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Endocytic traffic of the G-protein coupled receptor 17, a dualistic receptor implicated in oligodendrocyte differentiation

Settore disciplinare BIO/14

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ABSTRACT

Oligodendrocytes are glial cells that produce specialized membranes (myelin sheaths) that are wrapped around the neuronal axons of the central nervous system (CNS) which function is to ensure an efficient and fast conduction of electrical impulses along axons and the maintenance of axonal integrity. Several pathologies of the CNS are correlated to the loss of myelin, an event that can be a direct cause of disease like in multiple sclerosis or, in other situation, it can be a consequence of a more general damage like during ischemia. Within the CNS there are OPCs, that after appropriate stimulation, are able to migrate in the damaged area and completely mature producing new myelin sheaths. Among the factors necessary for the recruitment of precursors at the lesion, GPR17 seems to play a role as a sensor of the damage and regulator of OPC differentiation. GPR17 is a dualistic G-protein-coupled receptor that can be activated by both UDP-glucose and CysLTs, signaling molecules massively released in the brain upon different types of injury (Lecca et al., 2008; Fumagalli et al., 2011). Upon agonist binding the receptor expression, almost absent in OPCs, gradually increases in more mature precursors, it reaches a plateau in immature/preoligodendrocytes and then gradually decreases along with terminal differentiation. On the other hand inhibition of GPR17 expression causes impairment in oligodendrocyte differentiation and myelination in *in vivo* (Chen et al., 2009) and *in vitro* systems (Fumagalli et al., 2011). Altogether these data indicate that GPR17, acting in a specific time window, is implicated in OPCs maturation.

We characterized the expression of GPR17 in an immortalized oligodendrocyte precursor cell line (Oli-neu) (Jung et al., 1995): biochemical and immunofluorescence analyses of Oli-neu differentiation in neuronal conditioning medium, showed that GPR17 is almost absent in undifferentiated Oli-neu cells and reaches a peak after 72h differentiation, while myelin proteins begin to appear after 96h, when GPR17 levels decrease. When GPR17 was knocked down by means of RNA interference, fewer Oli-neu cells were capable of differentiating. These data demonstrate that Oli-neu cells are an helpful model to a further biochemical and biological characterization of GPR17.

Little information is available on the role of the agonists in regulating receptor availability at the cell surface and on GPR17 trafficking during cell-differentiation. As reported for many GPCRs, after ligand binding GPR17 may undergo to endocytosis and subsequent sorting into lysosomes for degradation and/or to recycling endosomes for delivery to the plasma membrane. In order to shed light on the mechanisms of GPR17

signaling and agonist induced activation, we investigate the endocytic trafficking of the native receptor in differentiating Oli-neu. To trace internalized GPR17, an antibody against an extracellular epitope of the receptor was generated to be used in internalization assays performed in Oli-neu cells. As shown by immunocytochemical and biotinylation assays, both UDP-glucose and LTD_4 increase the clathrin-dependent internalization of GPR17, though with different efficiency. Preliminary results seem to confirm that GPR17 is also differently internalized in OPC primary cultures. Confocal images demonstrated that, at early times after internalization, GPR17 is colocalized with the endosome markers transferrin receptor and Rab5 whereas at later times it shows colocalization with the lysosomal marker Lamp1. The intracellular accumulation of GPR17 in Lamp1 positive organelles increased after incubation with chloroquine, a lysosomotropic agent known to affect lysosomal degradation. On the other hand, the receptor colocalized to a very minor degree with Rab11, a marker of the long loop-recycling pathway.

In conclusion these results provide the first characterization of the endocytic fate of the native GPR17 in response to the two different classes of ligand activation and demonstrate that the receptor is mainly down regulated after stimulation in differentiated oligodendroglial cells.

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Abbreviations

ADP	*adenosine di-phosphate*
AMPA	*2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid*
AP-2	*adaptor protein AP-2*
ATP	*adenosine tri-phosphate*
BDNF	*brain-derived neurotrophic factor*
bFGF	*basic fibroblast growth factor*
BMP	*bone morphogenetic protein*
cAMP	*cyclic adenosine mono-phosphate*
CM	*conditioned medium*
Cng	*cangrelor*
CNS	*central nervous system*
CNP	*2',3'-cyclic nucleotide 3'-phoshodiesterase*
CP	*chlorpromazine*
$CysLTC_4$	*cysteinil-leukotriene* C_4
$CysLTD_4$	*cysteinil-leukotriene* D_4
$CysLTE_4$	*cysteinil-leukotriene* E_4
Db-cAMP	*dibutyryl-cAMP*
DTT	*dithiothreitol*
ECL	*enhanced chemiluminescence*
EGF-R	*endothelial growth factor receptor*
EndoF	endoglycosidase F
EndoH	endoglycosidase H
ER	*endoplasmic reticulum*
FGF	*fibroblast growth factor*
FGFR	*fibroblast growth factor receptor*
GABA	*Gamma-Amino Butyric acid*
GalC	*galactocerebroside*
GAP	*GTPase-activating protein*
GAPDH	*Glyceraldehyde 3-phosphate dehydrogenase*
GDP	*guanosine di-phosphate*
GFAP	*glial fibrillary acidic protein*
GFP	*green fluorescent protein*
GPCR	*G protein coupled receptor*
GPR17	*G protein receptor 17*
GRK	*G protein coupled Receptor Kinase*
GTP	*guanosine tri-phosphate*
Hsp70	*heat shock protein 70*
Iba1	*ionized calcium binding adaptor molecule 1*
ID2	*DNA-binding protein inhibitor 2*
ID4	*DNA-binding protein inhibitor 4*
IFNγ	Interferon γ
IGF	*insulin-like growth factor*
Ig-rb	*IgG rabbit*
Il-1β	*interleukin -1β*
Il-2	*interleukin -2*
Il-6	*interleukin -6*
LIF	*leukemia inhibitory factor*
LTC_4	*cysteinil-leukotriene* C_4
LTD_4	*cysteinil-leukotriene* D_4

LTE_4	*cysteinil-leukotriene E_4*
MAG	*myelin associated glycoprotein*
MBP	*myelin basic protein*
MCAo	*middle cerebral artery occlusion*
MOG	*myelin oligodendrocyte glycoprotein*
MTL	*montelukast*
NB	*neuronal basal medium*
NGF	*nerve growth factor*
NMDA	*N-methyl D-aspartate*
OPCs	*oligodendrocyte precursor cells*
PBS	*phosphate buffered saline*
PDGF-α	*platelet derived growth factor α*
PDGF-R	*platelet derived growth factor receptor*
PIP2	*phosphatidylinositol 4,5-bisphosphate*
PLP	*proteolipid protein*
pMN	*motor neuron progenitor*
PNGase	*peptide N-glycosidase*
PTX	*pertussis toxin*
PVDF	*polyvinylidene fluoride*
RA	*retinoic acid*
SDS	*sodium dodecyl sulfate*
SDS-PAGE	*sodium dodecyl sulfate*
Shh	*sonic hedgehog*
TBS	*tris buffered saline*
TEMED	*N,N,N,N-tetrametiletilendiamina*
TfR	*transferrin receptor*
TM	*transmembrane domain*
TNFα	*tumor Necrosis Factor α*
UDP	*uracil di-phosphate*
UDP-Gal	*uridine di-phosphogalactose*
UDP-Glc	*uridine di-phosphoglucose*
UTP	*uracil tri-phosphate*

1. INTRODUCTION

1.1 OLIGODENDROCYTES

Glial cells constitute the large majority of cells in the nervous system. Only recently their active role during the development and the physiology of the brain has been emphasized especially with respect to their dysfunction causing pathological conditions of the nervous system. Virchow in 1846 was the first to describe that there were cells other than neurons and called them “neuroglia”. Using techniques of metallic impregnation developed by Ramon y Cajal and Rio Hortega it was possible to characterize the different glial cell types.

Oligodendrocytes are post-mitotic cells which main function is to form the myelin sheath, a multilamellar, spirally wrapping extension of their plasma membrane, to envelope axons in the central nervous system. However other roles have been described: distribution of sodium channels along axons, participation in development and maintenance of axons but also inhibition of axonal growth and regeneration. oligodendrocyte can be distinguished from astrocytes for a number of features such as their smaller size, the density of the cytoplasm and dense chromatin in the nucleus and the presence of a large number of processes that contact and envelope axons. On a single axon the adjacent segments of myelin belong to different oligodendrocytes while the thickness and size of myelin sheets depend on the area of the CNS and species. During their maturation from precursors cells to myelin-forming cells, they pass through different developmental stages identified by morphological changes and expression of specific markers (Baumann and Pham-Dinh, 2001; Barres, 2008).

1.1.1 - Oligodendrocytes development and differentiation

Oligodendrocyte progenitor cells (OPCs) of the spinal cord are generated in the ventral neuroepithelium of the neural tube in early embryonic life and in particular from the motor neuron progenitor (pMN) domain that also produce motor neurons. OPCs derived from ventral pMN proliferate and migrate laterally and dorsally to occupy all areas of the spinal cord. Whereas at fetal stages OPCs are also produced from dorsal domain (Fig. 1). In the forebrain, OPCs originate from the ventral epithelium cells (in the region of the medial ganglionic eminence) and then migrate through the ventral telencephalon to occupy all ares including the cerebral cortex.

Different factors are implicated in the initial commitment of multipotent cells to oligodendrocyte lineage like BMP (bone morphogenetic protein) and Shh (sonic

hedgehog). BMP together with Wnt antagonize oligodendrocyte development, while Shh induces, in a concentration dependent manner, the activation of Olig2 that is a transcriptional factor required for a number of genes necessary for oligodendrocyte differentiation.

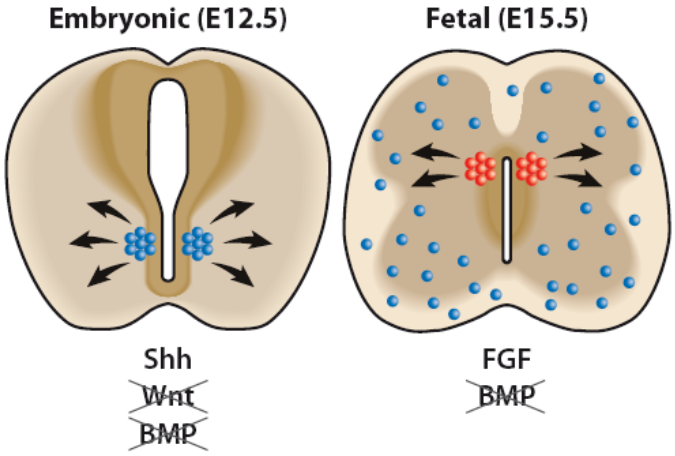


Figure 1. Oligodendrocytes production during development. Embryonic OPCs originate from the ventral pMN domain (blue cells) under the control of Shh. At later stages there is a dorsal production of OPCs (red cells) that do not require Shh but arise in a FGF-dependent manner. (Modified from Fancy et al., 2011).

After progenitors arise in these restricted areas, these cells migrate widely throughout the CNS to reach their target axon. Migration of OPCs is driven by chemo-attractant molecules like PDGF and semaphorin 3F or chemo-repellents signals like ephrins, CXCL1 and tenascin C (Miron et al., 2011). Once OPCs reach their axon, they receive specific signals that induce the maturation process consisting of successive stages, each stage is defined by specific markers and by a morphological change. OPCs are generated as bipolar cells and then develop more branches and finally form myelin sheaths tightly wound concentrically around the axon. OPCs can be identified initially through the expression of platelet-derived growth factor alpha (PDGF-αR), the receptor for the major mitogen PDFGα, the proteoglican NG2 and an isoform of the proteolipid protein (PLP) called DM-20. These cells are also GD3+ and A2B5+: the ganglioside GD3, whose expression disappears as the cell matures, is nevertheless in other glial cell types *in vivo* so it should be used with particular caution for the identification of cell types; the monoclonal antibody A2B5 recognizes several gangliosides that remain as yet uncharacterized, however it is downregulated as the cell differentiates into the mature oligodendrocyte.

The ability of migration and proliferation of OPCs is conserved also in the following stage of pre-oligodendrocytes. At this moment they are less motile and begin to express O4 while the expression of PDFGα-R is repressed. Pre-oligodendrocytes become immature oligogendrocytes characterized by the appearance of the marker GalC (galactosil ceramide) and the loss of expression of GD3 and A2B5 antigens on the cell surface. CNP (2',3'-cyclic nucleotide-3'- phosphohydrolase) is the earliest known myelin-specific protein to be synthesized by developing oligodendrocytes. In the mature stage, myelin protein like MAG (myelin associated protein) and MBP (myelin basic protein) are highly expressed even though mature oligodendrocytes are not still completely able to form myelin sheaths because they need to receive specific stimuli from axons. The latest myelin protein that appears in myelinating oligodendrocytes is MOG (myelin oligodendrocyte protein). All the markers typically expressed in each stage are represented in Fig.2.

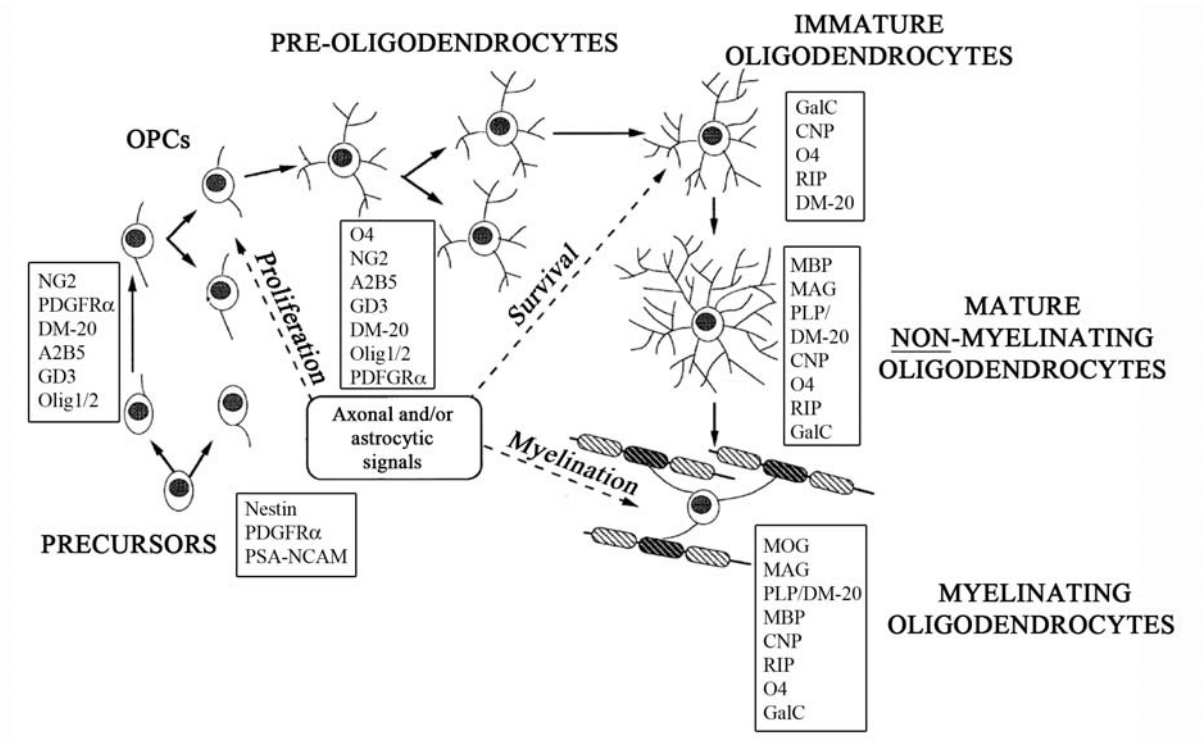


Figure 2. Different stages of oligodendrocyte maturation from precursors to myelinating cells. In box are listed the specific markers for each step. (Modified from Baumann and Pham-Dinh, 2001).

1.1.2 - Factors necessary for oligodendrocyte maturation and survival

Oligodendrocyte number, maturation and survival is determined by a balance between proliferating progenitors and programmed cell death (Barres et al., 1994). Oligodendrocytes differentiate in early postnatal day and they can be influenced by signals originating from the other surrounding cells of the CNS, like neurons and astrocytes. A lot of factors have been identified to promote oligodendrocyte maturation, survival and proliferation and it is thought that it is a combination of these that have effect on oligodendrocyte, rather than a single factor. Below a list of extrinsic factors that influence these steps is reported.

1. Growth factors

- *PDGF (platelet-derived growth factor)*

PDGF is secreted by neurons and astrocytes as active ligand. PDGFα-receptor transcripts are detected at very early stages of the developmental maturation of oligodendrocytes (Rowitch, 2004) and this receptor disappears at the O4+ stage due to its intracellular signaling blockade (Hart et al., 1989). It is an important survival factor for OPCs (Grinspan and Franceschini, 1995), as demonstrated by the impared oligodendrocytes development in the PDGF-αR- deficient mice (Fruttiger et al., 1999) in which there is a strong reduction in the number of oligodendrocytes and a dysmyelinating phenotype (tremor). It also stimulates motility of oligodendrocyte progenitors *in vitro* and it is chemoattractive.

- *bFGF (basic Fibroblast Growth Factor) o FGF2*

It upregulates the expression of PDGFR-α and therefore extend the developmental period during which oligodendrocyte progenitors or pre-oligodendrocytes are able to respond to PDGF (McKinnon et al.,1990). FGF2 expression increases postnatally in the CNS and its up regulation can inhibit oligodendrocyte progenitors differentiation *in vivo* that occurs through direct activation of fibroblast growth factor receptor (FGFR) signaling. Analysis of *FGF2* null mice indicated improved remyelination associated with removal of FGF2 inhibition of OPCs differentiation (Zhou and Armstrong, 2007).

- *IGF-I (Insulin Growth Factor I)*

In vitro studies revealed that IGF-I treatments have a proliferative effect on both oligodendrocytes and their progenitors. IGF-I exerts protective effects on oligodendrocytes by enhancing cell survival. It is also necessary as an inducer of differentiation to transform OPCs into differentiated myelin producing cells (Chesik et al., 2008).

2. Neurotrophins

This family of growth factors, which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5, was initially shown to stimulate growth and promote survival of neurons (Barde et al., 1982; Huang et al., 2001). In addition to this role, neurotrophins exercise control over various stages of glial development. For example NT-3 and BDNF impact the survival, proliferation and differentiation of oligodendrocytes (Rosenberg et al., 2006).

- *CNTF (ciliary neurotrophic factor*)

CNTF has been shown to enhance myelination by acting on the last stages of oligodendrocyte maturation. Although CNTF is synthesized by astrocytes (Stockli et al., 1991), it is unlikely to be available normally to oligodendrocytes *in vivo*: it lacks a secretory signal sequence and is not secreted by cells *in vitro* (Stockli et al., 1989).

3. Hormones

- *Thyroid hormone T3*

Participation of this hormone in oligodendrocyte differentiation and myelin synthesis was observed during hyperthyroidism, a condition that accelerates the myelination process while hypothyroidism decreases it (Walter and Morell, 1981). T3 receptors (TRs) are transcription factors that activate or suppress target gene expression, such as MBP. In addition it was demonstrated a role in the regulation of the proliferation since T3 arrests cell division and initiates differentiation at the appropriate time (Sarlième et al., 2004).

- *Retinoic acid (RA)*

Like thyroid hormone, also retinoic acid induces purified rat OPCs in culture to stop dividing and differentiate, an effect blocked by the expression of a dominant-negative form of the tumor suppressor p53 (Tokumoto et al., 2001). Moreover, both RA and thyroid hormone cause a transient, immediate early increase in several mRNAs encoding intracellular cell-cycle regulators and gene regulatory proteins. These results suggest that the thyroid hormone and RA activate the same intracellular pathway leading to oligodendrocyte differentiation and that this pathway depends on a p53 family protein.

4. Neurotransmitter

Neurotransmitter receptor are present on oligodendrocyte membrane and the locally release of factors from axons control the proliferation, differentiation and migration of oligodendrocytes. There is increasing evidence for a role of smaller transmitter molecules, particularly glutamate, adenosine and ATP: for example glutamate acts

through AMPA or kainate (but not NMDA) receptors on OPCs to decrease proliferation instead adenosine and ATP are modulators of late oligodendrocyte development and myelination (Gallo et al., 1996; Matute, 1998, 2006).

5. Others

A variety of other molecules promote proliferation of OPCs in normal and pathological conditions. The list of factors is continuously updated with molecules that exert a positive or negative effect on oligodendrocytes. Factors like Interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) may act on oligodendrocyte survival and, in contrast with CNTF, have secretory signal sequences and are secreted by astrocytes also *in vitro*. While IL-6 has a direct effect on OPCs proliferation, IL-1β acts indirectly to promote OPCs proliferation and survival by stimulating astrocytes to express CNTF or other growth factors (Herx et al. 2000; Liberto et al. 2004; McTigue and Tripathi, 2008).

1.1.3 - Mechanisms of demyelination

Loss of oligodendrocyte and OPCs is common to may CNS injury and diseases. Their death or dysfunction cause demyelination occurring in Alzheimer's disease, Huntington's disease and also in multiple sclerosis and spinal cord injury. The main effect of demyelination is that the saltatory conduction is delayed, the neuronal transmission is compromised, and moreover in the absence of the myelin sheaths axons are less protected. There are different mechanisms responsible for oligodendrocyte death and dysfunction that involve the oxidative stress, genetic alteration, excitotoxicity, pro-inflammatory cytokines and sphingomyelinase/ ceramide pathway.

Oxidative stress. The most frequent cause of oligodendrocyte loss is the oxidative injury. Myelin production requires a large amount of ATP, so these cells increase its production with the accumulation of hydrogen peroxide as a by-product of the metabolic pathway, that if not metabolized it can cause oligodendrocyte death (Wosik et al., 2003). In addition, oligodendrocytes are full of peroxisomes because of their need to produce lipids and these organelles are a source of hydrogen peroxide. Reactive oxygen species are highly toxic and in several pathological conditions including multiple sclerosis, Alzheimer's disease and CNS hypoxia it was noticed an association between oxidative stress and oligodendrocyte loss (Stankiewicz et al., 2007).

Genetic alterations. A large number of genetic alterations have been identified that lead to oligodendrocyte loss and CNS pathology. For example a mutation in PLP gene determines an accumulation of the protein in the endoplasmic reticulum impeding the correct myelination (Gow et al., 1994). Adrenoleukodystrophy is another example of genetic myelin disorder leading to oligodendrocyte loss: in this case there is a defect in ABCD1 gene causing the accumulation of long fatty acid chains that alter the oligodendrocyte membrane and render them vulnerable to cell death (Moser et al., 2004).

Excitotoxicity. This phenomenon is due to the presence of high extracellular concentration (50-100 μM) of glutamate, the principal excitatory neurotransmitter in the CNS. Oligodendrocyte and OPCs express glutamate receptors and in particular a form of AMPA receptor lacking the GluR2 subunit which increases the permeability of Ca^{2+}. Notably, excess of glutamate causes a prolonged activation of glutamate receptors and the increase of intracellular calcium levels that can, for example, disrupt mitochondria with the consequent production of reactive oxygen species (Deng et al., 2006). These events cause necrotic and apoptotic oligodendrocyte death (Tanaka et al., 2000).

Pro-inflammatory cytokines. These molecules are often present in pathological conditions in the CNS. *In vitro* experiments showed that cytokines like IL-1β, IL-2, Interferon γ (IFNγ) and TNFα (Tumor Necrosis Factor) are able to directly promote oligodendrocyte death (Hisahara et al., 1997). It was reported also an indirect mechanism by which macrophages and microglia are activated by IFNγ and TNFα and produce free radicals that can kill oligodendrocytes (Merrill and Scolding, 1999).

Sphingomyelinase/ceramide pathway. Sphingolipids are the most abundant lipid component of the plasma membranes and represent 20% of the dry weight of myelin (Baumann and Pham-Dinh, 2001; Morales et al., 2007). Sphingolipids contain ceramide cores that mediate the clustering of death receptors which function is to initiate apoptosis. Oligodendrocytes are rich in ceramide which increases the sensitivity of oligodendrocytes to death receptors signaling. Ceramide can also be released into the cell by enzymatic cleavage of membrane sphingolipids mediated by the sphingomyelinase. Once into the cell ceramide can ultimately activate oligodendrocyte apoptosis (Morales et al., 2007).

1.1.4 - Myelin regeneration

In certain conditions the lost oligodendrocytes can be replaced with new myelinating oligodendrocytes that are able to restore myelin (Fig.3). Remyelination involves the generation of new mature oligodendrocytes starting from the activation of OPCs that migrate in the damaged area and differentiate. Both developmental myelination and remyelination share the same key steps in OPCs migration, proliferation and maturation into myelin-forming oligodendrocyte. The main difference between these two events regards the length and thickness of myelin sheaths: in remyelination, myelin forms shorter and thinner sheaths around axons (Blakemore, 1974). These findings suggest a relationship between axon and myelin.

In the adult brain a significant number of OPCs persists after CNS development is complete and not all precursors differentiate during the development (Wolswiijk and Noble, 1989). These progenitors represent the 5-8% of the cells in the CNS and they express NG2, PDGF-αR and Olig2. Adult OPCs are in a quiescent state but they become responsive to mitogens and chemoattractants released from glia after an injury. This activation depends on the inflammatory reaction that occurs during demyelination, when astrocytes and microglia release growth factors, like PDGF and FGF, necessary for remyelination. Recruited OPCs exit the cell cycle, establish a contact with the neuron, express myelin protein and finally enwrap the axon. The efficiency of remyelination can be influenced by many factors including growth factors, cytokines, extracellular matrix molecules and even gender and age appear to contribute to oligodendrocyte genesis. The age-related effect on remyelination is due to a less efficient recruitment and differentiation of OPCs. With age, intrinsic and extrinsic factors promoting OPCs maturation are disregulated and the production of cytokines, chemokines and growth factors is decreased (Franklin and Ffrench-Constant, 2008; Franklin and Kotter, 2008). As regards the gender, it was found that females remyelinate more efficiently than male rats but the basis of this sex divergence is not clear.

In case of an injury or disease, the failure of remyelination is probably due to the presence of inhibitory factors originating from both the axon and within the OPC itself, that appear to prevent adult OPCs from reaching their potential to participate in myelin repair. These factors seem to contribute also to the maintenance of the precursor reservoir. Among these molecules it was recently identified an orphan G protein-coupled receptor 17 (GPR17): if overexpressed this receptor is able to counteract the activity of the transcription factor Olig1 and thereby inhibit

oligodendrocytes maturation both *in vitro* and *in vivo* (Samanta and Kessler, 2004; Chen et al., 2009), conversely knocking out GPR17 results in precocious myelination and accelerated OPCs differentiation (Chen et al., 2009). GPR17 expression in adult OPCs after demyelination deems it a valuable potential therapeutic candidate in demyelinating diseases.

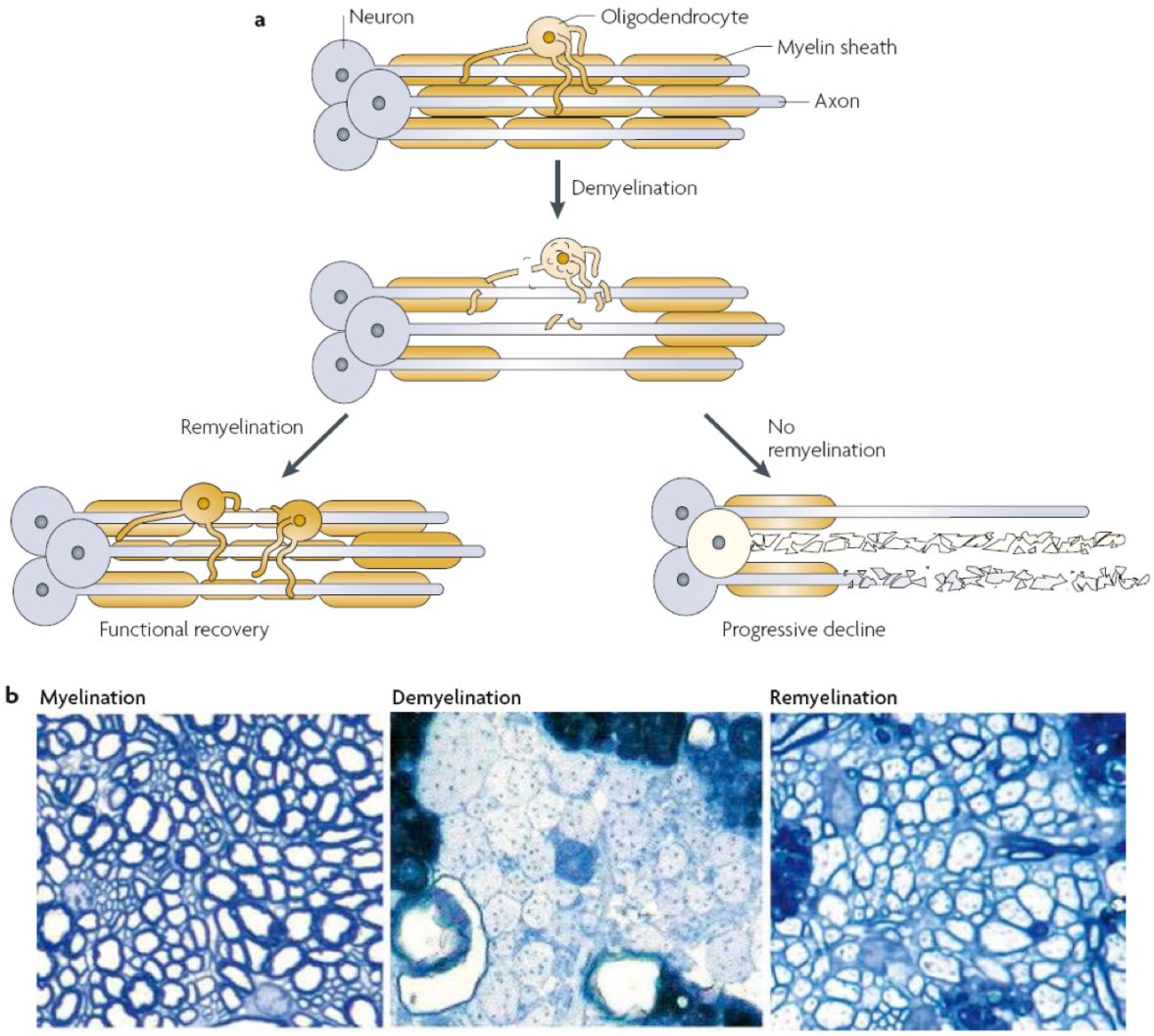


Figure 3. The fate of demyelinated axons. A) Following demyelination in the CNS, axons can be remyelinated. The myelin sheaths that are generated in remyelination are typically thinner and shorter than those that are generated during developmental myelination. Nevertheless, they are associated with recovery of function. In some circumstances as in multiple sclerosis, however, remyelination fails, leaving the axons and even the entire neuron vulnerable to degeneration. **B)** The images in this series are transverse sections from the adult rat cerebellar white matter, that show normally myelinated axons of various diameters in the left-hand panel, demyelinated axons following injection of ethidium bromide in the middle panel and remyelinated axons with typically thin myelin sheaths four weeks after the induction of remyelination in the right-hand panel. (From Franklin and Ffrench-Constant, 2008).

1.2 GPR17

1.2.1 Structure and pharmacological profile

The seven trans membrane domain G-protein-coupled receptors (GPCRs) represent the largest membrane receptor family encoded in the human genome with ~900 members. GPR17 was first described by Blasius and colleagues in 1998 while they were screening a library searching for a new P2Y receptor, however it was only recently deorphanized. GPR17 is a G*i* coupled receptor located at an intermediate phylogenetic position between P2Y receptors and the $CysLT_1$ and $CysLT_2$ receptors and it is specifically activated by both uracil nucleotides (UDP, UDP-glucose and UDP-galactose) and CysLTs (LTD_4 and LTC_4) (Ciana et al., 2006; Lecca et al., 2008). Consistent with this hybrid pharmacology, activation of GPR17 by uracil nucleotides was inhibited by the P2Y receptor antagonists cangrelor and MRS2179, whereas its activation by CysLTs was antagonized by montelukast and pranlukast, two already known CysLT receptor blockers. Dysfunctions of nucleotides and their receptors have been associated to various human diseases, including ischemic/inflammatory conditions (Burnstock and Knight, 2004; Abbracchio et al., 2003) Instead, cysteinyl-leukotrienes (CysLTs, such as LTC_4, LTD_4 and LTE_4) are inflammatory 5-lipoxygenase arachidonic acid mediators (Samuelsson, 2000) with established roles in bronchial asthma (Drazen, 2003) acting through $CysLT_1$ and $CysLT_2$ receptors.

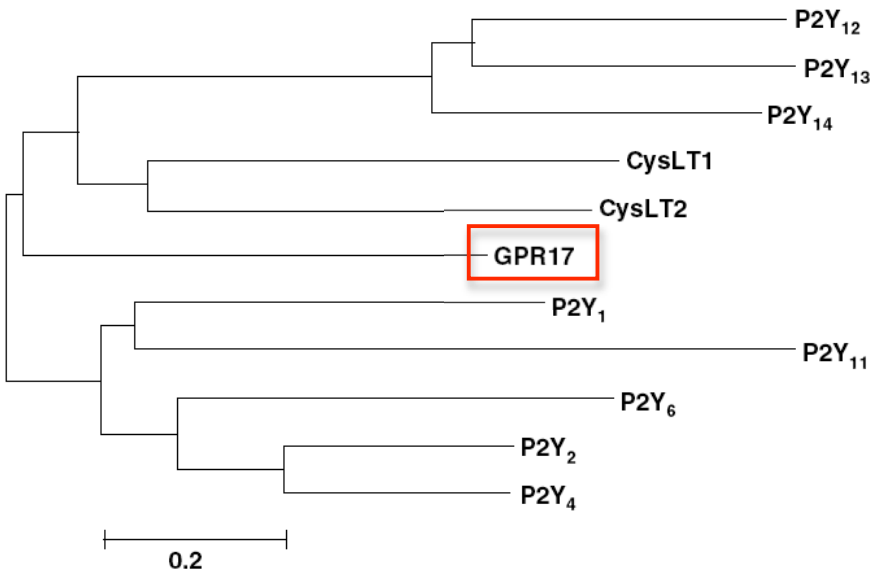


Figure 4. Phylogenetic tree showing the relationship between GPR17 and P2Y and CysLT receptors. (From Ciana et al., 2006).

Purinergic receptors are able to respond to extracellular signaling molecules and they are expressed in different human tissues to explicate a variety of functions. In particular, in the nervous system they can be found both in neurons and glial cells; they are involved both in short term events like neurotrasmission and neuromodulation, and in long term events like proliferation, growth and cellular differentiation. Purinergic receptor family can be divided in two groups: P1 receptors that respond to adenosin and P2 receptors that bind ATP, ADP, uracil nucleotide (UDP, UTP) and sugar nucleotides (UDP-glucose, UDP-galactose). P2 receptors are classified in 2 subgroups: the ionotropic receptors P2X and P2Y that are instead metabotropic receptors (Burnstock, 1972; Burnstock 1978; Abbracchio et al., 2006; Abbracchio et al., 2008; Burnstock 2008; Di Virgilio et al., 2009). Currently eight P2Y receptors (P2Y1,2,4,6,11,12,13,14 receptors) have been cloned and characterized. Human and rodent P2Y1, P2Y12, and P2Y13, and human P2Y11 preferentially bind ADP and ATP; human P2Y4 and P2Y6 respond to either UTP or UDP; P2Y14 respond only to the sugar nucleotides UDP-glucose and UDP-galactose and there is a group of receptors of mixed selectivity (human and rodent P2Y2, rodent P2Y4 and, P2Y11) (Parravicini et al., 2010).

Cysteinyl-leukotrienes (LTC_4, LTD_4 and LTE_4) are extracellular mediators released during inflammation, in fact they are mostly expressed on immune cells that triggers a proinflammatory response (Brink et al., 2003). They are generated by the 5-lipoxygenase pathway starting from the arachidonic acid and, contrary to the other leukotrienes, they contain the γ glutamyl-cysteinyl-glycine tripeptide in their sequence. Their receptors, classified as $CysLT_1R$ and $CysLT_2R$, have a seven transmembrane domain structure and belong to the G protein-coupled receptor family. Cysteinyl leukotrienes are implicated in several human diseases such as bronchial asthma, stroke and cardiovascular diseases (Parravicini et al., 2008; Maekawa et al., 2009).

From recent data the existence of a crosstalk between nucleotides and CysLT emerges, especially during inflammation where these molecules accumulate and, activating their receptors, stimulate their release (Ballerini et al., 2005). Another observation to support this hypothesis is that in human monocyte/macrophage-like cells $CysLT_1R$ desensitization is due to the extended exposure to extracellular nucleotides (Capra et al., 2005). Furthermore both types of receptors have the same structure and phylogenetically belong to the same “purine receptor cluster”, that also includes GPR17 and other several orphan receptors.

GPR17, like all the other GPCRs, is formed by seven transmembrane domains connected with intracellular and extracellular loops (Fig. 5, 6). The N-terminal end is in the extracellular portion of the protein and has a site for N-glycosylation that could be important for its intracellular transport from the endoplasmic reticulum to the plasma membrane; the C-terminal is in the intracellular compartment and it has a PDZ-1 like motif (X-S-X-∅) that could be involved in the interaction of the receptor with other proteins or in its internalization or recycling (Marchese et al., 2008). GPR17 exists in two splice variants in human and non-human primates (Fig. 5). These differ only with respect of the N-terminus, where the long human isoform hGPR17-L (367 aa), is 28 amino acids longer than the short (hGPR17-S) (Fig. 5). mGPR17 (murine) and rGPR17 (rat) sequences have 339 aminoacids like the short human isoform (Fig. 6). However hGPR17 shares 90.3% homology with mGPR17 and rGPR17. Sequences from rat, mouse and human show an almost complete overlapping of TM3, TM6 and TM7 domains and the conservation of a typical amino-acid motif in TM6 (H-X-X-R) that is usually present in several GPCRs including P2Y and CysLT receptors and probably necessary for ligand binding (Erb et al., 1995; Jiang et al., 1997; Jacobson et al., 2002) (Fig. 6). hGPR17-S was found to be the principal isoform in brain (Benned-Jensen and Rosenkilde, 2010), consistent with the reported expression pattern of rat and mice GPR17 (Blasius et al., 1998; Ciana et al., 2006; Lecca et al., 2008). The long isoform hGPR17-L is expressed at higher levels in heart and kidneys compared with brain.

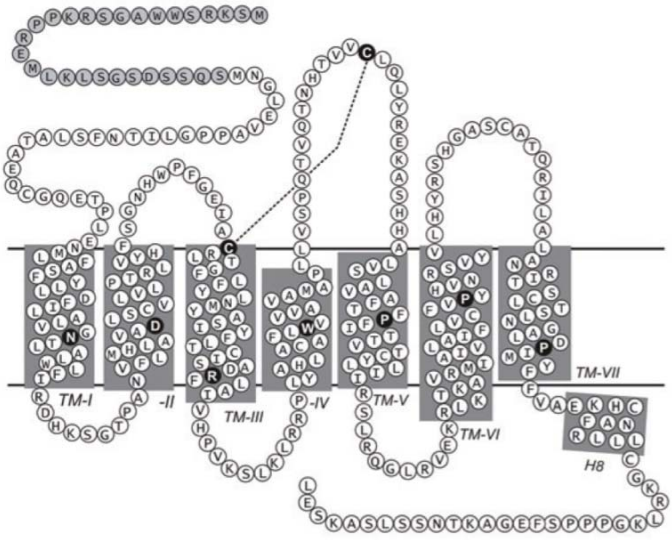


Figure 5. Long and short variants of hGPR17. Conserved disulphide-bridge between the extracellular loop 2 and the conserved cysteine residue in TM3 denoted as a stapled line. The 28 amino acids comprising the longer N-terminus of the hGPR17-L splice variant are indicated as grey circles. (From Benned-Jensen and Rosenkilde, 2010).

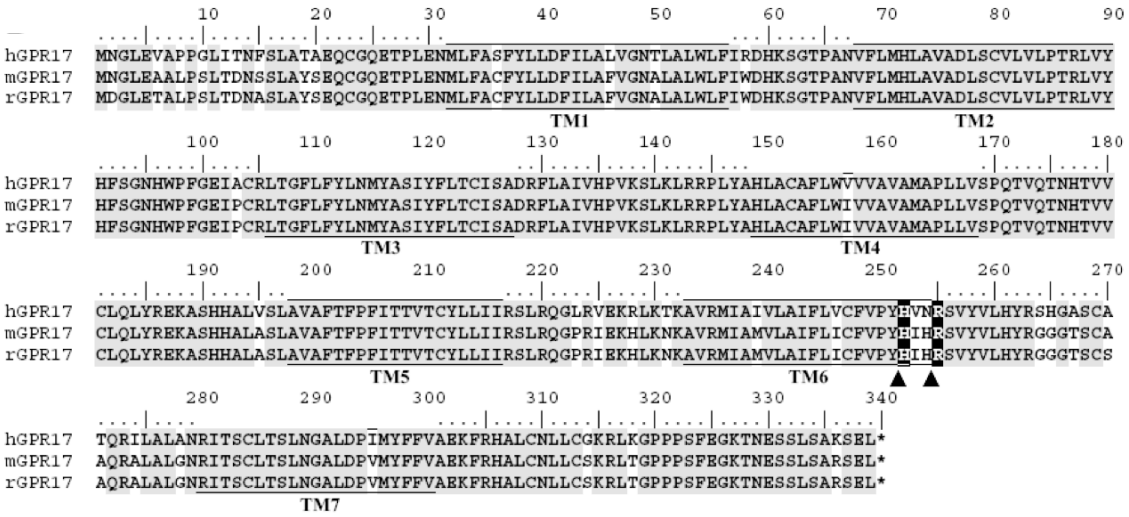


Figure 6. Alignment of short human, murine and rat GPR17 sequences. Seven transmembrane domains (TM) and the conserved motif in TM6 are highlighted. (From Ciana et al., 2006).

As GPCR stimulation results in increased binding of GTP to G-proteins, to better define the pharmacological profile of GPR17, the agonist-response of the receptor was determined measuring the [35S]GTPγS binding (Ciana et al., 2006). The assay was performed on purified membranes from transfected cells and the receptor activation was evaluated measuring the increase of [35S]GTPγS binding after stimulation with agonists and antagonists. The assay was performed in different cell types: COS-7 cells (not expressing CysLT receptors), HEK293 cells (not expressing P2Y receptors) and 1321N1 cells (not expressing P2Y nor CysLT receptors). In particular it was found that LTD4, LTC4, LTE4 and UDP, UDP-glucose and UDP-galactose stimulate the activation of GPR17 in a concentration-dependent manner, on the contrary other molecules like ATP, ADP, 2-metil-tioADP, UTP and guanosine did not have effect. From these experiments it resulted that the agonist-response profile was different from that of already known CysLT and P2Y receptors, with EC50 values in the nanomolar and micromolar range respectively. As regards the antagonists, cangrelor (antagonist of P2Y12 and P2Y13 receptors), MRS2179 (an antagonist of P2Y1 receptor) and montelukast and pranlukast, (antagonist of $CysLT_1$ receptor) have all shown to reduce [35S]GTPγS binding in a concentration dependent-manner. Furthermore blocking purinergic receptors and stimulating with CysLT agonists, and viceversa, the agonist-mediated response was not abolished suggesting the existence of two distinc binding sites for CysLT and nucleotides. In support of this hypothesis it was later published a paper in which, by docking and molecular dynamics simulations (Parravicini et al.,

2008), it was proposed the existence of two distinct binding pockets, one for nucleotides and one for leukotrienes. As both P2Y (Abbracchio et al, 2003; Fumagalli et al, 2003) and CysLT receptors (Brink et al, 2003; Capra et al, 2005) may couple to G-proteins of the Gi subfamily, it was investigated if also GPR17 is coupled to inhibitory Gi subunit. [35S]GTPγS binding assay performed in the presence of the Gi-protein inhibitor pertussis toxin (PTX) showed a strong inhibition of [35S]GTPγS binding upon agonist stimulation. In addition GPR17 activation is able to block adenylyl cyclase and cAMP synthesis. Contrasting data instead are published about the stimulation of intracellular Ca^{2+} release: in 2006 (Ciana et al., 2006) it was observed by single-cell calcium imaging a small increase of Ca^{2+} concentration after agonist stimulation even though Maekawa group (Maekawa et al., 2009) could not detect any calcium flux activated by GPR17. Furthermore, contrary to previous reports, Benned-Jensen and Rosenkilde (2009) reported that mouse or human GPR17 are activated by uracil nucleotides but apparently not by LTD_4 or LTC_4. The authors showed that LTD_4 did not significantly increase the internalization of FLAG-tagged hGPR17 in transiently transfected HEK293.

1.2.2 GPR17 expression in developing OPCs

In line with various papers (Blasius et al, 1998; Ciana et al. 2006; Benned-Jensen and Rosenkilde, 2010), both human and rat GPR17 seem to be highly present in the brain and in other organs typically undergoing ischemic damage like kidney and heart while it is very low expressed in the liver and lung. To determine GPR17 localization in mouse brain an antibody against the C-terminal region of the receptor was developed (Lecca et al., 2008). Different brain sections were immuno-labeled with the anti-receptor antibody in parallel with markers of neuronal or glial cells showing that GPR17 colocalizes with typical neuronal proteins like SMI-311 (marker of neurofilaments), β-tubulin (neuronal-specific tubulin) and NeuN (neuronal nuclear protein). Controversely, GPR17 was not find in other cell types like astrocytes, marked with GFAP (glial fibrillary acid protein) and S100b (glial neurotrophic factor), or in non stimulated microglia stained with Iba1 (ionized calcium binding adaptor molecule 1) antibody (Fig. 7). Examining the cerebral cortex another $GPR17^+$ cell type was identified that also expresses NG2, indicating an oligodendrocytic phenotype. Investigating more deeply it resulted that there are three subpopulation of cells: some are positive only for GPR17, others only for NG2 and others express both GPR17 and NG2. However $GPR17^+$ cells express also Olig2, but do not produce myelin protein

such as MAG, MBP or CNPase, suggesting that GPR17^{+} cells are quiescent oligodendrocytes in their first stage of differentiation (Fig. 7).

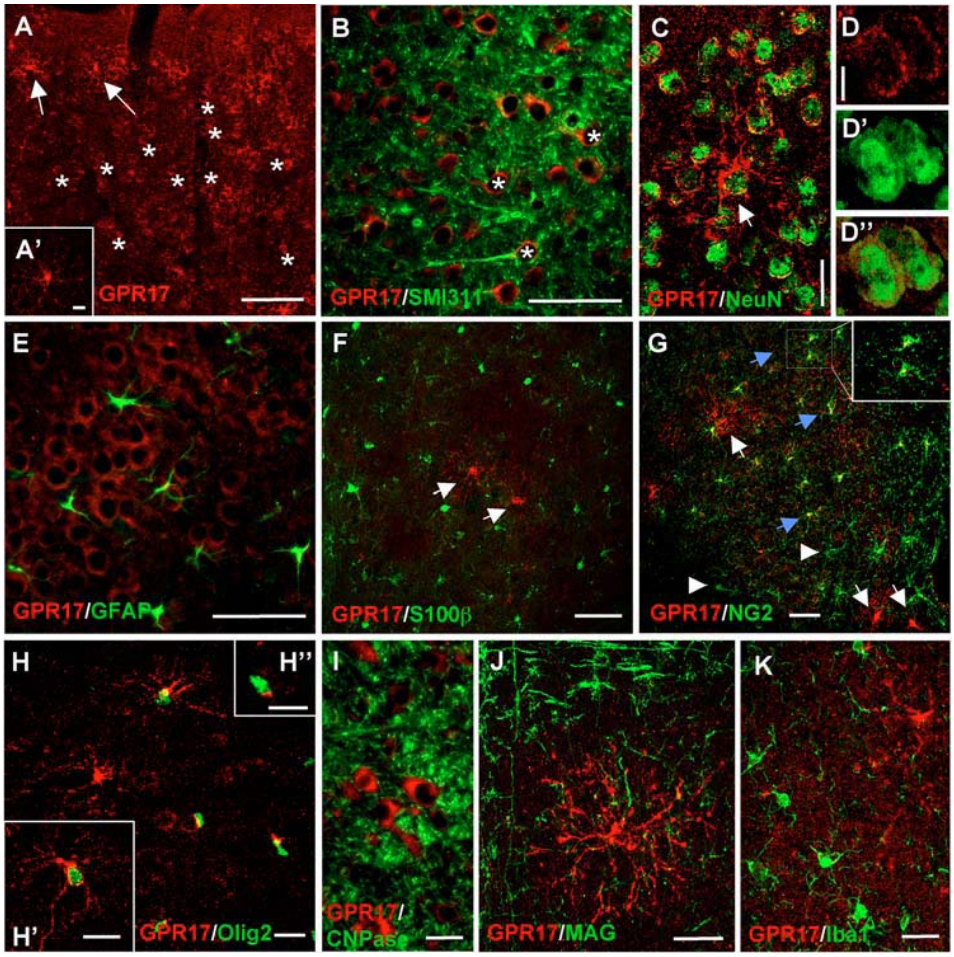
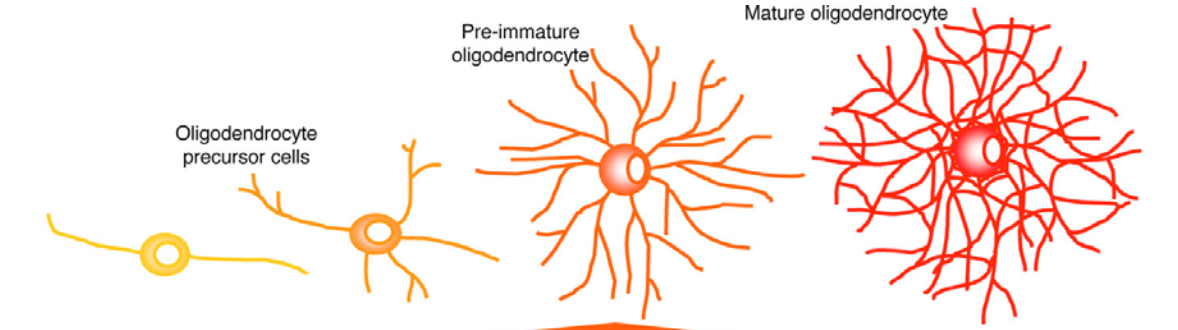


Figure 7 . Cellular localization of GPR17 in the intact mouse brain.
(A) In the intact cortex, GPR17 expression is detected in large round cells (asterisks) and in ramified cells (arrows). GPR17^{+} cells display co-expression of the neuronal proteins SMI311 (B) or NeuN (C). D panels show higher magnification images of two neurons postive for both GPR17 (red channel, D), and NeuN (green channel, D'); the merge is illustrated in D''. No co-localization is observed with the astroglial markers GFAP (E) and S100b (F). GPR17 expression is detected in NG2+ and Olig2+ oligodendrocytes (G and H, respectively), while it is absent in more mature oligodendrocytes expressing CNPase (I) and MAG (J). Some ''resting'' microglial cells positive for Iba1 were also found in cortex, but none of these express GPR17. (From Lecca et al., 2008)

At early stages in culture, OPCs showed a typical bipolar morphology and are positive for NG2 (stage 1, Fig.8). GPR17 mRNA is detected even if GPR17 protein was undetectable in the majority of cells, being present only in cells with a tripolar or more complex morphology (stage 2, Fig. 8). At this stage $GPR17^{+}$ cells co-express PDGFα-R, on the contrary DM-20 protein is not found. As OPCs mature, the level of GPR17 increases: at stage 3, pre-oligodendrocytes show the highest expression of GPR17, they become positive to DM-20 protein and O4 and, at the same time lose PDGFα-R. At later stages of maturation (stage 4) MBP immunoreactivity progressively increases towards the terminal differentiation while labeling for GPR17 declines (Fumagalli et al., 2011).

This timely regulated expression of GPR17 during oligodendrocyte maturation suggests a role of the receptor during this process. It was observed that *in vitro* and *in vivo* the loss of GPR17 function, in $GPR17^{-/-}$ mice, accelerates OPCs differentiation and myelination. On the contrary in a transgenic mouse over-expressing GPR17 the arrest of myelinogenesis or apoptosis occurred, and it was observed also the translocation into the nucleus of ID2 and ID4 (DNA-binding protein inhibitor), two potent repressors that physically interact with and sequester the bHLH factors Olig1 and Olig2 to block the progression of oligodendrocyte differentiation (Chen et al., 2009). These data suggest that GPR17 is an intracellular timer that negatively regulate the transition from immature to mature oligodendrocytes.



	Stage 1		Stage 2		Stage 3		Stage 4	
	mRNA	*protein*	*mRNA*	*protein*	*mRNA*	*protein*	*mRNA*	*protein*
GPR17	+	-	+	+	+	++	-	-
PDGFRα	+	++	+	++	-	-	-	-
NG2	+	++	+	++	+	+	-	-
O4	N.D.	-	N.D.	-	N.D.	++	N.D.	-
DM-20	+	-	+	-	+	+	+	N.D.
PLP	-	-	-	-	+	-	+	N.D.
MBP	-	-	-	-	+	-	+	++

Figure 8. GPR17 expression as a function of OPCs differentiation. The scheme summarizes the typical differentiation steps of OPCs, from the immature precursor to mature oligodendrocyte. Changes of GPR17 protein during differentiation are highlighted in a yellow-orange-red triangle, where orange represents the maximum expression level. (From Fumagalli et al., 2011)

1.2.3 GPR17 expression in pathological conditions

Given that GPR17 is highly expressed in tissues that undergo ischemia and that it is activated by both CysLTs and nucleotides, molecules that accumulate to a greater extent in ischemic brain (Ohtsuki et al, 1995; Ciceri et al, 2001; Burnstock and Knight, 2004), a possible role of the receptor is ipothesized in case of brain injury. GPR17 expression was investigated in an established model of ischemic damage (the permanent monolateral middle cerebral artery occlusion in the rat, MCAo), evaluating the expression of the receptor in the perilesioned area compared to the intact controlateral area. Upon brain injury CysLT concentration increases in the damaged area after 4h, while after only 20 min an increase in the nucleotides concentration is detected and it last even for days.

In MCAo 24h after injury, the amount of GPR17 first increases in neurons in the damaged area but after 48h decreases as a consequences of cell death and in fact these cells start to express Hsp70 (Armstrong et al., 1996) and change their morphology. Notably GPR17 knockdown determines survival of a large number of neurons (Ciana et al., 2006). After 72h from damage, also activated microglia in the perilesioned area starts to express, beyond isolectin IB4, also GPR17 becoming sensitive to both leukotrienes and nucleotides, highly concentrated in this area. At this stage it was found that GPR17 is upregulated in oligodendrocyte precursor cells near the damaged region, and, most importantly, these cells, start to mature and produce myelin proteins. In conclusion after its activation GPR17 seems to act in different ways: on one hand it determines neurons death but on the other hand it is necessary to recruit immune cells and to induce progenitors cells to differentiate. GPR17 can be considered a sort of sensor of the damage for its capacity to be activated by both nucleotides and CysLT, two important danger signals released by damaged cells after an injury. These findings propose GPR17 as a novel target for therapeutic manipulation to foster repair in demyelinating diseases like in multiple sclerosis. Even though the existing data about GPR17 concentrated on its expression and role in the CNS, very little is known about its signaling and the trafficking that regulate its expression at the surface membrane. One possible mechanism to control the distribution of the receptor at the plasma membrane and, consequently the ability to respond to extracellular stimuli, is the internalization and desensitization of the receptor.

1.3 GPCR trafficking

GPCRs belong to a superfamily of seven trans membrane spanning proteins that respond to diverse extracellular stimuli and control many physiological and pathological responses making these receptors the target of almost 50% of the drugs used in the treatment of various human diseases including neurodegenerative and psychiatric disorders, cardiovascular and pulmonary diseases and cancer (Marchese, et al., 2008). At the plasma membrane GPCRs bind their ligand that trigger a conformational change that activates heterotrimeric G-protein, Gα and Gβγ subunits, regulating intracellular second messenger levels. Different mechanisms exist to precisely regulate at the temporal level the activation of the receptor (Alphonse and Hanyaloglu, 2011) and a common property of most GPCRs is that ligand treatment decreases receptor responsiveness through a mechanism called “desensitization” that takes place in three phases: i) decoupling of heterotrimeric G proteins after receptor phosphorilation (Bouvier et al., 1988); ii) dynamin-dependent endocytosis of receptor/ligand complexes (Ferguson, 2001); iii) receptor downregulation through lysosomal degradation and/or reduced mRNA and protein synthesis. The large data accumulated demonstrate that the endocytic trafficking of GPCR play a key role in receptor down-regulation, resensitization by recycling to the plasma membrane or degradation in lysosomal compartments and the subsequent termination of signal. Endocytosis of GPCRs usually occurs through a clathrin-mediated mechanism by which the receptor is internalized in clathrin-coated pits, originated by invagination of the plasma membrane enriched in PIP_2 (Phosphatidylinositol 4,5-bisphosphate). A less common mechanism of endocytosis has been also described that is clathrin-independent and it is mediated by caveolin which associate to lipid rafts in the plasma membrane (Mayor and Pagano, 2007).

As regards the clathrin-mediated endocytosis, it proceeds via a two-step process that involve other effector proteins like GRKs (G-protein coupled receptor kinases) and β-arrestins. Serine/threonine phosphorylation of GPCRs is well known to promote receptor internalization (Hanyaloglu and von Zastrow, 2008; Reiter and Lefkowitz, 2006; Ferguson, 2001). After phosphorilation by GRK, β-arrestin are recruited from the cytoplasm and interaction with phospho-residues on the receptor induces a conformational change in β-arrestin that allows binding of clathrin and its adaptor proteins AP-2 (Fig. 9). Within the endosomes the receptor is dephosphorilated and the acid pH of these organelles induces a conformational change with the possible release

of the ligand. At this moment the receptor can follow distinct trafficking routes: it can be recycled back to the membrane or targeted to lysosomes (Pippig et al. 2005; Searichrist et al., 2002; Li et al. ,2000).

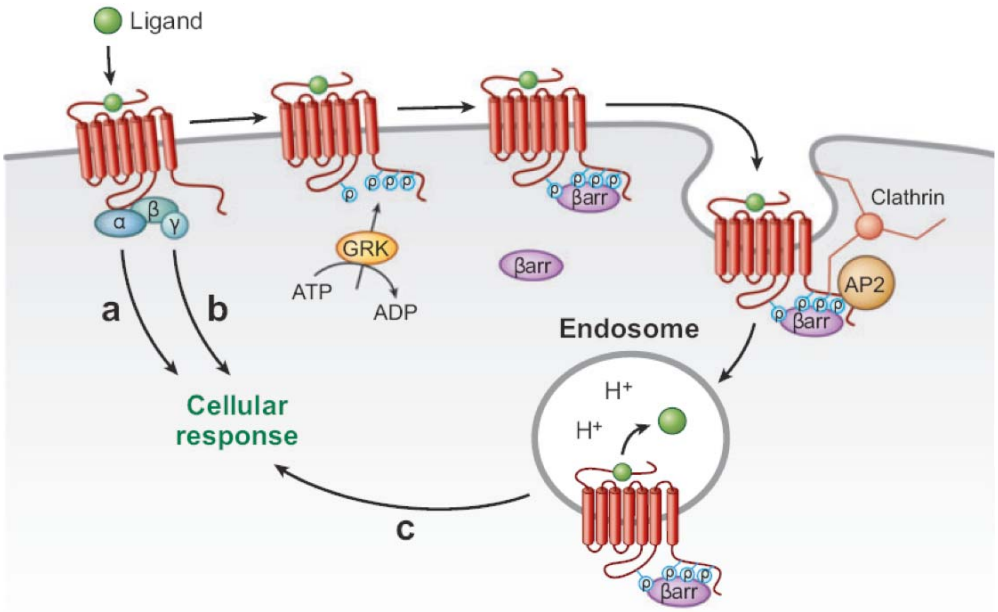


Figure 9. Rapid desensitization and endocytosis of GPCRs mediated by GRKs, arrestins, and clathrin-coated pits. Agonist-bound receptors initiate signaling by activating heterotrimeric G proteins at the plasma membrane, which signal to downstream effects via α and/or βγ subcomplexes (*arrows a* and *b*). Receptor phosphorylation by GRK promotes recruitment of arrestins from the cytoplasm, preventing subsequent interaction of receptors with G proteins effectively terminating the G protein-mediated signal and promoting receptor endocytosis via clathrin-coated pits. (From Hanyaloglu and von Zastrow, 2008).

Numerous studies have established a key role for Rab GTPases in regulating GPCR endocytosis and trafficking through different endocytic compartments. Rab proteins are recruited to distinct membrane-bound compartments to regulate transport between organelles; they are not randomly distributed on the organelle membrane but are clustered in distinct functional domains (Zerial and McBride, 2001). The Rab proteins belong to the Ras-like small GTPases, they are tightly associated to membranes by lipid groups attached to their C-termini and bind GTP in their active state. Hydrolysis of GTP to GDP is catalysed by GTPase-activating proteins (GAPs) and converts the Rab to an inactive form. Concerning GPCRs, an important role has been described in particular for Rab4, Rab5, Rab7 and Rab11 in regulating the endocytosis and trafficking of these receptors between early, late and recycling endosomes, and finally lysosomes. Rab5 is localized to the plasma membrane, clathrin-coated vesicles and early endosomes (Bucci et al.,1992); it was identified as a novel component required for the invagination of newly formed coated pits at the plasma membrane and as essential for clathrin-mediated endocytosis of transferrin receptor (TfR) (McLauchlan et al., 1998), a well-characterized marker used to follow recycling after transferrin binding. TfR, upon transferrin binding, is first internalized in early endosomes (Rab5+), then move sequentially through Rab4- and Rab11-positive structures (Ullrich et al., 1996; Peter van der Sluijs et al., 1992; Bucci et al., 1992) and finally it is targeted to the surface membrane. Therefore it is recognized that Rab5 determines the entry into the early endosomes, whereas the Rab4 and Rab11 domains contain the machinery necessary for sorting and recycling of transferrin receptor to the surface membrane. Thus Rab4 and Rab11 are involved in the regulation of receptor recycling back to the plasma membrane: Rab4 is considered to be involved in the fast recycling of receptors from early endosomes directly to the plasma membrane (Grimsey et al., 2011), while Rab11 is involved in the slow recycling of cargo via perinuclear endosomes. Finally Rab7 is localized in late endosomes to control aggregation and fusion of late endocytic structures and lysosomes (Bucci et al., 2000). These Rabs dynamically interact and participate in the temporal and organelle-specific distribution of selected cargo.

In conclusion the cell has a lot of mechanisms to finely tune the intracellular trafficking of receptors and to regulate the distribution at the plasma membrane. Characterization of GPR17 endocytic traffic is thus important to understand further the mechanisms of receptor signaling in oligodendrocytes. The internalization and subsequent sorting of receptor into the degradative or recycling compartments may

have important implications for the activation or silencing of signaling pathway(s) and, in turn, to be key events in OPCs differentiation and myelination.

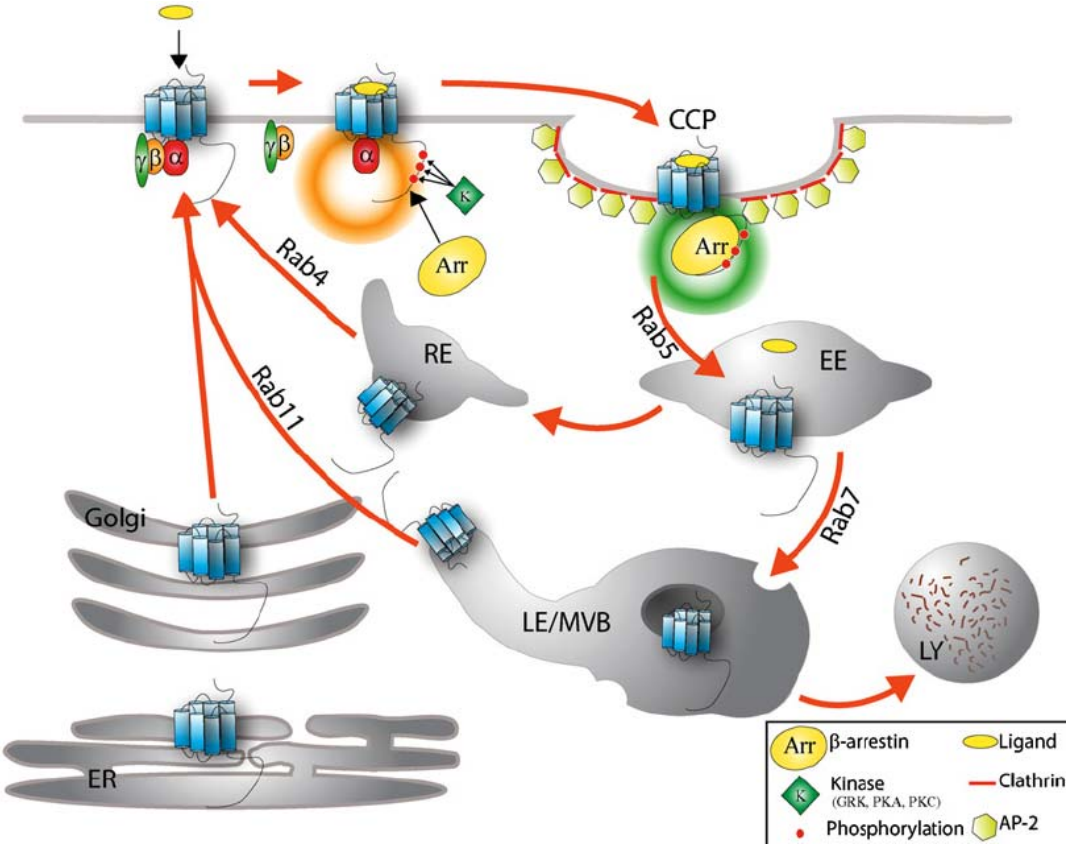


Figure 10. The trafficking life cycle of GPCRs. Following synthesis, GPCRs are transported through the endoplasmic reticulum (ER) and Golgi compartments where post-translational modifications and interactions with other proteins regulate their maturation and targeting at the surface. Once at the membrane the receptor is able to bind its ligand. Agonist bound receptors activate heterotrimeric G proteins witch initiate receptor intracellular signaling. Following activation, GPCRs are rapidly phosphorylated by kinases such as GRKs and arrestin (Arr) is then rapidly recruited from the cytosol to the activated GPCR, which uncouples the receptor from its G-protein and targets receptors to clathrin-coated pits (CCPs) for its internalization, resulting in rapid desensitization of G-protein signaling. In early endosomes (Rab5) the receptor can be trafficked to various endocytic compartments.The receptor can be sorted to lysosomal degradation pathways via Rab7 late endosomes. GPCRs can also traffic to rapid (Rab4) or slow (Rab11) recycling compartments for resensitization and recycling to the cell surface.
EE; early endosome; RE; recycling endosome; LE/MVB; late endosome/multivesicular body; LY; lysosome. Orange 'halo' at GPCR denotes heterotrimeric G-protein signals; green halo denotes β-arrestin-mediated, non-G-protein signaling. (From Alphonse and Hanyaloglu, 2011).

2. AIM OF THE STUDY

Oligodendrocytes are essential for the correct functioning of the CNS: their primary role is to form myelin sheaths, a fatty insulation composed of modified plasma membrane that surrounds axons and ensure an efficient and fast conduction of electrical impulses along axons, but it is also important for the maintenance of axonal integrity. Several pathologies of the CNS are correlated to the loss of myelin, an event that can be a direct cause of disease like in multiple sclerosis or in other situation it can be a consequence of a more general damage like during ischemia. Neurons are the cells that suffer more in case of oligodendrocyte malfunctioning. For this reason a great interest in remyelination has arisen. Remyelination is the regenerative process during which new myelin sheaths are restored. Within the CNS there are OPCs, that after appropriate stimulation, are able to migrate in the damaged area and completely mature producing new myelin sheaths. Among the factors necessary for the recruitment of precursors at the lesion, GPR17 seems to play a role as a sensor of the damage and regulator of OPC differentiation. Recent experiments highlighted the ability of GPR17 to activate OPCs differentiation in physiological and pathological conditions (Lecca et al. 2008; Fumagalli et al. 2011) upon agonist (UDP-glucose or CysLTs) binding: receptor expression, almost absent in OPCs, gradually increases in more mature precursors, it reaches a plateau in immature/preoligodendrocytes and then gradually decreases along with terminal differentiation. On the other hand inhibition of GPR17 expression causes impairment in oligodendrocyte differentiation and myelination in *in vivo* (Chen et al., 2009) and in vitro systems (Fumagalli et al., 2011). Altogether these data indicate that GPR17, acting in a specific time window, is implicated in controlling oligodendrocyte ontogenesis and suggest that the appropriate activation and deactivation of GPR17 are crucial steps for OPCs maturation.

As reported for many GPCRs, after ligand binding GPR17 may undergo to endocytosis and subsequent sorting into lysosome for degradation and/or to recycling endosomes for delivery to the plasma membrane. The balance of this dynamic intracellular trafficking is physiologically relevant as it modulates receptor levels on the cell surface (Ferguson, 2001; Marchese et al., 2008; Dunham and Hall. 2009; Ritter and Hall, 2009; Sorkin and von Zastrow, 2009) and may have important implications for the activation or silencing of GPR17-signaling pathway(s) and, in turn, for OPCs differentiation. Biochemical and molecular characterization of the mechanisms underlying the trafficking of GPR17 and the regulation of receptor signaling are largely

unknown. The aim of this study is to better define the trafficking of GPR17 in basal condition and upon activation. It has indeed been demonstrated in 1321N1 cell heterologously expressing hGPR17 that the agonists UDP-glucose and LTD_4 determine receptor internalization (Daniele et al., 2011). On the other hand, a previous study has demonstrated that GPR17 is not activated directly by agonists but may function exclusively as a negative regulator for the $CysLT_1R$ response to LTD_4 treatment (Maekawa et al., 2009). Furthermore, in a recent paper Benned-Jensen and Rosenkilde (2010) reported that mouse or human GPR17 are not activated by or bound by LTD_4 or LTC_4. Consistent with this result they showed that LTD_4 did not increase significantly the internalization of FLAG-tagged hGPR17 in transiently transfected HEK293.

In order to shed light on the mechanisms of GPR17 signaling and agonist induced activation, we investigate the endocytic trafficking of the native receptor. To this aim we develop a new antibody against an extracellular epitope of GPR17 to be used in internalization assays performed in Oli-neu cells, an immortalized mouse OPC line (Jung et al. 1995). These cells can be induced to differentiate toward a more mature phenotype and have been extensively used to investigate intracellular trafficking of myelin proteins (Jung et al. 1995; Winterstein et al. 2008). In this study we established the conditions to obtain a time regulated expression of GPR17 in Oli-neu and used these cells for the biochemical characterization of the native receptor and for the analysis of its trafficking in basal condition or after incubation with agonists. The study of the intracellular trafficking of the receptor may help to clarify (i) how the receptor levels at the plasma membrane and its signaling are regulated; (ii) how these mechanisms correlate to OPCs differentiation and myelin repair; (iii) whether and how the two different classes of GPR17 physiological ligands (i.e. uracil nucleotides or CysLT) regulate receptor responsiveness and desensitization.

3. MATERIALS AND METHODS

3.1 Materials and antibodies

The UDP-glucose, protease inhibitor cocktails, leupeptin, chloroquine (7-chloro-4- (4-diethylamino-1-methylbutylamino) quinoline), chlorpromazine hydrochloride, anti-rabbit and mouse IgG conjugated to horseradish peroxidase, and anti-rabbit IgG-light chain conjugated to horseradish peroxidase came from Sigma-Aldrich (Milan, Italy); LTD_4 was from Cayman Europe. Montelukast was a gift from Merck, and cangrelor was a gift from The Medicines Company (Parsippany, NJ). The sulfosuccinimidyl 2-(biotinamido) ethyl- 1,3' dithiopropionate (EZ-Link^{TM} , Sulfo-NHS-SS-Biotin) from Thermo Scientific (Milan, Italy) and streptavidin Plus UltraLink Resin from Pierce (Rockford, IL). The antibodies against MAG and NG2 came from Millipore (Billerica, MA, USA), the anti-MBP antibody from Covance (Rome, Italy), the anti-Lamp1 (lysosome-associated membrane protein-1) from BD Bioscience (Erembodegem, Belgium) and the anti-transferrin receptor (TfR) from Zymed Laboratories (San Francisco, CA, USA). An antibody against a C-terminal region of GPR17 (anti-Ct-GPR17) was raised in rabbits and affinity purified as previously described (Ciana et al., 2006). The fluorescein-, rhodamine- or Cy3-conjugated anti- mouse and/or IgG came from Jackson Immuno Research Laboratories (West Growe, PA, USA). The siRNA against GPR17 were designed and synthesised by Qiagen (Milan, Italy), and the transfection reagent INTERFERin^{TM} came from Polyplus-transfection (Illkirch, France).

3.2 Antibodies against the N-terminal region of GPR17

An antibody was raised in rabbit using a synthetic peptide corresponding to the NH_2-terminal sequence of mouse GPR17 (MNGLEAALPSLTDNSSLAYSEQC) coupled to keyhole-limpet haemocyanin (Taverna et al., 2007). Peptides were mixed with a complete Freund's adjuvant and injected subcutaneously at the final concentration of 250 μg per animal. Injection was repeated after 15 days. A blood sample was collected before immunization to use as a control during antibody characterization. After 30 days from the first immunization event the entire blood was collected and let few hours at room temperature to form the clot. The serum was then collected by centrifugation at 4°C for 20 min (1,000 rpm) and then the surnatant centrifuged at 4°C for 30 min (10,000 rpm). The antibodies (from here on referred to as anti-Nt-GPR17) were affinity purified and tested for their specificity in *in vivo* immunolabeling.

3.3 Cell line culture and differentiation

The Oli-neu murine OPC line was kindly provided by Prof. J. Trotter (University of Mainz, Germany) and cultured in Sato medium containing 1% horse serum. The neuron-conditioned medium (CM) was obtained from primary cultured neurons prepared from the cerebral cortex of 18-day-old rat embryos as previously described (Passafaro et al., 2001). These cultures are enriched in neurons but also contain a variable number of glial cells (mainly astrocytes). The CM was collected after 15 days and then passed through a 22 mm filter. The Oli-neu cells were cultured in normal medium or normal medium plus CM in a 1:1 ratio.

SATO MEDIUM	CONCENTRATION
DMEM powder (Invitrogen)	13.4 g/l
NaHCO3	2 g/l
Transferrin (Sigma-Aldrich)	10 µg/ml
Insulin (Sigma-Aldrich)	10 µg/ml
Putrescine (Sigma-Aldrich)	100 µM
Progesteron (Sigma-Aldrich)	200 nM
Tri-iodo-thyrodine (TIT- Fluka)	500 nM
Natriumselenit (Sigma-Aldrich)	220 nM
L-Thyroxine (Sigma-Aldrich)	520 nM
Gentamicin (Invitrogen)	25 µg/ml
Decomplementated Horse serum	1%

3.4 Bacterial culture and plasmid extraction

DH5α E. Coli were used to amplify Rab5-GFP and Rab 11-GFP plasmids. Competent bacteria were transformed by heat shock that allows plasmid DNA to enter and replicate in bacterial host cells. Competent cells and bacteria were mixed together and kept on ice for 30 min, then at 42°c for 90 s and at the end 90s on ice. To help the bacterial cells recover from the heat shock, the cells were briefly incubated with non-selective growth media. After 1h cells were plated on selective agar plates containing 30 μg/μl Kanamycin and incubated over night at 37°C. The day after, a single colony was again incubated over night at 37°C in LB medium with antibiotics. Plasmid DNA was prepared using plasmidPrep Midi Flow kit (GE Healthcare).

LB Medium

NaCl 170 mM

Bacto-tryptone 1%

Bacto-yeast 0.5%

3.5 RNA interference

For RNA interference, the Oli-neu cells were plated the day before and incubated in conditioned medium 6 h before the transfection. They were transfected without siRNA (mock transfection control) or with 2.5 nM of scrambled siRNA (negative control) or a siRNA designed to silence mouse GPR17 (Qiagen; target sequences) using the transfection reagent INTERFERin^{TM}. In accordance with the manufacturer's instructions, siRNA was diluted in DMEM without serum and mixed with the HiPerfect Reagent and incubated for 10 min at room temperature. The mixture was added to cells previously differentiated with CM. Twenty-four or 48 h after transfection, the cells were fixed for immunofluorescence analysis or extracted for immunoblotting.

3.6 Transfection with JetPei

The day before transfection cells were seeded on 24x24 mm glass coverslips (7.5×10^5 cells/coverslip) treated with poly-L-lysine (0.1mg/ml) and differentiated with neuronal CM. After 24h differentiation cells were transfected with N-terminally EGFP tagged cDNAs: GFP-Rab5a (0.75 μg/coverslip) and GFP-Rab11a (0.5 μg/coverslip) using JetPEI^{tm} (PolyplusTransfection). cDNAs were mixed with jetPEI^{tm} reagent in a 1:2 ratio and incubated for 20 min at room temperature and then gently added to the cells. After 24 h transfection, the medium was removed and fresh CM was added. Experiments were performed 48 h after transfection when Oli-neu reached 72h differentiation.

3.7 Pharmacological treatments and metabolic labeling

Agonists were added to differentiating Oli-neu cells at the following concentration 100 μm UDP- glucose and 50 nM LTD_4. Cells were then incubated at 37°C for the indicated times. When required, cells were preincubated for 10 min at 4 °C with antagonists (10 μM cangrelor; 10 μm montelukast) 100 μM chloroquine, 450 mM sucrose or 50 μM chlorpromazine. Pulse-chase experiments were performed essentially as previously described (Rosa et al., 1992). Briefly, the cells were pre-incubated for 1 h in methionine- and cysteine-free Oli-neu medium, and labeled with 200 μCi/ml of the Express ^{35}S-protein labeling mix ([^{35}S]-Methionine/Cysteine; Perkin-Elmer, Monza, Italy) for 90 min. They were then washed twice with complete medium containing a two-fold excess of methionine/cysteine, and chased in the absence or presence of the drugs for the times indicated in the Results. At the end of the chase, the cells were lysed in ice-cold buffer A and centrifuged at 20,000 g for 20 min. The extracts were immediately analyzed by gel electrophoresis, or after immunoprecipitation (see above). Gels were exposed to phosphor screens that were analyzed with the Storm phosphoimager (Molecular Dynamics). Band intensity was quantified with imageQuanta software.

3.8 Cell extracts

Oli-neu cells were lysed in buffer A (150 mM NaCl, 2 mM EGTA, 50 mM Tris-HCl, pH 7.5, and a Sigma-Aldrich protease inhibitor cocktail diluted 1:1,000) containing 1% TritonX-100, and then centrifuged 20,000 *g* for 20 min at 4°C. In order to prepare the total membrane fraction, the cells were scraped from the dishes in buffer A without Triton X-100, and centrifuged at 14,500 *g* for 1 h so that the fragments derived from broken processes could also be collected. They were then re-suspended in buffer A, homogenized by passing them through a 22G x $1^{1/2}$" 0.7 x 40 mm needle, transferred to ultracentrifuge tubes (Beckman, Brea, CA, USA), and centrifuged in a Beckman ultracentrifuge at 153,000 *g* for 1 h at 4°C. The pellets containing the total membranes were then extracted in buffer A with 1% TritonX-100 for 20 min at 4 °C and re-centrifuged briefly at 800 *g* to remove particulate.

3.9 Immunoprecipitation, endoglycosidase digestions and Western blotting

For immunoprecipitation of GPR17, aliquots of the cell extracts (100-200 μg of proteins) were incubated for 1 h with protein A or G sepharose beads. The beads were removed by centrifugation, and the 'pre-cleared' supernatants were added to protein A or G beads that had been pre-incubated with the primary antibodies or non-immune IgG for 2 h at 4°C. After overnight incubation, the beads were extensively washed with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.3 % (w/v) Triton X-100 . For the endoglycosidase digestions, immunoisolated proteins were eluted from protein A beads by heating at 65°C in a solution containing 0.5% SDS and 1% β-mercaptoethanol. Aliquots of each sample were then diluted with Nonidet P-40 (1% final concentration) and 1/10 concentrated endoglycosidase F (EndoF, also called PNGaseF) or endoglycosidase H (EndoH) reaction buffers (G7 or G5, New England Biolabs) in accordance with the manufacturer's instructions, and digested with 250 units of EndoF or EndoH for 1 h at 37°C or 0°C. The reactions were stopped by the addition of Laemmli sample buffer. Total cell extracts, immunoprecipitates and endoglycosidase samples were analyzed by SDS-PAGE followed by Western blotting using as secondary antibodies anti-rabbit IgG light chains or anti-mouse IgG conjugated to peroxidase (diluted 1:50,000). The peroxidase was revealed using a chemiluminescent substrate (Pierce). For quantitative analysis, unsaturated autoradiograms were acquired using an ARCUS II scanner (Agfa-Gevaert, Mortsel, Germany) and the density of each band was quantified using NIH Image J software (National Technical Information Service, Springfield, VA, USA).

3.10 Western blot

- *SDS-PAGE*

Proteins were separated by their molecular weight using polyacrylamide gel electrophoresis in presence of SDS as described by Laemmli, 1970. Polyacrylamide gel is a matrix made of a Stacking gel that is poured on top of the resolving gel. The percentage of acrylamide chosen depends on the size of the protein that one wishes to identify: 8% for GPR17 and 10% for the other proteins. Gels were prepared using mini-gel apparatus (Bio-Rad). Samples were prepared for gel loading by adding Laemmli sample buffer containing β-mercaptoethanol, that reduces disulfide bonds, and SDS, that causes protein denaturation. Samples resuspended in this buffer were heated 2 min at 65°C or 100°C.

Laemmli sample buffer

SDS 3%
Tris HCl, pH 6.8 125 mM
Glycerol 6.6%
β-Mercaptoethanol 3.3%
Bromophenol blue

Running gel (40 ml)	8% acrylammide	10% acrylammide
Resolving buffer	10 ml	10 ml
AMBA	10.64 ml	13.3 ml
H_2O bd	19.14 ml	16.48 ml
TEMED	20 ml	20 ml
APS	200 ml	200 ml

Stacking gel (20 ml)	3.75% acrilammide
Stacking buffer	5 ml
AMBA	2.5 ml
H_2O bd	12.28 ml
TEMED	20 μl
APS	200 μl

Resolving buffer

Tris HCl pH 6.8, 1.5 M

SDS 0.4%

AMBA

Acrylammide 30%

bis-acrylammide 0.8%

Running buffer

Tris, pH 8.8, 25 mM

Glycine 190 mM

SDS 0.1%

Stacking buffer

Tris HCl pH 8.8, 0.5 M

SDS 0.4%

APS

Ammonium peroxodisulfate 10%

TEMED (Tetramethylethylenediamine)

- *Immunoblotting*

After separation on 8% or 10% polyacrylamide gels, the proteins were blotted for 5h at 300mA onto Hybond^{TM}-PVDF membranes (GE Healthcare, Milano, Italy) that is soaked in methanol just before use. At the end the membrane was coloured with Ponceau S, a staining solution used to detect proteins on cellulose acetate because it is a negative stain which binds to the positively charged amino groups of the protein. Ponceau staining is reversibile, so it rinses out with distilled water until the background is clear.

BLOTTING BUFFER	PONCEAU S
Tris HCl pH 6.8, 20 mM	Ponceau S 0.1 %
Glycine 150 mM	Acetic Acid 5%
Methanol 20%	

After blocking PVDF membrane over night at 4°C in TBS 5% nonfat milk, it was washed 3 times in TBS 5% nonfat milk with 0.3% Tween-20 and then incubated 2h with primary antibody diluted in the same solution used for washing (Taverna et al., 2004; 2007). The excess antibody was removed washing three times with TBS 5% nonfat milk with 0.3% Tween-20 before incubation with secondary antibody for 1h. At the end the membrane was washed once for 10 min in TBS 5% nonfat milk with 0.3% Tween-20 and then twice for 10 min and 5 times for 5 min in TBS containing only 0.3% Tween-20. The primary antibodies were detected using anti-rabbit IgG or anti-mouse IgG conjugated to peroxidase (diluted 1:50,000). The peroxidase was detected using a chemiluminescent substrate (ECL, Pierce). For quantitative analysis, autoradiograms were digitized using Adobe Photoshop software and band intensities determined with NIH Image software.

TBS
Tris HCl, pH 7.4, 25mM
NaCl 15mM

PRIMARY ANTIBODY	FINAL DILUITION
α-GPR17	1:1,500 (1.38 μg/μl)
α-MAG	1:1,000
α-TfR	1:1,000
α-Actin	1:4,000
SECONDARY ANTIBODY	FINAL DILUITION
α- rabbit IgG	1:50,000
α- mouse IgG	1:50,000

3.11 mRNA extraction, reverse-transcription PCR, and real-time PCR

Total RNA was purified from tissue, platelets or cell cultures using the RNeasy Plus Mini Kit (Qiagen) in accordance with the manufacturer's instructions. One microgram of RNA was transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen), and aliquots (1 μL) of the total cDNA were amplified in 40 cycles of PCR prepared using TopTaq Master Mix Kit (Qiagen). The primers are indicated in Supplementary Table 1. The DNA fragments were then analyzed by means of agarose gel electrophoresis. The target sequences for the quantitative, reverse-transcription, real-time PCR were amplified from 1 μg cDNA; for the quantitative analysis of gene expression, we used the ABI Prism® 7000 Sequence Detection System, SDS software version 1.2.3 (Applied Biosystems, CA, USA). The target sequences were amplified using a pre-programmed thermal profile of enzyme activation at 50°C, followed by 40 identical cycles of denaturation at 95°C for 15 s, and annealing and amplification at 60°C for 1 min, conditions previously determined by Applied Biosystems as optimal. The TaqMan® primer and probe assays used were *P2Y2* (Mm00435472_m1), *GPR17* (Mm02619401_s1) and the endogenous control glyceraldehyde-3 phosphate dehydrogenase (GADPH: Mm99999915_g1). Control reactions performed in the absence of Quantiscript reverse transcriptase (Qiagen) excluded the possibility of genomic traces. The results were calculated using the $2^{-\Delta\Delta Ct}$ method, allowing normalization to GAPDH and the calibrator set to a value of 1 (Livak and Schmittgen, 2001).

3.12 Endocytosis Analysis

Live cells antibody labeling. Cells were plated at a concentration of 75,000 cells/cover slip, allowed to attach for 24 h and then were differentiated for 72h using neuronal CM. In internalization experiments cells were chilled at 4°C, medium was removed and the cells were washed once in ice-cold PBS with 0.1 mM $CaCl_2$,1 mM MgCl and 1 % Horse serum (HS, GIBCO). Cells were incubated at 4°C for 45 min with rabbit anti Nt-GPR17 antibody (10 μg/ml) diluted in ice-cold PBS with 0.1 mM $CaCl_2$,1 mM MgCl and 1 % HS. At the end of antibody incubation cells were washed 5 times with PBS with 0.1 mM $CaCl_2$,1 mM MgCl and 1 % HS and treated in Sato medium with or without agonists and/or reagents (UDP-glucose 100mM; LTD_4 500μM; sucrose 1M). Cells were incubated at 37°C for various times (8, 12, 15, 30 min). At the end cells were chilled at 4°C to block endocytosis and washed first in PBS with 0.1 mM $CaCl_2$,1 mM MgCl and then 3 times with HBSS with 0.1 mM $CaCl_2$,1 mM MgCl and 50mM glycine, pH2.5 to remove excess antibody. Before fixation with paraformaldehyde cells were washed again with PBS with 0.1 mM $CaCl_2$,1 mM MgCl and then processed for immunofluorescence.

Biotinylation assays. For biotinylation experiments it was used sulfosuccinimidyl 2-(biotinamido) ethyl- 1,3'dithiopropionate (EZ-Link™ Sulfo-NHS-SS-Biotin, Thermo Scientific), a reagent that is cleavable, water-soluble and is cell membrane impermeable due to its sodium sulfonate group. Sulfo-NHS-SS-Biotin has a long chain spacer arm separating the biotin group from the amino-reactive sulfo-NHS ester moity. The resulting biotinylated protein can then be detected when used in conjunction with streptavidin. It is possible to cleave the biotin group from the crosslinked target protein under reducing conditions, such as in the presence of 2-mercaptoethanol or DTT. Oli-neu cells were incubated in neuronal conditioned medium for 48 or 72 h, and then biotinylated using 0.3 mg/ml of. dissolved in PBS with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 30 min at 4°C. The labeled cells were washed three times for 10 min with 50 mM glycine in TBS (25 mM Tris, 85 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) to quench free biotin. When required, the cells were subsequently incubated in Oli-neu medium in the absence or presence of the drugs for the times indicated in the results. After a 4°C wash with ice-cold PBS with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, the remaining surface biotin labeling was removed by incubating the cells twice at 4°C with 50 mM dithiothreitol (DTT, Gerbu, Gaiberg, Germany) in PBS with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 15 min each time. DTT was neutralized with iodoacetamide (10 mM) in PBS with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, and the cells were then lysed in buffer A

containing 1% Triton X-100 and a protease inhibitor cocktail. After centrifugation (20,000 *g* for 20 min at 4°C), supernatants containing equal amount of protein were incubated with streptavidin beads to immunoprecipitate the biotinylated proteins (Martin and Henley, 2004; Winterstein et al., 2008). Following extensive washes in extraction buffer, proteins were eluted from the streptavidin beads by heating at 65 °C in Laemmli sample buffer and then analyzed by SDS-PAGE followed by Western blotting as descrbed above using antibodies against GPR17 (1:1,000) or transferrin receptor (1:1,000). Actin biotinylation was analyzed to ensure that there was no biotinylation of intracellular protein.

3.13 Immunofluorescence

The cells were fixed for 8-10 min at 37°C with 4% paraformaldehyde in phosphate buffer, pH 7.3, containing 4% sucrose and washed three times with PBS at room temperature. Cells were permeabilized for 5 min at room temperature in PBS containing 0.3% TritonX-100 , washed twice in glycine 0.1 M pH 7.4 for 15 minutes and, at the end, were rapidly washed with IF buffer before incubation with the primary antibody (2h).

PRIMARY ANTIBODY	FINAL DILUITION
α-GPR17	1:300
α-Lamp1	1:100
α-MAG	1:200
α-NG2	1:100
α-TfR	1:100
α-MBP	1:200

After 2 h cells were washed with IF buffer and incubated for 45 min with the anti-rabbit or mouse IgG secondary antibody, diluted 1:200, conjugated to a phluorofore. At the end coverlips were washed 5 times for 5 minutes with IF buffer followed by 2 washes in PBS that was finally removed by dipping in H_2O before mounting on mowiol. After immunostaining the images were recorded using an MRC-1024 laser-scanning microscope (Bio-Rad) equipped with a 60x objective. In order to compare the double-

stained patterns, the images from the fluorescein or rhodamine channels were acquired separately from the same areas, and superimposed. The images were processed using Photoshop (Adobe Systems, Mountain View, CA, USA).

PBS pH 7.4	IF Buffer	Mowiol (50 ml)
NaCl 137 mM	NaCl 300mM	Glicerol 6 g
KCl 3 mM	Gelatin 0.2%	Mowiol 2.4 g
Na_2HPO_4 x2H_2O 8mM	PBS	
NaH_2PO_4 xH_2O 2mM		

3.14 Image analyses and quantitation

We quantified the internalization of GPR17 in Oli-neu cells by measuring immunofluorescence intensity of the anti-Nt-GPR17 in cells before (time 0) or after incubations at 37°C followed by washes with glycine-buffer to strip the antibody bound at the cell surface (see Results). At least three independent experiments were performed and in each experiment about 180 cells were examined for condition. All images were recorded using identical parameters with an MRC-1024 laser-scanning microscope (Bio-Rad). Six serial sections were acquired for field and images were exported as TIFF files for analysis NIH Image J software. Values are expressed as fold increase over the intensity detected in the cells at time 0 (value assigned = 1). In order to assess co-localization of internalized GPR17 with Rab5 or Rab11, we analyzed sequentially acquired confocal sections from selected cells expressing similar levels of either GFP-Rab5a or GFP-Rab11a as revealed by the fluorescence intensity. The images were then processed using the Image J program equipped with a public domain co-localization plug-in (JACoP; Bolte and Cordelieres, 2006). The thresholds for each couple of images were estimated from the cytofluorograms as suggested by Cordelieres and Bolte[1]. The Menders' coefficients, giving an estimate of the amount of colocalizing signal from a channel over on other, were expressed as the percentage of co-localization between the red channel (anti-NtGPR17-antibody) and the green channel corresponding to either Rab5 or Rab11. The pooled results were expressed as mean values ± SEM.

Note 1: The conference proceedings he conference proceedings related the plugin available in http://imagejdocu.tudor.lu/lib/exe/fetch.php?media=plugin:analysis:jacop_2.0:just_another_colocalization_plugin:jacop_ijconf2008.pdf.

3.15 Primary mouse OPC culture

Primary cortical mixed cultures, also containing OPCs, were obtained from P3 mice, as described by Heins et al. (2002) with minor modification. Postnatal mice were anesthesised in ice and the heads were cut and transfered for dissection in PBS with 0.6% glucose . All meninges were removed and the cortices, finely chopped, were collected in cold Hank's Balanced Salt Solution HBSS with 0.1 mM CaCl, 2.1 mM MgCl (Sigma-Aldrich, Milan, Italy) and pelleted. The slices were incubated for 15 min at 37°C with 0.05% Trypsin, 0.025% EDTA and DNAse (1 μg/μl) and then centrifuged 10 min at 1,000 rpm. The pellet was resuspended in 10 ml basic medium (Dulbecco's Modified Eagles Medium plus F12 nutrient, DMDM-F12) containing 10% fetal bovine serum (FCS) and 5% horse serum (HS) both inactivated for 30 min at 56 °C. About $6x10^6$ cells were plated onto T75 flask pre-treated with poly-L-lysine (1.5 mg/ml) and were grown in basic medium for 7-8 days. Partially purified OPCs were isolated from the flasks by means of shaking as previously described in detail (Chen et al., 2007), and plated (about 7,000 cells) onto poly- L- lysine coated glass coverslips (13 mm diameter). After one day the cells were switched to Sato medium containing 1% horse serum (Invitrogen, Italy) and were analysed after 3 days of differentiation.

4. RESULTS

4.1 Endogenous expression and biochemical characterization of GPR17 in Oli-neu

First, we analyzed the expression of GPR17 in Oli-neu cells by confocal immunofluorescence microscopy after staining with an antibody directed against the C-terminal of GPR17 (anti-Ct-GPR17; Ciana et al., 2006) or MAG. Undifferentiated Oli-neu cells are mainly bipolar or have few cell processes and only very few cells (less than 5%) show some immunoreactivity for GPR17 (Fig. 1A, -CM). In order to promote their differentiation to a more mature phenotype and possibly modulate the expression of GPR17, we used various agents and culturing conditions. Many were ineffective or induced GPR17 expression inefficiently (db-cAMP and UDP glucose, data not shown) but Oli-neu cells showed rapid and substantial differentiation accompanied by the expression of GPR17 when they were cultured in the presence of a medium collected from primary cultures of cortical neurons (CM). After 10 h incubation in CM, immunoreactivity for GPR17 was already detected in an increasing number of Oli-neu cells. At this stage, the receptor was mainly localized in the biosynthetic pathway as demonstrated by colocalization with GS-28 (a SNARE protein localized in the Golgi cisternae; Fig. 1A, +CM 10 h). Few hours later (+CM 24 h) a considerable number of GPR17-positive cells were detected and after 48h about 60% of cells were intensely labeled. At this stage, GPR17-positive cells showed an increased number of cell processes and some of them started to co-express the myelin protein MAG (Fig. 1A +CM 48 h). To confirm the specificity of the immunostaining for GPR17, 48 h-differentiated cells were transfected with a short interfering (si) RNA specific for the silencing of mouse GPR17. As shown in Figure 1A, GPR17 labeling was largely reduced upon treatment with the specific siRNA (siRNAGPR17) but remained unchanged when the cells were transfected with a non-specific (scrambled) siRNA (siRNAsc). In addition we observed that after GPR17 knockdown few cells show differentiation with low amount of MAG and very few cell processes, il line with previous observations (Fumagalli et al., 2011).

In order to provide the first, previously unreported biochemical and molecular characterization of native GPR17 in OPCs, Oli-neu cell proteins were analyzed by Western blotting. The anti-Ct-GPR17-antibody recognized two polypeptides with a M_r of ~48 kDa and ~38 kDa only in differentiated cells (Fig. 1B). Expression of these two bands was largely reduced upon receptor knockdown with siRNA as shown by Western blotting and quantitative analysis (Fig. 1C and 1D). A band of ~46 kDa was also immunodetected in extracts from cultured rat OPCs (Fig. 1E), and inhibited by the

expression of a specific rat GPR17 siRNA. On the contrary, a polypeptide of ~57 kDa, which was recognized by the anti-GPR17-antibody in rat OPC cultures as well as in differentiated or undifferentiated Oli-neu cells, was not affected by GPR17 knock down, thus suggesting that it was a non-specific signal. We concluded that the mature receptor has a M_r of 48 - 46 kDa whereas the band of ~38 kDa detected in Oli-neu represents a precursor form of GPR17.

This conclusion was also supported by two different observations (Fig. 2). First, when the synthesis of GPR17 was analyzed in differentiated Oli-neu cells in pulse-chase experiments with ^{35}S-Met/Cys, two major ^{35}S-labeled polypeptides of 48 kDa and 38 kDa were immunoprecipitated with the anti-Ct-GPR17 antibody from 90 min labeled samples, while after a 5 h-chase, the 38-kDa polypeptide was not any more isolated, suggesting that it corresponds to a precursor form of the receptor converted to the mature receptor during the chase experiment (Fig. 2A, B). Second, when anti-Ct-GPR17 immunoprecipitates from Oli-neu cell extracts were digested with endoglycosidase F (to remove all N-linked carbohydrates) the polypeptides of 48 kDa and 38 kDa were both converted to a single band of 33 kDa (Fig. 2C). On the other hand, the 48-kDa band remained unchanged after endoglycosidase H digestion (that only removes high-mannose N-linked carbohydrates); conversely, the 38 kDa band diminished and a new band of 33 kDa appeared, thus confirming that the 38-kDa polypeptide is a precursor form of GPR17 carrying high mannose oligosaccharides chains. On the basis of these results, we also concluded that GPR17 is mainly an N-linked glycoprotein (Fig. 2C).

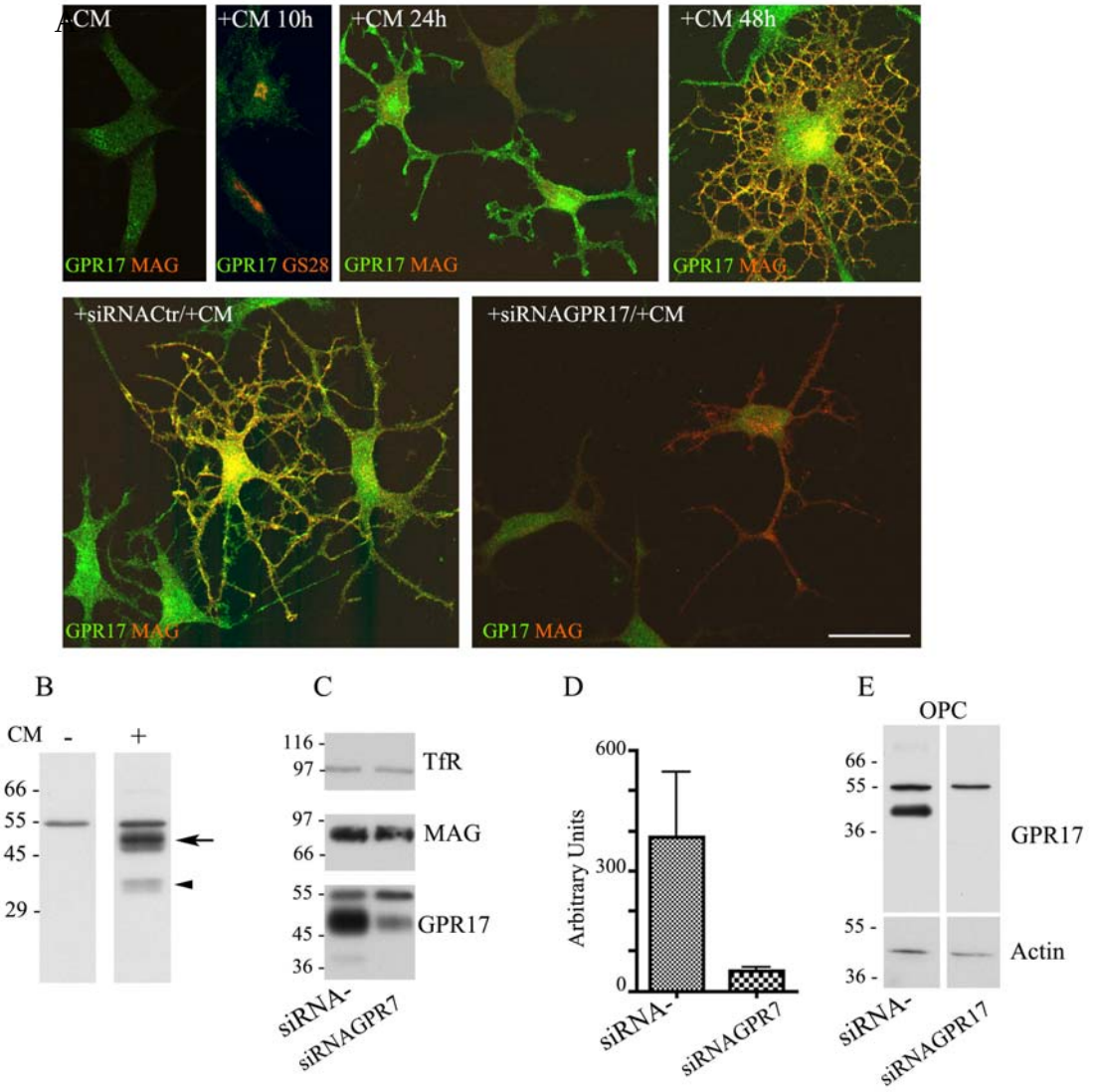


Figure 1. **GPR17 expression in Oli-neu cells**. **A**. Oli-neu cells were cultured in Sato medium (-CM), conditioned medium (+CM) for 10h, 24h or 48h, or transfected with either scrambled siRNA (+siRNAsc) or with a specific siRNA targeted against GPR17 (+siRNAGPR17) and then incubated in CM for 48 h (+CM). Cells were fixed and double immunolabeled with an affinity anti-Ct-GPR17 (green) antibody and a monoclonal antibody against GS28 or MAG (red), followed by fluorochrome-conjugated secondary antibodies. Images were recorded using a confocal microscope; merged images selected from four independent experiments are shown. Note the morphological changes and the increase of GPR17-labeling in Oli-neu maintained in Sato medium supplemented with CM. Immunoreactivity for GPR17 was largely reduced upon cell transfection with a specific siRNA against GPR17. Scale bar = 20 μm. **B**. Total membrane extracts (30 mg proteins) of Oli-neu cultured in Sato medium alone or supplemented with CM (indicated as CM - or +) were analyzed by western blotting using the anti-GPR17 antibody. The arrow and arrowhead indicate, respectively, bands of 48 kDa and 38 kDa specifically immunodetected in differentiated cells. **C**. Total membrane extracts (30mg/proteins) of Oli-neu cultured for 48 h in CM with scrambled siRNA (siRNAsc) or siRNA targeted against mouse GPR17 (siRNAGPR17) were immunoblotted-using antibodies against TfR, MAG or GPR17. Note reduction of the 38 kDa and 48 kDa bands after GPR17 knockdown. **D**. Quantitative analysis of the intensity of the 48-kDa bands detected in western blots in control and silenced cells: the data are expressed as arbitrary units and represent the mean values of three independent experiments. **E**. Total extracts from rat primary cultured OPCs transfected with either scrambled siRNA (siRNAsc) or a siRNA designed for silencing rat GPR17 (siRNAGPR17) were examined by immunoblotting using anti-GPR17 or actin antibodies. Note reduction of a specific 48 kDa band corresponding to the receptor protein but not of a non-specific band at 57 kDa after GPR17 knockdown.

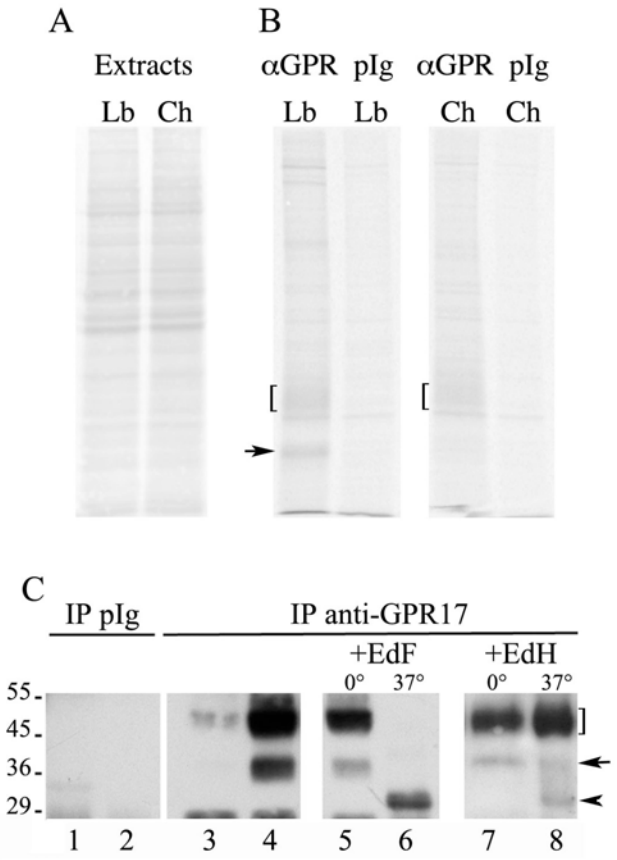


Figure 2. Synthesis and biochemical characterization of GPR17. **A.** Phosphoimager analysis of gels of total proteins from 72h differentiated Oli-neu cells labeled for 90 min with [^{35}S] Methionine/Cysteine (L) or "chased" (Ch) for 5h in medium supplemented with cold methionine **B.** Immunoprecipitates with anti-Ct-GPR17 (aGPR) or preimmune rabbit IgG (pIg) from aliquots (100 mg proteins) of cell extracts from labeled (Lb) or 5 h "chased" (Ch) samples. Square brackets = 48 kDa mature GPR17; arrow = 38 kDa immature form. **C.** GPR17 glycosylation. Proteins (100 mg) from Triton X-100 extracts of undifferentiated (lanes 1 and 3) or 72 h-differentiated (lanes 2, 4-8) Oli-neu were immunoprecipitated (IP) with anti-GPR17 or preimmune rabbit IgG (pIg). Before western blotting analysis, samples of lanes 5-8 were incubated with endoglycosidase F (EndoF) or H (EndoH) at 37 or 0 °C. Square bracket = 48 kDa band, mature GPR17; arrow = 38 kDa band, immature GPR17; arrowhead= 33 kDa band unglycosylated GPR17.

4.2 GPR17 expression is developmentally regulated in Oli-neu

Next, we investigated the expression of GPR17 and a myelin protein in Oli-neu cells at various stages of differentiation and found that GPR17 expression preceded that of the myelin proteins MAG and MBP. As shown in Fig. 3, strong immunoreactivity for GPR17 was detected in a significant number of cells (about 80%) after 48-72 h incubation in CM. On the other hand, the expression of GPR17 declined at later time and almost no GPR17-highly positive cells were detectable after 96-120 h incubation in CM (Fig. 3). On the contrary, the number of cells expressing detectable level of myelin proteins increased after 72 h of incubation in CM and after 96-120 h almost 80% of the cells were highly positive for MAG or MBP (Figs. 1, 3 and 4). These cells were in a resting phase, as shown by the absence of immunoreactivity for the Ki-67 protein, a marker of cell proliferation (data not shown).

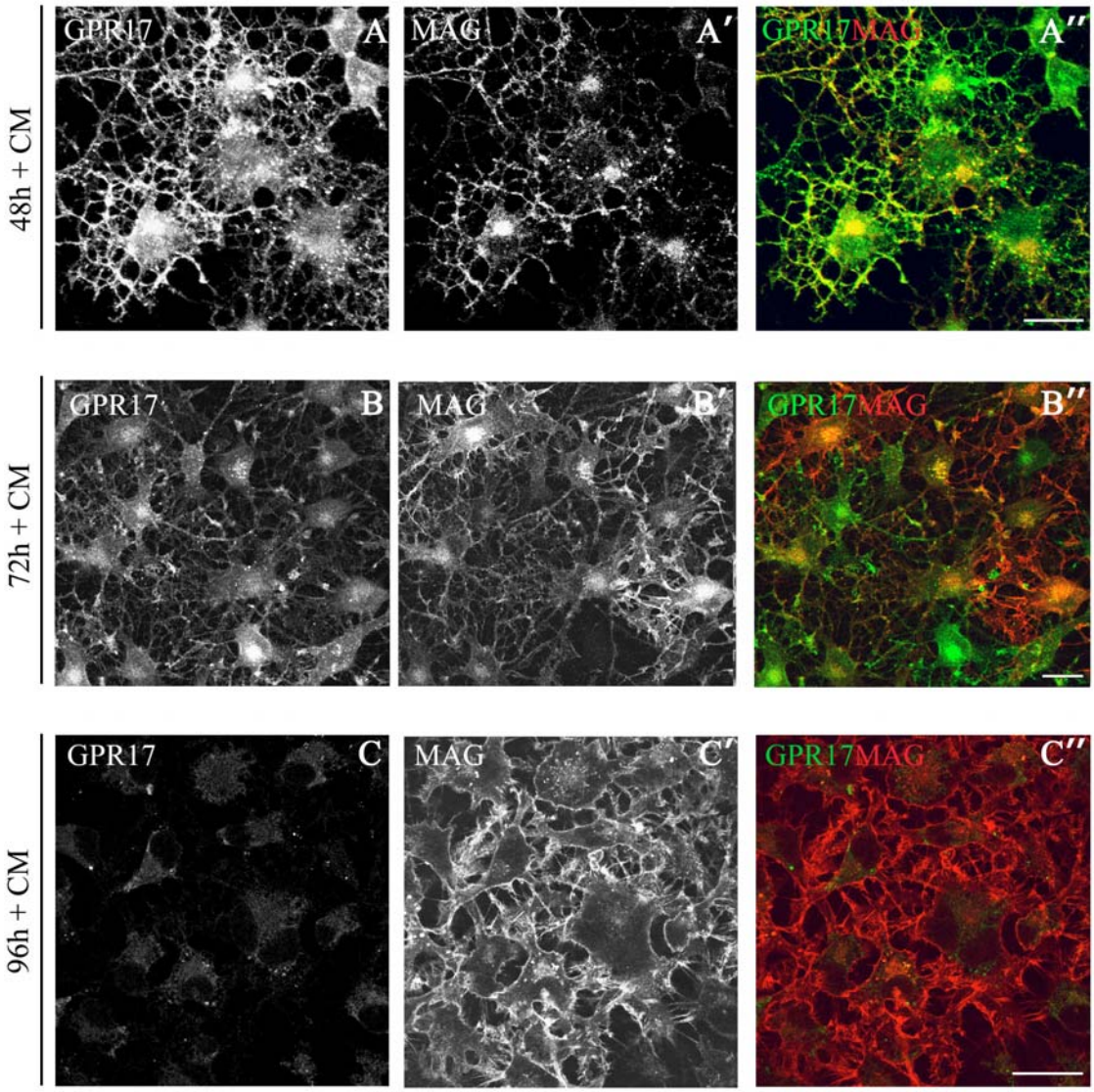


Figure 3. GPR17 in differentiating Oli-neu is time regulated. Oli-neu were cultured in CM for 48h, 72h and 96h (+CM) and then fixed and double immunolabeled with antibodies against GPR17 (A-C) or MAG (A'- C'). Images were recorded using a confocal microscope; double labeled fields and superimposed images (A''- C'', merges) are shown. Selected images from four independent experiments are shown. Scale bars = 20μm.

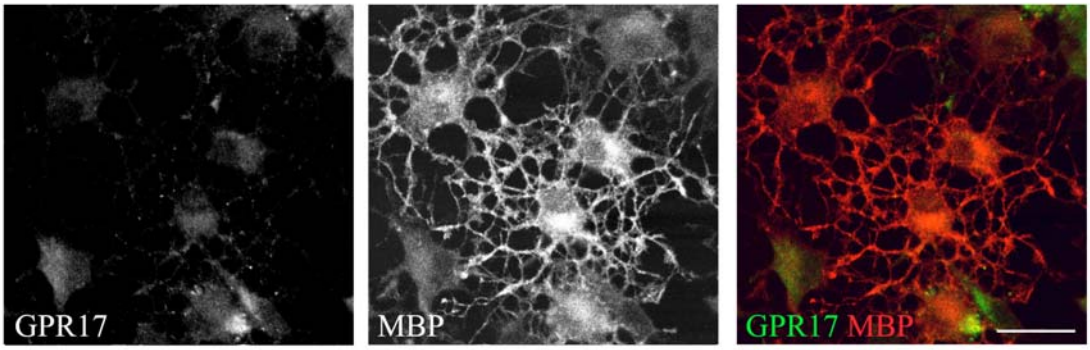


Figure 4. GPR17 and MBP immunoreactivity in differentiated Oli-neu. Cells were cultured with CM for 96 h and then fixed and double immunolabeled with the affinity purified anti-GPR17 antibody (green) and a monoclonal antibody against MBP (red). Images were recorded using a confocal microscope; selected double-labeled fields and superimposed images (merge) from two independent experiments are shown. Whereas GPR17 is barely detected, a large amount of MBP is detected in differentiated cells. Scale bar = 20 mm

The differences of GPR17 and MAG expression were also confirmed by Western blotting followed by quantitative analysis. As shown in Figures 5A and 5B, the peak of GPR17 protein preceded that of MAG and then rapidly declined, whereas the level of myelin protein continued to increase for up to 96 h. Notably, neurobasal medium supplemented with B27 (the medium used to culture rat cortical neurons) was less efficient in promoting GPR17 synthesis and Oli-neu differentiation (Fig. 6).

The changes in GPR17 protein expression after incubation with CM were consistent with changes in the level of GPR17 mRNA during Oli-neu cell differentiation as revealed by real-time PCR. As shown in Figure 5C, the mRNA coding for GPR17 was up-regulated during differentiation and increased 10-fold after 48 h of incubation with CM. In line with the protein levels, GPR17 messenger levels declined rapidly and returned to a lower level after 96 h. The mRNA levels of most of the other members of purinergic or CysLT receptor families detected in Oli-neu cells (i.e. $P2Y_1$, $P2Y_2$, $P2Y_{14}$ and, to a lesser extent, $P2Y_4$, $P2Y_6$ and $CysLT_2R$) did not significantly change after treatment with CM (Fig. 5D). The only exception was $P2Y_2$, whose mRNA increased upon incubation with CM and remained consistently high (Fig. 5C). Notably, $P2Y_{12}$ and $CysLT_1R$ mRNAs were undetected at each time.

Altogether these data indicate that Oli-neu cells can be induced to express consistent amount of GPR17 in a time regulated manner, as it has been observed in primary OPC culture (Lecca et al., 2008; Fumagalli et al., 2011). Accordingly, Oli-neu cultured for 60-72 hr in CM were selected to investigate the endocytic trafficking of native GPR17 upon agonist exposure.

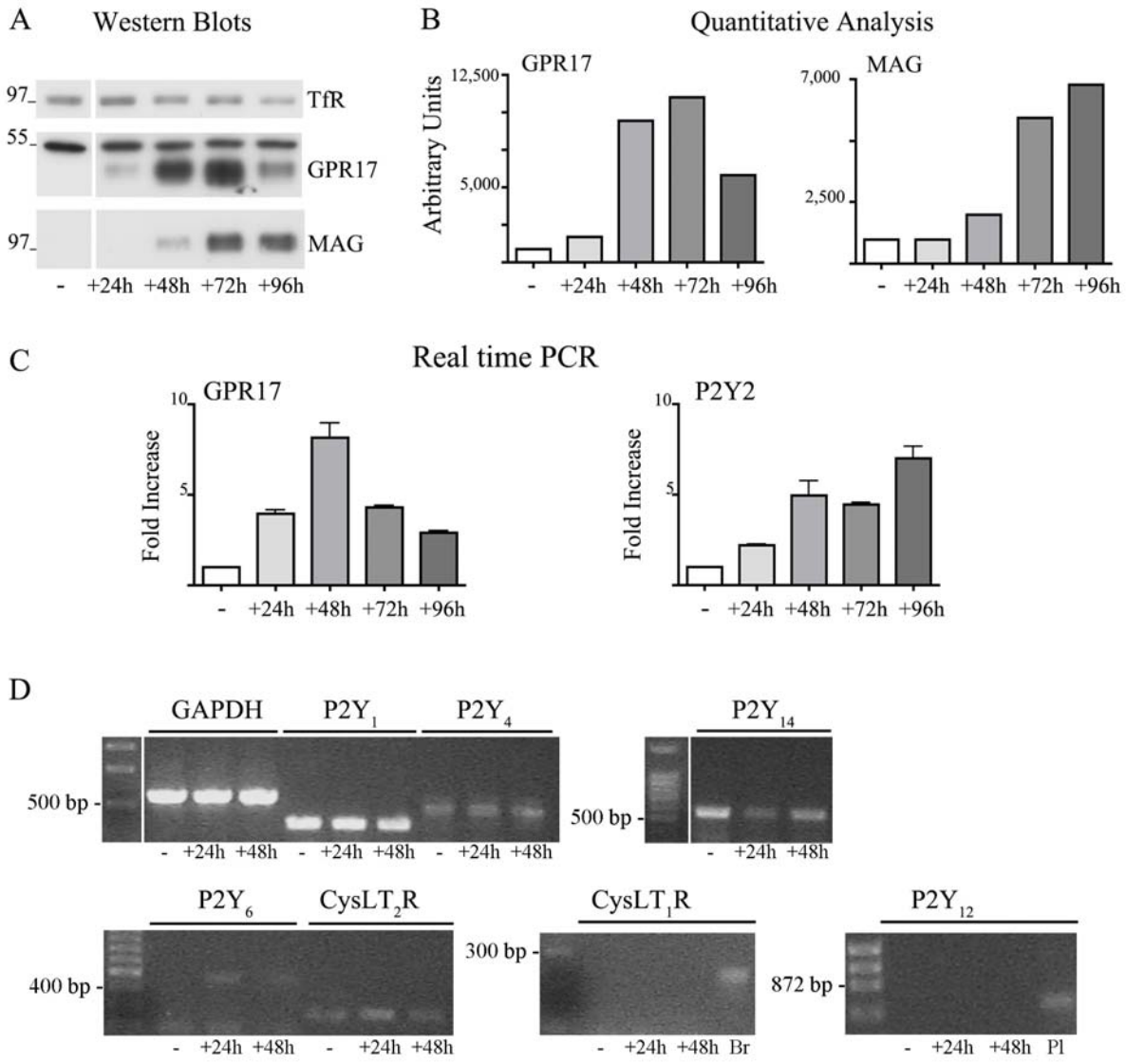


Figure 5. Neuron conditioned medium modulates the expression of GPR17 in Oli-neu.
A. Cell extracts from Oli-neu cultured either in Sato medium (-) or in medium supplemented with CM (+) for 24h, 48h, 72h or 96h were analyzed by western blotting using antibodies against the TfR, GPR17 or MAG. **B.** Quantitative analysis of western blots from two independent experiments; intensity of the bands measured with the ImageJ program is given as arbitrary units. **C.** Real time PCR analysis of GPR17 and of $P2Y_2$ gene expression in Oli-neu cultured in Sato medium (-) or exposed to CM (+) for 24h, 48h, 72h or 96h. GPR17 or $P2Y_2$ mRNA levels are relative to the housekeeping gene GAPDH and are expressed as fold increase with respect to the mRNA levels (=1) detected in Oli-neu maintained in Sato medium alone. Data are from 3 independent experiments and represent the means ± s.e.m. **D**. Reverse transcription-PCR amplification performed with specific primers, using 1 µg of RNA extracted from Oli-neu cultured in Sato medium alone (-) or in medium supplemented with CM (+) for 24h or 48h. RNA from brain (Br) and mouse platelet (Pl) was used as positive controls, as indicated and detailed in Experimental Procedures. GAPDH was used as an internal standard. The final products were separated on agarose gel (5 µl of the reaction mixtures were loaded on each lane). Bands revealed with ethidium bromide correspond to the predicted sizes of amplified DNA fragments.

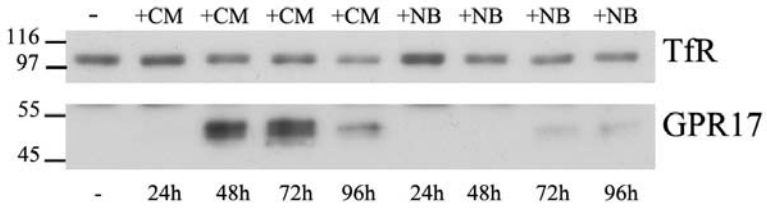


Figure 6. Changes in the expression of GPR17 in Oli-neu cultured in Neuron-conditioned medium or Neuronal medium. Oli-neu were cultured in Sato medium (-), or in Sato medium supplemented either with neuron conditioned medium (CM+) or with neuronal basal medium/B27 for 24h, 48h, 72h or 96h. Cell extracts (30 mg proteins) were analyzed by western blotting using antibodies against TfR or GPR17.

4.3 Endocytosis of native GPR17 in Oli-neu

To determine the endocytic fate of native GPR17, we employed protocols for tracking cell surface receptor using antibodies directed against extracellular epitopes. To this end, we raised an antibody directed against the extracellular N-terminal domain of GPR17 (anti-Nt-GPR17) in rabbits, and tested the antisera for their ability to recognize the receptor on the plasma membrane of living cells. As shown in Figure 7A, after incubation at 4 °C, the anti-Nt-GPR17 antiserum decorated the cell surface of 48 h-differentiated Oli-neu cells. Immunolabeling was either not observed on undifferentiated cells (data not shown) or was abolished by antiserum preadsorption with the antigen, confirming the specificity of the antibody (Fig. 7B). In addition, antibody labeling was completely abolished by washes with glycine buffer, pH 2.8 (acid wash), demonstrating that the antibody was exclusively localized on the cell surface after labeling at 4°C (Fig. 8).

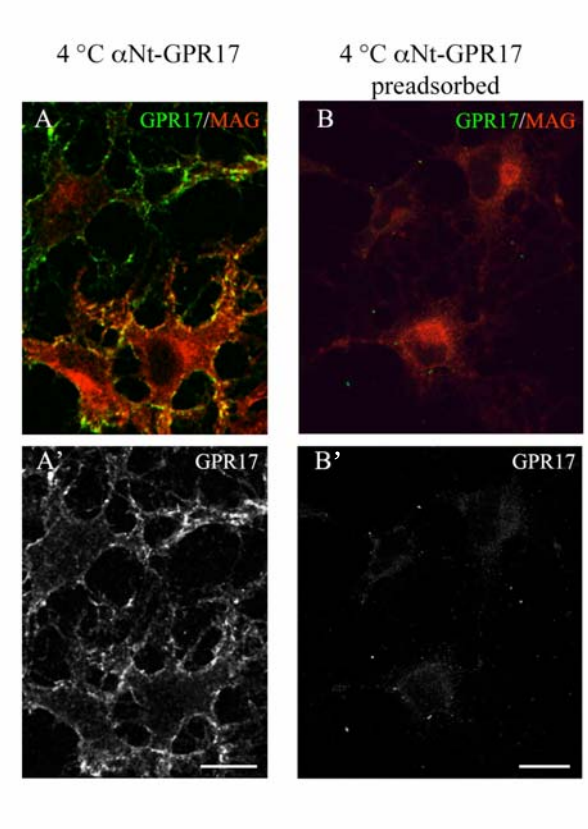


Figure 7. Anti-Nt-GPR17 antibodies label native GPR17. Cells were cultured with CM for 48 h and then incubated 45 min at 4 °C with the rabbit anti-Nt-GPR17 antiserum without or with preincubation with the antigen. After washing cells were fixed and labeled with a monoclonal antibody against MAG followed by incubation with anti-mouse IgG conjugated to fluorescein and with an anti-rabbit IgG conjugated to Cy3 to reveal the distribution of the anti-GPR17 antibody (GPR17). Note that the anti-Nt-GPR17 decorates the surface of cells that do not express or express little amount of MAG. Immunostaining for GPR17 was completely abolished by preincubating the antiserum with the peptide used for rabbit immunization. The merges are shown in A, B; GPR17 labeling is shown in A', B': Bars = 10 μm.

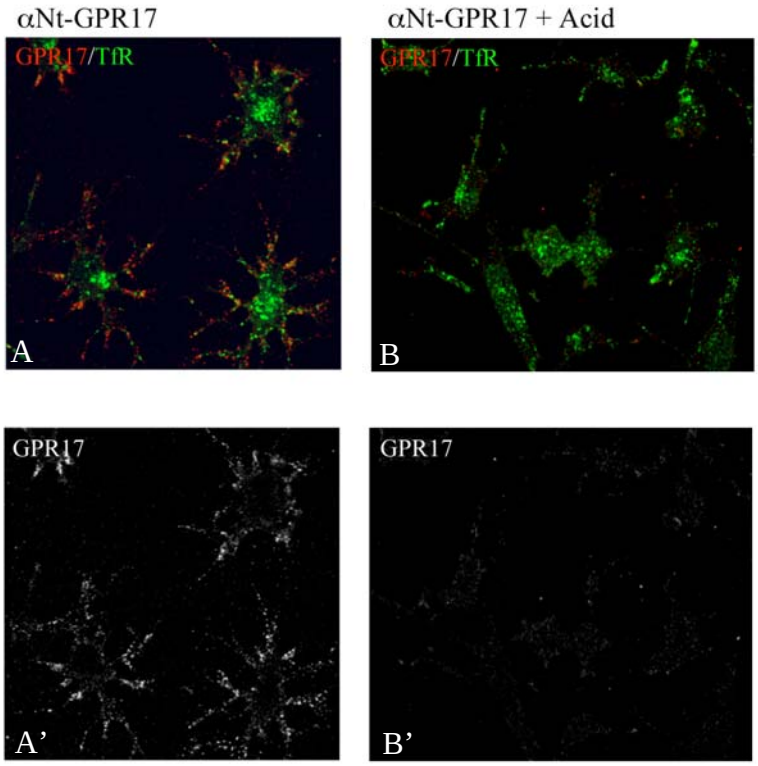


Figure 8. Labeling at 4 °C with anti-Nt-GPR17 is largely reduced by acid wash. Cells cultured with CM for 64 h were incubated 45 min at 4 °C with the rabbit anti-Nt-GPR17, washed three times with glycine buffer, pH 2.8, fixed and then labeled with a monoclonal antibody against TfR followed by incubation with anti-rabbit IgG conjugated to Cy3 and anti-mouse IgG conjugated to fluorescine. Immunostaining for GPR17 was almost abolished by acid wash confirming the distribution at the cell surface of the anti-Nt GPR17 upon incubation at 4°C. The merged images are shown in A, B; GPR17 labeling is shown in A', B'.

To perform internalization assays, after labeling with the anti-Nt-GPR17 antibodies, differentiated Oli-neu cells were incubated at 37 °C with or without agonists. In this study, we used micro molar concentrations of uracil nucleotides (100 μM UDPglucose) and nanomolar concentrations of CysLTs (50 nM LTD_4) as these agonist concentration were previously shown to activate the receptor in OPC primary cultures (Fumagalli et al., 2011). After incubation at 37 °C, cells were fixed, permeabilized and immunolabeled using monoclonal antibodies directed against MAG, TfR or Lamp1 followed by species-specific secondary antibodies directed against rabbit, mouse or rat IgG. According to confocal images, at early time points (4 min at

37 °C), labeling for the anti-Nt-GPR17 was still largely detected at the cell surface. Interestingly, labeling for GPR17 appeared more clustered in Oli-neu incubated in the presence of agonists suggesting a more efficient recruitment of the receptor in endocytic patches and vesicles upon stimulation (data not shown). At later times (8, 12 min at 37 °C), significant amounts of GPR17 appeared concentrated in the cytoplasm of Oli-neu cells. In these experiments, we traced the endocytic route of GPR17 by double labeling with antibody against protein markers of early/recycling endosomes or lysosomes and we performed also a quantitative analysis of the receptor internalized in the different conditions (Figs. 9-12). In order to allow a cleaner localization of the internalized receptor, after incubations at 37 °C, cells were acid-stripped in order to abolish the remaining cell surface antibody labeling. As shown in Figure 9, after 8 min, internalized GPR17 partially colocalized with TfR in small vesicles near the plasma membrane, whereas at later times (12 min), an aliquot of GPR17 was present in larger dot-like structures, some of which were labeled for the lysosome marker Lamp1 (Fig. 10). This was more evident in UDP-glucose stimulated cells with respect to LTD_4 treated or untreated cells. We next performed a quantitative analysis of internalized receptor by measuring the intensity of intracellular accumulated anti-Nt-GPR17 relative to the amount of antibody internalized after labeling at 4 °C followed by acid strip (time 0, intensity = 1). As shown in Figure 12, we found that after 8 and 12 min at 37 ° C, LTD_4 increased the intracellular accumulation of GPR17 of 2.9-3.4 fold over the level of receptor detected at time 0, but an even more pronounced effect (4-4.5 fold increase) was observed for UDP-glucose. A significant intracellular staining for GPR17 (1.7 to 2.1 fold increase over time 0) was also observed in the cells in the absence of stimuli, suggesting that the receptor was also internalized by a constitutive endocytosis. It has been reported that GPCRs may undergo clathrin dependent or independent endocytosis (Doherty and McMahon, 2009). To examine the pathway used by GPR17, after labeling with the antibody, cells were incubated with agonists in the presence of hypertonic sucrose to prevent assembly of clathrin lattices. Our results indicated that intracellular accumulation of GPR17 was largely inhibited by treatment with 0.45 M sucrose (Fig. 11). This was confirmed by quantitative analysis showing that the intensity of anti-GPR17 antibody accumulated in the cells after incubation with agonists was largely reduced in the presence of sucrose (see Fig. 12). This data strongly suggest that receptor is largely internalized by clathrin-mediated endocytosis after both LTD_4 and UDP-glucose stimulation.

4°C labeling + 10’ 37 °C

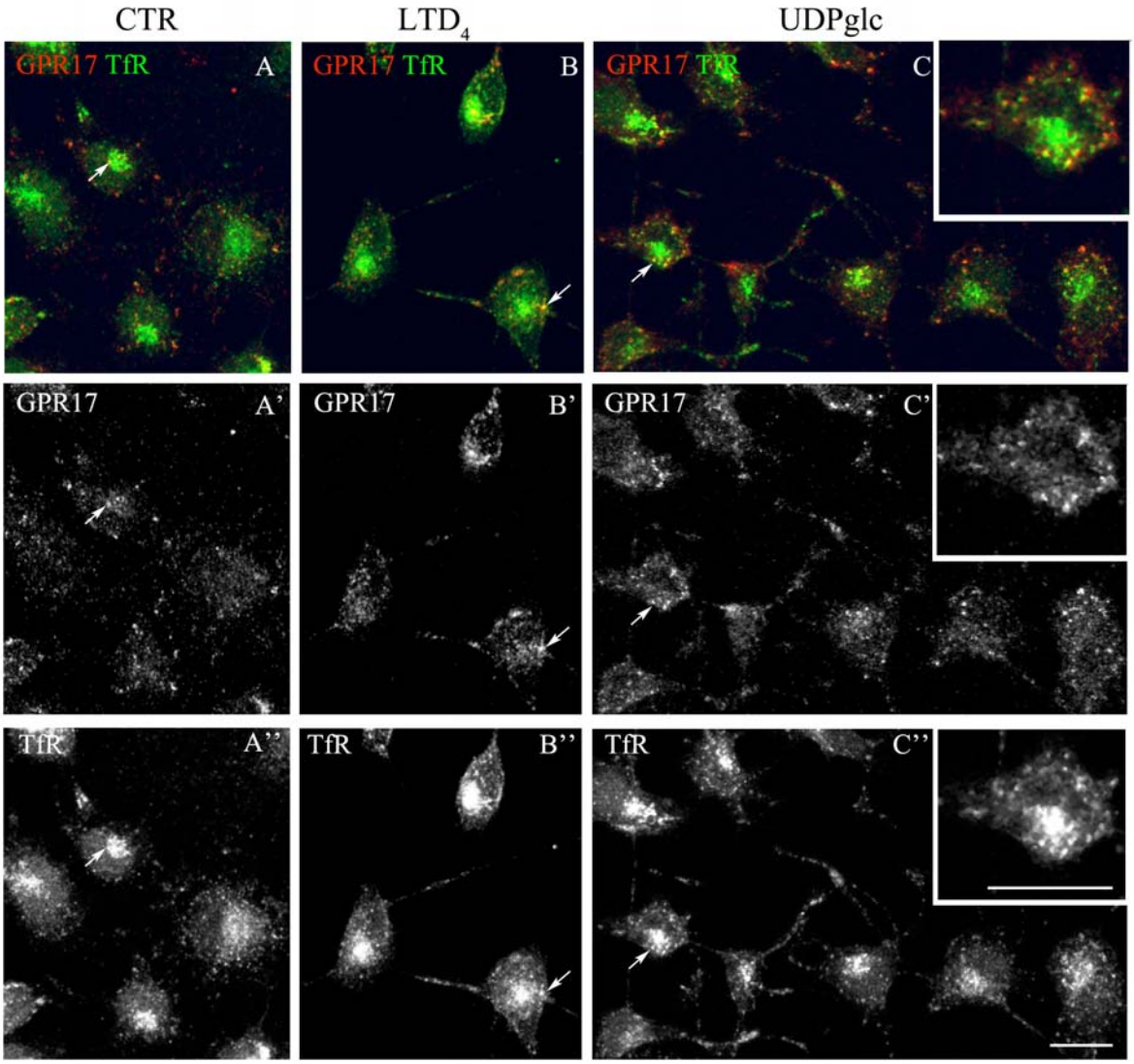


Figure 9. Internalization of GPR17 after 8 min of exposure to agonists. Oli-neu cells cultured 64 h in CM were labeled for 45 min at 4 °C with the affinity purified anti-Nt-GPR17 and then incubated without (control CTR) or with agonists (50 nM LTD_4 or 100 μM UDP glucose, UDPglc). After 8 min incubation at 37 °C, cells were washed with glycine buffer, fixed and immunostained with a monoclonal antibody against TfR followed by incubation with anti-mouse IgG conjugated to fluorescein and anti-rabbit IgG conjugated to Cy3 (GPR17). Confocal microscopy images show immunostaining for GPR17 in dot-like structures dispersed in the cytoplasm. Arrows indicate vesicles labeled for GPR17 and TfR (arrows). Images are representative of several experiments. The merges are shown in A, B, C. Bars = 20 μm

4°C labeling + 10’ 37 °C

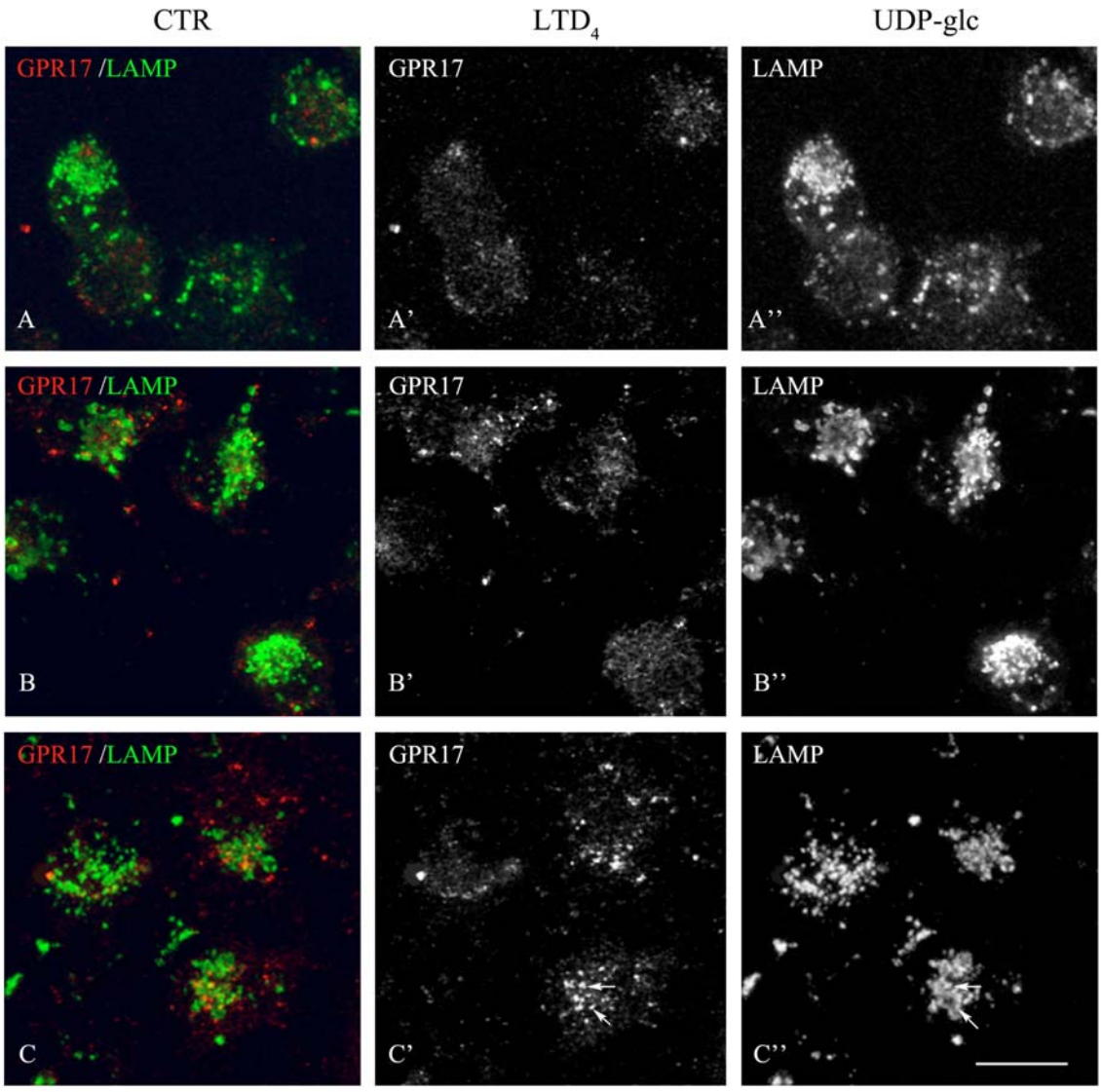


Figure 10. Internalization of GPR17 after 12 min of exposure to agonists. Oli-neu cells cultured 64 h in CM were incubated 45 min at 4 °C with the affinity purified anti-Nt-GPR17 and then incubated 12 min at 37 °C without (CTR) or with antagonists (50 nM LTD_4, 100 μM UDPglc). After washes with glycine buffer, cells were fixed and labeled with a monoclonal antibody against Lamp1 followed by incubation with anti-rabbit IgG conjugated to Cy3 and anti-rat IgG conjugated to fluorescein. Note colocalization of GPR17 with some Lamp1 positive vesicles in UDP-glucose stimulated cells (C’, C’’ arrows). The merges are shown in A-C. Images are representative of several experiments. Bars = 20 μm.

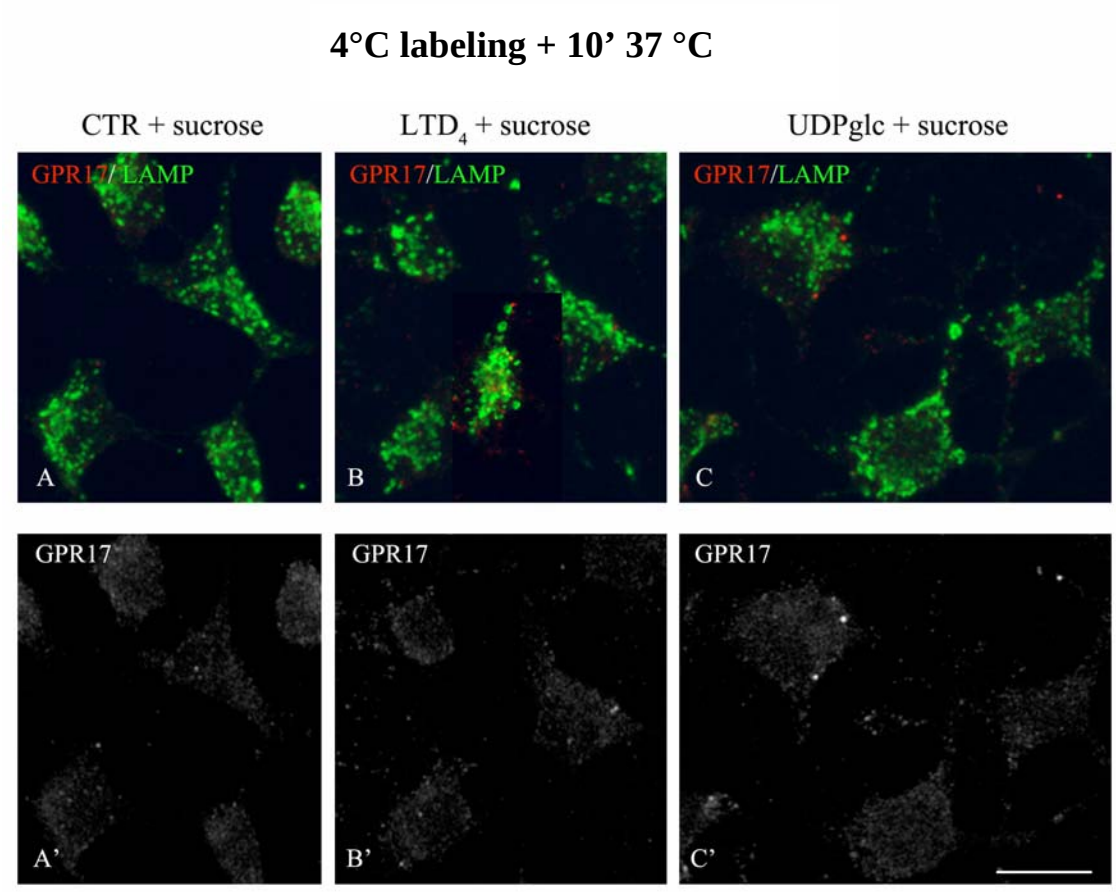


Figure 11. GPR17 is internalized through a clathrin-dependent pathway. Oli-neu cells were labeled for 45 min at 4 °C with the affinity purified anti-Nt-GPR17 and then incubated without (control Ctr) or with agonists in the presence of 450 mM sucrose. After 12 min incubation at 37 °C, cells were washed with glycine buffer, fixed and immunostained with a monoclonal antibody against Lamp1 (LAMP) followed by incubation with anti-rat IgG conjugated to fluorescein and anti-rabbit IgG conjugated to Cy3 (GPR17). Confocal microscopy images show the reduction of GPR17 immunostaining in cells upon hypertonic sucrose treatment. Images are representative of three experiments. The merged images are shown in A, B, C. Bar = 20 μm.

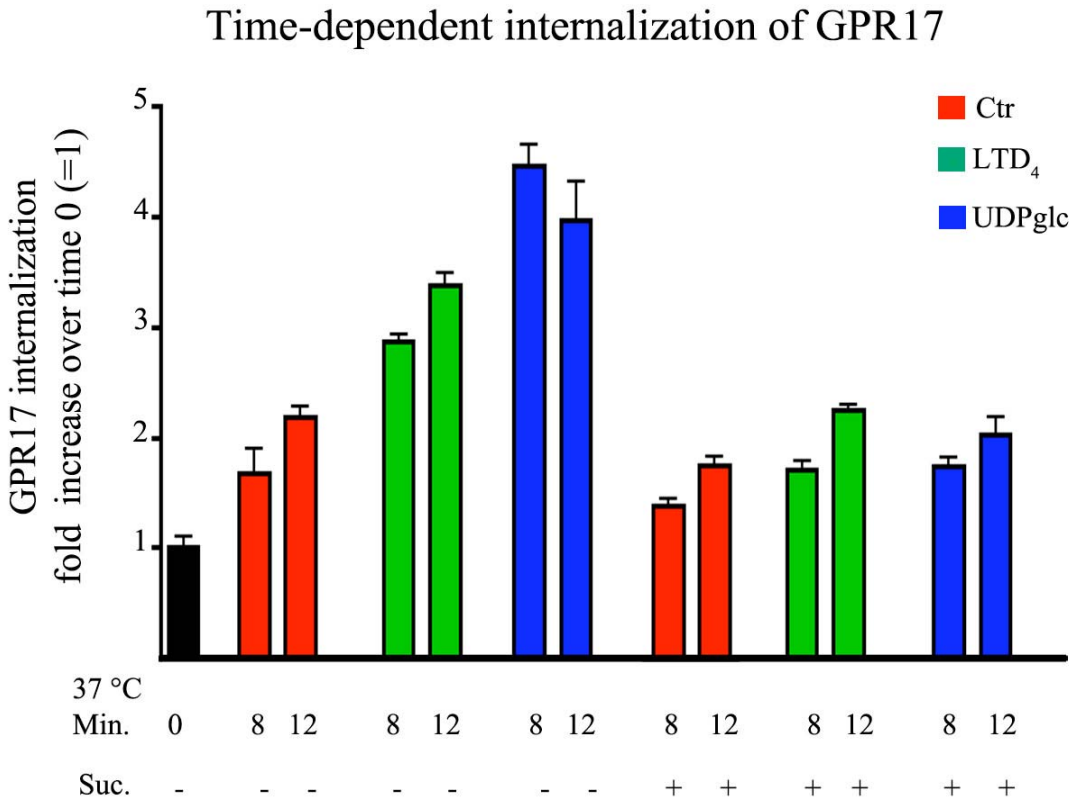


Figure 12. Quantitative analysis of agonist-induced internalization of GPR17. Oli-neu labeling with anti-Nt-GPR17 and agonist treatments were performed as described in Figs. 5 and 6 and 8. Confocal images were collected as described in Materials and Methods and the pixel intensity was determined by ImageJ. The graph represents the fold increase of the intensity of anti-Nt-GPR17 detected in the cells after incubations at 37 °C with respect to the intensity (=1) revealed after labeling at 4 °C followed by acid strip (= time 0). The values are the mean (±s.e.m) of at least three independent experiments. In each experiment about 180 cells were examined for condition. Intracellular accumulation of GPR17 increases over time and is higher in UDP glucose treated cells (UDPglc: 8 min = 4.46±0.20, p=0.0001; 12 min = 3.96±0.36, p=0.0014) than in cells incubated with LTD_4 (8 min = 2.87±0.06, p=0.0001; 12 min = 3.38±0.11, p=0.0001) or without agonists (Ctr: 8 min = 1.68±0.22, p=0.050; 12 min = 2.19±0.09, p=0.001). Hypertonic sucrose (450 mM sucrose) reduces the agonist-induced internalization of GPR17 (Ctr: 8 min = 1.38±0.7; 12 min = 1.76±0.09; LTD_4: 8 min = 1.7±0.08; 12 min = 2.24±0.06; UDPglc: 8 min = 1.73±0.10; 12 min = 2.02±0.17).

We next asked whether the internalized receptor could undergo degradation or recycling. Previous data have demonstrated that a number of Rab proteins regulate the trafficking of endocytosed membrane proteins, including GPCRs (Seachrist and Ferguson, 2003; Platta and Stenmark, 2011; Zerial and McBride, 2011). Internalized receptors are initially targeted to early/sorting endosomes that are enriched in Rab5/Rab4 proteins (Sonnichsen et al., 2000). From there, receptors are sorted to the late endosomes and then to lysosomes for degradation or recycled to the plasma membrane by a Rab11-positive, recycling pathway. To investigate the route of internalized GPR17, we transfected Oli-neu cells with Rab5a (Bucci et al., 1995) or Rab11 (Ullrich et al., 1996) NH2-terminally tagged with EGFP. After 18 hr incubation in CM, Oli-neu were transfected with 0.5-0.2 μg of the appropriate constructs, differentiated for additional 48 h, and then analyzed by confocal immunofluorescence to investigate GPR17 trafficking in the endocytic pathway. Cells expressing moderate levels of EGFP-Rabs (about 5% of total cells) were selected for analysis. Under this condition, Rab5 and Rab11 were localized on membrane bound organelles as previously indicated (Sonnichsen et al., 2000). According to confocal images (Fig. 13), GPR17 antibody colocalized partially with EGFP-Rab5 after 15 min in small vesicles while the co-immostaining diminished within 30 min incubation when more GPR17 antibody appeared in large dot-like structures, presumably late endosomes/lysosomes (Fig. 13). In contrast, GPR17 appeared colocalized only to a very limited degree with EGFP-Rab11 after 15 or 30 min incubation at 37 °C with or without agonists. We also performed a co-localization analysis using the JACoP plug-in of Image J (Bolte and Cordelieres, 2006) and the data quantification revealed that about 59-63% of anti-Nt-GPR17 antibody overlapped with Rab5 after 15 min incubation with agonists (ctr: 54.20%±1.68%, n=17; LTD4: 59.45%±2.64%, n=20; UDP-glucose: 63.04%±1.89%, n=22), whereas only the 41-38% of the receptor overlapped with the small GTP-binding protein after 30 min incubation (ctr: 34.74%±2.89%, n=15; LTD4: 40.46%±1.81%, n=18; UDP-glucose: 38.33%±1.96%, n=19). A smaller overlap between Rab11 and GPR17 was observed after 15 and 30 min incubations with or without agonists; data quantification revealed that approximately 35-40% of anti-GPR17 colocalize with Rab11 after 15 min-incubation (ctr: 34.74%±2.89%, n=11; LTD4: 41.18%±2.26%, n=12; UDP-glucose: 35.19%±2.34%, n=10) and similar values were found at later time (ctr: 32.35%±4.26%, n=12; LTD4: 34.48%±4.14%, n=15; UDP-glucose: 32.04%±2.39, n=15), thus suggesting that the receptor is mainly excluded from the “long loop” recycling pathway.

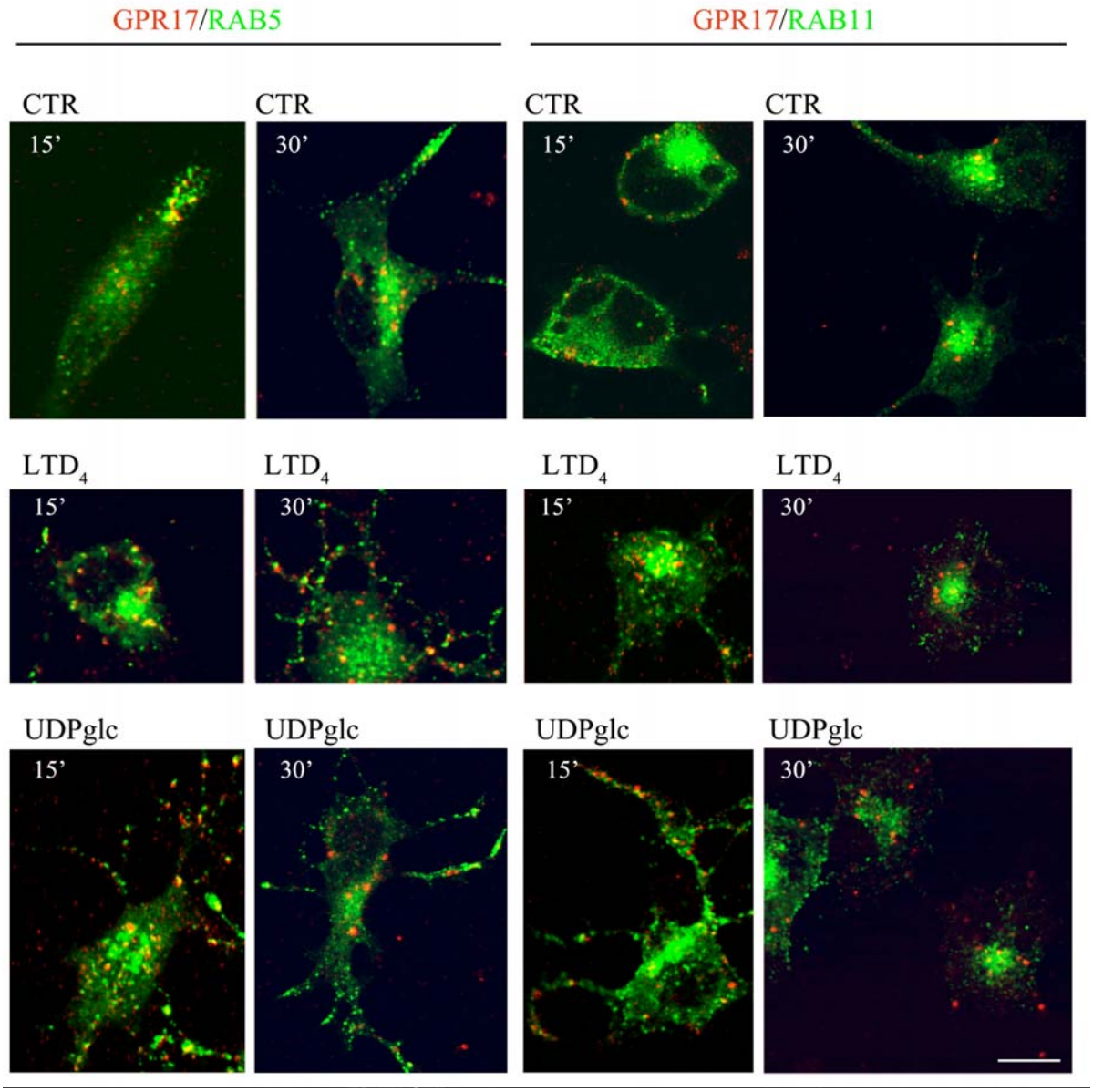


Figure 13. Analysis of GPR17 distribution in Rab5- or Rab11-positive compartments. Oli-neu cells were pre-incubated 12 h in CM and then transfected with 0.5 μg of cDNA coding for EGFP-Rab5 (Rab5) or EGFP-Rab11 (Rab11). After 48 h cells were labeled at 4°C with the anti-Nt-GPR17 and then incubated at 37 °C for 15 or 30 min without (CTR) or with agonist. Cells were then washed with glycine buffer, fixed and immunostained with the anti-rabbit IgG conjugated to Cy3 (GPR17). The merged images shown are representative of three experiments. Note a larger degree of colocalization of GPR17 with Rab5 after 15'incubation at 37°C. Bar = 10 μm

To further investigate whether GPR17 is delivered to the degradative compartment after stimulation, we incubated Oli-neu cells with chloroquine. Accumulation of this weak base inside intracellular acidic compartments has been reported to inhibit lysosomal enzymes (that require an acid pH) and also to prevent fusion of endosomes and lysosomes, thereby inhibiting the degradation of endocytosed proteins. In these experiments, after labeling at 0°C with anti-Nt-GPR17, cells were incubated for 30 min at 37 °C with or without agonists in the presence or absence of 100 μM chloroquine, fixed, and then co-immunolabeled for Lamp1. Accordingly, confocal images revealed an increase of GPR17-positive vesicles especially upon agonist treatments in the presence of chloroquine (Fig. 14). Furthermore, a number of these vesicles are also labeled for Lamp1 thus indicating that a consistent aliquot of GPR17 is delivered to lysosomes.

4°C labeling + 10’ 37 °C

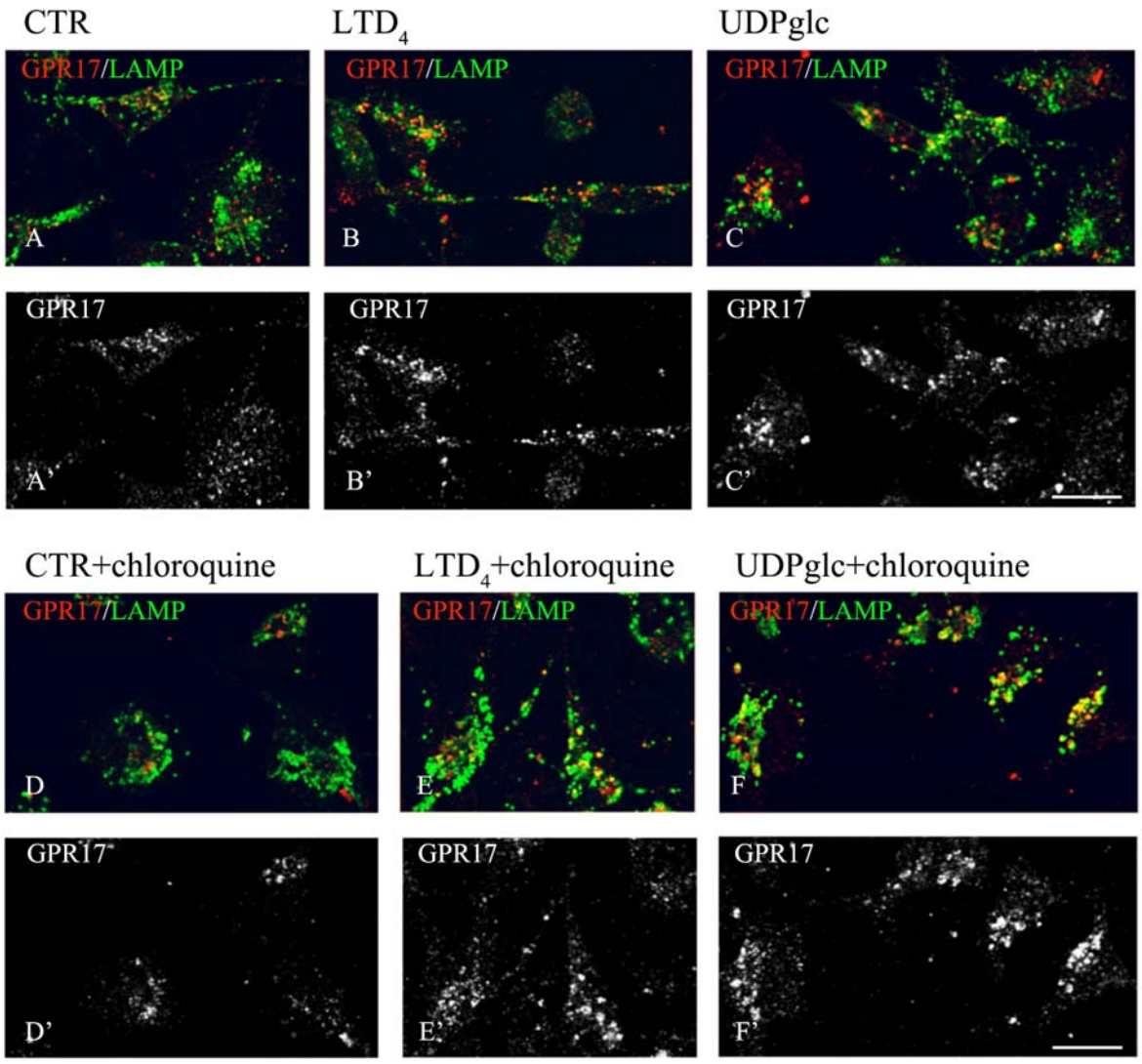


Figure 14. Effects of the lysosomotropic agent chloroquine on the metabolism of GPR17. Oli-neu cells cultured 64 h in CM were labeled for 45 min at 4 °C with the affinity purified anti-Nt-GPR17 and then incubated without (control, CTR) or with agonists in the absence (A-C’) or presence (D-F’) of 300 μM chloroquine. After 30 min incubation at 37 °C, cells were washed with glycine buffer, fixed and immunostained with a monoclonal antibody against Lamp1 (LAMP) followed by incubation with anti-rat IgG conjugated to fluorescein and anti-rabbit IgG conjugated to Cy3 (GPR17, A’-C’, D’-F’). Note increased accumulation of GPR17 after chloroquine treatment. Merged images (A-C, D-F) show colocalization of GPR17 with the lysosomal marker (yellow). Images are representative of two experiments. Bars = 20 μm.

In conclusion, these data demonstrate i) that GPR17 internalization is stimulated by both classes of agonists, although UDP-glucose determines a more efficient mobilization of the receptor into the endocytic pathway, and ii) that, upon stimulation, GPR17 is mainly down regulated via lysosomal degradation.

To further investigate the effects of UDP-glucose and LTD_4 and their antagonists on receptor internalization we performed biotinylation assays. In preliminary experiments, Oli-neu cells were incubated at 4°C (conditions that prevent plasma membrane internalization) with sulfo-NHS-SS-Biotin. After washing, cells were extracted immediately or after treatment with DTT in order to cleave biotin bound at the cell surface. Biotinylated proteins were isolated with streptavidin beads and analyzed by Western blotting using anti-Ct-GPR17 antibody. The specificity of cell surface biotinylation was monitored by assessing the labeling of TfR (as a marker of plasma membrane receptors) or actin (as a marker of cytosolic proteins). As shown in Figure 15, a consistent amount of TfR and GPR17 were biotinylated whereas actin was not (Fig. 15A). To test constitutive internalization, after biotinylation at 4°C Oli-neu were incubated 15 min at 37°C, cooled at 4 °C and washed with DTT (Fig. 15 B, C). Western blotting analysis revealed that under this condition, about 10% of the biotinylated receptor (10.82±6.6 % of total biotinylated GPR17; n=3 independent experiments) underwent internalization in the absence of agonists confirming that the receptor undergoes constitutive endocytosis.

We then used the biotinylation assays to investigate GPR17 internalization upon incubation with agonists or antagonists. As shown in Figure 15, the exposure of Oli-neu to UDP-glucose activated the endocytosis of GPR17, as shown by the fact that stimulated cells had more biotinylated internalized GPR17 than non stimulated cells (Fig. 15 D). This effect was counteracted by micro molar concentrations of cangrelor (Cng), an antagonist that has been previously shown to inhibit the UDP-glucose activation of GPR17 (Ciana et al., 2006; Lecca et al., 2008; Fumagalli et al., 2011). Furthermore, receptor internalization was largely inhibited by chlorpromazine, which is known to prevent assembly of the clathrin adaptor protein-2 on clathrin coated pits and thus to block clathrin-dependent endocytosis. Quantitative analysis of at least three independent experiments showed that, in comparison with non-stimulated cells, internalization of biotinylated GPR17 after UDP-glucose treatment increased to 392.9±97.2% ($p<0.0241$) with respect to the constitutively internalized receptor. When cells were probed with LTD_4, GPR17 underwent less efficient endocytosis: upon LTD_4 treatment, receptor internalization was indeed increased to 157.9± 12.21% ($p<0.0091$)

with respect to the constitutively internalized receptor. This internalization was inhibited by montelukast (MTL), an antagonist of the CysLT component of GPR17, and by chlorpromazine (Fig. 15C). In line with the data obtained with the antibody *in vivo* labeling, these results confirm that both UDP-glucose and LTD_4, though less efficiently, stimulate the internalization of GPR17 and, importantly, that GPR17 antagonists counteracted this effect.

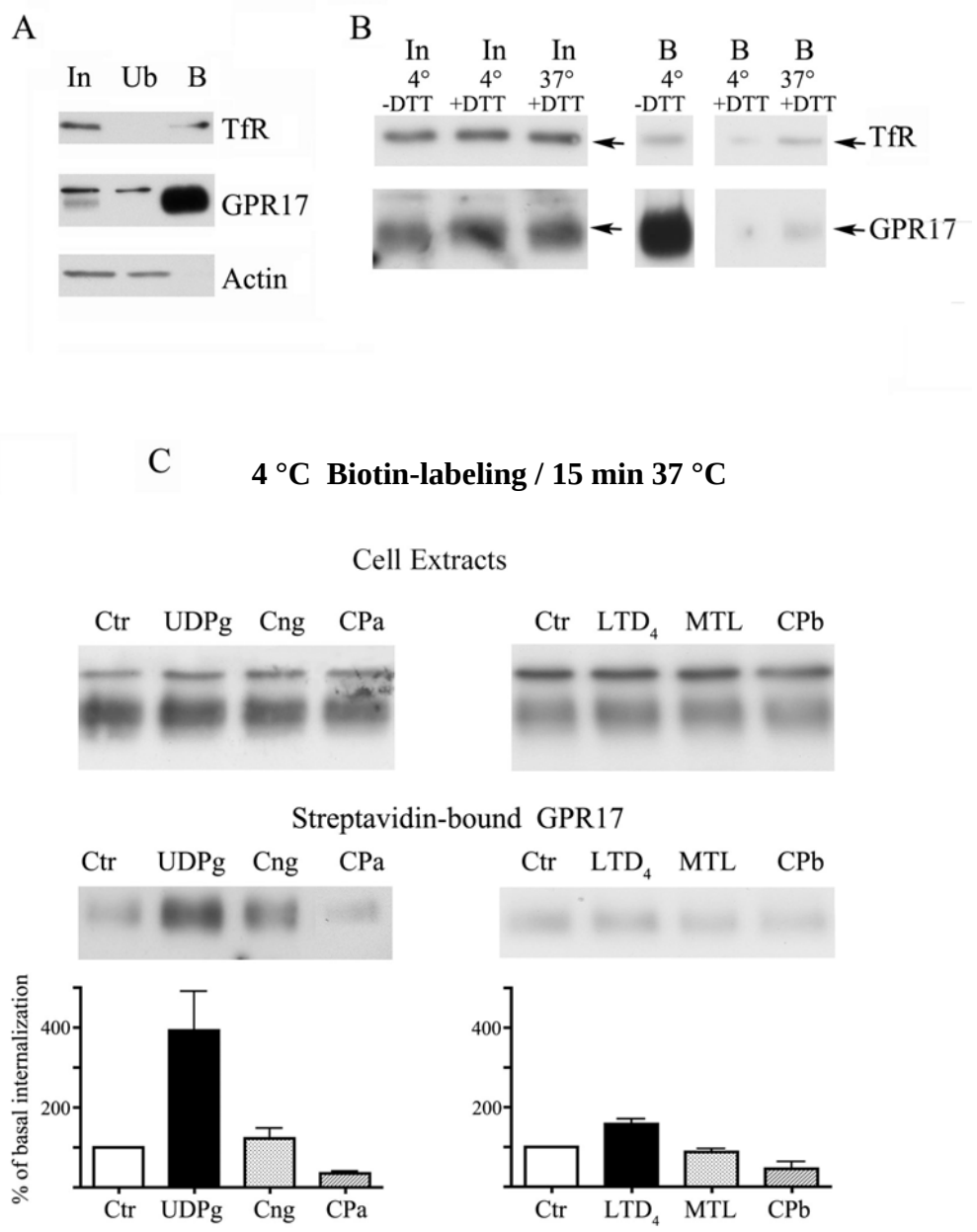


Figure 15. Internalization of biotinylated GPR17. **A**. 72 h-differentiated Oli-neu were labeled 30 min at 4 °C with biotin. Cell extracts (250μg) were incubated with streptavidin. Proteins bound to streptavidin-beads (B) as well as 5 % of total proteins used for streptavidin-beads incubation (input, In) and 5 % of proteins remaining in the supernatants after incubation (unbound- proteins, Un) were analyzed by western blotting with antibodies against TfR, GPR17 and actin. **B**. After biotin labeling at 4 °C, Oli-neu were extracted either without DTT treatment (4°C -DTT) or after DTT washes (4°C+DTT) or after incubated 15 min at 37°C followed by DTT treatments (37°C+DTT). Proteins bound to streptavidin-beads (*B*) as well as 5 % of inputs (In) were analyzed by western blotting with antibodies against TfR or GPR17. **C.** After biotin labeling, Oli-neu were incubated in the absence (Ctr) or presence of one of the following drugs: 100 μM UDP-glucose (UDPg); 100μM UDP-glucose and 10 μM cangrelor (Cng); 100 μM UDP-glucose and 50 μM chlorpromazine (CPa); 50 nM LTD_4 (LTD_4); 50 nM LTD_4 and 10 μM montelukast (MTL); 50 nM LTD_4 and 50 μM chlorpromazine (CPb). After 15 min at 37°C, cells were cooled at 4°C, treated with DTT and solubilized in extraction buffer. Cell extracts (250 μg) were incubated with streptavidin-beads. Bound proteins were analyzed by western blotting. The image shows representative blots of three independent experiments. Graphs represent the quantitative analysis of western blots. Data are expressed as % of biotinylated GPR17 internalized in non-stimulated cells (Ctr).

4.4 GPR17 endocytosis in OPCs

In order to support these results obtained in Oli-neu cells we reproduced the experiments in primary cultures, enriched in OPCs, isolated from P3 mouse brain cortex. The cells were grown for seven days in culture and then plated in Sato medium for three days. Isolated cells obtained from these cultures were first tested for the identification of OPCs expressing GPR17 after immunolabeling with the anti-Ct-GPR17 antibody. Several cells were found to highly express GPR17 with no or very little expression of MAG (Fig. 16A). Moreover they do not show colocalization with GFAP (Fig. 16B) nor Iba1 (data not shown). In three days cultures, some cells with high levels of MAG but negative for GPR17 were also detected (Fig. 16C), as reported for rat OPCs in Fumagalli et al., 2011.

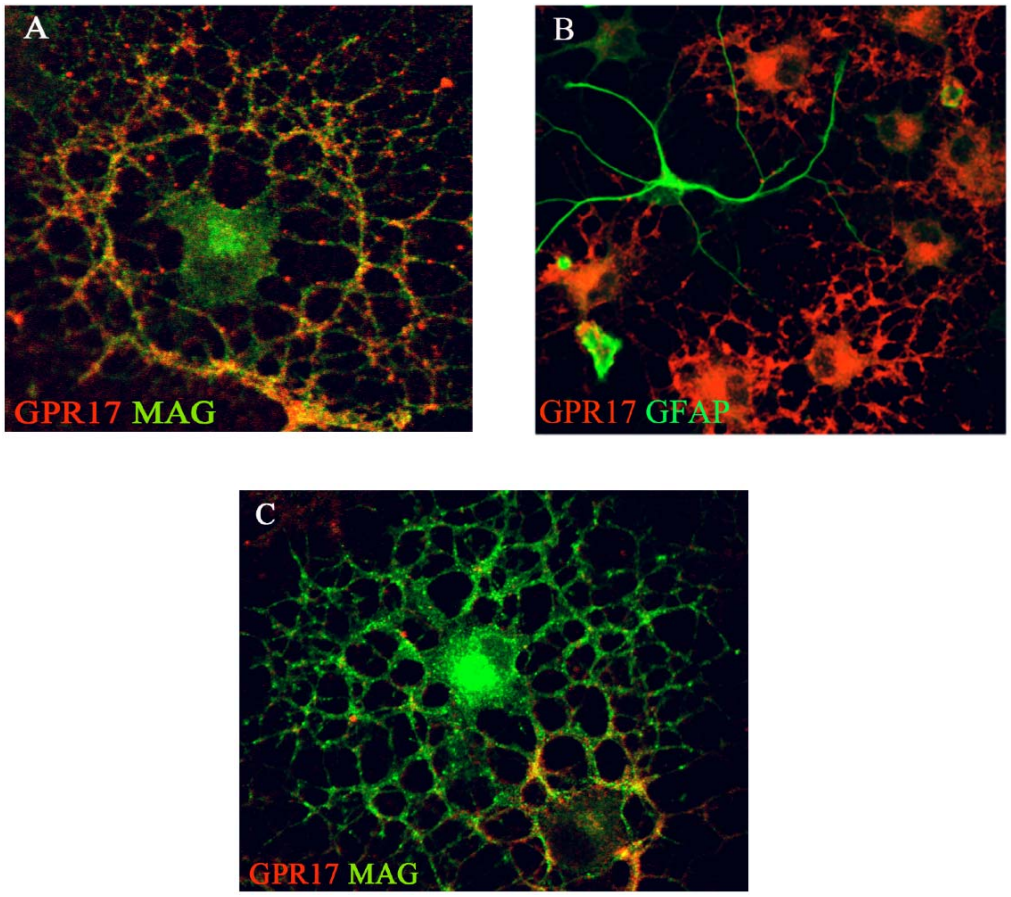


Figure 16. GPR17 expression in mouse OPC primary culture. Prymary culture were obtained from P3 mice as described in Material and Methods. Cells were fixed and double immunolabeled with anti-Ct-GPR17 antibody (green) and MAG (red). Cells at different differential stages were found in the culture, in A) cells are highly positive for GPR17 but express very little MAG, moreover cells do not colocalize with GFAP (B). In C) more mature cells show high levels of MAG but are negative for GPR17.

Internalization experiments were then performed in OPCs. First of all, we labeled the cells *in vivo* at 4 °C to reveal the GPR17 at the cell surface and after fixation the cells were immunolabeled with the specific secondary antibody. As shown in Figure 17 GPR17 decorates the surface membrane of many OPCs. To evaluate the endocytosis of the native receptor upon agonist stimulation, cells were labeled *in vivo* with anti-Nt-GPR17 antibody and then incubated at 37 °C to allow internalization, with or without agonists (Fig. 18). After 10 min incubation the cells were washed, fixed and stained using monoclonal Tfr antibody followed by specific secondary antibodies against mouse or rabbit IgG. At this time point significant amount of GPR17 was internalized in the early endosomes as shown by colocalization with TfR. As in Oli-neu, in UDP-glucose treated cells the endocytosis of the receptor seems to be more evident.

Anti-Nt-GPR17 4 °C labeling

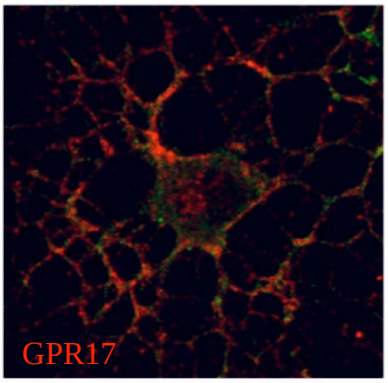


Figure 17. Anti-Nt-GPR17 antibodies label native GPR17. Cells were labeled with an affinity purified anti-Nt-GPR17 at 4 °C, washed, fixed and then stained with anti-rabbit IgG conjugated to Cy3 to reveal the distribution of GPR17.

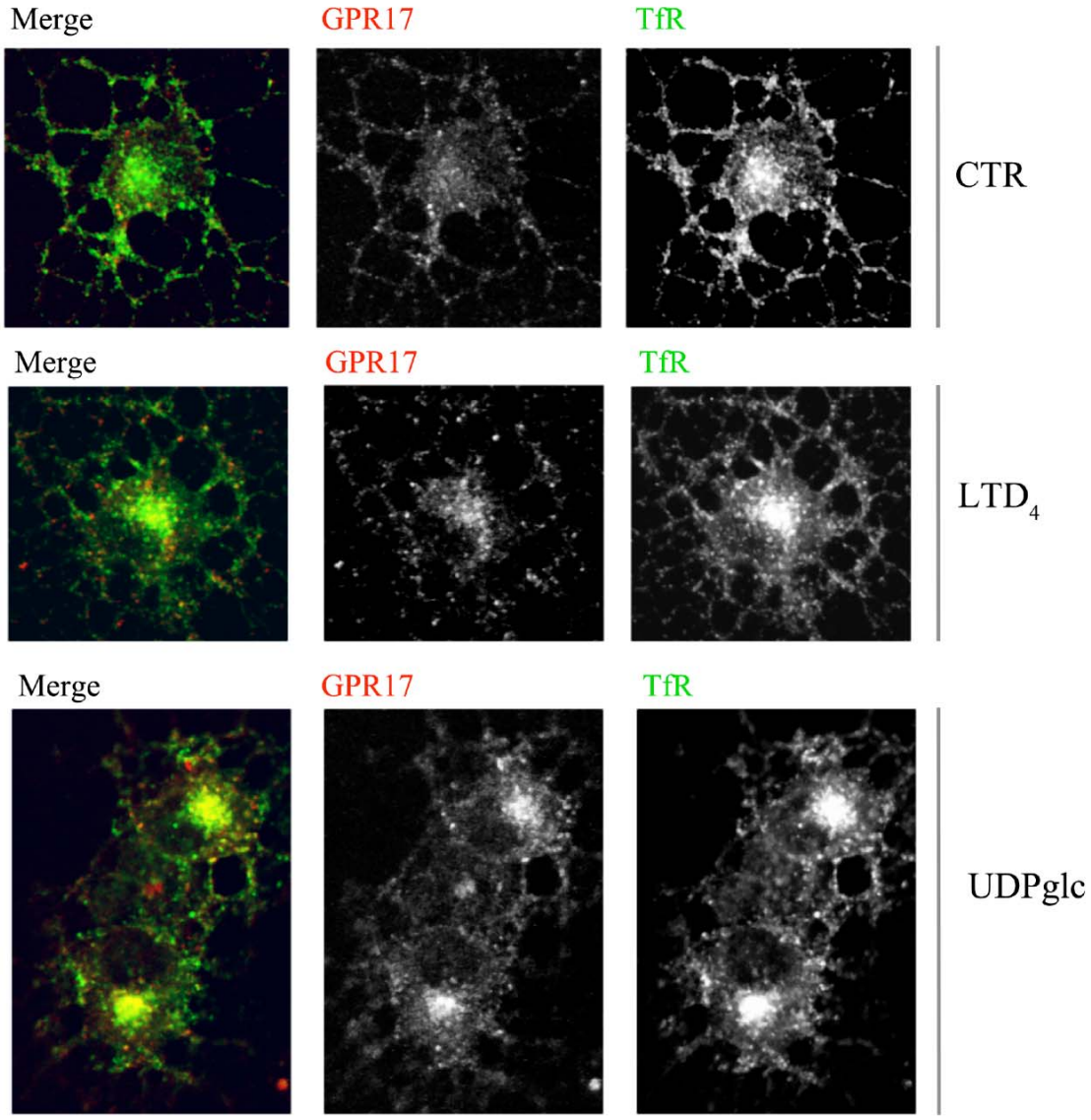


Figure 18. Internalization of GPR17 after 10 min of exposure to agonists. OPCs were labeled for 45 min at 4 °C with the affinity purified anti-Nt-GPR17 and then incubated for 10 min at 37 °C without (control CTR) or with agonists (50 nm LTD_4 or 100 μm UDP glucose, UDPglc). After incubation, cells were washed with glycine buffer, fixed and immunostained with a monoclonal antibody against TfR followed by incubation with anti-mouse IgG conjugated to fluorescein and anti-rabbit IgG conjugated to Cy3 (GPR17). Confocal microscopy images show that GPR17 colocalizes with Tfr.

5. DISCUSSION

Our study shows that the Oli-neu cell line is an appropriate model for investigating GPR17 in differentiating oligodendroglial cells. These cells indeed regulate GPR17 expression at distinct differentiation stages in a similar way to that observed in differentiating primary OPCs in culture (Lecca et al., 2008; Fumagalli et al., 2011), as well as in the CNS of young animals (Chen et al., 2009; Boda et al., 2011). Moreover, due to their lineage, these cells represent a more physiological milieu for receptor expression than heterologous expression systems. Using these cells we report here the first biochemical characterization of native mouse GPR17 by determining the molecular weight of the mature and precursor forms of the receptor and their glycosylation profile, and we investigate the endocytic trafficking of the receptor upon stimulation with agonists. Our data demonstrate that GPR17 undergoes clathrin-mediated endocytosis and sorting to lysosomes after exposure to UDP-glucose or LTD_4. This regulated internalization and subsequent sorting into the degradative compartment may have important implications for the down regulation of GPR17 signaling pathway(s) in differentiating OPCs.

GPR17 expression is time-regulated in differentiating Oli-neu

Oli-neu cells originally came from cultures enriched in OPCs after immortalization with the *neu* oncogene; it has been shown that about 50% of the cells maintained in normal medium express the O4 antigen, a marker of immature oligodendrocytes, and that very few express small amount of myelin proteins (Jung et al., 1995; Winterstein et al., 2008). In line with this data, the majority of Oli-neu cells express the basic helix-loop-helix transcription factor Olig2 required for oligodendrocyte specification and differentiation, as well as NG2, a proteoglycan present in oligodendrocyte precursors (not shown; Richardson et al., 2011 and refs therein). However, only 5% of the cells maintained in normal medium express detectable amounts of GPR17. In this study we demonstrate that medium from neuron primary cultures modulates the expression of GPR17 in Oli-neu while fostering cells towards a more differentiated phenotype. Accordingly, GPR17 is up regulated at early stages of differentiation and is then turned-down in cells with a more mature phenotype that express high levels of myelin proteins. Notably, this process resembles the time-regulated expression of GPR17 in primary OPC cultures. In these cultures, the peak of GPR17 expression occurs after 5-6 days in differentiation medium, when cells reach the stage of pre/immature oligodendrocytes (Fumagalli et al., 2011). In Oli-neu, the higher level of GPR17 is detected at 48-72 h

after incubation with CM and the peak of expression precedes that of myelin proteins, although some degree of co-expression of GPR17 with MAG can be found.

Taken together, these results support the conclusion that, under specific culture conditions, Oli-neu cells recapitulate the behavior of developing OPCs, at least in the early stages of their differentiation. Oligodendrocyte differentiation depends on the integration of multiple extracellular factors derived from neurons and astrocytes with a number of intrinsic determinants (Emery, 2010; Taveggia et al., 2010; and refs. therein). Our data suggest that Oli-neu cells retain a time-dependent differentiation programming that can be activated by extrinsic factors released by or derived from neurons and/or astrocytes. These factors are present in CM and induce Oli-neu cells to switch on their differentiation, which includes the regulated expression of GPR17. A further possibility is that factors present in the medium can counteract the signaling of the neu-oncogene, which has been transfected into the cells in order to immortalize them and foster their proliferation. Exit from the mitotic cycle may trigger the expression of GPR17 and promote differentiation. This hypothesis is supported by the observation that an erb B inhibitor induced the expression of GPR17 and the rapid (24 h) differentiation of Oli-neu cells (data not shown). In line with these findings, detailed proliferation studies performed with the DNA precursor base bromo-deoxy-uridine demonstrated that in culture as weel as in developing and adult mouse brain, OPCs start to express significant amounts of GPR17 as soon as they become post-mitotic suggesting that GPR17 specifically labels a subpopulation of OPCs that exits the cell cycle and becomes irreversibly committed to differentiation (Boda et al., 2011; Fumagalli et al., 2011).

GPR17 endocytic trafficking in oligodendroglial precursor cells

Previous pharmacological studies showed that GPR17 is a “dualistic” receptor activated by micromolar concentrations of uracil nucleotides (UDP-glucose) and nanomolar concentrations of CysLTs (Ciana et al., 2006; Lecca et al., 2008; Fumagalli et al., 2011). Moreover, studies in heterogously transfected cells demonstrated that, in a similar way to other GPCRs, upon prolonged exposure to its endogenous agonists, GPR17 can undergo loss of function, as shown by desensitization, i.e., inability to further respond to agonist stimulation (Daniele et al., 2011). However, in this study, the role of endocytosis in agonist-induced GPR17 desensitization was only marginally analyzed and, even more important, no cues were given on the occurrence and potential importance of this phenomenon in oligodendroglial cells, the cell type that mostly

expresses this receptor under physiological conditions.

Here, we demonstrate that native GPR17 undergoes a substantial endocytosis in differentiated oligodendroglial cells after UDP glucose as well as after LTD_4 stimulation, further supporting the conclusion that both classes of agonists activate GPR17. After ligand activated internalization, GPR17 is mainly routed into the degradative pathway as demonstrated by (i) a limited colocalization with Rab11, a marker of the long loop recycling pathway, (ii) the presence of substantial amounts of the receptor in lysosomes/late endosomes after incubation times higher than 30 min, and, (iii) further increased lysosomes/endosomes localization in the presence of chloroquine, a lysosomotropic agent perturbing lysosomal pH, enzymatic degradation as well as late endosomes and lysosomes fusion. As already mentioned, we found that LTD_4 also led to the internalization/degradation of GPR17, although with somehow less efficiency. Based on our demonstration that the $CysLT_1R$ is not expressed at any time in Oli-neu cells, we conclude that, in our system, prolonged LTD_4 exposure directly affects GPR17 responsiveness. The functional consequences of this different agonist-induced internalization of GPR17 upon exposure to either uracil or CysLT ligands are not yet known. A more efficient internalization obtained with uracil nucleotides may relate to the need of a more rapid desensitization and down regulation of the physiological responses elicited by GPR17 activation, whereas the less efficient internalization of GPR17 evoked by CysLTs may sustain a longer lasting receptor activation. It may be hypothesized that, depending upon specific pathophysiological conditions, distinct extracellular concentrations of uracil nucleotides or CysLTs are achieved, resulting in different levels of GPR17 activation/desensitization. It could be also possible that such difference of receptor internalization is due to activation of distinct signaling pathways, although, until now, upon binding to GPR17, both uracil nucleotides and CysLTs have been reported to activate the Gαi signaling pathway. However, occasionally, responses compatible with activation of the Gq protein have been reported (Ciana et al., 2006; Pugliese et al., 2009), suggesting that, depending on the type of the agonist bound to GPR17, a shift from one signaling pathway to the other may occur.

Our findings also show that in differentiating Oli-neu, GPR17 undergoes constitutive endocytosis, as indicated by *in vivo* labeling with the anti-Nt-GPR17 antibody and biotinylation experiments showing that internalization of a substantial aliquot of cell surface labeled receptors occurs in non-stimulated cells. It has been shown that various GPCRs, such as the alpha1a adrenergic receptor (Morris et al.,

2004), the GABA receptor, the metabotropic-glutamate receptors (Fourgeaud et al., 2003; Herring et al., 2003; Francesconi et al., 2009), the melanocortin 4 receptor (Mohammad et al., 2007) may undergo ligand-independent endocytosis, and it has been suggested that constitutive endocytosis contributes to receptor homeostasis maintaining an internal reserve pool capable of replenishing desensitized receptors after ligand binding (Morris et al., 2004). In addition, constitutive internalization may also play a role in regulating receptor distribution in distinct plasma membrane domains; for example, in neurons, this was observed in the case of soma versus axonal distribution of type 1 cannabinoid receptor (Leterrier et al., 2006). Both possibilities may be implicated in the regulation of GPR17 cell surface expression, also in view of the demonstration that, during both brain development and during the physiological OPCs maturation in the adult brain, GPR17 seems to be initially localized to the cell soma and then to extend to processes (Fumagalli et al., 2011; Boda et al., 2011).

In conclusion, this study provides the first characterization of endocytic trafficking of native GPR17 in differentiating oligodendroglial cells upon ligand exposure. Preliminary data show that agonist-induced internalization also occurs in OPCs from primary cultures, supporting the results on GPR17 endocytosis obtained with Oli-neu cell line. The dynamics of intracellular trafficking of the receptor is physiologically relevant as it modulates the receptor levels on the cell surface (Ferguson, 2001; Hanyaloglu and von Zastrow, 2008; Dunham and Hall, 2009; Ritter and Hall, 2009; Sorkin and von Zastrow, 2009). The internalization and subsequent sorting of the receptor into the degradative compartments may have important implications for activation/silencing of signaling pathway(s) in differentiating OPCs and, in turn, represent a key event necessary to allow OPCs to proceed to myelination. A similar process has been associated to the differentiation of other cell lineages, where the down regulation of membrane receptors via agonist activation has been proposed to be necessary to allow cells to proceed toward terminal differentiation (Walrafen et al., 2005). Furthermore, although the late endocytic compartment is mainly thought to down regulate signaling by sorting the receptors to lysosomes, it can also regulate specific signaling events. For example, it has been demonstrated that progression of the EGF receptor through late endosomes can facilitated ERK1/2-mediated signaling via the recruitment of the p14/MP1/MEK1 complex on late endosomes (Teis et al., 2006). We believe the characterization of GPR17 endocytic traffic is important to further understand the mechanisms by which receptor signaling can modulate oligodendrocytes differentiation.

Prospectives and pharmacological implications

Endocytosis is a mechanism implicated in the regulation of the expression and localization of GPCRs at the surface membrane, affecting their response to natural ligands or pharmacological agents. It has been ipothesized that in some cases different agents might drive GPCR to different endocytic pathways promoting recycling rather than degradation of the internalized receptor. This is the case for example of the human δ-opioid receptor, that could be differently targeted to endosomes or lysosomes by chemically distinct opioid agonists (Lecoq et al., 2004; Marie et al., 2003) suggesting that manipulating the endocytic machinery with different compounds could prevent the problem of tolerance due to repeated drug exposure. Moreover the cellular proteins that mediate GPCR trafficking may be considered as possible new pharmacological targets. GRK family of kinases, involved in desensitization, arrestin association and endocytosis of various GPCRs, is one of the most promising non-GPCR target.

The improved knowledge of the molecular mechanisms that regulate GPR17 trafficking during OPC differentiation may help the development of therapeutic approaches for receptor signaling modulation and, in turn, myelin formation. It would be, therefore, important to evaluate further the molecules (arrestins, GRKs) possibly involved in GPR17 internalization. Furthermore our data were obtained in Oli-neu in which GPR17 levels reached a plateau. It would be interesting to investigate whether different endocytic pathway is taken by receptor in more undifferentiated cells (i.e. 24-36h differentiated Oli-neu).

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