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Title

Regulation and signalling of the GPR17 receptor in oligodendroglial cells

Running title

GPR17 receptor in oligodendrocyte progenitors

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The authors declare that they have no conflict of interest.

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Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Abstract

Remyelination, namely the formation of new myelin sheaths around denuded axons, counteracts axonal degeneration and restores neuronal function. Considerable advances have been made in understanding this regenerative process that often fails in diseases like multiple sclerosis, leaving axons demyelinated and vulnerable to damage, thus contributing to disease progression. The identification of the membrane receptor GPR17 on a subset of oligodendrocyte precursor cells (OPCs), which mediate remyelination in the adult central nervous system (CNS), has led to a huge amount of evidence that validated this receptor as a new attractive target for remyelinating therapies. Here, we summarize the role of GPR17 in OPC function, myelination and remyelination, describing its atypical pharmacology, its downstream signalling and the genetic and epigenetic factors modulating its activity. We also highlight crucial insights into GPR17 pathophysiology coming from the demonstration that oligodendrocyte injury, associated to inflammation in chronic neurodegenerative conditions, is invariably characterized by abnormal and persistent GPR17 upregulation, which, in turn, is accompanied by a block of OPCs at immature pre-myelinating stages. Finally, we discuss current literature in light of the potential exploitment of GPR17 as a therapeutic target to promote remyelination.

Key words

GPR17, oligodendrocyte progenitors, signal transduction, neuroinflammatory diseases, remyelination

Main points

1. GPR17 signal transduction controls oligodendrocyte differentiation and lactate production

2. Transcription factors regulate GPR17 expression

3. GPR17 is dysregulated in neuroinflammatory diseases

4. Targeting GPR17 improves remyelination

1 Introduction

In 2006, the cloning, native and heterologous expression and initial characterization of the human and rat orphan receptor GPR17 was reported (Ciana et al., 2006). Two years later, the characterization of the mouse ortholog of GPR17 highlighted its extremely peculiar localization to oligodendrocyte precursor cells (OPCs), the normally quiescent stem-like cells that are found in both brain and spinal cord throughout life, and that are also known as NG2 glia, due to their expression of the typical proteoglycan NG2 (Lecca et al., 2008). At those times, NG2 glia were already known as key actors in physiological myelination during development as well as in the maintenance of myelin integrity during adulthood, due to their ability to slowly but significantly differentiate to mature, myelin-expressing oligodendrocytes, the cells that, by enwrapping neuronal endings, ensure rapid nerve impulses transmission and also exert trophic effects on axons (Nave, 2010; Nishiyama, Komitova, Suzuki, & Zhu, 2009). Evidence was also mounting at those times on the ability of these cells to rapidly react to toxic demyelinating insults with a robust proliferative response, that was, immediately later, followed by migration towards demyelinated lesions and generation of new remyelinating cells (Franklin & Ffrench-Constant, 2008). However, the molecules and factors involved in this rapid reparative reaction were still obscure. As a result of the discovery of the high expression of GPR17 by NG2 cells, in the subsequent years a number of studies was published by several different groups aimed at determining the role of this receptor in both physiological myelination as well as in the repair responses of the brain and spinal cord to myelin disruption (Boda et al., 2011; Ceruti et al., 2009; Chen et al., 2009; Fumagalli et al., 2011), thus validating this receptor as a new attractive target for remyelinating therapies. What made GPR17 so interesting was: (i) its expression at the plasma membrane of NG2 cells, i.e., at direct contact with the extracellular milieu, that made it easily accessible and amenable for regulation by pharmacological agents~~,~~ and, (ii) mounting evidence

indicating that demyelination was not only typical of multiple sclerosis (a disease in which oligodendrocytes are primarily affected), but indeed represented a common hallmark of several other neurodegenerative acute and chronic conditions like stroke, trauma, Alzheimer's and amyotrophic lateral sclerosis (Ferrer, 2018; Fumagalli, Lecca, & Abbracchio, 2016). It was thus evident that agents acting at GPR17 could represent a new class of neuroreparative therapeutic entities of potential interest in several incurable neurodegenerative conditions. Here, we revise the role of GPR17 signalling in OPC function, myelination and remyelination, as well as its spurious and atypical pharmacology and the genetic and epigenetic factors and mechanisms influencing its activity. We also discuss available data in view of the potential exploitment of GPR17 for the set-up of new remyelinating neurodegenerative therapies.

2 GPR17 involvement in oligodendrogenesis and myelination

As introduced above, in the healthy adult CNS tissue, the GPR17 receptor is mainly expressed by a relatively quiescent population of neural precursor cells of the oligodendrocyte lineage (NG2 glia). In detail, the receptor is present in two pools of progenitors labelling two distinct stages of oligodendrocyte differentiation: the early stage of proliferative OPCs expressing markers like NG2, A2B5 and PDGF receptor-alpha, and the subsequent phase of more ramified, pre-immature oligodendrocytes, characterized by NG2 downregulation and by expression of more advanced markers like O4 and O1. Interestingly, GPR17 expression peak coincides with the pre-oligodendrocyte phase and it is then gradually silenced in mature myelinating oligodendrocytes (Fumagalli et al., 2011; Lecca et al., 2008). During development, the expression of the receptor in the brain is low, but progressively increases to label the 80% of pre-immature oligodendrocytes at the end of the second week of life, after which GPR17 is downregulated while myelin development proceeds (Boda et al., 2011).

As a result of these data, GPR17 is now widely considered as a new useful marker to label progenitor cells at these two transition stages (Crociara, Parolisi, Conte, Fumagalli, & Bonfanti, 2013; Ferrara

et al., 2016; Mitew et al., 2014; Nakatani et al., 2013) and recognized as a putative regulator of myelination, both during early development and at adult stages. The latter consideration is corroborated by experimental evidence showing that any alterations of GPR17 precise expression pattern in OPCs result in myelination defects (Chen et al., 2009; Fumagalli et al., 2015, 2011). Receptor obliteration with small interfering RNAs in early OPCs profoundly reduced their ability to generate mature oligodendrocytes, suggesting a pivotal role of GPR17 in the initial phases of the differentiation process (Fumagalli et al., 2011). On the other hand, loss of GPR17 at advanced differentiation stages is necessary to enable cells to complete terminal maturation. Consistently, GPR17 forced overexpression in differentiating OPCs keeps cells at an immature phenotype that does not express CNPase. In line with this finding, myelinogenesis was found to be defective in transgenic mice overexpressing GPR17 under the promoter of 2',3'-Cyclic-nucleotide 30-phosphodiesterase (CNPase), a relatively advanced oligodendrocyte marker (Chen et al., 2009). In a similar way, under conditions where terminal OPC maturation is impaired, such as demyelinating diseases (see paragraph 3) or treatment with the mTOR inhibitor rapamycin, GPR17 is markedly upregulated (Fumagalli et al., 2015; Tyler et al., 2011) (see also below).

Recent data have corroborated the central role of GPR17 in orchestrating oligodendroglial differentiation and myelination. Single cell RNAseq analysis unveiled the existence of a wide heterogeneity in oligodendroglial subpopulations within the brain, each of them retaining specific spatial localizations and functional properties under both physiological and pathological conditions (Falcão et al., 2018; Jäkel et al., 2019; Marques et al., 2016). Interestingly, of 12 distinct cell subpopulations belonging to the oligodendrocyte lineage, GPR17 expression was detected only in three clusters defined as "differentiation committed precursors" (Marques et al., 2016). Moreover, a detailed spatial and functional analysis of OPC diversity, in zebrafish spinal cord, revealed that GPR17 expression is strictly present in cells localized in an axon-enriched environment and potentially able to myelinate axons, while it is virtually absent in OPCs housed in proximity of

neuronal somas, that never differentiate but seem to be more involved in network formation and synaptic activity (Hoche et al., 2019).

2.1 Signalling of GPR17 receptor in OPC functions

After its originally identification as an incomplete sequence of a human G protein-coupled receptor (GPCR) related to the P2Y receptor family (Bläsius, Weber, Lichter, & Ogilvie, 2002), GPR17 has been an orphan receptor for a long time. It was later recognized to be at an intermediate structural and phylogenetic position between already known P2Y, CysLT receptors and GPR99 (proposed as the third CysLT receptor) (Kanaoka, Maekawa, & Austen, 2013)), in the so called "purine receptor cluster" of class A GPCRs. GPR17 indeed displays the typical 7-transmembrane (TM) domain topology of GPCRs, with an amino acid identity with the known P2Y and CysLT receptors between 21 and 48% (Lecca et al., 2008).

Although the complex GPR17 pharmacological profile does not find complete consensus among the scientific community (Simon et al., 2017), a body of consistent evidence shows that GPR17 is activated by UDP, UDP-glucose and UDP-galactose and by cysteinyl-leukotrienes (LTC_4, LTD_4 and LTE_4) (Agier, Różalska, Wódz, & Brzezińska-Błaszczyk, 2017; Benned-Jensen & Rosenkilde, 2010; Ciana et al., 2006; Lecca et al., 2008), at ligand concentration ranges (i.e., μM and nM ranges, respectively) that are fully consistent with those necessary for these endogenous ligands to activate their already known cognate P2Y and CysLT receptors (Alexander et al., 2019). Activation by uracil nucleotides is reversed by some purinergic antagonists like the $P2Y_1$ antagonist MRS2179 or the $P2Y_{12}$ antagonist cangrelor, whereas, responses to cysLTs are inhibited by typical CysLT receptor antagonists like the already marketed drugs montelukast and pranlukast (Fumagalli et al., 2017).

Concentration-dependent inhibition of forskolin-stimulated adenylyl cyclase represents the main signal transduction mechanism that occurs after GPR17 activation in OPCs (Fumagalli et al., 2011).

The amount of intracellular cAMP, essential to determine the rate of oligodendrocyte differentiation (Malone et al., 2013), is fine-tuned by the signalling of several GPCRs expressed in these cells (Butt, Papanikolaou, & Rivera, 2019), including many purinergic receptors (Fumagalli et al., 2016).

High levels of cAMP suppress the expression of PDGFRα, thus inhibiting OPC proliferation (Li & Wang, 2011) and promote the expression of myelin proteins, such as CNPase and MBP (Clark, Miskimins & Miskimins, 2002). It can be hypothesized that, by reducing intracellular cAMP levels in early OPCs, GPR17 activation may slow down differentiation to allow cells fulfilling and completing all the metabolic changes needed to synthesize myelin lipids and get ready for terminal maturation. The expression of GPR17 in these phases may act as a "checkpoint" preventing inappropriate precocious myelination under physiological conditions. At later stages, when OPCs finally exit from the cell cycle and are committed to terminal maturation, GPR17 ligand-mediated downregulation and internalization allows cells to progressively escape GPR17 mediated cAMP inhibition in order to increase intracellular cAMP to the levels necessary to promote terminal maturation (Fumagalli et al., 2015). Accordingly, *in vitro* continuous exposure to either endogenous or synthetic GPR17 agonists promoted OPC differentiation into MBP-expressing cells and accelerated myelination in OPC-DRG co-cultures, likely due to receptor internalization (Capelli et al., 2019; Fumagalli et al., 2011) (as discussed in paragraph 2.3). Of note, GPR17 has been also shown to specifically mediate activation of delayed rectifier K^+ currents in a subpopulation of OPCs and pre-immature $O4^+$ oligodendrocytes, but not in mature oligodendrocytes. The transient nature of this effect is fully consistent with the expression pattern of GPR17, since K^+ currents themselves were shown to gradually disappear along with GPR17 physiological downregulation in late OPCs (Coppi et al., 2013). These findings suggest that these currents also correlate with the GPR17-mediated facilitating effect in OPC maturation. However, how this signalling pathway intersects with the cAMP production system in OPCs still remains to be determined.

In contrast with the above studies, the postulated GPR17 agonist MDL29,951 was shown to inhibit, rather than stimulate, oligodendrocyte maturation *in vitro* by reducing cAMP levels, (Hennen et al.,

2013). In OPCs, MDL29,951 rapidly mobilizes intracellular Ca^{2+} in a concentration-dependent manner and engages both $G_{\alpha i}$ and $G_{\alpha q}$. Moreover, MDL29,951-stimulated GPR17 effects are counteracted in a concentration-dependent manner by pranlukast and, to a lesser extent, by montelukast. However, it is worth noting that MDL29,951 is not selective for GPR17, but it has been originally developed as a ligand for the glycinergic site of the glutamate NMDA receptor, at which it shows very specific interaction in the nM range (Salituro et al., 1992). OPCs express NMDA receptors and their knock-down by specific silencing RNAs or by glutamate receptor antagonists result in a block of OPC maturation (Li et al., 2013). By acting at the glycinergic site of the NMDA receptor, MDL29,951 could markedly interfere with OPC differentiation, thus inhibiting myelination, independently of its action on GPR17.

Of great interest, signalling through GPR17 in oligodendrocytes has been recently shown to regulate food intake by modulating hypothalamic neuronal activities. Pharmacological inhibition of GPR17 by intracerebroventricular administration of the non-selective antagonist cangrelor, as well as GPR17 conditional knockout in AgRP neurons in the hypothalamus, reduced food intake by mimicking the effect of the ablation of FoxO1, a transcription factor intertwining insulin and leptin pathways with the release of feeding-regulating hormones by hypothalamic neurons (Ren, Cook, Kon, & Accili, 2015; Ren et al., 2012). However, it is worth noting that both the drug treatment and the knockout procedure performed in these studies are not strictly selective for hypothalamic neurons, as they may also affect other cell types, including oligodendroglial cells (Ou et al., 2019). Accordingly, ~~B~~ both GPR17-null mice and mice with an oligodendrocyte-specific knockout of GPR17 have lean phenotypes on a high-fat diet (Ou et al., 2019). As a potential mechanism at the basis of these effects, downregulation of GPR17 in oligodendrocytes results in increased activation of cAMP-protein kinase A (PKA) signalling, thus leading to upregulation of c-fos and pyruvate dehydrogenase kinase 1 (PDK1), which, in turn, pushes cellular metabolism towards lactate production. Elevated lactate levels in oligodendrocytes enhance the transfer of this metabolite to hypothalamic neurons, where the consequent activation of AKT and STAT3 signalling increases the synthesis of POMC peptides and

reduces AgRP/NPY peptides, finally inhibiting food intake. These findings uncover a critical role of GPR17 in metabolic homeostasis and suggest that modulation of its signalling might be a potential therapeutic approach for treating obesity (Ou et al., 2019).

GPR17 has been reported to also respond to emergency signals like oxysterols, in a similar way to other related receptors involved in inflammatory responses, like Epstein Barr virus induced gene receptor-2 and CXCR2 (Sensi et al., 2014). These data corroborate an unexpected heterogeneity and complexity in GPR17 recognition, thus challenging classical pharmacology paradigms on the 'monogamous' interaction between a specific class of natural ligands and a single GPCR (Haupt, Daminelli & Schroeder, 2013). More recent data also showed that the stromal derived factor 1 (SDF-1), also known as the endogenous ligand for CXCR4 and CXCR7 receptors, is able to transactivate GPR17 *in vitro*, specifically increasing the [^{35}S]GTPγS binding to the membrane of GPR17-expressing cells, with nanomolar affinity constant values (Parravicini et al., 2016). Globally, these data suggest that GPR17 may promiscuously respond to different signalling molecules depending on specific pathophysiological conditions and emergency situations. GPR17 responses may also vary depending upon its heterodimerization with other receptors, including P2Y and CysLT receptors (Maekawa, Balestrieri, Austen, & Kanaoka, 2009; Pillaiyar et al., 2019), which could help explain why ~~agonists~~ ligands of GPR17 have such diverse chemical structures. Since GPCR dimerization is also known to profoundly change pharmacological ~~responses~~ properties (Wang, Qiao, & Li, 2018), this could also explain the variability of GPR17 responses in different *in vitro* and *ex vivo* systems, and also be at the basis of the inability to reproduce some of its features in "artificial" reconstituted and recombinant systems. From a functional point of view, the ability of GPR17 to be activated by various chemically unrelated ligands makes this receptor a primary regulator of the oligodendrocyte response to pathological insults within the CNS. On the other hand, such abundance of activating molecules massively released after brain damage might easily lead to aberrant activation of GPR17 and to the consequent block of OPC maturation (see also paragraph 3).

2.2 Gene and epigenetic mechanisms regulating GPR17

In paragraph 2.1, we highlighted how the interaction between GPR17 and its ligands can regulate oligodendrocyte differentiation depending upon receptor expression and ligand-dependent downregulation, and how this may have implications from a pharmacological point of view. However, the correct expression timing of GPR17 is also the result of complex interactions between the intrinsically determined oligodendroglial differentiation program and extracellular stimuli possibly acting on the Gpr17 gene.

It is known that CNS cells release factors that drive OPCs to increase or reduce their differentiation or myelination rate via different effectors, such as glutamate receptors, adhesion molecules, gap-junctions and others (Elazar et al., 2019; He & Lu, 2013; Hughes & Appel, 2019; Rosa et al., 2019; Vejar, Oyarzún, Retamal, Ortiz, & Orellana, 2019). Stimulation of oligodendroglial cells with neuron-conditioned medium was able to increase Gpr17 promoter activity (Fratangeli et al., 2013), even if the specific signal has not been clearly elucidated.

The expression of the Gpr17 gene is regulated by a timely action of two families of transcription factors, namely the OLIG and the ID proteins. Olig1 and Olig2 are bHLH transcription factors and they are master regulators of oligodendrocyte differentiation. Although they are often co-expressed in oligodendroglial cells and have partially overlapping functions, it has been demonstrated that Olig2 is primarily involved in the differentiation of neural progenitors towards the oligodendroglial lineage, whereas Olig1 drives the maturation of OPCs to oligodendrocytes both during development and in the adult CNS (Li, Lu, Smith, & Richardson, 2007). Olig1 is also re-expressed in oligodendrocytes in the remyelination phase of cuprizone-induced demyelination (Arnett et al., 2004).

Both Olig1 and Olig2 can directly bind to a regulatory E-box region of the Gpr17 promoter, suggesting that they might directly regulate Gpr17 transcription (Chen et al., 2009). In Olig1 knock-out mice, GPR17 is downregulated, demonstrating a role for this transcription factor in controlling receptor expression. Conversely, the overexpression of Olig1 in OPCs has been described to downregulate Gpr17 and to promote MBP expression and oligodendrocyte maturation (Chen et al.,

2009). This apparent contradiction can be explained by the oligomerization of Olig1/2 with other transcription factors with modulating activity. This is particularly important, considering the transient expression of GPR17 and its role as a "timer" of myelination. Recent findings highlighted that, in chicken neural explants, Olig2 and Gli2/3, transcription factors activated by the morphogen Sonic hedgehog (Shh), can cooperate and promote the regulation of Gpr17 in the presence of the morphogen (Yatsuzuka et al., 2019). On the other hand, the transcription factor ID2 acts as an inhibitory signal of oligodendrocyte maturation through the interaction with the OLIG factors. When inactive, ID2 is localized in the cytosol. The sustained overexpression of GPR17 increased the overall expression of ID2, and significantly promotes its nuclear translocation, thus inhibiting Olig1 action on both Gpr17 and myelin genes (Chen et al., 2009). Therefore, GPR17 expression in the early phases of differentiation can be promoted by Shh signalling, and its inhibition in the late phases can be induced by ID2. During its transient expression, the downstream signalling of GPR17 prevents untimely myelination activating ID2, thus generating a negative feedback loop (Chen et al., 2009) (Fig. 1).

In the case of OLIG2, a different behaviour has been described under damage conditions. After lysolecithin (LPC) treatment *in vitro*, the interaction of OLIG2 with Gpr17 significantly increases in exon regions inducing its increased transcription, and an epigenetic mechanism has been demonstrated (Ou et al., 2016). The enhanced transcription of Gpr17 is sustained by higher acetylation levels of the lysine 27 in the histone 3 (H3K27) in the same regions of OLIG2 occupancy. The relevance of H3K27 acetylation in switching Gpr17 on during physiological differentiation has not been investigated, but other epigenetic mechanisms have been described. Indeed, a dynamic cooperation between transcription and epigenetic factors is a crucial event in OPC maturation, a complex process that requires a tight balance between proliferation, survival and differentiation. CHD7 and CHD8 are chromodomain helicase DNA-binding proteins, responsible for increasing or reducing the accessibility of promoters and other regulatory regions to transcription factors by opening or closing the chromatin structure. More than six thousand promoters are targeted by both CHD7 and CHD8, and many of them belong to genes involved in oligodendroglial differentiation and

myelination, including Olig1/2, Nkx2.2, Sox10 and Gpr17. It has been demonstrated that CHD7 directly binds to both the enhancer and the promoter regions of Gpr17, opens the chromatin and allows the recruitment and the timely action of Olig2 and Sox10 (Marie et al., 2018). These findings revealed that sequential levels of GPR17 regulation are required to orchestrate the timing of oligodendrocyte differentiation.

One single study has described that miR-146a-5p directly interacts with the 3'UTR of Gpr17 in neural cells transfected with a reporter construct (He et al., 2018), thus also highlighting a post-transcriptional regulation of the receptor. MiRNAs virtually regulate all the biological processes, including oligodendrogenesis, oligodendrocyte maturation and myelination, by fine-tuning the dosage of crucial genes at different levels (Marangon, Raffaele, Fumagalli, & Lecca, 2019). However, the direct regulation of Gpr17 by this or other miRNAs has not been investigated in oligodendrocytes so far.

2.3 Downstream mechanisms of GPR17 receptor regulation

The physical removal of a GPCR from the plasma membrane is an important process by which cells regulate their own state, their ability to respond to external stimuli, and their functions. Receptor desensitization is the first step, followed by internalization and receptor trafficking, that may result in either recycling or degradation (Bahouth & Nooh, 2017).

As described in paragraph 2, in the late stages of oligodendrocyte differentiation, GPR17 has to be downregulated to allow terminal maturation and eventually myelination (Fumagalli et al., 2015). This process, mediated by the interaction of the receptor with its ligand, leads to the rapid phosphorylation of the C-terminus promoted by G protein-coupled receptor kinases (GRKs) and the consequent recruitment of β-arrestin (Daniele et al., 2011; Daniele et al., 2014). Different ligands activate different desensitization pathways with different kinetics; it has been demonstrated that in both transfected cell lines and in OPCs, in case of stimulation with the cysteinyl-leukotriene LTD_4, GRK2

phosphorylates the receptor, promotes a transient association with β-arrestin and a G protein-dependent activation of CREB via ERK translocation into the nucleus. Instead, the stimulation with UDP-glucose induces a GRK5-mediated phosphorylation, and a stable association with β-arrestin that does not allow the nuclear translocation of ERK, thus preventing transcriptional events (Daniele et al., 2014). This different behaviour highlights that GPR17 functions strictly depend on extracellular stimuli and demonstrates the importance of biased agonism.

Intracellular effectors that interact with GPR17 after internalization determine the fate of the receptor (Fig. 2). In particular, the sorting-nexin 27 protein (SNX27) has been described to interact with a PDZ domain in the C-terminus of the receptor, thus preventing GPR17 lysosomal degradation and promoting its recycling to the membrane (Meraviglia et al., 2016). The timely function of SNX27 is crucial during oligodendrocyte differentiation; indeed, forced downregulation of SNX27 shifted GPR17 to the lysosomal compartment, accelerating its degradation and promoting earlier oligodendrocyte differentiation.

A proteomic analysis in cultured OPCs during differentiation also revealed a connection between GPR17 and the mTOR pathway (Tyler et al., 2011). The inhibition of mTOR with rapamycin elevates the expression of the receptor and maintains the cells in an immature stage. It has been shown that the mTOR pathway drives the localization of the ubiquitin ligase Mdm2 within the cells. Upon activation of GPR17 with its ligands, GRKs initiate the desensitization signal described above. When the mTOR pathway is active, Mdm2 translocates into the nucleus and promotes the degradation of nuclear factors. The inhibition of mTOR with rapamycin favours the cytosolic localization of Mdm2 and the consequent ubiquitination and degradation of cytoplasmic proteins, such as GRKs, thus preventing GPR17 desensitization (Fumagalli et al., 2015).

3 Dysregulation of GPR17 in neurological diseases

3.1 Pathological overexpression of GPR17 in various neurodegenerative paradigms

Most of the endogenous molecules activating GPR17 are damage signals. In the CNS, as a consequence of cells' breakdown, high levels of uracil nucleotides are massively found in the extracellular milieu (Lecca & Ceruti, 2008). Similarly, CysLTs are locally produced and released upon inflammatory responses (Gelosa, Colazzo, Tremoli, Sironi, & Castiglioni, 2017). Oxysterols are cholesterol metabolites of brain cells, including neurons, and can also act as oxidant and inflammatory signalling molecules (Griffiths et al., 2016). Finally, the chemokine SDF-1, a chemoattractant driving cell migration during development, can promote inflammation in several pathological contexts including multiple sclerosis-associated demyelination (Parravicini et al., 2016). Therefore, it is not surprising that altered expression of GPR17 and its signalling have been described in many pathological conditions such as brain ischemia (Ciana et al., 2006; Lecca et al., 2008), cuprizone- and LPC-induced demyelination (Coppolino et al., 2018; Nyamoya et al., 2019), traumatic brain injury and Alzheimer's like conditions (Boda et al., 2011), experimental autoimmune encephalomyelitis (EAE) (Chen et al., 2009; Coppolino et al., 2018). Marked GPR17 upregulation has been confirmed also in patients affected by multiple sclerosis (Chen et al., 2009), traumatic brain injury (Franke et al., 2013) and congenital leukoencephalopathy (Satoh et al., 2017). In both rodents and humans, early after CNS damage, GPR17 is upregulated also in cells that do not normally express the receptor, like dying neurons inside the lesion core and in infiltrating microglia/macrophages, suggesting a role in acute neuronal death and in the initial phases of the consequent immune cell response (Ceruti et al., 2009; Franke et al., 2013; Lecca et al., 2008; B. Zhao et al., 2012). However, in the case of activated microglia/macrophages in close proximity to the site of injury, it is not clear whether GPR17 staining could be the result of phagocytosis of dying cells. GPR17 knockdown was able to attenuate neuronal damage and detrimental microglia activation in mixed neuron-glia co-cultures exposed to oxygen-glucose deprivation (Zhao et al., 2018). In a similar fashion, very early pathological GPR17 upregulation in degenerating oligodendrocyte precursors inside the lesion might be linked to the activation of apoptotic processes within these cells (Ou et al., 2016). Accordingly, both genetic overexpression and pharmacological activation of GPR17 by non-selective agonist

MDL29,951 in oligodendrocytes have been demonstrated to induce upregulation of the pro-apoptotic gene Xaf1 and downregulation of c-Fos, a downstream factor in the PKA pathway regulating cell survival (Ou et al., 2016), thus confirming previous *in vitro* studies showing a link between GPR17 expression and reduced OPC survival in the presence of high concentration of ATP (Ceruti et al., 2011). To reconcile these data with the "trophic" and beneficial effect of GPR17 under pseudo-physiological conditions, we hypothesize that, when damage occurs, repair signalling molecules (like nucleotides, CysLTs, oxysterols and chemokines) acting as emergency signals are released to stimulate GPR17 expression and initiate OPC differentiation (see also 3.2). However, if these extracellular signals persist for long times, as it is the case for heavy or chronic damage, GPR17 becomes pathologically overexpressed, which prevents its physiological downregulation at late stages and eventually impedes maturation to myelinating phenotypes. This hypothesis is sustained by the demonstration that, under all the described neurodegenerative paradigms, GPR17-expressing OPCs do invariably show morphological features that are typical of immature phenotypes, suggesting that these cells are blocked at premyelinating stages (Fumagalli et al., 2016). As a consequence of this block and of the inability to resolve inflammation, cells are then committed to programmed cell death (Fumagalli, Lecca, Coppolino, Parravicini, & Abbracchio, 2017).

3.2 Role of GPR17 in remyelination

GPR17 plays a pivotal role in chronic tissue remodeling after injury. As summarized in paragraph 3.1, damaging insults stimulate GPR17 expression to initiate differentiation of normally quiescent OPCs. In this context, GPR17 overexpression in OPCs, taking place at a post-acute stage of disease, resembles the expression pattern of the receptor during developmental oligodendrogenesis and may represent an attempt to start remyelination by generating new mature oligodendrocytes (Boda et al., 2011). Fate mapping analysis performed in the recently generated GPR17-iCreER^{T2}:CAG-eGFP conditional reporter mouse line (Viganò et al., 2016) has indeed confirmed that, despite quiescence

under physiological conditions, the subpopulation of GPR17-expressing NG2-glia resumes its differentiation program after induction of a brain insult, indicating that these cells may represent a pool maintained in the brain parenchyma to quickly and efficiently drive regenerative processes once needed. Consistently, more or less at the same time after damage, GPR17-expressing OPCs start to proliferate and to migrate toward lesion boundaries and, in the presence of a permissive environment (see also 3.3), begin to express mature oligodendrocyte markers, proving that differentiation of these cells into myelinating oligodendrocytes is occurring (Bonfanti et al., 2017; Coppolino et al., 2018). However, despite this initial pro-regenerative response, GPR17 upregulation in OPCs persists over time, "freezing" these cells at an immature stage. A causal role of GPR17 pathological upregulation in remyelination failure is directly confirmed by the fact that GPR17-overexpressing transgenic mice exhibit the prototypic features of demyelinating disorders, displaying impaired formation of new myelin sheaths and reactive gliosis (Chen et al., 2009). On the other hand, GPR17 knockout mice manifested enhanced capacity to remyelinate lesions caused by LPC-induced demyelination (Lu et al., 2018; Ou et al., 2016), an effect that has been related to several intracellular pathways involved in oligodendrocyte differentiation, including activation of the cAMP/PKA/Epac1 pathway (Ou et al., 2016) and increased phosphorylation of Erk1/2 (Lu et al., 2018).

3.3 Relevance of neuroinflammation for GPR17-based therapeutic approaches

Among the possible causes underlying differentiation defects of GPR17-expressing OPCs, the local inflammatory environment is attracting great interest due to its widely recognized role in shaping structural and functional plasticity within the CNS in both physiological and pathological conditions (Peruzzotti-Jametti et al., 2014). Microglia switch toward a pro-regenerative phenotype has been proven to be essential for efficient remyelination by activated OPCs (Lloyd & Miron, 2019). On the contrary, it has been shown that inflammatory-activated microglia can hamper OPC-mediated myelin repair through many different mechanisms, including release of extracellular vesicles able to induce

harmful astrogliosis (Lombardi et al., 2019). Detrimental activation of microglia and astrocytes might participate to GPR17 overexpression and differentiation failure in the EAE model of multiple sclerosis, which is characterized by a very strong inflammatory substrate (Brambilla, 2019), while the same OPC subpopulation was able to efficiently differentiate in a context of less pronounced inflammation characterizing the cuprizone-induced demyelination model (Coppolino et al., 2018). Similarly, chronic inflammation may be responsible for aberrant GPR17 upregulation, leading to the impaired differentiation capability of GPR17-expressing OPCs observed in models of brain ischemia (Bonfanti et al., 2017) and LPC-induced demyelination (Ou et al., 2016). Based on this, therapeutic approaches aimed at targeting only GPR17 might be ineffective if a permissive local environment for efficient remyelination is not concomitantly favored. On the other hand, the simultaneous targeting of GPR17 and immune responses by combined therapies or multimodal modulators (like extracellular vesicles or miRNAs) may represent a better strategy to fully exploit GPR17 as a potential target to promote brain repair. In accordance, treatment with the non-selective GPR17 antagonist montelukast, a CysLTR antagonist, has shown to produce beneficial effects on brain connectivity and functional recovery of ischemic mice likely by simultaneously promoting GPR17-expressing OPC differentiation and by modulating microglial activation (Gelosa et al., 2019). Similarly, GPR17 inhibition by pranlukast treatment promoted ~~OL~~ oligodendrocyte differentiation after LPC-induced demyelination, also likely to a concomitant effect on brain resident immune cells (Ou et al., 2016). Of note, an akin strategy has been proven to be effective also for protecting the brain during physiological aging (Marschallinger et al., 2015), a process characterized by immune cell dysfunction and basal chronic inflammation as well (Colonna & Butovsky, 2017). Recently, the CysLTR2 antagonist HAMI3379 has been shown to promote oligodendroglial differentiation by exerting an inhibitory activity on GPR17, although a beneficial action on microglia expressing CysLTR2 requires further investigations (Merten et al., 2018). Very relevant to this topic, dual antagonists for both GPR17 and the pro-inflammatory purinergic receptor $P2Y_2$ have been generated (Pillaiyar et al., 2019). These compounds may represent multi-target drugs displaying high potential for the treatment

of inflammation-associated neurodegenerative diseases, but their impact on neuroinflammation and myelin repair remains to be evaluated. In summary, GPR17 overexpression at the site of brain damage represents a spontaneous attempt by glial cells to promote tissue repair. Despite that, persistent GPR17 upregulation at late disease stages stuns regenerative responses in the CNS and it is likely driven by unfavorable local inflammation. Thus, combined therapeutic approaches aimed at simultaneously promoting repair via GPR17 and attenuating endogenous inflammation may lead to novel advances in regenerative medicine.

4. Conclusions: implications for future therapeutic approaches targeting GPR17

Since its original identification in 2006, a large amount of evidence has been accumulating to support a key role for GPR17 in OPC function (not only differentiation, but also proliferation and migration) as well as in myelin formation and repair. A dual, time dependent role of GPR17 in OPC maturation has emerged, with a stimulatory effect in early progenitors, followed by its physiological downregulation in advanced, but still immature, OLs. These data suggest that GPR17 initially behaves as a trigger to shift proliferating progenitors towards differentiation, and then acts as a "brake" to prevent terminal maturation until cells are fully ready and prepared to synthesize myelin and myelinate axons.

Crucial insights in GPR17 pathophysiology have come from the demonstration that inflammation-associated chronic neurodegenerative conditions are invariably characterized by abnormal and persistent GPR17 upregulation, which is, in turn, accompanied by "freezing" of OPCs at immature pre-myelinating stages. Specifically, our data suggest that (i), during acute inflammation, in an initial attempt to induce repair, chemical mediators like CysLTs, extracellular nucleotides, cytokines and reactive species like oxysterols can all spuriously act to stimulate OPC maturation via GPR17; and (ii), if acute inflammation is not resolved, these attempts eventually fail, due to the fact that excessive and prolonged receptor activation prevents its downregulation and results in blockade of cells'

terminal maturation. Thus, chronic inflammation promotes an unfavorable local milieu contrasting the acquisition of fully mature functional oligodendrocyte phenotypes.

On this basis, as already mentioned, anti-inflammatory approaches could result in beneficial effects, since they would promote a more permissive local environment attenuating GPR17 overexpression and helping immature oligodendrocytes completing maturation. Of course, we envisage that, either utilized alone or in combination with anti-inflammatory agents, GPR17 ligands could favorably accelerate this process by finely regulating receptor activation.

However, there still is a lot of debate on the *type* of GPR17 ligand needed for beneficial effects. On one hand, antagonist ligands could succeed in triggering remyelination and repair, since they would contrast the excessive GPR17 overactivation associated to chronic inflammatory states. This is supported by the demonstrations that the potent, albeit not selective, GPR17 antagonist montelukast attenuated stroke associated damage and favored remyelination in the MCAo brain ischemia model (Gelosa et al., 2019), and proved able to contrast microglia activation, neuroinflammation and cognitive impairment in aged rats (Marschallinger et al., 2015). Similarly, pharmacological inhibition of GPR17 with pranlukast has been shown to promote remyelination in the LPC-induced demyelination model (Ou et al., 2016). On the other end, use of antagonists could dangerously contrast spontaneous OPC maturation and myelination in CNS areas not directly involved in inflammation, thus possibly causing side effects. Theoretically, mixed agonist/antagonist GPR17 ligands would represent ideal therapeutic entities, being able to adjust their effects to specific GPR17 receptor states. Such ligands would indeed be able to act as antagonists on cells overexpressing GPR17 inside and at the borders of demyelinated lesions, thus inducing receptor blockade and helping cells resuming maturation; at the same time, due to their intrinsic activity, such compounds would not completely block GPR17 activity on OPCs distal to the inflamed demyelinated area, thus avoiding any potential interference with physiological myelination. Several groups are currently developing ligands for GPR17 (Capelli et al., 2019; Eberini et al., 2011; Pillaiyar et al., 2019) which will

hopefully reveal adequate features to finely regulate the activity of this receptor and ameliorate endogenous OPCs reparative effects.

Author contributions

All authors: conception of idea, review of the literature, figure preparation and manuscript writing and editing.

Figure legends

Figure 1. Regulation of GPR17 expression at transcriptional level. In the early phases of OPC differentiation, the binding of the morphogen Shh with the receptor PTCH1 abrogates its inhibitory effect on the downstream receptor SMO (1), resulting in reduced conversion of the full-length transcription factors Gli2/3 into their repressor forms and in consequent enhanced translocation of the active Gli2/3 into the nucleus (2) (Yatsuzuka et al., 2019). The active transcription factors induce the expression of their target Olig1/2 (3), which in turn stimulate~~s~~ GPR17 expression, thus promoting increased levels of the receptor on the plasma membrane (4) (Yatsuzuka et al., 2019). Sustained expression of GPR17 and its downstream signalling triggers the expression of the transcription factor ID2 and promotes its nuclear translocation (5) (Chen et al., 2009). Nuclear ID2 acts as an inhibitory signal preventing Olig1 activity on GPR17 promoter (6), thus generating a negative feedback loop which may serve to keep OPCs in an immature stage (Chen et al., 2009).

Figure 2. Intracellular trafficking of GPR17 receptor. Upon interaction between GPR17 and its agonists, the C-terminus of the receptor is readily phosphorylated by GRKs with consequent recruitment of β-Arrestin (1) (Daniele et al., 2011; Daniele et al., 2014). This cascade of events triggers the internalization of the receptor (2), which may be followed by either recycling on the membrane or delivery to the lysosomes for degradation. Of note, interaction of SNX27 with the C-terminus of GPR17 is required for the efficient recycling of the receptor to the plasma membrane (3),

while in the absence of SNX27 the lysosomal degradative pathway seems to be preferred (4) (Meraviglia et al., 2016). The mTOR pathway plays a crucial role in orchestrating these processes. In fact, when mTOR is active, nuclear translocation of the ubiquitin ligase Mdm2 is favored (5). On the contrary, inhibition of mTOR by rapamycin (6) maintains high levels of Mdm2 in the cytoplasm, thus promoting the ubiquitination and consequent degradation of GRKs (7) that prevent ligand-induced GPR17 desensitization and internalization (Fumagalli et al., 2015).

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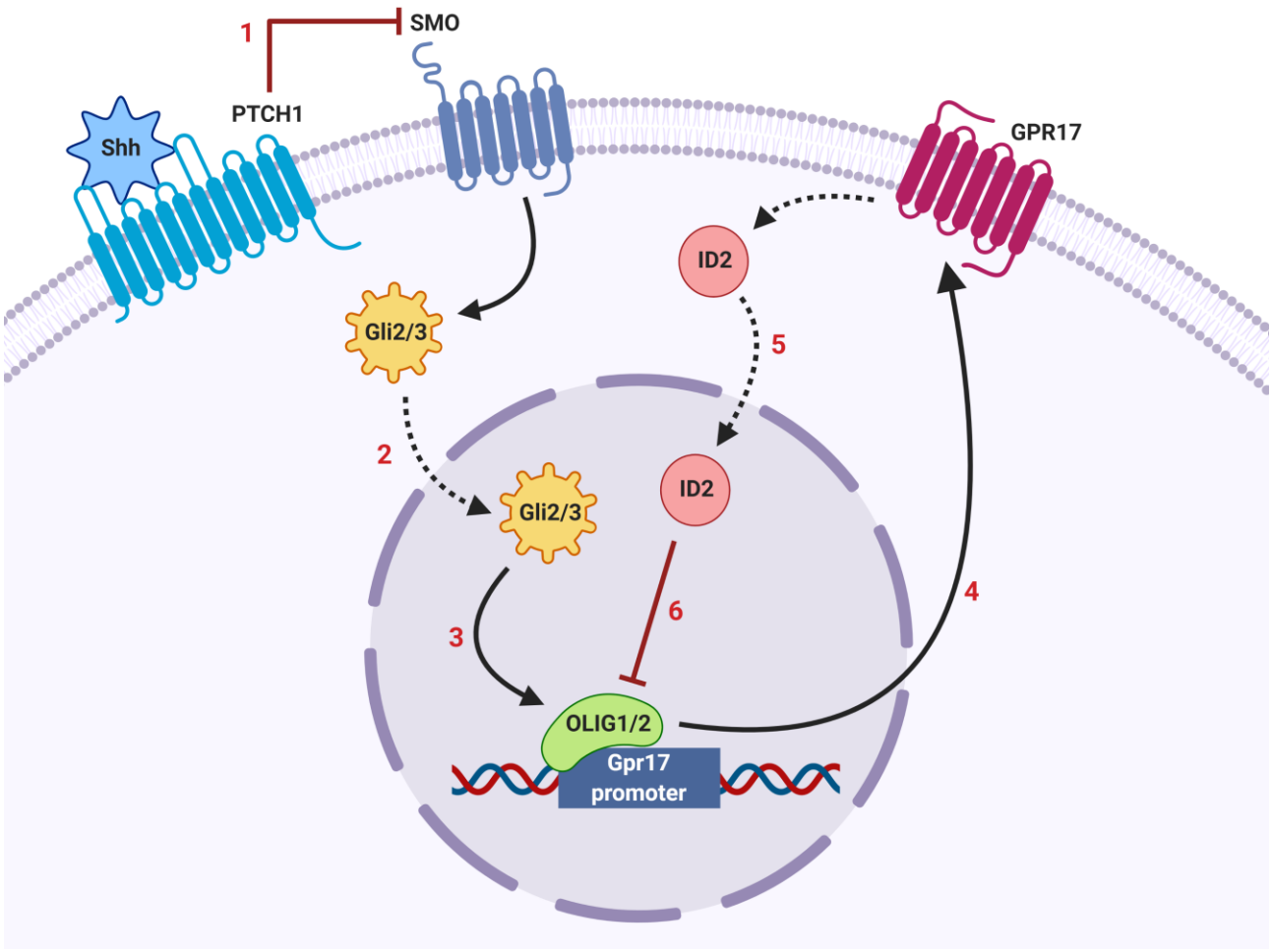

Figure 1.

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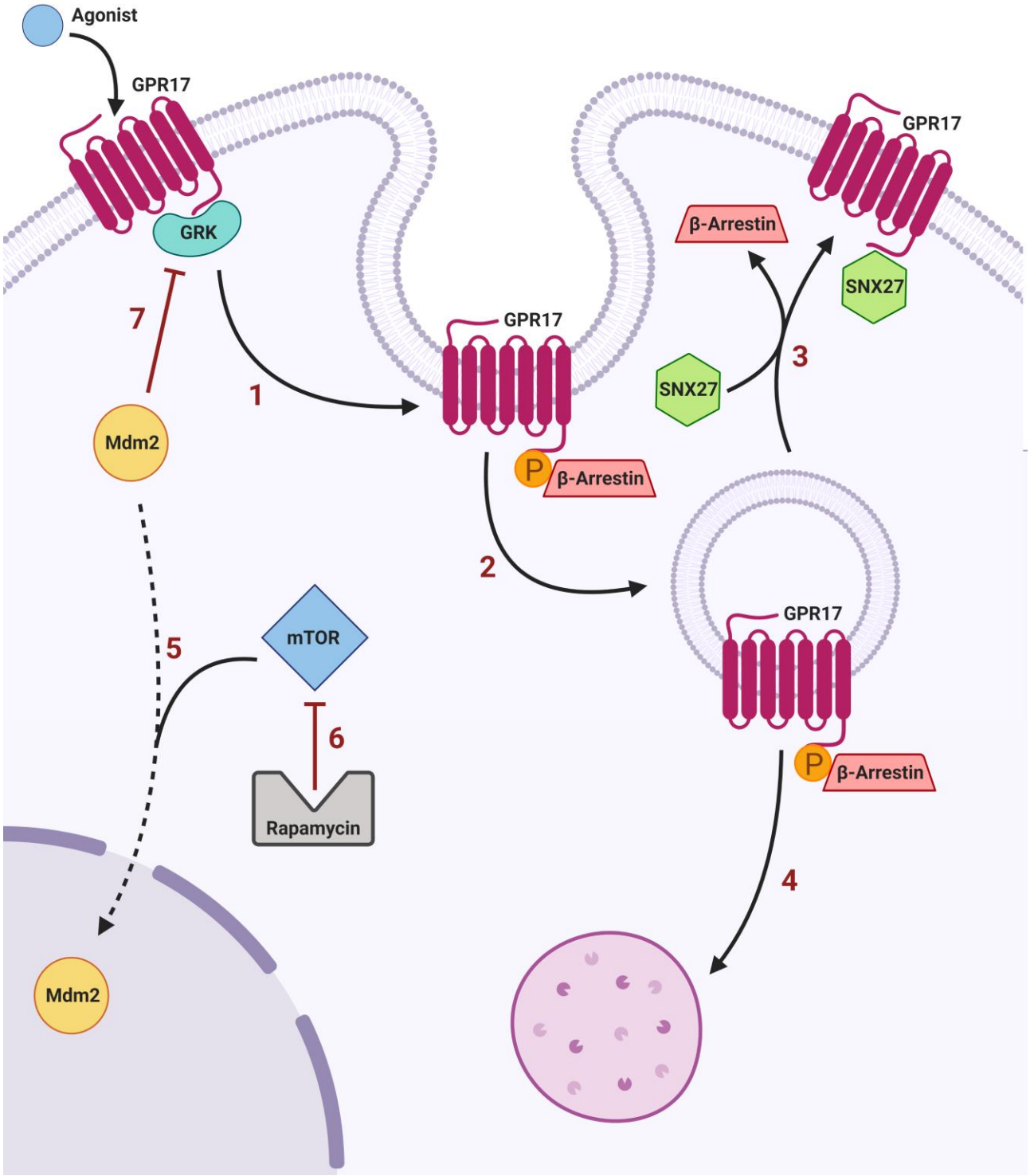

Figure 2.