The orphan G protein-coupled receptor GPR17: its pharmacology and function in recombinant and primary cell expression systems

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Abstract

The reconstitution of myelin sheaths, so called remyelination, represents an innovative therapeutic goal in multiple sclerosis (MS), the most common inflammatory-demyelinating disease of the central nervous system (CNS). Recently, the orphan G protein-coupled receptor (GPCR) GPR17, which is predominantly expressed in oligodendrocytes, has been identified as inhibitor of oligodendroglial differentiation, arresting oligodendrocytes in an immature, non-myelinating stage. Moreover, GPR17 expression is upregulated in human MS tissues, suggesting a key role of this receptor during the remyelination impairment that occurs in MS. However, the downstream signaling pathway connecting GPR17 to oligodendroglial maturation arrest is still poorly understood.

The present work confirms that GPR17 activation by the small molecule agonist MDL29,951 results in a reduction of mature oligodendrocytes in vitro, evident by their decreased intracellular myelin basic protein (MBP) levels. The GPR17-mediated maturation block is crucially triggered by the $G\alpha_{i/o}$ pathway, which leads to an inhibition of two signaling cascades: (i) the adenylyl cyclase (AC)-cyclic adenosine monophosphate (cAMP)–protein kinase A (PKA)–cAMP response element-binding protein (CREB), and (ii) the AC-cAMP-exchange protein directly activated by cAMP (EPAC). Remarkably, these findings demonstrate for the first time an involvement of EPAC on oligodendrocyte myelination. In conclusion, GPR17 but also its downstream signaling effectors as elucidated in this study provides several drug targets to influence oligodendrocyte differentiation.

Furthermore, knowledge about GPR17 pharmacology would benefit greatly from the identification of both its endogenous ligand(s) and/or selective antagonists. The activation of GPR17 by cysteinyl-leukotriene and purinergic ligands (UDP, UDP-gal, UDP-glc, LTC4, and LTD4) is ten years after its initial publication still controversially discussed. In line with other laboratories that did not validate GPR17 as dualistic receptor for both ligand families, the data of the present work obtained in (i) G protein rearrangement, (ii) β-arrestin recruitment, and (iii) ERK1/2 phosphorylation assays confirm that GPR17 must still be regarded as an orphan receptor. Additionally, the present work invalidates the P2Y12 antagonists ticagrelor and cangrelor as GPR17 inhibitors. However, investigations on two structurally related CysLT2 receptor antagonists, HAMI3379 and Cay10633, strongly suggest that their basic chemical scaffolds will serve as valuable starting point for the generation of more potent and selective GPR17 antagonists to inhibit signaling of this orphan receptor in MS.

Keywords: GPR17, orphan GPCR, protein kinase A (PKA), exchange protein directly activated by cAMP (EPAC), cAMP response element-binding protein (CREB), label-free dynamic mass redistribution (DMR)

Kurzfassung

Die Regeneration von Myelinscheiden, die Remyelinisierung, repräsentiert ein innovatives therapeutisches Ziel bei Multipler Sklerose (MS), der häufigsten inflammatorischen demyelinisierenden Erkrankung des zentralen Nervensystems (ZNS). Differenzierte Oligodendrozyten sind für die Bildung von Myelinscheiden verantwortlich. Der orphane G Protein-gekoppelte Rezeptor (GPCR) GPR17, der prädominant in Oligodendrozyten exprimiert ist, blockiert deren Differenzierung zu myelinisierenden Oligodendrozyten. GPR17 ist darüber hinaus im Gewebe von MS Patienten überexprimiert, was implizieren könnte, dass der Rezeptor eine zentrale Rolle als Inhibitor der Remyelinisierung bei MS spielt. Wie GPR17 Oligodendrozytendifferenzierung auf molekularer Ebene blockiert, d.h. welche intrazellulären Signalwege involviert werden, ist jedoch kaum bekannt.

Die vorliegende Arbeit bestätigt, dass die Aktivierung von GPR17 durch den synthetischen Agonisten MDL29,951 in vitro eine Reduktion der Reifung von Oligodendrozyten zur Folge hat, was durch eine verminderte Expression des intrazellulären Myelin Basic Proteins (MBP) detektierbar ist. Die GPR17-vermittelte Entwicklungsblockierung wird entscheidend durch den $G\alpha_{i/o}$ Signalweg beeinflusst. Dies beinhaltet die Hemmung von zwei Signalkaskaden: (i) die Adenylatzyklase (AC)-zyklischen Adenosinmonophosphate (cAMP) – Proteinkinase A (PKA) – cAMP response element-binding protein (CREB) und (ii) die AC-cAMP-Exchange Protein directly acitvated by cAMP (EPAC) Kaskade. Interessanterweise, zeigen die erhaltenen Ergebnisse zum ersten Mal eine Beteiligung des Guaninnukleotid-Austauschfaktors EPAC an der Myelinisierung durch Oligodendrozyten. Zusammenfassend bieten sowohl GPR17 als auch seine intrazellulären Effektorproteine diverse Ansatzpunkte, um die Differenzierung von Oligodendrozyten therapeutisch zu beeinflussen.

Zusätzlich würde die Identifizierung des endogenen Agonisten und/oder selektiver Antagonisten einen wertvollen Beitrag zur bisher rudimentären GPR17 Pharmakologie leisten. Die Aktivierung von GPR17 durch Cysteinyl-Leukotriene und Purin Liganden (UDP, UDP-gal, UDP-glc, LTC4 und LTD4) wird zehn Jahre nach der ersten Postulierung noch immer kontrovers diskutiert. Im Einklang mit anderen Arbeitsgruppen, die nicht belegen konnten, dass GPR17 durch die genannten Substanzen aktiviert wird, bestätigen die Daten der vorliegenden Arbeit, mittels Assays zur (i) G Protein-Umlagerung, (ii) β-Arrestin Rekrutierung und (iii) ERK1/2 Phosphorylierung, dass GPR17 immer noch als orphaner Rezeptor anzusehen ist. Darüber hinaus konnten die P2Y12 Antagonisten, Ticagrelor und Cangrelor, nicht als GPR17 Inhibitoren bestätigt werden. Im Gegensatz dazu konnten wir für zwei strukturell ähnliche CysLT2-

Rezeptor-Antagonisten, HAMI3379 und Cay10633, eine Hemmung von GPR17 nachweisen, wodurch diese Substanzen als neue Ausgangspunkte für die Entwicklung von potenten und selektiven GPR17 Antagonisten angesehen werden könnten, um somit den GPR17 Signalweg in MS zu blockieren.

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1 Introduction

1.1 Multiple sclerosis (MS): a severe neurological disease

Multiple sclerosis (MS) is an autoimmune disorder, which is characterized by inflammation-mediated demyelination of the nerve cells in the central nervous system (CNS). In consequence, nerve cells lose their electric insulation and thus their ability to communicate with high speed. This results in a range of neurological symptoms, such as loss of sensitivity, muscle spasms, visual problems and, furthermore, cognitive and psychiatric symptoms (Compston, Coles 2002).

Worldwide, approximately 2.5 million people suffer from MS, representing the chronic immune-mediated disorder with a high prevalence in young adults. In Germany alone, the annual incidence rate is 3.5 - 5 cases per 100.000 population for this devastating disease (Neusser et al. 2014). MS causes great afflictions for both patients and their families. Furthermore, the cost for drug therapy and nursing care represent a considerable burden for the global health care system. For instance, in the European Union alone, the estimated costs for the treatment of MS account 9 billion Euros every year (Franklin, Ffrench-Constant 2008).

Regrettably, no curative therapy for MS is available up to now. The current treatment strategies are aiming at elevating the life quality of patients, as the major part of the applied drugs belongs to the class of disease-modifying therapies. These treatments, as their name indicates, only help to control the progress of the diseases by modulation of the inflammatory processes. Thereby, the development of remedial therapies for MS represents a great challenge for the pharmaceutical industry (Aktas et al. 2010; Marinelli et al. 2016).

1.1.1 Remyelination: the challenging therapeutic goal

Mature oligodendrocytes and Schwann cells provide myelin in the CNS and in the peripheral nervous system (PNS), respectively. Axons are wrapped by a myelin sheath, which allows a saltatory conduction of action potentials. This permits a 20-100 -fold faster transmission of action potentials compared to non-myelinated axons (Miller 2002; Kremer et al. 2016). MS is characterized by an inflammatory damage of oligodendrocytes and, consequently, by the loss of myelin sheaths in the CNS. The demyelination cascade terminates in degeneration of the axon or even the entire neuron, except remyelination, a spontaneous myelin repair process, occurs (Franklin, Ffrench-Constant 2008). Although shorter and thinner myelin sheaths characterize myelin-repaired CNS

areas, they remain functional. Therefore, one important therapeutic goal against demyelinating/dysmyelinating diseases would be to enhance intrinsic mechanisms that specifically drive remyelination and thereby avoid axonal loss.

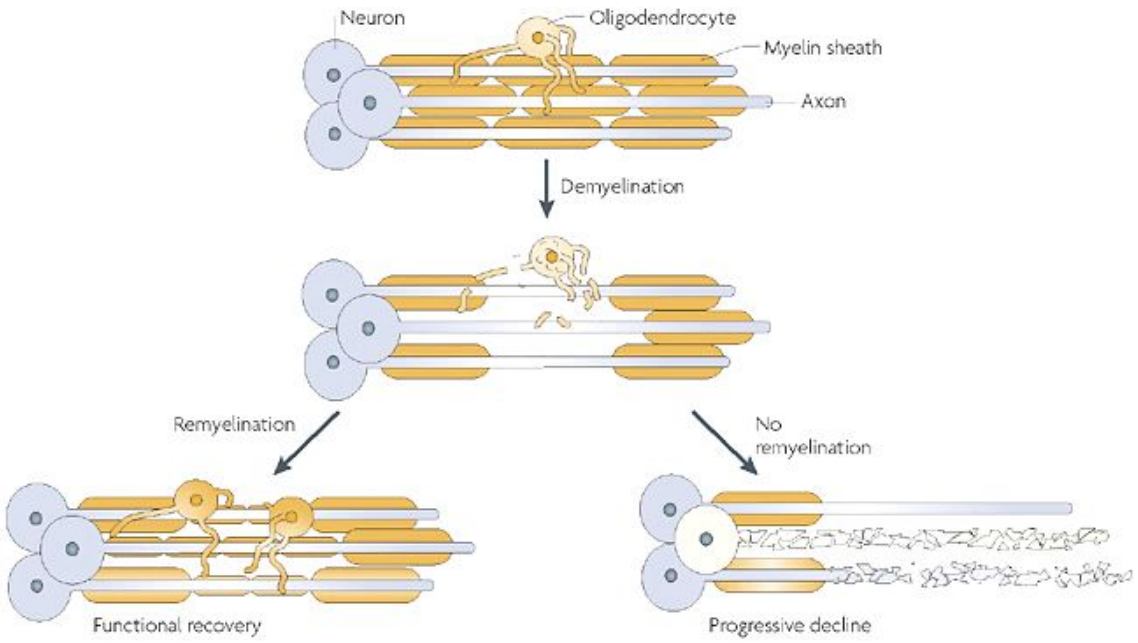


Figure 1. **Process of demyelination and remyelination on neuronal axons in the CNS**

Demyelination may be followed by spontaneous remyelination, but under pathophysiological conditions, such as in MS, remyelination fails. If no remyelination occurs, the axon and even the entire neuron perish because they are unprotected. In contrast, if remyelination occurs newly formed myelin sheaths remain functional although they are thinner and shorter than those that were initially generated during CNS development (modified from (Franklin, Ffrench-Constant 2008)).

Myelination and remyelination in the CNS require the proliferation of oligodendrocyte precursor cells (OPCs), their migration into the unmyelinated area, and their final differentiation into mature myelinating oligodendrocytes, the latter being probably of greatest relevance (Franklin 2002).

During differentiation, OPCs pass through specific stages, i.e. precursor cells, immature, and mature oligodendrocytes, which are characterized by a well-defined expression pattern of surface proteins and a change in morphology. The main molecular markers for the precursor cell stage are the cell surface receptor platelet-derived growth factor receptor alpha (PDGFRα) and the sulfated neural/glial antigen 2 proteoglycan (NG2), located in the plasma membrane. Immature oligodendrocytes are characterized by the expression of O-Antigens (O4), which are sulfatides expressed on the oligoden-

drocyte surface, and also NG2. Mature oligodendrocytes display a ramified morphology (Kremer et al. 2016) and surround axons with myelin sheaths, which are multi-lamellar sheets of their plasma membrane containing a characteristic composition of proteins and lipids. These include the myelin basic protein (MBP), the myelin-associated glycoprotein (MAG), the proteolipid protein (PLP) and the myelin oligodendrocyte glycoprotein (MOG) (Kremer et al. 2016).

Notably, analysis of multiple sclerosis plaques revealed limited or inadequate remyelination in MS, suggesting that intrinsic repair mechanisms that specifically drive oligodendrocyte-mediated remyelination occur in the demyelinated CNS areas but they fail to prevent axon degeneration (Kuhlmann et al. 2008; Franklin, Ffrench-Constant 2008). Therefore, MS therapy might benefit from the identification of oligodendrocyte intracellular mechanisms that allow overcoming MS remyelination failure, consequently becoming innovative targets to find groundbreaking pharmaceutical approaches to foster remyelination.

Recent findings suggest that oligodendrocyte differentiation is influenced by a wide range of intracellular molecular pathways. For instance, pharmacological studies employing an antagonistic antibody against the Leucine rich repeat and Ig-like domain-containing Nogo receptor interacting protein 1 (LINGO-1) and *in vivo* genetic analyses using a LINGO-1 knockout model (Mi et al. 2005; Mi et al. 2007; Pepinsky et al. 2011) showed that LINGO-1 has a critical role during myelination, blocking the development of oligodendrocytes. In fact, LINGO-1 is under clinical evaluation as a current innovative target to treat MS. In addition, enzymes like phosphodiesterases 4 (Syed et al. 2013) and 7 (Medina-Rodríguez et al. 2013), and a wide range of receptors are recognized as potential targets to promote the endogenous efforts towards myelin regeneration. For instance, nuclear estrogen receptor-β ligand treatment exhibited a direct neuroprotective effect on oligodendrocyte differentiation and myelination (Crawford et al. 2010). Furthermore, several guanine nucleotide-binding protein (G protein) coupled receptors (GPCRs) have been identified to have an impact on the pathogenesis of MS (Chen et al. 2009; Crawford et al. 2010; Ackerman et al. 2015). Thus, interference with these pathways may uncover new opportunities to treat and to cure demyelinating diseases in which physiological remyelination fails.

1.2 GPCR signaling and their role in differentiation of oligodendrocytes

The GPCR superfamily represents the largest and most diverse family of cell surface receptors. GPCRs, also known as seven transmembrane receptors (7TMs), comprise around 800 members and represent ≈3% of the human genome. They play critical roles in a variety of biological processes in the human body, such as metabolism, contractili-

ty, secretion, motility, transcription, and growth among others. Based on these widespread functions, aberrant GPCR function may lead to diseases including cardiovascular, metabolic, neurodegenerative, psychiatric disorders, cancer and infectious diseases (Neves et al. 2002; Duc et al. 2015). GPCRs respond to a wide range of ligands from single photons to ions, odorants, amino acids, fatty acids, neurotransmitters or peptides/polypeptides (Marinissen, Gutkind 2001; Insel et al. 2007; Millar, Newton 2010). The GPCR superfamily can be divided into five highly diverged classes: glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin class (GRAFS), whereby the rhodopsin class represents the largest group with around 670 family members, including 388 odorant receptors (Fredriksson et al. 2003; Lagerström, Schiöth 2008; Millar, Newton 2010).

Remarkably, about 36% of all available medications target GPCRs, which convert them into the most important class of drug targets (Rask-Andersen et al. 2011). However, so far marketed drugs target only approximately 10% of all known GPCRs, indicating their outstanding role in future drug discovery projects of pharmaceutical industry (Fang et al. 2015). In this context, Du and coworkers (Du, Xie 2012) have reviewed the expression of various GPCRs on different cell types in the CNS involved in the development and progression of MS, such as monocytes, macrophages, astrocytes, and oligodendrocytes, indicating the high potential of GPCRs as new drug targets in demyelinating diseases. For instance, Fingolimod (Gilenya®) represents an innovative immunomodulating drug for treatment of MS, targeting the sphingosin-1-phosphate (S1P) receptor. It was the first orally administered disease-modifying drug that has been introduced in the German market in 2011. In the human body, the prodrug Fingolimod is metabolized to FTY720-phosphate (FTY720-P), which blocks migration of T-lymphocytes into the CNS, thus preventing their contribution to the autoimmune reaction. FTY720-P mediates its effects by targeting the S1P receptors expressed on T-lymphocytes through a polypharmacological mechanism of action: initially the S1P1 receptor is activated by FTY720-P, but the activation is followed by the internalization of the receptor into the cell. This inhibits the T-lymphocyte's capacity to respond to the S1P and thereby causes their retention in the lymph nodes (Matloubian et al. 2004; Groves et al. 2013; Gonzalez-Cabrera et al. 2014).

GPCRs are also expressed during OPC migration, proliferation, and differentiation into mature myelinating oligodendrocytes, suggesting their involvement in the entire process of myelination, demyelination, and remyelination in the CNS. It is important to emphasize that the expression patterns of GPCRs change during oligodendrocyte development. Therefore, 7TM-receptors could represent ideal therapeutic targets to interfere with the well-define development cascade of oligodendrocytes at each stage. For instance, upon activation by ATP and ADP, the purinergic receptor P2Y1 triggers the migration of OPCs. Muscarinic acetylcholine receptors (mAChRs) and β-adrenoreceptors have a

positive and a negative regulatory effect on OPC proliferation, respectively. Moreover, the mAChR subclasses M1 and M3 (Deshmukh et al. 2013) and the serotonin receptor subtypes 5-HT1A and 5-HT2A (Marinelli et al. 2016) are associated with arrest of oligodendrocytes in immature stages. Interestingly, the number of factors known to attenuate oligodendrocyte differentiation exceeds the number of activators of oligodendrocyte maturation (Kremer et al. 2011). In line with the fact that a broad diversity of GPCRs are associated with differentiation of oligodendrocytes, several intracellular downstream effectors of GPCR signaling cascades have been also described to be involved during the development of oligodendrocytes.

1.2.1 Heterotrimeric G protein signaling and its downstream effectors

GPCRs are mediating their effects mainly by interacting with heterotrimeric guanine nucleotide–binding proteins (G proteins), thereby promoting a variety of intracellular signals. G proteins are composed of three subunits: Gα, Gβ, and Gγ. In its inactive conformation the Gα subunit is bound to GDP and forms a heterotrimer with the obligatory βγ dimeric complex. Agonist-stimulated or constitutively active GPCRs function as guanine-nucleotide exchange factor (GEF) and mediate the replacement of GDP for GTP. The conformational change within the heterotrimer is supposed to trigger dissociation of the Gα subunit from the Gβγ complex, although it is still under debate whether real dissociation or just conformational rearrangement occurs upon receptor activation (Hepler, Gilman 1992; Neves et al. 2002; Bünemann et al. 2003; Bodmann et al. 2015; Duc et al. 2015; Milligan, Kostenis 2006). Both the Gα and the Gβγ complex have the ability to trigger intracellular molecular cascades. Notably, both in the PNS and the CNS, myelination has been shown to be influenced by these intracellular signaling pathways.

To date, the Gα subunits are subdivided into four major subclasses $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{s}$, and $G\alpha_{12/13}$ due to their amino acid sequence homology (Pierce et al. 2002).

1.2.1.1 $G\alpha_q$

Activation of $G\alpha_q$ stimulates phospholipase Cβ (PLCβ) isoforms, enzymes which cleave phosphatidylinositol (4,5)-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). Cytosolic IP3 activates IP3 receptors at the endoplasmic reticulum which is followed by an increase of intracellular Ca^{2+} levels. In addition, DAG and intracellular Ca^{2+} levels can activate protein kinase C (PKC). The orphan very large G protein-coupled receptor 1 (VLGR1), also known as monogenic audiogenic seizure susceptible 1 (MASS1), is expressed in oligodendrocytes and has been associated with an increase in the myelination marker MAG *in vitro* via a PKA and PKC δ/θ-dependent pathway (Shin et al. 2013). Remarkably, in contrast, Baer and coworkers characterized PKC as negative modulator of oligodendrocyte differentiation (Baer et al. 2009). In the

latter study, a specific inhibitor of PKCα and β, Gö6976, counteracted the inhibitory influence of myelin basic extracts on oligodendrocyte development. These studies suggest different regulatory functions of the various PKC isoforms on the maturation of oligodendrocytes.

1.2.1.2 $G\alpha_s$ and $G\alpha_{i/o}$

Activated $G\alpha_s$ and $G\alpha_{i/o}$ stimulate or inhibit cAMP production, respectively, and thereby cAMP signaling. Recently, Mogha and co-coworkers investigated the role of the orphan adhesion GPCR GPR126 expressed on Schwann cells in the PNS. The receptor increases intracellular cAMP levels in a $G\alpha_s$-dependent manner to promote the development of Schwann cells (Monk et al. 2011; Mogha et al. 2013).

The lysophosphatidic acid receptor 1 (LPAR1), also expressed in Schwann cells, has been recently identified as a key player in migration and peripheral nerve development via $G\alpha_i$-dependent signaling (Anliker et al. 2013). Likewise, two GPCRs of the chemokine receptor family, CXCR2 and CXCR4, were verified as regulators of oligodendrocyte maturation. Remarkably, even if both receptors are known as activators of $G\alpha_i$ proteins, they show opposing effects on the development of oligodendrocytes: CXCR2 impairs differentiation and CXCR4 has a positive regulatory effect. However, the molecular mechanisms of both receptors within the cell are poorly understood (Kerstetter et al. 2009; Patel et al. 2010).

1.2.1.3 $G\alpha_{12/13}$

Activation of $G\alpha_{12/13}$ modulates the activity of the small GTPase RhoA via a RhoGEF-pathway (Siehler 2009). The orphan adhesion GPCR GPR56 promotes proliferation of OPCs via $G_{12/13}$ signaling, followed by RhoA activation. Consequently, stimulation of GPR56 impairs the transition of OPC to mature oligodendrocyte (Ackerman et al. 2015). Furthermore, the RhoA-Rho-associated kinase 2 (ROCK2) pathway was directly linked to less efficient maturation of oligodendrocytes (Baer et al. 2009). Importantly, the aforementioned LINGO-1 antibody (see 1.1.1) (Mi et al. 2005) acts by inhibiting RhoA to promote oligodendrocyte differentiation.

1.2.1.4 The second messenger cAMP and its downstream effectors

Intracellular accumulation of the second messenger cAMP is triggered by $G\alpha_s$ proteins and reduced by $G\alpha_{i/o}$ proteins. cAMP was the first discovered second messenger and mediates a wide range of cellular functions, such as calcium homeostasis, metabolism, secretion, muscle contraction, cell fate, and gene transcription (Schmidt et al. 2013). Importantly, cAMP triggers physiologically relevant processes by three cAMP effector proteins: (i) the protein kinase A (PKA), (ii) the exchange proteins directly activated by cAMP 1 and 2 (Epac1/2), and (iii) cyclic-nucleotide-gated ion channels (CNG channels)

(Vitali et al. 2015). Evidence to date demonstrates a major contribution of cAMP-associated pathways in the maturation cascade of oligodendrocytes. Increase of intracellular cAMP both directly by application of cAMP analogs and indirectly by activators of adenylyl cyclases and/or inhibitors of phosphodiesterases triggers the transition from OPCs to oligodendrocytes. In contrast, cAMP up-regulation has an inhibitory effect on OPC proliferation (Raible und McMorris 1993).

PKA, the best studied effector protein of cAMP, directly activates the transcription factor cAMP-responsive element-binding protein (CREB) upon alteration of intracellular cAMP levels. Worth mentioning, CREB was identified and characterized only one year after the identification of the cAMP response element (CRE) (Montminy et al. 1986; Montminy und Bilezikjian 1987). PKA directly phosphorylates CREB at Ser133. The CREB protein is ubiquituously expressed in the human body and is part of the leucine zipper transcription factor family (Ortega-Martínez 2015). A number of excellent studies demonstrate that upregulation of myelin proteins, such as MBP, largely depend on the activation of the transcription factor CREB (Sato-Bigbee, DeVries 1996; Afshari et al. 2001).

Further important cAMP targets are EPAC1 and 2, also named cAMP-GEFI and II, which have recently been discovered as essential cAMP targets by a database screen performed to elucidate the PKA-independent activation of the small G protein Rap (Rooij et al. 1998). EPAC is involved in several physiological processes, including cell proliferation, cell survival, and cell differentiation (Bos 2003; Roscioni et al.; Schmidt et al. 2013). Interestingly, a current study has described the involvement of EPAC in myelination via a GPR37-dependent mechanism (Yang et al. 2016).

1.2.2 β-arrestin recruitment and signaling

β-arrestins are multifunctional adaptor proteins which are important as GPCR activity modulators and indirect transducers. They are mostly known for their role in receptor desensitization and internalization. After phosphorylation of membrane-bound GPCRs by G protein-coupled receptor kinases (GRKs), β-arrestins are recruited, then sterically hindering further G protein coupling of the receptor (Shenoy, Lefkowitz 2011). Consequently, the receptor is desensitized. Furthermore, increasing evidence has accumulated to suggest that β-arrestins have the capacity to mediate GPCR signaling in a G protein-independent manner (Shukla et al. 2011).

Interestingly, GRK2, GRK5, β-arrestin1, and β-arrestin2 undergo a well-defined expression pattern during the maturation process in rat oligodendrocytes (Daniele et al. 2014). These data suggest that GRKs and β-arrestins could have a particular impact on the desensitization of GPCRs in the development of oligodendrocytes, but further studies are necessary to investigate this hypothesis (Daniele et al. 2014).

1.2.3 Extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling - mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) belong to the highly important class of protein kinases and act as serine/threonine kinases. In all eukaryotic cells, the MAPK pathways are involved in various cellular processes, for instance cell growth, division, differentiation or apoptosis (Pearson et al. 2001; Luttrell 2003; Roux, Blenis 2004; Lu, Xu 2006). A wide range of studies also connected activation of GPCRs with cellular growth and cell differentiation via MAPKs. Particularly in oligodendrocytes, several lines of evidence also implicated a key role of MAPKs, especially c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), extracellular signal-regulated kinase 5 (ERK5), and p38, during oligodendrocyte development (Chew et al. 2010; Xiao et al. 2012a).

ERK1 and ERK 2 have a molecular weight of 43 kDa and 41 kDa, respectively, and are also called p42MAPK und p44MAPK (Pearson et al. 2001). These kinases revealed 83% sequence homology and are ubiquitously expressed in the human body (Raman et al. 2007; Gonsalvez et al. 2015). ERK1/2 respond to a wide variety of stimuli, in particular to growth factors and phorbol esters, via receptor tyrosine kinase and PKC activation, but they are also involved in GPCR-mediated signaling (Roux, Blenis 2004; Raman et al. 2007). ERK1/2 activation has a critical role in oligodendrocyte development, myelination, remyelination, and survival (Gonsalvez et al. 2015). In particular, ERK1/2 long-term activation was associated with an increase of MBP expression (Xiao et al. 2012a; Xiao et al. 2012b) . Importantly, ERK1/2 has a direct impact on the thickness of the formed myelin sheath (Ishii et al. 2012). To date, there is no direct demonstration that GPCRs could alter the intracellular phosphorylation level of ERK1/2 during oligodendrocyte differentiation. However, the phosphodiesterase 4 (PDE4) inhibitor rolipram, which increases intracellular cAMP levels, activates maturation of oligodendrocytes *in vitro* partially increasing the phosphorylation levels of ERK1/2 (Sun et al. 2012).

Activated ERK1/2 may translocate into the nucleus to induce phosphorylation of transcription factors, as has been shown in various studies investigating the key role of ERK1/2 in the phosphorylation of the transcription factor CREB. The capacity to phosphorylate CREB represents the link between the cAMP-ERK1/2 pathway and upregulation of myelin proteins during maturation of oligodendrocytes (Costes et al. 2006; Won et al. 2015; Gonsalvez et al. 2015; Liu et al. 2016).

1.3 Orphan GPCRs

More than 140 of the non-odorant GPCRs are so-called orphan receptors with a not yet known endogenous ligand (Fang et al. 2015; Shore, Reggio 2015; Civelli 2005). Orphan

GPCRs are assumed to represent promising drug targets, which may allow for development of innovative pharmaceuticals with first-in-class mechanisms of actions (Shore, Reggio 2015). Orphan receptors are involved in a wide range of physio-pathologically relevant signaling cascades, implying that the discovery of their endogenous modulators will increase the knowledge about their relevance in the human body. One approach to investigate the physiological or pathophysiological role of an orphan receptor is to analyze knockout animals or transgenic animals (Tang et al. 2012).

The International Union of Basic and Clinical Pharmacology (NC-IUPHAR) defines the criteria for deorphanization of GPCRs. One of the highly important criteria is that the proposed ligand/ligands activity at an orphan receptor should be validated from at least two independent research groups in refereed papers. Furthermore, the potency of the putative ligand/ligands should be plausible with a physiologic function of the receptor (Davenport et al. 2013; Garland 2013).

The endogenous agonist identification of orphan GPCRs and consequently their deorphanization is one of the key goals to determine orphan GPCRs' potential as new drug targets. Therefore, in the last years several deorphanization bioinformatic and biochemical strategies have been developed: (i) identification of sequence similarities between receptors, (ii) definition of a relationship between receptor and the putative ligand expression profiles, (iii) identification of ligands in tissue extracts (also called orphan receptor strategy), and (iv) random testing of potential ligands on orphan receptors (also called reverse pharmacology) (Civelli et al. 1999; Civelli 2001; Tang et al. 2012).

Several lines of evidence have implicated an important role for orphan GPCRs expressed in oligodendrocytes or Schwann cells, such as GPR17, GPR56, GPR126 and VLGR1, in proliferation, myelination, remyelination and demyelination (Chen et al. 2009; Shin et al. 2013; Ackerman et al. 2015; Simon et al. 2016).

1.3.1 The G protein coupled receptor 17 (GPR17)

GPR17 belongs to the rhodopsin-like GPCRs and its gene was isolated for the first time in 1996 by Raport and co-workers using PCR-screenings on human genomic DNA as a template (Raport et al. 1996). The receptor sequence resembles those of the cysteinyl-leukotriene (CysLT) and purinergic receptors (PY2R), implicating a phylogenetic proximity between these two receptor classes and GPR17 (Bläsius et al. 1998; Ciana et al. 2006). In particular, GPR17 shares an amino acid similarity of 21 to 48% with receptors of the purinergic and cysteinyl-leukotriene cluster, respectively (Fumagalli et al. 2015b). Two splice variants of human GPR17 are known, whereby the long form is characterized by a 28 amino acid longer N-terminal part than the short form (Benned-Jensen, Rosenkilde 2010).

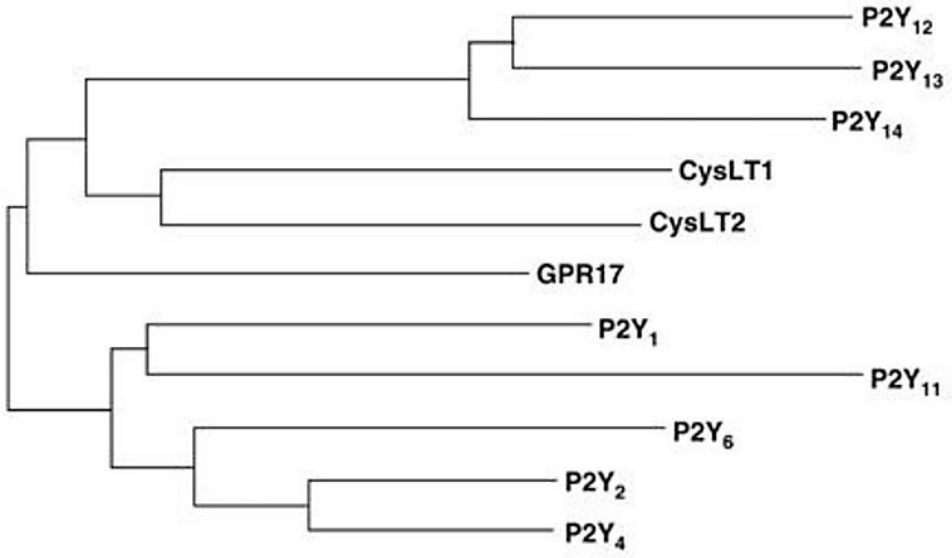


Figure 2. **Phylogenetic tree**

Phylogenetic tree pointing out the relation between the P2Y and CysLT receptor families and GPR17 (modified from (Ciana et al. 2006)).

1.3.1.1 Expression of GPR17

GPR17 is predominantly expressed in the CNS, in particular in oligodendrocytes at a certain stage of development. Strikingly, the expression of GPR17 on neurons is still under debate (Bläsius et al. 1998; Lecca et al. 2008; Chen et al. 2009; Benned-Jensen, Rosenkilde 2010; Ren et al. 2015). Moreover, GPR17 is also detectable in tissues, which typically undergo ischemic damage, more precisely in kidney and heart and, in addition, at a low level in liver and lung. Furthermore, immunostaining of human peripheral blood monocytes appears GPR17 positive (Ciana et al. 2006; Benned-Jensen, Rosenkilde 2010). In contrast, Chen and co-works reported a restriction of GPR17 expression to the brain (Chen et al. 2009).

The expression of GPR17 on rodent oligodendrocytes undergoes a well-defined time course in vitro. The receptor is absent in the early OPC stage. After this stage, GPR17 expression increases along with co-expressed O4, a marker for immature oligodendrocytes. GPR17 is downregulated in mature oligodendrocytes expressing typical myelination markers, such as MBP (Fumagalli et al. 2011). *In vivo,* the abundance of GPR17-positive cells in the murine spinal cord increases until postnatal day 7-14, but thereafter decreases drastically (Chen et al. 2009).

The fact that GPR17 expression is inversely correlated to the MBP abundance within the oligodendrocytes suggests a critical role of this receptor in the final differentiation from immature into myelinating oligodendrocytes (Fumagalli et al. 2011).

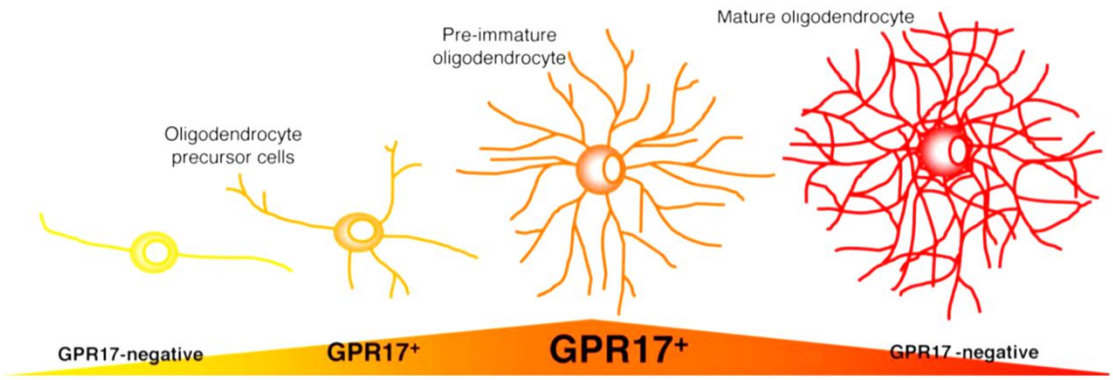


Figure 3. **GPR17 expression pattern in oligodendrocytes during development**

In an early stage of development, OPCs are GPR17-negative. During differentiation, oligodendrocytes start to express GPR17 and its expression reaches a maximum in pre-immature oligodendrocytes. Finally, no GPR17 expression occurs in mature myelinating oligodendrocytes (modified from (Fumagalli et al. 2011)).

1.3.1.2 GPR17 activation and signaling

GPR17 was initially deorphanizd by Ciana et al. in 2006 and described as the elusive dualistic receptor that can be activated by endogenous ligands of the uracil-nucleotide and cysteinyl-leukotriene receptor families, in particular UDP, UDP-gal, UDP-glc, LTD4, and LTC4 (Ciana et al. 2006). However, Bläsius et al. described in 1998 that GPR17 is not activated by the typical P2Y receptor agonists by utilizing classical second messenger assays (Bläsius et al. 1998). Furthermore, Heise and co-authors reported that GPR17 does not respond to LTD4 or LTC4 in 2000 (Heise et al. 2000). Additionally, several independent laboratories have not been supportive of the concept of GPR17 as dualistic receptor activated by uracil-nucleotides and cysteinyl-leukotrienes (Qi et al. 2013; Hennen et al. 2013; Köse et al. 2014). Thus, the nature of the endogenous ligand(s) of GPR17 is still under debate.

Recently, two additional classes of endogenous ligands have been proposed for this receptor. First, GPR17 activation by oxysterols has been reported. Oxysterols are described as agonists at both Epstein Barr virus-induced gene receptor-2 (EBI2) and chemokine receptor CXCR2 (Sensi et al. 2014). Recently, GPR17 has also been described to respond to stromal cell-derived factor 1 (SDF-1), a ligand of the two chemokine receptors CXCR4 and CXCR7 (Parravicini et al. 2016).

Noteworthy, MDL29,951 (2-carboxy-4,6-dichloro-1H-indole-3-propionic acid) has been recently reported as a small molecule synthetic agonist for GPR17. In particular, MDL29,951 specifically activated GPR17 in primary oligodendrocytes, which endogenously express a variety of purinergic receptors. However, MDL29,951 was not competent to activate the purinergic cluster receptors, the histamine 3 receptor (H3R), the dopamine 2 receptor (D2R) as well as ATP-responsive immortalized cells (Hennen et al.

2013). Moreover, a radioactive form of MDL29,951 showed no binding to CHO-K1 membranes, but specific binding to membranes stably expressing GPR17 (Köse et al. 2014). Taken together, these studies verify MDL29,951 as specific small molecule agonist at GPR17 in heterologous expression system and primary oligodendrocytes.

GPR17, activated by the proposed endogenous ligands, has originally been described as $G\alpha_i$-coupled receptor in 1321N1 cells (Ciana et al. 2006). Later, MDL29,951-activated GPR17 was characterized as promiscuous GPCR, engaging the entire set of intracellular adaptor proteins, which leads to intracellular second messenger alteration (Hennen et al. 2013). In immature oligodendrocytes, MDL29,951 rapidly mobilized intracellular Ca^{2+} in a $G\alpha_i$- and $G\alpha_q$-dependent manner and depressed cAMP concentration via $G\alpha_i$ activation (Hennen et al. 2013).

Considering the phylogenetic relationship of GPR17 to receptors of the purinergic and cysteinyl-leukotriene cluster, antagonists of both classes were tested as pharmacological tools to block GPR17 function. In the original deorphanization report, pranlukast and montelukast were reported to functionally inhibit LTD4-activated GPR17. Both are selective CysLT1 receptor antagonists and are used in a wide range of inflammatory conditions, in particular in asthma. Furthermore, cangrelor was shown to inhibit UDP-glc-induced GPR17 activity (Ciana et al. 2006). In a follow-up study by the same group, ticagrelor was also characterized as GPR17 inhibitor. Both substances, cangrelor and ticagrelor, block P2Y12 receptors expressed on thrombocytes and thereby act as antiplatelet drugs (Laine et al. 2016). Hennen and co-authors did not verify the antagonistic properties of montelukast, but instead confirmed pranlukast as a GPR17 antagonist, albeit with rather low potency (pIC50 = 5.57 in calcium release assays at hGPR17). Therefore, the identification of a potent antagonist on GPR17 still remains an open task for pharmaceutical research.

1.3.1.3 GPR17 and its physiological function

The high level of GPR17 expression in the brain likely implicates a significant role in physiological processes in the CNS.

Transgenic overexpression of GPR17, for example, results in myelinogenesis defects of developing mice in vivo. In line with this, GPR17 knockout revealed an early onset of oligodendrocyte myelination (Chen et al. 2009). In addition, pharmacological studies utilizing the small molecule agonist MDL29,951 uncovered an arrest of oligodendrocytes in a pre/immature stage mediated by GPR17 activation (Hennen et al. 2013). In former studies, Abbracchio and co-workers obtained contradictory results. Thus, GPR17 was first identified as promotor of oligodendrocyte differentiation by applying the putative endogenous ligands cysteinyl-leukotrienes and uracil nucleotides as well as by small interfering RNA (siRNA)-based gene silencing experiments (Ceruti et al.

2009). However, in a follow-up study, the same group described GPR17 as blocker of oligodendrocyte maturation, at least during the late stage of oligodendrocyte development (Fumagalli et al. 2015a).

Remarkably, whereas GPR17 is downregulated in spinal cord of adult mice, in demyelinating lesions of the murine experimental autoimmune encephalomyelitis (EAE) model a significant increase was detectable by quantitative RT-PCR (Chen et al. 2009). In line with the murine *in vivo* data, analysis of human multiple sclerosis samples revealed a significant increase in GPR17 expression in multiple sclerosis plaques (Chen et al. 2012). Taken together, these data support the notion that GPR17 impairs myelination of oligodendrocytes in the CNS; accordingly therapy of demyelinating diseases should benefit from a GPR17 antagonist (Hennen et al. 2013).

Besides the important role of GPR17 in myelination, studies revealed GPR17 as a significant protagonist in brain rejuvenation and food intake, as well as mediator of brain and spinal cord damage (Lecca et al. 2008; Ceruti et al. 2009; Ren et al. 2015; Marschallinger et al. 2015). An increased basal proliferative activity in proliferation assays was observed in a neurosphere culture system of GPR17 knockout cells, suggesting a negative influence of GPR17 on brain rejuvenation (Marschallinger et al. 2015). Moreover, analyses of an agouti-related peptide (AgRP) expressing neuron-specific GPR17 knockout mouse line suggested that GPR17 activation has a negative impact on food intake, leanness, and body fat (Ren et al. 2015). In contrast, Mastaitis and co-workers could not support the role of GPR17 in food intake (Mastaitis et al. 2015). Furthermore, GPR17 was recognized as a mediator of neuronal death at the early stage of ischemic damage by analyzing rodent ischemia and spinal cord injury models (Lecca et al. 2008; Ceruti et al. 2009).

In addition to the role of the receptor in the brain, GPR17 has been also described as immune modulator in inflammatory lung disease by a ligand-independent mechanism. *In vivo* and *in vitro* data showed that GPR17 acts as regulator of functionality and expression of the type 1 cysteinyl leukotriene receptor (CysLT1), which is an important mediator of human bronchial asthma (Maekawa et al. 2009; Maekawa et al. 2010; Qi et al. 2013).

Taken together, GPR17 exerts its main function in the brain. Particularly important for the investigations within the context of this thesis is its negative role in the maturation process of oligodendrocytes.

1.4 Aims of the study

In the last years, genetic and pharmacological studies verified the orphan GPR17 as negative modulator of oligodendrocyte differentiation and myelination. In addition,

GPR17 abundance is augmented in animal models of MS and active white matter plaques of MS patients. These two notions suggest an adverse impact of GPR17 on remyelination. However, the underlying molecular mechanisms engaged by GPR17 are still elusive. Detailed mechanistic insight into the GPR17 signal transduction circuitry from the plasma membrane to the nucleus is both desirable and required to firmly establish GPR17 as innovative, first-in-class target to foster remyelination in demyelinating diseases such as MS.

Therefore, the first aim of the present study is to unveil which members of the heterotrimeric G protein family including their downstream effector proteins are activated by GPR17 to arrest oligodendrocytes in an immature stage. To this end, two cell lines, primary rat oligodendrocytes and immortalized murine oligodendrocytes (Oli-neu cells) were utilized as model systems in conjunction with MDL29,251, a precious pharmacological tool and surrogate ligand, that specifically and reliably activates GPR17 in both heterologous expression systems and in oligodendrocyte cell cultures.

The identification of GPR17 endogenous activators would further support the development of pharmacological agents targeting GPR17 for the treatment of demyelinating diseases. GPR17 has been described as a dualistic receptor responding to the established ligands of CysLT and P2Y receptors, cysteinyl-leukotrienes and uracil-nucleotides, respectively. Although the original deorphanization report dates back to 2006, the scientific community is still in doubt about functionality of the proposed endogenous ligands. Thus, the second aim of the present work is to challenge the dubitable endogenous ligands. To monitor GPR17 functionality three different readouts, which analyze various endpoints in the signaling cascade were chosen: (i) $G\alpha_i$ protein rearrangement, (ii) β-arrestin recruitment, and (iii) ERK1/2-phosphorylation.

The negative influence of GPR17 on oligodendrocyte maturation strongly suggests that pharmacological inhibition may represent an innovative therapeutic strategy to foster “myelin repair”. Based on the phylogenetic relationship between GPR17 and P2Y12 as well as CystL1 receptors, selected antagonists for the latter (HAMI3379, CAY10633, ticagrelor, cangrelor) were investigated for their capacity to functionally interdict activation of GPR17 in recombinant and primary expression systems (third aim of this thesis).

2 Materials

2.1 Cell culture

2.1.1 Mammalian cell lines

Table 1. **Cell lines**

Name	*Biological source/Characteristic*	*Producer/Owner*
Oli-neu	brain OPC from mouse	Kindly provided by Prof. J. Trotter (University of Mainz)
HEK293	human embryonic kidney	Research group of Prof. Dr. Evi Kostenis, Institute of Pharmaceutical Biology, University of Bonn, Germany
3-HA-hGPR17-HEK293 (hGPR17-HEK293)	human embryonic kidney	Research group of Prof. Dr. Evi Kostenis, Institute of Pharmaceutical Biology, University of Bonn, Germany
mGPR17-HEK293	human embryonic kidney	Research group of Prof. Dr. Evi Kostenis, Institute of Pharmaceutical Biology, University of Bonn, Germany
rGPR17-HEK293	human embryonic kidney	Research group of Prof. Dr. Evi Kostenis, Institute of Pharmaceutical Biology, University of Bonn, Germany
FLAG-hCysLT1-HEK293 (CysLT1-HEK293)	human embryonic kidney	Research group of Prof. Dr. Evi Kostenis, Institute of Pharmaceutical Biology, University of Bonn, Germany
P2Y14-HEK293	human embryonic kidney	Research group of Prof. Dr. Evi Kostenis, Institute of Pharmaceutical Biology, University of Bonn, Germany

Name	*Biological source/Characteristic*	*Producer/Owner*
3HA-hGPR17-RLuc-GFP2-β-arrestin2-HEK293 (BRET-HEK293)	human embryonic kidney	Research group of Prof. Dr. Evi Kostenis, Institute of Pharmaceutical Biology, University of Bonn, Germany (Kindly provided by Stephanie Hennen)
1321N1	brain astrocytoma from human	Research group of Prof. Dr. Evi Kostenis, Institute of Pharmaceutical Biology, University of Bonn, Germany
hGPR17-1321N1	brain astrocytoma from human	Research group of Prof. Dr. Evi Kostenis, Institute of Pharmaceutical Biology, University of Bonn, Germany
Primary rat OPCs	newborn rat pups	Isolated from forebrains of Wistar rat pups at P0-P2

2.1.2 Cell culture medium

2.1.2.1 Media, buffer, supplements and growth factors

Table 2. **Media, buffer, supplements and growth factors**

Name	*Product Number*	*Company*
B27 supplement (B27)	17504-044	Thermo Scientific™
ciliary neurotrophic factor (CNTF)	450-50	PeproTech
Dulbecco's Modified Eagle Medium (DMEM)	41965	Thermo Scientific™
Fetal calf serum (FCS)	P30-3702	PANTM Biotech GmbH
G418, liquid (Geneticin, 100mg/ml)	Ant-gn-5	InvivoGen
Gentamicin	15750037	Thermo Scientific™
GlutaMAX	1674801	Thermo Scientific™
Hank's balanced salt solution (HBSS)	14025050	Thermo Scientific™
Horse serum, heat inactivated (HS)	26050070	Thermo Scientific™
Insulin	1258-014	Thermo Scientific™
L-thyroxine	T-2376	Sigma-Aldrich
N2 Supplement	1665870	Thermo Scientific™
Neurobasal Medium	21103049	Thermo Scientific™
Opti-MEM	1616013	Thermo Scientific™
Penicillin-Streptomycin solution	15140122	Thermo Scientific™
Recombinant human fibroblast growth factor-basic	100-18B	PeproTech
Recombinant human PDGF-AA	100-13A	PeproTech
Sodium selenite	S-5261	Sigma-Aldrich
3,3′,5-Triiodo-L-thyronine sodium salt (T3)	T-2752	Sigma-Aldrich
Trypsin-EDTA (0.05%)	25300054	Thermo Scientific™
UltraPureTM Distilled water	10977	Thermo Scientific™
Zeocin	R25001	Thermo Scientific™

2.1.2.2 Cell specific culture medium

Oli-neu cells

Constituent	*volume [ml]*	*final concentration*

Dulbecco's Modified Eagle Medium (DMEM)	500	
Horse serum (HS)	5	≈1%
Penicillin-Streptomycin	5	≈100 U/ml Penicillin, 0.1 mg/ml Streptomycin
Insulin	625 µl	5 µg/ml
Gentamicin	250 µl	25 µg/ml
N2 supplement	5 ml	≈1%
T3	400 µl	400 nM
sodium selenite	370 µl	190 nM
L-thyroxine	65 µl	520 nM

Mixed glia Medium

Constituent	*volume [ml]*	*final concentration*
Dulbecco's Modified Eagle Medium (DMEM)	500	
Heat-inactivated fetal calf serum (FCS)	50	≈10%
Penicillin-Streptomycin	5	≈100 U/ml Penicillin, 0.1 mg/ml Streptomycin

Proliferation Medium OPCs

Constituent	*volume [ml]*	*final concentration*
Neurobasal	50	
B27	1	≈2%
Penicillin-Streptomycin	0,5	≈100 U/ml Penicillin, 0.1 mg/ml Streptomycin
Glutamax	0,5	≈1%
Recombinant human fi-	50	10 ng/ml

broblast growth factor-basic (1µg/ml)		
Recombinant human PDGF-AA(1µg/ml)	50	10 ng/ml

Differentiation induction medium

Constituent	*volume [ml]*	*final concentration*
Neurobasal	50	
B27	1	≈2%
Penicillin-Streptomycin	0,5	≈100 U/ml Penicillin, 0.1 mg/ml Streptomycin
Glutamax	0,5	≈1%

Recombinant cell lines

1321N1 cells

Constituent	*volume [ml]*	*final concentration*
Dulbecco's Modified Eagle Medium (DMEM)	500	
Fetal calf serum (FCS)	50	≈10%
Penicillin-Streptomycin	5	≈100 U/ml Penicillin, 0.1 mg/ml Streptomycin

hGPR17-1321N1

Constituent	*volume [ml]*	*final concentration*
Dulbecco's Modified Eagle Medium (DMEM)	500	

Fetal calf serum (FCS)	50	≈10%
Penicillin-Streptomycin	5	≈100 U/ml Penicillin, 0.1 mg/ml Streptomycin
G418	4	800 µg/ml

HEK293 cells

Constituent	*volume [ml]*	*final concentration*
Dulbecco's Modified Eagle Medium (DMEM)	500	
Fetal calf serum (FCS)	50	≈10%
Penicillin-Streptomycin	5	≈100 U/ml Penicillin, 0.1 mg/ml Streptomycin

3HA-hGPR17-HEK293 (later named as hGPR17-HEK293)

Constituent	*volume [ml]*	*final concentration*
Dulbecco's Modified Eagle Medium (DMEM)	500	
Fetal calf serum (FCS)	50	≈10%
Penicillin-Streptomycin	5	≈100 U/ml Penicillin, 0.1 mg/ml Streptomycin
Zeocin	0.31	56 µg/ml

rGPR17-HEK293, mGPR17-HEK293, hCysLT1-HEK293 and P2Y14-HEK293

Constituent	*volume [ml]*	*final concentration*
Dulbecco's Modified Eagle Medium (DMEM)	500	

Fetal calf serum (FCS)	50	≈10%
Penicillin-Streptomycin	5	≈100 U/ml Penicillin, 0.1 mg/ml Streptomycin
G418	2.5	500 µg/ml

hGPR17-BRET-HEK293

Constituent	*volume [ml]*	*final concentration*
Dulbecco's Modified Eagle Medium (DMEM)	500	
Fetal calf serum (FCS)	50	≈10%
Penicillin-Streptomycin	5	≈100 U/ml Penicillin, 0.1 mg/ml Streptomycin
G418	2.5	500 µg/ml
Zeocin	0.31	56 µg/ml

2.2 Plasmids

Table 3. **Plasmids**

Plasmids	*Size (bp)*	*Host*
pcDNA3.1+	5428	Invitrogen™
hGPR17 in pcDNA3.1+	1020	Research group of Prof. Dr. Evi Kostenis, Institute of Pharmaceutical Biology, University of Bonn, Germany
$hG\alpha_{i1}$-91hRluc in pcDNA3.1+	2039	Kindly provided by M. Bouvier. Department of Biochemistry, Institute for Research in Immunology and Cancer, Université de Montréal, Montréal, Canada.
GFP10-$bG\gamma_2$ in pcDNA3.1+	958	Kindly provided by M. Bouvier. Department of Biochemistry, Institute for Research in Immunology and Cancer, Université de Montréal, Montréal, Canada.
$hG\beta_1$ in pcDNA3.1+	1032	Kindly provided by M. Bouvier. Department of Biochemistry, Institute for Research in Immunology and Cancer, Université de Montréal, Montréal, Canada.

2.3 Antibodies

2.3.1 Primary Antibodies

Table 4. **Primary antibodies**

Antibody	Species	Product Number	Company
Anti-β-actin	rabbit	BLD-622102	Biolegend
Anti-EPAC1	mouse	4155 S	Cell signaling Technology
Anti-EPAC2		4156 S	
Anti-pERK	mouse	5726	Cell signaling Technology
Anti-t-ERK	rabbit	9102	
Anti-p-CREB	rabbit	9198	Cell signaling Technology
Anti-t-CREB	mouse	9104	
Anti-$G\alpha_{i-3}$ (Anti-$G\alpha_i$)	rabbit	Sc-626	Santa Cruz Biotechnology
Anti-$G\alpha_o$	rabbit	SC-387	Santa Cruz Biotechnology.
Anti-$G\alpha_s$	rabbit	Sc-823	Santa Cruz Biotechnology.
Anti-$G\alpha_q$	rabbit	Sc-392	Santa Cruz Biotechnology.
Anti-GPR17	rabbit	CAY-17087-1	Cayman
Anti-MBP	mouse	LB-B8056-100	LifeSpan BioSciences

2.3.2 Secondary Antibodies

Table 5. **Secondary antibodies**

Antibody	Species	Product Number	Company
Anti-mouse-HRP	goat	A4416	Sigma
Anti-rabbit-HRP	goat	ABIN102010	Antikoerper-online

2.4 Chemicals

2.4.1 Chemicals used in the present work

Table 6. **Chemicals**

Substance	*Product Number*	*Company*
8-CPT-cyclic AMP	C010-100	BIIOLOG
8-CPT-2`-O-Me-cAMP	C 041	BIOLOG
Acrylamid, Rotiphorese Gel 40	A5115.1	Roth
Ammonium persulfate	A3678	Sigma-Aldrich

Adenosine 5′-triphosphate disodium salt hydrate (ATP)	A6419	Sigma
Calcium chloride, dihydrate	21097	Fluka
calcium ionophore A23187	1096801	Sigma-Aldrich
Cangrelor	3312.F08601	The Medicines Company
Carbachol	212385	Merck
CAY10633	10532	Cayman chemical
Coelenterazine 400A (Deep Blue C)	C-320-10	Gold Biotechnology
DMSO (cell culture)	A1584	AppliChem
Ethanol absolute	08-205	KMF
ESI-05	M 092-05	Biolog
Forskolin	1099	Tocris
FuGENE HD Transfection Reagent	E2312	Promega
Glycerin 99%	11052	Grüssimh GmbH
HAMI3379	10580	Cayman Chemical
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	54457	Fluka
Hydrochloric acid min. 37%	08-721	KMF
IGEPAL® Ca-630	13021	Sigma-Aldrich
Isobutylmethylxanthine (IBMX)	2845	Tocris
Kaleidoscope Prestained Standard	161-0324	Bio-Rad
LTC4	20210	Cayman Chemical
LTD4	20310	Cayman Chemical
MagicMark™ XP Western Protein Standards	LC5602	Invitrogen
Methanol	125678	Sigma-Aldrich
MDL29,951	SEWO6645SC	Maybridge
NuPAGE® Antioxidant	NP0005	Novex™ by Life Technologies
NuPAGE® LDS Sample Buffer (4X)	NP0007	Novex™ by Life Technologies
NuPAGE® MOPS SDS Running Buffer (20X)	NP0001	Novex™ by Life Technologies
NuPAGE™ Novex™ 10% Bis-Tris Protein Gels, 1.5 mm, 10-well	NP0315Box	Novex™ by Life Technologies

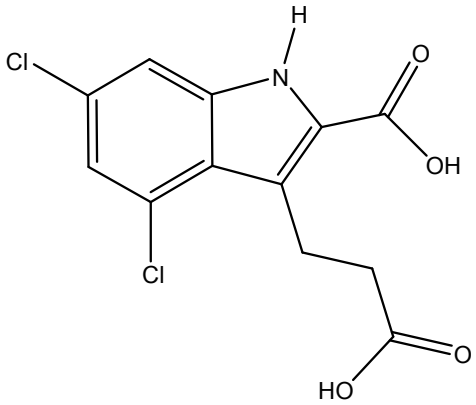
NuPAGE® Sample Reducing Agent (10X)	NP0009	Novex™ by Life Technologies
NuPAGE® Transfer Buffer (20X)	NP000P-6	Novex™ by Life Technologies
PD174265	513040	Calbiochem
Pertussis toxin (PTX)	2980	Sigma-Aldrich
Protease inhibitor cocktail	P8340	Sigma-Aldrich
Potassium chloride	5346.2	Roth
Potassium dihydrogen phosphate	1.04873.5000	Merck
Poly-L-ornithine hydrobromide (PLO)	P3655	Sigma-Aldrich
Poly-D-lysine hydrobromide (PDL)	P6407	Sigma-Aldrich
Pranlukast	10008319	Cayman chemical
Propan-2-ol	P-750715	Fisher Scientific
Lipofectamine® RNAiMAX Transfection Reagent	13778030	Thermo Scientific™
Roti®-Block	A151.2	Roth
Rp-8-CPT-cAMPS	C-011-05	BIOLOG
S.O.C. medium	15544034	Thermo Scientific™
SDS Pellets	CN30.3	Roth
Sodium chloride	10112640	Fisher scientific
Di-sodium hydrogen phosphate, anhydrous	71640	Fluka
Sodium hydroxide	12156	Grüssing GmbH
Sp-6-Bnz-cAMPS	B 040	BIOLOG
Temed	2367.3	Roth
Tris	A2264	AppliChem
Triton-X	93420	Fluka
Tween	P-1379	Sigma-Aldrich
UltraPure™ Distilled water	10977035	Thermo Scientific™
Uridine 5′- diphosphate disodium salt hydrat (UDP)	94330	Sigma-Aldrich
Uridine 5′-diphosphoglucose disodium salt (UDP-glc)	94335	Sigma-Aldrich

UDP-α-D-Galactose, Disodium Salt (UDP-gal)	6700111	Calbiochem
Yeast extract	2363.3	Roth

Table 7. **Not purchased compounds**

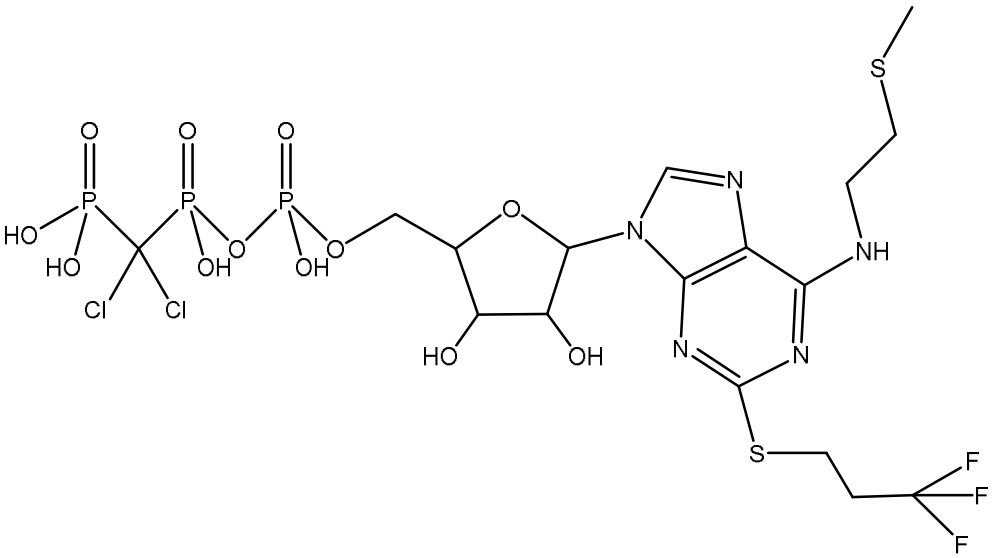
Substance	function	Source
FR900359	Selective inhibitor of $G\alpha q_{/11}$ (Schrage et al. 2015)	Kindly provided by AG König, Institute of Pharmaceutical Biology, University of Bonn, Germany
Ticagrelor	P2Y12 antagonist	Kindly provided by Sven Nylander

2.4.2 **Chemical structures**



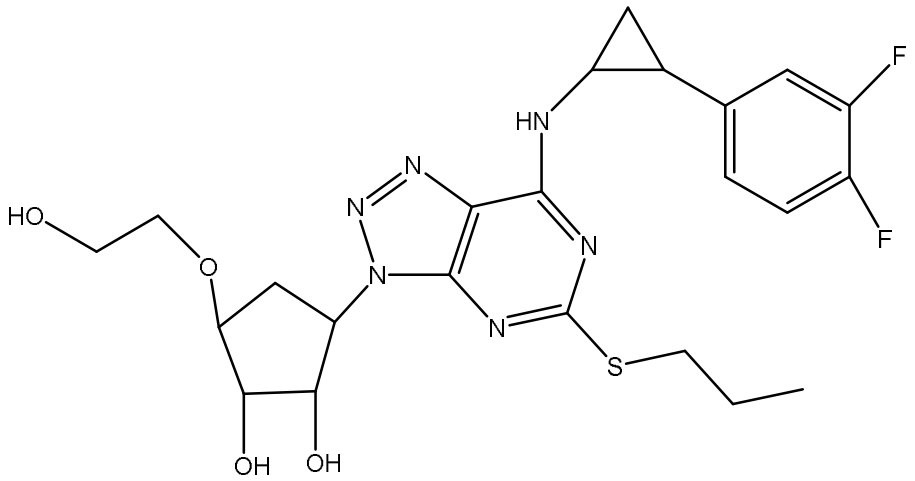
Chemical Formula: $C_{12}H_9Cl_2NO_4$

Structure 1. MDL29,951 (GPR17 Agonist) (Hennen et al. 2013)



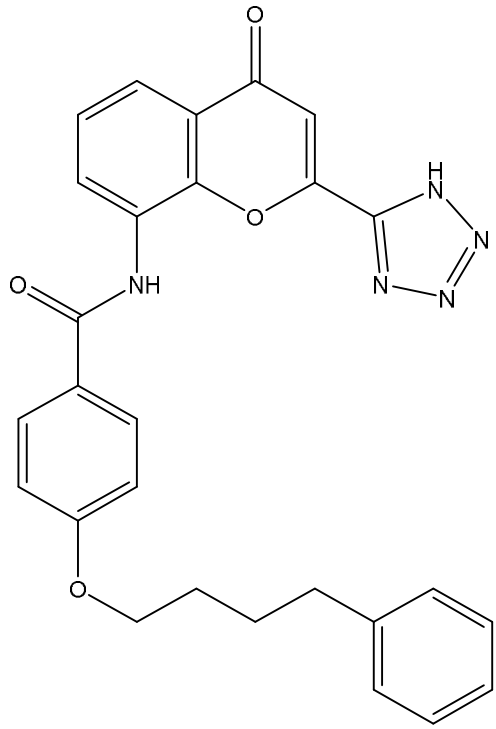
Chemical Formula: $C_{17}H_{25}Cl_2F_3N_5O_{12}P_3S_2$

Structure 2. Cangrelor (P2Y12 Antagonist) (Remko et al. 2016)



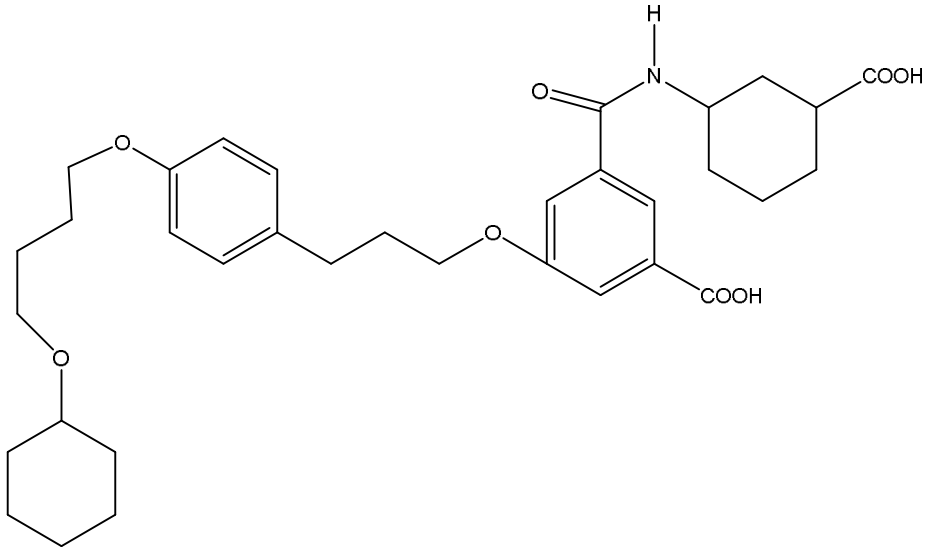
Chemical Formula: $C_{23}H_{28}F_2N_6O_4S$

Structure 3. Ticagrelor (P2Y12 Antagonist) (Remko et al. 2016)



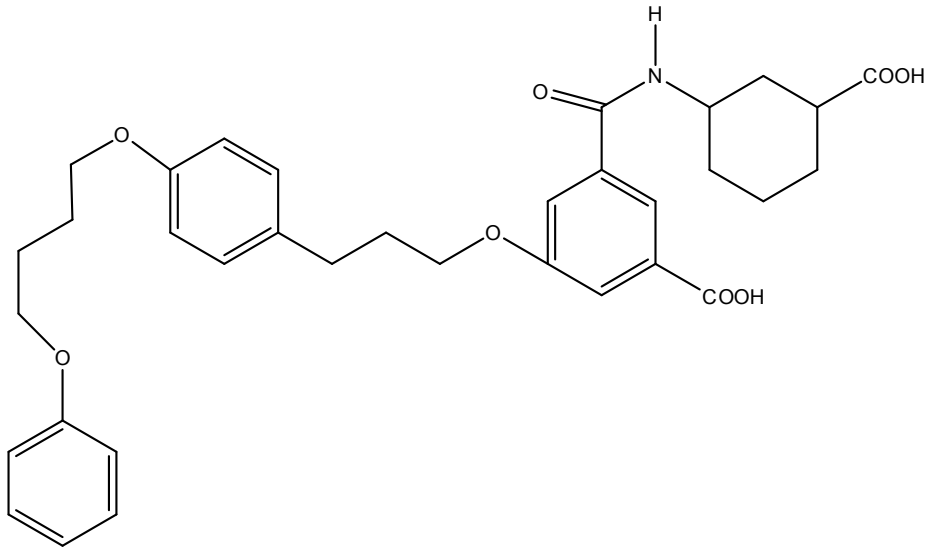
Chemical Formula: $C_{27}H_{23}N_5O_4$

Structure 4. Pranlukast (CysLT1 Antagonist) (Nakai et al. 1988)



Chemical Formula: $C_{34}H_{45}NO_8$

Structure 5. HAMI3379 (CysLT2 Antagonist) (Wunder et al. 2010)



Chemical Formula: $C_{34}H_{39}NO_8$

Structure 6. CAY10633 (=BayCysLT2) (CysLT2 Antagonist) (Ni et al. 2011)

2.5 Buffers and solutions

2.5.1 Water purification

Sterile UltraPure™ Distilled water was used for molecular and cell biological experiments. Purified water was obtained with the A Milli-Q Water System and was used for all media and solutions.

2.5.2 Solutions

Solution 1 APS 10% (Ammonium persulfate)

Component	*Total amount*	*Final concentration*
Ammonium persulfate	1 g	10%
dH2O	ad 10 ml	-

Aliquots were stored at -20 °C.

Solution 2 2 M Calcium Chloride (used for calcium phosphate transfection)

Component	*Total amount*	*Final concentration*
$CaCl_2$ x $2H_2O$	14.7 g	2 M
dH_2O	ad 50 ml	-

Sterile filtration was used to sterilized solutions and 10 ml aliquots were stored at 4 °C.

Solution 3 DeepBlueC stock solutions (Coelenterazine 400A)

Component	*Total amount*	*Final concentration*
DeepBlueC	50 µg	1 mM
Ethanol	ad 125 µl	-

Solution 4 DeppBlueC assay solutions

Component	*Total amount*	*Final concentration*
DeepBlueC	75 µl	50 µM
Ethanol	450 µl	30%
HBSS (+20 mM Heppes)	ad 1500 µl	-

Solution 5 Ethanol (70%) (Used for plasmid DNA preparation)

Component	*Total amount*	*Final concentration*
Ethanol absolute (96%)	730 ml	70%
dH2O	ad 1000 ml	-

Solution 6 2X HBS (Used for calcium phosphate transfection)

Component	*Total amount*	*Final concentration*
HEPES	5.95 g	50 mM
NaCl	8.18 g	280 mM
Na2HPO4	0.133 g	1.5 mM
dH2O	ad 500 ml	-

The solution was sterilized by sterile filtration and stored in aliquots of 50 ml at -20 °C.

Solution 7 1 M HEPES

Component	*Total amount*	*Final concentration*
HEPES	23.8 g	1 M
dH_2O	ad 100 ml	-
NaOH	q.s.	-

After regulation of the pH to 7.2-7.4 with NaOH, the solution was sterilized by sterile filtration and 10 ml aliquots were stored at -20 °C.

Solution 8 Lysis buffer

Component	*Total amount*	*Final concentration*
TRIS 1M, pH 7.4	1500 µl	25 mM Tris
NaCl 5M	1800 µl	150 mM
EDTA 0.5M	120 µl	1mM EDTA
Triton-X	600 µl	1%
IGEPAL®	600 µl	1%
dH2O	ad 60 ml	-

1.5 ml aliquots were stored at -20 °C.

Solution 9 1X PBS (used as wash puffer)

Component	*Total amount*	*Final concentration*
KCl	0.2 g	2.7 mM
KH_2PO_4	0.2 g	1.76 mM
Na_2HPO_4	1.44 g	10 mM
NaCl	8.0 g	137 mM
dH_2O	ad 1000 ml	-
HCl	q.s	

HCl was added up to a pH of 7.4. 50 ml aliquots were autoclaved and stored at room temperature.

Solution 10 20X PBS

Component	*Total amount*	*Final concentration*
KCl	4 g	54 mM
KH_2PO4	4 g	35 mM
NaCl	160 g	2.7 M
Na_2HPO_4 12 H_2O	57.60 g	160.2 mM
dH_2O	ad 1000 ml	
HCl	q.s	

Solution was stored at room temperature.

Solution 11 Poly-D-lysine solution (used for coating)

Component	*Total amount*	*Final concentration*
Poly-D-lysine	5.0 mg	0.1 mg/ml
dH_2O	ad 50 ml	-

Solution was sterilized via sterile filtration and at stored at 4 °C.

Solution 12 Poly-L-ornithine solution

Component	*Total amount*	*Final concentration*
Poly-L-ornithine hydro-bromide	5mg	0.1 mg/ml
dH_2O	ad 50 ml	-

Solution 13 Sodium hydroxid (0.1 M)

Component	*Total amount*	*Final concentration*
Sodium hydroxid	0.4 g	0.1 M
dH_2O	ad 100 ml	-

Solution 14 10% Sodium dodecyl sulphate (SDS)

Component	*Total amount*	*Final concentration*
SDS	10.00 g	10%
dH_2O	ad 100 ml	-

Solution was stored at room temperature.

Solution 15 Stripping buffer

Component	*Total amount*	*Final concentration*
SDS	10 g	10%
Glycin	1,88 g	25 mM
dH_2O	ad 1000 ml	-

Solution was stored at room temperature.

Solution 16 TE buffer (used for calcium phosphate transfection)

Component	*Total amount*	*Final concentration*
Tris-HCl	0.61 g	10 mM
EDTA	0.19 g	1 mM
dH_2O	ad 500 ml	-

Solution 17 Tris 1.5 M

Component	*Total amount*	*Final concentration*
Tris	181.785 g	1.5 M
dH_2O	ad 1000 ml	-

Adjusted pH to 8.8 with HCl and stored at room temperature.

Solution 18 Tris 1 M

Component	*Total amount*	*Final concentration*
Tris	121.19g	1 M
dH2O	ad 1000 ml	-

Adjust pH to 6.8 with HCl and stored at room temperature

Solution 19 Washing buffer (Western blot analysis)

Component	*Total amount*	*Final concentration*
Tween	1 ml	0.1%
1X PBS	ad 1000 ml	-

2.6 Consumables

Table 8. **Consumables**

Consumables	*Product Number*	*Company*
Assay plate, 384 well	784080	Greiner
Blue 1000 µl tips	686290	Greiner bio-one
Cell culture flasks, 25/75/175 cm^2	430168/430729/431079	Corning
Cell scraper	3926.90.97	Corning
Centrifuge tubes, 15ml/50ml	430791/430829	Corning
Compound plate, 384 well	3657	Corning
Costar® 6 / 12 / 24 well plates	3506 / 3512	Corning
Cryogenic vials	5000-1020	Nalgene® Thermo Fisher Scientific
Culture dishes 21/55 cm^2	430166 / 430167	Corning
Culture dishes (untreated)	430591	Corning
Disposable filter unit 0.2 µl	FB30/0.2 CA-s	Whatman®
EPIC cell assay plate, 384 well	9027.90.50	Corning
Gelloading 200µl tips	B71932	Bioplastics
Gel cassettes 1.5 mm	NC2015	Thermo Fisher Scientific
Microtubes snap lock, 1,5 ml/ 2ml	115105/115106	Labomedic
Nitrocellulose membranes HybondTM-C Extra	RPN203E	GE Healthcare
Parafilm™	1447011	Labomedic
Pasteur pipettes, glass	447016	Labomedic
Oxygen crystal tips 10 µl	110727	Labomedic
Whatman paper	GB005	Biometra
Yellow 200 µl tips	70.760.002	Sarstedt
Sponge Pad for Blotting	EI9052	Thermo Fisher Scientific
Stripette® serological pipettes	4486 – 4490	Corning
Tip, Gelloading	B71932	Bioplastics BV

2.7 Laboratory instruments and equipment

Table 9. **Instruments and equipment**

Instrument/equipment	*Type*	*Company*
Autoclave	Varioklav® H+P	Labortechnik

Balances	TE64 (precision balance) TE6101 BL310	Satorius
Camera	CoolSNAP HQ2 DFC 360 FX	Photometrics Leica
Centrifuges	MiniSpin Galaxy Mini Centrifuge 5810	Eppendorf VWR Eppendorf
CO_2 incubator (cell culture)	Heraeus®HERAcell® 240	Thermo Fisher Scientific
Counting chamber	Neubauer	Labomedic
Detection System	DeVision DBOX Detection-System	Decon
Dry block heater	Thermomixer® comfort	Eppendorf
Electronic pipet filler	Easypet®	Eppendorf
Fluorescence microscope	DM IL LED Fluo	Leica
objectives	HI PLAN I 10X/0.22 PH1 HI PLAN I 20X/0.30 PH1 HI PLAN I 40X/0.50 PH2	Leica
Freezer (-80 °C)	Heraeus®Herafreeze®	Thermo Fisher Scientific
Freezer (liquid nitrogen)	MVE 815P-190 System monitor MVETec 3000	Chart BioMedical Ltd. Chart BioMedical Ltd.
Microplate readers	Mithras LB940 Multimode Reader Tecan Sunrise (Photometer) FlexStation 3 Multimode Microplate Reader	Berthold Technologies r-biopharm Molecular Devices
Microscopes	CKX31SF	Olympus
pH electrode	InLab Sciences pro, Academia	Mettler Toledo
pH-meter	SevenEasy™	Mettler Toledo
Photo documentation systems	De Vision DBOX	Decon Sience Tec
Pipettes	0.5-10 µl; 10-100 µl; 100-1000 µl	Eppendorf
Safety cabinets	HeraSafe	Thermo Fisher Scientific

Semi-automatic pipettor	CyBi®-Selma	Cybio
UV/VIS spectrophotometer	SmartSpecTM Plus	BIO-RAD
Vacuum pump system	AP 15 Membrane Vacuum Pump	HLC BioTech
Vortex	Reax Top	Heidolph
Water purification	A Milli-Q Water System	Merck Millipore
XCell SureLock® Mini-Cell and XCell II™ Blot Module	EI0002	Thermo Fisher Scientific

2.8 Assay Kits

Table 10. **Kits applied in the following work**

Kit name	*Article number*	*Company*
Amersham ECL prime Western Blotting Detection	RPN2236	GE Healthcare
Cell Titer-Blue Cell Viability Assay	G8080	Promega
NucleoBondR Xtra Maxi	740414.10	Macherey-Nagel
Phospho-ERK (Thr202/Tyr204) Cellular Assay Kit (former name Cellul'erk kit)	64ERKPEI	Cisbio bioassays
IP-One HTRF® assay kit	62IPAPEJ	Cisbio bioassays
Cylic cAMP cell-based assay kit	62AM4PEC	Cisbio bioassays
FLIPR Calcium 5 Assay kit	38220000	Molecular devices
PierceTM BCA Protein Assay Kit	23225	Thermo Scientific

2.9 Sterilization method

To autoclave all heat-stable materials and solutions at 121 °C and 1.2 bar for 21 min, the Varioklav® (H+P Labortechnik AG, Oberschleisheim) was used. To sterilize temperature-sensitive buffers and solutions, they were filtered through sterile filter pore width of 0.2 µm.

2.10 Software

Table 11. **Software**

Software	*Company*
Application Suite 3.3.1	Leica
ChemDraw	PerkinElmer
MikroWin2000	Berthold Technologies GmbH & Co. KG
Gel-Pro Analyzer	Media Cybernetics, L.P
Gelscan software V6.0	Bioscitec
GraphPad Prism6	GraphPad Software
Microsoft Exel 2010	Microsoft® Corporation
Microsoft PowerPoint 2010	Microsoft® Corporation
Microsoft Word 2010	Microsoft® Corporation
XFluor4	Microsoft® Corporation/Tecan sunrise

3 Methods

3.1 Cell biological methods

All cells were kept in cell incubators with a constant 96% humidified atmosphere and 5% CO_2 at 37 °C. Cell culture solutions and media were pre-warmed to 37 °C before application. To work under aseptic conditions, for every procedure a safety cabinet with laminar air flow was used.

3.1.1 Passaging cell lines

Recombinant cell lines and Oli-neu cells were passaged at least two times a week. To detach the cells from the flask, a solution of the serine protease trypsin (Trypsin-EDTA (0.05%)) was used.

First, the medium was removed and adherent cells were washed once with 3 ml PBS. Then the Trypsin-EDTA solution was added to the cells (1-3 ml depending on the size of the flask) and flasks were incubated for at least 1 min at 37 °C. After detachment of the cells, trypsin reaction was stopped with a 4-fold higher volume of DMEM medium (containing 10% of either FCS (recombinat cells) or HS (Oli-neu cells)). An appropriated split ratio (1:2-1:10) was utilized depending on the cell line. In order to reach and maintain stable measurements, cells were used up to passage 80.

3.1.2 Cryopreservation and revitalization of cell lines

In cell biology laboratories the long term-storage of cells is required to revitalize and utilize cells at a later time point. To this end, cells were cryopreserved and stored at -196 °C.

Freezing medium was freshly prepared by supplementing pure FCS or growth medium (containing 20% FCS) with 10% DMSO. The addition of DMSO serves as frost protection agent that prevents high size ice crystal formation and therby, cell death. Cells were cultured up to a density of 80-90% in 75 cm^2 cell culture flasks and then detached as described (see 3.1.1). After trypsin reaction, cell suspension was centrifuged for at least 4 min at 800 g. Growth medium was removed and the cell pellet was resuspended in 1 ml freezing medium before being transferred into cryogenic vials covered by a styrofoam box and placed into a -80 °C freezer. This allows a maximal freezing rate of 1 °C/min. Afterwards, vials were transferred into the liquid nitrogen tank (-196 °C) for long-term storage.

To revitalize the respective cell lines after long-term storage in the liquid nitrogen tank, cells were rapidly thawn in the water bath (37 °C) and then transferred into 10 ml of prewarmed medium. After centrifugation, medium was removed and the cell pellet was resuspended in respective cell line-specific growth medium without antibiotics. The medium was then changed to medium with antibiotics after 24-48 h culture at 37 °C. The cells were cultivated for at least 5 days before seeding for cell-based assays or to isolate proteins for Western blot analysis.

3.1.3 Cell counting

The cells were counted with the Neubauer counting chamber (also known as hemocytometer) containing two compartments. The counting chamber is a thick crystal slide where a grid is added in the middle of each compartment. To count cells, the grid was covered by a cover glass and the compartments were loaded with 10 µl cell suspension. One compartment contains nine squares. Cells were counted in at least three squares by using a microscope and then the average was determined. Finally, the cell density was calculated by the following term: cell density [cells/ml] = counted cells x dilution factor x 10^4.

3.1.4 Coating cell culture dishes and plates

Before working with HEK293 and Oli-neu cells, culture dishes and plates were coated with poly-D-lysine (PDL). For primary rat oligodendrocytes poly-L-ornithine (PLO) was used as coating agent. The cell culture surfaces were completely covered with the solution and incubated for at least 1 h at 37 °C (up to 4 h for Oli-neu cells). Thereafter, PDL or PLO were removed and surfaces were washed at least twice with sterilized PBS or Milli-Q water and then dried under the safety cabinet with UV light. The prepared plates were kept for a maximum of seven days at 4 °C or were directly used.

3.1.5 Culture and differentiation of primary rat OPCs

To isolate primary rat OPCs from forebrains of Wistar rat pups at P0-P2, a differential detachment method was used as previously described (Chen et al. 2007; Hennen et al. 2013). Cerebra were mechanically separated with a syringe and two different cannulae (first 1.2 × 40 mm and then 0.60 × 30 mm). Tissue suspension was centrifuged for 5 min at 800 g. After removal of the medium, pellet was resuspended in growth medium and filtered through a 70 µm cell strainer. The suspension was then plated on PDL-coated 75 cm² culture flasks in growth medium and medium was exchanged every second day. To detach OPCs from astrocytes and microglia after 8-11 days in culture, mixed cultures were shaken (240 rpm) overnight. Then, the cell suspension was plated onto uncoated Petri dishes for 45 min to further purify the oligodendrocyte precursor

population (OPCs). OPCs were seeded into PLO-coated plates and cultured in proliferating Neurobasal medium supplemented with growth factors for 3-4 days. The medium was changed every second day. Thereafter, medium was switched to growth factor-free Neurobasal medium to induce spontaneous in vitro differentiation and GPR17 expression.

3.1.6 **Transient transfection of recombinant cells**

The term transfection describes a non-viral method to transfer DNA into eukaryotic cells. The method can be subclassified in stable and transient transfection. During a stable transfection the DNA is stably inserted into the host cell genome, while a transient transfection leads to a temporally limited transfer of plasmid DNA into the host cell.

3.1.6.1 Transient calcium phosphate transfection of HEK293 cells

The calcium phosphate precipitation method was used to transiently transfect HEK293 cells. HEK293 cells were seeded 24 h before transfection at a density of 3 x10^6 cells per 10 cm cell culture dish in growth medium to reach an approximate cell confluence of 60-70% at the next day. In line with the established protocol, 20 µg plasmid DNA were mixed with 500 µl TE buffer followed by the addition of 60 µl 2 M $CaCl_2$. The DNA/$CaCl_2$ solution was added slowly to a gently vortexed round bottom falcon containing 500 µl of 2X HBS-buffer to allow precipitation. After 10 min incubation time the solution was carefully added to the cells and the cell culture dish was prudently swiveled. To avoid cell toxicity, the medium was changed after 4-6 h. 48 h after transfection HEK293 cells were analyzed by cell-based assays.

3.1.6.2 Transient transfection of HEK293 cells with FuGENE HD (Promega)

The non-liposomal FuGENE® HD transfection reagent allows the transfection of eukaryotic cells. The reagent was applied according to the manufacturer`s instructions. 24 h before transfection, HEK293 cells were seeded on 6 cm cell culture dishes at a density of 2 x 10^6 cells to achieve 50 % confluence on the day of transfection. The transfection solution was prepared by adding 6 µg of the respective DNA to 500 µl Opti-MEM. Afterwards, 18 µl FuGENE® HD reagent was gently added to the solution. FuGENE® HD DNA/Opti-MEM solution was then incubated for 2-5 min. This mixture was applied to the cells and 48 h after transfection cell-based assays were performed.

3.1.7 **RNA interference**

RNA interference is a knockdown method to silence a gene by mRNA degradation. In the present study, this method was used to knockdown GPR17. Before transfection, Oli-neu cells were seeded and incubated for 24 h in the presence of 1 µM PD174265. Oli-

neu cells were transfected using the transfection reagent Lipofectamine RNAiMAX and 50-100 nM scrambled siRNA (Qiagen, Cat no.1027280) or a siRNA designed to silence mouse and rat GPR17 (5-CCCGGTTGGTTTATCACTTCT-3, Qiagen, Cat no. SI04661524) according to the manufacturer`s instructions. GPR17 knockdown of Oli-neu cells was analyzed after 24-48 h by Western blotting or Ca^{2+} flux assay.

3.2 Cell-based assays

In order to determine the signaling of GPR17, various cell-based assays were applied using recombinant cells stably expressing human, rat or mouse GPR17 and primary rat oligodendrocytes as well as Oli-neu cells, both endogenously expressing GPR17.

3.2.1 Label-free dynamic mass redistribution assay

The dynamic mass redistribution (DMR) method is a recently established holistic approach to analyze changes in the distribution of cellular contents or intracellular particles in living cells in real-time (Schröder et al. 2010; Schröder et al. 2011). In the present study this highly profound technology was used to investigate the MDL29,951-triggered whole cell signaling in primary rat oligodendrocytes and Oli-neu cells. The DMR method was recently applied to determine the signaling of various GPCRs in an immortalized human neural progenitor cell line and this has been performed in dependence of the differentiation stage of these cells (Pai et al. 2012). Primary cells are difficult to analyze by classical biochemistry methods, which are based on transfection with labeled constructs to verify intracellular signaling pathways, therefore the DMR technology is a suitable tool to study endogenously expressed GPCR signaling in both immortalized and primary cells. Moreover, due to a lower receptor level, the functional analysis of GPCRs endogenously expressed in primary cells require a highly sensitive assay, which is ideally fulfilled by the holistic DMR method.

3.2.1.1 Theoretical background of dynamic mass redistribution

The DMR method is applied to analyze ion channels, GPCRs and receptor tyrosine kinases (Sun et al. 2014). Stimulation/activation of these proteins results in cellular processes that are associated with mass relocations. The highly sensitive DMR technique detects these dynamic mass redistributions in the cellular matter by using a waveguide grating (RWG) biosensor that is localized at the bottom of every well of a special microtiter plate (Fang et al. 2006; Fang et al. 2007). Polarized broadband light is passed through the bottom of this plate and a change of the wavelength of outgoing light is recorded over minutes up to hours. Thereby, the reflected wavelength (resonant wavelength) depends on the optical density of the cell layer within approximately 150 nm from the well bottom. Consequently, after stimulation of cells by test compounds a dy-

namic mass redistribution is caused in the cellular matter and this in turn results in a shift of the outgoing resonant wavelength relative to the baseline value that is recorded in picometer (pm) (Schröder et al. 2011).

3.2.2 Experimental procedure of dynamic mass redistribution assay

DMR was recorded in real-time in differentiated Oli-neu cells and primary rat oligodendrocytes applying the Corning Epic system. In order to record optical traces, 3,000 OPCs per well were seeded for 4 days in proliferation medium on 384-well fibronectin-coated biosensor microtiter plates. To induce differentiation, the medium was changed to medium without growth factors. Oli-neu cells were seeded in the presence of 1 µM PD174265 at a density of 18,000 cells per well. On the assay day, cells were washed twice with HBSS containing 20 mM HEPES and leaved with an end volume of 30 µl buffer per well. To stabilize readings, the mircotiter plate was incubated for 1 h in the DMR reader at 28 °C. In the meanwhile, 4-fold higher concentrated compound solutions were prepared and transferred into a 384-well compound source plate and also incubated at 28 °C for 20 min to reach thermal equilibrium. The sensor plate was scanned to record a baseline optical signature for 5 min and then compound solutions were transferred into the biosensor plate by using the Cybi-SELMA semi-automated electronic pipetting system. Optical changes were recorded for at least 3,200 s. To inhibit $G\alpha_i$ signaling, pertussis toxin (PTX) (100 ng/ml) was added 16 h before the measurement.

3.2.3 Data determination

All DMR signals were buffer corrected using the GraphPad Prism 5 software. Representative DMR traces are illustrated from one experiment measured in triplicates. Each experiment was repeated at least three times. Concentration-response curves are derived from the maximum wavelength shift at each compound concentration.

3.2.4 Bioluminescence resonance energy transfer

Bioluminescence resonance energy transfer (BRET) is a biophysical method to study protein-protein interaction and dynamic changes in protein conformation in living cells in real-time.

The approach is based on a nonradioactive transfer of energy and represents a naturally occuring physical phenomenon used by marine animals. The energy transfer takes place between three components (i) a bioluminescence donor, (ii) an oxidated substrate, and (iii) an energy acceptor (Ward, Cormier 1979).

Energy transfer occurs after substrate oxidation when the distance between the energy donor and the energy acceptor amounts between 10-100 Å. The advantage of this ap-

proach over various biophysical methods is the possibility to measure protein-protein interaction in living cells in real time (Pfleger, Eidne 2006).

In the field of GPCR investigation, BRET studies are used to analyze the interaction of a receptor with G proteins, of a receptor with β-arrestin or of two receptors (hetero- or homodimerization) (Mercier et al. 2002; Pfleger, Eidne 2006).

3.2.4.1 Theoretical background of BRET^2

In the present study, the BRET^2 approach was used to study $G\alpha_i$ protein rearrangement and β-arrestin2 recruitment in living cells. BRET^2 and BRET^1, the latter also referred as original technique, differ in the choice of donor, substrate, and acceptor. BRET^2 uses Renilla reniformis luciferase (Rluc), originally isolated from the sea pansy, as energy donor with an emission peak at 400 nm after oxidation of the substrate DeepBlueC and GFP, firstly isolated from jellyfishes Aequorea Victoria, as an energy acceptor emitting light at 515 nm (Ward, Cormier 1979).

3.2.4.2 Experimental procedure of the BRET^2 assays

In order to detect β-arrestin2 recruitment, BRET^2 assays were performed in HEK293 cells stably expressing hGPR17-Rluc and GFP2-β-arrestin2 as already described by Hennen et al. (Hennen et al. 2013).

Furthermore, to analyze the direct interaction between the $G\alpha_i$ subunit and the γ subunit, HEK293 cells were transiently transfected by FuGENE HD transfection (see 3.1.6.2) with $G\alpha_{i1}$-91Rluc and GFP10-$G\gamma_2$ along with the complementary $G\beta_1$ subunit and hGPR17 (Gales et al. 2006) (see Table 12). Transiently transfected cells were analyzed after 48 h.

Table 12**. Transiently transfected DNA amount**

Plasmid	hGPR17	$hG\alpha_{i1}$-91hRluc	GFP10-$bG\gamma_2$	$hG\beta_1$	pcDNA3.1.
DNA amount	3,25	2,25	0,75	1,75	2,5

Before the assays, HEK293 cells were detached by trypsinization (see 3.1.1, counted, once washed with PBS and centrifuged. Thereafter, the cell pellet was resuspended in a suitable volume of assay buffer (HBSS, 20 mM Hepes) to reach a density of 1 x 10^6 cells per ml. To stabilize readings, cell suspension was distributed to 96-well white microtiterplate (170 µl/well) and incubated at 28 °C for 20 min while shaking (180 rpm). In the meanwhile, DeepblueC substrate (50 µM) was freshly prepared. After the incubation time, cells were shaken in the presence of agonist (10 µl) for (i) 1 min to detect

conformational change of the G-protein subunits or for (ii) indicated time points to measure the time-dependent β-arrestin2 recruitment after receptor activation. In order to measure the protein-protein interaction, 20 µl substrate (final concentration 5 µM) were injected to the cell suspension by using the multimode reader. Two seconds after substrate injection light emission at 400 and 515 nm was measured sequentially using the Mithras LB 940 reader.

3.2.4.3 Data evaluation of BRET^2 assay

MikroWin200 software was used to collect the data directly after measuring by the benchtop reader. Results were calculated as the ratio of emitted light by the energy acceptor GFP (515 nm) over Rluc catalyzed light emission (400 nm) (referred as BRET-ratio). To elaborate the ligand-promoted net BRET the Graphpad prism software was used to calculate the difference between the BRET ratio obtained in the absence (buffer value) and in the presence of indicated agonists. β-arrestin2 recruitment was pictured as kinetic.

3.2.5 HTRF technology (ERK1/2 phosphorylation, IP1 accumulation and cAMP alteration assay)

Homogeneous Time Resolved Fluorescence (HTRF) combines the standard fluorescence resonance energy transfer technology (FRET) with the time resolved measurement (TR). The significant advantage of the HTRF technique is the increased sensitivity and fewer false positive as well as false negative results (Degorce et al. 2009). Cisbio discovered various numbers of assay kits to investigate GPCR signaling (second messenger accumulation and downstream kinase assays), new biomarkers, and protein-protein interactions based on the HTRF technique. In order to explore the signaling behavior of GPR17, in the present study the HTRF method was used to determine the alteration in IP1 production, cAMP production, and ERK1/2 phosphorylation after activation of GPR17.

3.2.5.1 Theoretical background of HTRF technology

Emission of HTRF is detected at two different wavelengths, donor emission at 620 nm and acceptor emission at 665 nm. Based on FRET, an energy transfer to an energy acceptor occurs after excitation of an energy donor, required for the transfer is a close proximity between donor and acceptor (Degorce et al. 2009). As energy donor Europium ions or Terbium ions (Eu^{3+} or Tb^{3+}) are used, complexed with three molecules bispyridin to a cryptate, which build a three dimensional cage structure around the Eu^{3+} or Tb^{3+} . The cage structure is necessary as light collecting device, because Eu^{3+} or Tb^{3+} are not fluorescent by its own. Eu^{3+} or Tb^{3+} are excited by light with a wavelength of 320 nm. Europium and Terbium belong to the lanthanides and consequently to the rare

earth elements (Degorce et al. 2009). The long half-life of the fluorescent signal circumvents the measurement of the short-life auto fluorescence, the main disadvantage of the FRET technology. D2, an organic motif of approximately 1000 Da, was developed as an energy acceptor of the second generation ((Degorce et al. 2009). To avoid steric hindrance problems, it is 100 times smaller than the first generation of acceptors (Degorce et al. 2009).

3.2.6 ERK1/2 phosphorylation assay principle

The activation of several GPCRs leads to a downstream ERK1/2 phosphorylation. To investigate the increase of phosphorylated ERK1/2 after GPR17 stimulation the Cellul'erk kit was applied. This ERK1/2 kit utilizes the HTRF technology in a sandwich immunoassay. Anti-phospho-ERK1/2 antibodies labeled with d2 or Eu^{3+}-cryptate bind to pERK1/2 and the short distance between donor and acceptor consequently leads to an energy transfer based on FRET. The intensity of the emitted light is proportional to the amount of phosphorylated kinase.

3.2.7 IP1 accumulation assay principle

Activation of a $G\alpha_q$-coupled GPCR leads to an increase in the activity of the enzyme phospholipase C (PLC), which induces the inositol phosphate cascade. IP3 is one of the main cascade metabolites that triggers the release of calcium from intracellular stores. The measurement of the increase in IP3 as a second messenger of the $G\alpha_q$ pathway is limited due to its extreme instability. However, the degradation of the sub-metabolite inositol-1-phosphate (IP1) by inositol monophosphatase can be inhibited by lithium chloride addition (Trinquet et al. 2006). Based on this, IP1 represents a more suitable second messenger to investigate the $G\alpha_q$ pathway. The applied IP1 accumulation HTRF assay kit is based on a competitive immunoassay between in cells accumulating IP1 and d2-labeled IP1. Anti-IP1 antibody labeled with Tb^{3+}-cryptate is applied as fluorescence donor. The assay kit allows measurement of agonist and antagonist effects on $G\alpha_q$-coupled receptors in various cellular backgrounds (Zhang, Xie 2012).

3.2.8 cAMP alteration assay principle

GPCRs have the ability to activate $G\alpha_{i/o}$ and/or $G\alpha_s$ proteins. A class of intracellular enzymes, the adenylyl cyclases, is directly influenced by these G protein classes. The adenylyl cyclase catalyzes the production of cAMP. $G\alpha_s$ proteins lead to adenylyl cyclase activation, whereas $G\alpha_{i/o}$ proteins attenuate the cAMP production. Among intramolecular ring formation ATP is converted into cAMP and inorganic pyrophosphate (Marinissen, Gutkind 2001). In order to measure a decrease of the intracellular cAMP

levels, the cells were preincubated with forskolin to stimulate the intracellular cAMP production and in consequence to make the decrease in cAMP levels feasible. The cAMP assay kit is based on a competitive immunoassay between in cells accumulating cAMP and d2-labeled cAMP, similar to the IP1 assay kit. Anti-cAMP antibody labeled with Eu^{3+}-cryptate is used as a fluorescence donor.

Furthermore, intracellular cAMP levels are rapidly degraded by phosphodiesterases. To avoid the degradation during the measurement isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, was added to the respective assay buffer.

3.2.9 **Experimental procedure - ERK1/2 phosphorylation assay**

The HTRF Cellul'erk kit was applied to investigate receptor-mediated ERK1/2 phosphorylation in different cell lines (i) HEK293 cells stably expressing hGPR17, hCysLT1, and hP2Y12 receptor, (ii) 1321N1 cells stably expressing hGPR17 and native 1321N1 cells, (iii) Oli-neu endogenously expressing GPR17 after induction of differentiation, and (iv) primary rat oligodendrocytes as well endogenously expressing GPR17 in the differentiation stage.

The increase in phosphorylated ERK1/2 levels was measured according to the manufacturer's instructions (two plate assay protocol). In brief, cells were seeded into a clear 96-well cell culture plate coated with PLO or PDL. HEK293 cells, 1321N1 cells and Oli-neu were seeded at a density of 40,000 cells per well. To induce differentiation, Oli-neu cells were cultured for 24 h in medium containing 1 µM PD174265. OPCs were seeded in proliferating media at a density of 5,000 cells per well and cultured for 3-4 days and differentiation for two days. The different PTX added 18 h before the assay

Before the assay, cells were washed with HBSS buffer supplemented with 20 mM HEPES and starved for 4 h in serum-free medium. The starvation time was followed by agonist incubation at 37 °C for certain times. Thereafter, the agonist was removed and cells were lysed in 50 µl of the supplemented lysis buffer. Plates were incubated while shaking for 30 min at room temperature and then frozen overnight. After transfer of 16 µl lysate to a white 384-well plate, d2-labeled anti-phospho-ERK1/2 (2 µl) and Eu^{3+}-cryptate-labeled anti- ERK1/2 (2 µl) were added to the wells and the plate was incubated for 2 h in the dark at room temperature. Time-resolved FRET signals were measured after excitation at 320 nm. Data analysis was based on the fluorescence ratio emitted by the d2-labeled anti-phospho-ERK1/2 (665 nm) over the light emitted by the Eu^{3+}-cryptate-labeled anti-ERK1/2 (620 nm), measured by the Mithras LB 940 reader (Bock et al. 2012).

3.2.10 **Experimental procedure - IP accumulation**

Changes of the intracellular second messenger IP1 in HEK293 cells stably expressing hGPR17, rGPR17 and mGPR17 were quantified by following the manufacturer`s instructions. The cells were detached and washed once with PBS. After centrifugation (4 min 800 g), the cell pellet was resuspended in stimulation buffer (HBSS, 10 mM HEPES, 50 mM LiCl) and dispensed in a 384-well microplate at a density of 100,000 cells per well. In order to stabilize the readout, cells were preincubated for 30 min at 37 °C before compound addition.

To analyze the antagonist abilities of the different compounds, the cells were incubated at 37 °C with 3.5 µl of different concentrations (3-fold) of the proposed GPR17 antagonist diluted in stimulation buffer. After 30 min a fixed MDL29,951 concentration corresponding to the EC_{80} on the respective GPR17 receptor ortholog (hGPR17 = 1 µM, rGPR17 = 3 µM and mGPR17 = 10 µM) (Hennen et al. 2013) was added to the cells. The reaction was stopped after 30 min of agonist incubation by addition of lysis buffer containing the assay-specific components (d2-labeled IP1 and anti-IP1 antibody labeled with Tb^{3+}-cryptate). The plate was incubated for 60 min at room temperature and afterwards time-resolved FRET signals were measured after excitation at 320 nm using the Mithras LB 940 multimode reader.

3.2.11 **Experimental procedure - cAMP alteration**

Alteration of the intracellular second messenger cAMP was analyzed using the HTRF cAMP dynamic 2 kit according to the manufacturer's instructions in Oli-neu cells. 30,000 cells per well were cultured in PDL-coated 48-well tissue culture plates for 48 h using medium containing 1 µM PD174265. At the assay day, cells were treated for 20 min in assay buffer (HBSS supplemented with 20 mM HEPES and 1 mM IBMX) and afterwards stimulated with 3 µM forskolin in the presence of respective MDL29,951 concentrations. To stop the reaction after 20 min stimulation at 37 °C, 40 µl of HTRF assay lysate buffer were added to the cells. Then lysates were frozen overnight.

In order to detect intracellular cAMP levels, 10 µl of the samples were transferred to a 384-well plate and both assay components, d2-labeled cAMP and anti-cAMP antibody labeled with Eu^{3+}-cryptate, were added. Before time-resolved FRET signals were measured (excitation at 320 nm) by using the Mithras LB 940 reader, the plate was incubated for 60 min at room temperature.

3.2.12 **Data evaluation of HTRF assay kits**

According to the manufacturer`s instruction the measured data were elaborated utilizing the Microwin2000 and the Graph pad prism software.

Data analysis was made based on 10,000 × [emission (665 nm)/emission (615 nm)], meaning light emitted by d2 (acceptor fluorophore)-labeled component over the light emitted by the Eu^{3+}-cryptate-labeled antibody. Intracellular IP1 levels were normalized to the maximum of MDL29,951-mediated GPR17 activation. Levels of phosphorylated ERK1/2 were normalized to ERK1/2 phophorylation values obtained with the highest compound concentration. Alteration of intracellular cAMP levels was normalized to the values achieved with 3 μM forskolin.

3.2.13 **Calcium accumulation assay**

3.2.13.1 Calcium accumulation assay principle

In order to identify receptor-dependent calcium release from intracellular stores, such as the endoplasmic reticulum, the Calcium 5 assay kit was used. Calcium ions regulate a broad range of cellular processes and therefore they represent one of the key second messengers in mammalian cells. During a dye uptake step, the cytosol of the cells is loaded with the calcium-sensitive fluorescent dye. GPCR stimulation results in a receptor-dependent release of calcium ions from intracellular stores. The fluorescent signal increases proportionally to the intracellular calcium concentration.

3.2.13.2 Experimental procedure of calcium release assay

Intracellular Ca^{2+} mobilization was quantified according to the manufacturer's instructions with the Calcium 5 Assay kit. Oli-neu cells were seeded 24-48 h before the assay in the absence and in the presence of 1 μM PD174265 at a density of 70,000 cells per well into black 96-well PDL-coated tissue culture plates with clear bottom. Furthermore, to investigate the $G\alpha_{i/o}$ involvement in the GPR17-mediated calcium signal, 16 h before the assay PTX (100 ng/ml) was added to the cells. On the assay day, cells were loaded with the Calcium 5 indicator dye for 30 min and time-dependent calcium release was measured after the addition of respective concentrations of MDL29,951 by the FlexStation 3 Multimode Microplate Reader. Emission was measured at a wavelength of 525 nm (excitation 485 nm). In order to quantify the Ca^{2+} flux, changes in fluorescence units were converted in concentration-response curves.

3.2.14 **Cell viability assay**

3.2.14.1 Cell viability assay principle of the CellTiter-Blue kit

The applied cell viability assay (also known as resazurin reducing assay (RR-assay)) is based on a fluorescence method. The assay uses the capacity of living cells to reduce the redox dye resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) into resorufin (7-hydroxy-3H-phenoxazin-3-one), a fluorescent end product. Loss of the ability to metab-

olize the indicator dye is detectable by a decrease in fluorescence signal. The RR-assay represents an ideal system to identify anti-proliferating and cytotoxic compounds (Riss et al. 2015).

3.2.14.2 Experimental procedure of cell viability assay

CellTiter-Blue kit Oli-neu cells were seeded at a density of 25,000 cells per well into black 96-well PDL-coated plates with clear bottom. On the next day, cells were incubated with 0.1% dimethyl sulfoxide (DMSO) served as negative control (100% cell viability) or indicated compounds diluted in medium containing 1 µM PD174265 for 48 h. As a positive control etoposide, a topoisomerase 2 inhibitor, was used. To detect the capacity of the cell to reduce the redox dye as indicator for cell viability, cells were incubated for 3 h at 37 °C with 20 µL of the CellTiter-Blue reagent. Fluorescence emission was measured at 590 nm after excitation at 560 nm by using a FlexStation 3 Multimode Microplate Reader. The detectable fluorescence signal is proportional to the viability of the cells. Data were expressed as the percentage of cell viability relative to DMSO control.

3.3 Methods in molecular biology

3.3.1 Heat shock transformation

Transformed bacteria are used for plasmid storage and replication. Transformation is a non-viral molecular biology method to introduce DNA into competent bacteria cells. The method applies the natural competence of bacteria to insert external DNA. Therefore,

XL-blue E. coli cells were pretreated with rubidium chloride to force cell competence/enable permeation of DNA and then these chemical competent bacteria could bestored at -80 °C. For transformation 100 µl of competent XL-1 blue bacteria were thawed on ice and about 50 ng plasmid DNA was added to the bacteria. The DNA/cell suspension was mixed carefully and afterwards incubated for approximately 30 min on ice. The incubation time was followed by a heat shock at 42 °C in a water bath for 30-60 s. Thereafter, suspension was incubated for 2 more minutes on ice before the addition of 300 µl SOC medium. In order to develop encoded antibiotic resistance, suspension was incubated at 37 °C for 60 min while shaking (220 rpm) in an incubator. Then, a volume of 50 µl-300 µl was plated on 37 °C prewarmed LB agar plates, which were prepared in advance containing the respective antibiotic.

3.3.2 Cryoconservation of bacterial strains

To store bacterial host cell lines of the various plasmids, cells were cryoconservated by mixing 800 µl of bacterial suspension with 200 µl of glycerin and storing at -80 °C. In order to reactivate the bacterial cell line for preparative plasmid isolation, one drop of the glycerin cell suspension was added to prewarmed (37 °C) LB medium containing the appropriated amount of respective antibiotic.

3.3.3 Preparative isolation of plasmid DNA

The applied approach isolates plasmid DNA based on the anion change chromatography.

To purify plasmid DNA with a high yield for transfection experiments, plasmid isolation kit NucleoBond Xtra Maxi was utilized according to the manufacturer`s protocol. The kit contains all needed buffers and solutions. To this end, 300 ml LB (+ 100 µg/ml ampicillin) was inoculated with the bacteria harboring the respective plasmid (either one colony from LB agar plate or 100 µl of the glycerol stock) and incubated overnight at 37 °C under shaking (220 rpm). After removing of the supernatant bacteria were lysed for 5 min, neutralized and the clarified lysate was added to the prepared anion-exchange column. The plasmid DNA was bonded to the column and then impurities were washed out. Afterwards, the DNA was eluted from the column and precipitated with isopropanol. The isolated DNA was washed with 70% Ethanol, resuspended in DNAse/RNAse free water and stored at -20 °C for further use.

3.3.4 Determination of nucleic acid concentration by photometrical method

Commonly the photometrical method is applied to analyze DNA concentrations in solution. To determine the DNA concentration, the optical density of the solution at 260 nm is measured and used in the following term:

$$C\ (\mu g/ml) = OD260 \times D \times R$$

OD260 Optical density at 260 nm

D Dilution factor

R Conversion factor (for DNA 50)

To determine the purity of the DNA solution, the quotient of the optical density at 260 and 280 nm was calculated. A ratio between 1.6 to 2.0 is required to neglect impurities.

3.3.5 **Analysis of protein expression by Western blot**

3.3.5.1 Lysate preparation

In order to analyze protein expression by Western blot analysis, proteins were isolated from respective cell lines. OPCs were seeded in proliferating medium into 6- or 12-well PLO-coated tissue culture plates (80.000-120.000 cells per well). After 3-4 days the culture medium was changed to growth factor-free medium to induce differentiation and GPR17 expression. On the next day medium was supplemented with 20 ng/ml T3 and 10 ng/ml CNTF together with the analyzed compounds. Oli-neu cells were seeded at a density of 300,000 cells per well into 12-well PDL–coated tissue culture plates in Sato medium. On the next day, cells were incubated with indicated compounds and 1 µM PD174265. At specified times cells were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer supplemented with protease inhibitor mixture. Lysates were rotated 20 min at 4 °C and centrifuged at 15,000 g at 4 °C for 10 min.

3.3.6 **Determination of the protein content of lysates**

In order to analyze the proteins in a quantitative manner, protein amount of lysate samples was analyzed by Pierce^{TM} BCA Protein Assay Kit according to the manufacturer`s protocol. The assay kit is based on a two-step color reaction (known as bicinchoninic acid protein assay), first the protein biuret reaction and then the bicinchoninic acid (BCA) protein reaction by Smith 1985 (GORNALL et al. 1949; Smith et al. 1985). In the biuret reaction Cu^{2+} is reduced to Cu^{1+} by proteins in alkaline medium and results in a weak blue color. The second step is the chelation of Cu^{1+} and BCA leading to an intensive purple color.

In brief, 10 µl of each sample were diluted with 40 µl (1:5 dilution) of 0.1 M NaOH and a bovine serum albumin (BSA calibration) standard (0 µg/µl - 10 µg/µl BSA) was prepared. 200 µl of the freshly mixed protein detection reagent were added to each sample. After 30 min at 37 °C, the absorption of the BCA/Cu^{1+} complex was measured at 560 nm and the protein concentrations were calculated by using the BSA standard curve.

3.3.6.1 Production of separating gel and stacking gel

In order to analyze the protein lysates, the gels for electrophoresis were prepared. For two separating gel, the following solutions were mixed and added to an empty gel cassette.

Table 13. **10% separating gel** (amounts/quantities for two gels)

Component	*Total amount*
Milli Q H_2O	9.6 ml
Acrylamid	5 ml
TRIS (1.5 M)	5 ml
SDS (10%)	200 µl
APS (10%)	100 µl
Temed	10 µl

Table 14. **5% stacking gel** (amounts/quantities for two gels)

Component	*Total amount*
Milli Q H_2O	3.65 ml
Acrylamid	0.625 ml
TRIS (1 M)	0.630 ml
SDS (10%)	50 µl
APS (10%)	30 µl
Temed	5 µl

Gels were covered with isopropanol. After 30 min of polymerization time, the solutions from Table 2 were mixed, added as well to the gel cassette and a 10-tooth comb was applied.

3.3.7 SDS-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE)

After detection of the protein amount of lysates, 7.5 to 15 µg of protein were separated by 10% SDS-polyacrylamide gel electrophoresis. Therefore, protein samples were mixed with NuPage Sample Reducing Agent (10X) and sample buffer (2-4X). The running buffer was added to the chamber and in the inner chamber additionally blended with 500 µl NuPage Antioxidant. As molecular weight markers 5 µl Kaleidoscope standard and 2.5 µl Magic Marker standard were used. The prepared samples were added to the gel and separated for approximately 3 h at 60 V.

3.3.8 Western blot analysis

In order to transfer the proteins to nitrocellulose membrane, electroblotting was performed for 2 h at 25 V in Nupage Transfer Buffer (including 20% methanol) using the blotting module. After blotting, membranes were rinsed two times with washing buffer (PBS + 0.1% Tween 20) and then blocked with Roti-Block for at least 1 h at room temperature and then incubated overnight with antibody diluted in Roti-Block. The respective antibody dilutions are listed in Table 15.

Table 15. **Antibody dilutions**

Antigene	Dilution
MBP	1:5000
GPR17	1:5000
ERK	1:2500
p-ERK	1:2500
CREB	1:2500
p-CREB	1:1000
$G\alpha_s$	1:3000
$G\alpha_q$	1:3000
$G\alpha_o$	1:5000
$G\alpha_i$	1:5000
EPAC	1:1000
β-actin	1:2500
Secondary Antibody	
Anti-mouse-HRP	1:10000
Anti-rabbit-HRP	1:10000

On the next day membranes were washed three times with washing buffer and were then incubated for 1 h at room temperature with the respective horseradish peroxidase-conjugated secondary antibody diluted in Roti-Block (Table 15). The immunoreactive proteins were visualized by chemiluminescence using Amersham ECL Prime Western Blotting Detection Reagent according to the manufacturer`s recommendations and quantified by densitometry using Gelscan software. To normalize for equal loading and protein transfer, membranes were washed or stripped and reprobed with an antibody against the standard β-actin.

3.3.9 **Stripping of nitrocellulose filters**

Antibodies binding to nitrocellulose filters were removed (stripped) by washing for 20 min with stripping buffer at room temperature. Afterwards, membranes were washed

once with PBS for 10 min and then three times (5 min each) in washing buffer (PBS + 0.1% Tween 20) and subsequently blocked with Roti Block (60 min, RT).

4 Results

4.1 Oli-neu cells as a suitable model system

Recently, it has been reported that GPR17 overexpression *in vivo* and *in vitro* blocks differentiation of oligodendrocytes, and thus, GPR17 has been proposed as a cell intrinsic timer of myelination (Chen et al. 2009). Subsequently, this prediction has been confirmed by arresting primary mice oligodendrocytes in an immature stage, preventing their differentiation and maturation, by pharmacological stimulation of the receptor with MDL29,951 (Hennen et al. 2013).

Primary oligodendrocytes represent the perfect *in vitro* system to analyze GPR17 signaling and its influence on differentiation and myelination in a physiological cellular background. However, the low amount of cells after tissue preparation and their slow metabolism often limits experimental analyses of intracellular processes in this cell line (Winterstein et al. 2008; Fratangeli et al. 2013). Thus, to circumvent this difficulty, in the following study Oli-neu cells were applied as model system in addition to primary oligodendrocytes. Oli-neu cells are murine oligodendroglia precursor cells, which have the capacity to undergo differentiation after chemical induction (Jung et al. 1995) and to express GPR17 similar to primary oligodendrocytes (Fratangeli et al. 2013).

4.1.1 Induction of Oli-neu cell differentiation

Various chemical compounds, such as dexamethasone, di-butyryl-cyclic-AMP and PD174265, have the ability to induce Oli-neu cell differentiation to a different extent and thereby to trigger expression of myelin proteins (Jung et al. 1995; Gobert et al. 2009; Fratangeli et al. 2013). In the following study, the selective epidermal growth factor receptor inhibitor PD174265 was used to differentiate Oli-neu cells, as recently described by Gobert and co-workers (Gobert et al. 2009). Oli-neu cell differentiation is detectable by morphological changes (dendrocyte outgrowth) and expression of typical myelination markers, such as MBP and PLP (Dugas et al. 2006; Burda et al. 2013).

4.1.1.1 Morphological change of Oli-neu cells during differentiation

Burda et al. described the morphological shift in differentiating oligodendrocytes from simple bipolar to their complete ramified morphology (Burda et al. 2013). To investi-

gate the morphological change in the applied model cell system, bright field pictures were analyzed to identify the effects of PD174265 on dendrocyte outgrowth.

Oli-neu cells cultured in control medium (Sato medium) exhibited a typical bipolar oligodendrocyte progenitor cell (OPC) shape (Figure 4, A). After 48 h incubation with PD174265, Oli-neu cells stopped proliferating and displayed the characteristic polipolar morphology of differentiating oligodendrocytes (Figure 4, B), thus confirming Oli-neu cell differentiation after treatment with PD174265.

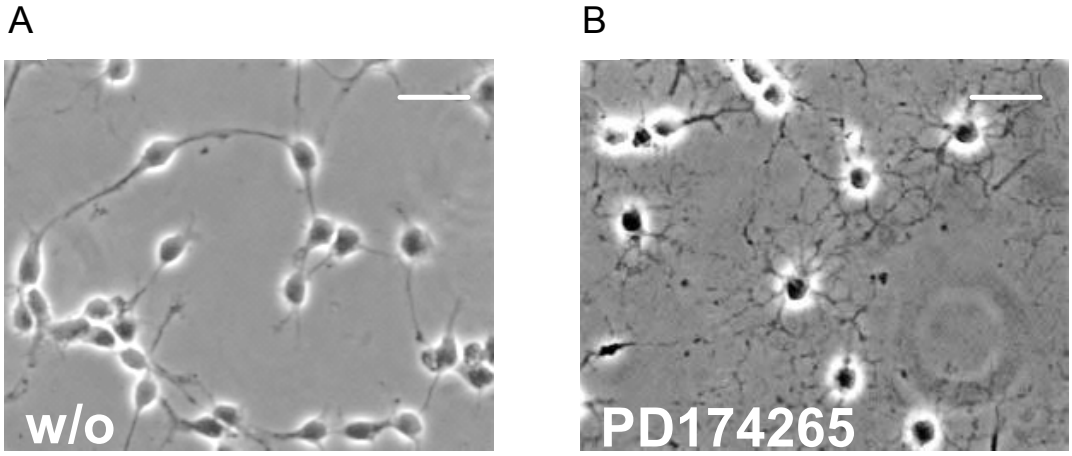


Figure 4. **PD174265-induced morphological changes in Oli-neu cells**

Bright field microscope pictures showing Oli-neu cells cultured for 48 h in Sato medium either untreated (*A*) or in the presence of 1 µM PD174265 (*B*). Scale bar = 20 µm.

4.1.1.2 Endogenous Expression of GPR17 in Oli-neu cells

Highly relevant for the following study is the GPR17 expression pattern in Oli-neu cells. Recently, Fratangeli and co-workers published that GPR17 is expressed in Oli-neu cells during differentiation in the immature stage, similar to the GPR17 expression observed in primary oligodendrocytes (Fumagalli et al. 2011; Fratangeli et al. 2013). To verify these data under the present experimental condition, GPR17 expression was analyzed by Western blotting, comparing lysates from Oli-neu cells cultured in proliferation (Sato medium) and differentiating culture conditions (Sato medium + 1µM PD174265), respectively.

Immunoblotting analysis disclosed an absence of GPR17 in proliferating Oli-neu cells cultured under control conditions without PD174265 over 72 h. In contrast, in cell lysates cultured in culture medium containing PD174265 a specific 47 kDa GPR17 immunoreactive band was initially detectable after 24 hours (approximately 23% of the

GPR17 expression after 72 h) and raised during the following days (Figure 5, A and B). After 48 h, the GPR17 expression amounted approximately 54% of the total expression measured after 72 h. The obtained data confirm the previous findings that GPR17 is up-regulated in Oli-neu cells in the early stage of differentiation (Fratangeli et al. 2013).

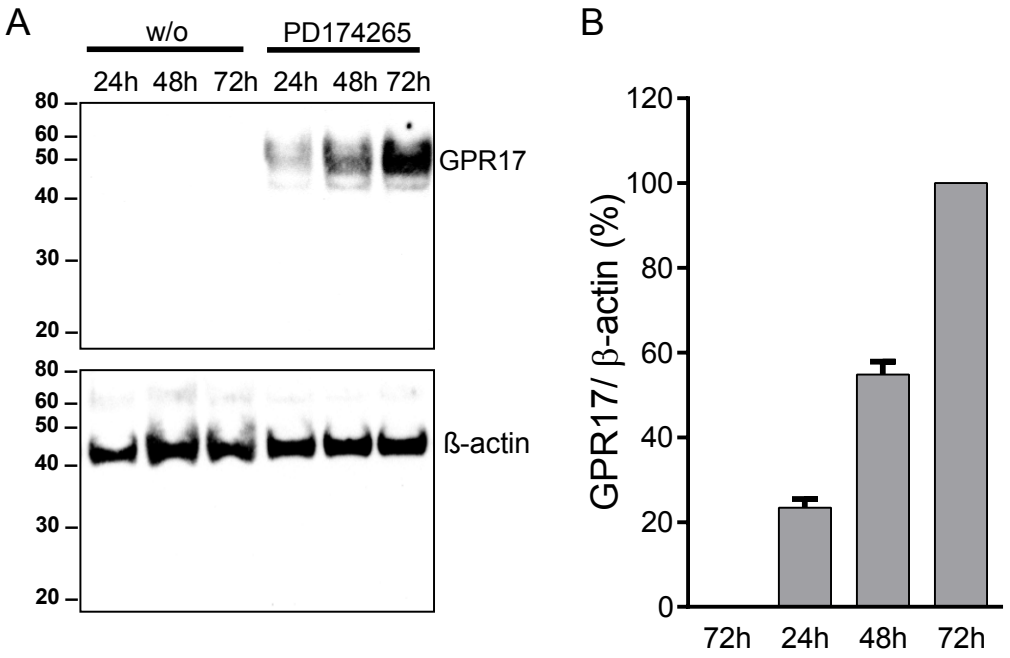


Figure 5. **GPR17 expression in Oli- neu cells after addition of PD174265**

(A) Illustrative Western blot analysis of GPR17 expression in Oli-neu cells cultured for 24 h, 48 h or 72 h in the absence and in the presence of 1 µM PD174265. (B) Quantitative evaluation of the intensity of the 47-kDa immunoreactive band corrected by beta-actin from three separated experiments. Data were kindly provided by Stephanie Hennen, Institute for Pharmaceutical Biology, University of Bonn, Germany.

4.1.1.3 Expression of the myelin basic protein in Oli-neu cells

Analysis of GPR17 role in myelination in the applied model cell system requires the expression of typical myelination markers, such as MBP. It has been previously described that after addition of PD174265, Oli-neu cells start to express MBP to a high extent (Fratangeli et al. 2013).

To validate the former findings, protein lysates of differentiating cells (presence of PD174265) and proliferating cells (absence of PD174265) were compared to determine MBP levels by protein blot analysis. As shown in Figure 6, quantitative comparison of protein samples revealed a lack of MBP in proliferating cell samples over 72 h. However, upon treatment of the cells with 1µM PD174265 for 72 h, MBP levels increased over the time. After 24 h the intracellular MBP levels amounted approximately 59% and af-

ter 48 h ≈73% of the 72 h-expression level. These data suggest that Oli-neu cells start to differentiate into myelinating MBP expressing cells after induction with PD174265.

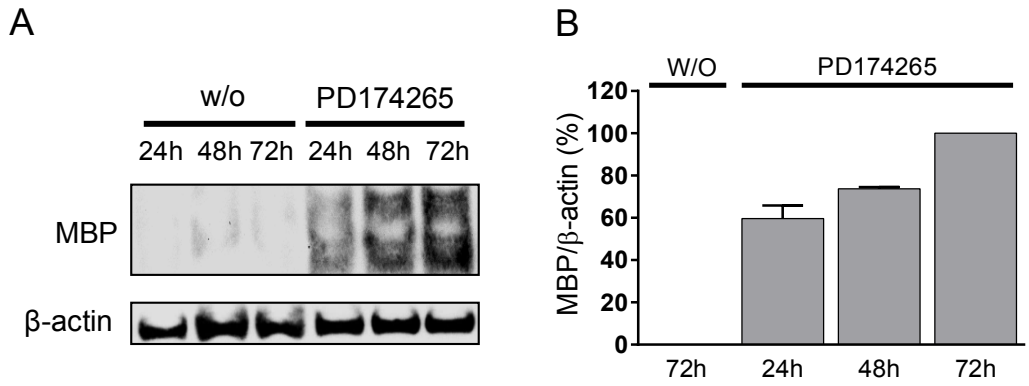


Figure 6. **Expression of the myelination marker MBP in Oli-neu cells**

(A) Representative protein blot of lysates prepared form Oli-neu cells cultured for 24 h, 48 h, and 72 h in the absence and in the presence of PD174265. Intracellular MBP levels were detected by anti-MBP antibody reprobed for beta-actin. (B) Quantitative evaluation of western blot from three separate experiments. Data were kindly provided by Stephanie Hennen, Institute for Pharmaceutical Biology, University of Bonn, Germany.

4.1.1.4 G protein expression during Oli-neu cell proliferation and maturation

A previous study has determined the expression of the entire set of Gα subunits in primary rat OPCs and oligodendrocytes by protein blotting and immunocytochemistry (Mir, Le Breton 2008). In order to analyze the expression of $G\alpha_i$, $G\alpha_q$, $G\alpha_s$, and $G\alpha_o$ in Oli-neu cells during proliferation and differentiation, Oli-neu cells were cultured for 48 h in the absence and in the presence of 1 µM PD174265 to induce differentiation and Gα subunit expressions were quantified by Western blot analyses with specific antibodies. In both stages of development - proliferating and differentiating - Oli-neu cells expressed the whole panel of Gα subunits (Figure 7, A-D). These data indicate that G protein signaling to all four main pathways is feasible in Oli-neu cells.

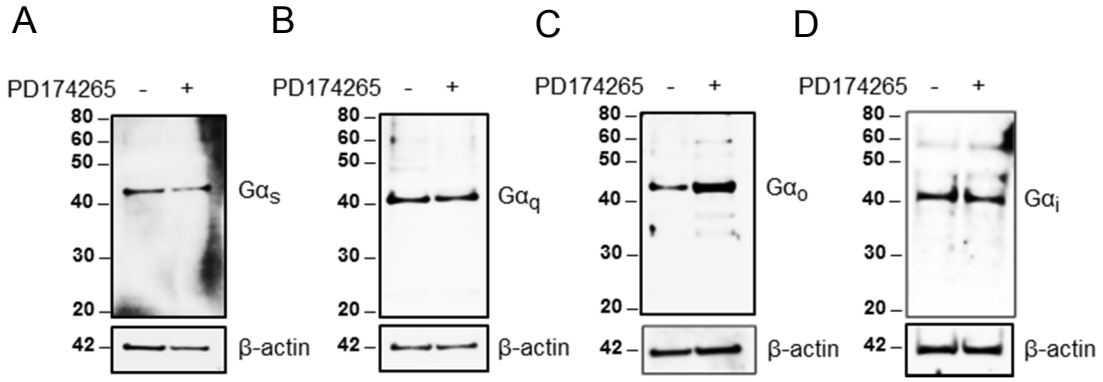


Figure 7. **G protein subunit expression in differentiating and proliferating Oli-neu cells**

Protein lysates of Oli-neu cells cultured in the absence and in the presence of 1 µMPD174265 for 48 h were analyzed by Western blot applying antibodies against (A) $G\alpha_s$, (B) $G\alpha_q$, (C) $G\alpha_o$, and (D) $G\alpha_i$.

4.1.2 Specific activation of GPR17 in Oli-neu cells by MDL29,951

To investigate the functionality of GPR17 in Oli-neu cells, we took advantage of MDL29,951, the first small molecule agonist of GPR17. MDL29,951 was recently described as a potent and specific agonist for GPR17 in both heterologous cell systems and primary rat oligodendrocytes (Hennen et al. 2013; Köse et al. 2014). A series of classic second messenger assays in Oli-neu cells were performed, to determine the capacity of MDL29,951 to activate GPR17 and to quantify the contribution of the different G proteins to the receptor signaling in this cell line.

4.1.2.1 GPR17 mediated calcium release in Oli-neu cells

Recently, Hennen and coauthors defined that the $G\alpha_q$ pathway activates the mobilization of Ca^{2+} from intracellular stores in primary rat oligodendrocytes in a GPR17-dependent manner. In order to investigate potential $G\alpha_q$-coupling of MDL29,951-activated GPR17 in Oli-neu cells, calcium release assays were performed. A robust and concentration-dependent Ca^{2+} release from intracellular stores was detectable in Oli-neu cells treated with increasing concentrations of MDL29,951 cultured in medium with PD174265 for 24-48 h. Ca^{2+} release was undetectable in proliferating Oli-neu cells cultured in the absence of PD174265, which did not have a detectable abundance of GPR17 (Figure 8, A).

Moreover, to analyze the contribution of the various G protein pathways to the calcium release, cells were incubated for 16 h with the $G\alpha_{i/o}$ pathway inhibitor PTX. Pretreatment with PTX decreased the GPR17 mediated calcium influx but did not completely

block calcium release (Figure 8, B). Taken together, these data suggest that, in contrast to primary oligodendrocytes (Hennen et al. 2013), GPR17 signals to both $G\alpha_{i/o}$ and $G\alpha_q$ proteins to release Ca^{2+} from intracellular stores in Oli-neu cells.

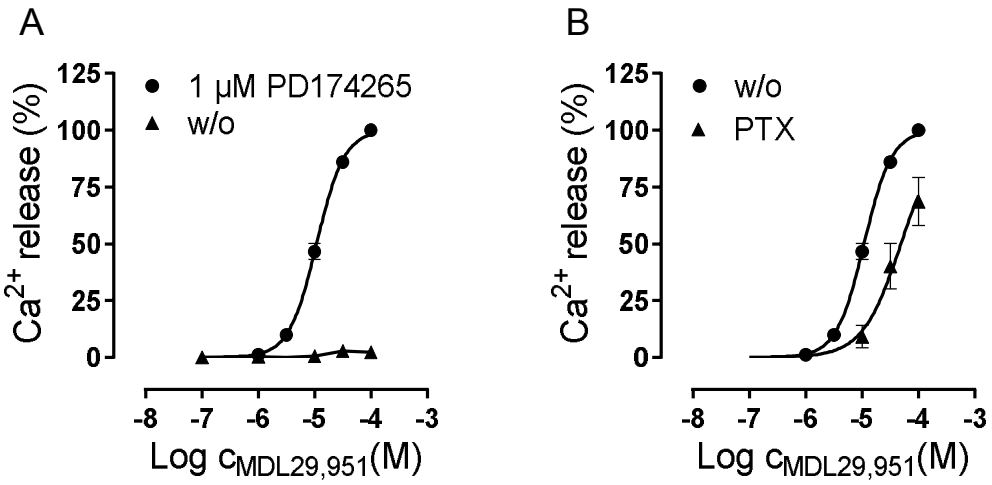


Figure 8. **Calcium release from intracellular stores in Oli-neu cells**

(A) MDL29,951 concentration-effect curves of intracellular Ca^{2+} release in Oli-neu cells cultured in the absence or in the presence of PD174265 derived from the maximum peak of real-time traces from three separate experiments. (B) PTX influence on MDL29,951-induced Ca^{2+} release in PD174265-differentiated Oli-neu cells. Data were kindly provided by Stephanie Hennen and Stefanie Blättermann, Institute for Pharmaceutical Biology, University of Bonn, Germany.

To further verify the agonist specificity of MDL29,951, GPR17 expression was decreased in differentiating Oli-neu cells by transfection with a small interfering siRNA. Analysis of GPR17 expression levels in control cells compared to GPR17 knockdown cells revealed a decrease in the intensity of the GPR17 immunoreactive band in cells transfected with siRNA specific for GPR17 (Figure 9).

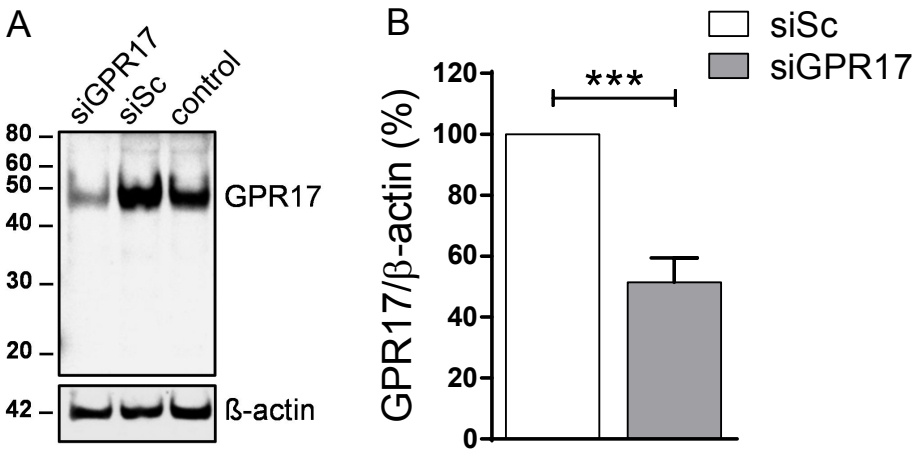


Figure 9. **GPR17 knockdown by siRNA transfection in Oli-neu cells**

(A) Effect of scrambled siRNA (*siSc*) and GPR17-siRNA (*siGPR17*) on GPR17 expression as pictured on a protein blot. (B) GPR17 expression levels represented in a bar diagram derived from Western blots upon GPR17 knockdown from five independent experiments. ***, $p<0.001$. Data were kindly provided by Stefanie Blättermann, Institute for Pharmaceutical Biology, University of Bonn, Germany.

Accordingly, MDL29,951-induced Ca^{2+} flux was reduced upon GPR17-siRNA cell transfection. Ca^{2+} release triggered by both the receptor-independent calcium ionophore A23187 and adenosine 5-triphosphate (ATP), through activation of endogenous P2Y receptors, was unaffected (Figure 10), indicating that siRNA transfection had no influence on the capacity of Oli-neu cells to release Ca^{2+} from intracellular calcium stores.

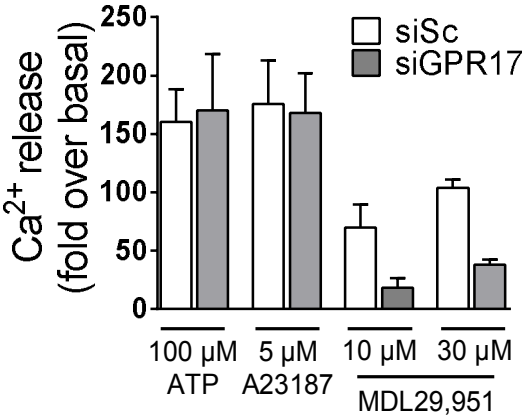


Figure 10. **Effect of GPR17-siRNA knockdown on calcium release from intracellular stores in Oli-neu cells**

Effect of scrambled siRNA (*siSc*) and GPR17-siRNA (*siGPR17*) knockdown on ATP-, A23187-, and MDL29,951-triggered Ca^{2+} release in Oli-neu cells treated with PD174265. Data were kindly provided by Stefanie Blättermann, Institute for Pharmaceutical Biology, University of Bonn, Germany.

4.1.2.2 GPR17-mediated inhibition of cAMP accumulation

Additionally, the influence of GPR17 on intracellular cAMP accumulation was analyzed in order to investigate the $G\alpha_{i/o}$ and $G\alpha_s$ coupling of GPR17.

The amount of forskolin-stimulated intracellular cAMP was concentration-dependently decreased by the GPR17 agonist MDL29,951 in Oli-neu cells cultured for 24 h with PD174265. In contrast, in proliferating Oli-neu cells (cultured without PD174265), which do not express GPR17, no decrease was detectable (Figure 11). This verified that MDL29,951-mediated lowering of cAMP levels is GPR17-dependent. Furthermore, cAMP accumulation was not detectable at higher concentrations of the GPR17 agonist. These data reveal that GPR17 activates $G\alpha_{i/o}$ but not $G\alpha_s$ protein signaling in Oli-neu cells endogenously expressing GPR17, although $G\alpha_s$ protein are present in this cellular background (Figure 7A).

Altogether, the data of the first part of the present work lend further support to the notion that GPR17 engages $G\alpha_{i/o}$ and $G\alpha_q$ proteins for downstream signaling in primary rat oligodendrocytes and Oli-neu cells. Importantly, for further investigations of the present study, Oli-neu cells express GPR17 in a similar time-dependent manner during differentiation similar to that seen in primary oligodendrocytes (Hennen et al. 2013), and MDL29,951 has the capacity to specifically activate GPR17 in Oli-neu cells. Furthermore, during differentiation, Oli-neu cells express the typical differentiation marker MBP. Thus, the obtained data validate Oli-neu cells as suitable model system to study the pathways engaged by GPR17 to arrest oligodendrocyte differentiation and myelination.

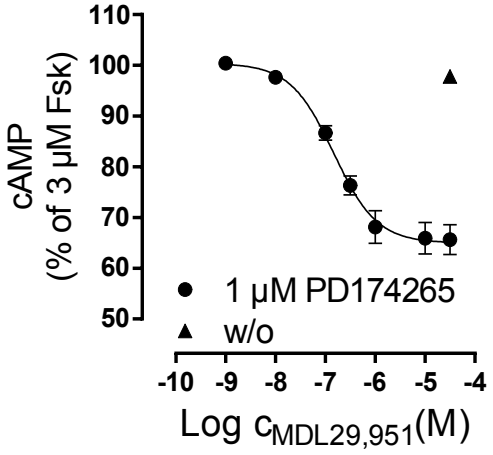


Figure 11. **MDL29,951-decreased cAMP accumulation in Oli-neu cells expressing GPR17**

Alteration of forskolin-stimulated intracellular cAMP-levels triggered by MDL29,951 in Oli-neu cells in the absence (n = 3) and in the presence (n = 6) of PD174265.

4.2 GPR17 signaling pathway in oligodendrocytes

4.2.1 $G\alpha_{i/o}$ proteins as major contributors to the GPR17 signaling in oligodendroglial cells

The results of the classical second messenger assays revealed a contribution of both $G\alpha_{i/o}$ and $G\alpha_q$ proteins and their effector proteins to GPR17 signaling in primary rat oligodendrocytes (Hennen et al. 2013) and Oli-neu cells. In order to determine whether both G protein pathways play equivalent roles after GPR17 activation in oligodendroglial cells, their unmitigated cell activity was inspected.

4.2.1.1 Global cell response analyzed by dynamic mass redistribution

To this end, GPR17 global cell responses in Oli-neu and primary rat oligodendrocytes were determined by dynamic mass redistribution (DMR). This non-invasive label-free method uncovers the whole cell activity after receptor stimulation and was described as ideal approach to analyze GPCR signaling in living cells, detecting the signaling pathways of all four G protein subfamilies (Schröder et al. 2010; Schröder et al. 2011). Furthermore, to analyze the contribution of $G\alpha_{i/o}$ proteins to the GPR17-mediated whole cell responses, PTX was applied to pharmacologically block $G\alpha_{i/o}$ signaling (Schröder et al. 2010).

In differentiated Oli-neu cells, DMR was analyzed after addition of MDL29,951 in increasing concentrations in the absence and in the presence of PTX. As shown in Figure 12, MDL29,951 triggered a concentration-dependent and robust DMR response in Oli-neu cells. Moreover, after incubation of the cells for 16 h with PTX, the whole-cell response was completely abolished (Figure 12, A and B).

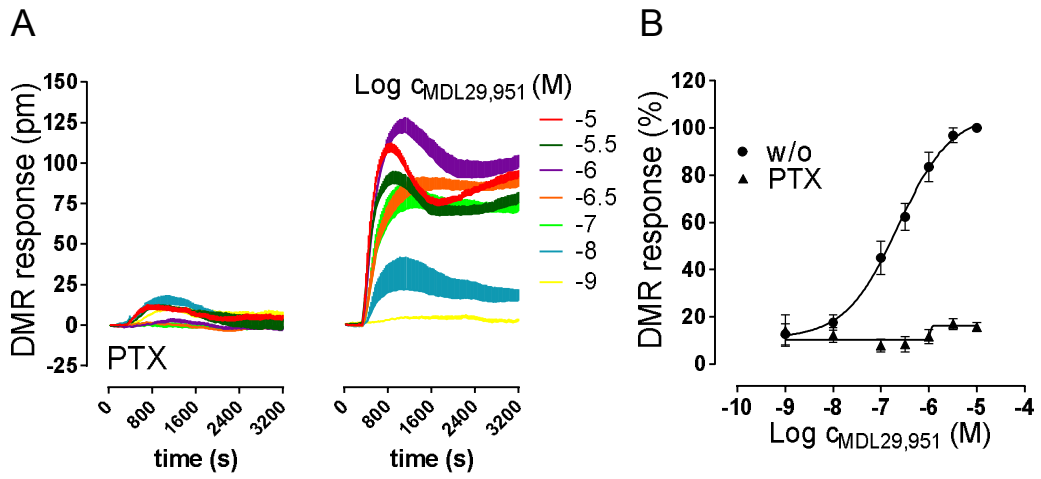


Figure 12. **MDL29,951-triggered DMR recordings in Oli-neu cells**

(A) Representative concentration-dependent DMR recordings of the GPR17 agonist MDL29,951 in Oli-neu cells cultured for 24 h with PD174265 in the absence and in the presence of PTX. (B) Concentration-response curves derived from different DMR traces as in (A) from seven independent experiments. Data were in part provided by Stephanie Hennen, Institute for Pharmaceutical Biology, University of Bonn, Germany.

Corresponding observations were made in primary rat oligodendrocytes incubated for 24 h in medium without growth factors to induce their differentiation. MDL29,951 prompted concentration-dependent stable optical traces that after incubation of the cells for 16 h with PTX were largely abrogated (Figure 13).

The obtained results support the notion that the wavelength shift resulted mainly from a $G\alpha_{i/o}$ -mediated signaling event in both cell lines. Thus, these data drive the hypothesis that in oligodendrocytes, the $G\alpha_{i/o}$ cascade is the main pathway triggered by GPR17, responsible for the negative impact of GPR17 on oligodendrocyte differentiation.

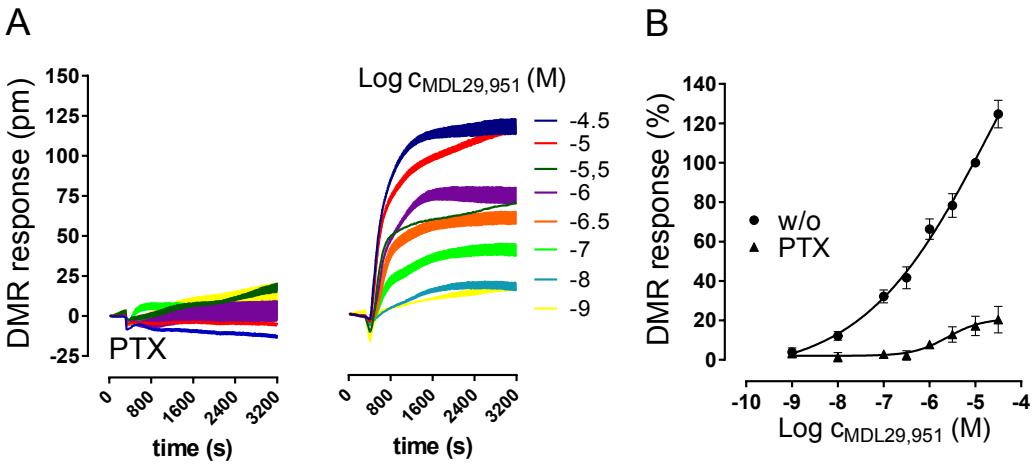


Figure 13. **MDL29,951-triggered DMR recordings in primary rat oligodendrocytes**

(A) Characteristic DMR traces of MDL29,951-triggered responses in primary rat oligodendrocytes in the absence and in the presence of PTX, cultured for 24 h in medium without growth factors to induce differentiation. (B) Concentration-response curves calculated from six independent experiments. Data were in part provided by Stephanie Hennen, Institute for Pharmaceutical Biology, University of Bonn, Germany.

4.2.2 GPR17 arrested oligodendrocytes in an immature stage via $G\alpha_{i/o}$

4.2.2.1 Influence of MDL29,951 on Oli-neu cell differentiation

To validate the hypothesis that GPR17 acts as a negative modulator of oligodendrocyte maturation via the $G\alpha_{i/o}$ signaling cascade, Oli-neu cells were first incubated in the absence and in the presence of 10 µM MDL29,951 for 48 h, while cultured with 1 µM PD174265 to induce their differentiation, and subsequently Western blot analyses were performed to quantify MBP expression. As shown in Figure 14, evaluation of the immunoblottings revealed an average of approximately 38% lower MBP abundance in Oli-neu cells treated with MDL29,951, which indicated that GPR17 activation mediated a maturation block in this cell model.

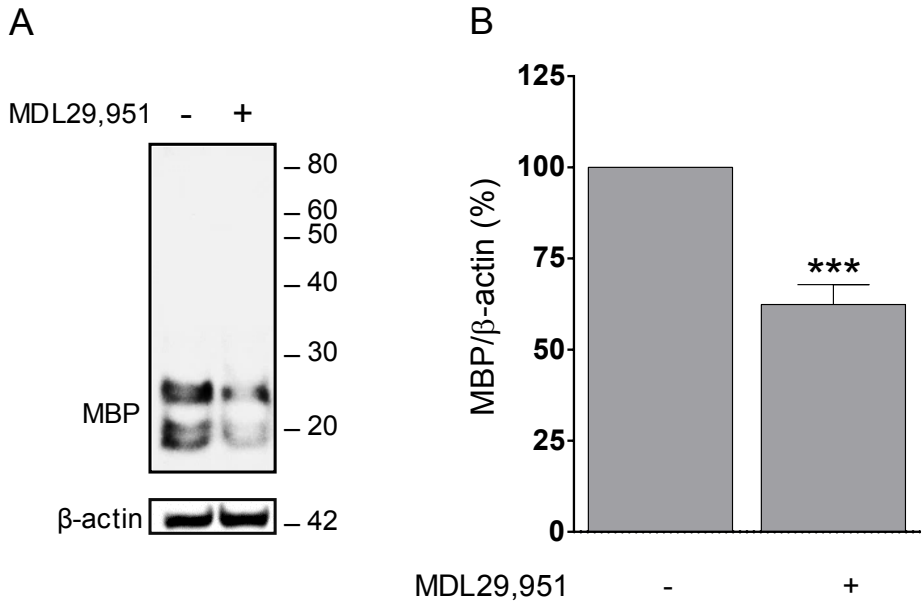


Figure 14. **GPR17-mediated decrease in MBP expression levels in Oli-neu cells**

(A) Illustrative Western blot of MBP downregulation mediated by 10 µM MDL29,951 compared with untreated cells in Oli-neu cells differentiated with 1 µM PD174265. (B) Densitometric analysis of MBP immunoreactive bands, quantified from eight independent experiments. ***, $p<0.001$.

4.2.2.2 Influence of MDL29,951 on primary rat oligodendrocyte differentiation

To further verify the obtained data, which assumed that GPR17 mediates a maturation block, immature primary rat oligodendrocytes were incubated for 48 h in the absence and in the presence of MDL29,951 in culture medium containing thyroid hormone T3 to induce differentiation. Densitometric analysis exhibited an average reduction of approximately 25% in MBP expression levels after addition of the GPR17 agonist MDL29,951 (Figure 15). Therefore, the obtained data in both cell lines confirmed that GPR17 activation arrests oligodendrocytes in an immature, less differentiated stage and, consequently, that GPR17 has a negative impact on oligodendrocyte development.

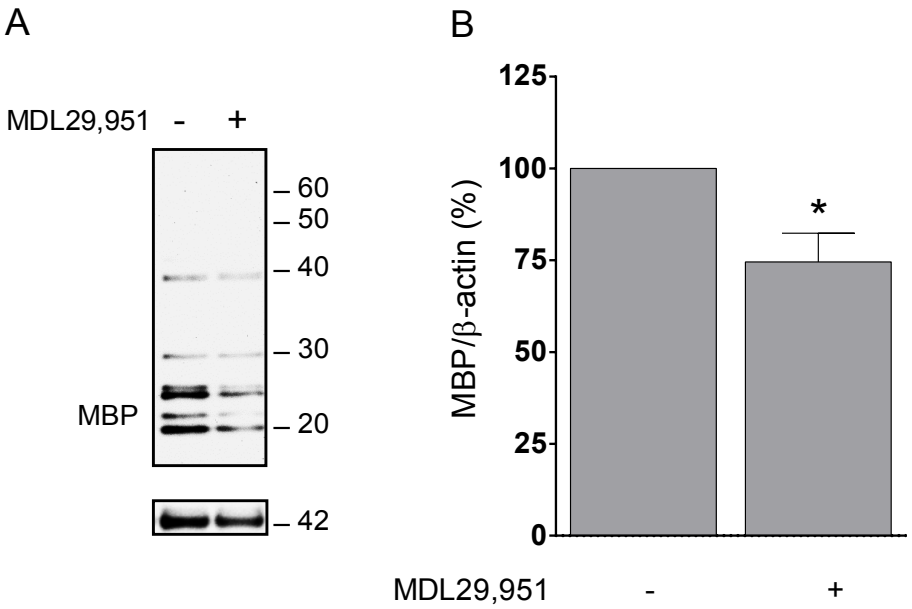


Figure 15. **Depression of MBP expression triggered by GPR17 in primary rat oligodendrocytes**

(A) Representative Western blot illustrating lower levels of MBP from primary rat oligodendrocytes cultured in differentiation medium containing T3 in the presence of 30 µM MDL29,951 in contrast to control cells. (B) Quantification from six independent protein blot analyses. *, $p<0.05$.

4.2.2.3 Engagement of $G\alpha_{i/o}$ on GPR17-mediated MBP decrease

Current evidence supports the idea that $G\alpha_{i/o}$-coupled GPCRs act as inhibitors of oligodendrocyte maturation, such as the chemokine receptor CXCR2 or the thrombin receptor PAR1 (Kerstetter et al. 2009; Yoon et al. 2015).

The DMR analysis in the present study verified that GPR17 mainly signals via $G\alpha_{i/o}$ proteins in oligodendrocytes. In order to investigate whether GPR17 blocks oligodendrocyte maturation by a $G\alpha_{i/o}$ protein-dependent pathway, the MBP expression of primary rat oligodendrocytes and Oli-neu cells was analyzed in the absence and in the presence of PTX.

Oli-neu cells were first incubated with PTX for 16-20 h, before induction of differentiation with 1µM PD174265 for 48 h. Immunoblotting quantification analyses of MBP expression displayed that incubation with PTX counteracted the decrease of MBP expression triggered by the GPR17 agonist MDL29,951 (Figure 16). The obtained data suggest that the $G\alpha_{i/o}$ pathway mainly triggers the arrest of differentiation mediated by GPR17 in Oli-neu cells.

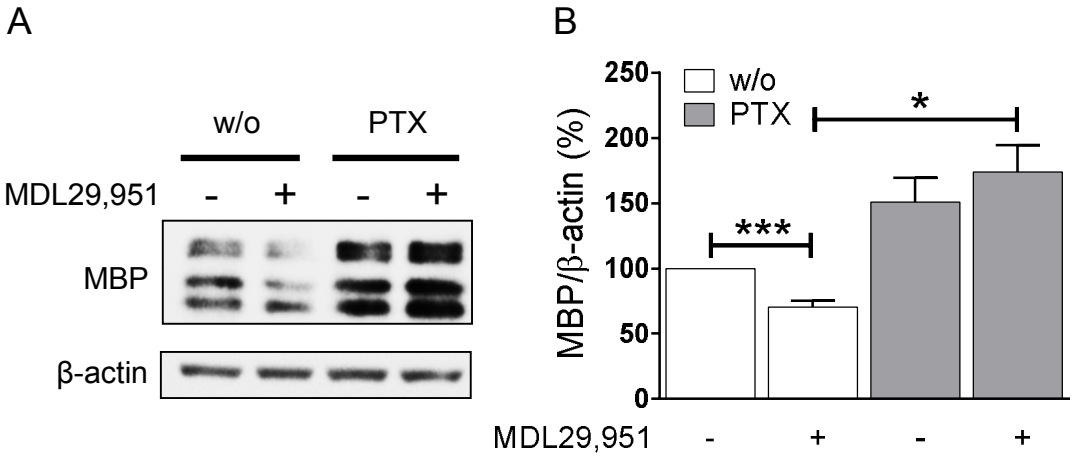


Figure 16 **Influence of PTX on MBP expression in Oli-neu cells**

(A) Western blot analysis of intercellular MBP levels representative for the counteracting effect of PTX on the GPR17-mediated effect on the MBP expression. (B) Qualitative evaluation of twelve independent protein analyses. *, p 0.05; ***, p 0.001.

Comparable results were obtained in primary rat oligodendrocytes, where PTX had the ability to counteract the MDL29,951-mediated reduction of MBP expression (Figure 17).

Together, these findings indicate that MDL29,951-activated GPR17 engages $G\alpha_{i/o}$ proteins to reduce MBP expression in primary rat oligodendrocytes and Oli-neu cells, to finally arrest oligodendrocytes in an immature stage. Because $G\alpha_{i/o}$ proteins mainly inhibit the enzyme adenylate cyclase, thus decreasing intracellular cAMP production, the present data suggest a major role of cAMP in the GPR17 signaling cascade to arrest oligodendrocyte differentiation.

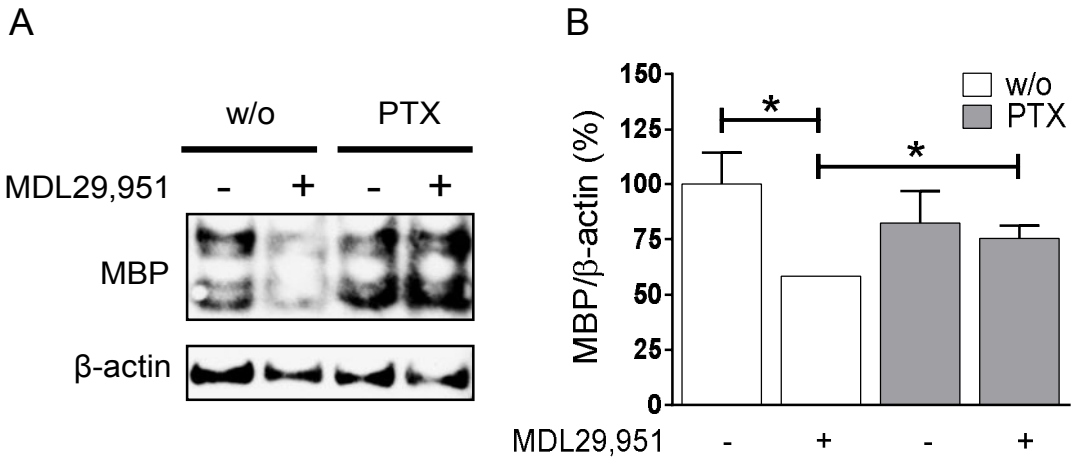


Figure 17. **Influence of PTX on MBP expression in primary rat oligodendrocytes**

(A) Representative Western blot analysis of MBP, then reprobed for beta-actin, in primary rat oligodendrocytes after 16 h of incubation with PTX in the absence and in the presence of 30 µM MDL29,951. (B) Quantitative analysis of MBP expression in primary rat oligodendrocytes from eight independent experi-

ments. *, $p<0.05$. Data were in part provided by Stephanie Hennen, Institute for Pharmaceutical Biology, University of Bonn, Germany.

4.2.3 GPR17-mediated regulation of intracellular cAMP levels affects MBP expression levels

cAMP is a second messenger involved in key signal transductions within the cell. Adenylate cyclase converts adenosine triphosphate (ATP) into cAMP and pyrophosphate (PPi), and thereby the intracellular cAMP concentration is increased. In the last years, different independent groups have evaluated the essential role of intracellular cAMP modulation on the demanding oligodendrocyte differentiation process. High intracellular cAMP levels trigger the transition of OPCs to mature oligodendrocytes, thereby inducing oligodendrocyte myelination (Pleasure et al. 1986; Raible, McMorris 1990; Pende et al. 1997). Consequently, indirect increase of the intracellular cAMP levels by phosphodiesterase inhibitors mediates a high abundance of myelinating oligodendrocytes in culture (Syed et al. 2013).

To further substantiate the GPR17-$G\alpha_{i/o}$-cAMP signaling pathway, the effect of cAMP on MBP expression upon GPR17 stimulation was investigated. To this end, differentiating Oli-neu cells were incubated together with MDL29,951 in the absence and in the presence of the membrane permeable, lipophilic, and non-hydrolysable cAMP analog 8-(4-chlorophenylthio)-adenosine-3´,5´-cAMP (8-CPT-cAMP). As seen in Figure 18, the cAMP analog overcame the decrease in MBP levels upon GPR17 activation, displaying no significant effects in its own right. Similar results were obtained in primary rat oligodendrocytes, where 8-CPT-cAMP - ineffective when applied alone - also counteracted GPR17-induced MBP depression (Figure 19).

Taken together, these results strongly suggest that GPR17 activation mediated a decrease of intracellular cAMP levels in order to keep oligodendrocyte in an immature, not myelinating stage, in line with the previous findings correlating cAMP concentration and oligodendrocyte differentiation. Interestingly, intracellular concentration of cAMP is an important stimulus to regulate the activity of two main effector proteins, the cAMP-dependent protein kinase A (PKA) and the Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP (EPAC). Consequently, a new analysis was initiated to uncover the putative role of both downstream effectors upon GPR17 activation that results in arrest of oligodendrocyte maturation.

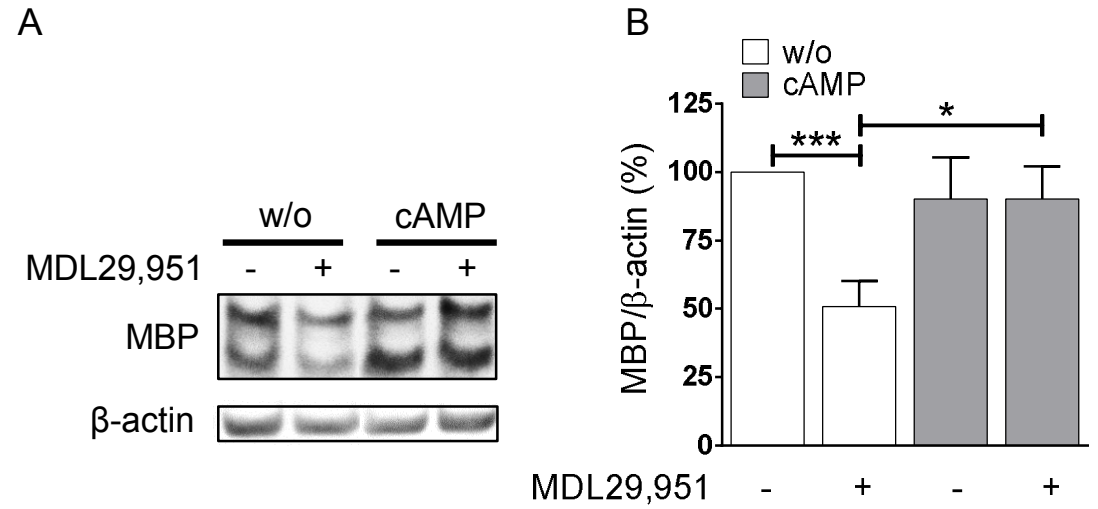


Figure 18. **Expression of MBP is promoted by treatment of Oli-neu cells with 8-CPT-cAMP in the presence of MDL29,951**

(A) Illustrative protein analysis of MBP levels in Oli-neu in the absence (*w/o*) and in the presence of 1 µM of the cAMP analog 8-CPT-cAMP (*cAMP*). (B) Quantitative analysis of ten independent protein blots. *, $p<0.05$; ***, $p<0.001$.

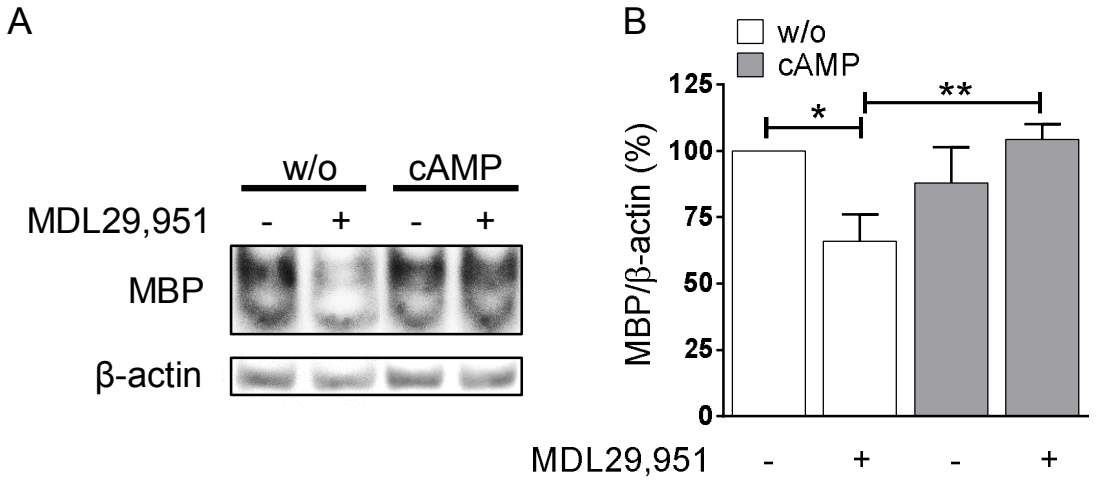


Figure 19. **MBP expression is triggered by 8-CPT-cAMP in the presence of MDL29,951 in primary rat oligodendrocytes**

(A) Representative Western blot of MBP levels in differentiating oligodendrocytes treated for 48 h in medium with triiodothyronine (T3) and 30 µM MDL29,951 in the absence (*w/o*) and in the presence of 1 µM 8-CPT-cAMP (*cAMP*). (B) Calculation of MBP levels from five isolated experiments in primary rat oligodendrocytes illustrating that the cAMP analog triggered MBP expression in the presence of MDL29,951. *, $p<0.05$; **, $p<0.01$.

4.2.4 Investigation of the PKA-pCREB pathway

PKA function has been extensively studied in the last years. Changes in the intracellular levels of cAMP regulate PKA activity, leading to phosphorylation of both cytosolic targets and nuclear transcription factors. Interestingly, recent studies have described the positive influence of PKA activity on oligodendrocyte differentiation (Sato-Bigbee et al. 1999; Shiga et al. 2005).

4.2.4.1 GPR17 stimulation results in decreased PKA activity

To investigate whether the alteration of cAMP levels mediated by GPR17 leads to a change in the PKA activity that subsequently primes a decrease in MBP expression, the effect of N6-benzoyladenosine-3,5-cyclic monophosphate (SP-6-Bnz-cAMPs) a selective cell-permeable PKA activator on GPR17-mediated MBP depression was analyzed by immunoblot quantification. To this end, both Oli-neu and primary rat oligodendrocytes were pre-incubated with SP-6-Bnz-cAMPs in two different concentrations (20 µM and 2 µM) in the absence and in the presence of the GPR17 agonist. As shown in Figure 20, both concentrations did not significantly affect MBP expression levels in their own right, but counteracted the MDL29,951-mediated decrease of MBP levels in Oli-neu cells. Equivalent results were obtained in rat oligodendrocytes (Figure 21).

Taken together, the data indicate that a downstream signaling consequence of GPR17 activation is a reduction of PKA activity, which finally impairs oligodendrocyte differentiation. These findings are in keeping with published observations describing a relationship between PKA activity and differentiation of oligodendrocytes (Sato-Bigbee et al. 1999; Shiga et al. 2005).

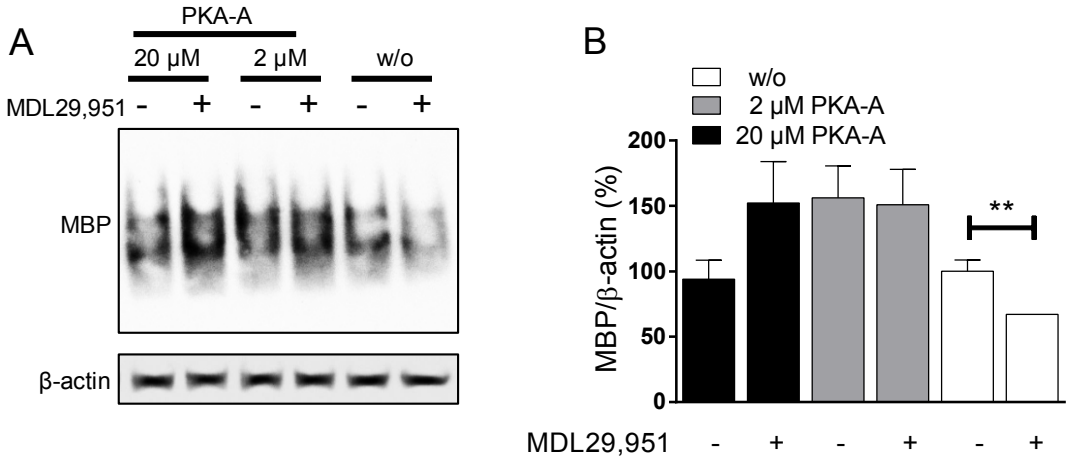


Figure 20. **PKA activation triggered MBP expression in Oli-neu cells and overcame the GPR17 agonistic effect**

(A) Illustrative western blot of MBP expression, afterwards reprobed for β-actin expression from Oli-neu cell lysates in the absence and presence of 10 µM MDL29,951 showing that SP-6-Bnz-cAMPS overcame

the negative effect of MDL29,951 on MBP expression. (B) Evaluation of MBP expression from eight independent experiments.

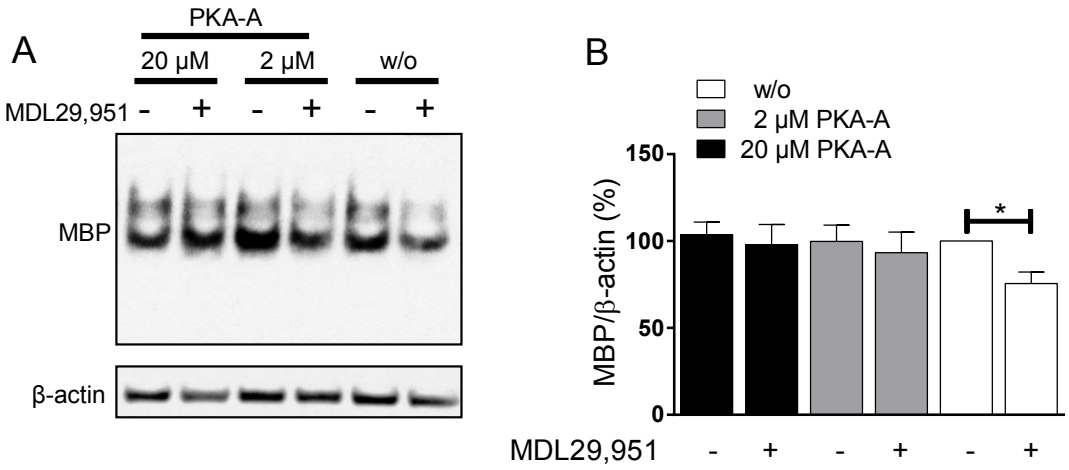


Figure 21. **Activated PKA stimulated MBP expression in primary rat oligodendrocytes cells and counteracted GPR17-mediated effect**

(A) Illustrative blot of MBP levels, the same membrane was as well reprobed for beta-actin, from oligodendrocytes incubated in the absence and presence of 20 µM or 2 µM Sp-6-Bnz-cAMPS for 48 h to counteract the MDL29,951 (30 µM) effect. Culture medium contained T3 to induce differentiation. (B) Evaluation of the relative MBP expression from nine independent experiments in oligodendrocytes differentiated for 48 h with T3. *, $p<0.05$.

4.2.4.2 Effects of GPR17 stimulation on CREB phosphorylation

Regulation of gene expression in oligodendrocytes is crucial to successfully control the challenging processes of maturation and differentiation. It has been discovered that levels of the intracellular second messenger cAMP affect gene expression via the activation of transcription factors that bind to DNA regulatory regions carrying specific consensus sequences (Sato-Bigbee et al. 1994; Sands, Palmer 2008).

An important pathway in oligodendrocyte differentiation is the phosphorylation of the transcription factor CREB by PKA at Ser133. This results in the binding of CREB to the DNA regulatory region cyclic AMP response element (CRE), which leads to the regulation of MBP gene expression (Sato-Bigbee, Yu 1993; Sato-Bigbee et al. 1994; Sato-Bigbee et al. 1999).

To investigate whether GPR17 stimulation in primary rat oligodendrocytes has an influence on CREB activation, the level of intracellular CREB phosphorylation in the absence and in the presence of 30 µM MDL29,951 was measured after 48 h treatment. A significant decrease of p-CREB in cells stimulated with the GPR17 agonist was detectable in comparison to untreated cells by immunoblotting quantification analyses (Figure 25). Notably, pre-incubation with different PKA activator concentrations (Sp-6-Bnz-cAMPS) counteracted the GPR17-dependent p-CREB reduction. Taken together, the

results revealed a key role for GPR17 in diminishing MBP expression by engaging the cAMP-PKA-CREB cascade. In particular, inhibition of the AC after GPR17 activation decreases intracellular cAMP levels, which in turn depresses PKA activity and, in consequence, reduces intracellular phosphorylated CREB levels.

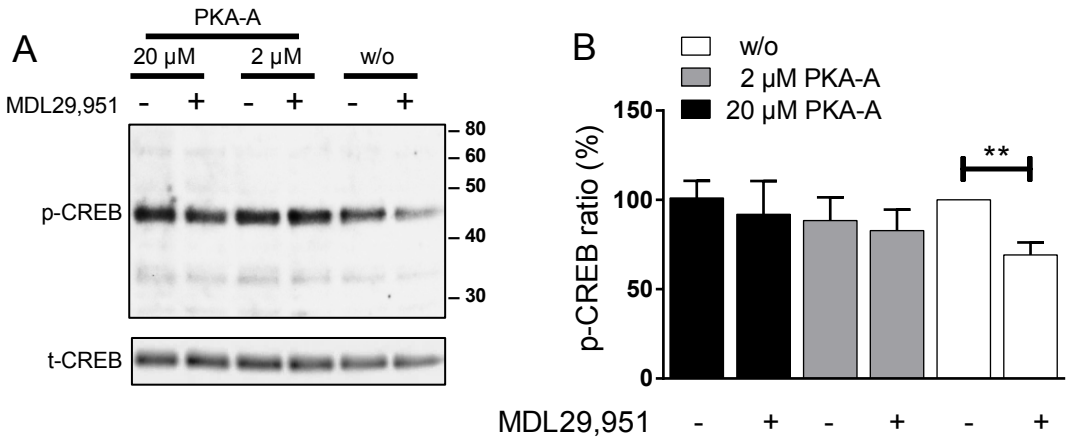


Figure 22. **Activation of PKA overcame the MDL29,951-mediated decrease in CREB phosphorylation**

(A) Representative Western blot of phosphorylated CREB (*p-CREB*), afterwards reprobed for total CREB levels (*t-CREB*). Primary rat oligodendrocytes were cultured with T3 in the absence and presence of 30 µM MDL29,951 with two different concentrations of Sp-6-Bnz-cAMPS for 48 h. Western blot analysis exhibited both the significant decrease of CREB phosphorylation upon GPR17 activation and promotion of CREB phosphorylation by cotreatment with the PKA activator. (B) Evaluation of phosphorylated to total CREB ratios from seven independent experiments in oligodendrocytes differentiated for 48 h. **, $p<0.01$.

4.2.5 Influence of GPR17 on ERK1/2 phosphorylation

PKA is also known to phosphorylate CREB through a second downstream mechanism, by activation of the mitogen-activated protein kinase (MAPK) cascade, which in turn phosphorylates CREB (Costes et al. 2006; Smith et al. 2010; Syed et al. 2013). Extracellular signal-regulated kinase 1 and 2 (ERK1/2) are members of the MAPK family of protein kinases, which are involved in several cellular processes such as survival, differentiation and proliferation (Costes et al. 2006). Interestingly, ERK1/2 are key signaling intermediates playing an essential role in promoting early development and myelination of oligodendrocytes (Xiao et al. 2012b; Fyffe-Maricich et al. 2011).

4.2.5.1 ERK1/2 phosphorylation in heterologous cell expression system

To investigate whether ERK1/2 are GPR17 effector proteins, GPR17-dependent ERK1/2 phosphorylation (pERK1/2) was first analyzed in a HEK293 cell background.

As shown in Figure 23, A, a robust time-dependent increase in ERK1/2 phosphorylation was detectable, peaking within 2 min and returning to baseline after 30 min.

Notably, after pre-incubation with the $G\alpha_q$-selective inhibitor FR900359, the ERK1/2 phosphorylation was completely abolished, whereas pre-incubation with the $G\alpha_{i/o}$ inhibitor PTX partially abolished the ERK1/2 phosphorylation (Figure 23, B). These results indicate that in HEK293 cells GPR17 used both $G\alpha_q$ and $G\alpha_i$ pathways to phosphorylate ERK1/2, suggesting an astounding order to regulate intracellular ERK1/2 phosphorylation: $G\alpha_q$-mediated ERK activation occurs upstream to that mediated by $G\alpha_i$.

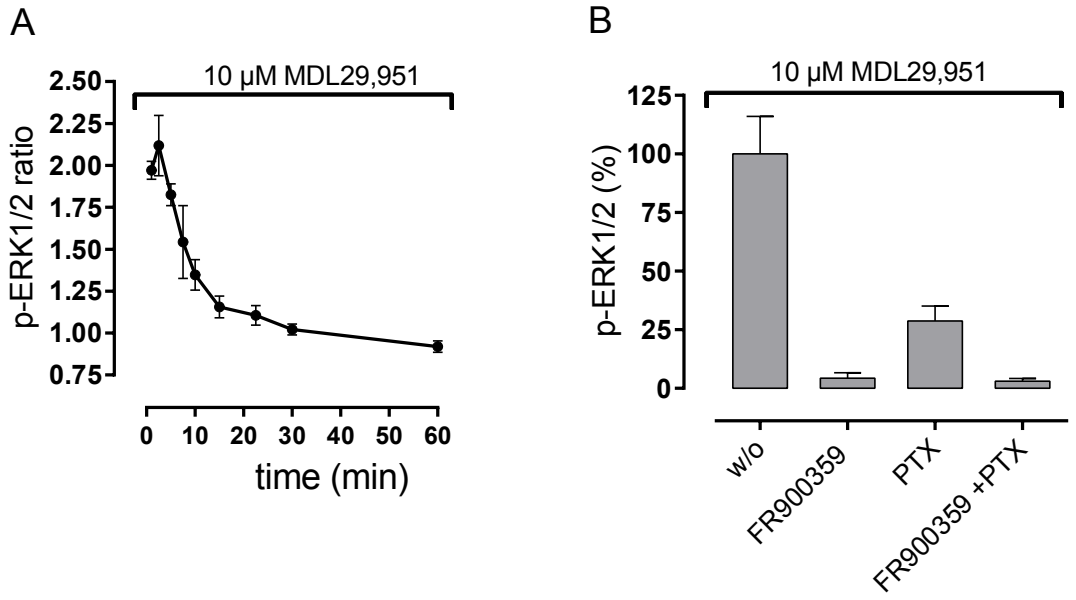


Figure 23. **GPR17-mediated ERK1/2 phosphorylation in heterologous cell expression system**

(A) Kinetics of ERK1/2 phosphorylation in HEK293 cells stably expressing GPR17 after treatment with 10 µM MDL29,951 (n = 3). (B) Phosphorylation of ERK1/2 in hGPR17 HEK239 cells after MDL29,951 stimulation, pretreated with FR900359 (1 µM), PTX (50 ng/ml), or both.

4.2.5.2 ERK1/2 activation in oligodendroglial cell lines

To further analyze whether activated GPR17 endogenously expressed in oligodendrocytes conducts modifications in intracellular p-ERK1/2 levels similar to those observed in HEK293 cells, primary rat oligodendrocytes and Oli-neu cells were stimulated with MDL29,951 over time.

In Oli-neu cells receptor activation resulted in a robust and rapid pERK1/2 increase of transient nature, with a maximum peak after 7-10 min that reversed quickly toward baseline (Figure 24, A). Comparable results were obtained in primary rat oligodendrocytes; MDL29,951 induced an earlier transient signal of pERK1/2 after 5 min, which

returned to baseline after 30 min (Figure 24, B). Therefore, these data confirm a transient ERK1/2 phosphorylation also in native expression systems after GPR17 activation.

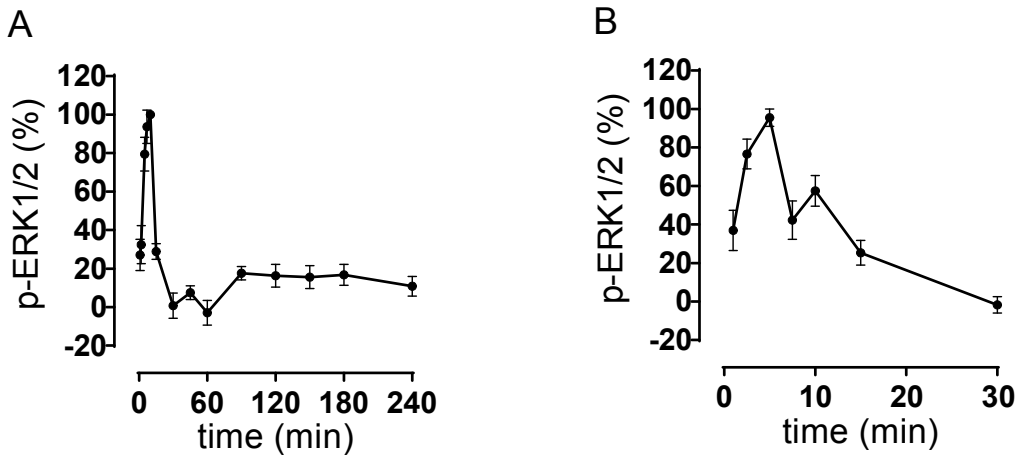


Figure 24. **Kinetics of the phosphorylation of ERK1/2 triggered by MDL29,951 in oligodendroglial cell lines**

Time-dependent ERK1/2 activation in Oli-neu cells (A) and primary rat oligodendrocytes (B) mediated by MDL29,951. Data in (B) were kindly provided by Stephanie Hennen, Institute for Pharmaceutical Biology, University of Bonn, Germany.

To determine the underlying molecular mechanism of short-term ERK1/2 phosphorylation in Oli-neu cells, cells were pre-incubated with the $G\alpha_q$ inhibitor FR900359 and the $G\alpha_{i/o}$ inhibitor PTX. Notably, pre-treatment with PTX completely abolished ERK1/2 phosphorylation whereas FR900359 showed no influence on the maximum peak of pERK1/2 after 10 min (Figure 25, A). Neither PTX nor FR900359 affected viability of Oli neu cells because FCS-mediated ERK phosphorylation was unaltered when cells were pre-treated with these inhibitors (Figure 25, B). In conclusion, in Oli-neu cells, GPR17-mediated ERK1/2 phosphorylation is completely $G\alpha_i$-dependent with the $G\alpha_q$ pathway having no impact on intracellular p-ERK1/2 levels.

Altogether, these results provide evidence that MDL29,951-stimulated GPR17 induces ERK1/2 phosphorylation in a G protein-dependent manner both in heterologous cell expression systems and in oligodendroglial cell systems endogenously expressing GPR17, albeit via engagement of different Gα subfamilies in a manner depending on the cellular background.

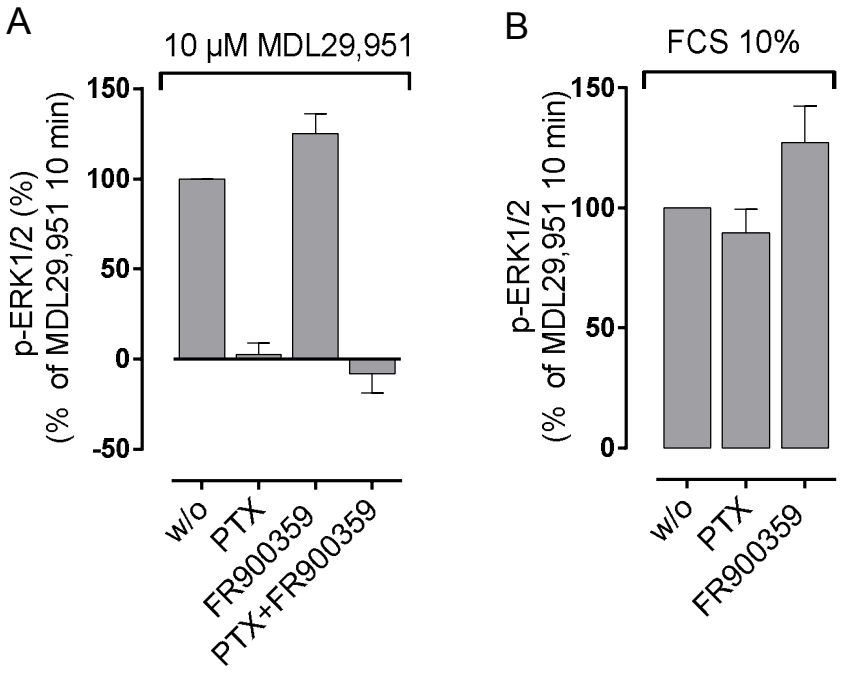


Figure 25. **Effect of G protein selective inhibitors on ERK1/2 phosphorylation triggered by GPR17 in Oli-neu cells**

(A) ERK1/2 phosphorylation in Oli-neu cells triggered by MDL29,951 after pre-treatment with FR900359 (1µM), PTX (50 ng/ml), or both. (B) ERK1/2 phosphorylation mediated by 10% fetal calf serum (*FCS*) in oligodendrocytes after pre-treatment with FR900359 (1 µM) or PTX (50 ng/ml) compared to buffer pretreatment (n = 2-3).

Recent publications have supported a direct correlation between long-term increase of intracellular phosphorylated ERK1/2 and oligodendrocyte differentiation (Fyffe-Maricich et al. 2011; Xiao et al. 2012a; Xiao et al. 2012b). To investigate whether long-term alteration of ERK1/2 phosphorylation in oligodendrocytes is also associated with activation of GPR17, p-ERK1/2 levels were quantified in primary rat oligodendrocytes and Oli-neu cells after incubation with MDL29,951 for 24 h and 48 h. However, in Oli-neu cells, no GPR17-mediated alteration on p-ERK1/2 levels was detectable after 24 h or 48 h (Figure 26).

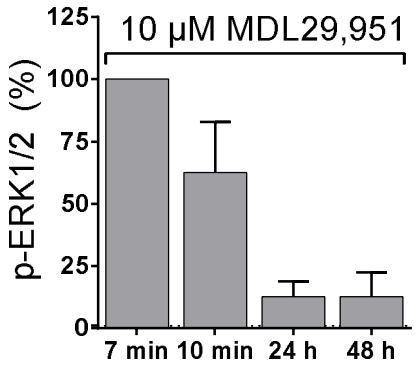


Figure 26. **ERK1/2 long-term activation in Oli-neu cells in the presence of MDL29,951**

(A) Long-term phosphorylation of ERK1/2 time course in Oli-neu cells mediated by 10 µM MDL29,951 for 10 min, 24 and 48 h compared with the phosphorylation peak detected upon 7 min treatment (n = 2-5).

Likewise, in primary rat oligodendrocytes, although over the time the phosphorylation of ERK1/2 surged, longer incubations with 30 µM MDL29,951 were not associated with an increase or decrease of pERK1/2 (Figure 27, A and B).

Taken together, these data provide evidence that MDL29,951-stimulated GPR17 triggers phosphorylation of ERK1/2 at short time points but does not induce sustained long-term ERK activation, which is considered as key factor for efficient enhancement of oligodendrocyte differentiation. Thus, GPR17 unlikely affects oligodendrocyte differentiation by an ERK1/2-dependent pathway.

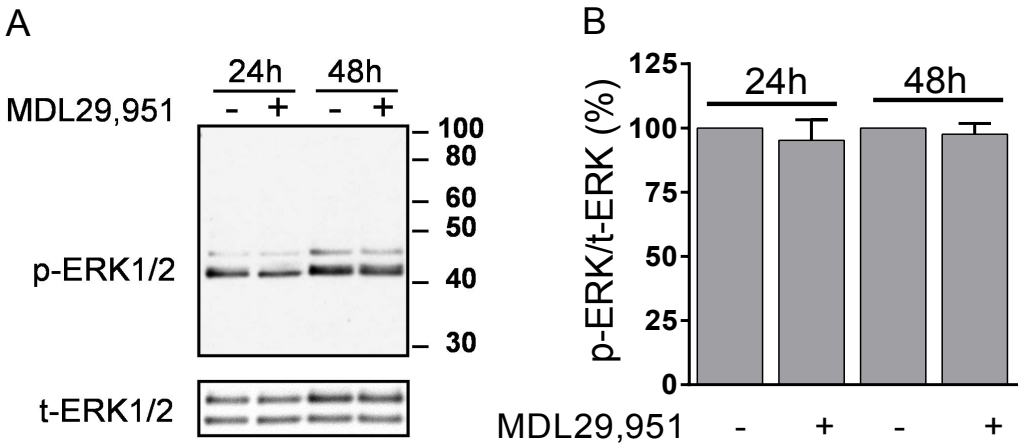


Figure 27. **ERK1/2 long-term activation in primary rat oligodendrocytes is not affected by MDL29,951**

(A) Representative Western blot analysis of alteration of phosphorylated ERK1/2 (p-ERK1/2), reprobed for total ERK1/2 levels (t-ERK1/2), in differentiating primary rat oligodendrocytes in the presence and absence of MDL29,951 after 24 and 48 h incubation. (B) Quantitative assessment of pERK1/2 alteration triggered by 30 µM MDL29,951 in primary rat oligodendrocytes (n = 5-6).

4.2.6 The exchange factor directly activated by cAMP (EPAC) as a downstream effector protein of GPR17 activation

For a long time PKA was described as the exclusive effector protein of cAMP, but in 1998 Rooij identified a second alternative cAMP target, the Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP (EPAC) (Rooij et al. 1998). The established existence of a distinct EPAC-dependent signaling pathway led to suggest involvement of this downstream effector in several cellular processes. In the last years, it has become obvious that a wide range of physiological and pathophysiological processes within the same cell can be synergistically or antagonistically coordinated by both PKA and EPAC. Because the presence of EPAC in oligodendrocytes and its potential impact on their development are unknown, the effect of EPAC on MBP expression was next analyzed in oligodendroglial cell lines.

4.2.6.1 Role of EPAC in oligodendrocyte differentiation

Initially, to examine whether EPAC is expressed in oligodendrocytes, EPAC levels in primary rat oligodendrocytes were determined after 24 h, 48 h, and 72 h of differentiation by Western blot analyses. As shown in Figure 28, specific immunoreactive bands were detectable at each time point, demonstrating that EPAC is expressed in oligoden-

drocytes over time during their differentiation. These results support the assumption that EPAC might also play a role during the differentiation processes of oligodendrocytes.

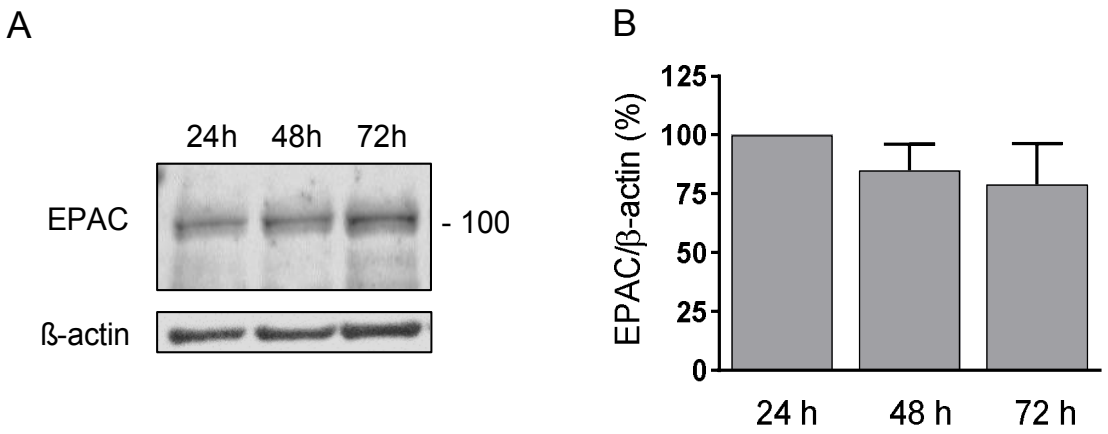


Figure 28. **EPAC expression during oligodendrocyte differentiation**

(A) Primary rat oligodendrocytes were differentiated with T3 for 24, 48, and 72 h; samples were then analyzed by Western blot for EPAC expression with specific antibodies against EPAC1/2 (EPAC). (B) Bar diagram of EPAC abundance during oligodendrocyte differentiation from four different experiments.

Next, to test whether EPAC activity is altered upon GPR17 stimulation, the influence of increasing concentrations of the cell permeable selective EPAC activator 8-(4-Chlorophenylthio)-2'-O-methyl-cAMP (8-CPT-2´-O-Me-cAMP) was tested on the MDL29,951-induced depression of MBP expression in primary rat oligodendrocytes. Treatment with 8-CPT-2´-O-Me-cAMP overcame the GPR17-mediated decrease on MBP expression without any influence on this oligodendrocyte differentiation marker on its own (Figure 29).

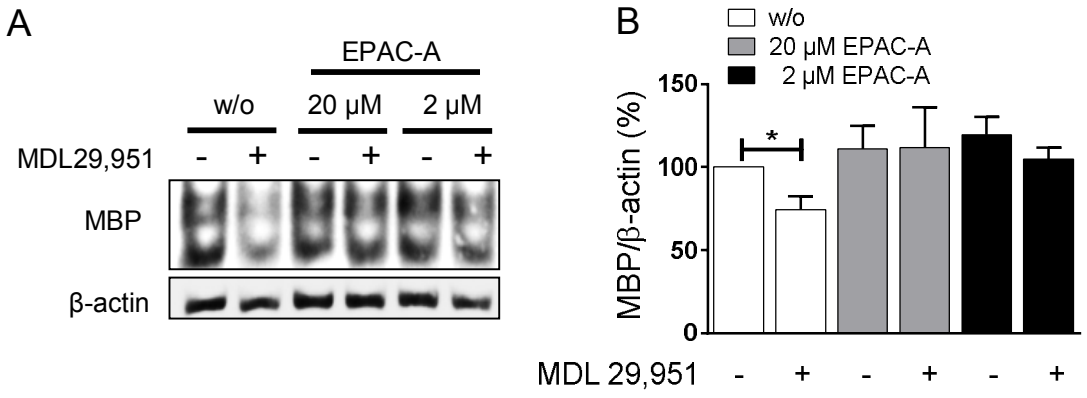


Figure 29. **8-CPT-2´-O-Me-cAMP, an activator of EPAC, overcame the GPR17-mediated depression of MBP expression in primary rat oligodendrocytes**

(A) Representative Western blot of MBP levels in differentiating primary rat oligodendrocytes incubated for 48 h with 30 µM MDL29,951 in the absence and presence of 20 µM and 2 µM of the EPAC activator 8-CPT-2´-O-Me-cAMP (*EPAC-A*). (B) Assessment of MBP levels from six independent experiments in primary rat oligodendrocytes displaying that treatment with 8-CPT-2´-O-Me-cAMP supports MBP expression in the presence of the MDL29,951. *, $p<0.05$.

Consistent with these data, the EPAC activator also rescued the decrease of MBP expression in Oli-neu cells (Figure 30). Therefore, the achieved data indicate that cAMP reduction triggered by MDL29,951 alters EPAC function as a downstream signaling consequence of GPR17 activation.

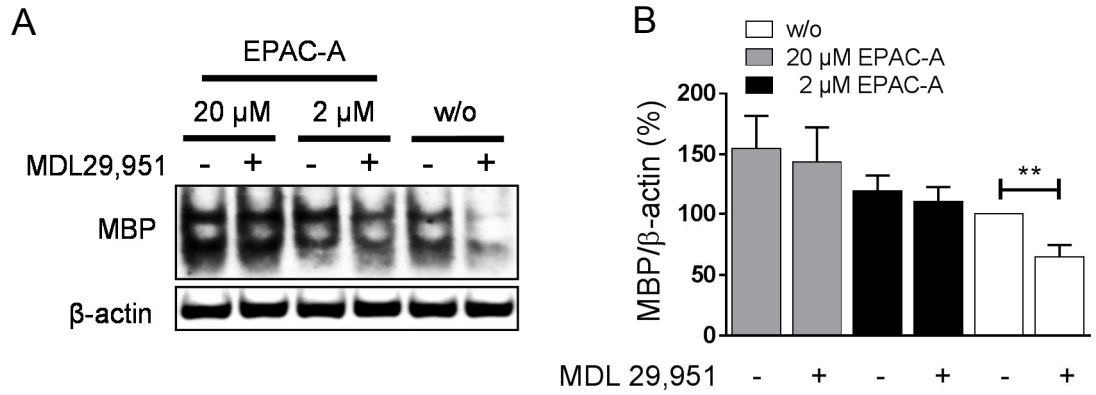


Figure 30. **The EPAC activator 8-CPT-2´-O-Me-cAMP counteracted the GPR17-induced reduction of MBP in Oli neu cells**

(A) Representative Western blot investigating MBP expression levels in differentiating Oli-neu cells treated for 48 h with 10 µM MDL29,951 in the absence (*w/o*) and presence of two different concentrations of 8-CPT-2´-O-Me-cAMP (*EPAC-A*) 20µM and 2 µM. (B) Calculation of MBP expression in Oli-neu cells from nine independent experiments. **, $p<0.01$.

4.2.6.2 Essential role of EPAC signaling in oligodendrocyte differentiation

The obtained data suggest that EPAC activation could play an important role during oligodendrocyte differentiation. Therefore, to confirm this hypothesis, immature primary rat oligodendrocytes were cultured for 24 h, 48 h, and 72 h in absence and in presence of 10 µM ESI-05 (4- methylphenyl- 2, 4, 6- trimethylphenylsulfone), a membrane-permeant specific EPAC inhibitor.

Incubation of primary rat oligodendrocytes for 48 h and 72 h with ESI-05 led to a significant decrease in MBP expression compared with the solvent-treated cells, thus indicating an arrest of oligodendrocytes in a less differentiated stage. Compared to parallel untreated cells, the EPAC inhibitor reduced the MBP levels by approximately 30% after 48 h treatment and by approximately 60% after 72 h (Figure 31).

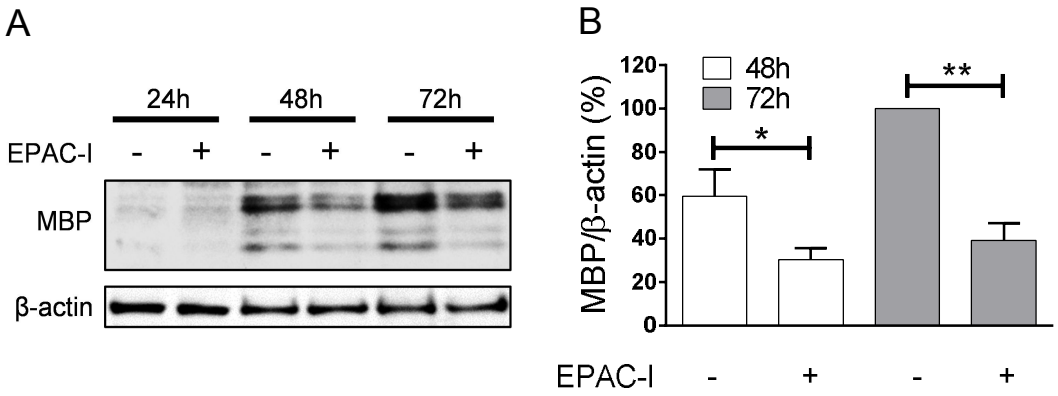


Figure 31. **Influence of the EPAC inhibitor ESI-05 on differentiation of primary rat oligodendrocytes**

(A) Illustrative Western blot displaying the influence of 10 µM ESI-05 (*EPAC-I*) on the expression levels of MBP, reprobed for beta-actin, in differentiating primary rat oligodendrocytes after 24 h, 48 h, and 72 h of culturing in medium containing T3 to facilitate maturation compared with control cells. (B) Quantification of the immunoreactive bands of MBP expressed in primary rat oligodendrocytes after 48 h and 72 h incubation with ESI-05. Average of five independent experiments. *, $p<0.05$; **, $p<0.01$.

To exclude that the negative effect on MBP expression is not due to a toxic effect of the compound, the influence of the EPAC inhibitor on Oli-neu cell viability was tested over 48 h. No influence of the EPAC inhibitor on cell viability was measured compared to the DMSO 0.1%-treated cells (negative control), thus confirming a true pathway inhibition and not just a toxic effect. The toxic effect of etoposide served as positive control, demonstrating a 30% decrease of oligodendrocyte viability (Figure 32).

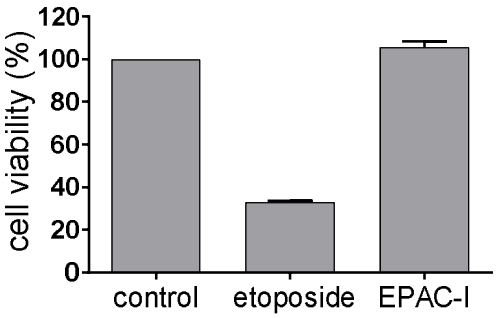


Figure 32. **ESI-05 does not affect cell viability of Oli-neu cells**

Analysis of viability of Oli-neu cells after treatment for 48 h with 10 µM of the EPAC inhibitor ESI-05 (*EPAC-I*) compared with the influence the topoisomerase II inhibitor etoposide (50 µM) as positive control.

Moreover, to verify the downstream role of PKA and EPAC in the signaling cascade upon the cAMP decrease triggered by GPR17 stimulation, MBP expression analyses were carried out in primary rat oligodendrocytes incubated for 48 h with medium containing T3 and MDL29,951. To counteract the MBP depression triggered by the GPR17

agonist, the stable cAMP analog 8-CPT-cAMP was added to the cells. In addition, cells were co-treated in the absence and in the presence of either the EPAC inhibitor ESI-05 or the selective PKA inhibitor, 8-(4-Chlorophenylthio) adenosine-3', 5'-cyclic monophosphorothioate, Rp- isomer (Rp-8-CPT-cAMPS). As shown in Figure 33, Western blot analyses confirmed again that 8-CPT-cAMP counteracted the negative influence of the GPR17 agonist on MBP expression (see also Figure 19).

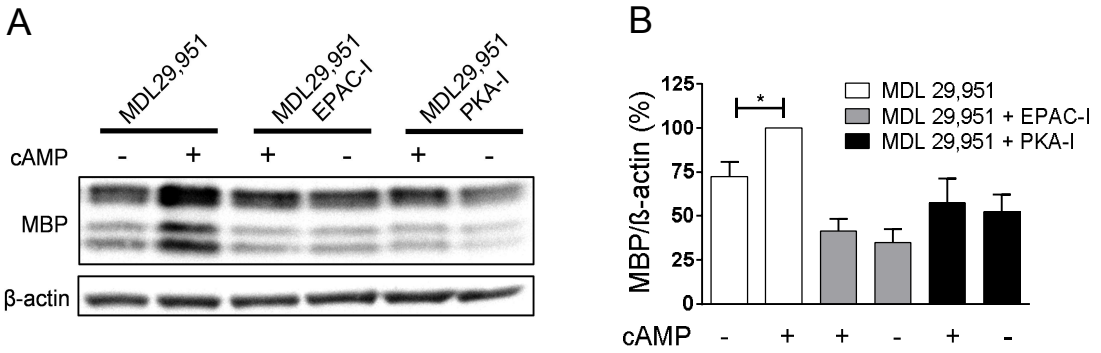


Figure 33. **EPAC and PKA involvement in the downstream signaling of GPR17**

(A) Western blot analysis of the influence of 10 µM of the EPAC inhibitor ESI-05 (*EPAC-I*) and 10 µM of the PKA inhibitor RP-8-CPT-AMPS (*PKA-I*) on MBP expression in primary rat oligodendrocytes cultured for 48 h in differentiation medium containing T3 and 30 µM MDL29,951, in the absence and in the presence of 1 µM 8-CPT-cAMP (*cAMP*). (B) Quantitative calculation of MBP immunoreactive bands, corrected by beta-actin, from five independent experiments. *, $p<0.05$.

Furthermore, both inhibitors, ESI-05 and Rp-8-CPT-cAMPS, blocked the counteracting effect of the cAMP-analog on GPR17-triggered MBP reduction to a similar extent. Therefore, these results confirm that PKA and EPAC are downstream effectors of cAMP and reveal that both proteins act as key modulators of MBP expression in the GPR17-dependent signaling pathway.

In summary, the obtained data lead to the assumption that the effector protein EPAC plays a not yet recognized and essential role in the challenging differentiation processes of oligodendrocytes. Furthermore, they also demonstrate that the low levels of cAMP triggered by GPR17 stimulation diminish both the PKA/CREB and EPAC signaling pathways in order to arrest oligodendrocyte differentiation and maturation.

4.3 Analysis of proposed GPR17 endogenous ligands

GPR17, which is phylogenetically related to purinergic and cysteinly-leukotriene receptors, has been described as being activated by compounds of both endogenous ligand classes (Ciana et al. 2006; Hennen et al. 2013). However, a number of independent studies did not or just partially confirm the original deorphanization research article

(Bläsius et al. 1998; Heise et al. 2000; Maekawa et al. 2009; Benned-Jensen, Rosenkilde 2010; Wunder et al. 2010; Qi et al. 2013; Hennen et al. 2013; Köse et al. 2014). Thus, pharmacology of the proposed endogenous ligands of GPR17 is still under debate.

In order to reveal whether GPR17 represents the elusive orphan receptor that is activated by both ligand classes, GPR17-mediated cell responses were monitored over time by using classical second messenger assays recording intracellular events such as $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$ activation, as well as β-arrestin recruitment.

4.3.1 Direct G protein rearrangement mediated by GPR17

To investigate the direct ligand-induced rearrangement of the Gαβγ complex mediated by GPR17, agonist-promoted decreases in $BRET^2$ signals were studied in HEK293 cells co-expressing the energy acceptor $G\gamma_2$-GFP (green fluorescent protein) and the partner energy donor $G\alpha_i$-Rluc (renilla reniformis luciferase enzyme) together with unlabeled hGPR17 and $G\beta_1$. $BRET^2$ signal-decrease is a consequence of the G protein heterotrimer reshaping triggered by GPR17 activation. This reshaping results in an increase of the distance between the N-terminus of $G\gamma_2$ and the $G\alpha_i$ subunit, creating the route for GDP exit and GTP entry (Gales et al. 2006). In this context it is important to note that GPR17 strongly couples to $G\alpha_{i/o}$ proteins in recombinant cell systems (Hennen et al. 2013). The advantage of this readout is that the agonist response occurs upstream and is not modulated by other signaling events that may affect measurements further downstream of the receptor. Consequently, this assay monitors early GPR17 activation of the putative endogenous ligands, which is then followed by enzyme activation involved in the second messenger production. Incubation of the HEK293 cells with 1 µM MDL29,951 exhibited a robust signal after 1 min. In contrast, the proposed endogenous ligands UDP, UDP-glc, UDP-gal, LTC4, and LTD4 did not lead to any heterotrimer conformational change, as shown in Figure 34.

Taken together, the obtained results confirmed the robust MDL29,951-mediated GPR17 activation, but could not verify pairing of the proposed endogenous ligands with GPR17.

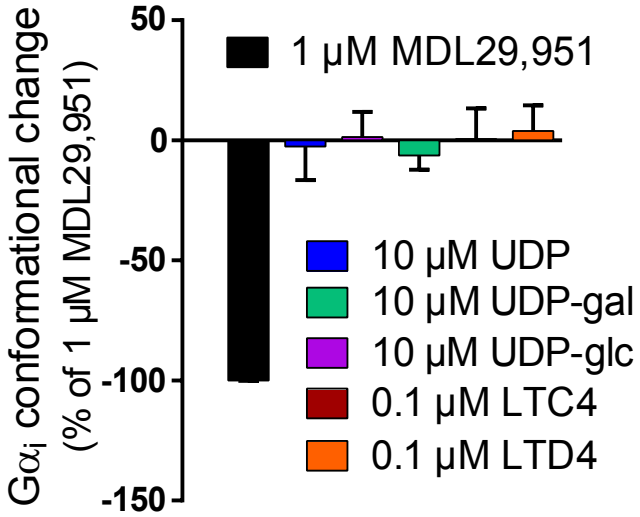


Figure 34. **Molecular rearrangement within the $G\alpha_i\beta\gamma$ heterotrimer**

GPR17-mediated conformational change of the $G\alpha_i$-subunit relative to the γ-subunit induced by tested compounds as an indicator of G protein activation. HEK293 cells were transiently transfected to co-express hGPR17, $G\alpha_i$1-91Rluc, and GFP10-$G\gamma_2$ along with the complementary subunit $G\beta_1$. The rearrangement was recorded after 1 min of compound incubation with indicated concentrations of the proposed endogenous ligands (10 µM UDP, 10 µM UDP-glc, 10 µM UDP-gal, 0,1 µM LTD4 and 0,1 µM LTC4). The data were compared to the recently characterized small molecule agonist at GPR17, MDL29,951. Maximal $BRET^2$ signal-decrease after incubation with MDL29,951 (1 µM) was set to -100%. Data are shown as means ± S.E.M. from three to eight independent experiments.

4.3.2 β-arrestin recruitment mediated by GPR17

β-arrestin was initially reported as a mediator of GPCR desensitization and downregulation. It has also been described as a scaffold able to orchestrate G protein signaling to various GPCR effector proteins, including MAPKs and phosphoinositide 3-kinase (PI3K) (Povsic et al. 2003; Rajagopal et al. 2010). Notably, β-arrestin recruitment by GPCRs is described as occurring both dependently and independently of G protein signaling (Rajagopal et al. 2005; Kenakin, Christopoulos 2013). Therefore, it might be possible that the proposed endogenous ligands for GPR17 could signal by a G protein-independent pathway.

It has been documented that MDL29,951-activated GPR17 mediates β-arrestin recruitment in a recombinant transfected cell model (Hennen et al. 2013). Accordingly, to detect G protein-dependent and -independent signaling of proposed endogenous ligand-activated GPR17, the β-arrestin recruitment after GPR17 activation was recorded. The receptor- β-arrestin interaction was analyzed by a $BRET^2$ assay in HEK293 cells co-expressing the energy donor hGPR17-Rluc and the energy acceptor β-arrestin2-GFP2. GPR17 fused to Rluc maintains functionality and is expressed in the cell membrane as

previously described by Hennen and coauthors in 2013 (Hennen et al. 2013). In agreement with previous findings, MDL29,951 recruited β-arrestin in a time-dependent manner, with a maximum peak after 1-5 min and returning to approximately 25% of the maximal value after 60 min. In contrast, neither uracil-nucleotides nor cysteinly-leukotrienes triggered significant GPR17-dependent β-arrestin recruitment over 60 min in the indicated high concentrations (Figure 35).

Taken together, the obtained data do not confirm that either uracil-nucleotides or cysteinly-leukotrienes activate GPR17 in this readout. This is in contrast to previous results by Daniele et al., which showed an association of GPR17 with β-arrestin after cell incubation with UDP-glc and LTD4 (Daniele et al. 2014).

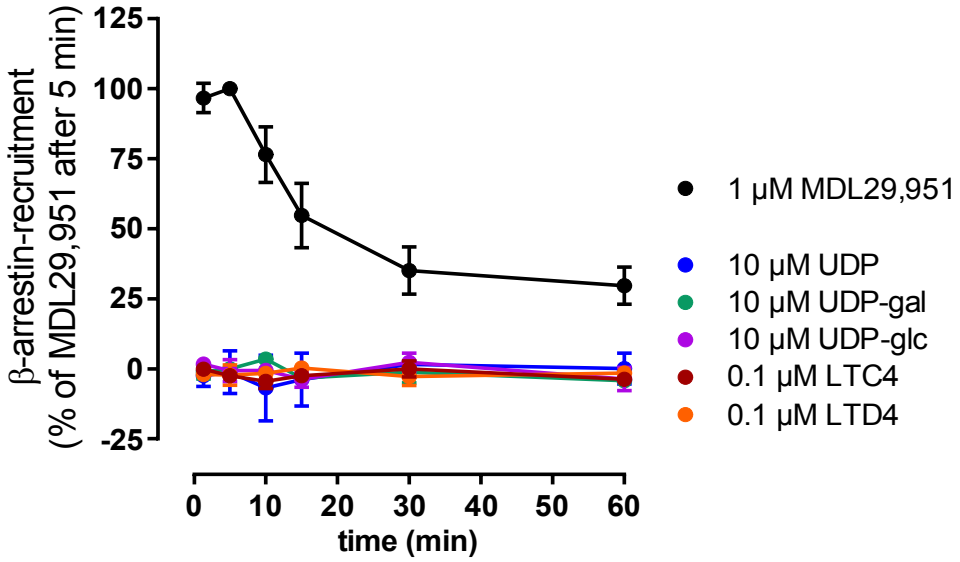


Figure 35. **β-arrestin recruitment in HEK293 cells stably expressing hGPR17**

β-arrestin recruitment was measured by a $BRET^2$ signal, which occurred between hGPR17-Rluc and GFP2–β-arrestin2 in stably transfected HEK293 cells at indicated time points (1.5, 5, 10, 15, 30, and 60 min). Cells were stimulated with 1 µM MDL29,951, 10 µM UDP, 10 µM UDP-gal, 10 µM UDP-glc, 0.1 µM LTC4 or 0.1 µM LTD4. Data are shown as means ± S.E.M. from three to four independent experiments.

4.3.3 Proposed endogenous ligands did not induce GPR17-dependent ERK1/2-phosphorylation

ERK1/2 belong to the class of mitogen-activated kinases, which are involved in a wide range of cellular processes, such as cell proliferation and differentiation. The MAP-kinase cascade can be activated by $G\alpha_{i/o}$, $G\alpha_s$, and $G\alpha_q$ as well as β-arrestin by direct or indirect mechanisms. This depends on the coupling ability of the upstream GPCR and

the cellular background (Zheng et al. 2008). Thus, a large variety of receptor upstream effects converge activating ERK1/2 as a detectable phosphorylation process. Hence, its measurement represents an ideal way to detect amplified low GPCR response.

MDL29,951-activated GPR17 mediated robust ERK1/2 phosphorylation in primary and immortalized cells, as previously shown in the present work. Accordingly, taking advantage of the ERK1/2 phosphorylation assay, further analysis of the controversial endogenous ligands of GPR17 was carried out. Based on previous data, which reported a time-dependent ERK1/2 phosphorylation by UDP-glc- and LTD4-activated GPR17 in 1321N1 cells (Daniele et al. 2014), these cells stably expressing hGPR17 were chosen as cellular background for this assay. 1321N1 cells expressing hGPR17 were incubated with the cysteinyl-leukotrienes and uracil-nucleotides, and the ERK1/2 phosphorylation was compared to cells challenged with MDL29,951 at different time points (Figure 36). In agreement with preceding results (Hennen et al. 2013), MDL29,951 stimulated a robust and rapid ERK1/2 phosphorylation over time with a maximum peak after 1-5 min in cells expressing hGPR17. After 30 min the signal declined to the basal level. In contrast, GPR17 stimulation with the putative endogenous agonists did not lead to an increase in ERK1/2 phosphorylation over 60 min.

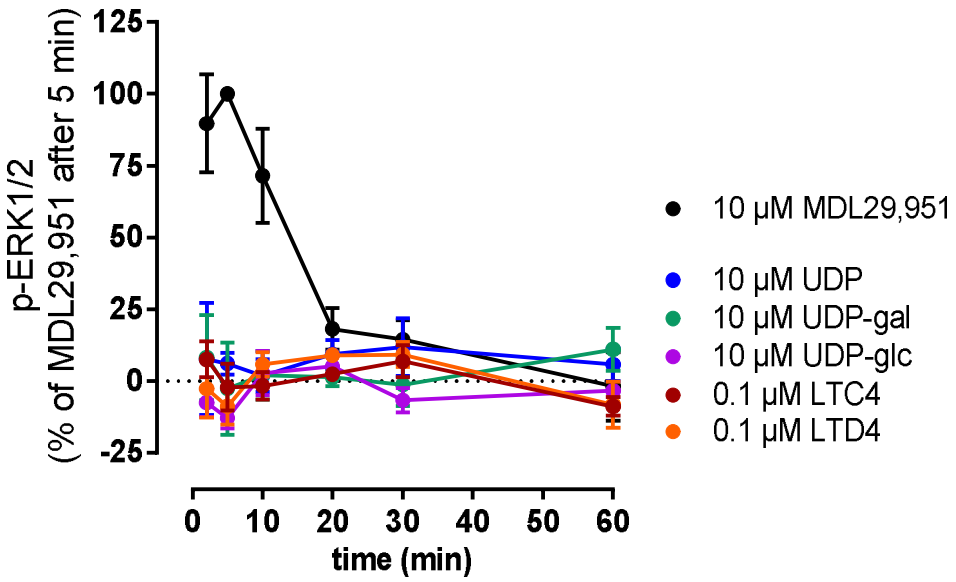


Figure 36. **1321N1 cells stably expressing hGPR17 were unresponsive to the proposed endogenous ligands in the ERK1/2 phosphorylation assay**

ERK1/2 phosphorylation kinetics (time points: 1, 5, 10, 20, 30 and 60 min) were measured in hGPR17-1321N1 cells after treatment with 10 µM MDL29,951 or the proposed endogenous ligands (10 µM UDP, 10 µM UDP-gal, 10 µM UDP-glc, 0.1 µM LTC4 and 0.1 µM LTD4). Data are shown as means ± S.E.M. from four independent experiments.

As a control, native 1321N1 cells were incubated with 10 µM MDL29,951 over 60 min to analyze the receptor specificity of MDL29,951 signal. As shown in Figure 37, MDL29,951 did not induce ERK1/2 phosphorylation. The correctness of assay conditions and viability of the analyzed cells were controlled by stimulation with fetal calf serum (FCS) and carbachol. Carbachol activates endogenously-expressed muscarinic receptors while FCS components activate endogenous growth factor receptors. As shown in Figure 37, in contrast to MDL29,951 both FCS and carbachol induced a robust ERK1/2 signal after 5 min in native 1321N1 cells.

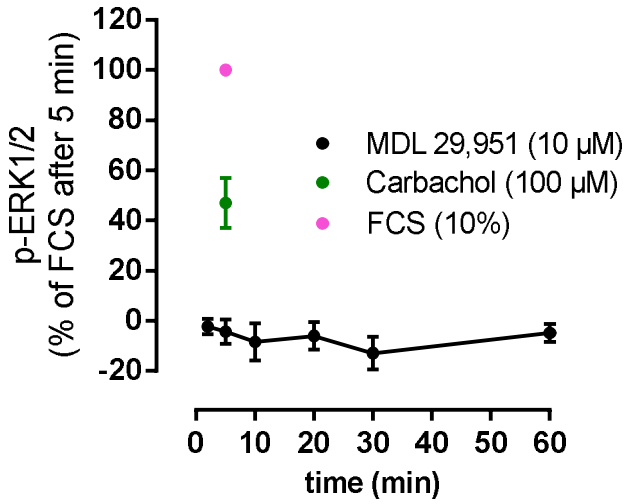


Figure 37. **Increase of intracellular ERK1/2 phosphorylation in native 1321N1 cells**

1321N1 cells were incubated with a medium containing 100 µM of the muscarinic agonist carbachol, 10% FCS or 10 µM MDL29,951. Native 1321N1 cells responded to carbachol and serum but did not respond to MDL29,951. ERK1/2 phosphorylation was recorded at indicated time points. Data are shown as means ± S.E.M. from two to three independent experiments.

In order to confirm the activity of LTC4 and LTD4, HEK293 cells stably expressing the CystLT1 receptor, which represents the established target for LTD4 and LTC4, were incubated with both ligands and ERK1/2 phosphorylation was determined after indicated time points. As expected, LTD4 and LTC4 triggered an increase in phosphorylated ERK1/2 mediated by the CysLT1 receptor with a maximum peak after 2 min and 5 min, respectively. Then, ERK1/2 phosphorylation declined to basal levels after 60 min of incubation (Figure 38, A). In order to analyze the receptor specificity of the ERK1/2 signal, native HEK293 cells were stimulated for 2 min with LTD4 and for 5 min with LTC4. Neither LTD4 nor LTC4 triggered any ERK1/2-phosphorylation in native HEK293 cells, demonstrating the CysLT1 specificity for these compounds (Figure 38, B).

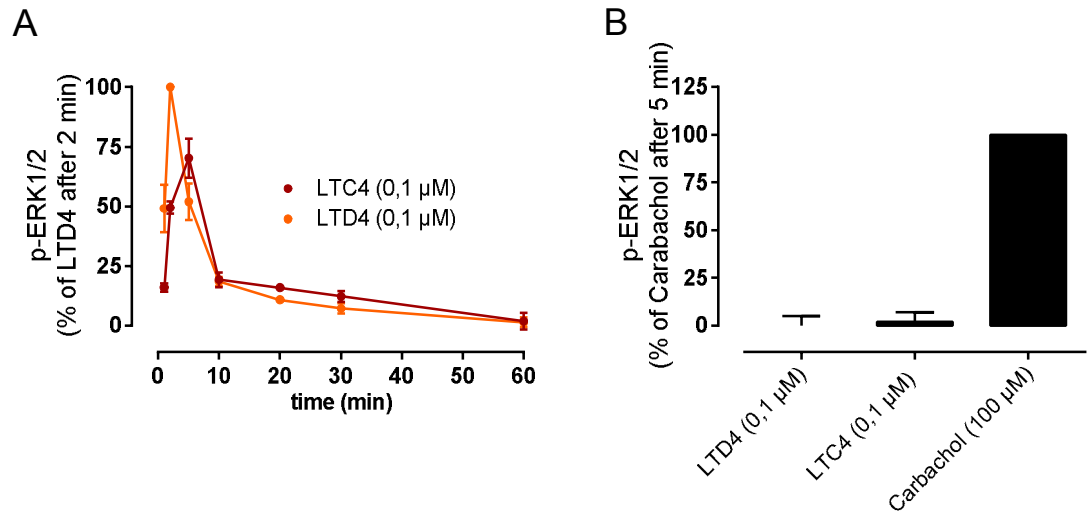


Figure 38. **ERK1/2 phosphorylation in HEK293 cells stably expressing CysLT1 receptor over the time (A) and in native HEK293 (B).**

(A) ERK1/2 phosphorylation kinetics (time points: 1, 5, 10, 20, 30, and 60 min) were recorded in CysLT1-HEK293 cells after treatment with 0,1 µM LTD4 and LTC4. (B) ERK1/2 phosphorylation was measured in native HEK293 cells after incubation with LTD4 for 2 min or LTC4 for 5 min and was compared to the carbachol-mediated ERK1/2 activation. Data are shown as means ± S.E.M. from four independent experiments.

Likewise, to confirm the uracil-nucleotides functionality and to test the specificity of the receptor response, the signal of HEK293 cells stably expressing P2Y14, which represents an entrenched target for uracil-nucleotides, was compared with the signal of native HEK293 in the ERK1/2 phosphorylation assay. Both cell lines were incubated for 5 min with either 10 µM UDP, UDP-gal or UDP-glc.

In contrast to the lack of signal detected in native HEK293 cells, the P2Y14 receptor stably expressed in the same cell system triggered an intracellular activation of the MAP-kinase cascade, measured as a significant increase of phosphorylated ERK1/2, after stimulation with UDP, UDP-gal, and UDP-glc (Figure 39). This demonstrated that the tested uracil-nucleotides are functional under the present assay conditions.

Taken together, the obtained data indicate that neither uracil-nucleotides nor cysteinyl-leukotrienes have the capacity to activate ERK1/2 in a cell system stably expressing GPR17, which is in contrast to the effect observed with MDL29,951.

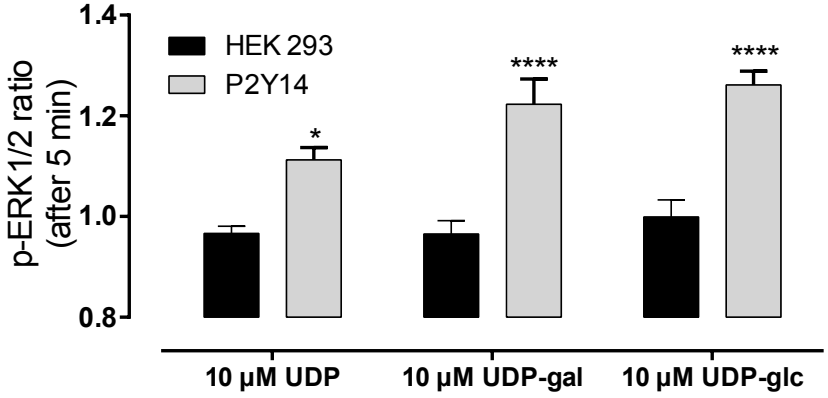


Figure 39. **Level of phosphorylated ERK1/2 in native HEK293 cells compared to P2Y14-HEK293 cells**

Incubation with UDP, UDP-gal, and UDP-glc (10 µM) resulted in a phosphorylated ERK1/2 increase in P2Y14-HEK293 cells, in contrast native HEK293 cells did not respond to the uracil-nucleotides. Data are shown as means ± S.E.M. from four independent experiments. *, $p<0.05$; ****, $p<0.0001$.

In conclusion, the results obtained in: (i) G protein rearrangement, (ii) β-arrestin recruitment, and (iii) ERK1/2 phosphorylation assays are in line with previous studies which could not confirm that GPR17 responds to UDP, UDP-glc, UDP-gal, LTC4, and LTD4. Therefore, these findings support all literature refuting GPR17 putative endogenous ligands and, consequently, GPR17 as a dualistic receptor activated by uracil-nucleotides and cysteinyl-leukotrienes (Bläsius et al. 1998; Heise et al. 2000; Maekawa et al. 2009; Wunder et al. 2010; Benned-Jensen, Rosenkilde 2010; Qi et al. 2013; Hennen et al. 2013; Köse et al. 2014).

4.4 Analysis of potential antagonists of GPR17

Recent studies recognized GPR17 as a negative modulator of oligodendrocyte maturation and differentiation; consequently, GPR17 was proposed as an innovative therapeutic target in demyelinating diseases like MS. In particular, the therapy of demyelinating diseases would benefit from a potent GPR17 antagonist.

4.4.1 P2Y receptor inhibitors as potential antagonists for GPR17

The original GPR17 deorphanization report by Ciana and coauthors identified the P2Y12 receptor antagonist cangrelor, the P2Y1 receptor antagonist MRS2179 and the CysLT1 receptor antagonists pranlukast and montelukast as potent inhibitors of UDP-glc-activated human and rat GPR17 (Ciana et al. 2006). The experimental procedure adopted by the authors was the [^{35}S]GTPγS binding assay. Cangrelor has been characterized as a potent antagonist with an IC50 value in the nanomolar range. Moreover, a

second P2Y12 receptor antagonist, ticagrelor, was also described as a GPR17 inhibitor (Gelosa et al. 2014).

Several follow-up studies by the same group confirmed the antagonist capacity of the described P2Y receptor and CysLT1 receptor antagonists. On the other hand, Hennen and coauthors confirmed that pranlukast effectively attenuates the activation of mouse and rat GPR17 receptors triggered by MDL29,951, but failed to recapitulate the antagonist properties of montelukast at MDL29,951-activated GPR17 (Hennen et al. 2013). Therefore, the true potential of these compounds to act as antagonists is currently unclear.

In the initial studies, the antagonist properties were investigated based on uracil-nucleotide- and cysteinyl-leukotriene-activated GPR17 signaling. However, both cysteinyl-leukotrienes and the uracil-nucleotides have been shown to be inactive GPR17 agonists in the present work. Therefore, further investigations of the real nature of the proposed antagonists are necessary. Toward this goal, cangrelor and ticagrelor have been here analyzed as potential antagonists of MDL29,951-mediated GPR17 signaling. Three GPR17 receptor orthologs were tested: the human, the rat, and the mouse. Pranlukast, which was shown as inhibitor of MDL29,951-mediated GPR17 signaling at all three receptor orthologs (Hennen et al. 2013), has been selected as reference antagonist in the following experiments.

In order to analyze the potency of cangrelor and ticagrelor to antagonize MDL29,951-activated GPR17 response, a classical second messenger assay was chosen, the IP1 accumulation assay. It has been described that MDL29,591-activated mGPR17, rGPR17, and hGPR17 robustly couple to $G\alpha_q$ proteins to increase intracellular IP1 levels (Hennen et al. 2013).

4.4.1.1 Analysis of the antagonist properties of cangrelor and ticagrelor at the three GPR17 orthologs

Initially, hGPR17-HEK293 cells were pre-incubated (30 min, 37 °C) with pranlukast and the two purine receptor antagonists, followed by a stimulation with MDL29,951 (1 µM = EC80) for 30 min. As depicted in Figure 40, pranlukast caused a partial inhibition of the receptor response but could not completely abolish the GPR17 signaling. In contrast, cangrelor and ticagrelor exhibited no significant reduction of the GPR17-mediated intracellular IP1 accumulation. Thus, the obtained results confirmed the antagonist potency of pranlukast but did not verify the published antagonist capability of cangrelor and ticagrelor at the hGPR17.

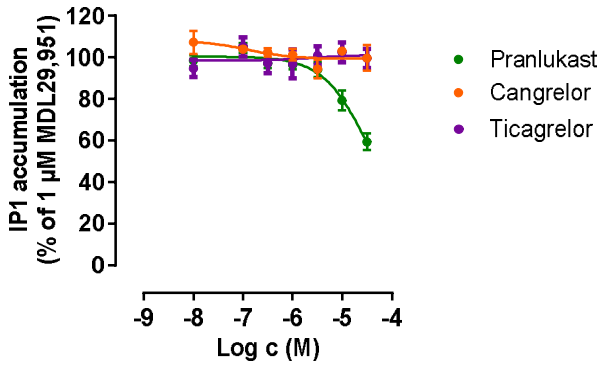


Figure 40. **Alteration of IP1 levels in HEK293 cells stably expressing human GPR17**

Concentration-dependent inhibition of intracellular IP1 levels by ticagrelor, cangrelor, and pranlukast were analyzed in HEK293 cells stably expressing hGPR17 upon stimulation by MDL29,951. Data were expressed as percentages of IP1 levels induced by 1 µM MDL29,951 in the absence of the potential antagonists. Data are shown as means ± S.E.M. from three to four independent experiments.

The current literature describes noticeable differences between ligand potencies among species-specific orthologs of GPCRs, which complicates use of compounds from *in vivo* studies at the human to *in vivo* studies in animal models (Milligan 2011; Strasser et al. 2013). Accordingly, in the next experiment the P2Y12 receptor antagonists cangrelor and ticagrelor were tested in HEK293 cells stably expressing the mouse and the rat GPR17 receptor orthologs. The antagonists were tested against the approximated EC80 of MDL29,951 at the rat and the mouse receptors (Hennen et al. 2013). In line with the data obtained from hGPR17-HEK293 cells, pranlukast, but not cangrelor or ticagrelor, effectively inhibited the function of MDL29,951-activated mouse or rat GPR17 (Figure 41). The obtained results confirmed pranlukast as an effective antagonist for all three receptor orthologs, but neither cangrelor nor ticagrelor could be verified as MDL29,951-activated GPR17 inhibitors.

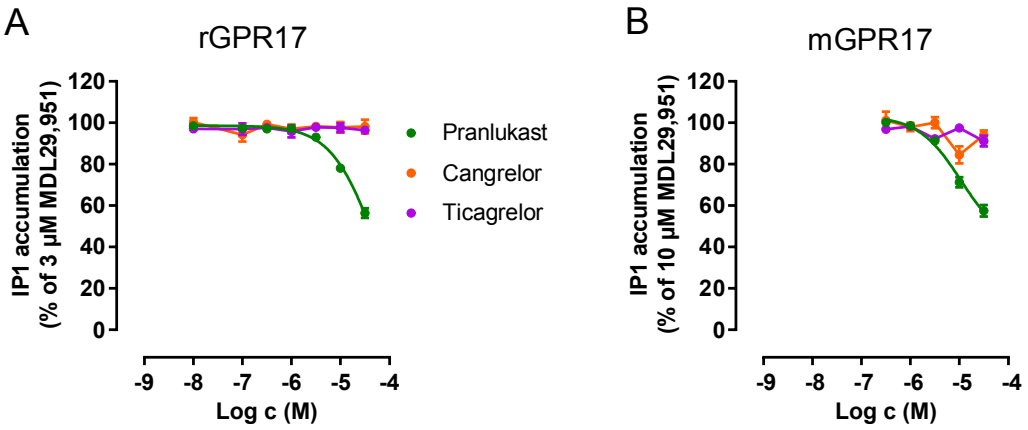


Figure 41. **Intracellular IP1 levels in HEK293 cells stably expressing rat GPR17 (A) or mouse GPR17 (B)**

MDL29,951-mediated IP accumulation was recorded in the presence of increasing concentrations of cangrelor, ticagrelor or pranlukast in HEK293 cells stably expressing (A) rGPR17 or (B) mGPR17. Data were expressed as percentages of IP1 levels induced by 3 µM MDL29,951 at rat receptor (A) or 10 µM MDL29,951 at the mouse receptor in the absence of the potential antagonists. Data are shown as means ± S.E.M. from three to five independent experiments.

4.4.2 CysLT2 receptor antagonists as candidates for GPR17 inhibition

A potent inhibitor of GPR17 may represent an excellent drug candidate to reduce the GPR17-mediated arrest of oligodendrocyte maturation and, in consequence, to foster remyelination in demyelinating diseases. However, in the present study, the proposed antagonists either showed a low potency to inhibit MDL29,951-stimulated GPR17 (pranlukast) or were completely inactive (ticagrelor and cangrelor). Thus, identification of a potent GPR17 antagonist remains an open challenge.

Recently, two selective and potent CystLT2 receptor antagonists have been described in the literature, HAMI3379 and Cay106333. HAMI3379 was defined in 2010 as the first potent and selective antagonist at the CysLT2 receptor by Wunder and co-authors (Wunder et al. 2010). Furthermore, CAY106333, also known as BayCysLT2, was characterized as selective CysLT2 receptor antagonist in 2011 by Ni and co-workers (Ni et al. 2011). Based on the phylogenetic relationship of GPR17 to receptors of the CysLT receptor cluster and the inhibition of GPR17 by pranlukast, known as a CysLT1 receptor antagonist, these two selective CysLT2 receptor antagonists were tested for their inhibitory properties at GPR17.

4.4.2.1 Potential antagonist effects of the CystLT2 receptor antagonist at the three GPR17 receptor orthologs

In order to assess the potency of HAMI3379 and CAY106333, intracellular IP1 levels of HEK293 cells stably transfected with the hGPR17 were recorded after incubation with these compounds. Compared to pranlukast, the two specific CysLT2 receptor antagonists exhibited a more potent inhibition of the human GPR17-mediated signal (Figure 42). As shown in Table 16, the rank order of the IC50 values of the three investigated substances was: HAMI3379 ≈ CAY10633 > pranlukast. Although the IC50 values of HAMI3379 and CAY10633 are comparable, HAMI3379 almost completely inhibited the effect of hGPR17 on IP1 accumulation. In contrast, after incubation with 30 µM CAY10633, hGPR17 still retained ≈40% of its activity. The obtained results clearly highlight HAMI3379 as the most promising hGPR17 antagonist followed by CAY10633.

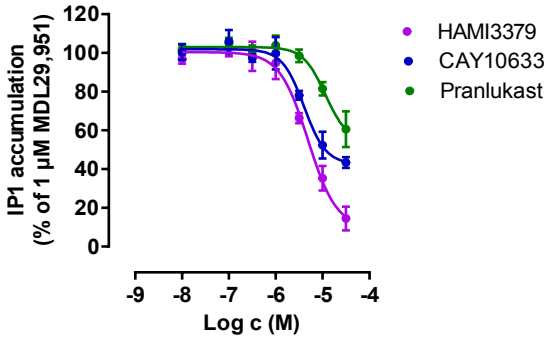


Figure 42. **Alteration of IP1 levels in HEK293 cells stably expressing hGPR17**

IP1 accumulation inhibition curve of HAMI3379 and CAY10633 in the presence of 1 µM MDL29,951 (EC80) was recorded in hGPR17-HEK293 cells after pre-incubation with the indicated concentrations of the proposed inhibitors for 30 min. Data were expressed as percentages of IP1 levels induced by 1 µM MDL29,951 in the absence of an antagonist. Data are shown as means ± S.E.M. from three to seven independent experiments.

Table 16. **LogIC50 values of the potential antagonists**

Substance	HAMI3379	CAY10633	Pranlukast
LogIC50	-5,3	-5,4	-4,9

Pharmacological study of potential medical compounds combines *in vitro* analysis with *ex vivo* and *in vivo* experiments. Therefore, in drug development it is essential to analyze both the human receptor and the rodent receptor orthologs to verify whether the compounds are suitable for animal models. Consequently, the antagonist potencies of HAMI3379 and CAY10633 were next analyzed at the mouse and the rat GPR17 in the

IP1 accumulation assay, and compared to the result achieved by incubating the cells with pranlukast. As shown in Figure 43, HAMI3379 and CAY10633 inhibited GPR17 signaling mediated by MDL29,951 on both receptor orthologs, with HAMI3379 showing the highest potency. However, at both receptor orthologs neither HAMI3379 nor CAY10633 completely abolish MDL29,951-mediated signaling in the investigated concentrations. As indicated in the Table 17, although both CysLT2 receptor antagonists inhibited the mouse and the rat receptor, they showed highest potency at the human receptor. The rank order of the substances is characterized by a minimal difference in the IC50 values: (i) mGPR17: HAMI3379 > pranlukast > CAY10633, (ii) rGPR17: HAMI3379 > pranlukast > CAY10633. The rank order at the mouse and the rat receptor is similar to that obtained at the hGPR17. Taken together, our data show that HAMI3379 exhibited the best antagonist properties at all three receptor orthologs.

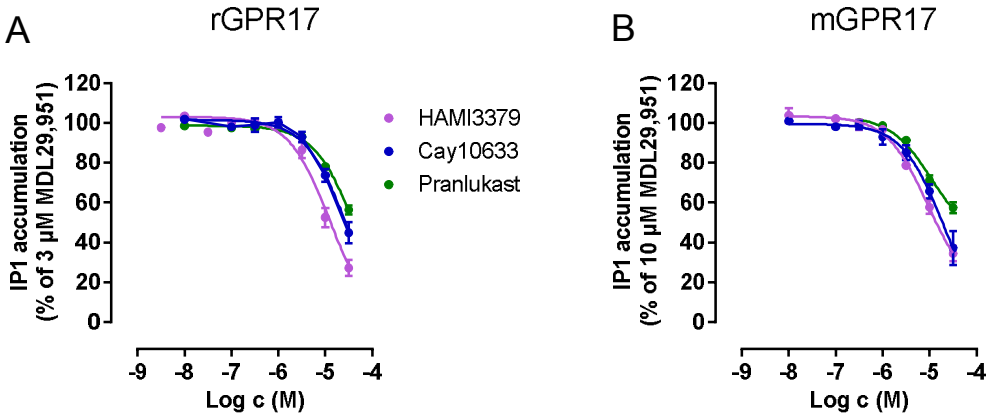


Figure 43. **Decrease of intracellular IP1 levels in HEK293 cells stably expressing rat GPR17 (A) and mouse GPR17 (B)**

The inhibitory effects of increasing concentrations of the CysLT2 receptor antagonist set (CAY10633 and HAMI3379) were analyzed at MDL29,951-stimulated rGPR17 (A) or mGPR17 (B) by IP1 accumulation assays. Data were expressed as percentages of IP1 levels induced by 3 µM MDL29,951 at rat receptor (A) and 10 µM MDL29,951 at the mouse receptor (B) in the absence of the potential antagonists. Data are shown as means ± S.E.M. from three to five independent experiments.

Table 17. **LogIC50 values of the potential antagonists at rGPR17 and mGPR17 expressed in HEK293 cells**

Substance	HAMI3379	CAY10633	Pranlukast
rGPR17 (LogIC50)	-4,9	-4,6	-4,4
mGPR17(LogIC50)	-5,1	-4,7	-5,0

In summary, the achieved results obtained by IP1 accumulation assays show for the first time that HAMI3379 and CAY10633 act as antagonists at all three GPR17 receptor orthologs. Further studies are needed to evaluate the potential of these compounds in primary cell systems and to elucidate whether their chemical structures can be used as lead for designing GPR17 antagonists with higher potency.

5 Discussion

Demyelination is a pathophysiological process that occurs in inflammatory demyelinating diseases, such as MS, and is characterized by the damage of the specific multilayered membrane located around neuronal axons (Franklin, Ffrench-Constant 2008). Current treatment strategies for demyelinating diseases focus on immunological attack modulation. However, long-term functional recovery requires successful myelin repair. In consequence, over the last few years it has become evident that remyelination represents an attractive therapeutic approach in neuroinflammatory demyelinating diseases (Franklin, Ffrench-Constant 2008; Kotter et al. 2011). Generation of myelin in the CNS requires transition from OPCs to myelinating/mature oligodendrocytes. A precise molecular mechanistic cascade regulates this maturation process and is influenced by a wide range of positive and negative stimuli. However, the molecular mechanisms that inhibit remyelination under pathological conditions are poorly understood. Thus, a better understanding of the signaling cascades and identification of novel targets regulating OPC differentiation could provide new avenues for therapeutic approaches.

5.1 GPR17: its signaling in oligodendrocytes

The orphan receptor GPR17 impairs differentiation of immature oligodendrocytes to myelinating oligodendrocytes *in vivo* and *in vitro*. In particular, genetic studies of GPR17 in mice revealed that development of oligodendrocytes is arrested at an immature stage in animals overexpressing GPR17. On the other hand, mice with a deficiency in GPR17 expression showed an early onset of myelination (Chen et al. 2009; Hennen et al. 2013). In addition, pharmacological receptor stimulation blocks oligodendrocyte differentiation in *in vitro* studies (Hennen et al. 2013).

Importantly, qRT-PCR analyses revealed a GPR17 upregulation in demyelinated lesions of MS patients that is not present in white matter samples from non-neurological probands. Furthermore, GPR17 abundance increases in an experimental autoimmune encephalomyelitis (EAE) model (Chen et al. 2009). Thus, GPR17 inhibition by an antagonist or antagonizing antibody has been proposed as a potential approach to treat demyelinating diseases.

The role of GPR17 in myelination is specific to the CNS; no evidence to date has shown its expression on Schwann cells in the PNS. This fact further supports the concept of multiple signaling systems regulating CNS and PNS myelination independent of each other (Brinkmann et al. 2008). GPR17 affects myelination by triggering nuclear translocation of the differentiation inhibitors 2 and 4 (ID2 and ID4) and by upregulating the

expression of ID2 (Chen et al. 2009). Beyond this, very little is known about the intracellular signaling events after GPR17 activation which impair the transition from immature to mature oligodendrocytes.

Consequently, the establishment of GPR17 as a therapeutic target would benefit from the identification and rigorous validation of GPR17 effector proteins. In addition, this may provide a deeper insight into the GPR17-mediated myelination block, and may allow to unravel additional innovative therapeutic candidates to treat demyelinating diseases, such as MS.

5.1.1 GPR17: a negative regulator of myelination and maturation of oligodendrocytes

In the current literature, an increasing number of *in vitro* and *in vivo* studies are investigating the impact of GPCRs expressed on the oligodendrocyte cell surface on oligodendrocyte myelination. For instance, the adhesion orphan receptor GPR56 blocks oligodendrocyte differentiation in an early stage of development (Ackerman et al. 2015). In contrast, other GPCRs, such as the cannabinoid receptors CB1 and CB2, act as positive modulators of the oligodendrocyte maturation process (Gomez et al. 2011; Tomas-Roig et al. 2016). In the present work, the pharmacological investigation of the activated GPR17 reveals an attenuation of intracellular MBP expression in primary rat oligodendrocytes and Oli-neu cells. The impairment of MBP expression is known to be an indicator of opposing action on oligodendrocyte maturation. Consistent with former findings by Hennen et al. and Chen et al. (Chen et al. 2009; Hennen et al. 2013), the obtained data characterized GPR17 as inhibitor of oligodendrocyte differentiation.

5.1.2 Specific G protein coupling of GPR17 in oligodendrocytes

A previous study (Hennen et al. 2013) investigated the coupling of MDL29,951-activated GPR17 in heterologous cell systems. In HEK293 cells, the entire repertoire of G protein pathways ($G\alpha_{i/o}$, $G\alpha_q$, $G\alpha_s$) is activated by hGPR17 (Hennen et al. 2013). In contrast, although $G\alpha_i$, $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$ are expressed in primary rat oligodendrocytes (Mir, Le Breton 2008), GPR17 couples only to $G\alpha_{i/o}$ and $G\alpha_q$ (Hennen et al. 2013). In line with these former findings, GPR17 exclusively couples to $G\alpha_{i/o}$ and $G\alpha_q$ proteins in Oli-neu cells, which endogenously express GPR17 and the four main Gα-subunits. This has been demonstrated by an MDL29,951-induced cAMP decrease and a partially PTX-insensitive calcium flux. Non-appearance of intracellular cAMP level increase confirms the absence of $G\alpha_s$ signaling after GPR17 activation. Taken together, the obtained data confirm a cellular background-dependent coupling of GPR17. Cellular background-dependent pharmacology is a widely spread and well-known phenomenon. Differences

in the stoichiometry of the number of signaling proteins may cause a functional discrimination of ligands across cell lines (Kenakin, Christopoulos 2013).

The coupling preference of GPR17 expressed in oligodendroglial cells is in line with the already described negative impact of $G\alpha_i$ and $G\alpha_q$ proteins on oligodendrocyte development in the CNS. For instance, CXCR2, a $G\alpha_i$-coupled chemokine receptor, is a negative regulator of oligodendrocyte maturation (Hall et al. 1999; Kremer et al. 2011). In addition, the muscarinic M1 and M3 receptors, which both preferentially couple to $G\alpha_q$, are also obstructers of oligodendrocyte differentiation. Accordingly, in a PLP-induced EAE animal model, the muscarinic receptor antagonist benztropine induces remyelination (Deshmukh et al. 2013). Moreover, inhibition of protein kinase C (PKC), which is a known sensor of $G\alpha_q$ activation, promotes OPC differentiation (Baer et al. 2009).

5.1.3 GPR17 engages $G\alpha_{i/o}$ protein to regulate MBP expression

In the present work, the analysis of the MDL29,951-induced DMR signals verifies a major contribution of the $G\alpha_{i/o}$-cascade in both oligodendroglial cell lines to the GPR17 signaling. This contribution was evidenced by a large reduction of the DMR response after preincubation with the $G\alpha_{i/o}$ inhibitor PTX. Accordingly, down-regulation of MBP protein levels triggered by pharmacologically-activated GPR17 was rescued in both oligodendroglial cell lines after treatment with PTX. Taken together, the experimental data confirmed the predominant role of $G\alpha_{i/o}$ proteins in the differentiation arrest mediated by the GPR17 signaling cascade.

5.1.4 GPR17-mediated influence of intracellular cAMP levels on MBP expression

The crucial role of the $G\alpha_{i/o}$ pathway engaged by GPR17 supports the hypothesis that an alteration of intracellular cAMP levels may be a major cause of the oligodendrocyte differentiation blockade triggered by GPR17. Elegant studies spanning decades of work with many different biochemical methods robustly demonstrated the positive influence of increased intracellular cAMP concentration on oligodendrocyte differentiation. In particular, Sato-Bigbee and co-workers determined a positive influence of the cAMP-analog dibutyryl-cAMP on myelination by quantifying the MBP expression. Consistent with this study, a favorable impact of AC activators on differentiation induction has also been confirmed in Oli-neu cells (Joubert et al. 2010). In addition, PLP and MAG, which are also typical markers of oligodendrocyte differentiation and myelination, are upregulated by increasing intracellular concentrations of cAMP (Ye et al. 1992). In line with these findings, myelin protein extracts have been shown to attenuate oligodendrocyte differentiation by a cAMP-decreasing mechanism because the effect was rescued by

addition of dibutyryl-cAMP or the phosphodiesterase-4 inhibitor rolipram (Syed et al. 2013).

In agreement with these former investigations, in the present study a stable cAMP analog (8-CPT-cAMP) rescues the negative impact of activated GPR17 on the intracellular MBP levels. The obtained data verified that GPR17 involves the second messenger cAMP to control myelination of oligodendrocytes. Interestingly, a very recent study determines a similar cAMP-dependent mechanism to control oligodendrocyte myelination/maturation elicited by another GPCR named GPR37 (Yang et al. 2016)

5.1.5 GPR17-mediated influence of PKA on myelination in oligodendrocytes

cAMP triggers physiological and pathophysiological effects by activating PKA and the more recently discovered enzymes EPAC1 and 2. Previous studies revealed a positive influence of PKA on oligodendrocyte development (Sato-Bigbee et al. 1999; Shiga et al. 2005). Interestingly, PKA is also an important enzyme affecting oligodendrocyte precursor cell migration into demyelinated lesions (Takahashi et al. 2013). The results of the present study show that incubation of primary rat oligodendrocytes and Oli-neu cells with Sp-6-Bnz-cAMPs, a PKA activator, rescues the MDL29,951-mediated MBP decrease. In line with previous studies, the obtained data confirm the positive influence of PKA activity on myelination and link GPR17 to the inhibition of the cAMP-PKA cascade in oligodendrocytes.

Therapeutic approaches targeting the cAMP system are still capturing the attention of the pharmaceutical industry. The main focus is the manipulation of the cAMP-degradation system with selective or non-selective PDE inhibitors (Zhang et al. 2005). Roflumilast, for instance, was the first selective PDE4 inhibitor marketed in Germany for the treatment of the chronic obstructive pulmonary disorder (Wedzicha et al. 2016). Furthermore, phosphodiesterases were already recognized as drug targets for the treatment of neurodegenerative disorders of the CNS (Menniti et al. 2006). In addition, the cAMP effector, PKA, has been documented as a promising target in the treatment of cancer, heart disease, and dysfunctional immune cells (Skalhegg et al. 2005; Dhalla, Müller 2010; Sapio et al. 2014). However, the drug development of medicaments influencing the cAMP pathway remains challenging because cAMP and PKA are ubiquitously expressed in the human body and are involved in a broad spectrum of intracellular processes. Thus, direct alteration of cAMP or rather PKA signaling in drug therapy may result in potential adverse effects (Zhang et al. 2005)

Nevertheless, pharmacological therapy may benefit by targeting the cAMP pathway in a tissue-specific manner by cAMP-modulating GPCRs. In demyelinating diseases, the inhibition of GPR17, which is highly expressed in the CNS in MS, may represent an

excellent approach to increase intracellular cAMP levels and therefore, the PKA activity in a tissue-specific manner to foster remyelination.

5.1.6 CREB a major transcription factor in myelination

Elevation of intracellular cAMP concentrations triggers generation of active transcription factors and MAP kinases (MAPKs). Both cAMP effector classes, for instance, phospho-CREB and phospho-ERK1/2 are known to be involved in the mature oligodendrocyte development. Transcription factors control gene expression by binding to DNA regulatory regions, which are carrying specific consensus sequences (Sato-Bigbee et al. 1994; Sands und Palmer 2008).

In oligodendrocytes, phosphorylated CREB activates the CRE sequence present in the MBP promoter to trigger its expression. In 1993, Sato-Bigbee and co-workers described for the first time the expression of the transcription factor CREB in oligodendrocytes. Their work formed the basis for the investigation of the major role of CREB in myelination (Sato-Bigbee, Yu 1993). In addition, the direct influence of phosphorylated CREB on MBP expression was demonstrated by utilizing antisense RNA against CREB (Sato-Bigbee, DeVries 1996). Accordingly, compromised CREB phosphorylation has been observed in the presence of myelin associated inhibitors (Syed et al. 2013). Moreover, the impaired oligodendrocyte differentiation and myelination after GSK3β-(glycogen synthase kinase 3 beta) pathway activation is linked to the CREB phosphorylation (Azim, Butt 2011). In line with the crucial role of PKA in oligodendrocyte development, an increase in CREB phosphorylation levels has been shown to be a consequence of enhanced PKA activity in oligodendrocytes (Sato-Bigbee et al. 1999; Afshari et al. 2001).

In accordance with these findings, in the present study GPR17 activation was linked to a decrease in CREB phosphorylation in primary rat oligodendrocytes. In addition, the achieved data reveal that the selective PKA activator Sp-6-Bnz-cAMPs has the capacity to counteract the GPR17-triggered negative effect on CREB phosphorylation. Taken together, the obtained results confirm the activation of CREB by PKA and associate GPR17 to the PKA-CREB pathway.

5.1.7 ERK1/2 long-term phosphorylation in primary oligodendrocytes

MAPKs, in particular ERK1/2, are key players in oligodendrocyte development. Interestingly, ERK1/2 long-term activation, but not short-term activation, is directly linked to the MBP expression in oligodendrocytes. Accordingly, ERK1/2 was identified as an important component in oligodendrocyte differentiation (Xiao et al. 2012a; Xiao et al. 2012b). Remarkably, deletion of ERK2, but not of ERK1, altered the timing of oligodendrocyte maturation in vivo (Fyffe-Maricich et al, 2011). It is also known that

ERK1/2 utilize CREB-phosphorylation to increase oligodendrocyte differentiation (Syed et al. 2013).

In the present study, activated-GPR17 in oligodendroglial cells does not mediate any long-term alteration of ERK1/2 phosphorylation. Importantly, the obtained results verify that ERK1/2 phosphorylation is not involved in the GPR17 triggered oligodendrocyte maturation arrest. Consequently, the data confirm the assumption of a direct GPR17-$G\alpha_{i/o}$-adenylyl cyclase-PKA-CREB pathway.

5.1.8 ERK1/2 short-term phosphorylation in various cellular backgrounds

Although no GPR17-dependent ERK1/2 long-term activation occurs, a robust GPR17-dependent short-term increase of ERK1/2 levels was detectable in primary rat oligodendrocytes and mouse Oli-neu cells, similar to that observed in HEK293 cells over-expressing the human GPR17 (Hennen et al. 2013). The investigation of the molecular mechanism that underlies the short-term ERK1/2 phosphorylation reveals an engagement of different G protein pathways in Oli-neu cells compared with HEK293 cells. In HEK293 cells, the ERK1/2 phosphorylation is hierarchically mediated: $G\alpha_q$-mediated phosphorylation arises upstream to that mediated by $G\alpha_i$. In contrast, in Oli-neu cells ERK1/2 short-term activation is $G\alpha_i$-mediated and completely $G\alpha_q$-independent. Thus, GPR17 engages different G proteins to regulate ERK1/2 activation, depending on the cellular background (Hennen et al. 2013).

5.1.9 EPAC expression in oligodendrocytes

In addition to PKA, the EPAC activity is also influenced by intracellular cAMP level alteration. Remarkably, increasing intracellular cAMP concentration can independently activate either PKA, EPAC, or both, depending on the cellular background. A growing number of recent studies describe both synergistic and antagonistic effects of EPAC and PKA on a variety of processes in different cells, such as axon guidance and phosphorylation of AKT in primary cortical neurons (Nijholt et al. 2008; Murray et al. 2009). In addition, EPAC has a regulatory role in the differentiation of several cell types, for instance in osteoclasts and Schwann cells (Mediero et al. 2014). Interestingly, activated EPAC alters cAMP-mediated effects from a proliferative to an anti-proliferative/neurite outgrowth-promoting signal in neuroendocrine PC12 cells (Kiermayer 2005).

In consequence, the present work investigated the influence of EPAC on oligodendrocyte myelination. Previous studies unveiled the expression pattern of EPAC1 and EPAC2 in different tissues (Rooij et al. 1998). While EPAC1 is ubiquitously expressed, EPAC2 expression is predominant in the brain and in secretory cells, including the pancreas and testis. Remarkably, EPAC1 is broadly expressed at low levels in the adult brain, but it is strongly and selectively expressed in regions of the developing brain. On

the contrary, EPAC2 is robustly expressed in the mature as well as in the neonatal brain (Rooij et al. 1998). The results of the present study determined that EPAC1 and 2 are expressed in oligodendrocytes during their differentiation. This is a first indication for an involvement of EPAC in oligodendrocyte development.

5.1.10 EPAC plays a functional role during OPC differentiation

Boomkamp and co-authors previously reported the positive influence of EPAC on myelination in spinal cord injury (Boomkamp et al. 2014). Furthermore, it was shown that EPAC is a positive modulator of Schwann cell differentiation and myelination in the PNS (Bacallao et al. 2013). However, until the end of 2015, precedent literature provided no direct demonstration of EPAC as effector protein in oligodendrocyte differentiation.

The present study shows that pharmacological EPAC activation rescues the GPR17-mediated MBP decrease in primary rat oligodendrocytes and Oli-neu cells. Furthermore, the selective EPAC inhibitor (ESI-05) diminishes the MBP expression in a significant manner. The obtained results demonstrated for the first time an influence of EPAC on oligodendrocyte differentiation. The achieved findings have received further support by a recent study that identified GPR37 as regulator of oligodendrocyte differentiation by an EPAC-dependent mechanism (Yang et al. 2016). Taken together, both pathways: (i) cAMP-PKA-CREB and (ii) cAMP-EPAC enhanced differentiation and myelination of oligodendrocytes. In contrast, a previous study showed that EPAC and PKA do not have any synergistic effect on Schwann cell differentiation in the PNS, EPAC activation has a positive impact on Schwann cell maturation, and alteration in PKA activity has no effect on Schwann cell differentiation (Bacallao et al. 2013). In contrast, Mogha and coworkers showed the important role of GPR126 on Schwann cell myelination through $G\alpha_s$-cAMP-PKA dependent mechanism (Mogha et al. 2013).

These findings are also in line with the role of known EPAC effector proteins. EPAC has been shown to directly influence the RhoA pathway and the PI3 kinase-AKT cascade, and to be a link between cAMP and the p38 MAPK signaling cascade (Chen et al. 2012). For instance, the G protein-coupled bile acid receptor (TGR5) inhibits the RhoA/Rho kinase pathway by an EPAC-dependent mechanism to induce smooth muscle relaxation (Rajagopal et al. 2013). EPAC enhances AKT functionality in primary cortical neurons (Nijholt et al. 2008) and controls the neuronal excitability in cerebellar neurons by a p38-dependent pathway (Ster et al. 2007). Interestingly, all three signaling pathways: i) the RhoA pathway, ii) the PI3 kinase-AKT pathway, and iii) the p38 pathway have been recognized as key players in orchestrating oligodendrocyte maturation and myelination (Bhat et al. 2007; Flores et al. 2008; Pedraza et al. 2014). On the other hand, GPR37 blocks oligodendrocyte differentiation by an EPAC-ERK1/2-dependent

mechanism (Yang et al. 2016). However, our results showed that GPR17 did not influence ERK1/2 long-term phosphorylation, thus indicating that GPR17 does not engage an EPAC-ERK1/2 dependent cascade. In addition, an increasing number of studies describe a marked difference between the roles played by each EPAC isoform (EPAC1 and EPAC2) in different cell types. An interesting example is the positive impact of EPAC2 on astrocytic differentiation (Seo and Lee 2016). On the other hand, EPAC1 controls vascular inflammation in an isoform-specific manner (Liu et al. 2015; Parnell et al. 2015). In summary, a closer examination should reveal whether one of the already mentioned signaling cascades (RhoA, PI3K-AKT or p38) or an unknown pathway is engaged by EPAC1 and/or EPAC2 to trigger oligodendrocyte maturation after GPR17 activation. Therefore, further studies are required to clarify the precise downstream signaling pathway of EPAC-activation mediated by GPR17.

The achieved findings strongly suggest that EPAC might provide an attractive target for therapeutic strategies to promote oligodendrocyte remyelination, potentially allowing other cellular functions mediated by the cAMP-PKA signaling pathway to continue undisturbed. Moreover, in 2015, Parnell and co-workers reviewed the available data for promising EPAC-targeted therapies and concluded that a drug therapy may also benefit from selective targeting either EPAC1 or EPAC2 without influencing other pathways. This should result in reduced side-effects in drug therapy. The different expression patterns of EPAC1 and EPAC 2 in the developing brain (Rooij et al. 1998) may suggest a distinguished role of both isoforms in oligodendrocyte differentiation. Thus, it would be of high interest to analyze whether a separate function of EPAC 1 and EPAC 2 is responsible for oligodendrocyte maturation and, therefore, whether an isoform-specific drug therapy may provide an innovative therapeutic approach to treat demyelinating diseases, such as MS.

5.1.11 Proposed model of GPR17-mediated pathway to impair oligodendrocyte differentiation

In conclusion, the present work demonstrates that the $G\alpha_{i/o}$ coupling of GPR17 in oligodendrocytes elicited by the synthetic agonist MDL29,951 is directly linked to decreased activity of the two cAMP effector proteins EPAC and PKA. Therefore, the present study reveals for the first time a key player role of EPAC in oligodendrocyte development. Additionally, diminished PKA activity reduces phosphorylation of the transcription factor CREB after GPR17 activation. Consequently, both pathways, (i) cAMP-PKA-CREB and (ii) cAMP-EPAC lessen the expression of MBP (Figure 44), which is a key marker for oligodendrocyte differentiation and myelination.

Clearly, follow-up studies are required to verify to which extent the PKA and the EPAC pathways contribute to the oligodendrocyte maturation *in vitro* and *in vivo* in the CNS.

The sequential nature of both GPR17-activated pathways offers multiple target points at which the signaling cascade could be regulated. This provides the possibility of influencing the cascade by various pharmacological interventions to promote oligodendrocyte differentiation in the presence of negative stimuli and to avoid side-effects in pharmacological treatment.

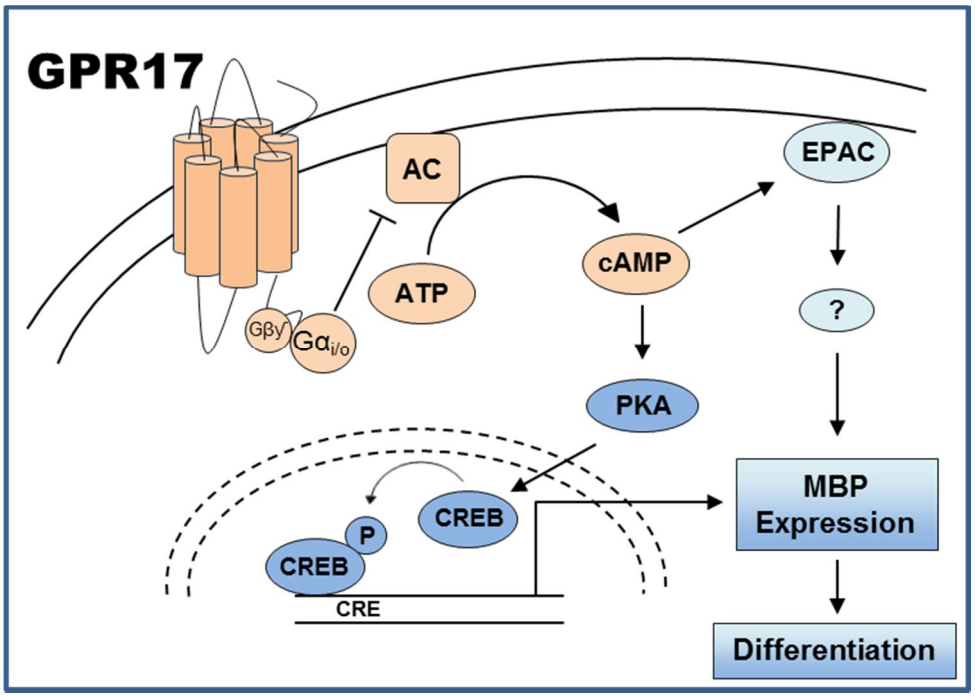


Figure 44. **Proposed model of GPR17-mediated pathway to impair differentiation of oligodendrocytes**

Stimulated GPR17 couples to $G\alpha_{i/o}$ proteins in oligodendrocytes to inhibit adenylyl cyclase (AC). In consequence, this reduces intracellular cAMP levels and leads to a decreased PKA and EPAC activity. Less PKA activity diminishes CREB phosphorylation. Therefore, both pathways (i) cAMP-PKA-CREB and (ii) cAMP-EPAC impair oligodendrocyte differentiation, evidenced by lower MBP expression.

5.2 GPR17: Still an orphan receptor?

The current literature describes an increasing number of deorphanization reports, of which, unfortunately, several could not be verified by independent laboratories. An excellent example for such kind of failure represents the GPR39 deorphanization report. In 2005, Zhang and co-workers reported obestatin as the endogenous ligand for GPR39 (Zhang et al. 2005). However, several other laboratories could not confirm these results (Chartrel et al. 2007; Holst et al. 2007). Interestingly, in 2007 the original report was retracted by its authors (Zhang et al. 2007; Civelli et al. 2013) but the real nature of obestatin as GPR39 ligand is still controversially discussed (Zhang et al. 2008).

5.2.1 Concept of dualistic receptor

The sequence similarity identification between receptors represents a common way to deorphanize GPCRs. However, prior literature describes the limits of GPCR deorphanization by the pairing of orphan receptors with the known receptor ligands of the same phylogenetic cluster (Civelli et al. 2013). For instance, the opioid receptor-like 1 receptor (ORL-1) now called Nociceptin/Orphanin FQ (N/OFQ) peptide (NOP) receptor was paired with dynorphin A, the endogenous agonist of the kappa-opioid receptor, considering the phylogenetic similarities of the receptors. However, poor affinity of dynorphin A was shown at the NOP receptor. Shortly afterwards the cognate eptadecapeptide N/OFQ, the first endogenous ligand discovered by reverse pharmacology, was independently identified as NOP receptor agonist by two different laboratories (Meunier et al. 1995; Reinscheid et al. 1995). On the contrary, lysophosphatidic acid (LPA) activates both lysophosphatidic acid receptor 1 (LPA1) and GPR23 (also termed as LPA4), although these receptors share only 10% amino acid similarity (Civelli et al. 2013).

GPR17 is phylogenetically located between CysLT and P2Y receptors and described as new dualistic receptor activated by uracil nucleotides and cysteinyl-leukotrienes, considering the sequence similarity identification deorphanization approach (Civelli et al. 2013). In particular, (i) the P2Y receptor agonists UDP, UDP-glc, UDP-gal and (ii) the CysLT receptor agonists LTD4 and LTC4 have been reported to activate GPR17 with (i) EC50 values in the micromolar and (ii) nanomolar range, respectively. Several follow-up studies by the same laboratory further confirmed the dualistic nature of GPR17 (Daniele et al. 2011; Fratangeli et al. 2013). Recently, oxysterols, known as activators of Epstein Barr virus induced gene receptor-2 (EBI2) and the chemokine receptor 2 (CXCR2), were reported as a third class of endogenous GPR17 activators (Sensi et al. 2014). A recent report also demonstrated an activation of GPR17 by stromal cell-derived factor 1 (SDF-1), a chemokine receptor (CXCR4 and CXCR7) ligand, increasing the spectrum of proposed GPR17 activators (Parravicini et al. 2016).

In contrast, different independent groups could not or just partially confirm GPR17 activation by cysteinyl-leukotrienes and uracil-nucleotides. Already in 1998, Bläsius and co-workers reported that GPR17 is not activated by typical P2Y receptor agonists (Bläsius et al. 1998). Furthermore, Heise and co-authors found no evidence that GPR17 responds to LTD4 or LTC4 (Heise et al. 2000). This was confirmed by a second independent group (Wunder et al. 2010). Although Benned-Jensen and co-workers supported in their study a GPR17 activation by uracil nucleotides in the micromolar range, they could not confirm a response to cysteinyl-leukotrienes (Benned-Jensen, Rosenkilde 2010). Furthermore, the putative endogenous ligands established for CysLT receptors were unable to activate calcium flux in 1321N1 cells stably expressing GPR17 (Maekawa et al. 2009). Qi et al. failed to recapitulate the activation of GPR17 by UDP,

UDP-gal, UDP-glc, and LTC4 in five different immortalized recombinant cell lines expressing the human GPR17 by both inhibition of forskolin-stimulated cAMP accumulation and promotion of inositol phosphates alteration (Qi et al. 2013). In a later study, Hennen and co-workers were unable to measure an activation of the human GPR17 long version by the uracil nucleotides and cysteinyl leukotrienes by applying the holistic label-free DMR method (Hennen et al. 2013). Moreover, in a radioligand binding study with tritium-labeled MDL29,951 no competition of the tracer by UDP, UDP-glc or LTC4 could be detected, when membranes from Chinese hamster ovary (CHO) cells recombinantly expressing the receptor were used. However, the authors of this study cannot preclude that the proposed endogenous ligands may bind to another receptor binding site (Köse et al. 2014). Accordingly, the GPR17 activation by uracil-nucleotides and cysteinyl-leukotrienes is still controversially discussed by the scientific community.

In the present study, neither uracil nucleotides nor cysteinyl-leukotrienes activate GPR17 in a direct G protein rearrangement assay, an early receptor activation readout. In contrast, MDL29,951-acivated GPR17 mediates a robust intramolecular G protein rearrangement. Furthermore, no evidence was found that GPR17 activated by the P2Y agonist or the CysLT agonist triggers G protein-dependent or -independent β-arrestin recruitment, although GPR17 mediates a time-dependent β-arrestin recruitment activated by MDL29,951. To amplify a possible low GPR17 response mediated by uracil nucleotides and cysteinyl-leukotrienes, the ERK1/2 phosphorylation assay was used. However, no ERK1/2-phosphorylation is detectable after receptor activation by UDP, UDP-glc, UDP-gal, LTC4, LTD4 in contrast to a strong ERK1/2 phosphorylation mediated by MDL29,951-activated GPR17. Together, the obtained results of different assays, (i) $G\alpha_i$ protein rearrangement, (ii) β-arrestin2 recruitment, and (iii) ERK1/2 phosphorylation, which anaylze three different events in the receptor downstream cascade, could not confirm an activation of GPR17 by uracil-nucleotide and cysteinyl-leukotriene. Therefore, in line with the mentioned negative results of several independent laboratories, our findings did not verify the deorphanization of GPR17. Thus, following the deorphanization definition of the IUPAR: (i) confirmation of the endogenous activator by at least two independent working groups and (ii) potency of the proposed ligand should be plausible with a physiologic function of the receptor, we conclude that GPR17 still remains an orphan receptor. Consequently, more contribution of the scientific community would be desirable to clarify the true nature of the cognate GPR17 ligand.

5.2.2 P2Y12 receptor antagonist and their effectiveness at GPR17

GPR17 acts as negative regulator of oligodendrocyte differentiation and was identified as innovative target to treat MS. Therefore, pharmacological therapy of MS may benefit

from identification of a potent GPR17 antagonist to foster remyelination in demyelinated lesions.

Considering the phylogenetic relationship between GPR17 and the receptors of the P2Y and CysLT receptor families, Ciana and co-workers identified CysLT1 and P2Y receptor inhibitors as antagonist at the uracil-nucleotides- and cysteinly-leukotrienes-activated GPR17 (Ciana et al. 2006). In particular, montelukast and pranlukast, both marketed as anti-asthmatic drugs targeting CysLT1, are reported to act as GPR17 antagonists (Ciana et al. 2006). Hennen and co-workers confirmed pranlukast as MDL29,951-activated GPR17 antagonist. However, the IC50 value did not reach the reported nM range of the original study. In contrast, Hennen et al. failed to recapitulate the inhibition capacity of montelukast (Hennen et al. 2013).

Additionally, cangrelor and ticagrelor, targeting the P2Y12 receptor to inhibit platelet aggregation, were as well identified as GPR17 antagonists (Ciana et al. 2006; Gelosa et al. 2014). Cangrelor was shown as potent antagonist at the rat, mouse, and human GPR17 (Lecca et al. 2008). Furthermore, cangrelor inhibits UDP-glc induced patch-clamp recordings mediated by GPR17 (Coppi et al. 2013) as well as UDP-glc and LTD4-induced cAMP inhibition in immature oligodendrocytes (Fumagalli et al. 2011).

The curiosity about the functional properties of the published antagonists was driven by the inactivity of the proposed endogenous ligands at GPR17 in the present study, and this led to the investigation of cangrelor and ticagrelor.

The achieved data demonstrated that ticagrelor and cangrelor do not have the capability to inhibit the functionality of the MDL29,951-activated human, rat, and mouse GPR17 in IP1 accumulation assays. Accordingly, the present study could not confirm the reported antagonistic effects of P2Y12 inhibitors at GPR17 and revealed additionally a discrepancy between the obtained data and the previous results about reported GPR17 modulators.

Consequently, the question arises: why does the inconsistency in the GPR17 literature occur? Although initially the proposed endogenous agonists and synthetic antagonists were mainly characterized by [^{35}S]GTPγS binding assays in an artificial cellular background, in several follow-up studies by the same group the pharmacology of the putative ligands and inhibitors was then tested in second messenger assays using immortalized cells and primary rat oligodendrocytes (Ciana et al. 2006; Daniele et al. 2011; Fratangeli et al. 2013; Daniele et al. 2014). Except 1321N1 cells, a high number of immortalized and native cells express P2Y receptors (Atwood et al. 2011). In particular, primary OPCs express the P2Y receptor subtypes 1, 2, 4, 6, 11, 12, 13, and 14 and, in addition, the CysLT2 receptor (Fumagalli et al. 2011; Fratangeli et al. 2013; Fumagalli et al. 2015b). Furthermore, P2Y12 receptor was found in premature oligodendrocytes by RT-PCR quantification (Fumagalli et al. 2011). Following this line of thought, de-

termination of receptor activity in an environment with various endogenous receptors is often problematic. In consequence, this could lead to potentially incorrect pairing of GPR17 with ligands of the P2Y and CysLT receptor classes.

Furthermore, P2Y receptors are extensively reported to build hetero-oligodimers that may change the receptor pharmacology compared to the parent receptor (Abbracchio et al. 2006). It has been published that CysLT1 and GPR17 built heteromers, whereby the functionality of the CysLT1 receptor is modulated by GPR17 (Maekawa et al. 2009). Thus, GPR17 activation by P2Y and CysLT agonists and inhibition by antagonist of both receptors classes observed in other studies might be explained by the occurrence of GPR17 heteromers in the used cell system.

Taken together, seven years after the GPR17 deorphanization report was published, the receptor is still reported as orphan receptor by the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) (Davenport et al. 2013) and this was confirmed in the present study. Thus, the pairing of GPR17 with its real natural modulator remains as an open challenge. In addition, the obtained results could not verify cangrelor and ticagrelor as GPR17 inhibitors.

5.2.3 GPR17 is inhibited by CysLT2 receptor antagonist

As mentioned before, pharmacological GPR17 inhibition is observed in vitro with pranlukast, a drug synthesized to target the CysLT1 receptor (Ciana et al. 2006; Hennen et al. 2013). In addition to the high receptor analogy between the human GPR17 and the human CysLT1 receptor (31%), GPR17 shows further 36% receptor similarity to the human CysLT2 receptor (Ciana et al. 2006; Maekawa et al. 2009). The phylogenetic proximity supports the hypothesis that CysLT2 antagonists may also effectively block GPR17 function.

In 2010 Wunder and co-workers described HAMI3379 as the first potent and selective antagonist at the CysLT2 receptor, without any effect at the CysLT1 receptor (Wunder et al. 2010). *In vivo*, HAMI3379 blocks ischemic brain injury by acting as a CysLT2 antagonist (Shi et al. 2015). One year later, Ni and co-workers pharmacologically characterized a second selective CysLT2 antagonist, CAY10633 (BayCysLT2). Interestingly, the authors described an attenuation of myocardial ischemia/reperfusion injury in mice after CysLT2 inhibition by CAY10633 (Ni et al. 2011).

Both HAMI3379 and CAY10633 are isophthalic acid derivatives. Their structural difference is only an exchange of a benzyl ring to a cyclohexyl ring. Nevertheless, CAY10633 is described to be 10-fold less potent than HAMI3379 to inhibit LTD4-induced CysLT2 receptor signaling (Ni et al. 2011).

Wunder et al. already hypothesized an antagonistic effect for HAMI3379 at GPR17. However, they could not prove their assumption because they failed to activate GPR17 with the proposed endogenous ligands LTD4 and LTC4 (Wunder et al. 2010).

Taking advantage of the GPR17 small molecule agonist MDL29,951, in the present study, the inhibitory potency of HAMI3379 and CAY10633 was measured at GPR17 in comparison with the already known GPR17 antagonist pranlukast. Both CysLT2 antagonists successfully block activated-human GPR17 in a recombinant cell system. Moreover, HAMI3379 evokes the most potent inhibition of MDL29,951 activity at the rodent GPR17 orthologs, closely followed by CAY10633 and pranlukast. Nevertheless, both CysLT2 antagonists show the highest potency at the human receptor. The activity of the human GPR17 is almost completely blocked by HAMI3397.

Based on their similar capacity to inhibit GPR17, it could be envisaged that the common basic chemical structure of both CysLT2 antagonists may serve as lead for the discovery of higher potent GPR17 inhibitors. In addition to the ability of CysLT2 antagonists to counteract GPR17 function *in vitro*, further studies have also to exhibit whether the compounds are suitable to promote oligodendrocyte differentiation in primary cell cultures and *in vivo* by acting as GPR17 antagonists. It should be emphasized that the use of CysLT2 antagonists in primary oligodendrocytes might be problematic due to the co-expression of CysLT2 receptor and GPR17 during oligodendrocyte development (Fumagalli et al. 2011).

Drug targeting of receptors in the CNS requires the penetration of the not intrathecal applied compounds through the blood brain barrier (BBB). The BBB is a selective physiological barrier, which protects the CNS against unwanted pathogens. Accordingly, the BBB represents a substantial hurdle for drugs targeting CNS receptors, such as GPR17 (Banks 2009). Interestingly, in a pharmacological study HAMI3379 reaches functional concentrations in the CNS after intraperitoneal injection in rats, which verified a successful penetration trough the BBB (Shi et al. 2015). This further supports the idea to use HAMI3379 as a lead structure in the GPR17 antagonist development.

Taken together, for the first time, the achieved data exhibit a potent GPR17 inhibition by applying CysLT2 receptor antagonists. Further studies are required to evaluate whether the CysLT2 antagonists are suitable to block GPR17 activation in primary cells and *in vivo*.

6 Summary

Multiple sclerosis (MS) is an inflammatory demyelinating disease, which is accompanied with oligodendrocyte damage and terminated in axonal loss. A curative therapeutic goal is replacement of the lost myelin sheath because in MS, physiological remyelination, which requires mature myelinating oligodendrocytes, is either inadequate or fails completely (Franklin, Ffrench-Constant 2008). Accordingly, drug development would benefit by identification and characterization of CNS targets that promote oligodendrocyte differentiation and myelination. The G protein-coupled receptor GPR17 was identified as such an innovative target (Chen et al. 2009; Hennen et al. 2013). GPR17 arrests oligodendrocytes in a pre/immature stage and thereby blocks development of myelinating oligodendrocytes in the CNS.

To gain more insight into GPR17 function, the first part of the present study focusses on the analysis of the GPR17 signaling pathway in primary rat oligodendrocytes and the immortalized mouse oligodendrocyte cell line Oli-neu. Consistent with the role of GPR17 in primary oligodendrocytes, the obtained data demonstrate a reduction of the oligodendrocyte myelination marker myelin basic protein (MPB) levels after GPR17 activation by the recently discovered small molecule agonist MDL29,951 (2-carboxy-4,6-dichloro-1H-indole-3-propionic acid) (Hennen et al. 2013). The achieved data identify the $G\alpha_{i/o}$ signaling pathway, one of the four main G protein pathways, as crucial for the GPR17-mediated arrest of oligodendrocytes in an immature stage. GPR17-activated $G\alpha_{i/o}$ proteins inhibit adenylate cyclase (AC) activity to decrease intracellular cAMP levels, and this impairs protein kinase A (PKA) and exchange factor directly activated by cAMP (EPAC) activity, two known cAMP sensors. In consequence, depressed PKA activation reduces the phosphorylation of the transcription factor CREB, known to control MBP transcription levels. In conclusion, the present study verifies GPR17-dependent $G\alpha_{i/o}$ signaling as inhibiting stimulus for the AC-cAMP-PKA-CREB and the AC-cAMP-EPAC signaling cascades. Inhibition of both pathways blocks oligodendrocyte myelination and maturation, evident by the decrease of intracellular MBP levels. Consequently, the sequential nature of the signaling cascades provides multiple target points to foster remyelination of the CNS. Remarkably, this study also identifies for the first time EPAC as key player in oligodendrocyte differentiation. This provides the hypothesis to investigate EPAC as new target in demyelinating disease to foster repair. Clearly, further studies are required to characterize the EPAC signaling pathway promoting oligodendrocyte maturation in more detail.

Identification of the endogenous ligand of GPR17 would further help to clarify its physiological role and provide insight into receptor pharmacology. Based on its phylogenetic

relationship to P2Y and CysLT receptors, GPR17 has been described as a dualistic GPCR activated by ligands of both receptor classes: UDP, UDP-gal, UDP-glc, LTC4, and LTD4 (Ciana et al. 2006). However, the deorphanization could not be confirmed by several independent groups and thus, the real nature of the proposed endogenous agonists for GPR17 is still enigmatic within the scientific community (Bläsius et al. 1998; Heise et al. 2000; Maekawa et al. 2009; Benned-Jensen, Rosenkilde 2010; Wunder et al. 2010; Qi et al. 2013; Hennen et al. 2013; Köse et al. 2014). In consequence, the second part of the present work investigates the putative endogenous ligands of GPR17. The achieved data demonstrate that neither uracil nucleotides (UDP, UDP-gal, and UDP-glc) nor cysteinyl-leukotrienes (LTC4 and LTD4) activate GPR17 signaling, which is shown by: (i) G protein rearrangement, (ii) β-arrestin recruitment, and (iii) ERK1/2 phosphorylation assays. In contrast, robust activation is obtained across all assays when the receptor is stimulated with MDL29,951. Therefore, the obtained results confirm those reports which could not verify uracil nucleotides or cysteinyl-leukotrienes as endogenous ligands for GPR17. In consequence, the present study supports the view that GPR17 is still an orphan receptor with unknown endogenous agonist.

Based on the GPR17 signaling pathway, GPR17 inhibition may represent a novel strategy to foster remyelination in the CNS. Therefore, the drug therapy of demyelinating diseases may benefit from potent GPR17 antagonists. The third part of the present study examines the potential of different compounds to antagonize GPR17 function. Both CysLT1 and P2Y receptor antagonists have been proposed as potent GPR17 inhibitors (Ciana et al. 2006; Gelosa et al. 2014), considering the phylogenetic relationship between GPR17 and the two receptor classes. Therefore, the potential of cangrelor and ticagrelor, both known as P2Y12 receptor inhibitors, was first analyzed. Surprisingly, neither cangrelor nor ticagrelor have the capability to block MDL29,951-activated GPR17 signaling in an inositol monophosphate (IP1) accumulation assay in recombinant cells. In contrast, pranlukast, a CysLT1 receptor antagonist, inhibits the GPR17 activity but with a low potency. Together, the obtained results do not confirm cangrelor or ticagrelor as GPR17 antagonist. Thus, identification of a GPR17 antagonist remains an existing challenge. Hence, two CysLT2 receptor antagonists with a high structural similarity, HAMI3379 and CAY10633 (Wunder et al. 2010; Ni et al. 2011), were subsequently analyzed as potential GPR17 inhibitors. Remarkably, both HAMI3379 and CAY10633 potently inhibited GPR17-dependent IP1 accumulation in recombinant cells. Thus, the present study shows for the first time an inhibition of GPR17 by CysLT2 inhibitors. Further studies are required to analyze whether these or chemically similar compounds will be suitable to efficiently block GPR17 function in primary oligodendroglial cells and *in vivo* models.

In summary, the present study provides detailed molecular insight into the GPR17 signaling pathways: (i) AC-cAMP-PKA-CREB and (ii) AC-cAMP-EPAC, which block

oligodendrocyte differentiation. Furthermore, EPAC is identified for the first time as key player orchestrating oligodendrocyte maturation and myelination. In addition, the present work provides further support for the notion that GPR17 is not the elusive dualistic orphan receptor responding to uracil nucleotides and cysteinyl-leukotrienes. Finally, cangrelor and ticagrelor are characterized as ligands that do not dampen functionality of GPR17 as opposed to the CysLT2 antagonists HAMI3379 and CAY10633, capacity of which to antagonize GPR17 function has been shown herein for the first time. Thus, both CysLT2 inhibitors represent new exciting starting points for the development of GPR17 antagonists with higher potency and selectivity.

7 List of abbreviations

AC	adenylate cyclase
ad	Latin word for up to
ATP	adenosine 5'-triphosphate
BRET	bioluminescence resonance energy transfer
CB	Cannabinoid receptors
CRE	cAMP response element
CREB	cAMP response element-binding protein
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CRC	concentration-response curve
CNG channels	cyclic-nucleotide-gated ion channels
CysL	cysteinyl-leukotriene
CysLT1 receptor	type 1 cysteinyl-leukotriene receptor
CysLT2 receptor	type 2 cysteinyl-leukotriene receptor
CXCR	chemokine receptors
DAG	diacylglycerol
dH_2O	demineralized water
DMR	dynamic mass redistribution
D2R	dopamine 2 receptor
DRC	dose-response curve
EAE	experimental autoimmune encephalomyelitis (animal model of multiple sclerosis)
EC_{50}	concentration of half maximum effect
EPAC	cAMP-exchange Protein directly acitvated by cAMP
EBI2	Epstein Barr virus induced gene receptor-2
ERK	extracellular-signal regulated kinase

et al.	et alii, Latin word for and others
FRET	fluorescence resonance energy transfer
FSK	forskolin
g	acceleration by gravity
g	gram
GDP	guanosine 5′-diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
G protein	guanine nucleotide-binding protein
GPR17	G protein-coupled receptor 17
GRK	G protein-coupled receptor kinases
h	human
h	hour(s)
H3R	histamine 3 receptor
HTRF®	homogeneous time resolved fluorescence
IC_{50}	concentration of half maximum inhibition
NC-IUPHAR	International Union of Basic and Clinical Pharmacology
IP1	inositol monophosphate
IP3	inositol 1,3,4-trisphosphate
JNK	c-Jun N-terminal kinase
LINGO-1	Leucine rich repeat and Ig-like domain-containing Nogo receptor interacting protein 1
log M	logarithm of molar concentration to base 10
LTC4	leukotriene D4
LTD4	leukotriene D4
LPAR1	lysophosphatidic acid receptor 1
M	molar concentration (mol/liter)
m	murine/mouse

mAChRs (M)	Muscarinic acetylcholine receptors (Muscarinic receptors)
MAG	myelin associated glycoprotein
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MASS1	monogenic audiogenic seizure susceptible 1
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
ms	millisecond
µl	microliter
µM	micromolar
nm	nanometer
nM	nanomolar
NG2	neural/glial antigen 2 proteoglycan
O4	O-Antigens
OD	optical density
Oli-neu cells	immortalized murine oligodendrocytes
OPC	oligodendrocyte precursor cell
PCR	polymerase chain reaction
PDGFRα	platelet-derived growth factor receptor alpha
PDE4	phosphodiesterase
PI	phosphatidylinositol
PI3	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLP	proteolipid protein
PNS	peripheral nervous system
PTX	Pertussis toxin
PY2 receptors	purinergic receptors

q.s.	quantum satis (as much as needed)
RhoA	Ras homolog gene family, member A
ROCK2	RhoA-Rho-associated kinase 2
RLuc	Renilla luciferase
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
RWG	resonant waveguide grating
s	second(s)
SCI	spinal cord injury
S.E.M	standard error of mean
S1P	spingosine-1-phosphate
SDF-1	stromal cell-derived factor 1
siRNA	small interfering RNA
TGR5	G protein-coupled bile acid receptor
7TMR	seven transmembrane receptor
UDP	Uridine 5′- diphosphate
UDP-gal	Uridine 5′-diphosphogalactose
UDP-glc	Uridine 5′-diphosphoglucose
VLGR1	very large G protein-coupled receptor 1
w/o	without

8 Register of publications

Research articles:

Bock A, Merten N, Schrage R, Dallanoce C, Bätz J, Klöckner J, Schmitz J, Matera C, **Simon K**, Kebig A, Peters L, Müller A, Schrobang-Ley J, Tränkle C, Hoffmann C,De Amici M, Holzgrabe U, Kostenis E, Mohr K. (2012) The allosteric vestibule of a seven transmembrane helical receptor controls G-protein coupling. *Nature Communications*,.3:1044. doi: 10.1038/ncomms2028.

Hennen S, Wang H, Peters L, Merten N, **Simon K**, Spinrath A, Blättermann S, Akkari R, Schrage R, Schröder R, Schulz D, Vermeiren C, Zimmermann K, Kehraus S,Drewke C, Pfeifer A, König GM, Mohr K, Gillard M, Müller CE, Lu QR, Gomeza J, Kostenis E. (2013) Decoding signaling and function of the orphan G protein-coupled receptor GPR17 with a small-molecule agonist. *Science Signaling*, 22;6(298):ra93. doi: 10.1126/scisignal.2004350.

Christiansen E, Watterson KR, Stocker CJ, Sokol E, Jenkins L, **Simon K**, Grundmann M, Petersen RK, Wargent ET, Hudson BD, Kostenis E, Ejsing CS,Cawthorne MA, Milligan G, Ulven T (2015). Activity of dietary fatty acids on FFA1 and FFA4 and characterisation of pinolenic acid as a dual FFA1/FFA4 agonist with potential effect against metabolic diseases. *British Journal of Nutrition*,2015; 113(11):1677-88. doi: 10.1017/S000711451500118X. Epub 2015 Apr 28.

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Klepac K, Kilić A, Gnad T, Brown LM, Herrmann B, Wilderman A, Balkow A, Glöde A, **Simon K**, Lidell ME, Betz MJ, Enerbäck S, Wess J, Freichel M, Blüher M, König G, Kostenis E, Insel PA, Pfeifer A. (2016) The Gq signalling pathway inhibits brown and beige adipose tissue. *Nature Communications*, 9;7:10895. doi: 10.1038/ncomms10895

Poster presentations:

Simon, K.; Hennen, S.; Merten, N.; Schröder, R.; Peters, L.; Schrage, R.; Vermeiren, C.; Mohr, K.; Müller C.E.; Gillard, M.; Gomeza, J.; Kostenis, E. (2014) GPR17: still an orphan GPCR? The Purines International Conference on Nucleotides Nucleosides and Nucleobases (Bonn, 23.-27. Juli)

Simon, K.; Hennen, S.; Merten, N.; Schröder, R.; Peters, L.; Schrage, R.; Vermeiren, C.; Mohr, K.; Müller C.E.; Gillard, M.; Gomeza, J.; Kostenis, E. (2014) GPR17: the elusive dual uracil nucleotide/cysteinyl- leukotriene receptor or still an orphan? DPhG Jahrestagung 2014 / Annual meeting (Frankfurt, 24.-26. September)

Simon, K.; Hennen, S.; Preis, P. Merten, N.; Gomeza, J.; Kostenis, E. (2015) GPR17: An orphan GPCR, awaiting the identification of its true native ligand. Molecular Pharmacology Gordon Research Conference Connecting G Protein-Coupled Receptor Mechanisms to Physiological Functions (Ventura, Californien, USA, 1.-6. Februar 2015)

Simon, K.; Hennen, S.; Merten, N.; Blättermann, S.; Gomeza, J.; Kostenis, E. (2015) GPR17: understanding its signaling and physiological function. DPhG Jahrestagung 2015/ Annual meeting (Düsseldorf, 23.-25. September)

Simon, K.; Hennen, S.; Merten, N.; Blättermann, S.; Gomeza, J.; Kostenis, E (2015) GPR17: understanding its signaling and physiological function. Summer Academy Pharmacology 2015 (GRK1873), (Bonn,27.-29 September)

Simon, K.; Hennen, S.; Merten, N.; Kostenis, E., Gomeza, J. (2016) GPR17: A negative regulator of oligodendrocyte differentiation. G Protein-Coupled Receptors: Structure, Signaling and Drug Discovery (Keystone, Denver, USA 21. -25. Februar)

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