

UNIVERSITÀ DEGLI STUDI DI MILANO

Graduate School in Pharmacological Sciences /
Scuola di Dottorato in Scienze Farmacologiche

XXVI ciclo

Dipartimento di Scienze Farmacologiche e Biomolecolari



**TIME-DEPENDENT AND CRITICAL ROLE OF THE
GPR17 RECEPTOR DURING OLIGODENDROCYTE
DIFFERENTIATION: IMPLICATIONS FOR THE
DEVELOPMENT OF NEW REMYELINATION
STRATEGIES**

Settore disciplinare BIO/14

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Anno Accademico 2012/2013

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Chapter 1

INTRODUCTION

1.1 Oligodendrocytes: the myelin-forming cells in the central nervous system (CNS)

The CNS contains two major types of specialized cells, neurons and glial cells (Figure 1.1). 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 guiding 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 central nervous system that ensure a rapid signal conduction in the white matter. 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.

As mentioned before, the main function of OLs is to produce myelin and to form the myelin sheath around neuronal processes of CNS which is essential to ensure the insulation of axons and to allow the salutatory conduction of nerve impulses. 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).

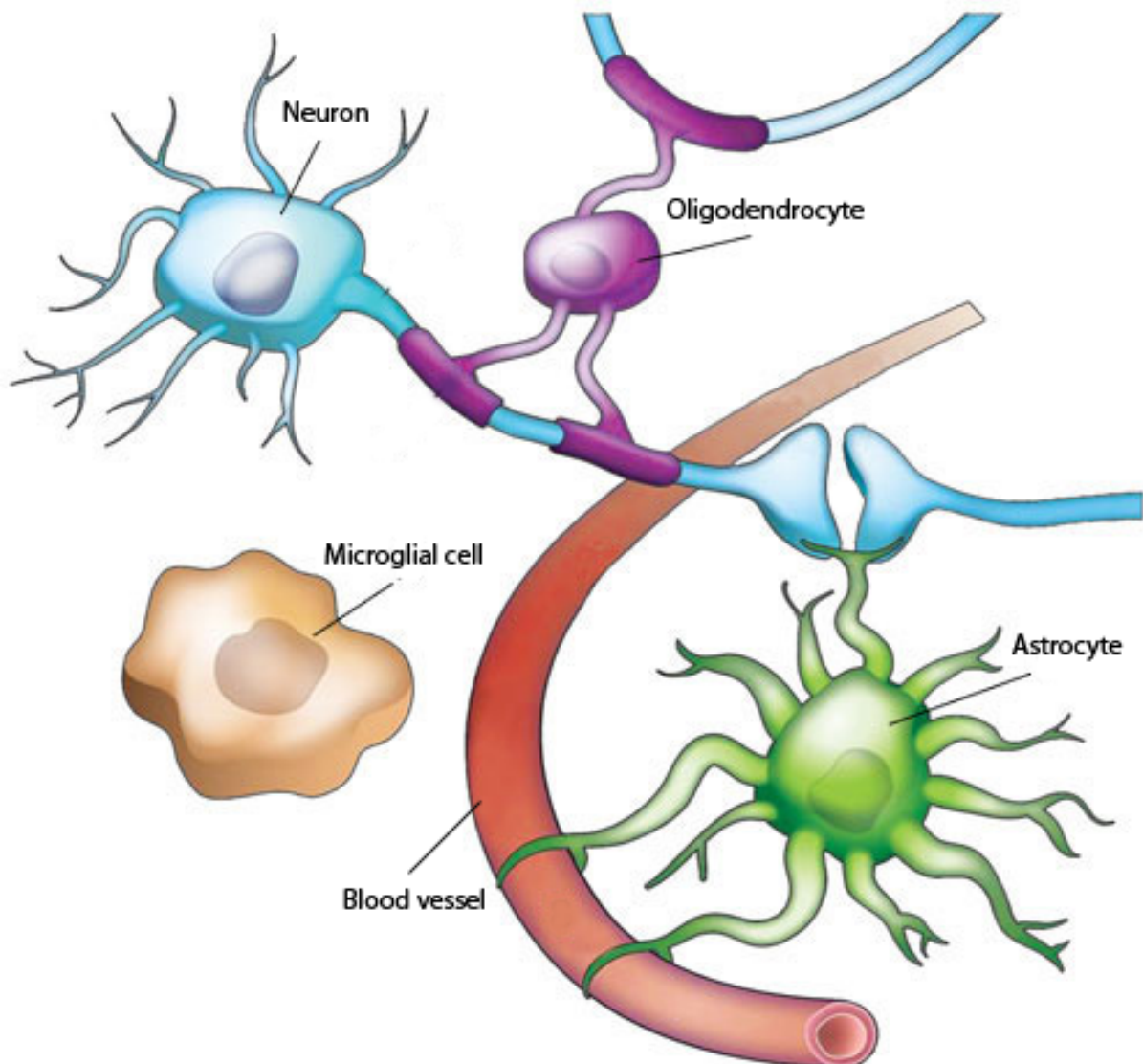


Figure 1.1 - Glial populations within the CNS

Schematic representation of the different types of glial cells in the CNS and their interactions, among themselves and with neurons. Astrocytes are stellate cells with numerous processes contacting several cell types in the CNS: soma, dendrites and axons of neurons, soma and processes of OLs, and other astrocytes; astrocytic feet also ensheath endothelial cells around blood capillaries forming the blood-brain barrier. OLs are the myelinating cells of the CNS; they are able to myelinate up to 50 axonal segments, depending on the region of the CNS. Microglia, which represent the 20% of the total glial population within the CNS, keep the brain under surveillance for damage or infection.

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 white matter. 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, it will be described and discussed the embryonic and postnatal development of OLs and their functional roles in physiological and pathological conditions.

1.1.1 Embryonic and postnatal development of OLs

OLs are ubiquitous in both the white and grey matter of brain and spinal cord. They 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. 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 (PDGFR α) 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 1.2). 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.

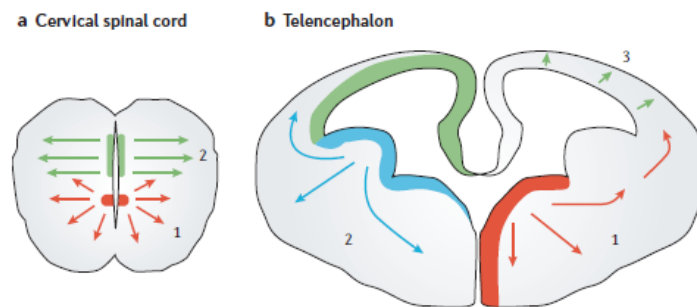


Figure 1.2 - Origins and migration of oligodendrocyte precursors in the rodent cervical spinal cord and telencephalon.

(a) In the mouse spinal cord, ~85% of oligodendrocyte precursors 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).

After the formation, OPCs migrate extensively from oligodendroglial 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).

To migrate and disperse correctly into the CNS parenchyma, OPCs interact with a plethora of environmental factors (adhesion molecules in the extracellular matrix, mitogens and

secreted growth factors, chemokines, hormones and neurotransmitters) via their surface receptors (de Castro et al., 2005; Cohen, 2005). The growth factor FGF2 and PDGF-AA are examples of mitogenic factors important for OPC movement. They act via separate and highly specific pathways in a non-synergistic manner: PDGF controls OL migration by inducing alterations in intracellular Ca^{2+} levels (Simpson and Armstrong 1999) which, in turn, result in the activation of intracellular elements necessary for the detachment from the substratum at the rear, generation of motile force, and attachment at the leading edge process (Marks et al., 1991; Fay et al., 1995; Elferink and de Koster, 1995); FGF2 acts as a chemoattractant by forming a complex with its receptor FGFR1 whose activity is impaired by anosmin-1, a protein expressed by retinal axons that is defective in the X-linked form of human Kallmann syndrome (Bribian et al., 2006). The direct consequence of the interaction of these growth factors and morphogens with molecules from the extracellular matrix (fibronectin, merosin, tenascin-C and PSA-NCAM) is the modification of OPC migration.

Another remarkable group of contact-mediated molecules regulating OPC migration in the CNS is the ephrins (Ephs). Unlike ephrin A5 (that acts via EphA), the membrane-anchored B2 and B3 ephrins (that both interact with EphB receptors) reduce the adhesion of embryonic OPCs (isolated from the diencephalon), consequently decreasing their motility (Prestoz et al., 2004; Petros et al., 2006). Indeed, EphB2 receptor over-expression has been shown to increase the invasiveness of migrating glioblastoma cells *in vitro* and *in vivo*, while blocking EphB produces the opposite result (Nakada et al., 2004).

Interestingly, OPCs also have metalloproteinases that allow cells to move in the extracellular environment and let them extend processes to take contact with axons (Baumann and Pham-Dinh, 2001).

Besides the molecules that promote OPC migration there are some “stop signals” which inhibit this process. In this respect, tenascin-C has been reported to block OL precursors at the optic nerve in conjunction with other signals (Bartsch et al., 1994; Kiernan et al., 1996), such as netrin-1 that is transiently expressed at the optic nerve head and it serves as a repulsive cue to stop the migration of precursors into retina (Deiner, 1997). Another candidate stop signal is the chemokine CXCL1, which is secreted by astrocytes and has been proposed to regulate spatial and temporal patterning of spinal cord myelination through inhibiting neonatal OPC motility during spinal cord development (Miller et al., 2002; de Castro et al., 2013).

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.

1.1.2 Oligodendroglialogenesis

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. At the beginning, OPCs have a bipolar morphology and express PDGFR- α , the proteoglycan NG2 (glial antigen 2), the isoform DM-20 of the proteolipid protein (PLP) and the gangliosides A2B5 and GD3 (Baumann and Pham-Dinh, 2001) (Figure 1.3). 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 oligodendrocyte 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 one 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), 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).

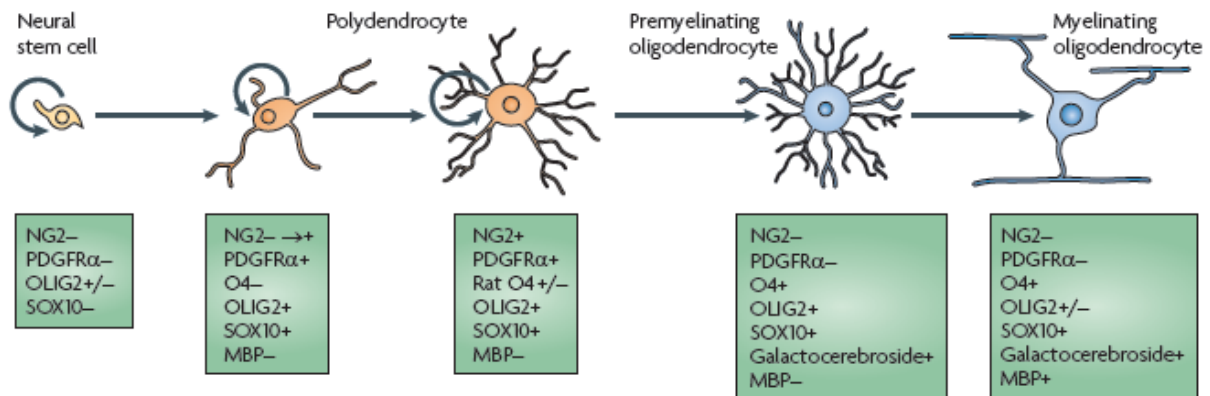


Figure 1.3 – OPC differentiation process

NG2 and PDGFR α are not expressed by neural stem cells but are expressed by proliferating progenitor cells (proliferation is indicated by the semicircular arrows) of the OL lineage. These OPCs differentiate into premyelinating OLs, they lose the expression of NG2 and PDGFR α and begin to express the immature OL antigen O4. The myelinating stage is characterized by the expression of myelin proteins such as MBP, MAG and PLP (not listed here). SOX10 is expressed throughout development, whereas Olig2 seems to be down-regulated in the mature OLs (adapted by Nishiyama et al., 2009).

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 and GD3. Pre-oligodendrocytes further progress to the mature stage during which they synthesize the myelin basic protein (MBP), the myelin associated glycoprotein (MAG) and the mature isoform of PLP1 (PLP). 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).

1.1.2.1 Factors necessary for OL survival and maturation

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 the factors known to be involved in the control of OL survival and differentiation.

Growth and trophic factors

Growth and neurotrophic factors have been known to be involved in the regulation OPC differentiation since the 1990s. These group of molecules include platelet-derived growth factor (PDGF), basic fibroblastic growth factor (bFGF), insulin like growth factor 1 (IGF1) and ciliary neurotrophic factor (CNTF).

- PDGF is synthesized during development by both astrocytes and neurons. It is a survival factor and a potent mitogen for OPCs, although it triggers only a limited number of cell division. Its receptor, the PDGFR- α can be considered a typical marker of OPCs since the receptor disappears at the O4 stage of OL maturation. PDGF also stimulates *in vitro* the motility of OPCs and is chemoattractive. Of note, it is able alone to stimulate multipotent neuronal staminal cells to differentiate towards the oligodendroglial lineage by inducing the Olig2 expression (Hu et al., 2008). During differentiation, OLs become insensible to PDGF because of the blockade of its intracellular signaling pathway and, then, of its synthesis. (Hart et al., 1989)
- bFGF (also called FGF 2) is also a mitogen for neonatal OPCs. It up-regulates the expression of PDGFR- α and therefore increases the developmental period during which OPCs or pre-oligodendrocytes are able to respond to PDGF. Pre-oligodendrocytes can even revert to the oligodendrocyte progenitor stage when cultured with both PDGF and bFGF. This inhibition of OL differentiation can be overridden by the presence of astrocytes (Baumann et and Pham-Dihn, 2001).
- IGF1 has been reported to promote proliferation and survival of both OPCs and pre-oligodendrocytes, by interacting with the Type I receptor (IGF-1R), a heterotetramer with intrinsic tyrosine kinase activity and by activating two important intracellular downstream signaling pathways, the MEK/ERK and PI3K/Akt cascades (Bibollet-Bahena and Guillermina, 2009). On the other hand, the effect on OL development is related to the stimulation of protein synthesis through the phosphorylation not only of the MEK/ERK and PI3K/Akt cascades but also of the *mammalian target of rapamycin* (mTOR), which regulates protein translation through the phosphorylation of key-factors involved in this process (Bibollet-Bahena and Almazan, 2009). Moreover, *in vivo* studies performed in

transgenic mice that overexpressing IGF1 have shown that the percentage of myelinated axons and the thickness of the myelin sheaths were significantly increased. On the contrary, mice that ectopically express IGF binding protein-1 (IGFBP-1), a protein able to inhibit IGF1 action had a decreased number of myelinated axons, together with a reduction of the myelin sheaths thickness. In addition, IGF1 could be involved in both the increase in OL number and in the amount of myelin produced by each OL (Carson et al., 1993). In this respect a phase II pilot study of the tolerability and efficacy of subcutaneously administered recombinant human IGF1 (CEP-151) in patients with Multiple Sclerosis (MS) has been completed, although no results have been reported yet.

- CNTF is a neurotrophic factor that can also act as comitogen with PDGF. Animals deficient in CNTF have a reduced number of mitotic glial progenitors. It has also been demonstrated that CNTF promotes OL survival *in vivo* (Barres et al., 1993).

Neurotrophins

Neurotrophins are a family of proteins which include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and NT-4/5. These molecules were initially shown to be important in the development of specific neuronal population (Barde et al., 1982; Huang and Reichardt, 2001). However, it is now recognized that neurotrophins also modulate cell-fate decisions (McTigue et al., 1998) and control various stages of glial development.

- NT-3 is a mitogen for optic nerve oligodendroglial precursors only when added with high levels of insulin, with PDGF, or with a combination of them. NT-3 also promotes OL survival *in vitro*. Its receptor is the tropomyosin-related kinase C (TrkC) receptor that is expressed in OLs. *In vivo* studies demonstrated that, mice lacking NT-3 or its receptor TrkC, exhibit profound alterations in CNS glial cells, particularly in OPCs; there is an important reduction in the spinal cord diameter, thereby suggesting that cell populations other than neurons are affected (Kahn et al., 1999). It has been shown that NT-3 in combination with BDNF is able to induce proliferation of endogenous OPCs and the subsequent myelination of regenerating axons in a model of contused adult rat spinal cord (McTigue et al., 1998).

Neuregulins

Neuregulins are a family of four structurally related proteins that are part of the EGF family of proteins. These proteins have been shown to have diverse functions in the development of the nervous system, including Schwann cells and OL differentiation (Taveggia et al., 2010). Among neuregulins, the glial growth factor (GGF) has been shown to modulate OPC differentiation. It is a member of the neuregulin family of growth factors generated by alternative splicing, including Neu, heregulin, and the acetylcholine receptor-inducing activity (ARIA); it is a neuronal factor, mitogenic on oligodendrocyte precursors; it is also a survival factor for these cells. It delays differentiation into mature oligodendrocytes. In mice lacking the family of ligands termed neuregulins, OLs in spinal cord failed to develop (Vartanian et al., 1999). This failure can be rescued *in vitro* by the addition of recombinant neuregulin to explants of spinal cord. In the embryonic mouse spinal cord, neuregulin expression by motoneurons and the ventral ventricular zone is likely to exert an influence on early OPCs. Neuregulin is a strong candidate for an axon-derived promoter of myelinating cell development (Barres and Raff, 1999).

Neurotransmitters and their receptors

It has long been known that neurotransmitters have trophic functions on glial cells, besides their main role in neurotransmission. According to this, different receptors for neurotransmitters have been found on OL plasmatic membranes. Once activated, these receptors can have positive or negative effects on OPC survival and maturation.

Several data suggest that glutamate, the most abundant excitatory neurotransmitter in the mammalian brain is an excellent candidate signal for the neuronal regulation of OPCs development. Accordingly, the AMPA/Kainate subtype of glutamate receptors has been reported to be expressed and to play an important role in the early development of OPCs (Gallo et al., 1996; Gudz et al., 2006). Moreover, recent evidence shows that electrically active axons can induce myelin formation by vesicular release of glutamate that signals nearby OLs to start local production of a myelin-building protein (Wake et al., 2011; Araque et al., 2011). Although the responsible glutamate receptors in this process remain unexplored, these data suggest that glutamate may also be involved in the late development of OPCs and provide a novel insights into how experience can influence brain development.

The other important subtype of ionotropic glutamate receptor, the NMDA receptor (NMDAR), has been reported to be extremely important for CNS function. Also NMDARs exist in the OL lineage cells and they contribute to myelin damage in pathological conditions

(Karadottir et al., 2005; Salter and Fern, 2005; Micu et al., 2006; Manning et al., 2008). In a recent work it has been demonstrated that NMDARs activation promotes OPC differentiation *in vitro* and remyelination in an *in vivo* model of demyelination, providing these receptors as a potential targets for the treatment of demyelination diseases (Li et al., 2013).

The dopamine D3 receptor (D3R) has been found to be expressed by precursors and immature OLs, but not in mature OLs. Interestingly, by confocal microscopic analysis, it has been shown that D3R is associated with cell bodies and cell membranes, but not with the processes emanating from cell soma. Moreover, immunohistochemistry has revealed the presence of D3R in some OLs located mainly within parts of the *corpus callosum* during myelinogenesis. Treatment of glial cultures with a dopamine agonist altered the normal pattern of OL maturation and elaboration of myelin sheath (Bongarzone et al., 1998). Another dopamine receptor, D2R, is also present in a subset of mature interfascicular OLs in the rat *corpus callosum* (Howard et al., 1998). Although the identification of these receptors in OLs, their physiological role in these non-neuronal cells is still not known.

OLs also express opioid receptors. μ -receptors are present since the earliest stages of OL development, while κ -receptors are detected later at the time that MBP is expressed. μ -receptors stimulation has been shown to trigger a proliferative response in these cells (Knapp et al., 2000).

Finally, extracellular ATP has been identified as an important activity-dependent axonal signal that, when non-synaptically released from electrically stimulated axons, activates purinergic P2 receptors on neighboring OPCs, promoting their differentiation. According to this, both ATP and adenine nucleotide such as ADP and uracil nucleotide such as UDP are able to trigger Ca^{2+} increases in OLs (Butt et al., 2006; Fumagalli et al., 2011). These cells, in fact, express different subtypes of P1 and P2 receptors (Agresti et al., 2005; Verkhratsky et al., 2009). Interestingly, non-synaptically released ATP has been shown to stimulate astrocyte release of the pro-myelinating cytokine leukemia inhibitory factor (LIF), that, acting on OLs, accelerates their terminal differentiation (Ishibashi et al., 2009). For further details see paragraph 1.1.4.

Hormones

It has also been demonstrated that hormones can influence the myelination process. According to this, the discovery that hyperthyroidism accelerates the myelination process and hypothyroidism decreases it, assumes that the thyroid hormone 3,3,5-triiodothyronine (T_3) may be involved in OL development. Moreover, OL differentiation as well as the degree of

myelin synthesis is increased by thyroid hormone. In fact, *in vitro* studies, demonstrated that OPCs stop dividing and differentiate in the presence of T₃. This hormone also promotes the morphological and functional maturation of postmitotic OLs, thus increasing the number of mature OLs. The effect of T₃ is mediated by its interaction with specific receptor isoforms (TR). Only three TR isoforms are functional: α 1, β 1, and β 2. OPCs mainly express α 1 isoform, although β 2 may also be present, whereas mature OLs express α 1 and β 1. The expression of different receptors in relation to the maturation state of the OLs may mean that these effects are independently regulated by thyroid hormone. It has been shown that T₃ regulates the gene expression of MBP (Farsetti et al., 1991), PLP, MAG, and CNP (Ibarolla et al., 1997).

To the same family of nuclear receptor of TR belong retinoid X receptors (RXRs). Recently, it has been shown that these receptors are positive regulators of OPC differentiation (Huang et al., 2011). RXRs are nuclear receptors that regulate cell proliferation and differentiation (Ahuja et al., 2003; Germain et al., 2006; Lefebvre et al., 2010). There are three members in the RXR family, RXR α , RXR β and RXR γ , which form homodimers or heterodimers with other nuclear receptors, including retinoic acid receptors (RARs), TR, vitamin D receptors (VDRs), peroxisome proliferator activator proteins (PPARs), and liver X receptors (LXRs), to control transcription of target genes. Following CNS injury, all three members of RXR are highly expressed in lesions (Schrage et al., 2006). Interestingly, it has been recently found that RXR γ is highly expressed in OLs during the regenerative phase of CNS remyelination (Huang et al., 2011). In acute and remyelinating MS lesions, RXR γ is highly expressed by OLs, macrophages and astrocytes, but its expression is very low in chronic inactive lesions, suggesting that RXR γ is an active component of remyelination. Moreover, the *in vitro* transfection of cultured OPCs with siRNAs generated against RXR γ , resulted in less morphologically differentiated OLs. Based on these results, RXR γ emerges as a regulator of OL differentiation and as a promising pharmacological target for regenerative therapies of CNS (Huang et al., 2011).

Micro-RNA (miRNAs)

In recent years, increasing evidence indicate that also epigenetic mechanisms take part into OL development. These mechanisms include chromatin remodeling by histone deacetylases (HDACs), DNA methylation and, above all, gene silencing by miRNAs (Mehler, 2008; Liu and Casaccia, 2010; Yu et al., 2010). MiRNAs are a class of endogenous small non-coding

RNA that consist of about 22 nucleotides processed from endogenous genomic loci that bind primarily to the 3' Untranslated region (UTR) of target mRNAs through an imperfect match to repress their translation and stability (Bartel, 2009). It is known that they play a critical role in various biological processes, including cell proliferation, differentiation, apoptosis and tumorigenesis (Zheng et al., 2010; 2012). Recently, several studies have demonstrated that miRNAs are required in controlling OL differentiation and myelination (Dugas et al., 2010; Letzen et al., 2010; Zhao et al., 2010). Specifically, a number of stage-specific miRNAs have been identified. Some of them are directly involved in the regulation of OPC proliferation, others promote the transition from OPCs to mature/myelinating OL, affecting both OPC differentiation and myelination.

Concerning the control of the number of oligodendroglial cells, it has been shown that the miR-17-92 cluster, processed from common precursor transcripts, including miR-17, miR-18a, miR-19a, miR-20a, miR19b, miR-92a (Lau et al., 2008), and especially miR-19b, play essential roles in this process. Both *in vitro* and *in vivo* experiments demonstrated that these miRNAs promote OPC proliferation through the inhibition of PTEN, a phosphatase involved in the regulation of the cell cycle (Budde et al., 2010). Besides miR-17-92, other miRNA are involved in the regulation of OPC proliferation. Among these, miR-219, miR-338 and miR-138 take part to this regulation by targeting OPC-expressed genes, (e.g. PDGFR α , Hes-5 e Sox-6), whose expression is necessary to promote the cessation of proliferation coupled to OL differentiation (Zhao et al., 2010; Dugas et al., 2010). They also inhibit proliferation signaling molecules such as PDGFR α and FGFR, as well as neuronal differentiation factors (e.g. Zfp238 and FoxJ3) (Dugas et al., 2010; Zhao et al., 2010). MiR-219 may also regulate OL terminal maturation and myelin maintenance by targeting fatty acid elongases such as ELVOL7 (Shin et al., 2009). The only difference among miR-219, miR-338 and miR-138 is that, while miRNA 219 and miR-338 promote the expression of CNPase, MBP and MOG of both early and late OL differentiation stages, miR-138 promotes the expression of CNPase and MBP but not of MOG (Dugas et al., 2010; Zhao et al., 2010).

Although the functional significance of these miRNAs is beginning to emerge, these results confirm the fact that the miRNA play multiple roles at various stages of OL development, including the initial production of fate-specified OPCs, the differentiation of mature OLs and generation of compact CNS myelin during development, and in the maintenance of functional myelin sheaths. New knowledge about these small regulatory molecules will offer novel therapeutic interventions by which disease-related miRNAs could be antagonized.

1.1.3 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 allow the myelin sheath to support the fast nerve conduction in the thin fibers in the vertebrate system. High-speed conduction, fidelity of transfer signaling on long distances, and space economy are the three major advantages conferred to the vertebrate nervous system by the myelin sheath, in contrast to the invertebrate nervous system where rapid conduction is accompanied by increased axonal calibers. 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).

1.1.3.1 Myelin sheath composition

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 insulating properties of the myelin sheath, which favor rapid nerve conduction velocity, are largely due to its structure, its thickness, its low water content, and its richness in lipids. 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).

Myelin proteins comprise 30% dry weight of myelin. 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. 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 the proteolipid proteins 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. Interestingly, MAG function has been studied by generating MAG gene knock-out mice in which, surprisingly, CNS myelin forms almost normally even though the absence of MAG; however, a prominent defect of the mutant myelin sheaths is an abnormal formation of the periaxonal cytoplasmic collar that is lacking in most of the internodes; moreover, myelin sheaths contain cytoplasmic organelles between lamellae, indicating a delay or block of myelin compaction and 10% of axons, versus 3% in wild-type mice, received from two to four sheaths around a single axon, suggesting that MAG may have a role in helping OL processes to distinguish between myelinated and unmyelinated axons in the CNS (Li et al., 1994; Montag et al., 1994)

MOG was first identified by a polyclonal antibody directed against an antigen called M2 that induces autoimmune encephalomyelitis 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).

1.1.3.2 Myelination

Myelination consists of the formation of a membrane with a fixed composition and specific lipid-protein interactions, allowing membrane compaction and the formation of the dense and intraperiodic lines of myelin. Therefore, myelination also needs activation of numerous enzymes of lipid metabolism necessary for the synthesis of myelin lipids, of synthesis and transport of specific protein components of myelin or their mRNAs to the 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 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 1.1.3) and, then, ready for myelination (Figure 1.4).

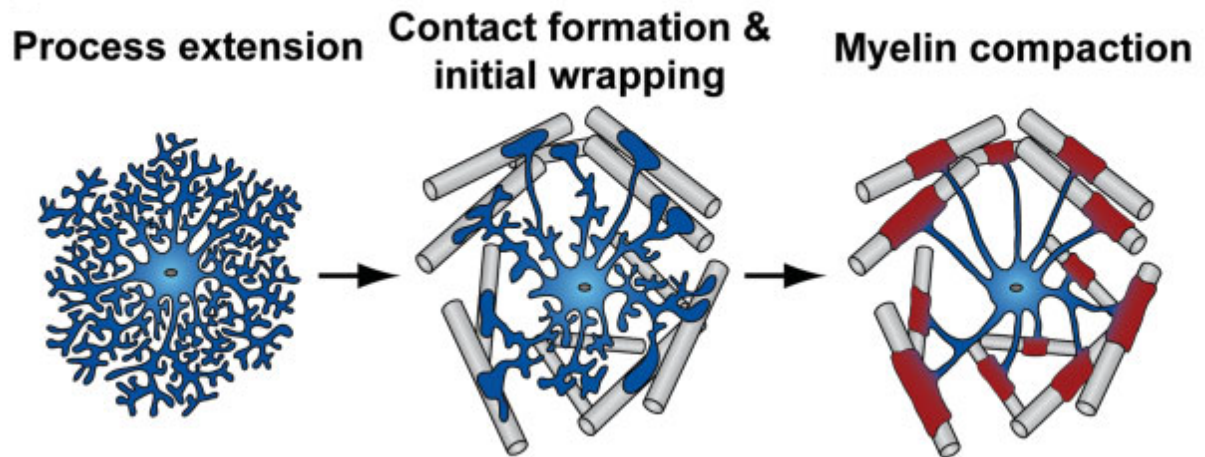


Figure 1.4 – Scheme representing the myelin sheath formation process

Myelination process is characterized by three phases: (i) OLs differentiate and extend highly ramified processes; (ii) OLs take contact with neuronal axons and start wrapping them; (iii) the ensheathment becomes compact and myelin sheath is formed (adapted from Bauer et al., 2009).

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 place. Two models have been proposed (Figure 1.5). 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 al., 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.

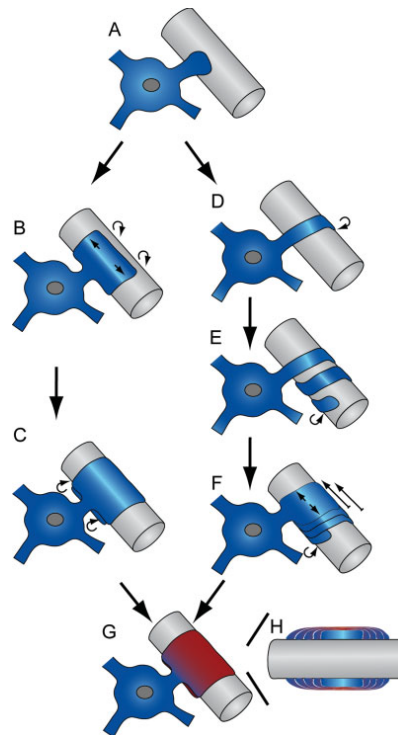


Figure 1.5 – Scheme representing the two models of myelination.

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).

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-glia contact; the second one 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).

The development of OLs and myelination of individual axon is a highly regulated process controlled by a number of mechanisms. These include axonal surface ligands, secreted molecules and axonal activity (Emery, 2010).

Candidates for axonally derived soluble-factors include FGFs and thyroid hormone, while-axonal cell surface molecules such as L1, MAG, NCAM and N-cadherin have been reported to regulate formation of the myelin sheath (Miller et al., 2002). As already mentioned in the paragraph 1.1.2.1, OL maturation is also influenced by neuregulins that are expressed on many axons and induces morphological changes in cultured OLs (Vartanian et al., 1994).

Different signaling pathways have been also reported to regulate myelination. In this respect, Wnt signaling via the canonical pathway is transiently activated in OPCs concurrent with the initiation of terminal differentiation. Both β -catenin activity and the expression of Tcf4/Tcf7l2 (a transcription factor that mediates the transcriptional effects of the Wnt/ β -Catenin pathway) are subsequently down-regulated in mature OLs (Fancy et al., 2009; Fu et al., 2009). This down-regulation of Wnt signaling may be necessary for OL differentiation, as mutant mice with elevated Wnt/ β -catenin signaling in the OL lineage display blocked differentiation and hypomyelination (Fancy et al., 2009). Paradoxically, however, deletion of the Wnt effector Tcf4 does not cause precocious OL differentiation as may be expected, but also blocks OL differentiation (Fu et al., 2002, Ye et al., 2009). Wnt signaling may, thus, exert complex roles in myelination, acting in conjunction with Tcf4 to promote the initial stages of OL differentiation, but preventing subsequent differentiation steps and myelination unless down-regulated (Emery et al., 2010)

Two other signaling pathways have been shown to be involved in regulation of myelination: Notch-1 and LINGO-1. Notch-1 and its ligands are signaling molecules that are involved in gene regulation mechanisms including those that induce neuronal development. Non myelinating OLs express Notch1 receptors, and neurons/axons express its ligand, Jagged1 or contactin (Wang et al., 1998; Nakahara et al., 2009). Binding of Jagged1 to Notch induces expression of the transcription factor Hes5, which blocks the maturation of Notch1-expressing cells, facilitating the migration of OPCs to white-matter tracts of the CNS. As development proceeds, down-regulation of Jagged1 is associated with the maturation of OPCs and myelination. These data suggest that Notch-1 may inhibit differentiation of OPCs in order to facilitate their migration to the white matter (Patel and Klein, 2011). LINGO-1 is a transmembrane protein that is abundantly expressed in the cortex of CNS and has been implicated in the inhibition of axon regeneration (Mi et al., 2004). It also regulates remyelination in the adult CNS by inhibiting OL differentiation. In 2009, using three different animal models of de/remyelination: EAE, cuprizone induced demyelination, and lysophosphatidylcholine (LPC) induced demyelination, Mi and coworkers demonstrated that LINGO-1 antagonism enhanced OPC differentiation and promoted remyelination of

demyelinated axons. Furthermore, LINGO-1 antagonism using monoclonal antibody 1A7 in the EAE model showed improvement in axonal integrity and the formation of new myelin sheath. In this respect, in a Phase I trial of BIIB033 (Biogen Idec), a LINGO-1 antagonist administered by infusion is currently recruiting (NCT01244139).

Besides an axonal regulation of myelination process, it is believed that much of the regulation of OL behavior is intrinsic in nature, with mechanisms such as an internal “clock” limiting the number of cell divisions in OPC cultures grown in the absence of neurons. According to this, recent findings provide evidence for the involvement of transcriptional regulators. Some of them are reported below.

- MRF (*Myelin gene regulatory-factor*) is a transcriptional factor that promotes the expression of many genes important in the production of myelin. It is therefore of critical importance in the development and maintenance of myelin sheaths. The expression of this transcriptional regulator is specific to mature, myelinating OLs. According to this, *in vivo* studies have shown that mice lacking-MRF in the oligodendroglial lineage continue to generate OLs but these cells do not fully mature and display defects in myelin gene-expression and myelin internode formation (Emery et al. 2009).
- YY1 (*Yin Yang 1*) is a transcriptional factor multifunctional, ubiquitously expressed, zinc finger protein that can act as a transcriptional activator, repressor, or initiator element binding protein. Previous studies have shown that YY1 modulates the activity of reporter genes driven by the myelin proteolipid protein (PLP1/Plp1) promoter and mice expressing a loss of function of YY1 generate OLs that do not fully mature to form proper myelin segments (He et al. 2007). YY1 is thought to repress transcriptional inhibitors by recruiting HDACs (He et al. 2007). HDACs normally remove acetyl groups from histones to allow for chromatin compaction, which subsequently silences transcription. Recent evidence suggests that this mechanism may be responsible for the repression of pathways that normally prevent OL differentiation (Li et al. 2010, Shen et al., 2005).
- Sox10 is a transcriptional factor characterized by a high-mobility-group DNA-binding domain (Wegner et al., 2001). Up to now, more than 20 different Sox proteins have been identified in mammals. In OPCs, Sox10 expression starts earlier than the expression of other commonly employed markers for these precursors such as PDGFR α and DM-20, but slightly later than Olig-2 (and possibly Olig-1). Once turned on, Sox10 remains expressed in OLs throughout the development and in the

adult. While the role of Sox10 in both development and differentiation of peripheral glia has been clearly established, its function in the OL lineage is still not known. So far, the best evidence for an essential role of Sox10 in OL development comes from the analysis of a human patient (Inoue et al., 1999). This patient carries a Sox10 allele in which deletion of a 12-bp segment at the end of the open reading frame has led to loss of the stop codon and elongation of the open reading frame by 82 additional amino acids. This mutation leads to severe myelin deficiencies in both the PNS and CNS, indicating that the mutant protein interferes with both Schwann cells and OPC development. Further studies are ongoing in order to clarify the role of this transcriptional factor.

- Nkx2.2 is a transcriptional factor that is expressed by OPCs during development (Qi et al., 2001). Experiments carried on in Nkx2.2 null mutants demonstrated that OPC differentiation is dramatically retarded in these animals suggesting that this transcriptional factor is involved in the regulation of this process. Interestingly, absence of Nkx2.2 expression also leads to a ventral expansion of the Olig1/Olig2 expression in neuroepithelial cells with a consequent increase in the production of Olig1/Olig2⁺- and PDGFR α ⁺-OPCs. These results strongly suggest that Nkx2.2 regulates the differentiation and/or maturation, likely affecting MBP and PLP expression (Qi et al., 2001) but not the initial specification, of oligodendrocyte progenitors.

1.1.3.3 Demyelination

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. Too little is, at present, 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 present 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 perioxosomal 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).

Another important myelin disease in the CNS is periventricular leukomalacia. This is a major cause of cerebral palsy and is thought to result from oligodendroglial loss or, at later stages of fetal development, damage secondary to ischemia or infection. However, it cannot be classified as a disease that causes primary demyelination for two reasons. First, the time of greatest risk (24–32 weeks of gestation) corresponds to the time of myelin formation, with a predominance of progenitor and pre-OLs seen in the CNS. Second, there may be significant axonal damage, with secondary rather than primary oligodendroglial defects resulting from abnormal axo–glial communication. It is more accurate therefore to classify it as a disease of hypomyelination.

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+} pompe 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 synthesizing neurotrophic factors such as

CNTF, IGF and glial cell-derived neurotrophic factor (GDNF) (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. In the brain, the main source of reactive oxygen species (ROS) are mitochondria, nitroxide synthase, monoamine oxidase and P450 enzymes. The high metabolic rate of neurons requires a certain basal production of ROS since myelin production is an energy dependent process, which needs large amounts of ATP and oxygen. 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. Hydrogen peroxide is also produced by peroxisomes, which are plentiful in OLs because of their need to produce large quantities of lipids. Cellular metabolism also creates reactive oxygen species, which are highly toxic and induce lipid peroxidation and DNA damage (McTigue and Tripathi, 2008). Another reason for OL susceptibility to oxidation is that many metabolic and myelin synthetic enzymes require iron as co-factor. Both OLs and OPCs have the largest stores of iron in the adult brain. While this metal is necessary for myelin production, it is also highly reactive and can evoke free radical formation and lipid peroxidation. This, together with high metabolism and numerous peroxisomes make OLs particularly vulnerable to oxidative damage. Moreover, paradoxically, OLs have low concentrations of glutathione (GSH), a robust antioxidative enzyme. This low glutathione concentration would allow intracellular iron levels rise. Thus, healthy cerebral cells need high levels of antioxidant defenses, both enzymatic (as the $\text{Cu}^{2+}/\text{Zn}^{2+}$ superoxide dismutase, glutathione peroxidase and the catalase) and non enzymatic (as glutathione, ascorbic acid and vitamin E). If these defenses are not able to keep 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).

In addition to risks associated with myelin synthesis, certain constituents of the myelin membranes can be damaging to OLs. Accordingly, the sphingomyelinase/ceramide pathway has been shown to be involved in the demyelination. These enzymes catalyze the breakdown of sphingomyelin to ceramide and phosphorylcholine. Once released into the cell, ceramide acts as a second messenger through intracellular signaling molecules in two different biological processes: cellular differentiation of tissues and programmed cell death. As previously described, brain is enriched in sphingolipids, which are the major lipid components of plasma membranes and comprise up to 20% of the dry weight of myelin. These molecules were long thought to be important for structural support but, now, it is also clear that they play key roles in cellular signaling. Ceramide contained in sphingolipids can coalesce to form domain enriched in death receptor (DRs), so called because they can activate programmed cell death. Thus, OLs, having an high percentage of sphingolipids, are susceptible to DRs effects. Moreover, sphingomyelinase (SMase), which is normally inactive, gets activated by signals commonly involved in CNS injury such as irradiation, infectious agents, interleukin (IL)-1 β , tumor necrosis factor α (TNF α), or nerve growth factor (NGF) (McTigue and Tripathi, 2008).

1.1.3.4 Remyelination.

As previously described, the loss of OLs and of the myelin sheath has dramatic consequences in the CNS. However, 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, salutatory conduction is reinstated and functional deficits are resolved (Figure 1.6). 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

The question of 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).

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 provide compelling evidence for a key role of neuroinflammation in remyelination. 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 have always 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.

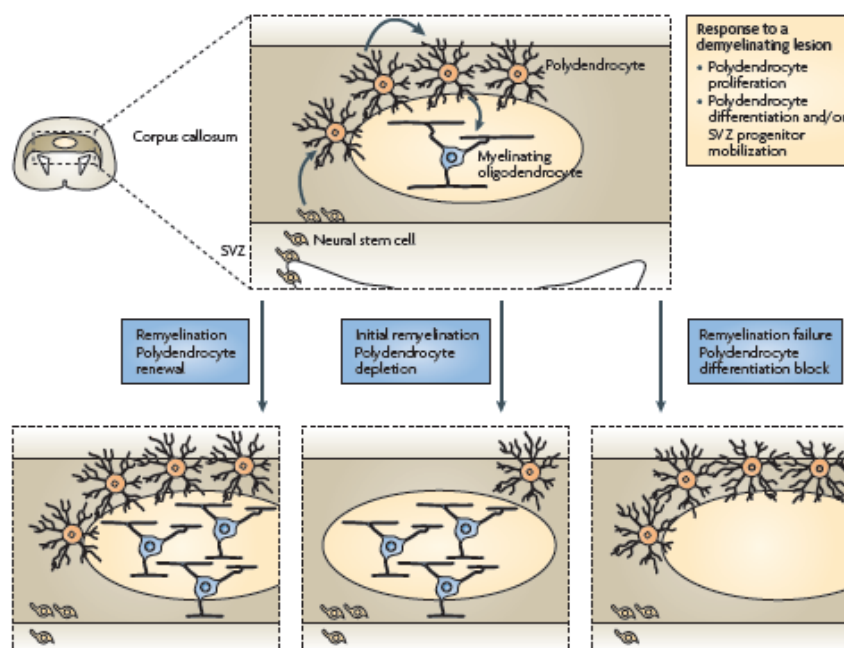


Figure 1 6 - Events that occur in response to a demyelinating lesion in the corpus callosum.

Following a demyelinating lesion (yellow oval), polydendrocytes that reside in the corpus callosum (dark green area) or those that are recruited from the subventricular zone (SVZ) proliferate around the lesion and differentiate into OLs, which myelinate demyelinated axons in the lesion (top panel). The extent of remyelination can therefore be affected by both the ability of the polydendrocytes to self-renew and their ability to differentiate into myelinating OLs. Successful remyelination might occur when polydendrocytes proliferate and differentiate into OLs, and in this scenario the polydendrocyte population would be replenished (left-hand bottom panel). Successful remyelination might also occur in the absence of substantial proliferation, in which case the polydendrocytes would not be repopulated and a second demyelinating lesion would therefore not be successfully remyelinated (central bottom panel). In the other possible scenario, remyelination would not occur because proliferated polydendrocytes fail to differentiate into OLs, or newly differentiated OLs fail to ensheath axons (right-hand bottom panel) (from Nishiyama et al., 2009).

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

Like all regenerative processes, the efficiency of remyelination decreases with age. This manifests as a decrease in the rate at which it occurs and is likely to have a profound bearing on disease progression (which, in the case of MS, can occur over many decades). The consequences of slow remyelination 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. The basis of the aging effect is likely to lie in age-associated changes, in both the extrinsic environmental signals to which OPCs are exposed in remyelinating lesions, and intrinsic determinants of OPC behavior. 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 depth the knowledge concerning this topic and try to answer many questions still unresolved.

1.1.3.5 A deeper insight into the biological features of polydendrocytes

Historically, it was believed that the mammalian CNS contains only three types of glial cells: astrocytes, OLs and microglia. However, observations made over the past two decades have revealed another major glial cell population that is distributed throughout the developing and mature CNS. As mentioned above, these cells are known as NG2⁺ or polydendrocytes to reflect the multi-processed morphology and the lineal relationship to OLs that characterize this type of glial cells. Polydendrocytes are defined as CNS parenchymal cells (non-vascular cells) that express the NG2 proteoglycan (Nishiyama et al., 2009).

The NG2 chondroitin sulphate proteoglycan is a single membrane-spanning chondroitin sulphate proteoglycan with a large extracellular domain and a short cytoplasmic tail. It was first identified and characterized on the cellular membrane of rat neurons in the late 1970s (Stallcup, 1981) and, subsequently, it has also been identified in vascular mural cells,

including pericytes, cells of mesenchymal lineages, such as immature chondrocytes, osteoblasts and myoblasts and stem cells in the skin (Trotter et al., 2010).

The first evidence that polydendrocytes are OPCs came from the observation that OPCs purified by immunopanning with A2B5 antibody were 95% positive for NG2. Moreover, these cells are positive for PDGFR α and, when cultured in differentiation media, these cells differentiate into OLs that express galactocerebroside (Levine and Stallcup, 1987; Stallcup and Beasley, 1987). On the other hand, polydendrocytes are antigenically distinct from astrocytes (they do not express glial fibrillary acidic protein -GFAP- or the glial glutamate-aspartate transporter -GLAST-) and from resident microglia (They do not express the microglial antigens F4/80 and CD11b or bind GSA-IB₄) (Nishiyama et al., 2009)

Since NG2⁺ cells make up 5-10 % of all glia in the developing and adult CNS, the comprehension of the physiological role of these cells is crucial. Surely, as a mitotically active population, the capability of these cells to generate new OLs is a known function. However, these cells participate in homeostasis in the adult CNS. For example, numerous evidences indicate that these cells are involved in glutamate signaling, as NG2⁺ cell processes can interdigitate between pre- and post-synaptic terminals (Bergles et al., 2000; Ong and Levine, 1999). Furthermore, excitatory synapses mediated by AMPA receptors have been identified on NG2⁺ cells and such cells have been shown to respond to neuronally released glutamate (Bergles et al., 2000). In addition to the expression of glutamate receptors, cells of the OL lineage have been shown to transport glutamate actively (Reynolds and Herschkowitz, 1987) and express glutamate transporter proteins (Kugler and Schmitt, 1999; Domercq et al., 1999). Together these data indicate that NG2⁺ cells have a key role in glutamate neurotransmission (Polito and Reynolds, 2005).

Besides the presence of glutamate receptors, NG2⁺ cells express functional GABA-A receptors (Lin and Bergles, 2004). These receptors alter the physiological properties of NG2⁺ cells by modifying ion concentrations within these cells. Accordingly, *in vitro* experiments showed that activation of these receptors on developing progenitor cells induce Ca²⁺ influx, inhibiting their proliferation and progression along the OL lineage.

High relevant is that NG2⁺-cells also react to many pathological conditions by active proliferation, hypertrophy, NG2 up-regulation and contribution to glial scar formation, suggesting that they actively participate in the neuroinflammatory events of the injured nervous system (Fumagalli et al., 2011).

A growing interest toward the NG2⁺ cells comes from both *in vitro* and *in vivo* studies, showing their ability to give rise to neurons and astrocytes, thus highlighting their intrinsic

multipotent capacity, even though this concept is currently a highly debated topic (Raff et al., 1983; Kondo and Raff, 2000; Guo et al., 2009; 2010).

Concerning the astroglial fate of NG2⁺ cells, early cell-culture studies showed that OPCs purified from rat optic nerves by immunopanning with antibodies against A2B5 ganglioside differentiate not only into OLs but also into process-bearing “type-2 astrocytes” in the presence of serum factors, which led to the concept of bipotential oligodendrocyte-type-2 astrocytes (O-2A) progenitors cells (Raff et al., 1983). More recently, A2B5⁺ cells from embryonic rat spinal cord were shown to differentiate into OLs, type-1 astrocytes and type-2 astrocytes in culture and have been called glial restricted progenitor (GRP) cells (Rao and Mayer-Proschel, 1997). Conversely, the search for *in vivo* correlates of O-2A progenitor cells or GRP cells has not revealed a clearly defined cell population. These observations led to the currently widely accepted view that the generation of type-2 astrocytes from OPCs was an *in vitro* artefact. In contrast to this, recent observations have indicated that polydendrocytes might be bipotential glial progenitors that can generate both OLs and astrocytes when transplanted into a glia-depleted environment (Franklin et al., 1995). This suggest that polydendrocytes are inherently capable of differentiating into astrocytes bur are prevented from fulfilling their astroglial fate in the normal *in vivo* environment (Nishiyama et al., 2009).

Concerning the neuronal fate of polydendrocytes, several observations suggest that polydendrocytes can also generate neurons. Accordingly, it has been demonstrated that neuron-like cells were generated in explant cultures of segments from neonatal rat optic nerves (Omlin and Waldmeyer, 1989) and that treatment of OPCs from postnatal rat optic nerves with bone morphogenetic protein2 (BMP2) and PDGFAA was shown to convert them into multipotent neural stem cell-like cells enabling the generation of neurons, astrocytes and OLs through epigenetic mechanisms (Kondo and Raff, 2004; Liu et al., 2007). As for the astroglial fate of polydendrocytes, also the *in vivo* capability to generate neurons is not so clear. This topic is currently studied by several laboratories through fate-mapping studies using transgenic animals in which Cre expression is driven by different promoters. Up to now, the results of these studies are not so clear. Some works claim that no neurons are generated from NG2 precursors (Dimou et al., 2008; Zhu et al., 2007); others demonstrated the neurogenic capability of these precursors (Rivers et al., 2008). The differences of these studies might be due to differences in the type of transgenic mice used. Surely, further studies will be addressed in order to clarify this debated topic

Research aimed at clarifying the functional role of NG2⁺ cells in the CNS are currently active within the scientific community since, up to now, some interesting questions related to

their heterogeneity, their capability to intervene after a cerebral injury, their multipotency and their role in the neural network remain still open.

1.1.4 Purinergic signaling and oligodendroglialogenesis

Few and recent studies have revealed a role for purinergic signaling in OL development and death (Butt, 2006; Agresti et al., 2005; Fumagalli et al., 2011). 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).

Recently, 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). In fact, 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). More recently, 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, Ca²⁺ influx and loss of Na⁺ and K⁺ gradients. Thus, its activation can amplify glutamate-receptor mediated excitotoxicity. A recent report (Matute et al., 2007) has demonstrated that ATP signaling can trigger OL excitotoxicity through the activation of P2X₇ receptors. 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 the experimental autoimmune encephalomyelitis (EAE), a model of MS (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 pyrimidineric 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) recently identified as 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 1.2.5.

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).

1.2 The G protein coupled receptor GPR17

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 a 80% homology in the amino acid sequence to the hGPR17, (Figure 1.7) (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 GPR17).

Here we aim at reporting the main structural, pharmacological, functional characteristics and expression profiles of GPR17 receptor so far known.

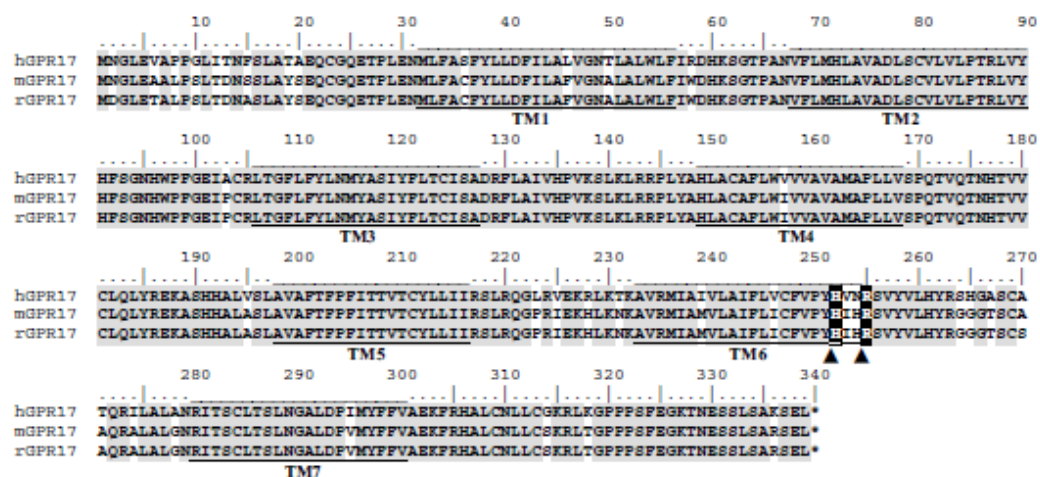


Figure 1.7 – Alignment of the human, the mouse and rat amino acidic sequence.

Note the highly conserved transmembrane domains (TM) and the H-X-X-R motif in T6 conserved in all GPCR receptors (from Ciana et al., 2006).

1.2.1 GPR17 receptor: structure and pharmacological characteristic

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 Gpr17) has two different exons and only a small portion of the second exon encodes for the GPR17 protein. So far nothing is known about mGpr17 promoter or regulatory sequence, neither for the human gene nor for the rat one. The majority of the studies have so far focused on GPR17 protein and its function.

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 1.8). 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 monophosphate), 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. GPR17 shows 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).

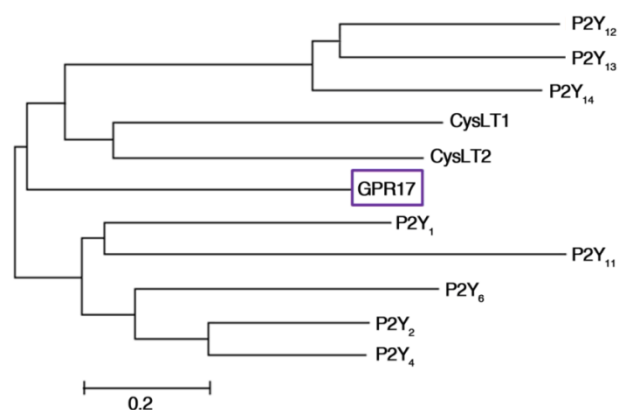


Figure 1.8 – Phylogenetic tree.

The figure represents the relationships between GPR17 and P2Y and CysLT receptors (from Ciana et al., 2006).

1.2.1.1 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]GTP γ S 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).

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 (μ M)) and for cysteinyl-leukotrienes; in fact, for the first, the EC₅₀ is comprised in a range while for the cysteinyl-leukotrienes (nanomolar (nM)).

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; Nørregaard 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.

1.2.1.2 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]GTP γ S 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-

metiladenosina 3',5'-bisphosphate) which antagonizes P2Y₁ (Jacobson et al. 2002) were assayed; both molecules inhibit in a concentration-dependent manner 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, ATPβS, 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 a concentration-dependent manner blocking 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.

1.2.1.3 Identification of new GPR17 ligands

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 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.

In the case of receptors such as GPR17 whose molecular structure is not yet known, 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, there are two homology models of hGPR17. The first model is entirely based on the crystal structure of bovine rhodopsin (bRh), the only high-resolution crystal structure of a GPCR available at time of publication of the model, and then the only useful "template" for the homology modeling. 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). Recently, thanks to protein engineering, a new GPR17 structure has been built (Figure 1.9) This is based on different templates: the human adenosine A_{2A}

receptor (hA_{2A}R), the human β_2 -adrenergic receptor (h β_2 AR), the turkey β_1 -adrenergic receptor (t β_1 AR) and squid Rh (Eberini et al., 2011).

The availability of a more accurate model of the receptor, 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).

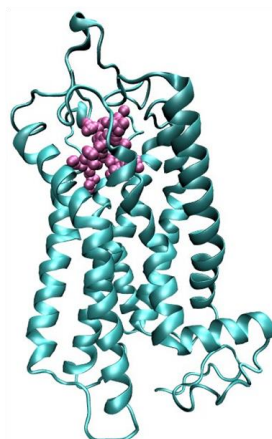


Figure 1.9 – Tridimensional model of human GPR17 receptor.

The color spheres represent the binding site predicted for extracellular nucleotides on the receptor.

Recently another molecule has been proposed as potential modulators of this receptor, the MDL29,951 (Hennen et al., 2013). Specifically, using different targeted pharmacological assays (measurement of cAMP accumulation, [³⁵S]GTP γ S incorporation, inositol phosphate accumulation, Ca²⁺ 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 II messengers and their reciprocal cooperation. Specifically, through cAMP assay, they showed a dual modulation of the adenylyl cyclase, suggesting an engagement of either the G_s and G_i mediated pathways. The coupling to G_i subunit after treatment with MDL29,951 was further demonstrated blocking the inhibition of forskolin-induced cAMP increase with the pertussis toxin (PTX). Moreover, functional GPR17-mediated G_{αq} activity was confirmed by a specific inhibition of Ca²⁺ 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).

1.2.2 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 G_i protein that inhibits the activity of the enzyme adenylylate cyclase and consequently the formation of cAMP (Ciana et al., 2006). It was also demonstrated the activation of G_q 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 1.5.2), 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, Ca²⁺-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 Ca²⁺ and membrane depolarization, which follow an acute ischemic stroke, by increasing K⁺ efflux, rapidly hyperpolarizing the membrane and reducing further voltage-dependent Ca²⁺ influx (Gribkoff et al., 2001).

1.2.3 GPR17 expression profile

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 transcriptome 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), β -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, myelin-associated glycoprotein (MAG) and myelin basic protein (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 autaptic 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 autaptic 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 (Figure 1.10). 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).

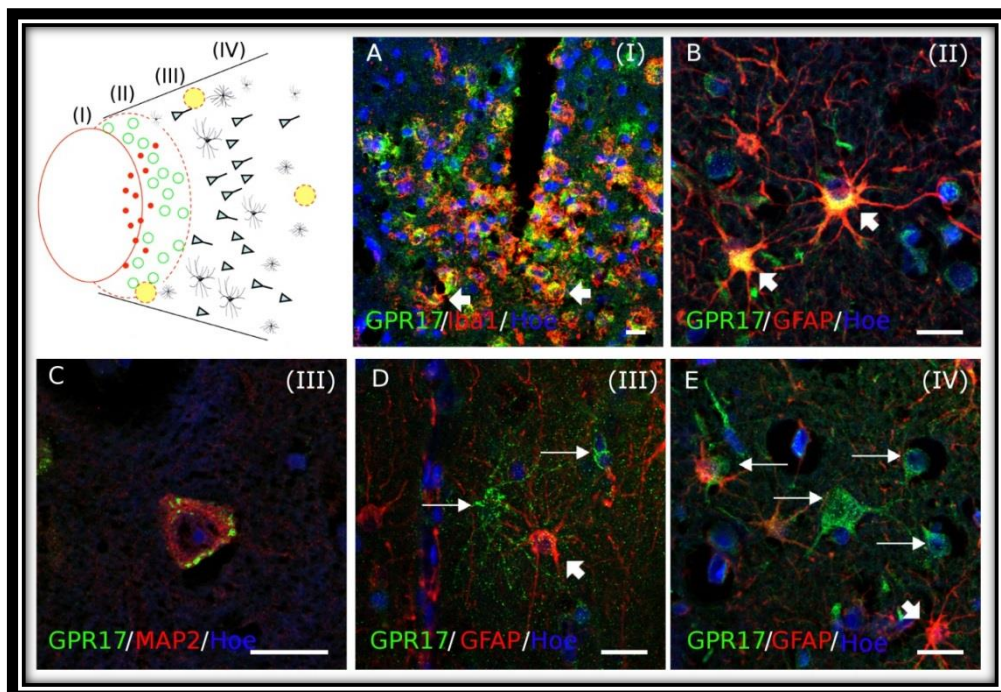


Figure 1.10 - Spatial gradient of GPR17 expression in various types of neural cells in autoptic specimens from TBI patients.

GPR17 expression was very strong in cells inside and at the borders of the necrotic core (zones I and II, also indicated as PCZ in the central drawing) and then gradually declined in zones (III) and (IV). Dense immunoreactive red spots in zones (I) and (II) of the central drawing likely represent shrunk dying or already dead cells, or fragments of necrotized cells. Green circles in zone (II) represent cells costaining for both GPR17 and the microglial marker Iba1 (a), thus representing activated globose microglia. In zone (III), stellate GPR17/GFAP double-positive reactive astrocytes were found (yellow cells indicated by arrows in b). GFAP-positive, GPR17-negative astrocytes (red cells in b; see also cells indicated by thick short arrows in d) were also present. Starting from zone (III) and throughout zone (IV), many pyramidal cells costaining for GPR17 and the neuronal marker MAP2 were present (c). In these zones, several GPR17-expressing cells that, however, did not stain for anyone of the previously mentioned markers (including GFAP) were also detected (green fluorescent cells indicated by long thin arrows in d and e). The morphology of these cells was reminiscent of that of OPCs. Nuclei are labeled with Hoechst 33258 dye (Hoe, blue fluorescence). Scale bars=10 μ m (a, b, d, e) and 20 μ m (c) (from Hanke et al., 2013).

1.2.4 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. GPR17 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.

The fact that GPR17 may act as sensor for white matter damage has also been confirmed in a recent work in which, an animal model of periventricular leukomalacia (PVL), that is the most common ischemic brain injury in premature infants, has been used. 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).

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.

1.2.5 Role of GPR17 in OL differentiation

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 first one corresponds to early, morphologically immature slowly proliferating NG2⁺ precursor cells that also express Olig2, PDGFR α , and the immature PLP isoform DM-20; the second one corresponds to more ramified, still immature pre-oligodendrocytes that are losing NG2 and PDGFR α immunoreactivity and already express O4, O1, and the two splicing variants of the myelin protein PLP (Figure 1.11). 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 μ M) or LTD₄ (100 nM) for 72 hours, induces a significant increase in the number of MBP⁺-cells (Lecca et al., 2008; Fumagalli et al., 2011). Additional *in vitro* data also show that, GPR17 antagonists (for example, cangrelor) or knock-down by siRNAs impaires 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 OLGs, 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 (I_K), in mature OL inwardly rectifying currents (K_{ir}) prevail. Altogether these data suggest the enhancement of I_K currents after UDP-glucose exposure is responsible for the GPR17-mediated facilitating effect in OPC maturation (Coppi et al., 2013).

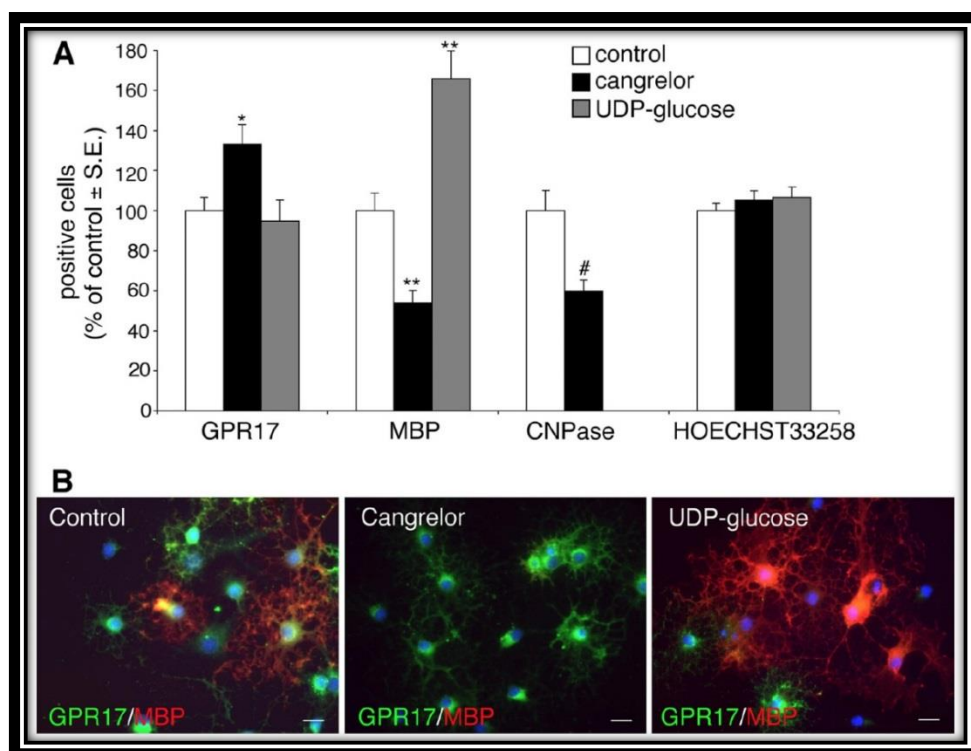


Figure 1.11 - Cangrelor delays and UDP-glucose accelerates OPC differentiation in vitro.

After GPR17 agonists treatment, cells were fixed and immunostained with anti-GPR17, anti-MBP, and anti-CNPase antibodies. **A**, histograms show quantification of the percentage of cells expressing the indicated OL markers in control and treated cells (with vehicle-treated control cells set to 100%). Hoechst 33258 was used to label cell nuclei. Data are the mean \pm S.E. (**, $p < 0.001$; *, $p < 0.05$ compared with control, one-way ANOVA, followed by Bonferroni's multiple comparison test. #, $p < 0.01$ compared with control, Student's t test). **B**, representative images of control, cangrelor-, or UDP-glucose-treated cells show double immunostaining with anti-GPR17 and anti-MBP antibodies. Nuclei were labeled with Hoechst 33258. Scale bars, 15 μ m. (from Fumagalli et al., 2011).

It was also observed that the receptor has a different intracellular localization depending on the differentiation stage: during the first day in culture, it is present within the compartments of synthesis of OPCs, while in more advanced differentiation stages it is found on the cell

surface and within the endosomal compartments. This observation suggests the hypothesis of a possible link between the subcellular distribution of GPR17 and the maturation process of these cells. In this regard, a recent *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. It has been propose that this is a key event necessary to allow OPCs to proceed to myelination (Fratangeli et al., 2013).

Recently, in astrocytes-OPCs 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).

A recent work has also 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).

An *in vivo* study has shown that, in cerebral cortex, during ontogeny, 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 which 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 (Figure 1.12). Finally, according to Chen and co-workers, GPR17 prevents OPC differentiation allowing the translocation into the nucleus of two potent repressors: ID2 and ID4. They belong to the family of transcription factors bHLH (basic proteins HLH) and they block OPC differentiation. Accordingly, *in vitro* studies showed that there is an over-expression of ID2 in the 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 go 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. As a result OPC differentiation is inhibited. 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 an OL-expressing CNP1 promoter. It means that, when CNPase is produced, GPR17 is transcribed (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 oligodendroglialogenesis, 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. In fact, it was also observed that, both in patients with MS and in animal models of EAE (Experimental Autoimmune Encephalomyelitis), that is a well established model of MS, 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 in order to take part to the reparative processes (Lecca et al., 2008; Ceruti et al., 2009; Boda et al., 2011).

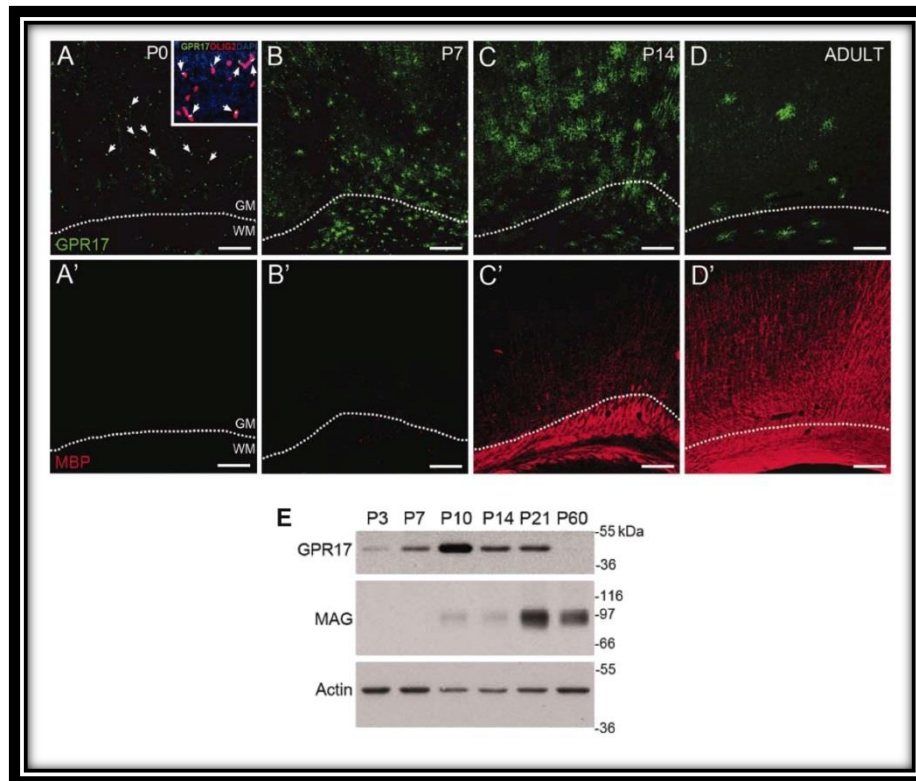


Figure 1.12 - Maturation pattern of postnatal GPR17 positive cells.

(A–D’): Changes in GPR17 and MBP expression from birth to adult stages. GPR17-positive cells were already present at birth (A). GPR17 expression increased over time (B and C) and declined in the adult cortex (D). MBP protein was instead not detectable until P7 (A’ and B’), whereas its expression level augmented at later time points (C’ and D’). (E) Western blot analysis of GPR17 and MAG proteins revealed the presence of GPR17 before MAG production has started. Furthermore, GPR17 protein appeared down-regulated before massive MAG protein production (modified from 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 sustained 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).

Although 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.

1.3 The mammalian target of rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) was identified and cloned (Brown et al. 1994; Chiu et al. 1994; Sabatini et al. 1995) shortly after the discovery of the two yeast genes, TOR1 and TOR2, in the budding yeast *Saccharomyces cerevisiae* during a screen for resistance to the immunosuppressant drug rapamycin (Kunz et al. 1993; Helliwell et al. 1994). Rapamycin was originally identified as an antifungal compound derived from *Streptomyces hygroscopicus*, present in soil samples collected from Easter Island (Dennis et al., 1999). It acts by forming an inhibitory complex with its intracellular receptor, the FK506-binding protein, FKBP12, which binds a region in the C terminus of TOR proteins termed FRB (FKB12–rapamycin binding), thereby inhibiting TOR activity (Chen et al. 1995; Choi et al. 1996).

mTOR is a highly evolutionarily conserved serine/threonine protein kinase belonging to the phosphoinositide 3-kinase (PI3K) family. It is ubiquitously expressed in cells and it regulates multiple cellular functions including survival, proliferation, organogenesis and differentiation of numerous cell types (Hwang et al., 2008). Mammalian genomes encode a single TOR protein with a similar structure exhibiting ~42% amino acid sequence identity to the yeast TOR proteins. TORs are high molecular weight proteins that contain several distinct and conserved structural domains. mTOR contains 2549 amino acids and comprises several conserved structural domains (Figure 1.13). The N-terminus possesses 20 tandem HEAT (for Huntignton, EF3, A subunit of PP2A, TOR1) repeats. Each HEAT repeat consists of two helices of ~40 amino acids, each with a specific pattern of hydrophobic and hydrophilic residues. The C-terminal half of mTOR contains the kinase domain. Immediately upstream of the catalytic domain is the FRB domain, which is involved in the interaction with FKBP12, the intracellular receptor of rapamycin. In addition, mTOR contains a relatively large FAT (for FRAP, ATM, TRAP) domain, which is also present in other PIKK proteins (Bosotti et al. 2000). The C-terminal end contains another FAT domain, designated FATC that is absolutely necessary for mTOR activity, and the deletion of even a single amino acid from this domain abrogates its activity (Peterson et al. 2000; Takahashi et al. 2000). mTOR also contains a putative negative regulatory domain (NRD) between the catalytic and FATC domains.

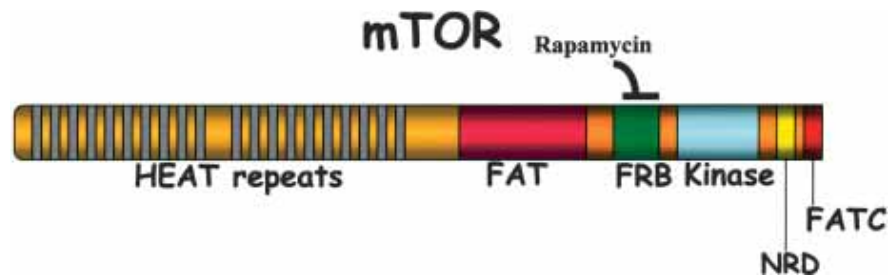


Figure 1.13 – Scheme representing the structural domains of mTOR kinase.

mTOR is a highly evolutionarily conserved serine/threonine protein kinase whose structure contains: the HEAT domain consisting of a flexible structure found in several cytoplasmic proteins; the FAT (for FRAP-ATM-TRAPP kinases) domain that is a homology region found in PI3-Kinase-related kinases; the FATC (FAT COOH-terminal) domain that is a highly conserved COOH-terminal domain, which regulates the function of mTOR; the FRB (FKBP12-rapamycin binding) domain that is the region recognized by the immunosuppressant drug rapamycin bound to immunophilin FK506 binding protein 12 (FKBP12) (from Hay and Sonenberg, 2004).

mTOR is linked to diverse cellular physiological functions that ultimately control cell and body growth. In the whole organism, mTOR plays a role in development, metabolism, memory and aging. At the cellular level, this kinase responds to the presence of nutrients and other growth cues (see below for details). It functions to regulate translation initiation, ribosome biogenesis and autophagy through mTORC1 and actin cytoskeleton reorganization through mTORC2 (see paragraph 1.3.1). Recently, evidence has indicated that mTOR is also involved in transcriptional regulation and RNA processing (Hannan et al., 2003; Cunningham et al., 2007; Willis and Moir, 2007; Kantidakis et al., 2010; Shor et al., 2010). Deregulation of mTOR signaling pathway is associated with the onset of pathological conditions including cancer, immune-related diseases, diabetes, cardiovascular and neurological disorders.

1.3.1 mTORC1 and mTORC2

mTOR exists in two functionally distinct complexes, the mTORC1 (rapamycin-mTOR complex) and the mTORC2 (rictor-mTOR complex). mTORC1 (Figure 1.14) is a heterotrimeric protein complex that functions as a nutrient/energy/redox sensor and controls protein synthesis (Hay and Sonenberg, 2004). mTORC1 is composed of mTOR itself, regulatory-associated protein of mTOR (Raptor), which positively regulates mTOR activity and acts as a scaffold for the recruitment of mTORC1 substrates (Hara et al., 2002; Kim et al., 2002), mammalian lethal with SEC13 protein 8 (MLST8), whose role remains unclear at present and the recently identified PRAS40 and DEPTOR (Kim et al., 2002; 2003; Wullschlegel et al., 2006). The activity of this complex is controlled by hormones, growth factors, cytokines and oxidative stress (Kim et al., 2002; Fang et al., 2001). In addition to extracellular stimuli, mTORC1 is responsive to internal signals. For example, when cellular

energy is low, the AMP kinase (AMPK) blocks mTORC1 activity, via activation of the inhibitory protein TSC (tuberous sclerosis complex) 2. Moreover, the activity of mTORC1 is also sensitive to nutrient availability. Increasing the cellular concentration of amino acids, such as leucine, activates mTORC1, although the molecular mechanisms are not fully elucidated (Garelick and Kennedy, 2011).

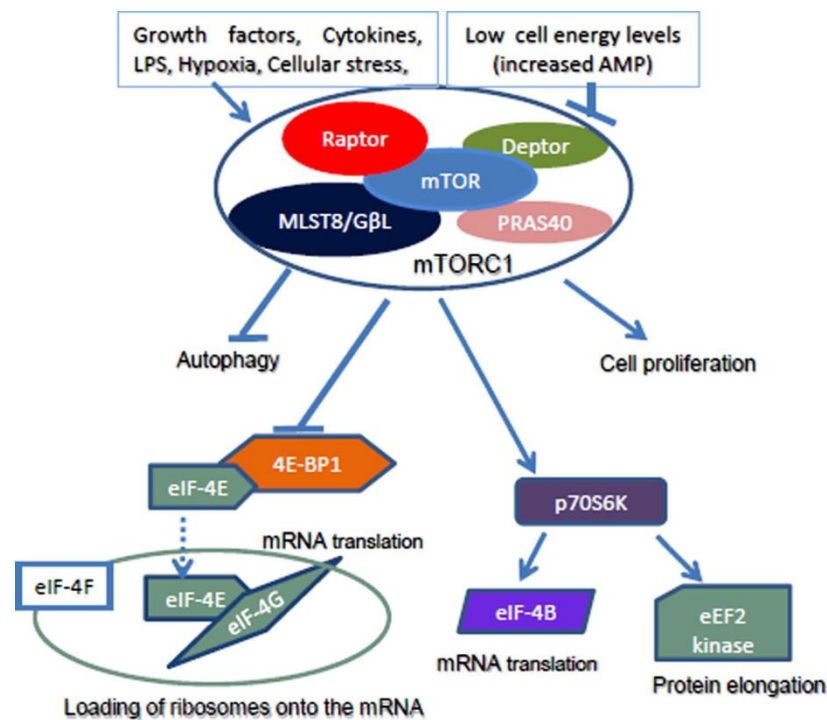


Figure 1.14 - Scheme representing the molecular partners of mTORC1 and its main intracellular targets.

The complex is critically involved in the regulation of protein translation. Phosphorylation of the eukaryotic initiation factor 4E-binding protein 1, 4E-BP1, by mTORC1 enables the elongation initiation factor, (eIF)-4E to associate with eIF-4G leading to the formation of eIF-4F, which facilitates the loading of ribosomes onto the mRNA. Phosphorylation of the ribosomal S6 protein kinase 1, p70S6K, by mTORC1, is followed by the phosphorylation of eIF-4B and the eukaryotic elongation factor 2 (eEF2) kinase, which facilitate protein synthesis (from Dello Russo et al., 2013).

The activation of mTORC1 generally increases the cellular capacity of protein generation. The two main downstream targets on mTORC1, the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and the ribosomal S6 protein kinase 1 (p70S6K), are key components of the protein translation machinery (Ma and Blenis, 2009). In fact, the regulation of the initiation process of protein translation is the most well characterized action regulated by the mTORC1 pathway. Phosphorylation of 4E-BP1 by mTORC1 induces the dissociation of 4E-BP1 from the eukaryotic translation initiation factor (eIF)-4E. This allows eIF-4E to associate with eIF-4G leading to the formation of eIF-4F, which facilitates the loading of ribosomes onto the mRNA. Phosphorylation of the p70S6K by mTORC1 also promotes the initiation of

protein translation by the phosphorylation of eIF-4B and regulates protein elongation through the phosphorylation of the eukaryotic elongation factor 2 (eEF2) kinase. The activation of p70S6K facilitates the role of the eEF2 kinase in the elongation process (Proud, 2007). Finally, the cellular capacity of protein generation is also controlled by mTORC1-mediated suppression of macroautophagy (autophagy).

mTORC2 complex is composed of mTOR, rapamycin-insensitive companion of mTOR (Rictor), GβL, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1) (Weichhart and Säemann, 2009) (Figure 1.15). It appears to be regulated by insulin, growth factors, serum, and nutrient levels (Frias et al., 2006). Originally, mTORC2 was identified as a rapamycin-insensitive entity, as acute exposure to rapamycin did not affect mTORC2 activity or Akt phosphorylation (Sarbasov et al., 2005). However, subsequent studies have shown that, at least in some cell lines, chronic exposure to rapamycin, while not affecting pre-existing mTORC2, promotes rapamycin inhibition of free mTOR molecules, thus inhibiting the formation of new mTORC2 (Sarbasov et al., 2006).

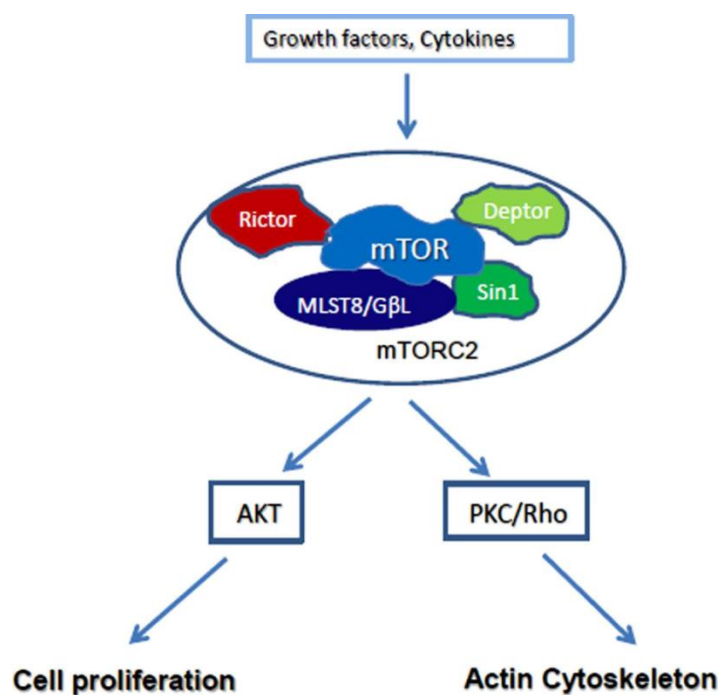


Figure 1.15 - Scheme representing the molecular partners of mTORC2 and its main intracellular targets.

The complex is involved in the regulation of cell proliferation through the activation of Akt signaling pathway and cytoskeleton modification through the activation of PKC (from Dello Russo et al., 2013).

In comparison with mTORC1, the biological function of mTORC2 is less well understood. It is known that its main target is Akt kinase that is phosphorylated by this protein complex at Ser⁴⁷³ (Hresko and Mueckler, 2005). Phosphorylation of this serine stimulates Akt

phosphorylation at a threonine T308 residue by PDK1 and leads to full Akt activation. As consequences, nutrient uptake and cell survival are promoted (Sarbasov et al., 2005). Every components of mTORC2 are essential; deletion or knock-down of rictor, Sin1, or mLST8/GbL has a dramatic effect on mTORC2 assembly and activation of Akt (Guertin et al., 2006). Interestingly, mTORC2 provides a quality control in the process of protein production by phosphorylating amino acid residues in nascent polypeptide chains, as it occurs during Akt protein translation (Oh et al., 2010). This complex is also involved in the regulation of actin cytoskeleton dynamics (Jacinto et al., 2004), by promoting the phosphorylation of protein kinase C and paxillin (Sarbasov et al., 2004). In conclusion, mTORC1 and mTORC2 contribute to integrate signals from growth factors, nutrients and stress, with the final effect of promoting anabolic and inhibiting catabolic intracellular processes (Foster and Fingar, 2010).

1.3.2 mTOR in OL differentiation

In the past three years, different *in vivo* and *in vitro* studies have demonstrated that the kinase Akt plays an important role in the regulation of OL differentiation and myelin formation in the CNS. Specifically, it has been shown that in transgenic mice over-expressing constitutively active Akt in OLs have increased expression of mTOR and increased myelination (Flores et al., 2008). In this mice, long-term exposure to rapamycin prevents the hypermyelinating phenotype, but has no effect on myelin maintenance in the normal adult CNS (Narayanan et al., 2009). The involvement of mTOR signaling in the regulation of OL functions was confirmed by *in vitro* studies. Tyler and co-workers demonstrated that inhibition of mTOR by rapamycin interferes with the development of GalC positive immature OLs in purified OPC cultures. They also proposed different functions for the two mTOR complexes during OL differentiation. While mTORC1 regulates myelin protein synthesis by interfering with protein translation, mTORC2 interferes with mRNA transcription of several myelin genes. Interestingly, intense mTOR activation was found during postnatal rat brain development, particularly in the white matter as myelination peaked in the forebrain. The involvement of mTOR in OL differentiation, albeit in a later stage of this process, was demonstrated also by Guardiola-Diaz et al., (2012). However, this group demonstrates that the mTOR inhibition by rapamycin does not impair the differentiation from late progenitors $O4^+/GalC^-$ to immature OL $O4^+/GalC^+$, but that this pathway blocks only the late stage of differentiation, from immature to mature OLs (Guardiola-Diaz et al., 2012). This different observation is likely due to the *in vitro* conditions used in the experiments. Moreover,

activation of mTOR signaling was found to be critical for cytoskeletal organization and the expression of normal levels of major myelin proteins *in vitro*, and for the onset of myelination in the postnatal mouse brain.

Thus, both *in vivo* and *in vitro* works underlie the importance of the mTOR signaling in driving terminal differentiation of OLs and myelination. However, the specific targets of mTOR in differentiating OLs remain to be defined. Some aspects of the differentiation and myelination programs have been proposed to be regulated by the mTOR signaling. These include nuclear regulation of differentiation, cytoskeletal rearrangements involved in morphological maturation and processes necessary for myelin synthesis (Wood et al., 2013).

Concerning the nuclear regulation, it is known that the initial stages of OPC differentiation require chromatin remodeling and transcriptional regulation. During these events, inhibitors of differentiation are down-regulated and, then, factors necessary for myelin gene transcription are up-regulated (Li et al., 2009). Several negative regulators of OL differentiation have been identified including the ID (inhibitor of DNA binding/differentiation) helix–loop–helix members ID2 and ID4 and TCF4/TCF7L2 (T cell factor 4). It has been demonstrated that rapamycin-treated cultures have elevated levels of ID2, ID4 and TCF4/TCF7L2 RNA expression, which correlate with a block in OPC differentiation (Tyler et al., 2009). Based on these data, it has been proposed that the mTOR signaling has a role in transcriptional regulation of OL differentiation, specifically by directly decreasing these negative transcriptional regulators or indirectly suppressing BMP signaling which is able to increase mRNA and protein levels of ID2 and ID4 (Wood et al., 2013; Samanta and Kessler, 2004; Cheng et al., 2007).

Concerning the cytoskeleton modification, it is known that the OPC differentiation implies profound morphological changes which are the consequences of continuous cytoskeleton rearrangements. A recent report supports the hypothesis that mTOR signaling underlies this event. Proteins identified and confirmed in the mTOR-regulated proteome with known roles in cytoskeleton include Fyn tyrosine kinase, Sirt2, β IV-tubulin, Gap43 and BASP-1 (Tyler et al., 2011). As discussed previously, a major mechanism for mTOR regulation of cytoskeleton in developing OLs is likely via mTORC2 (Tyler et al., 2009; 2011).

Finally, it is believed that the mTOR pathway regulates numerous processes necessary for myelin synthesis. For example, mTOR induces expression of multiple lipogenesis proteins as OLs differentiate (Tyler et al., 2011). According to this, it has been demonstrated that Fasn (fatty acid synthase) is expressed in OLs late in brain development and is dramatically decreased in mTOR-inhibited OLs (Saito et al., 2009; Tyler et al., 2011). Similarly, *fdft1*

(farnesyl-diphosphate farnesyltransferase 1), a cholesterol synthetic enzyme essential for myelin production (Saher et al., 2005), is also part of the mTOR-regulated proteome (Tyler et al., 2011). Several other lipid biosynthetic proteins including the cholesterol biosynthesis proteins *Idi1* (isopentenyl-diphosphate d-isomerase), *Fdps* (farnesyl pyrophosphate synthetase) and *Hmgcs1* (hydroxymethylglutaryl-CoA synthase) and the *Acs13* and *Acs14* (fatty acid biosynthesis enzymes long-chain-fattyacid- CoA ligase 3 and 4) (Tyler et al., 2011) were also identified in the mTOR proteome screen. Taken together, these studies support an essential role for mTOR signaling in lipogenesis in maturing OLs initiating myelin production (Wood et al., 2013).

To conclude, altogether these data indicate the existence of a link between mTOR and OL differentiation and myelination. Further studies, also the ones described in this thesis, will help to better understand the physiological role of this interaction and to clarify whether this pathway could be a new pharmacological target for remyelination strategies.

1.4 G protein-coupled receptor desensitization

G protein-coupled receptors (GPCRs) constitute a superfamily of seven transmembrane spanning proteins that respond to a huge variety of stimuli (both sensory and chemical). Once activated, they transduce the information provided by these stimuli into intracellular second messengers that are interpreted as meaningful signals by the cell. This process involves the coupling of agonist-activated GPCRs to different effector systems via their interaction with heterotrimeric guanine nucleotide binding proteins (G proteins). The binding of agonist to a GPCR, selects for a receptor conformation state that promotes the exchange of GDP for GTP on the G protein α -subunit and is presumed to allow the dissociation of the G protein $G\alpha$ - and $G\beta\gamma$ -subunits (Neer, 1995; Surya et al., 1998). Subsequently, the activated $G\alpha$ - and $G\beta\gamma$ -subunits positively and/or negatively regulate the activity of effector enzymes and ion channels (Neer et al., 1995; Gautam et al., 1998).

GPCR activity represents a coordinated balance between molecular mechanisms governing receptor signaling, desensitization, and resensitization. Receptor desensitization, represents an important physiological “feedback” mechanism that protects against both acute and chronic receptor overstimulation. This process is the result of the activation and of the recruitment of three families of regulatory molecules: second messenger-dependent protein kinases, G protein-coupled receptor kinases (GRKs) and arrestins (Ferguson, 2001).

GPCRs desensitization involves different mechanisms: (i) the uncoupling of the receptor from heterotrimeric G proteins in response to receptor phosphorylation, (ii) the internalization

of cell surface receptors to intracellular membranous compartments and (iii) the down-regulation of the total cellular complement of receptors due to reduced receptor mRNA and protein synthesis, as well as both the lysosomal and plasma membrane degradation of pre-existing receptors (Ferguson, 2001) (Figure 1.16).

The uncoupling of GPCRs from heterotrimeric G proteins is the consequence of a covalent modification of the receptor (phosphorylation) mediated by intracellular kinases. It is generally accepted that both second messenger-dependent protein kinases (cAMP-dependent protein kinase (PKA) and protein kinase C (PKC)) and GRKs phosphorylate serine and threonine residues within the intracellular loop and carboxyl-terminal tail domains of GPCRs.

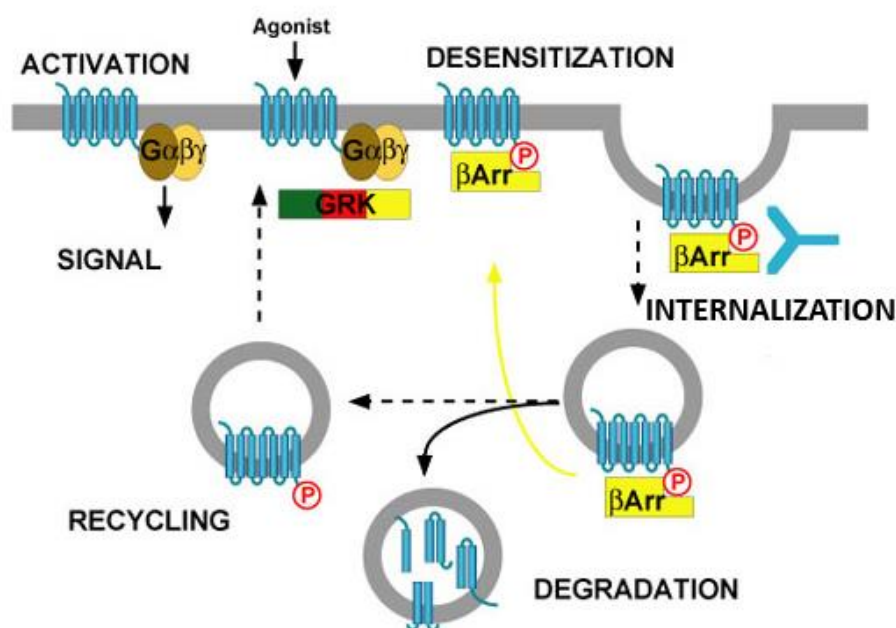


Figure 1.16 - Molecular mechanisms involved in the GRK- and β -arrestin dependent desensitization and internalization of GPCRs.

As shown in the scheme, desensitization, internalization and recycling of GPCRs are intimately connected. The whole process is initiated by binding of an agonist to the receptor, which triggers a signal (usually via heterotrimeric G-proteins). However, agonist binding also converts the receptor into a substrate for a family of kinases, the G-protein-coupled receptor kinases (GRKs). These kinases phosphorylate only agonist-activated receptors. Subsequently, the phosphorylated receptor becomes a binding partner for arrestins. Arrestins are normally cytosolic proteins, but they recognise agonist-activated, phosphorylated receptors and bind them. This binding makes the receptor inaccessible for G-proteins and it targets the receptor for internalisation. This is because arrestins do not only bind receptors, but they also bind components of clathrin-coated pits. Thus, arrestin-bound receptors move into clathrin-coated pits and are then internalised.

The GRK family of kinases is comprised of seven family members that share significant sequence homology (see paragraph 1.5). Each of the GRKs shares a similar functional organization with a central catalytic domain, an amino-terminal domain that is thought to be important for substrate recognition and that contains an RGS-like domain, and a carboxyl-

terminal domain that contributes to the plasma membrane targeting of the kinase. GRKs phosphorylate agonist-activated receptors, thereby promoting the binding of cytosolic cofactor proteins called arrestins, which sterically uncouple the receptor from heterotrimeric G protein (Benovic et al., 1987; Lohse et al., 1990, Pippig et al., 1993).

The second messenger-dependent protein kinases, PKA and PKC, are phosphotransferases that catalyze the transfer of the γ -phosphate group of ATP to serine and threonine residues contained within specific amino acid consensus sequences of proteins. Second messenger-dependent protein kinase are activated in response to GPCR-stimulated increases in intracellular second messengers such as cAMP, Ca^{2+} , and diacylglycerol and participate in GPCR signaling by mediating the phosphorylation of downstream target proteins. However, these kinases also feedback phosphorylate GPCRs at phosphorylation consensus sites within their intracellular loops and carboxyl-terminal tail domains. In contrast to GRKs, second messenger-dependent protein kinases not only phosphorylate agonist-activated GPCRs, but also indiscriminately phosphorylate receptors that have not been exposed to agonist (Hausdorff et al., 1989; Lohse et al., 1990). Thus, agonist-independent phosphorylation is a property that has generally been ascribed only to second messenger-dependent protein kinases and not GRKs (Lefkowitz, 1993). Nevertheless, it is now recognized that GPCRs spontaneously isomerize to an activated conformation in the absence of agonist, which suggests that GRKs may also contribute to the regulation of basal GPCR activity (Pei et al., 1994; Rim and Oprian, 1995).

After the association with the phosphorylated GPCRs, β -arrestins undergo a conformational change which allows them to bind clathrin and the adaptor protein complex AP-2, both of which are critical components of receptor-mediated endocytosis. The binding occurs between the amino terminal region of the clathrin heavy chain and the carboxyl terminal region of β -arrestins, and between the β 2 subunit of AP-2 and the extreme carboxyl terminal region of β -arrestins. This interaction facilitates the entry of desensitized receptors into clathrin-coated pits for subsequent internalization (Goodman et al. 1996, Laporte et al. 1999). The destiny of the internalized GPCRs is different according to the type of the receptor. Studies have revealed that specific determinants within the cytoplasmic tails of GPCRs determine whether GPCRs are either recycled back to the plasma membrane or are retained within the intracellular compartment of the cell and/or targeted to lysosomes (Innamorati et al., 1998; Trejo and Coughlin, 1999; Oakley et al., 1999; Anborgh et al., 2000). Many GPCRs are internalized, but are not recycled back to the cell surface (Hermans et al., 1997; Trejo and Coughlin, 1999; Groarke et al., 1999; Oakley et al., 1999; Zhang et al., 1999;

Anborgh et al., 2000; Bremnes et al., 2000). These internalized GPCRs, are predominantly targeted to lysosomes for degradation (Trejo and Coughlin, 1999; Bremnes et al., 2000). Other internalized GPCRs are not necessarily sorted to lysosomes, but may be retained within the endosomal compartment (Zhang et al., 1999; Anborgh et al., 2000). As a consequence, by virtue of the fact that some receptors do not recycle, they will mediate transient responses to agonist. Finally, some internalized GPCRs can be resensitized. The physiological importance of receptor resensitization in the maintenance of normal tissue homeostasis is obvious since prolonged or irreversible receptor desensitization would leave a cell unable to respond appropriately to extracellular stimuli. Just as GPCR desensitization provides a mechanism protecting cells against receptor overstimulation, GPCR resensitization protects cells against prolonged receptor desensitization. The mechanism by which the resensitization of many GPCRs is achieved is thought to be the agonist-stimulated internalization of receptors to an intracellular membrane compartment (endosomes) enriched in a GPCR-specific phosphatase activity. During this process the receptor is released from β -arrestin, becomes dephosphorylated, is sorted and recycled back to the cell surface.

Taken together, the information summarized in this paragraph highlights the complex inter-relationship between mechanisms involved in GPCR desensitization, internalization and resensitization. The activation of distinct GPCR subtypes is translated into diverse receptor-specific patterns for GPCR desensitization and resensitization. This diversity may be exploited for the development of strategies to therapeutically manipulate GPCR function in diseases associated with altered GPCR signaling.

1.5 G protein-coupled receptor kinases

G protein-coupled receptor kinase (GRKs) are serine/threonine kinases that mediate the process of GPCR desensitization, the physical uncoupling of the G protein from the cognate receptor. These kinases phosphorylate intracellular domains of activated receptors, leading to the recruitment of the multifunctional adaptor proteins, arrestins, to the receptors and the attenuation of intracellular G protein-dependent signaling (Lefkowitz and Shenoy, 2005).

Based on sequence similarity and gene structure, vertebrate GRKs are classified into three subfamilies: visual GRK subfamily (GRK1, rhodopsin kinase and GRK7, cone kinase), the β -adrenergic receptor kinase subfamily (GRK2/GRK3) and the GRK4 subfamily (GRK4, GRK5 and GRK6). All GRKs are multi-domain proteins which share a common structural architecture (Figure 1.17) comprising a highly conserved, centrally located catalytic domain of approximately 270 residues, similar to that of other serine-threonine kinases, flanked by an

N-terminal domain of approximately 185 amino acids and a variable-length carboxyl-terminal domain (105-230 amino acids). Both the N-terminal and the C-terminal domains are involved in the regulation of GRK targeting to the membrane and activity. The N-terminal domain, which has been proposed to be important for receptor recognition and intracellular membrane anchoring, harbors several regulatory motifs, including a regulator of G protein signaling homology (RH) domain. The C-terminus of GRKs contains structural elements responsible for their membrane targeting, for the interactions with lipids and membrane proteins and for the control of the subcellular distribution of these kinases (Pitcher et al., 1998; Penela et al., 2010).

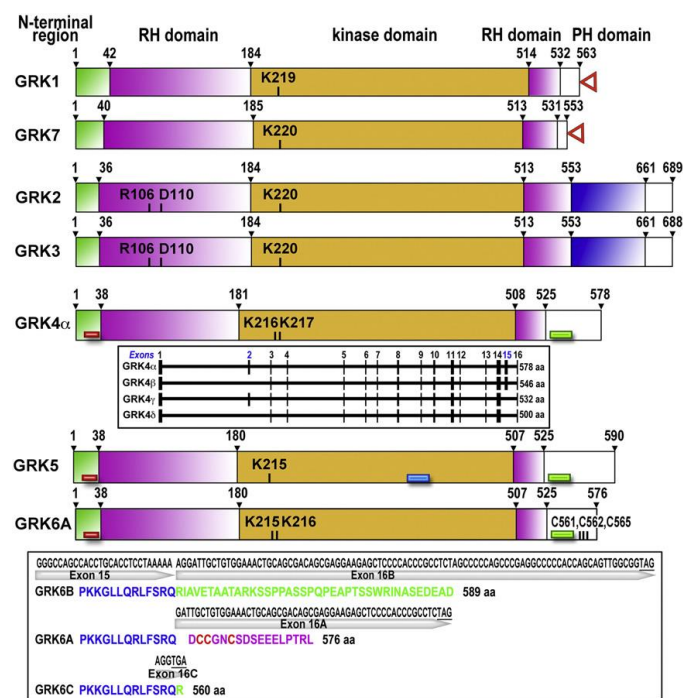


Figure 1.17 - Domain structure of GRKs.

All GRKs share a common structure comprising a highly conserved, centrally located catalytic domain of approximately 270 residues, similar to that of other serine-threonine kinases, flanked by an N-terminal domain of approximately 185 amino acids and a variable-length carboxyl-terminal domain (105-230 amino acids) (from Gurevich et al., 2012).

Subcellular localization of GRKs has mainly been described in cultured cells, focusing on mechanisms of their membrane recruitment. However *in vivo*, cells are highly compartmentalized and contain multiple types of specialized membranes absent in cultured cells. This is particularly obvious in neurons, which arguably have complex morphology and perform more signaling than any other cell type (Bisegna et al., 2008; Caruso et al., 2011). It is therefore important to investigate how the various isoforms of GRKs are addressed in the

various compartments of the cell membrane as the different subcellular localization could contribute to the various cellular functions even independently from their receptors

As previously described, the ability to phosphorylate activated GPCRs was the first GRK function to be discovered. Receptor phosphorylation by itself can decrease G protein coupling (Wilden, 1995) and enables high-affinity binding of arrestin, which stops G protein-mediated signaling by blocking the cytoplasmic surface of the receptors and preventing further binding and activation of the same receptors and inducing their desensitization (Krupnick et al., 1997; Ferguson et al., 2001). After the binding to β -arrestins, the phosphorylated receptors are endocytosed through a specific process which involved proteins called clathrins. This mechanism allows the receptor resensibilization and their recycle in the membrane (Reiter and Lefkowitz, 2006; Moore et al., 2007). The mechanism of GRKs' action is shown in Figure 1.18.

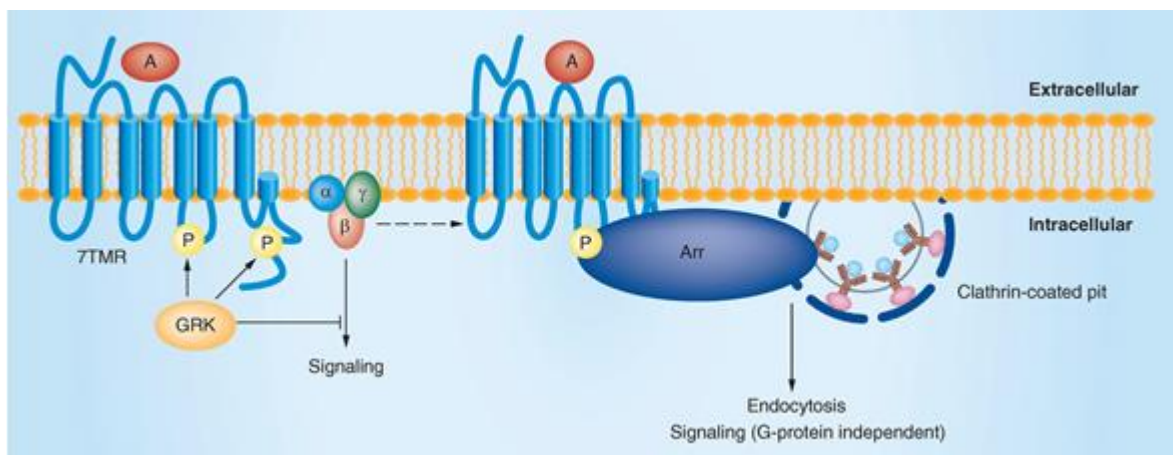


Figure 1.18 – Representative scheme of GPCR desensitization induced by GRKs.

In the figure it is shown the agonist-induced GPCR desensitization. After the agonist-induced receptor activation (A) the intracellular C-terminal domain of the receptor is phosphorylated by a GRK. This phosphorylation increases the receptor affinity for the arrestins which, then recruit clathrins inducing receptor internalization (from Lympelopoulos et al., 2013).

The most striking feature distinguishing GRKs from other kinases is that their activity depends on the functional state of the target: GRKs effectively phosphorylate active GPCRs. However, they are clearly capable of phosphorylating other targets at the membrane in response to receptor activation. An increase of the availability of receptor phosphorylation sites upon their activation does not appear to play a role, because active GPCRs can enhance GRK phosphorylation of exogenous peptide substrates, indicating that docking with the active receptor directly activates the GRK (Palczewski et al., 1991; Chen et al., 1993). This receptor-dependent activation functions with non-cognate pairs, as demonstrated by the fact that GRK2

robustly phosphorylates rhodopsin in strictly light-dependent fashion (Benovic et al., 1986). A shared activation mechanism of GRKs relying on common structural features presented by active GPCRs thus seems to allow relatively few GRKs to phosphorylate hundreds of structurally different receptors (Palczewski, 1997). Interestingly, GRK4 is the only GRK isoforms (GRK4 α splice variant) reported to be constitutively active and capable of phosphorylating unstimulated GPCRs (Ménard et al., 1996; Rankin et al., 2006).

Most GRKs require the presence of negatively charged lipids to phosphorylate GPCRs efficiently, and they also require the cytoplasmic surface of the active receptor to form a pocket that is not accessible in the inactive state (Choe et al., 2011; Rasmussen et al., 2011; Standfuss et al., 2011) into which the GRK will dock. The size and physical properties of this pocket are expected to be highly conserved among receptors, while the size and sequence of the more exposed cytoplasmic loops of the receptor are clearly not. According to this model of GPCR-mediated GRK activation is one that involves the activated receptor in its native lipid environment forming a surface that is complimentary in shape and charge to that of GRKs in their active and closed conformation. Such a mechanism would allow GRKs to recognize many GPCR substrates. Once docked, the kinase adopts a closed conformation that allows it to phosphorylate any substrate in close proximity. The molecular details of how GRKs initially recognize receptors remain still unclear, but one hypothesis is that their intrinsically disordered N-termini form an initial low-affinity interaction in what has been termed “fly-casting”, which is believed to kinetically favor complex formation (Shoemaker et al., 2000; Cortese et al., 2008).

In recent years it has been demonstrated that GRKs can also interact with a variety of proteins other than GPCRs and that in many cases they are able to phosphorylate them. Up to now, the list of non-GPCR substrates includes single transmembrane domain tyrosine kinases (PDGFR β), single transmembrane domain serine/threonine kinases, death receptors, toll-like receptors, transcription factors and adapter proteins. It remains unclear whether this mode of GRKs acting on non-GPCR-linked signaling pathways is an exception or a rule. If indeed GRKs participate not only in desensitization, but also in signaling via such a huge variety of targets, they might play a role in processes such as cell growth and proliferation, cell death and motility, immunity, cancer, and development.

GRKs have also been reported to phosphorylate a diverse collection of receptor-associated proteins, changing their binding to the receptor and/or their activity, which might lead to desensitization of G protein dependent or independent signaling pathways (Gurevich et al., 2012). Other known targets of GRKs are nuclear proteins, such as class II HDACs (Martini et

al., 2008), multiple transcription factors and cytoskeleton proteins, such as tubulin that was the first non-GPCR substrate described (Gurevich et al., 2012).

As many other enzymes in the cell, the expression of GRKs is tightly regulated by various factors and is altered in pathological conditions. For example, chronic or even acute administration of GPCR agonists can increase the level of GRKs in the brain, which may lead to tolerance to drugs (Hurle, 2001; Diaz et al., 2002; Fan et al., 2002; Rubino et al., 2006; Schroeder et al., 2009). Administration of GPCR antagonists or removal of endogenous agonists also can affect the GRK concentration too (Hurle, 2001; Diaz et al., 2002; Bezard et al., 2005; Ahmed et al., 2008, 2010). In some cases, transcriptional regulation is involved, whereas in others alterations in the protein concentration are not accompanied by changes in the mRNA levels, suggesting the regulation at post-transcriptional levels.

Insufficient or excessive activity of GRKs was implicated in a variety of human disorders, which include heart failure, depression, Parkinson's disease and pain. As key regulators of GPCR-dependent and -independent signaling pathways, GRKs are emerging drug targets and promising molecular tools for therapy (Gurevich et al., 2012).

1.5.1 GRK2

Among all isoforms of GRKs, GRK2 is one of the most studied member. It is known that cells of the immune system express particularly high levels of GRK2 protein and its expression level is precisely regulated (De Blasi et al., 1995; Chuang et al., 1992). *In vivo*, the expression of GRK2 protein in cells of the immune system is altered in a number of disease states, including hypertension, cardiac failure, and the chronic inflammatory disease rheumatoid arthritis (Gros et al., 2000; Penela et al., 2001) and, recently, reduced levels of GRK2 have been found in leukocytes of patients with multiple sclerosis (MS), a demyelinating chronic inflammatory disease of the CNS (Vroon et al., 2005). The pathogenesis of this disease is characterized by the infiltration of activated T lymphocytes and macrophages into the brain and spinal cord and the family of G protein-coupled receptors (GPCR) plays an important role in regulation of inflammation by a variety of ligands, including chemokines, leukotrienes, prostaglandins, neurotransmitters, and adrenergic agonists (Lombardi et al., 2002). As described in the previous paragraph, the agonist-induced activation of these receptors triggers a series of signals that are rapidly attenuated by a phenomenon called homologous receptor desensitization. This phenomenon occurs as a consequence of receptor phosphorylation by GPCR kinases (GRK) and results in the

uncoupling of the receptor from the G protein, which abrogates receptor signaling. In addition, GRK-mediated receptor phosphorylation facilitates subsequent binding of arrestins, which promotes GPCR internalization (Ferguson 2001).

Since GRK2 can modulate the activity of multiple GPCR involved in regulation of inflammation and that chronic inflammation can reduce GRK2 levels, Vroon and co-workers hypothesize that changes in GRK2 expression will contribute to the pathogenesis of MS, suggesting the important role of this kinase in the disease (Vroon et al., 2005).

Apart from the recruitment of β -arrestin to the activated GPCR, it has been demonstrated that GRK2 also utilizes β -arrestin-independent mechanisms to mediate receptor internalization. Recent studies have shown that GRK2 associates with a growing number of protein partners with known roles in receptor internalization and signaling. In some cases, this interaction interferes with normal receptor down-regulation by blocking the kinase activity of GRK2. However, in other reported cases, the interaction between GRK2 and its protein partner directly affects receptor endocytosis or internalization.

Evidence suggests that GRK2, as other GRKs, can also regulate signaling mediated by other membrane receptor families such as tyrosine kinase receptors for IGF1, Insulin, PDGF or EGF (Hupfeld and Olefsky, 2007; Cipolletta et al., 2009). Other substrates of GRK2 are non-GPCR and non-plasma membrane receptor substrates such as tubulin, synucleins, phosphocin, ribosomal protein P2, the inhibitory g subunit of the type 6 retinal cyclic guanosine monophosphate (cGMP) phosphodiesterase, a subunit of the epithelial Na^+ channel, the ERM family protein ezrin, the calcium-binding protein DREAM, IKappaB α or the p38 MAPK (Peregrin et al., 2006; Ribas et al., 2007; Patial et al., 2010). These data suggest that GRK2 may act as “effector”, participating in the regulation of diverse cellular phenomena through the phosphorylation of substrates (Figure 1.19).

GRK2 may also contribute to modulate cellular responses in a phosphorylation-independent manner thanks to its ability to interact with a plethora of proteins involved in signaling and trafficking. In fact, this kinase has been reported to associate with PI3K, clathrin, GIT, caveolin, MEK, Akt, and RKIP (Ferguson 2007; Ribas et al., 2007). Very recent reports have identified novel interactions of GRK2 with the RalA GTPase in HEK293 cells (Aziziyeh et al., 2009) and with the APC protein in osteoblasts (Wang et al., 2009).

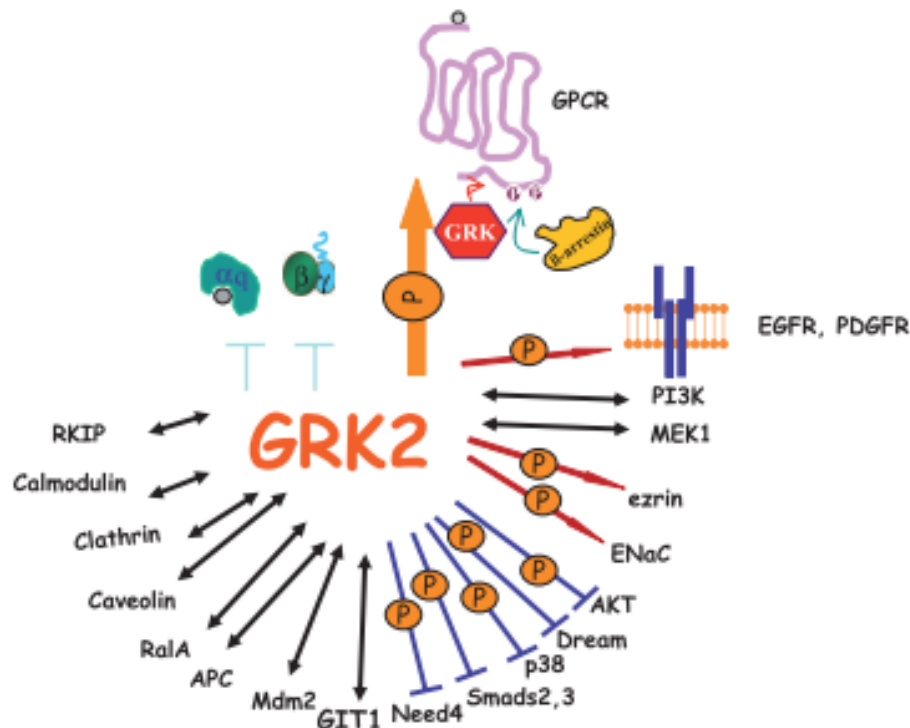


Figure 1.19- The complex GRK2 interactome.

The complex GRK2 interactome. In addition to its 'classical' role triggering GPCR phosphorylation and β -arrestin binding, GRK2 can modulate cell signaling by interacting with $G\alpha_q$ and $G\beta\gamma$ subunits. Furthermore, emerging evidence indicate that GRK2 phosphorylates diverse non-GPCR substrates and displays a complex network of functional interactions with proteins involved in signal transduction. The P symbol denotes that GRK2 has been shown to phosphorylate the indicated proteins (from Penela et al., 2010).

Other functional interactions have been shown to be involved in the regulation of GRK expression levels, localization, and activity (Penela et al., 2003; Reiter and Lefkowitz, 2006; Premont and Gainetdinov, 2007). The association of GRK2 with α -actinin, clathrin, calmodulin, caveolin or RKIP participates in controlling GRK2 activity and in determining the complex subcellular distribution of the kinase. It has been demonstrated that the phosphorylation by different kinases enhances (PKA, PKC, Src) or decreases (ERK) membrane targeting and/or the catalytic activity of GRK2 (Sarnago et al., 1999; Elorza et al., 2000; Penela et al., 2003), thus opening the possibility of transmodulation by different signaling pathways. A recent work has put forward S-nitrosylation of GRK2 as a novel mechanism to inhibit its activity (Whalen et al., 2007). Phosphorylation of GRK2 at given tyrosine or serine residues is also emerging as a key mechanism to dynamically modulate its interaction with cellular partners. Tyrosine phosphorylation by c-Src appears to enhance the interaction of GRK2 with $G\alpha_q$ (Ribas et al., 2007) and with the GIT1 scaffold protein. On the other hand, ERK1/2 phosphorylates GRK2 on S670, strongly impairing the GRK2/ $G\beta\gamma$

interaction (Pitcher et al., 1999) and inhibiting kinase translocation and catalytic activity towards receptor membrane substrates, while also modulating GRK2 interaction with GIT1 (Penela et al., 2008).

Regarding the regulation of GRK2 expression little has been reported about the mechanisms governing GRK transcription. For example, in aortic smooth muscle cells it has been shown that agents inducing physiological vasoconstriction and hypertrophy enhance GRK2 promoter activity, whereas inflammatory cytokines have the opposite effect (Ramos-Ruiz et al., 2000).

Probably, regulation of GRK2 stability may provide an important mechanism for modulating its expression levels. According to this, it has been demonstrated that GRK2 is rapidly degraded by the proteasome pathway after β_2 -adrenergic receptor (β_2 -AR) or of the chemokine receptor CXCR4 (Penela et al., 1998, 2001). This degradation is a complex process that is the result of phosphorylation of GRK2 by c-Src and MAPK (Penela et al., 1998; 2001; Elorza et al. 2003). More recently, Mdm2, an E3-ubiquitin ligase involved in the control of cell growth and apoptosis, has been shown to play a key role in GRK2 degradation (Salcedo et al., 2006). Mdm2 and GRK2 association and subsequent proteolysis are facilitated by the β -arrestin scaffold function upon β_2 -adrenergic receptor stimulation. On the contrary, activation of the PI3K/Akt pathway by agonists such as IGF1 alters Mdm2 phosphorylation and triggers its nuclear localization thus hampering Mdm2-mediated GRK2 degradation and leading to enhanced GRK2 stability and increased kinase levels (Salcedo et al., 2006).

Recently, studies carried on by Cobelens and co-workers (2007) have shown that the treatment with hydrogen peroxide in C6 rat glioma cells alters GRK2 translation by a novel mechanism involving the activity of calpain and the cyclin-dependent kinase, Cdk1 and the mTOR signaling. These studies suggest that H_2O_2 -induced Cdk1 activation triggers inactivation of a translational component which results in inhibition of GRK2 protein synthesis. In this work, a number of Cdk1 targets involved in the silencing of gene expression have been identified including RNA polymerase II, elongation factor 1 γ (Gottesfeld et al., 1997) and 70-kDa ribosomal protein S6 kinase (S6K1) (Shah et al., 2003). S6K1 is an important regulator of translation that is under the control of the rapamycin-sensitive mTOR pathway, which is considered a master modulator of protein synthesis. Indeed, the mTOR complex controls the activity of other translational factors, as the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) and the elongation kinase (Tee and Blenis, 2005). Moreover, Cdk1 not only modulates the mTOR downstream factor S6K1 as mentioned, but also the initiation factor 4E-BP1 (Heesom et al., 2001). It has been hypothesized that the mTOR

pathway is impaired after oxidative stress via Cdk1-induced modulation of molecules downstream from mTOR. Moreover, rapamycin treatment resulted in a time-dependent decrease of GRK2 expression (Cobelens et al., 2007).

The increasingly complex GRK2 “interactome” puts forward this kinase as a relevant signaling node of the cellular transduction network. The intricacy of this network of functional interactions and the participation of this protein in basic cellular processes as migration and cell cycle progression or cardiovascular cell functionality predicts that alterations in GRK2 levels and/or activity, as those reported in several relevant cardiovascular, inflammatory or cancer pathologies, may have important effects in human disease. For this reason a better comprehension of the mechanisms involved in the regulation of GRK2 levels and activity may represent a promising strategy for the development of new pharmacological tools.

Chapter 2

AIM

In the adult central nervous system (CNS), OLs play a role of paramount importance in allowing saltatory conduction of nerve impulses and in maintaining neuronal function and survival (McTigue and Tripathi, 2008). In spite of this, they are remarkably vulnerable to a number of damaging conditions, including excitotoxicity, oxidative stress and the presence of pro-inflammatory cytokines. A plethora of acute CNS injuries (including trauma, ischemia, infections and intoxications) and chronic degenerative and psychiatric diseases (such as Alzheimer's disease, Parkinson's disease, Huntington's disease, schizophrenia) are characterized by oligodendrocytes (OL) loss and myelin breakdown (McTigue and Tripathi, 2008).

In the adult intact and injured CNS, OL turn-over is guaranteed by a subset of NG2⁺ called oligodendrocyte precursor cells (OPCs) (Polito and Reynolds, 2005), that are able to spontaneously differentiate into mature OL. However, this process is efficient only under specific conditions, but often, such as in MS, remyelination fails. An uncontrolled environmental inflammatory response may, indeed, cause a profound impairment on the extent of repair (Fumagalli et al., 2011). On this basis, the identification of signals/molecules that block or promote OPC differentiation represents an important starting point for the development of new therapeutic strategies able to exploit the intrinsic reparative potential of these precursors.

In this scenario, the GPR17 receptor emerges as a new and promising therapeutic target to promote myelin repair (Lecca et al., 2008; Chen et al., 2009; Boda et al., 2011). Since its discovery, the receptor has proven to be interesting both concerning the possibility to exploit it pharmacologically and for its role in the pathogenesis of demyelinating diseases.

GPR17 is a G protein-coupled receptor that can be activated by two distinct classes of endogenous ligands, the uracil nucleotides (UDP, UDP-glucose and UDP-galactose) and the cysLTs (LTC₄, LTD₄ and LTE₄) (Ciana et al., 2006). Both these two types of molecules are released in large quantities after CNS injury (Ciceri et al., 2001; Melani et al., 2005). It has been proposed that, under pathological conditions, these factors may act as danger signals, promoting reparative mechanisms aimed at restoring damaged neuronal circuits through the activation of specific membrane receptors (Di Virgilio et al., 2009). GPR17 is involved in these mechanisms and acts as a molecular sensor of damage (Lecca et al., 2008). In fact, this receptor is expressed by a subpopulation of OPCs dispersed both in the white and grey matter of the CNS and, similarly, *in vitro*, this receptor specifically identifies the stages of transition at which the OPCs exit the cell cycle and are directed towards differentiation, while the

receptor is never expressed by mature myelinating OL (Fumagalli et al., 2011; Boda et al., 2011).

It has also been reported that both the pharmacological and biotechnological manipulation of this receptor modifies the normal differentiation program of OPCs by either promoting or inhibiting cell terminal maturation (Fumagalli et al., 2011). Moreover, both *in vivo* and *in vitro* data showed that, to complete OPC maturation, the receptor has to be turned off, thus highlighting the existence of a precise time window at which GPR17 must be expressed during the differentiation of these cells (Chen et al., 2009; Fumagalli et al., 2011; Fratangeli et al., 2013). Together with the demonstration of persistent aberrant expression of GPR17 in OPCs under demyelinating conditions (Chen et al., 2009; Boda et al., 2011), these findings raise the hypothesis that alterations leading to excessive/prolonged functional expression of GPR17 in OPCs may contribute to the failure of precursors to efficaciously remyelinate.

Since the factors that take part to the regulation of GPR17 expression throughout OPC differentiation are only partially understood, the experiments carried on in this thesis were designed to investigate (i) the consequences of GPR17 agonists activation at early stages of the differentiation process; (ii) the physiological meaning of GPR17 down-regulation at late differentiation stages and (iii) the mechanisms underlying this down-regulation.

Concerning the first aim, based on the fact that previous experiments demonstrated that, in OPCs, GPR17 activation by UDP-glucose is able to promote cell differentiation towards a mature phenotype (Fumagalli et al., 2011), specific experiments were designed in order to evaluate the pro-differentiating effects of other known GPR17 agonists (UDP, LTD₄ and LTE₄) in the same cellular model. Then, since OPC differentiation and the subsequent myelination are strictly correlated, we decided to also assess the effects of some GPR17 agonists (UDP and UDP-glucose) on the formation of myelin segments. To this purpose, we set up another *in vitro* model, the OPC/DRG co-cultures, which gave us the possibility to directly quantify myelination.

Concerning the second aim, previous data also clearly showed that GPR17 is not present in morphologically mature MBP⁺-cells, suggesting that the receptor has to be turned down at critical stage of maturation. To better understand the consequences of this physiological down-regulation, we specifically designed an experiment aimed at interfering with this process through the receptor over-expression. In this respect, we took advantage of fluorescent reporter plasmids for GPR17 over-expression that allowed us to trace the cells by fluorescent microscopy throughout all OPC differentiation process.

Finally, we investigated the mechanisms underlying GPR17 down-regulation. In general, it is known that GPCRs are down-regulated either through the homologous desensitization induced by its own agonists and/or by the intracellular kinases. In this respect, we first tested the capability of two GPR17 agonists (UDP-glucose and LTD₄) to induce GPR17 desensitization and, then, we evaluated the involvement of GRK2, a G-protein coupled receptor kinase highly expressed in the CNS, that usually actively participates in GPCRs homologous desensitization. Finally, since recent data have shown that the mTOR signaling pathway plays an important role in regulating the process of OPC maturation (Tyler et al., 2009; Guardiola-Diaz et al., 2012), by also modulating GPR17 (Tyler et al., 2011), we performed specific experiments to investigate this hypothesis. Specifically, we treated OPCs with rapamycin, an immunosuppressant drug that inhibits the activity of this kinase. Moreover, to assess whether the two mechanisms described above are interconnected to each other, we measured GRK2 protein levels after rapamycin treatment and, through a cAMP assay, examined the capability of GPR17 agonists to induce receptor desensitization after mTOR inhibition by rapamycin.

Altogether these experiments will help us to better understand the role of GPR17 in OL maturation, shedding light on the mechanisms underlying its physiological regulation in order to develop new pharmacological and biotechnological strategies to promote/implement the reparative potential of the OPC precursors, that are still present in the adult brain.

Chapter 3

MATERIALS AND METHODS

3.1 Primary rat oligodendrocyte precursor cells (OPC) cultures

3.1.1 Dissection and plating of rat cortical cortices

Primary mixed glial cultures were obtained from postnatal day 2 Sprague Dawley rats (Charles River). In a laminar flow hood, pups were anesthetized with ether, decapitated and, then, brains were extracted from the skull. Olfactory bulbs and cerebellum were cut off and the two cerebral hemispheres were divided along the midline for the cerebral cortex isolation. After the removal of meninges, cortical tissues were diced with a sterilized razor blade and transferred into a 50 ml sterile centrifuge tube (Euroclone) containing ice-cold Hanks balanced salt solution with calcium and magnesium (HBSS, Euroclone). Afterwards, HBSS was aspirated and tissues were washed with HBSS without calcium and magnesium and incubated with 10 ml trypsin-EDTA solution (final concentration 0.5%, 0.2% in HBSS without calcium and magnesium) containing 1% DNase I (final concentration 0.01 mg/ml) (Sigma-Aldrich) for 30 minutes in a water bath at 37°C for tissue disaggregation. After the incubation, trypsin was inactivated with HBSS containing 10% of fetal bovine serum (FBS, Euroclone) and tissues were further triturated mechanically with a Pasteur pipet. The cellular suspension was passed through a 100 µm cell strainer (BD) in order to eliminate undissociated tissue residues, collected into a 50 ml sterile centrifuge tube and centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in the medium for cortical astrocytes containing:

- DMEM high glucose (Euroclone)
- 2 mM L-glutamine (Sigma-Aldrich)
- 1 mM Sodium pyruvate (Sigma-Aldrich)
- Penicillin 100 U/ml-Streptomycin 100 µg/ml (Euroclone)
- 2,5 µg/ml Fungizon (Euroclone)
- 20% FBS

The cellular suspension was spread over the entire T75 poly-D-lysine (final concentration 10 µg/ml, Sigma-Aldrich) -coated flask surface. Then, flasks were put in a tissue culture incubator (Steri Cult 200) at 37°C, 5% CO₂ and 95% relative humidity. The medium was changed every 2-3 days until cell confluence (about 8 days after the plating).

3.1.2 Isolation and culture of rat OPCs

Eight days after the plating, mixed glial cultures reached the confluence and phase-dark, process-bearing OPCs and microglial cells started appearing on the top of phase grey bed layers of astrocytes (Figure 3.1). At this time point the cultures were ready to be shaken to obtain OPCs. For OPC isolation we followed the protocol described by Chen and co-workers (Chen et al., 2007) with some modifications.

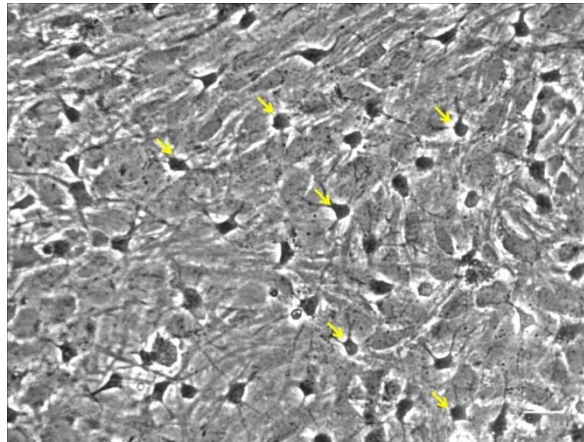


Figure 3.1 - Mixed glial cultures from rat cerebral cortex.

The picture shows the oligodendrocyte precursor cells and the microglia attached on the monolayer of cortical astrocytes (40x magnification; scale bar 15 μ m). As described in the paragraph, 8 days after the seeding, mixed glial cultures reach the confluence and phase-dark, process-bearing OPCs and microglial cells started appearing on the top of phase grey bed layers of astrocytes. OPCs have a polygonal soma with some small ramifications (see yellow arrows).

Culture flasks were removed from the incubator, well fixed with the plug-seal cap tightly screwed on a horizontal orbital shaker and shaken for 20 minutes at 200 rpm at room temperature to remove microglial cells. The medium containing microglial cells was discarded from the flasks by aspiration and 12 ml of fresh DMEM containing 10% of FBS were added to each flask. The flasks were put again on the shaker for further 3-4 hours to isolate OPCs. The cell suspension from each flask was then collected by a pipette, transferred to an untreated Petri-dish and incubated for 20 minutes in tissue culture incubator at 37 °C for differential adhesion of contaminating microglia and astrocytes. At the same time, 12 ml of fresh DMEM containing 10% of FBS were added to each flask and returned to the incubator. The culture medium was completely changed every 2-3 days to allow more OPCs to grow on the astrocyte layer so that they could be shaken a second time after 3-4 days using the same procedure.

The cell suspension was transferred into a 50 ml tube and centrifuged at 1200 rpm for 15 minutes. The supernatant was carefully discarded and the pellet was resuspended and dissociated in a small amount of Neurobasal (Life Technologies) containing 2 mM L-Glutamine, 1% of Penicillin 100 U/ml-Streptomycin 100 µg/ml and 2% of B27 (Life Technologies). Living cells were counted using the Trypan blue which allows the exclusion of death cells. The OPC suspension was diluted to the desired concentration with the same medium used for the pellet resuspension and plated onto poly-D,L-ornithine-coated plates (final concentration 50 µg/ml, Sigma-Aldrich) to a specific density according to the subsequent experiments (see successive paragraphs) in the presence of PDGF-BB (final concentration 10 ng/ml, Sigma-Aldrich) and bFGF (final concentration 10 ng/ml, Space import-Export) to allow cell proliferation. OPCs were maintained in this medium for 2-3 days and, then, switched to a differentiation medium lacking growth factors and containing the thyroid hormone T₃ (final concentration 10 ng/ml, Sigma-Aldrich) to allow cell differentiation to a mature OL phenotype.

3.2 Myelinating co-cultures

3.2.1 Dissection and isolation of mouse dorsal root ganglia (DRG)

Mouse DRG were isolated from E14.5 embryos using the protocol as described in Taveggia et al., 2005. Mouse mother was sacrificed by dislocation and placed in a supine position. Abdominal skin was cut using medium-size scissors (2 Biological) in an “I” pattern to expose abdominal muscles; uterus was removed from abdominal cavity and embryos were isolated from uterus by cutting through tissue surrounding amniotic fluid. All embryos were put in a 10 cm Petri dish containing L-15 (Life Technologies) and individual embryo were transferred to a 6 cm Petri dish with a thin layer of L-15 to isolate DRG. Under a dissecting microscope, the head of each embryo was cut off at the cervical flexure and the tail was cut off just caudal to the hind-limbs; the ventral portion was removed in order to isolate the dorsal structures containing the spinal cord. Using micro-dissecting scissors (2 Biological), vertebral column was cut proceeding caudally in order to expose spinal cord and DRG. The same procedure was repeated for all embryos. After isolating all spinal cords, DRG were plucked off using dissecting forceps (2 Biological) and deposited onto the center of collagen-coated (final concentration 0.5 mg/ml diluted with glacial acetic acid diluted 1:1000 in sterile dH₂O, Cultrex Trevigen) glass coverslips previously covered with 180 µl of C-media composed by:

- MEM (Life Technologies)

- 4 mg/ml Glucose (Sigma-Aldrich)
- 10% FBS
- 2 mM L-Glutamine
- Penicillin 100 U/ml-Streptomycin 100 µg/ml
- 50 ng/ml NGF (Harlan).

3.2.2 Generation of DRG explant cultures

DRG cultures were obtained using the same protocol described in Taveggia et al., 2007. The following day, C-media was removed and neuronal cells were cycled with 250 µl of two different media, NBF and NB (see respective composition below), for 20 days following a specific scheme (Figure 3.2) to allow DRG growth and to eliminate all non-neuronal cells.

NBF	NB
<ul style="list-style-type: none"> • Neurobasal • 2% B27 • 4 mg/ml Glucose • 2 mM L-Glutamine • Penicillin 100 U/ml-Streptomycin 100 µg/ml • 50 ng/ml NGF • Fluorodeoxyuridine (FUDR): 12,3 mg fluorodeoxyuridine (FdU, Sigma-Aldrich) + 12,2 mg uridine (Sigma-Aldrich) dissolved in 0.5 ml MEM 	<ul style="list-style-type: none"> • Neurobasal • 2% B27 • 4 mg/ml Glucose • 2 mM L-Glutamine • Penicillin 100 U/ml-Streptomycin 100 µg/ml • 50 ng/ml NGF

	Monday	Wednesday	Friday
Week 1		NBF	NB
Week 2	NBF	NB	NBF
Week 3	NB	NB	NB

Figure 3.2 – Protocol of scheme for the generation of DRG cultures

The day after the plating, DRG were cycled with two different media: NBF (containing fluorodeoxyuridine) which allows the elimination of non neuronal cells and NB (without fluorodeoxyuridine). Both media were supplemented with NGF which promotes the neuritis outgrowth.

After 20 days, neuritis were well extended radially from DRG explants and they were ready to be myelinated by OPCs. Purified OPCs, separated by orbital shaking (as described in paragraph 3.1.2) and immunopanned using Ran-2 (see paragraph 3.2.3), were added to each DRG in culture (35000 cells/DRG) and kept in M1 medium containing:

- MEM
- 10% FBS
- 4 g/L Glucose
- 2 mM L-Glutamine
- 100 U/ml Penicillin-100 µg/ml Streptomycin

The following day myelination was induced by the addition of 1 µg/ml recombinant chimeric TrkA-Fc (Space Import-Export) which binds to NGF, that has been shown to inhibit myelination by OLs, to the culture medium. The myelinated segments started appearing 8 days after OPCs seeding and became more consistent at day 11.

3.2.3 Immunopanning

The immunopanning technique was used to eliminate non-oligodendroglial cells from the cell population obtained after the mixed glial cell cultures shaking. This procedure was essential to prevent astrocytes and microglia contamination in OPC-DRG co-cultures according to the protocol described in Taveggia et al. 2007.

The day before the shaking, two 10 cm Petri dish were incubated overnight with 10 ml goat anti-mouse IgG (final concentration 0.5 µg/ml, Cappel) diluted in 50 mM Tris HCl pH 9.5 (Sigma-Aldrich). The following day, dishes were rinsed 3 times with sterile PBS (Euroclone) and incubated at room temperature for 3 hours with primary antibody Ran-2 hybridoma supernatants (kind gift of dr. Taveggia) diluted in BSA Medium containing:

- MEM
- 0.1% BSA (Sigma-Aldrich)
- 2.5 % Hepes 1N (Sigma-Aldrich)

The cell suspension, obtained after shaking of mixed glial cell cultures for 3 hours, was transferred into a 50 ml tube and centrifuged at 1200 rpm for 7 minutes. The supernatant was carefully discarded and the pellet was resuspended and dissociated in 6.5 ml of NM15 containing:

- MEM
- 15% FBS

- 2 mM L-Glutamine
- 6 mg/ml Glucose
- Insulin (Sigma-Aldrich)
- Penicillin 100 U/ml-Streptomycin 100 µg/ml

The cell suspension was incubated for 20 minutes at room temperature with one of Ran-2 coated-dishes which were previously rinsed 3 times with sterile PBS. Non-attached cells were collected and re-incubated for further 20 minutes with a second Ran-2 coated-dish. After incubation the cell suspension was centrifuged at 1200 rpm for 10 minutes. Finally, the supernatant was discarded and the pellet was resuspended in a small amount of M1 medium for cell counting.

3.3 OPCs transient transfection

3.3.1 GPR17 over-expression

OPCs were seeded on 13 mm poly-D,L-ornithine-coated coverslips (2×10^4 cells/well) and maintained for 2 days in Neurobasal supplemented with B27 and proliferative factors (PDGF-BB and bFGF) as described in paragraph 3.1.2. Afterwards, OPC differentiation was induced by removing proliferative factors and by adding T_3 . After 3 days in differentiation medium, cells were transfected with two different fluorescent plasmids previously generated in the lab:

- pEGFP-N1 (an empty vector which allows the identification of cells incorporating the plasmids since they become fluorescent);
- pEGFP-GPR17-N1 (a vector in which GPR17 receptor coding sequence was cloned in frame with EGFP so that all cells incorporating the plasmids over-expressed GPR17).

The transfection was performed by using the transfection reagent NeuroFECT™ (Genlantis), a biodegradable cationic polymer indicated for transfection of neuronal cells, according the manufacturer's protocol. NeuroFECT and DNA were prepared in separate tubes. Briefly, 4 µg of NeuroFECT and 1 µg of DNA (both pEGFP-N1 and pEGFP-GPR17-N1) were diluted respectively in 50 µl of Neurobasal. Then, the diluted NeuroFECT was added to diluted DNA in a drop wise fashion for the formation of NeuroFECT/DNA complex. The complex was incubated for 15 minutes at room temperature. During this time, culture medium was replaced with 400 µl of fresh Neurobasal supplemented with 2% of B27 and with 10 ng/ml of T_3 . After 15 minutes, the NeuroFECT/DNA complexes (100 µl/ well) were

added to the cells. Cells were, finally, incubated at 37°C in 5% CO₂ for 48 hours and fixed with 4% paraformaldehyde (Sigma-Aldrich) for immunocytochemistry (see paragraph 3.5).

3.3.2 GPR17 knock-down

OPCs were seeded on poly-D,L-ornithine-coated 6-well plates (10 x 10⁴ cells/well) and maintained for 3 days in Neurobasal supplemented with B27 and proliferative factors (PDGF-BB and bFGF) as described in paragraph 3.1.2. After 3 days in proliferating medium, differentiation was induced by adding T₃ and cells were transfected with a siRNA specifically designed for silencing rat GPR17 (Qiagen): CCGTATAGAGAAGCACCTCAA (target sequence). The sequence was designed to minimize homology to any known vertebrate transcript. This sequence has already been successfully utilized to knock-down GPR17 (Fumagalli et al., 2011). In parallel, an ineffective randomly designed RNA sequence was used as negative control (Qiagen). In the present study, siRNAs were transfected with Lipofectamine RNAiMAX reagent (Invitrogen) as described in Fumagalli et al., 2011 to a final concentration of 100 nM/well following the manufacturer's protocol. After 2 days from the transfection, cells were treated with UDP (100 µM) for 48 hours. Cells were, then, lysed for Western blot analysis (see paragraph 3.6).

3.3.3 GRK2 knock-down

OPCs were seeded on poly-D,L-ornithine-coated 6-well plates (15 x 10⁴ cells/well) and maintained for 3 days in Neurobasal supplemented with B27 and proliferative factors (PDGF-BB and bFGF) as described in paragraph 3.1.2. After 3 days in proliferating medium, differentiation was induced by adding T₃ and cells were transfected with a pool of four siRNA specifically designed for silencing rat GRK2 (Thermo Scientific). In parallel, an ineffective randomly designed RNA sequence was used as negative control (Thermo Scientific). Both siRNA were resuspended in 1X siRNA Buffer (Thermo Scientific) at a final concentration of 20 µM. siRNA were transfected with Lipofectamine RNAiMAX reagent (Life Technologies) to a final concentration of 50 nM (6-well cell culture plate) following the manufacturer's protocol. siRNAs (5 µl) and lipofectamine (6 µl) were diluted respectively in 195 µl of OptiMEM (Life Technologies). Then, the diluted siRNA was added to diluted lipofectamine and incubated at room temperature for 15 minutes. After 15 minutes, 400 µl of the mix was added to each well and cells were incubated at 37°C in 5% CO₂ for 48 or 96 hours in order to

evaluate the efficiency and the consequences of GRK2 silencing at two different time points of OPC differentiation. Finally, cells were lysed for Western blot analysis (see paragraph 3.6)

3.4 Pharmacological treatments

3.4.1 GPR17 endogenous ligands

GPR17 endogenous ligands were tested both in primary OPCs culture and in OPC-DRG co-cultures to assess their pro-differentiation and pro-myelination effect respectively.

According to the type of culture, a specific protocol of treatment was set up (Figure 3.3). For primary OPCs, cells were seeded on 13 mm poly-D,L-ornithine-coated coverslips (2×10^4 cells/well) or on poly-D,L-ornithine-coated 6 cm dishes (35×10^4 cells/dish) and maintained for 2 days in Neurobasal supplemented with B27 and proliferative factors (PDGF-BB and bFGF) as described in paragraph 3.1.2. Afterwards, OPC differentiation was induced by removing proliferative factors and by adding T_3 (day 0) and pharmacological treatments with GPR17 agonists UDP (100 μ M, Sigma-Aldrich), LTE_4 (10 nM, Cayman Chemical) and LTD_4 (100 nM, Cayman Chemical) was performed the following day. After 48 hours, cells were fixed for immunocytochemistry (see paragraph 3.5) or lysed for Western blot analysis (see paragraph 3.6).

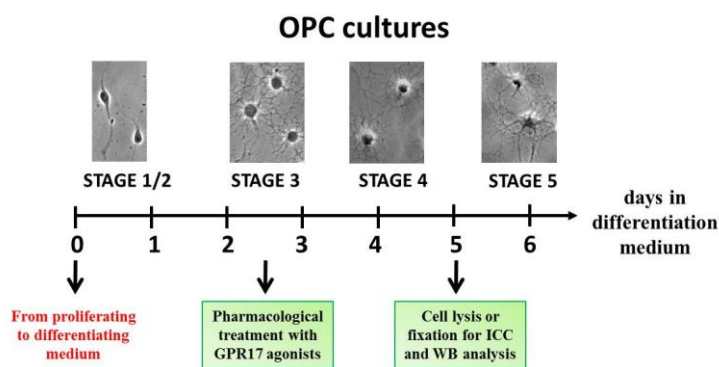


Figure 3.3 - Schematic representation of the experimental protocol followed for the pharmacological treatment of OPC cultures with GPR17 agonists.

Primary purified OPCs were cultured in presence of PDGF and of bFGF for two days. Cell differentiation was induced by adding the thyroid hormone T_3 in the medium ("day 0"). After two/three days in the differentiating medium, when the majority of cells expresses GPR17 (stage 3), cells were treated with GPR17 agonists (UDP μ M; LTE_4 10 nM; LTD_4 100 nM). After 48 hours cells were lysed for WB analysis or fixed for ICC.

For OPC-DRG co-cultures (Figure 3.4), UDP-glucose (100 μ M, Sigma-Aldrich) and UDP (100 μ M) were added to cultures at day 4. The pharmacological treatment was repeated every 2 days up to day 11, when cells were fixed for immunocytochemistry (see paragraph 3.5).

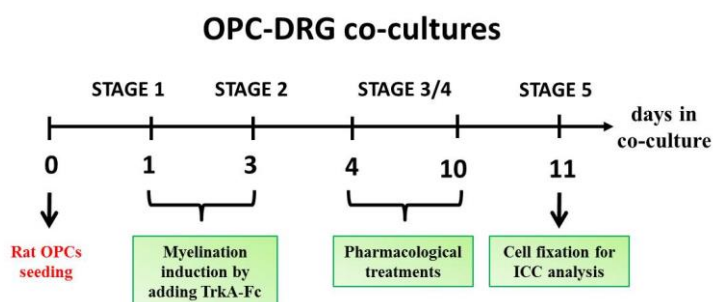


Figure 3.4 - Schematic representation of the experimental protocol followed for the pharmacological treatment of OPC-DRG co-cultures with GPR17 agonists.

Dorsal Root Ganglia (DRG) from E14.5 mouse embryos were plucked off from spinal cord, put in culture in Neurobasal supplemented with B27 in the presence of NGF (100 ng/ml) and cycled with fluorodeoxyuridine to eliminate all non-neuronal cells. After 20 days, when neuritis were well extended radially from DRG explants, purified OPCs were added to each DRG (day “0” in co-culture) and kept in M1 medium. Myelination was induced the following day by the addition of recombinant chimeric TrkA-Fc to the culture medium. GPR17 agonists were added starting from day 4 (stage 3). At day 11 all cells were fixed for ICC analysis.

3.4.2 GRK2 inhibitor

To verify the involvement of GRK2 in the LTD₄-induced pro-OPC maturation, primary OPCs, cultured in differentiating medium, were treated with LTD₄ (100 nM) in the absence (control) or in the presence of 1 μM of the specific GRK2 inhibitor (KRX29, Gomez-Monterrey et al, 2013) as shown in Figure 3.5. After 48 hours, cells were fixed for immunocytochemistry (see paragraph 3.5).

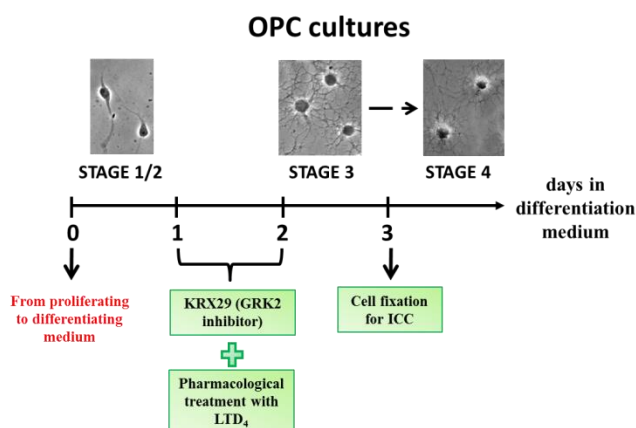


Figure 3.5 - Schematic representation of the experimental protocol followed for the pharmacological treatment of OPC cultures with GRK2 inhibitor.

Primary purified OPCs were cultured in presence of PDGF and of bFGF for two days. Cell differentiation was induced by adding the thyroid hormone T₃ in the medium (“day 0”). At day 0, the GRK2 inhibitor KRX29 (1 μM) was added in the medium. At day 1 and day 3 in the differentiating medium, cells were treated with LTD₄ (100 nM). At day 4 and at day 6, cells were fixed for ICC.

3.4.3 mTOR inhibitor

To test the effect of mTOR pathway inhibition on OPC differentiation, cells were treated with rapamycin (15 nM, Sigma-Aldrich), an immunosuppressant drug which is able to inhibit mTOR (Brown et al., 1994). The concentration of rapamycin was chosen on the basis of already published data and cell treatment was performed according to the protocol shown in Figure 3.6.

To assess the consequences of rapamycin on OPC terminal maturation, cells were maintained for 2 days in Neurobasal supplemented with B27 and proliferative factors (PDGF-BB and bFGF) as described in paragraph 3.1.2. Afterwards, OPC differentiation was induced by removing proliferative factors and by adding T_3 (day 0). At day 1, cells were treated with rapamycin and at day 3 the pharmacological agent was re-added in the medium. At day 6, cells were fixed for immunocytochemistry (see paragraph 3.5) or lysed for western blot analysis (see paragraph 3.6).

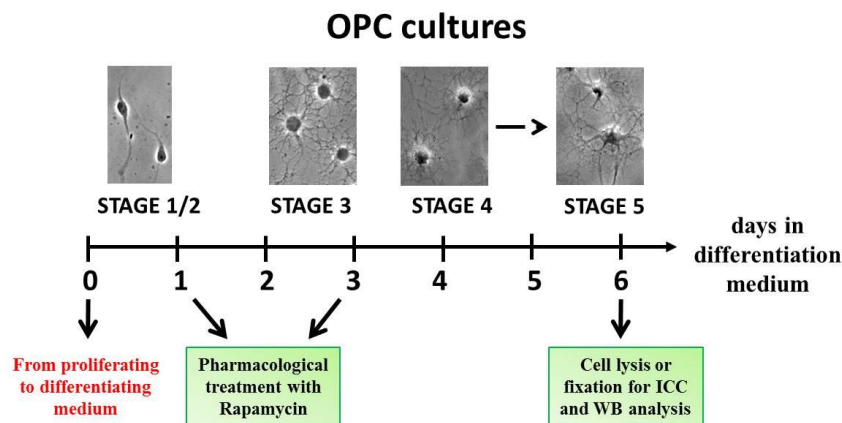


Figure 3.6 - Schematic representation of the experimental protocol followed for the pharmacological treatment of OPC cultures with rapamycin.

Primary purified OPCs were cultured in presence of PDGF and of bFGF for two days. Cell differentiation was induced by adding the thyroid hormone T_3 in the medium ("day 0"). At day 1 and day 3 in the differentiating medium, cells were treated with rapamycin (15 nM). To investigate the consequences of rapamycin on the transition from immature (stage 4) to mature stage (stage 5), cells were lysed for WB analysis or fixed for ICC at day 6.

To evaluate the effects of rapamycin on the transition from pre-oligodendrocytes to immature OLs, cells were also lysed and fixed at day 4 (Figure 3.7).

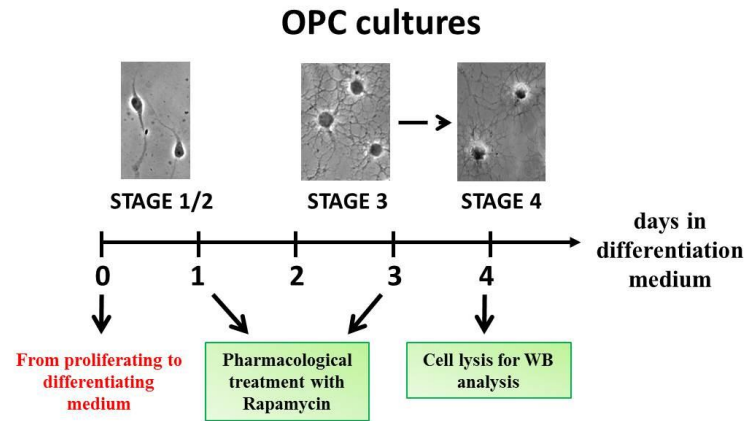


Figure 3.7 - Schematic representation of the experimental protocol followed for the pharmacological treatments of OPC cultures with rapamycin.

Primary purified OPCs were cultured in presence of PDGF and of bFGF for two days. Cell differentiation was induced by adding the thyroid hormone T_3 in the medium ("day 0"). At day 1 and day 3 in the differentiating medium, cells were treated with rapamycin (15 nM). To investigate the consequences of rapamycin on the transition from pre-oligodendrocyte (stage 3) to immature stage (stage 4), cells were lysed for WB analysis at day 4.

3.5 Immunocytochemistry

Both primary OPCs and OPC-DRG co-cultures were fixed at room temperature with 4% paraformaldehyde in 0.1 M PBS (Euroclone) containing 0.12 M sucrose (Sigma-Aldrich). Cells were subsequently incubated for 20 min at room temperature with Goat Serum Dilution Buffer (GSDB; 450 mM sodium chloride and 20 mM sodium phosphate buffer, pH 7.4, 15% goat serum, and 0.3% Triton X-100), before exposure to the primary antibodies diluted in GSDB (see Table 3.1). After an overnight incubation at 4°C or 2.5 hours incubation at room temperature, cells were rinsed three times for 10 min in a high salt buffer solution (500 mM sodium chloride and 20 mM sodium phosphate buffer, pH 7.4), and then incubated (1 hour, room temperature) with secondary goat anti-rabbit and goat anti-mouse antibodies conjugated to AlexaFluor[®]488 or AlexaFluor[®]555 (Life Technologies) diluted 1:600 in GSDB. Subsequently, nuclei were labeled with the fluorescent dye Hoechst-33258 (Life Technologies) diluted 1:10,000 in high salt buffer solution. Cells were rinsed three times in high salt buffer, three times in low salt buffer and, finally, once in 5 mM sodium phosphate buffer, pH 7.4. Coverslips were mounted with Fluorescent Mounting Medium (Dako), and analyzed using an inverted fluorescence microscope (200M; Zeiss) connected to a PC computer equipped with the Axiovision software (Zeiss) or a confocal microscope (LSM510 META, Zeiss).

Table 3.1 – Primary antibody used in ICC

ANTIBODY	COMPANY	DILUTION	INCUBATION
Rabbit anti-GPR17	Home-made	1:100	o/n (4°C) or 2,5 hours (RT)
Rat anti-MBP	Millipore	1:200	o/n (4°C) or 2,5 hours (RT)
Rabbit anti-GRK2	Santa Cruz	1:50	o/n (4°C)
Mouse anti-SMI 31	Cell Signalling	1:500	2.5 hours (RT)
Mouse anti-SMI 32	Cell Signalling	1:500	2.5 hours (RT)
Rabbit anti-NG2	Millipore	1:200	o/n (4°C)
Mouse anti β -Tubulin III	Promega	1:500	o/n (4°C)

For OPC cultures, the quantitative analysis was done with ImageJ software, counting the total number of positive cells in 20 fields under 20X magnification (3 coverslips for each experimental condition). Depending on the experiment and on the parameters to be estimated, at least 700 cells/condition were counted.

For OPC-DRG co-cultures, the quantification of myelin segments was performed with ZEISS LSM Image Browser in 6 random fields of 3-6 coverslips for each experimental condition. Briefly, stacks of images of MBP and Smi31/Smi32 positive cells were taken at 40X magnification; images in the stack were merged at each level and pixels overlapping in the red and green fields above a predefined threshold intensity value were highlighted in white. The amount of myelin per axon (myelination index), was calculated as the ratio between the white pixels area and the green pixels area.

3.6 Western blot

Cells were lysed with 50 μ l of lysis buffer (20 mM Tris pH=7.2, 0.5% DOC, 1% Triton, 0.1% SDS, 150 mM NaCl, 1 mM EDTA and 1% proteases inhibitors Sigma-Aldrich) and, then, approximately 25-30 μ g aliquots from each protein sample were loaded on 12.5% sodium-dodecylsulphate polyacrylamide gels (for MBP and CNPase) or on 8.5% sodium-dodecylsulphate polyacrylamide gels (for GPR17, NG2 and GRK2), and blotted onto nitrocellulose membranes (MBP and CNPase) or PVDF membranes (GPR17, GRK2 and NG2) (Bio-Rad Laboratories). Membranes were saturated with 10% non-fat dry milk in Tris-buffered saline (TBS; 1 mM Tris-HCl, 15 mM NaCl, pH 8) for 1 h at room temperature and incubated overnight at 4°C with primary antibodies diluted in 5% non-fat dry milk in TBS

(Table 3.2). The following day, membranes were washed in TBS-T (TBS plus 0.1% Tween20[®]), incubated for 1 h with goat anti-rabbit or goat anti-mouse or goat anti-rat secondary antibodies conjugated to horseradish peroxidase (1:4000, 1:2000 and 1:2000 in 5% non-fat dry milk in TBS respectively; Sigma-Aldrich). Detection of proteins was performed by enhanced chemiluminescence (ECL, Thermo Scientific) and autoradiography. Non-specific reactions were evaluated in the presence of the secondary antibodies alone.

The quantification of protein bands was done with ImageJ software. Films were scanned and a horizontal rectangular was drawn around the bands. To each band corresponds a specific peak and the area under the peak represents the density of Western blot band. α -Tubulin expression was analyzed from the same sample as an internal control and it was used for data normalization.

Table 3.2 – Primary antibody used in WB

ANTIBODY	COMPANY	DILUTION
Rabbit anti-GPR17	Home-made	1:1000
Rat anti-MBP	Millipore	1:500
Rabbit anti-GRK2	Santa Cruz	1:200
Mouse anti-NG2	Millipore	1:1000
Rabbit anti-CNPase	Santa Cruz	1:250
Mouse anti-Tubulin	Sigma Aldrich	1:1500

3.7 Total RNA isolation and Real time PCR

A semiquantitative analysis of RNA was done with real-time PCR (TaqMan[®] Gene Expression Assays, Applied Biosystem). This technology exploits the 5'-3' exonuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorophore-based detection. As in other quantitative PCR methods, the resulting fluorescence signal permits quantitative measurements of the accumulation of the product during the exponential stages of the PCR. In the TaqMan[®] system, three oligonucleotides are used for quantifying each sequence. Two of them are the primers for the PCR amplification. The third oligonucleotide (the probe) is designed to hybridize specifically to the amplified sequence. It has a reporter fluorescent dye (R) at the 5'-end and a quencher dye (Q) at the 3'-end. When the probe is intact, it is non-fluorescent, due to fluorescence quenching by Q when it is close to R. Q acts in this way as a free resonance

energy transfer (FRET) across the space. During the process of primer extension of PCR, the DNA polymerase displaces and cleaves the probe using its 5' to 3' exonuclease activity. Cleavage separates the dye and the quencher so that the dye becomes now fluorescent. The increase in fluorescence is thus directly correlated to the accumulation of the PCR product, and can be used to monitor the formation of the PCR product in real time. For every sample, an amplification plot is generated, showing the increase in the reporter dye fluorescence (ΔR_n) with each cycle of PCR. From each amplification plot, a threshold cycle (C_t) value is calculated, representing the PCR cycle number at which the fluorescence becomes detectable above a threshold value, based on the variability of base line data in the first 15 cycles. The C_t values were elaborated with the Comparative CT method ($\Delta\Delta CT$) which allows the relative quantification of template comparing the expression levels of the interested gene with the ones of the housekeeping gene.

Total RNA was extracted using the TRIZOL® reagent (Life Technologies) according to the manufacturer's instructions. RNA was then pre-treated with RQ1 DNase (Promega) for eliminating genomic DNA contamination. Retrotranscription of 1 μ g RNA was performed with Superscript II RNaseH Reverse Transcriptase (200 U per sample; Life Technologies) using 100 pmoles of OligodT (Life Technologies), as primers. The quality of RNA sample was assessed with amplification reaction (PCR).

For real time PCR, several mix were prepared according to the number of interested genes. The housekeeping gene GAPDH was used as control. Each mix included:

- Master mix (2X)
- 250 nM Probe (specific for each gene, Life Technologies) (Table 3.3)
- Sterile H₂O
- 50 ng of cDNA

Table 3.3 – Probes used in Real time PCR

TARGET cDNA	PROBE
NG2	Rn00578849_m1
GPR17	Rn03020713_s1
MBP	Rn01399619_m1
SIRT2	Rn01457502_m1
GAPDH	Rn01775763_g1

3.8 Immunoprecipitation

OPCs were treated with medium alone (basal) or with UDP-glucose (5 μ M) or LTD₄ (50 nM) for 5 minutes, and then lysed for 60 min at 4 °C by the addition of 200 μ l RIPA buffer (9.1 mM NaH₂PO₄, 1.7 mM Na₂HPO₄, 150 mM NaCl, pH 7.4, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS, protease inhibitor cocktail). 1 mg of cell lysate was incubated with anti-GPR17 antibody overnight at 4 °C under constant rotation. Probes were then immunoprecipitated with protein A-Sepharose (2-3 h at 4 °C). Immunocomplexes, after being washed, were resuspended in Laemmli solution and boiled for 5 min, resolved by SDS-PAGE (8.5%), transferred to PVDF membranes and probed overnight at 4°C with the specific primary antibodies: anti-GRK2 (1:100); anti-GPR17 (1:1000). The primary antibodies were detected using anti-rabbit or anti-mouse IgG light chains conjugated to peroxidase (diluted 1:10000 and 1:5000, respectively).

3.9 Receptor phosphorylation

OPCs were treated with medium alone (control) or with 100 μ M UDP-glucose or 100 nM LTD₄ for different times (5, 30 or 90 min), and then lysed for 60 min at 4 °C by the addition of 200 μ l RIPA buffer (see paragraph 3.8). Extracts were then quantified by protein assay, and 1 mg of cell lysate was precleared with protein A-Sepharose (1 h at 4 °C) to precipitate and eliminate IgG. Samples were then centrifuged for 10 min at 4 °C (14,000 g). The supernatants were incubated with anti-GPR17 antibody overnight at 4 °C under constant rotation and then, immunoprecipitated with protein A-Sepharose (2 h at 4 °C). Immunocomplexes, after being washed, were resuspended in Laemmli solution and boiled for 5 min, resolved by SDS-PAGE (8.5%), transferred to PVDF membranes and probed overnight at 4 °C with primary antibody anti-Phosphothreonine (Millipore, 1:750) or anti-Phosphoserine (Millipore, 1:750). The primary antibodies were detected using anti-rabbit IgG light chains conjugated to peroxidase (diluted 1:10.000). The peroxidase was detected using a chemiluminescent substrate (ECL, Perkin Elmer).

3.10 Measurement of cyclic AMP levels

The intracellular cAMP assay was performed to test the ability of GPR17 agonists to induce homologous desensitization. Intracellular cAMP levels were measured using a competitive protein binding method as reported (Daniele et al, 2011 Fumagalli et al, 2011).

Briefly, purified OPCs were seeded on poly-D,L-ornithine 24-well plates (1.5×10^4 cells/well) in 0.5 ml of medium (Neurobasal + B27) and maintained in culture for 5–6 days (peak of GPR17 expression). For the assay, the entire medium was removed, and cells were incubated at 37°C for 15 min with 0.4 mL of DMEM in the presence of the phosphodiesterase inhibitor, Ro20-1724 (20 μ M). Cells were pre-treated with high concentration of UDP-glucose (5 μ M) or LTD₄ (50 nM) for different times (5–120 min) and then, cells were washed with 400 μ l of saline and stimulated with the same agonists but at low concentration (UDP-glucose 500 nM; LTD₄ 5 nM) and their ability to inhibit cAMP accumulation stimulated by 10 μ M forskolin was assessed. Reactions were terminated by medium removal and addition of 200 μ l of 0.4 N HCl. After 30 min, lysates were neutralized with 50 μ l of 4 N KOH, and the suspension was centrifuged at 800 rcf for 5 min. For determination of cAMP, cAMP-binding protein isolated from bovine adrenal glands was incubated with [³H]cAMP (2 nM), 50 μ l of cell lysate or cAMP standard (0–16 pmol) at 4 °C for 150 min, in a total volume of 300 μ l. Bound radioactivity was separated by rapid filtration through GF/C glass fiber filters and washed twice with 4 ml of 50 mM Tris-HCl, pH 7.4. Radioactivity was measured by liquid scintillation spectrometry.

To assess the involvement of GRK2 and of mTOR pathway in GPR17 homologous desensitization, the same experiment was repeated, in the absence (control cells) or in the presence of 1 μ M of the specific GRK2 inhibitor or of 15 nM of rapamycin.

3.11 Statistical analysis

All results were expressed as mean \pm s.e. of at least three independent experiments. Statistical analysis was done with *non-linear multipurpose curve-fitting Graph-Pad Prism* program (Graph-Pad). The statistical test used was chosen according to the type of experiment performed and was indicated in the legend of the figure. Three degrees of significance were considered: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)

Chapter 4

RESULTS

4.1 Effects of GPR17 activation by its endogenous ligands on oligodendrocyte precursor cell (OPC) maturation and myelination

4.1.1 GPR17 agonists regulate myelin proteins expression during OPC differentiation *in vitro*

As described in detail in the “*Introduction*”, OPCs are widely distributed in the grey and in the white matter of the adult brain and spinal cord, constituting about the 5% of all cells in the CNS (Pringle et al., 1992). These precursors are extremely important within the adult nervous system, since they represent an endogenous source of remyelinating oligodendrocytes (OLs) in diseases characterized by demyelinated lesions.

OPC differentiation into mature OL is a complex process that requires cell cycle exit and the coordinate expression of specific genes and proteins required for maturation and myelination. During this spontaneous process, OPCs progress through distinct morphological and antigenic stages that have been characterized extensively both *in vitro* and *in vivo* (Warrington et al., 1993; Pfeiffer et al., 1993; Miller, 2002; Emery, 2010). In this respect, recent studies carried on in our laboratory have proposed GPR17 as a new functional OL marker (Lecca et al., 2008; Fumagalli et al., 2011, Boda et al., 2011). Both *in vivo* and *in vitro* experiments have shown that this receptor (both its transcript and protein) is natively expressed by OPCs with a precise spatio-temporal pattern. Indeed, as depicted in Figure 4.1, despite the presence of GPR17 transcript in early bipolar NG2⁺-progenitors of stage 1, the protein starts to appear in more mature precursors of stage 2, reaching a plateau in pre-oligodendrocytes of stage 3, then gradually decreasing in post-mitotic immature OL of stage 4. After the O4/O1 differentiation stage, GPR17 is progressively turned down and never found in fully mature myelinating OL of stage 5. Moreover, by means of pharmacological and biotechnological approaches, it has been previously demonstrated that OPC exposure to the GPR17 endogenous agonist UDP-glucose (100 μ M) promotes their differentiation towards more mature cells, as indicated by the increased number of MBP⁺-OL in culture. Of note, the pro-differentiation effect of this endogenous ligand was specifically mediated by GPR17 since the receptor knock-down completely abrogates this effect (Fumagalli et al., 2011).

To widen these data, we first evaluated the pro-differentiating properties of other known endogenous GPR17 agonists (i.e. UDP, LTD₄ and LTE₄) (Fumagalli et al., 2011) in purified primary OPCs. The ability of these compounds to act as agonists on GPR17 has been previously established by cAMP assay (see “*Introduction*”), but their effects on OPC maturation have not been evaluated yet.

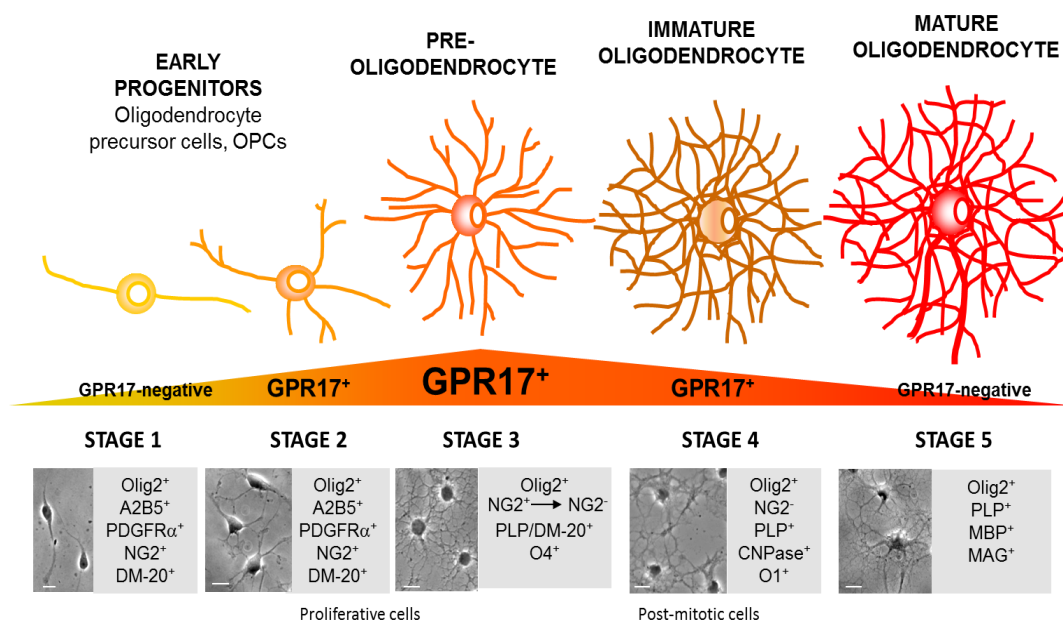


Figure 4.1 - Schematic representation of the OPC differentiation process

The drawing shows the different stages of OPC differentiation process, starting from immature NG2⁺-precursors to mature OL of stage 5. Specific stage markers and some representative brightfield microscope images of cell morphology at each maturation step are reported. As indicated by the yellow/orange triangle, GPR17 expression is restricted to a specific temporal window, with a peak in the pre-oligodendrocytes of stage 3 (image modified from Fumagalli et al., 2011)

To this purpose, OPCs were cultured for 2 days in presence of PDGF and bFGF in order to promote proliferation and, then, after 3/4 days in differentiating medium (stage 2/3) cells were treated with UDP (100 μM), LTE₄ (10 nM) and LTD₄ (100 nM). After 48 hours, cells were fixed for ICC analysis or lysed for WB analysis. As shown in Figure 4.2 (E), UDP, LTE₄ and LTD₄ strongly increased the percentage of MBP⁺-cells compare to vehicle-treated control (CTRL: 100 ± 6.83%; UDP: 158.4 ± 11.49%; LTE₄: 165.2 ± 8.80%; LTD₄: 171 ± 21.70%), indicating an acceleration in OPC maturation, as also confirmed by appearance of a myelinating phenotype in culture (Figure 4.2 B, C and D). No changes in the total number of cells in culture were observed in all experimental conditions, indicating that these ligands did not affect cell proliferation and/or survival (data not show). In line with these data, also WB analysis confirms the pro-differentiating effects of UDP and LTE₄ on OPC differentiation (Figure 4.2 F and G). Indeed, quantitative analysis of CNPase protein showed that both these ligands significantly increased CNPase expression with respect to control (CTRL: 100 ± 5.06%; UDP: 137.2 ± 6.47%; LTE₄: 126.1 ± 5.79%).

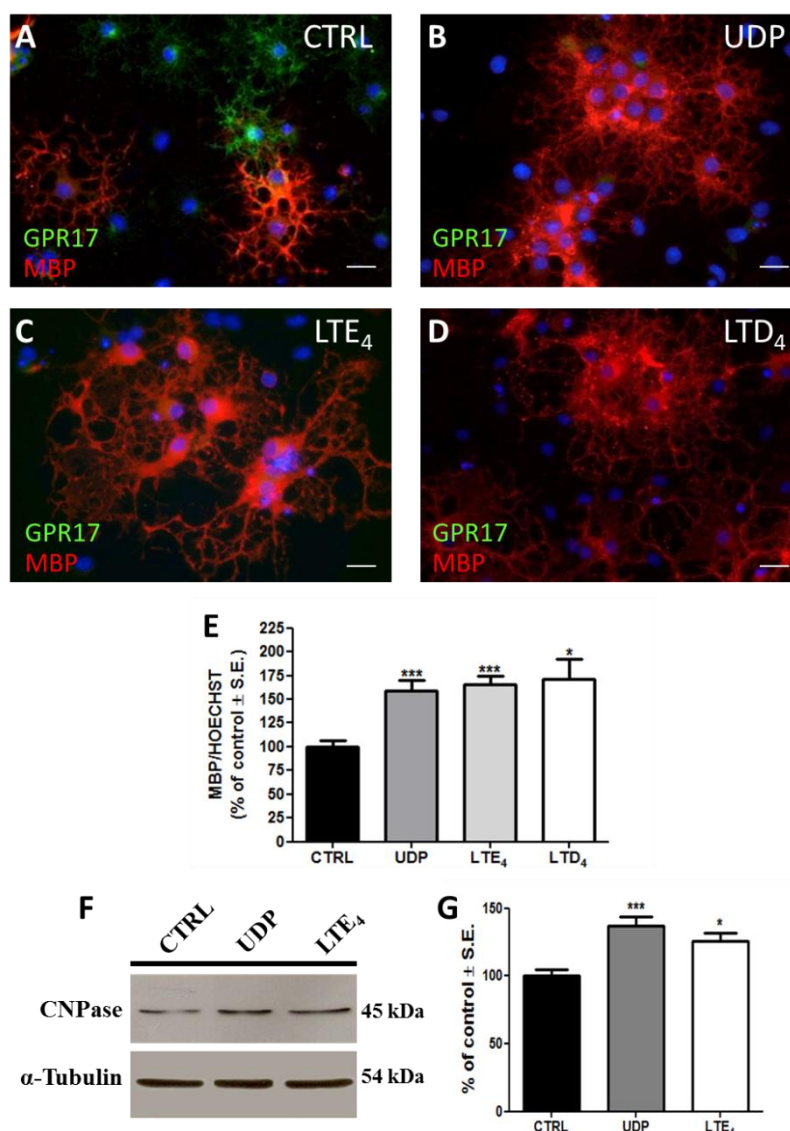


Figure 4.2 - Activation of GPR17 by its endogenous ligands accelerates OPC maturation.

Primary purified OPCs were cultured in differentiating medium containing T_3 . At day 3/4, GPR17 endogenous ligands UDP (100 μ M), LTE₄ (10 nM) and LTD₄ (100 nM) were added to cultures. After 48 h, cells were fixed and immunostained with anti-GPR17 (green) and anti-MBP (red) antibodies or lysed for WB analysis. (A-D) Representative images of CTRL (A), UDP (B), LTE₄ (C) and LTD₄ (D) treated cell. (E) Histograms show quantification of the percentage of MBP positive cells in control and treated cells (with vehicle-treated control cells set to 100%). Hoechst 33258 was used to label cell nuclei. The number of positive cells was counted in 10 optical fields under a 10X magnification. Data are the mean \pm S.E. of cell counts from a total of 10 coverslips/condition from three independent experiments (CTRL: 100 \pm 6.83%; UDP: 158.4 \pm 11.49%; LTE₄: 165.2 \pm 8.80%; LTD₄: 171 \pm 21.70%). ***, $p < 0.001$; *, $p < 0.05$ compared to control; one-way ANOVA, followed by Bonferroni's multiple comparison test. Scale bars: 15 μ m. (F) CNPase expression was assessed by western blot after UDP and LTE₄ treatment. (G) Histograms show the quantification of CNPase protein levels in CTRL and in UDP or LTE₄-treated cultures. Scanning densitometry was quantified and normalized to control (set to 100%) on the same WB. α -tubulin expression was analyzed from the same samples as an internal control. Data are expressed as mean \pm S.E. of three independent experiments (CTRL: 100 \pm 5.06%; UDP: 137.2 \pm 6.47%; LTE₄: 126.1 \pm 5.79%). *, $p < 0.05$; ***, $p < 0.001$ compared to CTRL, non parametric Mann-Whitney test.

To univocally confirm that the pro-differentiating effect of these ligands was due to GPR17 activation, we took advantage of siRNAs specifically designed for silencing rat GPR17. Both the efficiency and the efficacy of these siRNAs to specifically act against rat GPR17 were already tested in the same experimental model of purified OPC cultures (Fumagalli et al., 2011). Using the same protocol of transfection described in this publication, we repeated the knock-down experiments. Based on these premises, OPCs were cultured in differentiating medium and transfected with either an ineffective randomly designed siRNA (negative control, “Neg”) or with siRNAs specifically designed against rat GPR17 (siRNA) as described in Fumagalli et al., 2011. 48 hours after transfection, cells were cultured in differentiation medium in the absence or presence of UDP (100 μ M) for another 2 days and, then, lysed for WB analysis. As shown in Figure 4.3 A and B, WB analysis confirmed the remarkable decrease of GPR17 in cells transfected with GPR17 siRNAs with respect to cells transfected with ineffective siRNAs (Neg: $100 \pm 3.62\%$; siRNA: $43.10 \pm 0.91\%$).

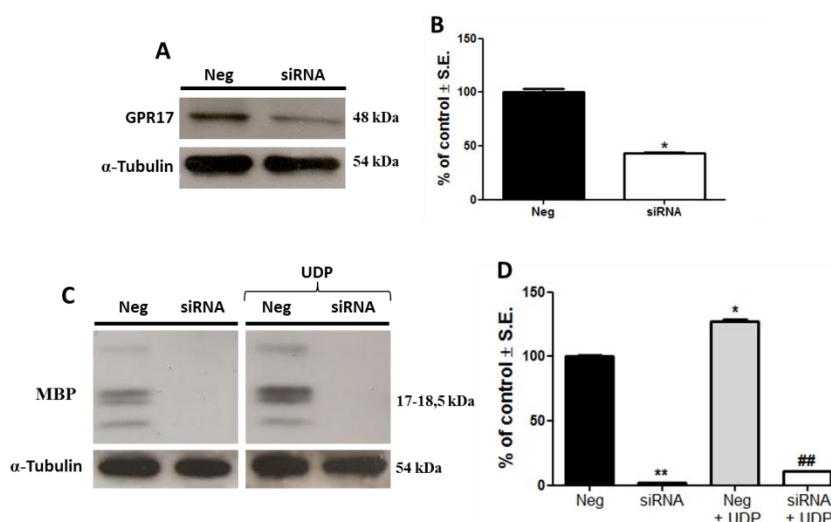


Figure 4.3 - GPR17 knock-down abolishes the UDP pro-differentiating effect.

Primary purified OPCs were cultured in presence of PDGF and of bFGF for 2 or 3 days and, then, cell differentiation was induced by adding T_3 in the medium. Concomitantly, cells were treated with either siRNAs (50 nM) specifically designed against rat GPR17 (siRNA) or ineffective randomly designed siRNA utilized as a negative control (Neg). For 48 hours after transfection, UDP (100 μ M) was added in the culture medium and was left for another 48 hours. (A, C) GPR17 and MBP expression was assessed by WB analysis. (B) Histograms show the quantification of GPR17 protein levels in cells transfected with the ineffective siRNA and in siRNA GPR17-treated cultures. Scanning densitometry was quantified and normalized to CTRL (set to 100%) on the same WB. α -tubulin expression was analyzed from the same samples as an internal control. Data are expressed as mean + S.E. of two independent experiments (Neg: $100 \pm 3.62\%$; siRNA: $43.10 \pm 0.91\%$). *, $p < 0.05$ compared to Neg, unpaired T-test. (D) Histograms show the quantification of MBP protein levels in cells transfected with the ineffective siRNA and in siRNA GPR17-treated culture in presence or absence of UDP. Scanning densitometry was quantified and normalized to CTRL (set to 100%) on the same western blot. α -tubulin expression was analyzed from the same samples as an internal control. Data are expressed as mean + S.E. (siRNA: $1.98 \pm 0.13\%$; siRNA+UDP: $10.81 \pm 1.10\%$). **, $p < 0.01$ compared to Neg; *, $p < 0.05$ compared to Neg; ###, $p < 0.01$ compared to siRNA+UDP, unpaired T-test.

Moreover, as expected, UDP treatment increased MBP protein levels compared to non-treated cultures (Neg: $100 \pm 1.21\%$; Neg+UDP: $127.6 \pm 1.10\%$). In contrast, GPR17 knock-down in siRNA-transfected cultures blocked OPC maturation (siRNA: $1.98 \pm 0.13\%$) and, in these cultures, the pro-differentiation effect of UDP was completely lost (siRNA+UDP: $10.81 \pm 1.10\%$), indicating that, as for UDP-glucose, also in this case the effect was specifically mediated by GPR17. These data confirm that GPR17 plays a functional role in OPCs differentiation and its activation at early differentiation stages induces OPCs to undertake their differentiation program towards a mature phenotype.

4.1.2 Characterization of OPC/DRG co-cultures

To evaluate whether, besides favouring the acquisition of a myelinating phenotype, GPR17 ligands can influence the formation of myelin segments, purified OPCs were cultured with DRG. Different protocols for this *in vitro* model have been described in literature and they mainly differ for the time length for which DRG are maintained in cultures before adding OPCs. Indeed, DRG can be cultured as dissociated or undissociated neurons (Jarjour et al., 2012). Both models are largely accepted within the scientific community, but we decided to follow the second approach, since our preliminary data indicate that the dense neurite beds extending from intact ganglia favours myelin sheath formation.

As described in detail in the paragraph 3.2.1, DRG explants from E14.5 mice were directly put in culture after being plucked off from embryo spinal cords. Then, they were grown in presence of NGF, a neurotrophic factor which is known to promote neuritis outgrowth and in presence of FUDR in order to remove all non-neuronal cells. After 20 days, when neuritis were well extended radially from DRG explants, OPCs were seeded onto DRG and myelination was induced by the adding of TrkA-Fc which is a recombinant factor able to sequester NGF that has been demonstrated to inhibit OPC myelination (Chan et al., 2004). To fully characterize how OPC differentiation proceeds in this *in vitro* model, also in relation to GPR17 expression, cells were fixed at different times and immunostained with specific differentiation stage markers. As shown in Figure 4.4, after 4 days in co-culture (A), the majority of OPCs expressed NG2 and they were distributed along neuronal filaments; after 6 days (B), all the cells expressing GPR17, displayed an highly ramified morphology and some of them started taking contact with axons; after 8 days in co-culture (C), cells started expressing MBP and establishing contacts with neurofilaments, wrapped them and forming

the myelin sheath. The number of myelinating OL progressively increased during the following days.

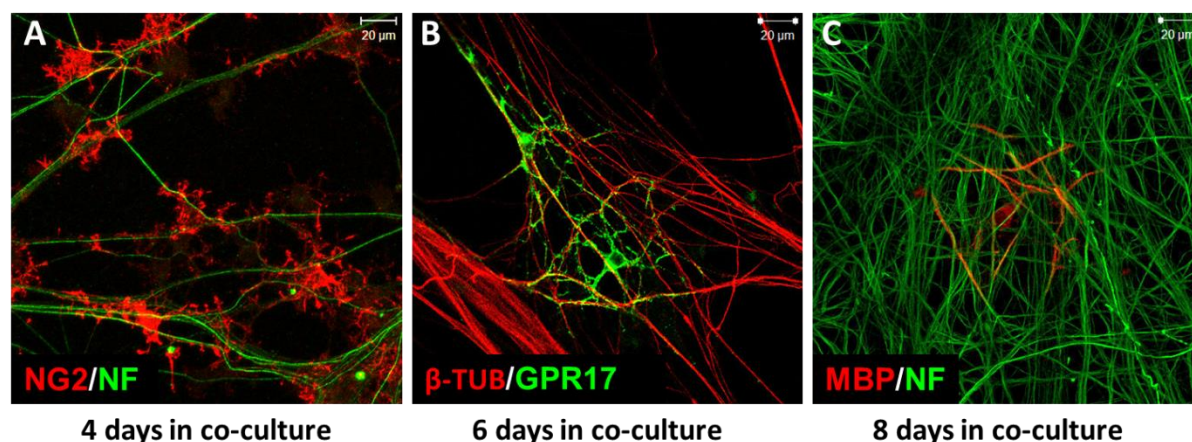


Figure 4.4 - Characterization of OPC/DRG co-cultures.

DRG were maintained in culture in presence of NGF for 20 days. When neurites were well extended radially from DRG explants, purified OPCs were added to each DRG in culture. In order to characterize how OPC differentiation proceeds in this *in vitro* model, cells were fixed and immunolabeled with different stage-specific markers at different times. (A) After 4 days in co-culture, the majority of OPCs expressed NG2 (in red); neurofilaments (NF) are immunolabeled with SMI 31, which specifically recognized the phosphorylated neurofilament H and with SMI 32, which recognized the non-phosphorylated neurofilament H (in green). (B) After 6 days in co-culture, the majority of OPCs expressed GPR17 (in green); neurofilaments are immunolabeled with anti- β -tubulin III (in red). (C) After 8 days in co-culture, cells started expressing MBP (in red) and some of them started myelinating neuronal axons (NF, in green). The number of myelinating OL progressively increases during the following days (data not shown). Images were taken under a confocal microscope at 40X magnification. Scale bars: 20 μ m.

Since this type of culture is extremely complex and variable, in the last decade several strategies have been proposed for setting a reliable method for the quantification of the myelination process. These approaches include the measurement of CNPase activity (Birgbauer et al., 2004; Roth et al., 1995) or of the immunofluorescence staining for MBP (Ghoumari et al., 2003; Mi et al., 2009; Miron et al., 2010). However, it has to be highlighted that both these two methods do not allow a direct quantification of myelination. In fact the former is based on the measurement of a marker of OL differentiation, rather than the presence of myelin sheaths; the latter allows only the quantification of MBP which is present in OL processes and cell bodies as well as in myelin sheaths. Moreover, neither approach considers the number of axons present in the culture that is a prerequisite for myelination. The need of a fast, accurate, objective and automatic quantification of the amount of myelin is crucial for the study of factors influencing myelination and remyelination. In this respect, Zhang and co-workers have recently developed a new method for myelin quantification which is based on the possibility to directly quantify the myelin amount in an *ex vivo* slice culture

system using the immunohistochemistry approach and a confocal microscope. Through this method, the structural myelin protein (MBP) and the axonal protein neurofilaments (NF) are stained with two different fluorophores and, then, only the overlapping between these two staining, which represents the myelin sheath, is measured. This approach allows the exclusion of OL cell bodies and of non-myelinating processes which are also stained for MBP (Zhang et al., 2011).

We took advantage of this new method of analysis and we adapted this approach to the quantification of myelination in our *in vitro* system (Figure 4.5). The OPC/DRG co-cultures were maintained in culture for 11 days and, then, cells were fixed and immunostained for NF and for myelin. NF (in green) were immunolabelled with two different antibodies: anti-Smi 31, which specifically recognized the phosphorylated neurofilament H and anti-Smi 32, which recognized the non-phosphorylated neurofilament H; myelin was immunolabelled with anti-MBP antibody (in red).

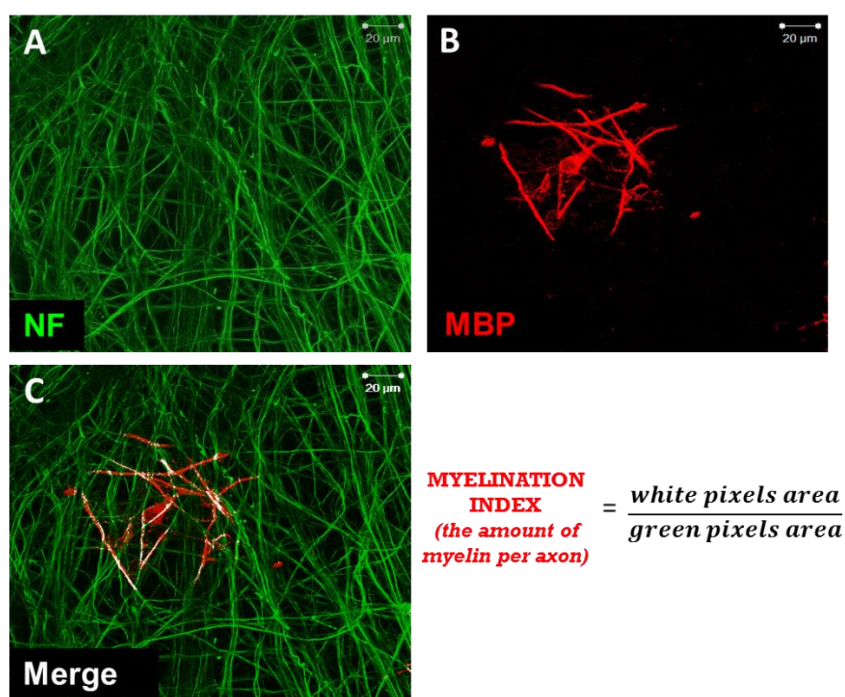


Figure 4.5 - Automatic quantification of myelination in OPC/DRG co-cultures.

(A) OPC-DRG co-cultures were maintained in culture for 8 days and then fixed and immunostained for neurofilaments with anti-Smi 31 and anti-Smi 32 antibodies (in green), and (B) for myelin with an anti-MBP antibody (in red). (C) To perform the quantification of myelination, stacks of images of MBP and NF positive cells were taken under confocal microscope Zeiss LSM 510 META at 40X magnification. The ZEISS LSM Image Browser was utilized to automate quantification of the myelination index. Images in the stack were merged at each level and pixels overlapping in the red and green fields above a predefined threshold intensity value were highlighted in white. The myelination index, which represents the amount of myelin per axon, is the ratio between the white pixels area and the green pixels area.

Quantification of myelination was performed under the confocal microscope. Stacks of images of MBP and NF positive cells were taken at 40X magnification and, then, images within the stack were merged at each level. The ZEISS LSM Image Browser was utilized to automate quantification of the myelination: pixels overlapping in the red and green fields above a predefined threshold intensity value were counted (white pixels area) and divided by the number of green pixels (NF). This ratio is the “Myelination Index” and represents the amount of myelin per axon.

4.1.3 GPR17 agonists promote the myelination in OPC/DRG co-culture system

To determine whether GPR17 activation can also affect the myelination process, we performed a series of experiments using the OPC/DRG co-culture system. As described in the previous paragraph, OPCs co-cultured with DRG spontaneously differentiate reaching a mature phenotype starting from day 8. UDP-glucose (100 μ M) and UDP (100 μ M) were added to OPC/DRG co-cultures at day 4 when OPCs started expressing GPR17 and the pharmacological treatment was continued up to 11 days in order to have a high number of myelinating OL. After this time, DRG displayed a general “suffering” state due to a huge increased number of OPCs which made the system unsuitable for the automatic quantification. After fixation, cells were immunostained for MBP and NF and myelin segments were quantified as previously described.

As shown in Figure 4.6 (D), GPR17 activation mediated by both UDP-glucose and UDP significantly promoted the formation of myelinated segments as shown by the increased value of the “Myelination Index” (CTRL: $0.011 \pm 0.0015\%$; UDP-glucose: $0.026 \pm 0.008\%$; UDP: $0.027 \pm 0.007\%$). This increase was due to the presence of more MBP⁺-myelinated axons in treated-cultures (Figure 4.6 B and C) with respect to vehicle-treated cells (Figure 4.6 A).

This result clearly demonstrated that, besides regulating the spontaneous OPC differentiation program as shown above, agonists-mediated GPR17 activation also enhanced OPC myelination.

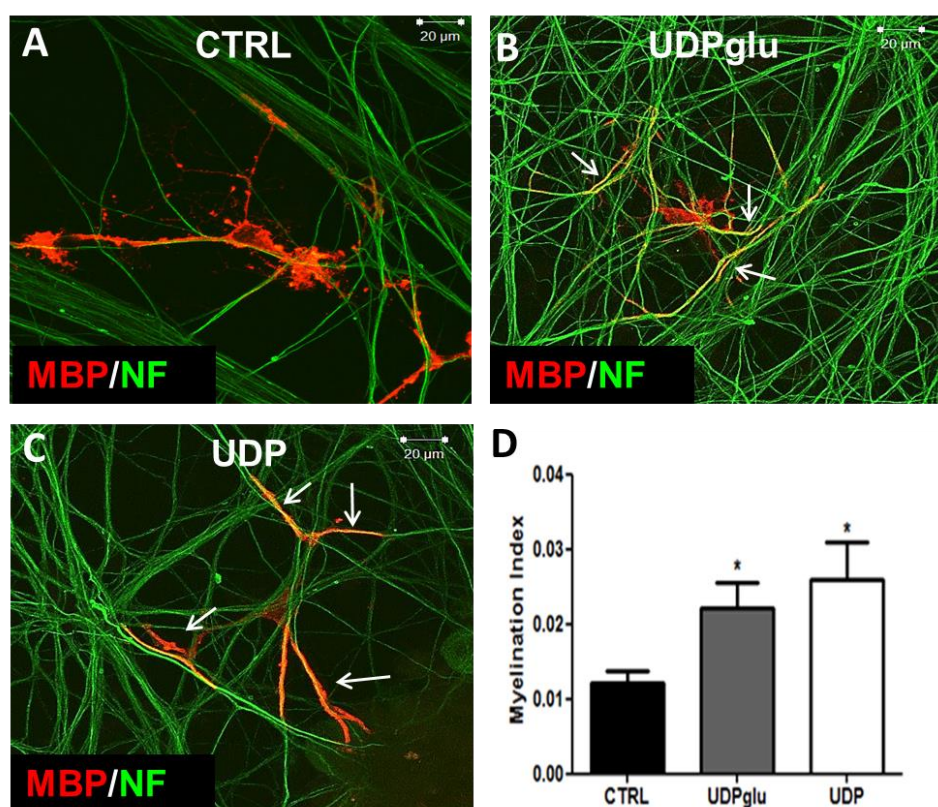


Figure 4.6 - The GPR17 agonists UDP-glucose and UDP increase myelination in OPC/DRG co-cultures.

OPC/DRG co-cultures were exposed for 96 h to UDP-glucose (100 μM) or UDP (100 μM). Representative images of control (CTRL, A), UDP-glucose (UDPglu, B) or UDP (C) treated cells show double immunostaining for anti-MBP antibody (red) and anti-neurofilament antibody (NF, green), scale bar: 20 μm. (D) Histograms show the quantification of the myelin segments (Myelination index). Both UDP-glucose and UDP increase myelination with respect to control. Data are the mean ± S.E. of the myelin index obtained from the analysis of 6 random fields of 3 coverslips for each experimental condition from two independent experiments (CTRL: 0.011 ± 0.0015%; UDP-glucose: 0.026 ± 0.008%; UDP: 0.027 ± 0.007%). *, $p < 0.05$ Student's T-test.

4.2 Down-regulation of GPR17 at late OPC differentiation stages is needed to complete cell maturation

Our previous *in vitro* data demonstrated that GPR17 transiently labels specific early OPC differentiation stages, reaching a maximal expression in cells that exit the cell cycle and become irreversibly committed to differentiation. On the contrary, mature OLs expressing the myelin proteins PLP, MAG and MBP do no longer express the receptor (see paragraph 1.2.5 for details) (Fumagalli et al., 2011). Accordingly, a detailed *in vivo* study also showed that, in rodents, during postnatal development, GPR17-expressing OPCs are already present at birth; receptor expression increases over time during postnatal development clearly preceding the peak of myelination, and then declines in the adult brain while myelination proceeds (Boda et

al., 2011). Altogether these results suggest that the down-regulation of GPR17 at an advanced differentiation stage is a prerequisite to allow cells to proceed to terminal maturation.

On this basis, to verify this hypothesis, OPCs were transfected with a fluorescent plasmid in which the coding sequence for GPR17 has been cloned in frame with the sequence coding for GFP (GPR17-GFP). In parallel, as a control we used the same empty vector (GFP). These plasmids gave us the possibility to follow the destiny of the cells thanks to the green fluorescence, in order to compare OPC maturation of control cells, with cells in which GPR17 has been over-expressed. As shown in figure 4.7, we demonstrated that most cells incorporating a GFP-GPR17 fusion vector for GPR17 over-expression did not express the mature marker CNPase (B). Conversely, the majority of cells transfected with the control plasmid (containing only GFP) normally underwent *in vitro* maturation and expressed CNPase (A).

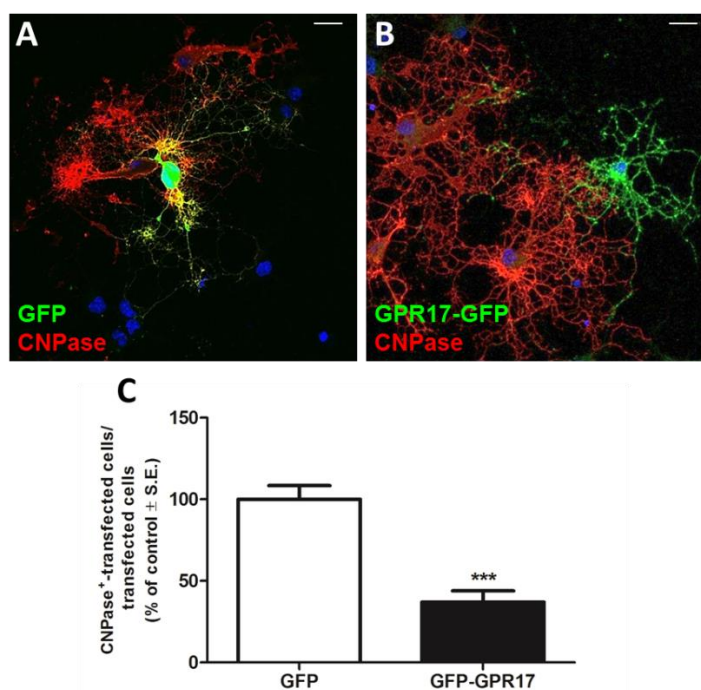


Figure 4.7 – Forced GPR17 expression at late OPC differentiation stages impairs cell terminal maturation.

(A) shows representative culture transfected with an empty GFP-fluorescent plasmid. The majority of these GFP-positive cells (in green) normally undergoes *in vitro* maturation, as shown by expression of the mature marker CNPase (in red). On the contrary, most cells incorporating a GFP-GPR17 fusion vector for GPR17 over-expression (B) do not mature and do not express CNPase. (C) Histograms show quantification of the percentage of transfected cells expressing CNPase over the total transfected cell population in the two conditions analyzed: transfection with the control plasmid GFP set to 100% ($100 \pm 8.46\%$, number of counted cells: 1654) and transfection with GFP-GPR17 ($37.03 \pm 6.84\%$, number of counted cells: 720). ***, $p < 0.001$ compared to control, Student T-test.

For the quantification, the total number of transfected cells expressing CNPase was expressed as the percentage over the total transfected cell population (C) (GFP: $100 \pm 8.46\%$; GFP-GPR17: $37.03 \pm 6.84\%$). These data clearly suggest the existence of a timely restricted expression of GPR17 and that interferences with this physiological down-regulation of the receptor in OPCs result in permanently elevated GPR17 levels and impairment of OPC terminal differentiation.

4.3 GPR17 homologous desensitization in primary OPCs

4.3.1 GPR17 agonists switch the receptor off inducing its desensitization/internalization

The mechanisms underlying GPR17 physiological down-regulation during OPC differentiation still remain to be elucidated. Their characterization is quite important, since alterations in these mechanisms leading to excessive/prolonged expression of GPR17 in OPCs may contribute to the limited remyelination after demyelinating insults. According to this, GPR17 has been found markedly up-regulated in rodents in which focal cerebral demyelination was induced through the local injection of lysolecithin (Boda et al., 2011) and in MS patients (Chen et al., 2009), suggesting that impaired GPR17 down-regulation may contribute to the defective remyelination observed in these pathological conditions.

In this respect, to explain GPR17 transient expression in OPCs and its effects on cell maturation, a current hypothesis is that, by binding to GPR17, its endogenous ligands can first induce early precursor cells to undergo differentiation and then switch the receptor off by agonist-induced desensitization, thus allowing cell terminal maturation. Thus, by controlling the active state of receptors at the plasma membrane, agonist-mediated GPR17 desensitization/internalization finely regulates cell progression along their differentiation pathway.

Previous studies performed either in a recombinant system (Daniele et al., 2011) or native GPR17⁺-cells (Olineu cells) (Fratangeli et al., 2013) have already demonstrated that both uracil nucleotides and cystenyl-leukotrienes are able to induce GPR17 desensitization/internalization with different kinetics. In the present work, by using primary cultured OPCs, we investigated in detail the molecular mechanisms underlying GPR17 desensitization. To this aim, we first performed a cAMP assay as previously described in Daniele et al., 2011. Cells were maintained in culture up to the pre-oligodendrocyte stage (which corresponds to the peak expression of GPR17) and, then, treated for different times (5-120 min) with 50 nM LTD₄ or 5 μM UDP-glucose (almost 10 times over their EC₅₀ values).

After extensively washes, cells were stimulated with the same agonists, but at low concentration (UDP-glucose 500 nM; LTD₄ 5nM) and their ability to inhibit cAMP accumulation stimulated by forskolin was measured. As shown in Figure 4.8, both UDP-glucose and LTD₄ decreased GPR17 responses after an initial challenge with a high concentration of either agonist. These effects occurred in a time-dependent manner, resulting in almost complete inhibition (>85%) of GPR17 activation after 120 minutes of pre-incubation with both agonists.

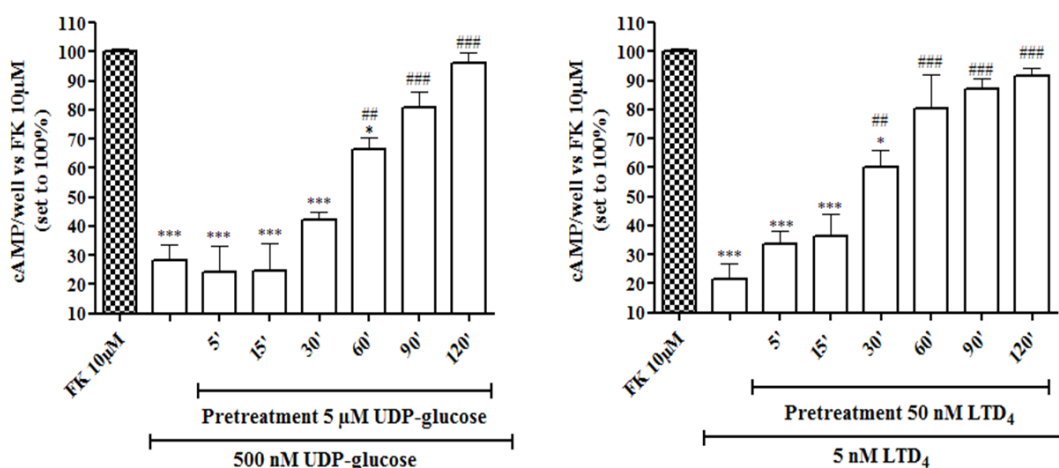


Figure 4.8 - Effects of UDP-glucose and LTD₄ on GPR17 desensitization in OPCs.

Cells were treated with 5 µM UDP-glucose or 50 nM LTD₄ for different time periods (5-120 min). After extensive washing, cells were treated for 15 min with 500 nM UDP-glucose or 5 nM LTD₄ in the presence of 10 µM forskolin (FK) and intracellular cAMP levels were measured. Data are expressed as the percentage of FK-stimulated cAMP levels, set to 100%, and represent the means ± S.E. of four independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs FK alone; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ vs 500 nM UDP-glucose or 5 nM LTD₄; one-way ANOVA, followed by Bonferroni's multiple comparison test.

4.3.2 GRK2 expression and physical association with GPR17 receptor

As extensively described in the paragraph 1.3, agonist induced GPCRs desensitization classically involves rapid receptor phosphorylation by second messenger-dependent and/or G-protein coupled receptor kinases (GRKs) (Gurevich et al., 2012). Among GRK isoforms, the mammalian GRK 2, 3, 5 and 6 represent the kinases primarily involved in GPCR regulation (Ribas et al., 2007; Yang and Xia, 2006). Agonists' stimulation switches the receptor in an active state, triggers its phosphorylation by GRKs and primes the recruitment of regulatory proteins termed β-arrestins, leading to receptor desensitization from G protein and to the subsequent internalization (Ferguson, 2001). Based on this evidence, we decided to investigate whether GPR17 agonists-mediated homologous desensitization requires GRKs.

Among all GRKs isoforms, we focused on GRK2 since this isoform is widely expressed within the brain (Arriza et al. 1992) and alteration of its expression have been detected in leukocytes of patients with MS (Vroon et al., 2005), suggesting a possible role in demyelination.

As a first step, using real time PCR in combination with ICC and WB experiments, we performed a detailed analysis of GRK2 mRNA and protein expression, in parallel with that of GPR17 and MBP during spontaneous *in vitro* OPC differentiation. As shown in figure 4.8 (B and C), according to our previous data, GPR17 expression (both mRNA and protein) was maximal in pre-oligodendrocytes (stage 3) and completely segregated from that of mature myelin. In contrast, MBP expression started later: MBP mRNA was already detectable in late precursors of stage 2 and MBP protein dramatically increased during the transition from pre-oligodendrocytes of stage 3 and mature OL of stage 5. Both GRK2 mRNA and protein were already present at early differentiation stages. According to these observations, all OPCs expressed this kinase (Figure 4.8 A). Interestingly, as GPR17, GRK2 expression reached a maximal peak in pre-oligodendrocyte; then, in parallel to GPR17, GRK2 levels progressively declined, reaching their lowest levels in the terminal phases of differentiation (Figure 4.8 B and C). This pattern of expression may reflect a functional link between GPR17 and GRK2 function during OPC maturation.

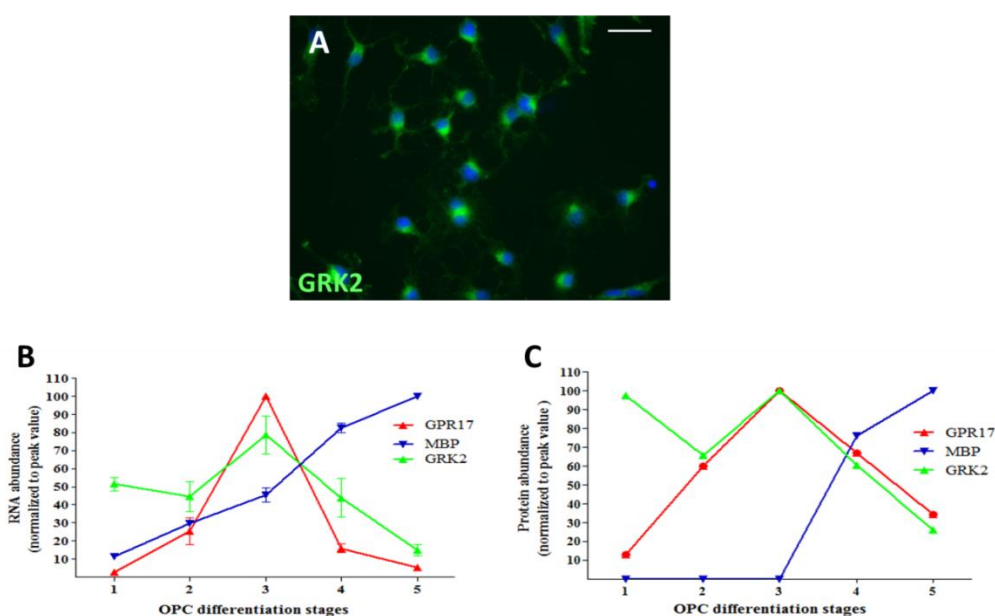


Figure 4.9 - GRK2 expression during OPC differentiation.

(A) Representative image of OPCs at stage 1. Cells were immunolabelled with anti-GRK2 antibody (green). Hoechst 33258 was used to label cell nuclei. Image was taken at 40X magnification. Scale bar: 25 μ m. (B, C) Graph shows mRNA and protein levels of GRK2 (green line), GPR17 (red line) and MBP (blue line). Both RNA and protein were obtained from rat OPCs at different maturation stages (from stage 1 to 5). Fold changes are expressed as percentage of the peak value of each gene or protein, set to 100%.

To verify whether agonists-induced GPR17 homologous desensitization involves GRK2, we evaluated its recruitment and association upon GPR17 stimulation by co-immunoprecipitation experiments. To this aim, OPCs were exposed to high concentration of either UDP-glucose (5 μ M) or LTD₄ (50 nM) and, then, GRK2/GPR17 association was measured. As shown in figure 4.10, under basal conditions, GPR17 weakly associated with GRK2. This association was remarkably increased after UDP-glucose stimulation and, to more extent, after LTD₄ stimulation (Basal: $1 \pm 0.01\%$; UDP-glucose: $4.93 \pm 0.63\%$; LTD₄: $8.37 \pm 0.43\%$). These data clearly confirmed the involvement of GRK2 in agonist-induced GPR17 homologous desensitization, particularly after LTD₄ stimulation.

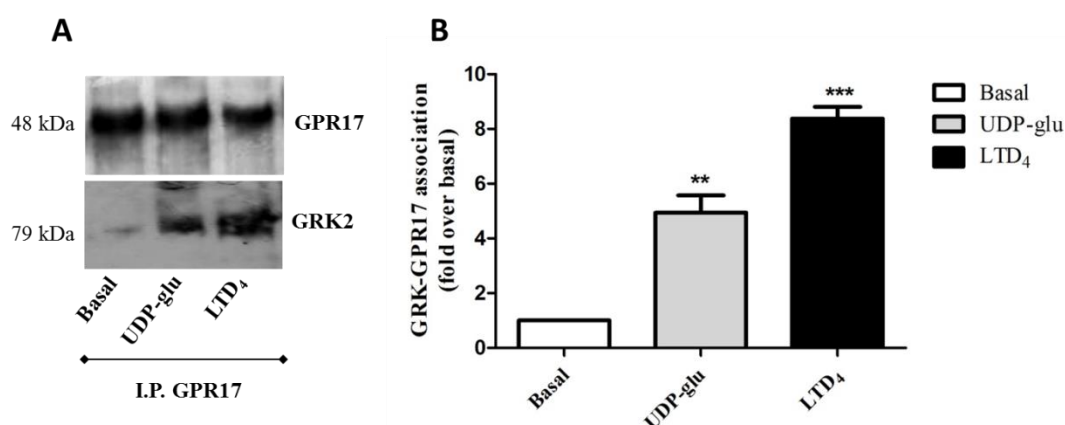


Figure 4.10 - UDP-glucose and LTD₄ induce GRK2 association to GPR17.

Cells, isolated and differentiated at the pre-oligodendrocytes (stage 3), were treated with medium alone or with 5 μ M UDP-glucose or 50 nM LTD₄ for 5 min. Rat GPR17 was immunoprecipitated using an anti-GPR17 antibody and immunoprecipitates were probed with anti-GRK2 antibody. (A) Representative immunoblots of GPR17 and GRK2 in different experimental conditions. (B) Histograms show the result of densitometric analysis. Data are expressed as percentage versus basal value and represent the mean \pm S.E. of three independent experiments (Basal: $1 \pm 0.010\%$; UDP-glu: $4.93 \pm 0.63\%$; LTD₄: $8.37 \pm 0.43\%$). **, $p < 0.01$; ***, $p < 0.001$ vs basal value; one-way ANOVA, followed by Bonferroni's multiple comparison test.

4.3.3 GRK2 mediates LTD₄-induced GPR17 homologous desensitization

The uncoupling of GPCRs from heterotrimeric G proteins, which occurs during agonists-induced receptor desensitization, is the consequence of a covalent modification of the receptor (phosphorylation) mediated by intracellular kinases. On this basis, we decided to evaluate whether GPR17 agonists modify the phosphorylation state of GPR17. To this purpose, OPCs were treated with medium alone (basal) or with UDP-glucose (5 μ M) or LTD₄ (50 nM) and, then, cells were lysed and incubated with anti-GPR17 antibody for immunoprecipitation experiments. The phosphorylation level of GPR17 was assessed using primary antibodies

which can specifically recognize the phosphorylated serine (p-Ser) and threonine residues (p-Thr).

As depicted in Figure 4.11, UDP-glucose and LTD₄ similarly induced GPR17 phosphorylation on threonine and on serine residues (for p-THR, Basal: $1 \pm 0.058\%$; UDP-glu: $1.57 \pm 0.042\%$; LTD₄: $2.12 \pm 0.038\%$; for p-SER, Basal: $1 \pm 0.058\%$; UDP-glu: $1.98 \pm 0.038\%$ LTD₄: $1.95 \pm 0.032\%$), suggesting that this modification is a prerequisite to the subsequent desensitization.

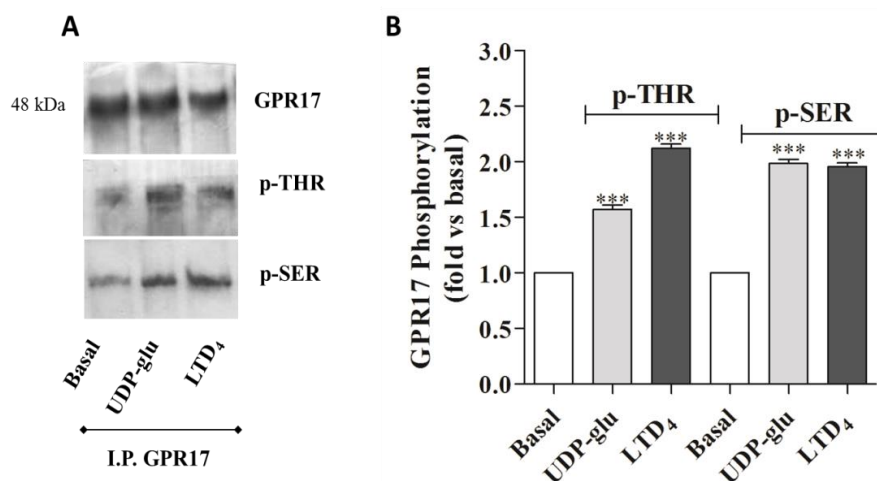


Figure 4.11 - UDP-glucose and LTD₄ mediated GPR17 phosphorylation on Ser and Thr residues. OPCs were treated with 5 μ M UDP-glucose or 50 nM LTD₄ for 5 minutes. Following incubation, GPR17 was immunoprecipitated using an anti-GPR17 antibody, and immunoprecipitates were probed with anti-phosphothreonine or anti-phosphoserine antibodies. (A) Representative immunoblots of GPR17, phospho-Thr (p-THR) and phospho-Ser (p-SER) protein levels in different experimental conditions. (B) Histograms show the result of densitometric analysis. Data are expressed as percentage of basal phosphorylation levels (set to 1) and represent the mean \pm S.E. of two independent experiments (For p-THR, Basal: 1 ± 0.058 ; UDP-glu: 1.57 ± 0.042 ; LTD₄: 2.12 ± 0.038 . For p-SER, Basal: 1 ± 0.058 ; UDP-glu: 1.98 ± 0.038 ; LTD₄: 1.95 ± 0.032). ***, $p < 0.001$ vs basal value; one-way ANOVA, followed by Bonferroni's multiple comparison test.

To further confirm the specificity of GRK2 in LTD₄-mediated GPR17 desensitization in OPCs, we repeated the cAMP assay in presence of KRX2929, a selective GRK2 inhibitor, which has been previously characterized (Gomez-Monterrey et al., 2014). Cells pre-treated with the GRK2 inhibitor impaired LTD₄-mediated GPR17 desensitization (Figure. 4.12 black bars), suggesting that GRK2 selectively mediated GPR17 homologous desensitization following to the exposure of this receptor agonist.

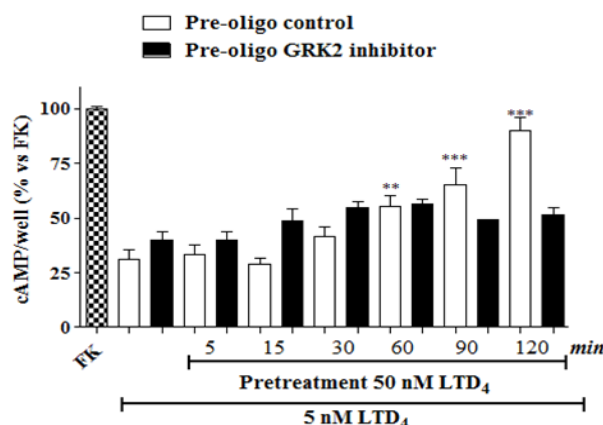


Figure 4.12 - GRK2 pharmacological inhibition prevents LTD₄-mediated GPR17 desensitization in OPCs.

OPCs, isolated and differentiated at pre-oligodendrocytes (stage 3), were treated with 50 nM LTD₄ for different times (5-120 min), in the absence (white bars) or in the presence (black bars) of the selective GRK2 inhibitor, KRX29 (1 μM). After extensive washing, cells were treated for 15 min with 10 μM FK, in the absence or in the presence of 5 nM LTD₄. Intracellular cAMP levels were evaluated as reported in the method section. Data are expressed as the percentage of FK-stimulated cAMP levels, set to 100%, and represent the means ± E.S. of six independent experiments. **, $p < 0.01$; ***, $p < 0.001$ vs 5 nM LTD₄; one-way ANOVA, followed by Bonferroni's multiple comparison test.

4.3.4 Role of GRK2 in GPR17 agonist-mediated OPC maturation

To dissect the actual involvement of GRK2 in OPC myelination, we assessed the effects of GRK2 knock-down on cell maturation. After 3 days in proliferation medium, OPC differentiation was induced by adding T₃ in the culture medium and GRK2 silencing was obtained by transfecting cells with a pool of four siRNA specifically designed against GRK2. In parallel, control cultures were transfected with an ineffective randomly designed pool of siRNA. The silencing efficiency of GRK2 and its consequences on OPC differentiation were evaluated by WB analysis after 48 and 96 hours, which correspond to pre-oligodendrocyte and immature OL stages respectively. As depicted in Figure 4.13, at both times after RNA interference, cells transfected with GRK2 siRNAs (siRNA) showed a significant reduction of GRK2 protein levels with respect to cultures transfected with ineffective siRNAs (CTRL) (CTRL 48h: 100 ± 1.19%; siRNA GRK2 48h: 52.14 ± 8.91%; CTRL 96h: 100 ± 2.13%; siRNA GRK2 96h: 66.91 ± 3.27%).

Once established the efficacy of GRK2 siRNAs, we analyzed the effects of GRK2 knock-down on the expression of GPR17, CNPase and MBP both in pre-oligodendrocytes and in immature OL. As shown in Figure 4.14, GRK2 knock-down did not alter GPR17 expression in pre-oligodendrocytes, but induced a significant increase in immature OL (CTRL 48h: 100

$\pm 1.71\%$; siRNA GRK2 48h: $115.3 \pm 20.19\%$; CTRL 96h: $100 \pm 3.29\%$; siRNA GRK2 96h: $171.4 \pm 34.10\%$).

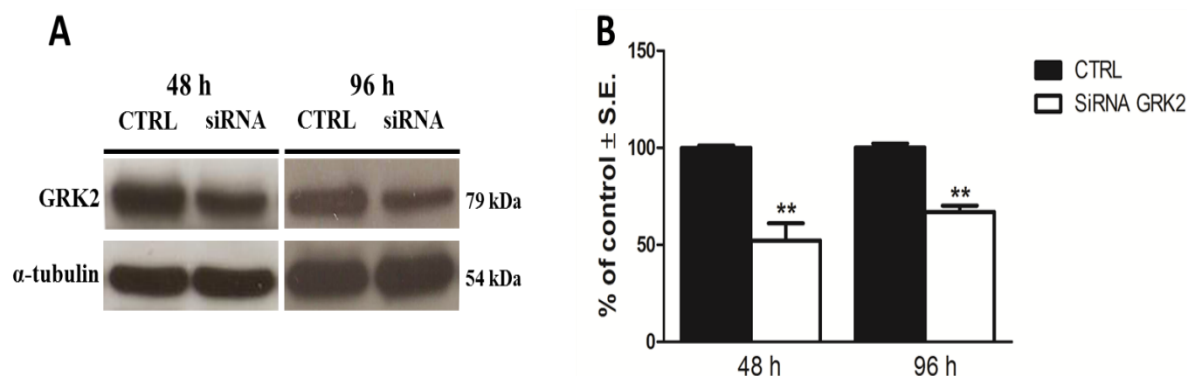


Figure 4.13 - GRK2 knock down by specific siRNA in primary OPCs.

Primary purified OPCs were cultured in presence of PDGF and of bFGF for 2 or 3 days and, then, cell differentiation was induced by adding T_3 in the medium. Concomitantly cells were treated with either siRNAs (50 nM) specifically designed against rat GRK2 (siRNA GRK2) or ineffective randomly designed siRNA utilized as a negative control (CTRL). (A) GRK2 expression was assessed by WB analysis, 48 (pre-OL) and 96 hours (immature OL) after transfection. (B) Histograms show the quantification of GRK2 protein levels in CTRL and in siRNA GRK2-treated cultures. Scanning densitometry was quantified, corrected for the corresponding α -tubulin band on the same WB and normalized to CTRL (set to 100%). Data are expressed as mean + S.E. of three independent experiments (CTRL 48h: $100 \pm 1.19\%$; siRNA GRK2 48h: $52.14 \pm 8.91\%$; CTRL 96h: $100 \pm 2.13\%$; siRNA GRK2 96h: $66.91 \pm 3.27\%$). **, $p < 0.01$ compared to CTRL, non parametric Mann-Whitney test.

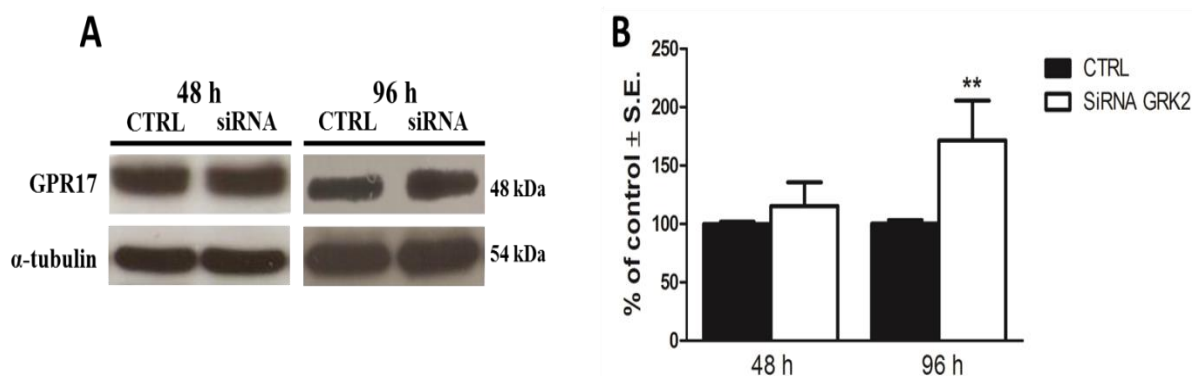


Figure 4.14 - GRK2 knock down by specific siRNA prevents GPR17 down-regulation at immature OL stage.

Primary purified OPCs were cultured in presence of PDGF and of bFGF for 2 or 3 days and, then, cell differentiation was induced by adding T_3 in the medium. Concomitantly, cells were treated with either siRNAs (50 nM) specifically designed against rat GRK2 (siRNA GRK2) or ineffective randomly designed siRNA utilized as a negative control (CTRL). (A) GPR17 expression was assessed by WB analysis in indicated condition after 48 (pre-OL) and 96 hours (immature OL) from transfection. (B) Histograms show the quantification of GPR17 protein levels in CTRL and in siRNA GRK2-treated cultures. Scanning densitometry was quantified and normalized to control (set to 100%) on the same WB. α -tubulin expression was analyzed from the same samples as an internal control. Data are expressed as mean \pm S.E. of three independent experiments (CTRL 48h: $100 \pm 1.71\%$; siRNA GRK2 48h: $115.3 \pm 20.19\%$; CTRL 96h: $100 \pm 3.29\%$; siRNA GRK2 96h: $171.4 \pm 34.10\%$). **, $p < 0.01$ compared to CTRL, non parametric Mann-Whitney test.

Also for the mature markers CNPase and MBP, no variation was detected in cells transfected with GRK2 siRNA after 48 hours (for CNPase, CTRL 48h: $100 \pm 0.51\%$; siRNA GRK2 48h: $102.6 \pm 8.15\%$; for MBP, CTRL 48h: $100 \pm 1.95\%$; siRNA GRK2 48h: $101.9 \pm 24.59\%$). On the contrary, after 96 hours, both CNPase and MBP expression resulted markedly reduced in cells transfected with GRK2 siRNAs with respect to control cultures (for CNPase, CTRL 96h: $100 \pm 0.72\%$; siRNA GRK2 96h: $80.68 \pm 3.03\%$; for MBP, CTRL 96h: $100 \pm 1.34\%$; siRNA GRK2 96h: $64.26 \pm 11.74\%$) (Figure 4.15).

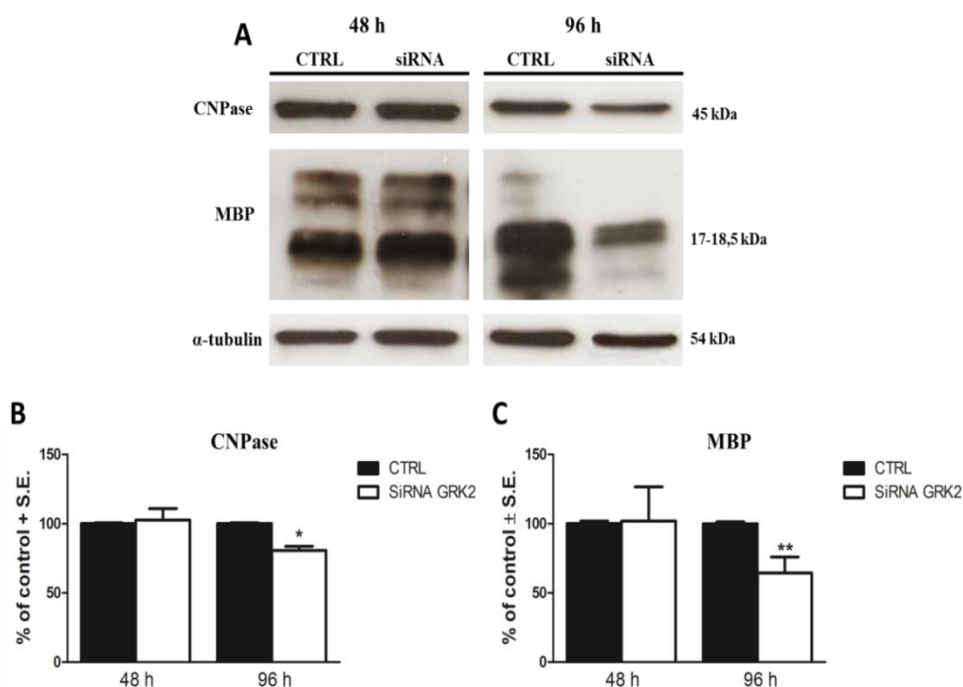


Figure 4.15 - GRK2 knock down by specific siRNA blocks OPC maturation

Primary purified OPCs were cultured in presence of PDGF and of bFGF for 2 or 3 days and, then, cell differentiation was induced by adding T_3 in the medium. Concomitantly, cells were treated with either siRNAs (50 nM) specifically designed against rat GRK2 (siRNA GRK2) or ineffective randomly designed siRNA utilized as a negative control (CTRL). (A) MBP and CNPase expression was assessed by WB analysis in indicated condition after 48 (pre-OL) and 96 hours (immature OL) from transfection. (B-C) Histograms show the quantification of MBP and CNPase protein levels in CTRL and in siRNA GRK2-treated cultures. Scanning densitometry was quantified and normalized to control (set to 100%) on the same WB. α -tubulin expression was analyzed from the same samples as an internal control. Data are expressed as mean \pm S.E. of three independent experiments (for CNPase, CTRL 48h: 100 ± 0.51 ; siRNA GRK2 48h: $102.6 \pm 8.15\%$; CTRL 96h: $100 \pm 0.72\%$; siRNA GRK2 96h: $80.68 \pm 3.03\%$; for MBP, CTRL 48h: $100 \pm 1.95\%$; siRNA GRK2 48h: $101.9 \pm 24.59\%$; CTRL 96h: $100 \pm 1.34\%$; siRNA GRK2 96h: $64.26 \pm 11.74\%$). *, $p < 0.05$; **, $p < 0.01$ compared to CTRL, non parametric Mann-Whitney test.

Globally, these data indicate a significant impairment of OPC maturation after GRK2 knock-down, which is detectable starting from the immature OL stage, suggesting an active involvement of this kinase in the transition from pre-oligodendrocytes to immature OL.

Finally, having established that, in primary cultured OPCs, LTD₄-mediated GPR17 desensitization occurs through the primary recruitment of GRK2, we asked whether GRK2 pharmacological inhibition had any effects on cysLT-promoted cell maturation. To this purpose, OPCs were exposed to LTD₄ in absence (CTRL) or in presence of the selective GRK2 pharmacological inhibitor.

As shown in Figure 4.16, OPC exposure to LTD₄ resulted in appearance of a myelinating phenotype in culture and in an increase of the number of MBP⁺-OL (C, E), demonstrating, as expected, an acceleration of cell maturation. In contrast, in presence of the specific GRK2 inhibitor, LTD₄-mediated differentiation was significantly inhibited (Figure 4.16 D, E), as showed by a similar number of MBP⁺-cells in these experimental conditions with respect to control culture. No differences in the number of MBP⁺-OL was observed in cultures treatment with only GRK2 inhibitor (B) (CTRL: 100 ± 11.15%; GRK2 inhibitor: 99.38 ± 4.83%; LTD₄: 171.7 ± 12.89%; LTD₄ + GRK2 inhibitor: 93.94 ± 9.0%). Also the total number of cells (Hoechst 33258 staining) were unaffected by pharmacological treatment (data not shown).

Globally, these data clearly demonstrate that GRK2-mediated desensitization of GPR17 is indeed necessary to enable OPCs to complete their differentiation program.

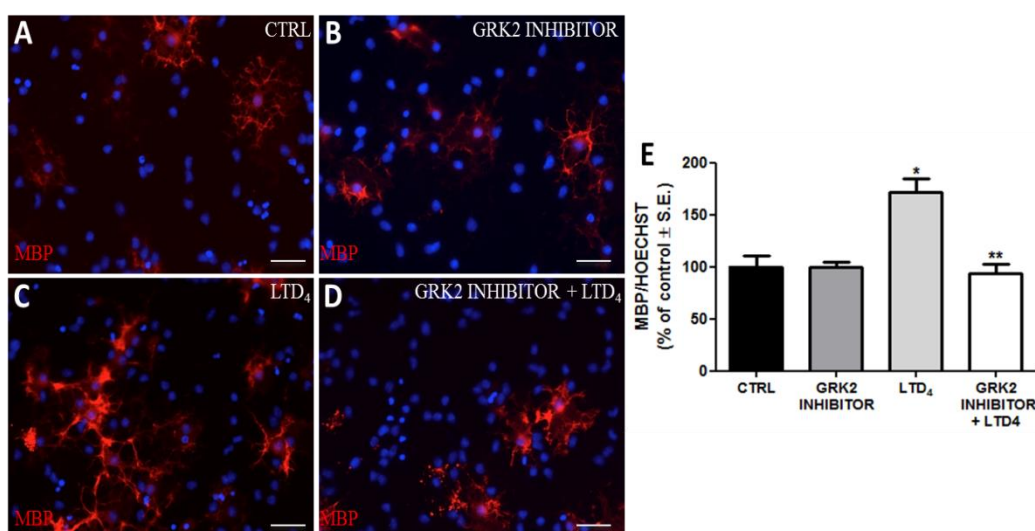


Figure 4.16 – GRK2 pharmacological inhibition blocks LTD₄-mediated OPC differentiation.

OPCs, maintained in differentiating medium, were treated with LTD₄ (100 nM) in the absence (CTRL) or in the presence of 1 μM of the specific GRK2 inhibitor (KRX29). After 48 hours, cells were fixed to determine their maturation degree by immunostaining with anti-MBP antibody. Hoechst 33258 was used to label cell nuclei. The number of positive cells was counted in 50 optical fields under a 40X magnification (~2000 cells/coverslip in the control condition). (A-D) Representative images of CTRL (A), GRK2 inhibitor (B), LTD₄ (C) and GRK2 inhibitor + LTD₄ (D) treated-cells. (E) Histograms show the quantification of the percentage of cells expressing the mature marker MBP in control and treated cells (with CTRL set to 100%). Data are the mean ± S.E. of cell counts from three independent experiments (CTRL: 100 ± 11.15%; GRK2 inhibitor: 99.38 ± 4.83%; LTD₄: 171.7 ± 12.89%; LTD₄ + GRK2 inhibitor: 93.94 ± 9.0%). *, $p < 0.05$ vs CTRL; **, $p < 0.01$ vs LTD₄; non parametric Mann-Whitney test.

4.4 Role of the mTOR pathway in the regulation of GPR17 expression

As mentioned in the previous paragraphs, the most rapid mechanism through which GPCRs are uncoupled from heteromeric G proteins is the covalent modification of the receptor as a consequence of phosphorylation by intracellular kinases (both GRKs and second messenger-dependent protein kinases). We have already demonstrated above that GRKs are involved in GPR17 receptor down-regulation after agonist exposure. However, recruitment of these kinases to the receptor may be also independent of agonist stimulation. This has been shown for second messenger-dependent protein kinases that do not only phosphorylate agonist activated GPCRs, but, under some circumstances, can also phosphorylate receptors that have not been exposed to agonists, thus representing an alternative or additional pathway to regulate the response of membrane receptors to extracellular signals (Hausdorff et al., 1989; Lohse et al., 1990). Nevertheless, it is now recognized that GPCRs spontaneously isomerize to an activated conformation in the absence of agonist, which suggests that GRKs may also contribute to the regulation of basal GPCR activity (Pei et al., 1994; Rim and Oprian, 1995). Therefore, the identification of further intracellular signaling pathways that can modulate the activity of these kinases in an agonists-independent manner is crucial. On this basis, we decided to investigate other intracellular signaling pathways involving kinases that, by modulating GPR17 expression/trafficking and/or GRK2 expression/function through modifications of its phosphorylate state, may also control OPC maturation.

In this respect, recent studies have shown that a possible pathway involved in the regulation of OPC differentiation is the mTOR signaling cascade (Tyler et al., 2009; 2011; Guardiola-Diaz et al. 2012). As widely described in paragraph 1.3.2, this signaling pathway is essential for OPC differentiation, since it regulates the transition from pre-oligodendrocytes to immature OLs and the subsequent acquisition of a mature myelinating phenotype. In particular, mTOR forms two distinct signaling complexes, termed mTORC1 and mTORC2 which are both involved in controlling OPC maturation, but through distinct mechanisms: mTORC2 transcriptionally controls key genes required for OPC differentiation, whereas mTORC1 influences the generation of mature OLs through a post-transcriptional mechanism (Tyler et al., 2009). Thanks to a proteomic analysis, the same group also demonstrated that GPR17 is one of the protein whose expression is altered after OPC treatment with rapamycin, a selective inhibitor of mTOR activity (Tyler et al., 2011), suggesting that this transduction signaling cascade may be involved in the regulation of GPR17 expression. This result has been an important starting point for improving and expanding our knowledge of the mechanisms underlying GPR17 physiological regulation. We thus performed different

experiments in order to understand the role of the mTOR pathway in regulating GPR17 expression.

4.4.1 mTOR pathway inhibition blocks OPC terminal maturation preventing GPR17 down-regulation

In order to evaluate the effects of mTOR inhibition on OPC terminal maturation, cells were maintained for 2 days in proliferating medium and, after 24 hours in differentiating medium, they were treated with rapamycin (15 nM). At day 6, when the majority of cells reached the mature phenotype, cells were fixed for ICC analysis.

As shown in Figure 4.17 (A and B), at stage 5 (mature OL) control cultures displayed, as expected, a low number of GPR17⁺-cells. In contrast, in rapamycin-treated cultures, the number of GPR17⁺-cells was significantly increased and, in parallel, the number of MBP⁺-cells was dramatically decreased. Histograms in C, summarizing the cell counts of the ICC experiments, clearly show that inhibition of the mTOR signaling pathway induced by rapamycin treatment slowed OPC maturation and increased GPR17 expression (for GPR17, CTRL: $100 \pm 7.92\%$; RAPA: $138.6 \pm 8.06\%$; for MBP, CTRL: $100 \pm 9.25\%$; RAPA: $46.26 \pm 7.59\%$). No variation in the total number of cells (Hoechst 33258 staining) was observed after treatment.

To confirm the results obtained with the ICC experiments and to extend the analysis to other specific markers of OPCs, WB studies were also performed. To this purpose, control and rapamycin-treated cells were lysed (according to the procedure described in the “*Materials and Methods*” section) at day 6 in culture and protein lysates were loaded on a polyacrylamide gel for electrophoretic separation. GPR17 protein levels were measured in parallel to those of the proteoglycan NG2, CNPase and MBP.

As depicted in Figure 4.17 (D, E), WB analysis confirmed that, at stage 5, cells exposed to rapamycin had a higher protein content of GPR17 compared to untreated cells. Furthermore, cells treated with rapamycin and maintained in culture up to stage 5 also showed increased expression of the proteoglycan NG2 compared to control cells (for NG2, CTRL: $100 \pm 3.45\%$; RAPA: $141.7 \pm 9.79\%$; for GPR17, CTRL: $100 \pm 2.07\%$; RAPA: $191.5 \pm 33.63\%$). In parallel, as shown in figure 4.17 (F, G), the same cultures had a reduced protein content of CNPase and MBP (for CNPase, CTRL: $100 \pm 0.88\%$; RAPA: $65.60 \pm 2.32\%$; for MBP, CTRL: $100 \pm 0.64\%$; RAPA: $31.32 \pm 6.23\%$).

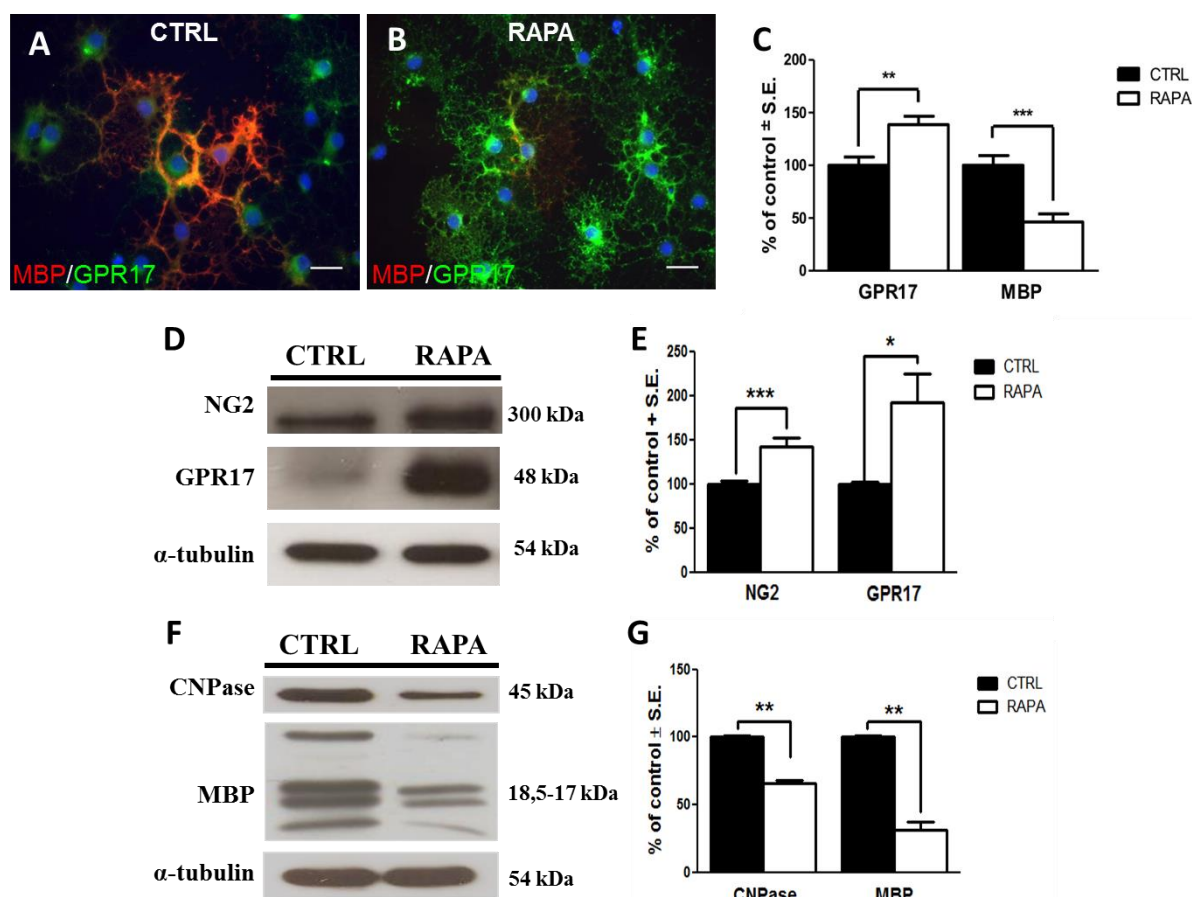


Figure 4.17 – mTOR inhibition by rapamycin induces a reduction of the mature markers MBP and CNPase and in parallel increases GPR17 and the precocious markers NG2.

Primary purified OPCs were cultured in presence of PDGF and of bFGF for two days. Cell differentiation was induced by adding T_3 in the medium. Rapamycin (15 nM) was added at day 1 and day 3. At day 6 (mature OL) cells were fixed for ICC analysis or lysed for WB analysis. (A, B) Representative images of control (CTRL) (A) and rapamycin (RAPA) (B) treated-cells, showing double immunostaining with anti-GPR17 (green) and anti-MBP (red) antibodies. (C) Histograms show quantification of the percentage of GPR17 and MBP positive cells in control and treated cells (with vehicle-treated control cells set to 100%). Hoechst 33258 was used to label cell nuclei. The number of positive cells was counted in 10 optical fields under a 20X magnification. Data are the mean \pm S.E. of cell counts from a total of 3 coverslips/condition from three independent experiments (for GPR17, CTRL: $100 \pm 7.92\%$; RAPA: $138.6 \pm 8.06\%$; for MBP, CTRL: $100 \pm 9.25\%$; RAPA: $46.26 \pm 7.59\%$). **, $p < 0.01$; ***, $p < 0.001$ compared to CTRL, Student T-test. (D, F) Representative immunoblots of GPR17, NG2, CNPase and MBP protein levels in different experimental conditions. (E, G) Histograms show the result of densitometric analysis. Scanning densitometry was quantified and normalized to control (set to 100%) on the same WB. α -tubulin expression was analyzed from the same samples as an internal control. Data are expressed as mean \pm S.E. of three independent experiments (for NG2, CTRL: 100 ± 3.45 ; RAPA: 141.7 ± 9.79 ; for GPR17, CTRL: 100 ± 2.07 ; RAPA: 191.5 ± 33.63 ; for CNPase, CTRL: $100 \pm 0.88\%$; RAPA: $65.60 \pm 2.32\%$; for MBP, CTRL: $100 \pm 0.64\%$; RAPA: $31.32 \pm 6.23\%$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to CTRL, non parametric Mann-Whitney test.

To gain insights into the mechanisms by which mTOR regulates GPR17 receptor expression, we first measured GPR17 mRNA levels, in parallel to those of MBP and NG2 by

quantitative PCR. To this aim, total RNA isolated from control and rapamycin-treated cultures at stage 5 was used to generate cDNA that was subjected to real-time PCR. Inhibiting mTOR by rapamycin resulted, as expected, in a strong reduction of MBP mRNA levels (Figure 4.18 B); in contrast, NG2 mRNA levels were markedly increased in rapamycin-treated cultures (Figure 4.18 C), whereas GPR17 mRNA levels only displayed a trend of increase (Figure 4.18 A) (for MBP, CTRL: 1 ± 0.02 ; RAPA: 0.57 ± 0.12 ; for NG2, CTRL: 1 ± 0.07 ; RAPA: 3.78 ± 1.02 ; for GPR17, CTRL: 1 ± 0.04 ; RAPA: 1.57 ± 0.18).

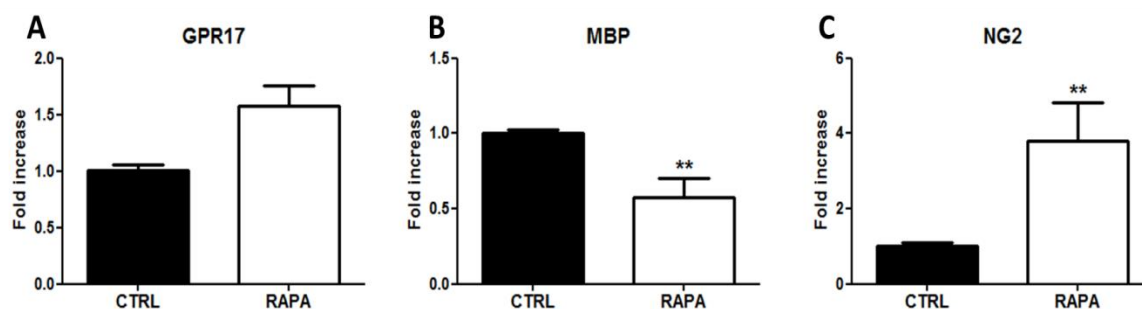


Figure 4.18 – Rapamycin significantly reduces mRNA levels of MBP and increases mRNA levels of NG2.

Primary purified OPCs were cultured in presence of PDGF and of bFGF for two days. Cell differentiation was induced by adding T_3 in the medium. Rapamycin (15 nM) was added at day 1 and day 3. At day 6 (mature OL stage) total RNA was extracted from control (CTRL) and rapamycin (RAPA) treated cells for Real Time PCR analysis. (A-C) GPR17, MBP, and NG2 mRNA levels were quantified using 7000 System Software. Fold changes are expressed as mean \pm S.E. (with CTRL set to 1) of data obtained from three independent experiments (for GPR17, CTRL: 1 ± 0.04 ; RAPA: 1.57 ± 0.18 ; for MBP, CTRL: 1 ± 0.02 ; RAPA: 0.57 ± 0.12 ; for NG2, CTRL: 1 ± 0.07 ; RAPA: 3.78 ± 1.02). **, $p < 0.01$ compared to CTRL, non parametric Mann-Whitney test.

4.4.2 mTOR signaling pathway controls OPC terminal maturation by regulating the transition from pre-oligodendrocytes to immature OL

As already mentioned, the data in literature and those so far shown in this thesis clearly demonstrate that the mTOR pathway is required for OPC terminal differentiation. However, it has to be highlighted that there are conflicting data on the involvement of this kinase in regulating the transition from pre-oligodendrocyte of stage 3 to immature OL of stage 4 (Tyler et al., 2009; 2011; Guardiola-Diaz et al., 2012). To deeply investigate this issue, the consequences of rapamycin treatment on this specific phase of pre-oligodendrocytes' development was assessed by immediately exposing OPCs to rapamycin. Cells were then maintained in culture until day 3 or 4, when the majority of cells reached the immature OL stage. In parallel, other cells treated or untreated with rapamycin were maintained in culture until day 6 (stage 5) in order to confirm the data already shown.

As shown in Figure 4.19 (B), at day 4, we already detected a significant increase in GPR17 protein levels in the cells exposed to the drug compared to the control condition (CTRL: $100 \pm 2.52\%$; RAPA: $138.7 \pm 5.22\%$). Moreover, as expected, the protein levels of the receptor still remained high at stage 5 (CTRL: $100 \pm 4.58\%$; RAPA: $156.7 \pm 11.81\%$), confirming the data already described in the previous paragraph. Also for the early marker NG2 (Figure 4.19 C), a significant change in protein levels was observed starting from stage 4 in cells subjected to rapamycin treatment (CTRL: $100 \pm 1.83\%$; RAPA: $122.5 \pm 3.50\%$). Of note, as for GPR17, the increased protein levels of this early marker persisted until day 6 (CTRL: $100 \pm 0.47\%$; RAPA: $230.2 \pm 32.99\%$), stage at which this modulation became much more visible.

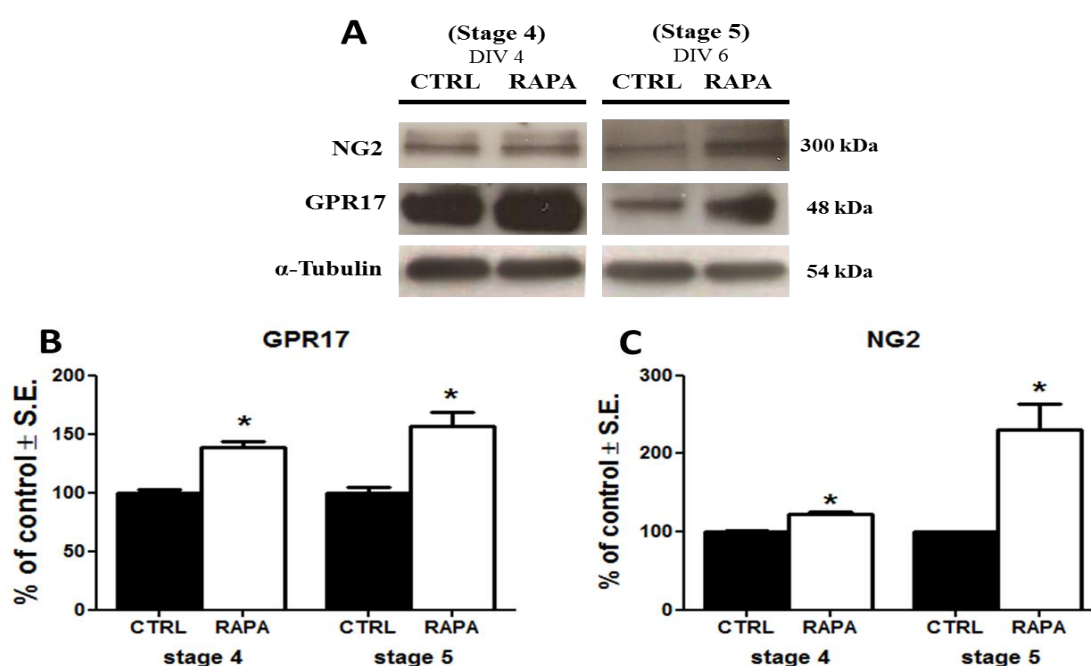


Figure 4.19 - Rapamycin increases NG2 and GPR17 protein levels both in immature OLs and mature OL.

Primary purified OPCs were cultured in presence of PDGF and of bFGF for two days. Cell differentiation was induced by adding T_3 in the medium. rapamycin (15 nM) was added at day 1 and day 3. At day 4 (immature OL) and 6 (mature OL) cells were lysed for WB analysis. (A) Representative immunoblots of GPR17 and NG2 protein levels in control (CTRL) and in Rapamycin (RAPA) treated cells. (B, C) Histograms show the result of densitometric analysis. Scanning densitometry was quantified and normalized to control (set to 100%) on the same WB. α -tubulin expression was analyzed from the same samples as an internal control. Data are expressed as mean \pm S.E. of two independent experiments (for GPR17 stage 4, CTRL: $100 \pm 2.52\%$; RAPA: $138.7 \pm 5.22\%$; for GPR17 stage 5, CTRL: $100 \pm 4.58\%$; RAPA: $156.7 \pm 11.81\%$; for NG2 stage 4, CTRL: $100 \pm 1.83\%$; RAPA: $122.5 \pm 3.50\%$; for NG2 stage 5, CTRL: $100 \pm 0.47\%$; RAPA: $230.2 \pm 32.99\%$). *, $p < 0.05$ compared to CTRL, non parametric Mann-Whitney test.

Furthermore, the reduction of MBP protein levels (CTRL: $100 \pm 3.43\%$; RAPA: $53.20 \pm 9.31\%$) observed at stage 4 in cells exposed to rapamycin (Figure 4.20 B) also confirmed that

OPC differentiation was already compromised at the immature OL stage, suggesting that mTOR pathway regulates the transition from pre-oligodendrocytes to immature OL.

Globally, these data demonstrate that the mTOR signaling pathway is required for the complete maturation of pre-oligodendrocytes. Results also demonstrate that blockade of this transduction cascade determines a persistent over-expression of GPR17 that might prevent pre-oligodendrocytes from reaching terminal maturation.

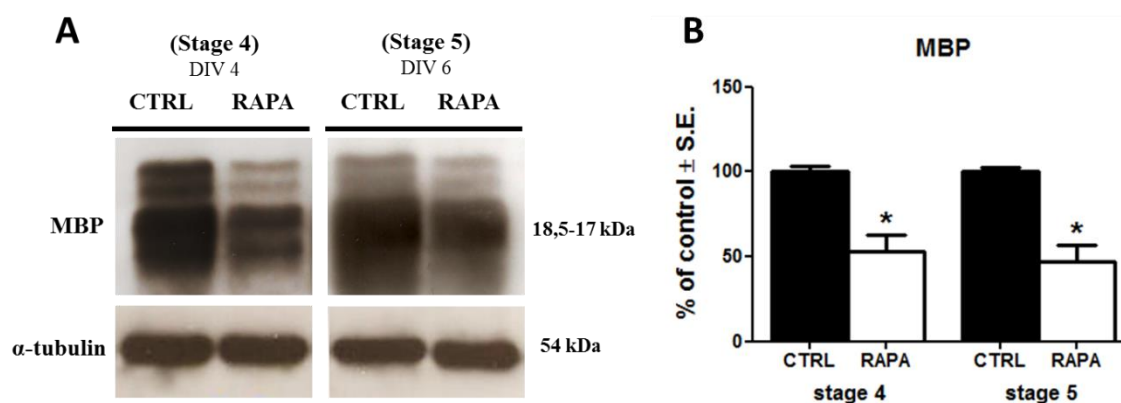


Figure 4.20 - Rapamycin reduces MBP protein levels both in immature OLs and mature OLs.

Primary purified OPCs were cultured in presence of PDGF and of bFGF for two days. Cell differentiation was induced by adding T_3 in the medium. Rapamycin (15 nM) was added at day 1 and day 3. At day 6 (mature OL) cells were lysed for WB analysis. (A) Representative immunoblots of MBP protein levels in control (CTRL) and in rapamycin (RAPA) treated cells. (B) Histograms show the result of densitometric analysis. Scanning densitometry was quantified and normalized to control (set to 100%) on the same WB. α -tubulin expression was analyzed from the same samples as an internal control. Data are expressed as mean \pm S.E. of two independent experiments (For MBP stage 4, CTRL: 100 \pm 3.43%; RAPA: 53.20 \pm 9.31%. For MBP stage 5, CTRL: 100 \pm 2.55%; RAPA: 46.68 \pm 9.72%). *, $p < 0.05$ compared to CTRL, non parametric Mann-Whitney test.

4.4.3 Involvement of GPR17 in OPC cytoskeletal changes during maturation

As described in the “Introduction”, OPCs undergo continuous cytoskeletal alterations prior to acquire the typical mature OL morphology. Indeed, during differentiation, OPCs with a simple bipolar morphology progress through different stages acquiring a more complex branched morphology. Notably, at immature OL stage, cellular branched processes appear to intertwine to form a ring-like structure. This particular cellular shape has been classified by Guardiola-Diaz and co-workers as a more advanced stage of immature OLs (Guardiola-Diaz et al., 2012). Since this morphology anticipates the mature OL stage, it has been proposed that it may contribute to both establishing interactions with axons to start myelination and to be important for microtubule-dependent distribution of myelin proteins, lipids and mRNAs to form myelin multiple layers (Bauer et al., 2009).

Based on this evidence, it is clear that the cytoskeleton plays a key role in OPC differentiation. However, the intracellular mechanisms regulating the cytoskeletal modifications are still poorly understood. Recent data have demonstrated an involvement of the mTOR pathway. Indeed, Guardiola-Diaz and co-workers showed that OPCs treated with rapamycin do not acquire the ring-like structure and that their terminal maturation is blocked at the immature OL stage.

Interestingly, we observed that dot-like structures intensively staining for GPR17 are clearly visible in immature OL rings (Fig 4.21A), suggesting that the receptor may actively take part to the formation of this “ring-like” circular structure around cell body of immature OLs.

Moreover, as shown in Figure 4.21 (C), we found that the number of cells with this ring-like morphology was significantly reduced in rapamycin-treated cultures, confirming that the mTOR pathway has a role in these morphological changes (CTRL: $100 \pm 11.03\%$; RAPA: $44.44 \pm 10.08\%$). Highly relevant, interference with GPR17 by rapamycin also clearly reduced the number of GPR17⁺-cells that displayed this characteristic morphology, besides preventing both ring formation and OPC maturation (Fig. 4.21 CTRL: $100 \pm 14.11\%$; RAPA: $31.09 \pm 7.24\%$). In addition we observed that, in almost all control GPR17⁺ cells, the receptor specifically segregates to these ring-like formations, whereas, in rapamycin treated culture, GPR17⁺-cells displayed a simpler multiprocess, branched morphology, with the receptor distributed to both the somata and the processes. Altogether, these data indicate that the mTOR-GPR17 axis may actively take part to cytoskeleton organization, crucial for a proper cell maturation.

In this respect, to further strengthen the link between cytoskeleton organization and mTOR pathway activation, we focused on Sirt2, a NAD-dependent histone deacetylase (North et al., 2003), that is highly expressed in myelin sheath and in OLs and that takes part to the microtubules turn-over and to the subsequent cytoskeleton organization (Li et al., 2007). It has been demonstrated that its over-expression significantly inhibits the oligodendroglial arborization and tubulin acetylation resulting in the block of OPC differentiation (Li et al., 2007). Moreover, a recent work has showed that Sirt2 is one of the cytoskeleton-related proteins regulated by the mTOR pathway (Tyler et al., 2011). To this purpose, we measured the Sirt2 mRNA levels in OPCs after rapamycin treatment. As shown in Figure 4.21 (E), Sirt2 mRNA levels were remarkably increased after rapamycin exposure (CTRL: 1 ± 0.06 ; RAPA: 2.31 ± 0.39), suggesting that its up-regulation may contribute to impairing the acquisition of

the mTOR/GPR17-mediated “ring-like” structures, resulting in a block of OPC terminal maturation.

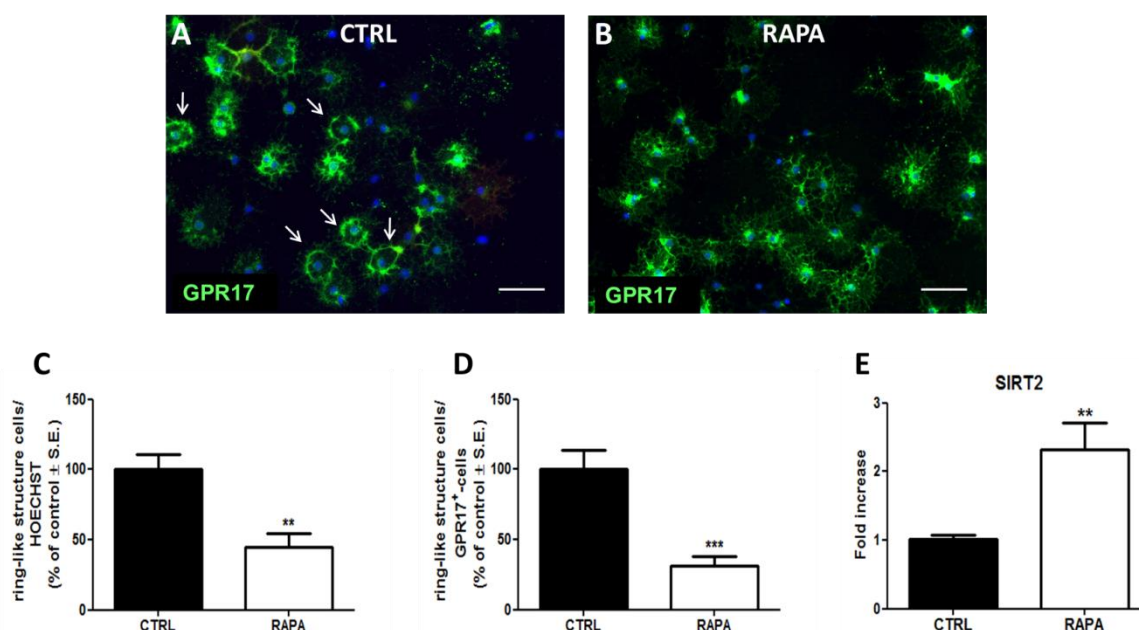


Figure 4.21 – Involvement of GPR17 in OPCs cytoskeletal changes during maturation

Primary purified OPCs were cultured in presence of PDGF and of bFGF for two days. Cell differentiation was induced by adding T_3 in the medium. Rapamycin (15 nM) was added at day 1 and day 3. At day 6 (mature OL) cells were fixed for ICC analysis. (A, B) Representative images of control (CTRL) (A) and rapamycin (RAPA) (B) treated-cells, showing double immunostaining with anti-GPR17 (green) antibody. GPR17 staining is very different in the two experimental conditions: in controls, the majority of the GPR17⁺-cells display the ring-like morphology indicating that they are in a more advance stage of immature OL, while in rapamycin-treated cultures, GPR17⁺-cells display a simpler multiprocess, branched morphology typical earlier stage of immature OL with the receptor distributed to both the somata and the processes. (C) Histograms show quantification of the percentage of the number of cells with the ring-like morphology on the total number of cells (Hoechst 33258 staining, C) and on on the total number of GPR17⁺-cells (green, D) in control and treated cells (with vehicle-treated control cells set to 100%). The number of cells with ring-like structure was counted in 10 optical fields under a 20X magnification. Data are the mean ± S.E. of cell counts from a total of 3 coverslips/condition from three independent experiments (for cells with ring-like morphology, CTRL: 100 ± 11.03%; RAPA: 44.44 ± 10.08%) (for GPR17⁺-cells with ring-like morphology, CTRL: 100 ± 14.11%; RAPA: 31.09 ± 7.24%). **, $p < 0.01$; ***, $p < 0.001$ compared to CTRL, Student T-test. (E) Sirt2 mRNA levels were quantified using 7000 System Software. Fold changes are expressed as mean ± S.E. (with CTRL set to 1) of three independent experiments (For SIRT2, CTRL: 1 ± 0.06; RAPA: 2.31 ± 0.39). **, $p < 0.01$ compared to CTRL, non parametric Mann-Whitney test.

4.4.4 mTOR signaling pathway regulates GPR17 down-regulation modulating GRK2 expression and/or activity

Based on the clear involvement of the mTOR pathway in regulating GPR17 expression, we decided to investigate the underlying mechanisms. In this respect, it has been hypothesized that in OPCs, similarly to other cellular system, mTOR is able to regulate GPCRs expression/trafficking by modulating GRK2 expression or activity that, in turn, might

interfere with the physiological down-regulation of GPCRs, which GRKs are associated to. This regulation has been already demonstrated in rat glioma cells (Cobelens et al., 2007).

To assess whether the mTOR pathway is involved in the regulation of GRK2 also in our cellular model, we cultured OPCs in presence or absence of rapamycin up to day 4 or 6 which correspond to stages 4 and 5 of the differentiation process in order to evaluate the effects of mTOR pathway inhibition both in immature and mature OL. Cells were then lysed for WB analysis. As shown in figure 4.22 (B), GRK2 protein levels were significantly reduced in rapamycin-treated cultures both at stage 4 (CTRL: $100 \pm 0.60\%$; RAPA: $69.62 \pm 7.95\%$) and at stage 5 (CTRL: $100 \pm 1.74\%$; RAPA: $87.54 \pm 1.60\%$) compared to control cultures.

These data suggest, albeit indirectly, that the reduced expression of GRK2 that is observed after rapamycin treatment may be the cause of the persistent expression of GPR17 that occurs under these experimental conditions.

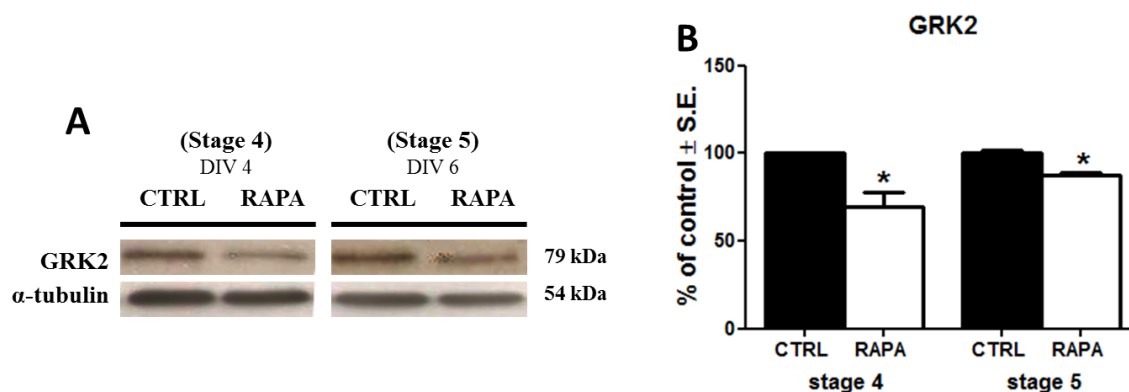


Figure 4.22 - Rapamycin reduces GRK2 protein levels both in immature OLs and mature OL.

Primary purified OPCs were cultured in presence of PDGF and of bFGF for two days. Cell differentiation was induced by adding T_3 in the medium. rapamycin (15 nM) was added at day 1 and day 3. At day 6 (mature OL) cells were lysed for WB analysis. (A) Representative immunoblots of GRK2 protein levels in control (CTRL) and in Rapamycin (RAPA) treated cells. (B) Histograms show the result of densitometric analysis. Scanning densitometry was quantified and normalized to control (set to 100%) on the same WB. α -tubulin expression was analyzed from the same samples as an internal control. Data are expressed as mean \pm S.E. of two independent experiments (for GRK2 stage 4, CTRL: $100 \pm 0.60\%$; RAPA: $69.62 \pm 7.95\%$; for GRK2 stage 5, CTRL: $100 \pm 1.74\%$; RAPA: $87.54 \pm 1.60\%$). *, $p < 0.05$ compared to CTRL, non parametric Mann-Whitney test.

To test this hypothesis, we examined the involvement of the mTOR pathway in LTD₄-mediated GPR17 desensitization in OPCs, by analyzing the functional responsiveness of the receptor to LTD₄ in the absence or presence of rapamycin, using the cAMP assay. Cells pre-treated with rapamycin displayed an impaired LTD₄-mediated GPR17 desensitization (Figure. 4.23 black bars), suggesting that the mTOR pathway takes part to LTD₄-mediated GPR17 homologous desensitization likely selectively regulating GRK2 activity and/or expression.

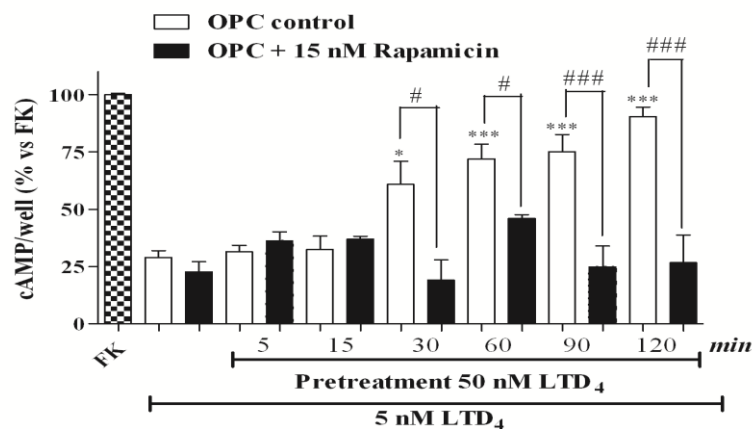


Figure 4.23 - mTOR inhibition prevents LTD₄-mediated GPR17 desensitization in OPCs.

OPCs, isolated and differentiated at pre-oligodendrocytes (stage 3), were treated with 50 nM LTD₄ for different times (5-120 min), in the absence (white bars) or in the presence (black bars) of rapamycin (15 nM). After extensive washing, cells were treated for 15 min with 10 μ M FK, in the absence or in the presence of 5 nM LTD₄. Intracellular cAMP levels were evaluated as reported in the method section. Data are expressed as the percentage of FK-stimulated cAMP levels, set to 100%, and represent the means \pm E.S of six independent experiments. *, $p < 0.05$; ***, $p < 0.001$ vs 5 nM LTD₄; #, $p < 0.05$; ###, $p < 0.001$ vs rapamycin untreated cells, one-way ANOVA, followed by Bonferroni's multiple comparison test.

Chapter 5

DISCUSSION

Demyelination is a pathological process that is characterized by the loss of myelin sheath, the multilayered membrane that wraps and insulates axons, ensuring the rapid propagation of electrical signals. This highly specialized structure is produced and maintained by a specific glial cell population: the oligodendrocytes (OLs). As described in the “*Introduction*”, these cells originate from precursors, the NG2⁺ oligodendrocyte precursor cells (OPCs), that during postnatal development differentiate into mature myelinating oligodendrocytes (OLs). However, it has to be highlighted that, as already mentioned in paragraph 1.1.5, a significant percentage of these NG2⁺-precursors is still present in the adult CNS (both in white and grey matter) and constitutes the primary endogenous source of new OLs in case of demyelination. In fact, it is known that, after a demyelinating insult, subsequent to infectious, metabolic or immune perturbation, these precursors undergo a switch from an essentially quiescent state to a regenerative phenotype. This process is called remyelination and allows the generation of new OLs, that are able to produce a new myelin sheath.

Normally, the remyelination process occurs spontaneously; however, in some conditions, such as in multiple sclerosis (MS), this process fails or is inadequate (Siffrin et al., 2010; Franklin et al., 2008; Fancy et al., 2011; Aktas et al., 2010; Stys et al., 2012). The causes of this failed remyelination are not so well understood. It is believed that OPC differentiation block and impaired repair may be due to local inflammatory inhibitory signals, intrinsic alterations of cell differentiation program, or both (Nishiyama et al., 2009). Whatever the mechanism, it is believed that implementation of an effective remyelination strategy represents an important goal in a therapeutic perspective for MS and other demyelinating diseases.

In this respect, several approaches could be used to promote endogenous remyelination, like (a) enhancing OPC recruitment into demyelinated areas, (b) promoting myelination via a specific action on key molecules involved in OPC differentiation, and, (c) removing the factors that inhibit remyelination. Regarding the second approach, in the last few years, the discovery, in our laboratory, of a new molecular target to be exploited to promote OPC differentiation has attracted our attention.

Our laboratory has, indeed, deorphanized and characterized a new P2Y-like receptor, GPR17 (Ciana et al., 2006; Pugliese et al., 2009; Temporini et al., 2009), demonstrating its active role in orchestrating OL differentiation and maturation (Lecca et al., 2008; Fumagalli et al., 2011; Boda et al., 2011). Specifically, our recent data have indicated that, *in vivo*, GPR17 specifically decorates a subset of small cells with fine radiating processes dispersed in both grey and white matter, that may represent a source of pre-oligodendrocytes able to turn into

myelinating cells when necessary (Lecca et al., 2008). Moreover, our *in vitro* data demonstrated that early GPR17 activation by UDP-glucose promotes OPC maturation toward more mature myelinating cells and that its early inhibition by either receptor antagonists (e.g, cangrelor or montelukast) (Ciana et al., 2006; Lecca et al., 2008), or specific silencing RNAs markedly impairs OPCs differentiation program (Fumagalli et al., 2011).

In order to extend the pharmacological characterization of GPR17, in the first part of this thesis we used rat primary OPC cultures to analyze the consequences of the pharmacological treatment with other known GPR17 endogenous ligands, that have already been shown to activate the receptor, by reducing intracellular cAMP levels (Fumagalli et al., 2011). To this purpose, we assayed the pro-differentiating effects of the uracil nucleotide, UDP, and of two cysLTs, LTD₄ and LTE₄. As depicted in figure 4.2, all the tested agonists markedly increased the number of mature myelinating MBP⁺-cells and also showed an increased protein expression of the mature marker CNPase. These results are consistent with our previous observations and confirm that the activation of this receptor induced by these two classes of endogenous mediators accelerates OPC differentiation.

Moreover, since OPC differentiation and myelination are intimately related step during the development of the CNS, we decided to examine whether agonist-induced GPR17 activation also affects the myelination process. In this respect, we took advantage of the *in vitro* model OPC/DRG co-cultures and, after an initial characterization of this system, we used it to test the capability of either UDP-glucose or UDP to promote myelination. We find that the number of MBP positive myelin segments is significantly increased in the presence of both GPR17 endogenous ligands, suggesting that the activation of this receptor enhances the myelination process.

These results are at odds with a recent *in vitro* study, that suggests that both the presence and activation of GPR17 inhibit the OL maturation program (Hennen et al., 2013). In this paper, in a series of experiments primary OLs isolated from heterozygous GPR17^{+/-} mice were treated with a new postulated ligand (the small molecule MDL29,951), and receptor activation evaluated through a pathway-unbiased screen based on dynamic redistribution of cellular mass. Results led to the opposing view that GPR17 inhibits, instead of stimulating, OL maturation (Hennen et al., 2013). Such a main difference is likely related to the way OPCs were manipulated and the time at which the pharmacological treatment with the GPR17 agonist was performed. A different shaking-off procedure to obtain early progenitors from brain cortex was used in the two studies; moreover, while we plated OPCs in defined media immediately after the shaking, Hennen and coworkers plated only pre/immature OLs purified

by immunopanning from mixed glial cell populations that were already positive for the more advanced marker O4⁺. As a consequence, also the timing at which cells were exposed to the GPR17 agonist is different. In fact, while we treated cells at an early stage at which GPR17 starts to be expressed at the membrane, the group of Hennen treated cells at a more advanced stage, when GPR17 is expressed at high levels and at which the intracellular mechanisms responsible for GPR17 down-regulation are already activated. Thus, the use of already committed cells suggests that both their intrinsic maturation programs and their abilities to respond to GPR17 agonists are different from those of our precursors. It could indeed be hypothesized that, at the O4⁺ stage, agonists would induce a complete and precocious down-regulation of the receptor, which could, in turn, be at the basis of the observed inhibition of myelination. Alternatively, as nevertheless suggested by authors themselves, MDL29,951 can also have other GPR17-independent effects. Ongoing experiments have been planned to investigate this issue in more detail.

Although GPR17 exact pharmacological profile is still under debate (Ciana et al., 2006; Benned-Jensen and Rosenkilde, 2010; Maekawa et al., 2009; Qi et al., 2013; Hennen et al., 2013), altogether these data suggest that therapeutic promise exists for drug-targeting of GPR17 in demyelinating diseases. In this respect, our data confirm the pivotal role of GPR17 in the initial phases of the differentiation process and further support our hypothesis according to which the functional presence of the receptor at early differentiation stages is important to keep cells at an immature state, which is necessary to prepare them for myelination.

Moreover, our previous published data showing that GPR17 is not present in morphologically mature MBP positive cells (Fumagalli et al., 2011) arise the hypothesis that loss of GPR17 at a relatively advanced differentiation stage is a prerequisite to allow cells to proceed to terminal maturation. The results presented in this thesis validate this hypothesis. In fact, GPR17 over-expression at a stage during which normally OLs down-regulate it, determined a block in the differentiation process at the pre-oligodendrocyte stage. In this respect, previous published data showed that myelinogenesis is defective in the CNS of CNP-GPR17 transgenic mice, a mouse line in which the receptor is over-expressed in CNP-positive OLs (Chen et al., 2009). Based on these results, the same authors concluded that GPR17 is a negative regulator of OL myelination, but this simplistic conclusion has been then re-interpreted at the light of more recent data that significantly contributed to defining the specific differentiation stages at which OPCs express GPR17 (Fumagalli et al., 2011). In this respect, the first results presented in this thesis clearly corroborate the existence of a timely

restricted expression of GPR17 and that interference with this pattern of expression completely alters the differentiation program of these cells.

This conclusion is particularly important at the light of exciting recent demonstration that GPR17 has been found up-regulated in a lysolecithin-induced demyelination animal model (Boda et al., 2011) and in MS patients (Chen et al., 2009), suggesting that impairment of its physiological down-regulation can be associated to the block of differentiation observed in these pathological conditions.

Certainly, OPC differentiation in general and the transient nature of GPR17 expression in particular are the result of a fine-tuned program based on the integration of both extrinsic and intrinsic molecular signals. The actual mechanisms that take part in this regulation are only partially understood. Our current hypothesis to explain GPR17 transient expression in OPCs is that, by binding to GPR17, its endogenous ligands can first induce early precursor cells to undergo differentiation, as shown above, and, then, switch the receptor off by agonist-induced desensitization and its physical removal from the membrane, thus allowing OPCs to proceed to myelination. A similar process has been associated with specification of other cell lineages, where the down-regulation of membrane receptors for trophic or differentiation factors has been proposed to be necessary to allow cells to proceed toward terminal differentiation (Walrafen et al., 2005). For example, during erythrocyte maturation, stimulation of erythropoietin receptors by erythropoietin is necessary to induce erythroid precursor cells to proceed to the erythroblast stage; however, at this stage, erythropoietin receptors have to be removed from the cell membrane to allow precursors to become functional erythrocytes (Flint-Ashtamker et al., 2002). In a similar way, the results described in this thesis indicate that GPR17 undergoes phosphorylation and time-dependent desensitization upon a challenge with purinergic and cysLT agonists, supporting what was already demonstrated in a recombinant system (Daniele et al., 2011) and in cellular lines natively-expressing GPR17 (Fratangeli et al., 2013).

Furthermore, for the first time, our results point to the involvement of GRK2 in this process. This is a G-protein coupled receptor kinase that is widely expressed within the brain whose altered expression levels in peripheral blood circulating cells have been correlated to MS (Vroon et al., 2005). As other GRKs, GRK2 is able to phosphorylate the intracellular domain of active GPCRs mediating its desensitization. Here, we showed that this kinase, whose expression (both protein and transcript) paralleled that of the receptor during the *in vitro* differentiation process reaching the highest levels at the pre-oligodendrocyte stage, is primarily involved in the transition from pre-oligodendrocytes to immature OL, since its

knock-down by siRNA prevents GPR17 down-regulation and concomitantly blocks OPC terminal maturation. Moreover, we demonstrated that recruitment of GRK2 by GPR17 is strictly related to receptor activation induced by LTD₄; in fact, the pharmacological inhibition of this kinase, by blocking GPR17 down-regulation, prevents LTD₄-induced OPC differentiation.

It is important to highlight that GRKs activity (and the subsequent GPCRs trafficking within the cell) can be also regulated in an agonist-independent manner (Pei et al., 1992; Rime and Oprian, 1995). To shed light on this possibility, we decided to investigate other intracellular pathways that, through modulation of the expression or activity of regulatory molecules, may be involved in GRK dependent processes and take part to OPC differentiation.

In this respect, we focused on the mTOR pathway since its involvement in the regulation of OPC terminal maturation has been already partially suggested by previous studies showing the role of the serine-threonine kinase Akt in supporting myelination (Zeger et al., 2007; Flores et al., 2008). However, only recently, the group of Tyler (2009; 2011) and the group of Guardiola-Diaz (2012) have independently demonstrated the key role of the mTOR pathway in the regulation of OPC differentiation process. Highly relevant for our studies was the finding that GPR17 is up-regulated after mTOR inhibition, as demonstrated by screening through a iTRAQ mass spectrometry-based approach (Tyler et al., 2011).

The results described in this thesis are consistent with the data present in literature. In fact, cells exposed to the immunosuppressant drug, rapamycin, and analyzed at the mature OPC stage 5, displayed low protein and mRNA levels of the mature markers CNPase and MBP, in parallel to a higher content of the proteoglycan NG2 (both transcript and protein). Of note, these cells also had a higher content of GPR17 (only protein), suggesting that the mTOR signaling pathway controls OPC terminal maturation by regulating its functional expression.

It has been reported that mTOR regulates OPC differentiation through two distinct downstream signaling systems. Indeed, as already described in the “*Introduction*”, thanks to its association with distinct accessory proteins, rictor and raptor, mTOR can give rise to two different complexes: mTORC1, that has been demonstrated to regulate OPC differentiation mainly by a post-translational mechanisms and is able to interfere with protein translation to regulate the synthesis of myelin proteins, of enzymes involved in the synthesis of lipids and cholesterol and of cytoskeletal proteins; mTORC2 that, by interfering with the processes of mRNA transcription, controls the expression of several transcription factors (e.g. ID2, ID4 and TCF4) and myelin genes (such as MBP and PLP) (Tyler et al., 2009; 2011).

Since in literature there are conflicting data regarding the involvement of mTOR in the regulation of the transition from pre-oligodendrocyte of stage 3 and immature OL of stage 4 (Tyler et al., 2009; 2011; Guardiola-Diaz et al., 2012), specific experiments were designed to investigate this effect. To this purpose, immediately after OPC preparation, cells were exposed to rapamycin and maintained *in vitro* until the majority of cells reached the immature OL stage (stage 4). As shown in Figure 4.20, at stage 4, GPR17 and NG2 protein levels were already significantly increased in cultures exposed to the drug compared to control cultures. In parallel, a reduction of MBP protein content was observed (Figure 4.21), indicating that terminal differentiation is already altered at this stage.

The fact that mTOR pathway inhibition blocks OPC differentiation at the pre-OL stage is also confirmed by the appearance of alterations in cytoskeleton reorganization that commonly occurs during this process. Indeed, increased process extension and branching complexity are morphological features typical of cells in transition from OPC/pre-OL to immature OL and initiate the program to become mature OLs. Our data confirm that the mTOR pathway is related to the cytoskeleton maturation of OLs. As already shown by Guardiola-Diaz and co-workers, also in our cultures, rapamycin exposure did not arrest the progression of early progenitors but inhibited their acquisition of the characteristic ring-like structure (see Figure 4.19), an event that occurs during the normal progression of the immature OL. This particular morphology is considered a more advanced stage of immature OL that immediately precedes the mature OL stage. Interestingly, we also observed that dot-like structures intensively staining for GPR17 are clearly visible in immature OL rings (Fig 4.21A), suggesting that the receptor may actively take part to the formation of this “ring-like” circular structure around the cell body of immature OLs. The biological role of this ring-like structure is not yet known, but several hypothesis have been put forward: (i) it may contribute to process extension and branching complexity that are essential to allow cells establishing interactions with neuronal axons to start myelination; (ii) it may contribute to the cytoskeleton-dependent transport of myelin proteins, lipids and mRNA. Concerning this second point, it is known that microtubules and microfilaments, which compose the OL cytoskeleton, act as intracellular transport rails along which myelin-specific proteins and lipids are transported, and that this transport is essential to form the multiple layers of myelin sheath (Bauer et al., 2009). In this respect, our preliminary data show that GPR17 may play an active role in the cytoskeletal changes involved in the local synthesis of myelin proteins. Based on this observation, we speculate that mTOR pathway inhibition, by preventing GPR17 down-regulation may, in turn,

be responsible for a dysfunction in the formation of this structure, thus resulting in impairment of terminal maturation.

The involvement of mTOR pathway in the cytoskeleton reorganization was already suggested by Tyler and co-workers in 2011. Through a proteomic analysis, this group identified several cytoskeleton proteins and factors whose expression was altered upon rapamycin treatment. Specifically, in parallel to the down-regulation of myelin protein, cholesterol and lipid synthesis enzymes, they reported alterations in Sirt2, β IV-tubulin, Gap-43 and (BASP)-1 (Tyler et al., 2011). Concerning Sirt2, also our results suggest a link between the mTOR signaling and this cytoskeletal enzyme. However, while Tyler reported a reduction of the Sirt2 expression, we observed increased mRNA levels of Sirt2 after rapamycin treatment. This discrepancy may be related to the fact that we measured mRNA levels while the group of Tyler directly measured the protein; however, it may also be related to the timing at which the analysis has been performed. In fact, it has been reported that Sirt2 expression is finely regulated during OPC differentiation. Specifically, this enzyme is found at high levels in myelin sheaths and mature OLs. Moreover, its expression correlates with the appearance of significant morphological changes like highly ramified processes and concomitantly increased CNPase expression, suggesting that Sirt2 clearly marks cells entering into the pre-myelination stage (Li et al., 2007). Our results are consistent with these findings and are also supported by previous data showing that Sirt2 over-expression significantly inhibits oligodendroglial arborization and tubulin acetylation, thus slowing down cell maturation (Li et al., 2007). Furthermore, in a similar way to Sirt2, also GPR17 expression at late maturation stages mainly localizes to oligodendrocytic processes and not to the soma; thus, our hypothesis is that a direct link between Sirt2 and GPR17 might exist. In this respect, future experiments will be designed to better clarify this relationship.

Globally, our data confirm the fact that GPR17 down-regulation, either induced by agonist-binding and/or by the activation of mTOR pathway, is required for OPC terminal maturation. We do not rule out the possibility that both these mechanisms take part together to this process. Indeed, the capability of the mTOR pathway to regulate GRKs function has already been demonstrated in other cellular systems. To assess whether this regulation was also present in OPCs, we measured GRK2 proteins levels after mTOR pathway inhibition. WB analysis showed that GRK2 protein levels are significantly lower in rapamycin-treated cells compared to control cultures, both in immature OL of stage 4 and in mature OL of stage 5. Moreover, the fact that the two mechanisms proposed to be responsible for GPR17 down-regulation may be interconnected to each other is confirmed by the fact that, in the presence

of rapamycin (i.e., after mTOR inhibition), LTD₄ is not more able to induce GPR17 homologous desensitization, indicating that, as observed in presence of a specific GRK2 pharmacological inhibitor, blockade of GRK2 function prevents the removal of GPR17 from the cellular membrane. These data indicate that the reduced expression of GRK2 that is observed after rapamycin treatment may be the cause of the persistent expression of GPR17 that occurs under these experimental conditions. Also in this case, experiments are currently ongoing to assess whether the effect of mTOR on GPR17 expression is the consequence of a direct modulation of GRK2 expression/activity or whether this regulation requires the involvement of other molecules. In this respect, our current hypothesis is that Mdm2, an E3 ubiquitin ligase, which participates in the process of GRK2 ubiquitination by modulating its degradation and cellular levels (Salcedo et al., 2006), may be involved in GPR17 physiological down-regulation. Preliminary data showed that the formation of GRK2/Mdm2 complexes is strongly enhanced by rapamycin treatment, suggesting that the mTOR pathway controls GPR17 down-regulation by directly modulating Mdm2 expression/trafficking. Experiments are currently in progress to assess to what extent this mechanism is necessary for OPC maturation.

The role of the GRK2/Mdm2 and of the mTOR pathway in GPR17 physiological regulation and in OPC differentiation opens a new scenario for possible pharmacological treatments for demyelinating diseases. In this regard, it will be important to evaluate whether, in parallel to the GPR17 up-regulation observed in ischemia, traumatic brain or spinal cord injury and lysolecithin-induced demyelination (Lecca et al., 2008; Ceruti et al., 2009; Boda et al., 2011), there are alterations of the mTOR/GRK2/Mdm2 pathway.

In conclusion, the results described in this thesis contribute to understanding how GPR17 expression is regulated during OPC maturation and represent an important starting point for the development of new pharmacological or biotechnological approaches to implement the endogenous regenerative potential of quiescent NG2⁺-precursors within the adult brain.

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