinal cord parenchyma suggest that, in a similar way to the brain, also in this part of the CNS GPR17 likely makes OPCs able to react to insults. These conclusions are also supported by the data obtained in the first inducible GPR17 GFP-fluorescent reporter mouse, in which successful tamoxifen-induced recombination took place in GFP^{+}-OPCs and immature oligodendrocytes, confirming that, both in brain and spinal cord parenchymas, there is a subset of adult oligodendrocytes on which the promoter regulating GPR17 expression is active, even under physiological conditions.

Specifically, GPR17 expression pattern is significantly altered during EAE induction. As previously reported by Chen and colleagues (Chen et al., 2009), the mRNA for GPR17, in EAE mice, was found to be upregulated mostly at inflammatory areas showing infiltration of blood born cells, indicating that its expression is specifically induced in a subset of cells reacting to the insult. In a similar way to what already shown in the brain (Viganò et al., 2015), these quiescent GFP^{+} cells are able to strongly enhance their proliferation rate in response to inflammation/demyelination. After acute EAE, we observed a 3-fold increase in the number of GFP^{+} cells in the areas characterized by infiltrating cells compared to controls. In particular, GFP^{+}-$NG2^{+}$ were very reactive in line with literature data showing that after injury OPCs actively proliferate to enhance the pool of progenitors in order to support the differentiation and maturation of some of them to substitute the injured ones. Previous time-lapse imaging has revealed how $NG2^{+}$ cells are recruited to sites of focal CNS injury and rapidly migrate and proliferate to restore their density (Hughes et al., 2013). As already mentioned, in different mouse models of acute brain injury and ischemia, it has been recently demonstrated that $GPR17^{+}$-$NG2^{+}$ cells represent a rapidly reacting reserve pool (Viganò et al., 2015). Our data support a very similar behaviour of spinal cord OPCs after acute EAE, when $GPR17^{+}$ cells undergo activation and proliferation in order to counteract the insult. In a recent interesting study, it was also demonstrated that activated OPCs show a neonatal-like transcriptome, enhanced differentiation and migration capabilities, in parallel to increased expression of several genes including chemokines (e.g. IL-1b, CCL2) and purinergic receptors (e.g. P2Y2R) (Moyon et al., 2015), providing further evidence that OPC population is heterogeneous and differentially reacts to insults and it is influenced by the environment.

Here we report that the $GPR17^{+}$-$NG2^{+}$ subpopulation of cells in the WM reacts to the demyelinating insult by expanding their pool and also that this behaviour is restricted to the region where demyelination occurs, demonstrating that, also in the spinal cord, OPCs are not homogeneous.

It is well established that, in the postnatal and adult brain, NG2-glia represent the largest population of endogenous/resident progenitor cells (4–8% of total cells, depending on the brain region), capable of rapidly "reacting" to any type of injury and with a strong potential to repopulate areas of lesion (Aguirre and Gallo, 2007; Filous et al., 2014; Scafidi et al., 2014; Simon et al., 2011). NG2-glia cells morphology and their functional properties

are distinct in different brain region and interactions between NG2-glia and other neural cell types may vary between brain areas. Fate-mapping studies showed region-dependent differences in NG2-glia differentiation: e.g. the majority of adult NG2-glia located in the WM of the cerebral cortex differentiate mostly into mature, myelinating oligodendrocytes, while GM NG2-glia generate fewer mature oligodendrocytes (Dimou and Gallo, 2015). Further studies are necessary to fully understand how distinct subpopulations of NG2-glia display differences in their properties and functions.

In this respect, due to its "promiscuity" and ability to respond to different type of ligands accumulating at injury sites (Parravicini et al., 2016; Sensi et al., 2014), GPR17 could have a key role in "sensing" changes in the extracellular environment and contributing to the adaptive properties of the NG2-glia.

GPR17 is a sensor of neuro-inflammatory damage

At variance from the EAE model, in the cuprizone-induced demyelination model, that provides a highly reproducible system of primary OL apoptosis and secondary demyelination and where adaptive immune responses are not involved, we didn't observe any Gpr17 up-regulation. We instead found that Gpr17 expression levels, after their initial down-regulation during the demyelination phase, are re-established to basal levels during re-myelination and that these changes followed the kinetics of other myelin genes (Fig.6.5). We thus suggest that, in a more complex disease like EAE, further components, such as the adaptive immune system or inflammatory factors released by immune cells, are present at lesion sites and may be responsible for the detected up-regulation of Gpr17 expression levels under these conditions.

The explanation of this behaviour could be linked to intrinsic GPR17 properties: GPR17 is indeed placed at an intermediate structural and phylogenetic position between already known P2Y and CysLT receptors and has been demonstrated to respond to UDP, UDP-glucose and UDP-galactose in the micromolar range (Benned-Jensen and Rosenkilde, 2010; Ciana et al., 2006; Daniele et al., 2014; Lecca et al., 2008) fully consistent with the concentrations at which these endogenous ligands activated their already known cognate P2Y receptors (Abbracchio et al., 2006). Interestingly, GPR17 is also activated by cysteinyl-leukotrienes (LTC_4, LTD_4 and LTE_4), inflammatory mediators released upon damage (Ciana et al., 2006). More recently, GPR17 has been also reported to respond to emergency signals like oxysterols, or to cytokines, like CXCL12 (Parravicini et al., 2016), in a similar way to other

related receptors involved in inflammatory responses, like Epstein Barr virus induced gene receptor-2 and CXCR2 (Sensi et al., 2014). That is possible because GPR17 also shares some structural, phylogenetic and functional properties with some chemokine receptors (CXCRs), according to other recent data in literature that are challenging the currently accepted dogma that each receptor responds to a single endogenous ligand or a single family of related signalling molecules. Both GPR17 and CXCR2 are operated by oxysterols, and both GPR17 and CXCR ligands have been demonstrated to have a role in orchestrating inflammatory responses and OPC differentiation to myelinating cells in acute and chronic diseases of the CNS.

Interestingly, in our in vitro experiments, although CXCL12 was present in the TH1 cocktail, we only reported an up-regulation of GPR17 without any effect on the proportion of maturing OLs. It might be that when CXCL12 is present at excessively high concentrations and in combination with other cytokines, like in EAE/MS lesions, it does not induce differentiation, but instead causes blockade of OPCs at immature stages. This hypothesis is in line with the present findings in the EAE model and with previous data in other neuro-inflammatory conditions like brain trauma or ischemia (Boda et al., 2011; Lecca et al., 2008; Ceruti et al., 2009) showing that when GPR17 is up-regulated on OPCs by high levels of “emergency signals” (e.g. CysLTs, oxysterols, CXCL12) cells are blocked at immature stages and do not proceed to terminal differentiation.

Taken together, these findings support the emerging hypothesis that GPR17 may promiscuously respond to different signalling molecules depending on specific pathophysiological conditions and emergency situations.

GPR17 responses may also vary depending upon its heterodimerization with other receptors, including P2Y and Cys-LT receptors (Maekawa et al., 2009), which could help explain why agonists at GPR17 have such diverse chemical structures. This also suggests that GPR17 may function as an adaptor protein for enhancing the agonist repertoire of other GPCRs, consistent with the conclusion that MDL29,951, an agonist of GPR17, can engage Gq as well as Gi proteins (Fumagalli et al., 2011; Hennen et al., 2013).

The ability of GPR17 to respond to different classes of GPCR ligands suggests that this receptor modifies its function depending on the extracellular milieu changes occurring under specific pathophysiological conditions suggesting GPR17 as a strategic target for neurodegenerative diseases with an inflammatory/immune component. In this respect,

investigating GPR17 downstream will be interesting in order to describe "activated" OPC phenotype under pathological conditions like MS.

This study could increase the knowledge of signalling mechanisms in resolution, opening new directions to treat inflammation-associated diseases, such as MS where the chronicity of inflammatory can be associated with inadequate engagement of resolving pathways by DMTs; this approach may lead to combined therapies that, on the one hand, control inflammation and neurodegeneration, and on the other hand, foster re-myelination.

In human MS active lesions GPR17 is expressed by cells involved in inflammatory responses

Dysregulated expression of GPR17 has been described also in human samples from patients with traumatic brain injury (Franke et al., 2013). In both neurosurgical and autopsy specimens, GPR17 expression was evident inside the contused core and progressively declined distally according to a spatio-temporal gradient. Inside and around the core, GPR17 labelled dying neurons, reactive astrocytes, and activated microglia/macrophages. In peri-contused parenchyma, GPR17 decorated OPCs indicating re-myelination attempts.

GPR17 was found overexpressed in MS plaques as compared with white matter from non-neurological donor samples and normal-appearing white matter from MS donors (Chen et al., 2009), but the cellular distribution of the receptor was not investigated yet. Here we show that, indeed, in human brain GPR17 is expressed in oligodendroglial cells, but in active lesions it clearly decorates reactive microglia cells expressing HLA, and reactive astrocytes. These data suggest that in human MS, GPR17 is involved in reparative processes, exclusively at the lesion site, in a more complex fashion compared to that observed in rodents. Its expression in different cells suggests a cell type-specific expression and regulation of the receptor. In fact, it is known that, in humans GPR17 is physiologically present in OPCs and immature oligodendrocytes but the significance of its expression in other cell types is still elusive (Franke et al., 2013). Our hypothesis is that GPR17 could enable microglia and astrocytes to detect changes in their local environment and respond to release of inflammatory mediators within minutes. Our observations are related to active lesions but, in the next future, it will be interesting investigating whether GPR17 is involved in the evolution and in the progression of the MS lesions.

It is worth to mention that although human GPR17 is highly similar to the rodent receptor, in humans, the receptor is also present in a longer isoform, bearing 28 additional amino-

acids at the N-terminus (Pugliese et al., 2009). It has been previously demonstrated that both isoforms are functional when transfected in non-native systems, and even if they are both activated by the same ligands, their pharmacological profile and their signalling are different (Pugliese et al., 2009; Benned-Jensen and Rosenkilde 2010).

It is possible that, during evolution, primates developed a longer isoform to introduce new variability in this gene enabling a differential expression of GPR17 in non-oligodendroglial cells, such as activated microglia and astrocytes.

Our hypothesis is that GPR17 could be part of inflammation resolution process in the CNS. Its promiscuity and its cell-type specific expression including microglia and astrocytes, cells directly involved in the inflammatory reaction to the demyelinating insult, make this receptor a good candidate to shed light on molecular mechanisms occurring in the evolution of human MS plaques.

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