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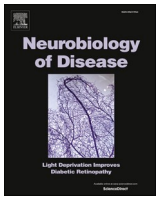

G protein-coupled receptor 17 is regulated by WNT pathway during oligodendrocyte precursor cell differentiation

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ABSTRACT

G protein-coupled receptor 17 (GPR17) and the WNT pathway are critical players of oligodendrocyte (OL) differentiation acting as essential timers in developing brain to achieve fully-myelinating cells. However, whether and how these two systems are related to each other is still unknown. Of interest, both factors are dysregulated in developing and adult brain diseases, including white matter injury and cancer, making the understanding of their reciprocal interactions of potential importance for identifying new targets and strategies for myelin repair. Here, by a combined pharmacological and biotechnological approach, we examined regulatory mechanisms linking WNT signaling to GPR17 expression in OLs. We first analyzed the relative expression of mRNAs encoding for GPR17 and the T cell factor/Lymphoid enhancer-binding factor-1 (TCF/LEF) transcription factors of the canonical WNT/β-CATENIN pathway, in PDGFRα$^+$ and O4$^+$ OLs during mouse post-natal development. In O4$^+$ cells, *Gpr17* mRNA level peaked at post-natal day 14 and then decreased concomitantly to the physiological uprise of WNT tone, as shown by increased *Lef1* mRNA level. The link between WNT signaling and GPR17 expression was further reinforced *in vitro* in primary PDGFRα$^+$ cells and in Oli-neu cells. High WNT tone impaired OL differentiation and drastically reduced GPR17 mRNA and protein levels. In Oli-neu cells, WNT/β-CATENIN activation repressed *Gpr17* promoter activity through both putative WNT response elements (WRE) and upregulation of the inhibitor of DNA-binding protein 2 (*Id2*). We conclude that the WNT pathway influences OL maturation by repressing GPR17, which could have implications in pathologies characterized by dysregulations of the OL lineage including multiple sclerosis and oligodendroglioma.

1. Introduction

In the central nervous system (CNS), myelination is the result of a highly regulated developmental program beginning with specification of oligodendrocyte (OL) precursor cells (OPCs) during late embryonic gestation. Later on, OPCs proliferate, migrate and differentiate, leading to the expression of 2′,3'-Cyclic-nucleotide 3′-phosphodiesterase (CNPase), a myelin-associated enzyme, and a large amount of myelin-associated proteins including myelin basic protein (MBP) to start the assembly of myelin sheaths enwrapping axons (Barateiro and Fernandes, 2014; Nawaz et al., 2015). In humans, CNS myelination is a long-lasting process starting during the fourteenth week of fetal development and lasting at least until late adolescence, whereas in mice, the majority of CNS myelination occurs within the first two postnatal months. OL maturation from OPCs into mature and myelinating OLs involves a sequence of different developmental stages each characterized by an

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increasingly complex morphology, different metabolic profile (Marangon et al., 2022) and the expression of stage-specific markers ($NG2^+$/$PDGFR\alpha^+$ OPCs, $NG2^+$/$PDGFR\alpha^+$/$O4^+$ pre-OLs, $O4^+$/CNP^+ immature OLs, $O4^+$/CNP^+/MBP^+ mature OLs) (Marinelli et al., 2016). OL specification and differentiation toward fully-myelinating cells is under the control of a number of intrinsic and extrinsic factors (Mitew et al., 2014). Among them, the G protein-coupled receptor 17 (GPR17) has emerged as an intrinsic key timer which displays a promiscuous pharmacological profile ((Lecca et al., 2020), review). GPR17 is maximally expressed by a subset of $NG2^+$ OPCs during their transition to immature OLs and is then downregulated in OLs before terminal maturation (Fumagalli et al., 2011). Accordingly, GPR17-expressing OPCs are already present in mice at birth, increase over time, reaching the highest protein expression level between post-natal day (P)10 and P14 before the peak of myelination, and then decline (Boda et al., 2011). Any alteration in GPR17 precise expression pattern results in myelination defects both *in vitro* and *in vivo* (Fumagalli et al., 2011; Chen et al., 2009; Fumagalli et al., 2015). In primary rat OPC cultures, GPR17 silencing at early stage of differentiation profoundly affected their ability to generate mature OLs (Fumagalli et al., 2011). Conversely, forced *in vitro* GPR17 expression throughout the entire differentiation program impaired maturation, confirming that precisely time bounded expression of GPR17, followed by its downregulation at late stages, is necessary for OPC differentiation (Fumagalli et al., 2015). In line with this result, *CNP-GPR17* transgenic mice aberrantly and untimely overexpressing GPR17 in immature CNP^+ OLs during late stage of oligodendrocyte maturation showed myelination defects (Chen et al., 2009). The OL lineage is also subjected to extrinsic regulators including WNT (Wingless-type MMTV integration site family) (Guo et al., 2015; van Tilborg et al., 2016; van Tilborg et al., 2018). WNT/ β-CATENIN signaling, also named as the canonical WNT pathway, activates the TCF/LEF (T cell Factor/ lymphoid enhancer factor family) transcription factor family which encompasses four members: TCF7, TCF7L1, TCF7L2 (transcription factor 7 and transcription factor 7-like 1 and 2) and LEF1 (Lymphoid enhancer-binding factor 1) all containing a β-catenin-binding domain at the N-terminus and a high-mobility group (HMG) DNA-binding domain (Cadigan and Waterman, 2012). WNT/β-CATENIN signaling has distinct roles in oligodendrogenesis, OL differentiation, and myelination in a context-dependent manner, depending both on the CNS region and the developmental stage. A strong consensus has been reached that, in a similar way to GPR17 (Lecca et al., 2020), any dysregulation of WNT/β-CATENIN signaling activation exerts an inhibitory effect on post-natal differentiation of OLs (Guo et al., 2015). An OL "high WNT tone" associated to myelination impairment has been defined as a pathological hallmark of developmental white matter diseases in hypoxic-ischemic encephalopathy and periventricular leukomalacia as well as in multiple sclerosis (MS). Of note, glioma cell development has been concomitantly linked to aberrant activation of WNT signaling and dysregulation of GPR17 expression: WNT pathway genes overexpression has been associated with increased tumor growth and invasion *via* the maintenance of stemcellness leading to poor prognosis in glioma patients; on the contrary, GPR17 levels in tumor samples are reduced, and its activation inhibits glioma formation. Thus the balance between WNT and GPR17 might be dysregulated in glioma (Lee et al., 2016; Liu et al., 2021).

Understanding the intimate interplay between the GPR17 and the WNT systems during oligodendrogenesis is of outmost importance to fully understand the myelination process under normal and pathological conditions. In this study, we used a combination of *ex vivo* analysis of murine OPCs, pre-OLs and immature OLs, combined to *in vitro* mechanistic analyses in primary OPC cultures and Oli-neu cell line, to investigate the effect of WNT signaling pathway on the expression of GPR17 and its consequences on OL maturation. Our results suggest that high WNT tone disrupts OL maturation by upregulating the inhibitor of DNA-binding protein 2 (ID2), which, in turn, participates to GPR17 downregulation, whose expression is necessary to allow pre-OLs to proceed to fully mature myelinated cells.

2. Materials and methods

2.1. Animals

Experiments were performed with male OF1 strain mice pups from adult females purchased from Charles River (L'Arbresle, France). Samples of tissue has been collected according the directive 2010/63/EU of the European Parliament.

2.2. FACS analysis

After removing the cerebellum and olfactory bulbs, the brains were dissociated into single cell suspensions using the Neural Tissue Dissociation Kit containing papain (Miltenyi Biotec, Germany) (Post-natal day <10) or Adult Brain dissociation kit (Miltenyi Biotec, Germany) (Post-natal day 10 to 21) and the gentleMACS Octo Dissociator with Heaters (Miltenyi Biotec, Germany). Cells were filtrated on cell strainers (70 μm, Miltenyi Biotec, Germany).

After counting and resuspension at 10×10^6 cells/ml in FACS buffer (Dulbeccos's Phosphate Buffer Saline; Gibco Life Technologies, UK), 2 mM Ethylenediaminetetraacetic acid (EDTA; Sigma Aldrich, USA), 0.5% Bovine Serum Albumin (BSA; Miltenyi Biotec GmbH, Germany), cells were incubated with Fc blocker (BD Biosciences, France), then fluorophore-conjugated antibodies against mouse O4 and PDGFRα (both Miltenyi Biotec) or their corresponding control isotypes (Miltenyi Biotec), and viability marker (FVS 780, BD Biosciences) at concentrations recommended by the manufacturers or calculated after titration. Cells were washed with FACS buffer, resuspended in PBS (Phosphate Buffer Saline) and FACS analysis was done within 24 h.

After doublets exclusion based on morphological parameters, we analyzed the percentages of $PDGFR\alpha^+$, $O4^+$, and $PDGFR\alpha^+$/$O4^+$ cells.

2.3. O4+ and/or PDGFRα cell sorting

Brain cell suspensions were obtained as described above (see FACS analysis). $O4^+$ positive cells were first isolated using anti-O4 MicroBeads (Miltenyi Biotec, Germany) and negative fractions were then incubated with anti-PDGFRα MicroBeads (Miltenyi Biotec, Germany) to isolate PDGFRα-positive cells according to manufacturer instructions and as previously described (Boccazzi et al., 2021; Schang et al., 2022).

2.4. Primary $PDGFR\alpha^+$ OPC cultures

Brain single cell suspensions from post-natal day 5 pups were prepared using Neural Tissue Dissociation Kit containing papain (Miltenyi, Germany). PDGFRα-positive cells were isolated using the anti-PDGFRα MicroBeads (Miltenyi Biotec, Germany). PDGFRα-positive cells were cultured on poly-D-lysine-coated 24-well plates (30,000 cells/well) in proliferation medium consisting of Neurobasal (Gibco, France) supplemented with 2% B27 (Gibco, France), 1% penicillin/streptomycin, 20 ng/ml PDGF-AA, and 40 ng/ml bFGF (Sigma-Aldrich, France). After 3 days in proliferation medium, cells were switched to differentiation medium consisting of DMEM/High Glucose (Gibco, France) supplemented with 1% N2, 2% B27, 1% penicillin/streptomycin, 0.01% BSA, 10 nM d-biotin, 5μg/ml *N*-acetyl-cysteine and 40 ng/ml T3 (Sigma-Aldrich, France). Cells were treated with WNT Agonist II (SKL2001, Calbiochem, USA), at different concentrations (3 or 10 μM) or with vehicle and fixed after 24, 48 and 72 h for immunocytochemistry (ICC) analysis (see below).

2.5. Oli-neu cell line cultures

The OL precursor cell line Oli-neu was cultured in the proliferative medium DMEM Glutamax high glucose (Gibco, France) supplemented

with 1% horse serum, 1% of N2 supplement (Gibco, France), 10 μg/ml insulin, 0.35 μg/ml 3′,5-Triiodo-L-thyronine, 0.4 μg/ml L-Thyroxine, and 1% penicillin/streptomycin (Sigma-Aldrich, France). When cells reached 60% of confluence, 1 μM of PD174265 (EGFR tyrosine kinase inhibitor, ChemCruz, USA) was added to the medium to differentiate cells. Proliferative cells were maintained in proliferative medium containing 0.1% of DMSO.

2.6. Pharmacological modulation of WNT Pathway in Oli-neu cell line

Vehicle (DMSO 0.1%) or pharmacological treatments with WNT agonist II (Calbiochem, USA), CT99021 (Selleck Chemicals, USA) and XAV939 (Sigma-Aldrich, France) were added with PD174265 at the beginning of differentiation. Vehicle corresponded to medium containing 0.1% of DMSO.

2.7. Plasmid constructs and transfection assays in Oli-neu cell line

The human *GPR17* promoter luciferase construct was previously described (Fratangeli et al., 2013). The mouse *Gpr17* promoter was cloned and subsequently modified in the PGL4.17 vector (Promega, France) upstream to the firefly luciferase reporter gene using the NEBuilder technology (New England Biolabs, France). The mouse 1.1 kb promoter is the antisense mm10 sequence Chr18:31,949,371-31,950,469, corresponding to the −2460/−1362 region relative to the transcription start site, the A of the ATG codon being considered at position +1 (mm10 Chr18:31,948,009). The sequence was inserted between the *Hin*dIII and *Xho*I sites. Constructs comprising different mutated promoters (targeted mutations) were generated using Q5 Site-directed mutagenesis kit (New England Biolabs, France) according to manufacturer's protocol. Two targeted mutations were generated: WRE1 (TCTTTG to CCCTCG at position −2416/−2411) and WRE2 (CAAAGA to CCAGGG at position −1850/−1845). To overexpress GPR17, a plasmid encompassing the coding sequence of the mouse *Gpr17* gene under the control of the CMV promoter, referred as pcDNA3.1-GPR17 (Lecca et al., 2008), was used. pcDNA3.1-EGFP (Promega, France) plasmid was used as a control.

For transient transfection assays of luciferase plasmids and siRNA, human *GPR17* or mouse *Gpr17* promoter luciferase fusion constructs (100 ng/well) were mixed with pRL-SV (10 ng/well in 96-well plates). For modulation of *Id2* expression, a mix of siRNA containing anti-*Id2*-siRNA (Qiagen, SI010727–43, −50, −57, −64) and Scramble siRNA (Qiagen, Ctrl siRNA 102,781) was used to obtain 0, 5, 10, 25 or 50 nM of anti-*Id2*-siRNA. Scramble siRNA was used as negative control siRNA to reach 50 nM of total siRNA in each condition. The siRNA mixes were added to luciferase plasmids or used alone for RT-qPCR analysis. Plasmid DNA and/or siRNA were mixed with MACSFectin reagent (Miltenyi Biotec, Germany) in OptiMEM (Gibco, France) for 20 min according to manufacturer's protocol (0.5 μl MACSFectin and 50 μl OptiMEM per well for luciferase experiments in 96 well plates, 4 μl MACSFectin and 200 μl OptiMEM per well for RT-qPCR experiments in 12 well plates). Transfection mix was added to Oli-neu suspension at 1.5×10^5 cells/ml just before plating in poly-L-lysine coated 96-well plates (luciferase experiments, 50 μl transfection mix added to 100 μl cell suspension) or 12 well plates (RT-qPCR, 200 μl transfection mix added to 1 ml cell suspension). After 48 h in proliferation medium, transfected cells were switched to differentiating medium containing 1 μM PD174265 and simultaneously exposed to 3 μM of WNT agonist II or DMSO (0.1%) during 24 h. Firefly and Renilla luciferase activities were evaluated using DualGlo Luciferase Assay System (E2920, Promega, France) according to manufacturer's protocol in a microplate luminometer (Berthold, Germany).

For transient transfection assays of the expression vectors, pcDNA3.1-GPR17, named GPR17 plasmid (Lecca et al., 2008), and pEGFP·N1, named CTRL plasmid, were used. Both *Gpr17* and *egfp* coding sequences inserted in these plasmids were under the control of CMV promoter. Cells were plated on poly-L-lysine coated glass coverslips in 24 well plates at the density of 32,000 Oli-neu cells per well. On the next day, cells were transfected with 1 μg plasmid using 2 μl of MACSFectin (Miltenyi, Germany) per well according to manufacturer's protocol. Briefly, plasmid and MACSFectin were mixed in 100 μl of Opti-MEM, incubated 20 min and added to the cell culture medium. 4 to 5 h after transfection, the medium was changed with proliferation medium. After 48 h in proliferation medium, transfected cells were switched to differentiating medium containing 1 μM PD174265 and simultaneously exposed to 3 μM of WNT agonist II or DMSO (0.1%) during 48 h. At the end of the treatment, cells were fixed with 4% paraformaldehyde during 20 min for subsequent immunostaining.

2.8. RT-qPCR

Total RNA was extracted with the NucleoSpin® RNA plus XS (Macherey-Nagel, Germany) according to the manufacturer's instructions. RNA concentration was assessed by spectrophotometry with the NanodropTM apparatus (ThermoFisher Scientific). Total RNA (100–500 ng) was subjected to reverse transcription using the iScriptTM cDNA synthesis kit (Bio-Rad). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed in triplicate for each sample using SYBR® Green Supermix (Bio-Rad) for 40 cycles with a 2-step program (5 s of denaturation at 95 °C and 10 s of annealing at 60 °C). Amplification specificity was assessed with a melting curve analysis. Primers were designed using Primer3 plus software (sequences are provided in **Supplementary Table 1**). Specific mRNA levels were calculated after normalization of the results for each sample with those for *Rpl13a* mRNA (reference gene). The data are presented as relative mRNA units with respect to control group (expressed as fold change over control value).

2.9. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and subjected to immunocytochemistry (ICC) as previously described (Ceruti et al., 2011). Mouse anti-CNPase (1/250; Millipore MAB326), mouse anti-NG2 (1:200; Abcam, UK), rabbit anti-GPR17 (1:100, custom antibody produced by PRIMM, Milan, Italy or, for Oli-neu ICC, CAYMAN #10136) and/or mouse anti-MBP (1:200, Chemicon MAB 382, Japan) were incubated overnight (4 °C) and the secondary antibodies goat anti-rabbit, goat anti-rat or goat anti-mouse antibodies conjugated to AlexaFluor®488 or AlexaFluor®555 were incubated 1 h at room temperature (1:600, Life Technologies, USA). Nuclei were counterstained with DAPI (Sigma-Aldrich, France). Cells were finally analyzed by a fluorescent microscope (Nikon Eclipse Ti-E).

The total number of PDGFRα$^+$ cells in each coverslip was evaluated by counting DAPI$^+$ nuclei, and the number of GPR17$^+$, MBP$^+$, or NG2$^+$ cells with the ImageJ (NIH) software. Number of cells was determined in 20–25 optical fields chosen according to a pre-established scheme to represent the entire slide under a × 20 magnification using Nikon Eclipse Ti-E microscope. For CNPase-IR, images were acquired using Nikon Eclipse Ti-E microscope. The number of DAPI$^+$ nuclei and the mean density of CNPase-IR were determined using an appropriate macro design with the ImageJ (NIH) software. 29–31 optical fields per group repeated on 2 independent experiments were analyzed.

2.10. Statistical analysis

Data were expressed as mean value with standard error of the mean (SEM). Using GraphPad Prism Software, data were tested for normality using the Kolmogorov–Smirnov normality test. Multiple comparisons in the same data set were analyzed by One way analysis of variance (ANOVA) with the Dunnett's or Bonferroni post-hoc test or Two way ANOVA with the Bonferroni *post hoc* test. Single comparisons were made using Student's *t*-test.

3. Results

3.1. *Gpr17* and *Tcf/Lef* transcription factors are modulated during OL differentiation

We first analyzed and quantified the PDGFRα$^+$ and O4$^+$ cell populations in the post-natal developing mouse cerebrum by a flow cytometry approach. As expected, PDGFRα$^+$ cells (OPC) were the first detectable cell population at P1 (Fig. 1a,b). Double PDGFRα$^+$/O4$^+$ cells (Pre-OLs) and O4$^+$ cells (immature OLs) appeared quickly (P4) with a gradual reduction of PDGFRα$^+$/ O4$^-$ cells becoming significant at P7, underlining the transition from OPCs to pre-OLs. Double PDGFRα$^+$/O4$^+$ cells (Pre-OLs) disappeared at P10 indicating the transition from pre-OLs to immature O4$^+$ OLs. At P14, only O4$^+$ immature OLs were detectable and their number continued to increase between P14 and P21.

We next performed sequential magnetic cell sorting of i) O4$^+$ cells, including double PDGFRα$^+$/O4$^+$ cells until P21; ii) PDGFRα$^+$ cells sorted from the negative fraction of O4$^+$ cells until 10 (time until which PDGFRα$^+$ cells were expressed). Thus, the O4$^+$ fraction includes pre-OLs and immature OLs, and the PDGFRα$^+$ fraction corresponds to O4$^-$ OPCs. As expected, in both fractions, we evidenced a significant increase in *Mbp* mRNA level between P1 and P7 by RT-qPCR, which was prolonged and amplified in the O4$^+$ subpopulation until P21 (Fig. 1c). We then analyzed factors controlling OL differentiation *i.e.* GPR17 and canonical WNT pathway by quantification of the relative expression of mRNA encoding GPR17 and TCF/LEF transcription factors in both PDGFRα$^+$ and O4$^+$ fractions. *Gpr17* mRNA level peaked in PDGFRα$^+$ and O4$^+$ cells at P7 and P14, respectively (Fig. 1d). In both cell populations, the increase *of Gpr17* mRNA was concomitant with significant up-regulation of *Mbp* mRNA level. While in PDGFRα$^+$ cells *Gpr17* expression already started to decrease at P7 (consistent with the reduction of this population along with development), in O4$^+$ cells, the *Gpr17* mRNA level

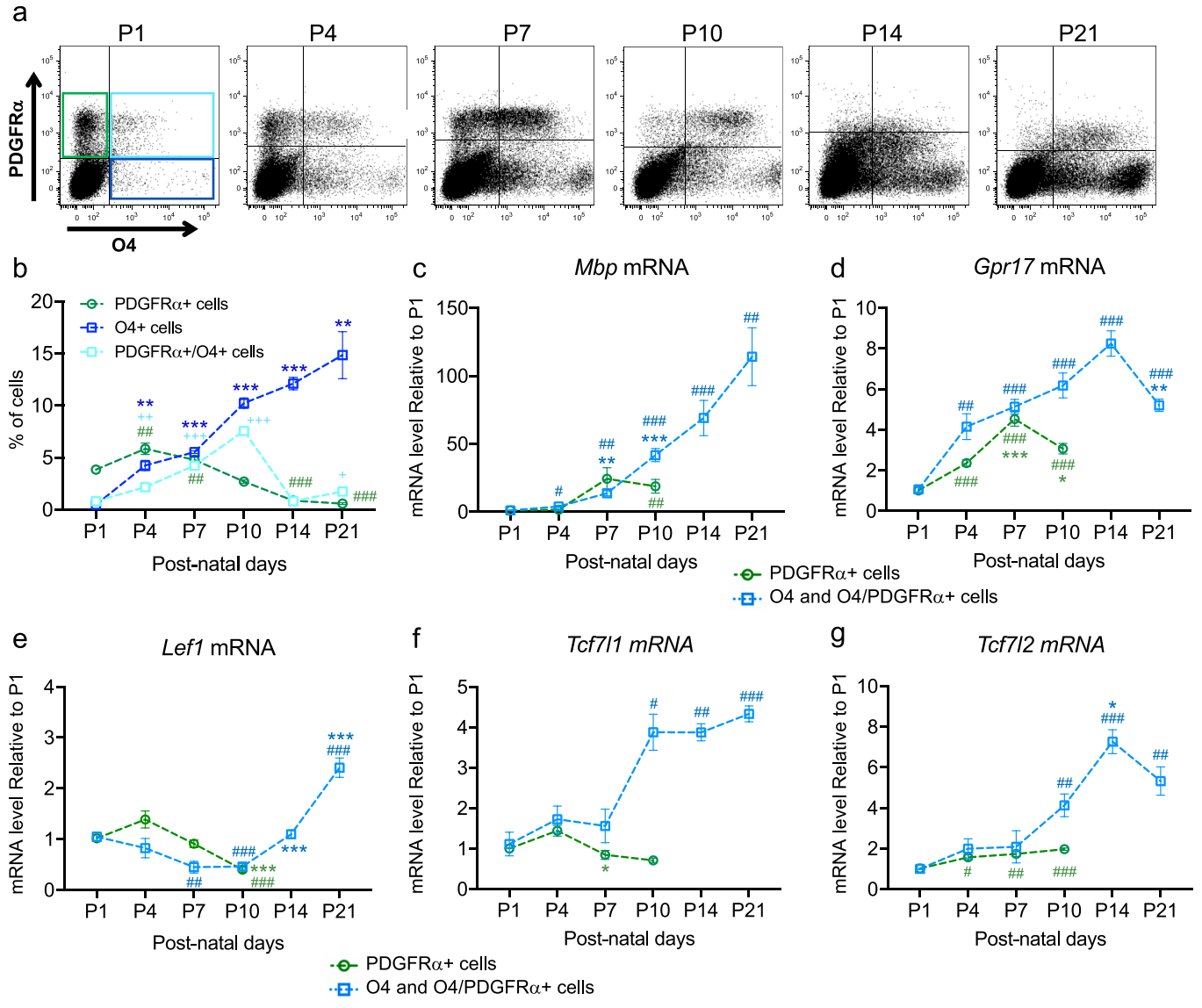

Fig. 1. *Gpr17* and *Tcf/Lef* expression during OL differentiation.
(**a**) Representative flow cytometric dot plot of PDGFRα-positive (+) cells and/or O4$^+$ cells from post-natal days (P) 1 to 21 in mouse brain. (**b**) Percentage (% of total alive cells) of PDGFRα$^+$ and/or O4$^+$ cells in P1 to P21 mouse brain. $N = 6$–7/group, Mean+/-SEM, One way ANOVA with Dunnett's post-hoc test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (**c-g**) RT-qPCR analysis of *Mbp* ($n = 9$–20/group), *Gpr17* (n = 9–21/group), *Lef1* ($n = 11$–21/group), *Tcf7l1* ($n = 4$–6/group) and *Tcfl2* ($n = 5$–13/group) mRNA levels in magnetically isolated PDGFRα$^+$ cells and O4$^+$ cells from P1 to 21 mouse brain. Relative expression to control group (P1), mean+/-SEM, One way ANOVA with Dunnett's post-hoc test: (#) means a significant difference with P1, $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$, (*) means a significant difference with the previous stage, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

decreased between P14 and P21 and was paralleled by a further increase in *Mbp* mRNA levels, signing the transition toward mature OLs. *Lef1* mRNA, a downstream mediator of the WNT/β-CATENIN signaling pathway, displayed a biphasic regulation during post-natal development with decreased expression until P10 in both populations. In $O4^+$ cells, *Lef1* mRNA significantly increased from P10 to P21 (Fig. 1e) concomitantly to increased myelination and the decrease of *Gpr17* mRNA expression. In addition, we assessed the expression of *Tcf7l1* and *Tcf7l2,* two other effectors of the WNT pathway. As expected, *Tcf7l2* mRNA, known to transiently and positively regulate post-natal OL differentiation independently of the WNT/β-CATENIN pathway (Hammond et al., 2015), was also significantly up-regulated in $O4^+$ cells (Fig. 1g). Finally, *Tcf7l1* mRNA expression level increased during oligodendrocyte differentiation (Fig. 1f).

3.2. WNT inhibits GPR17 expression and OPC differentiation

To analyze *Gpr17* mRNA regulation under canonical WNT pathway activation, we first used an *in vitro* model of PDGFRα$^+$ OPCs. Primary OPCs exposed to differentiation medium for 96 h expressed a significant increase in *Mbp* mRNA level overtime (Fig. 2a). As expected *Gpr17* mRNA level peaked at 72 h after differentiation and then started to decrease (Fig. 2b). We next exposed differentiating OPCs to the WNT agonist II, a selective inhibitor of GSK-3 (Glycogen synthase kinase-3), a key element of the multiprotein β-CATENIN destruction complex. WNT agonist II acts as WNT activator promoting β-CATENIN-induced transcription (Gwak et al., 2012). Induction of WNT signaling disrupted OPC differentiation as demonstrated by significant down-regulation of *Mbp* and *Cnp* mRNA in OPCs maintained in differentiating condition for 48 h and exposed to WNT agonist II 3 or 10 μM compared to differentiating cells receiving vehicle (Fig. 2c). A significant down-regulation of *Gpr17* mRNA concomitantly to the increase of *Lef1* mRNA occurred in WNT agonist II-treated differentiating cells (Fig. 2d). *Tcf7l1* and *2* mRNAs were significantly down-regulated in WNT agonist II-treated differentiating cells confirming that *Tcf7l1* and *2* transcriptional regulations do not depend on the WNT pathway activation (Fig. 2d). Finally, inhibitor of DNA-binding 2 (*Id2*) mRNA, that inhibits the differentiation of oligodendrocytes (Tiane et al., 2019) was significantly up-regulated in WNT agonist II-treated differentiating cells (Fig. 2d). At the protein level, WNT agonist II drastically decreased the number of GPR17$^+$ cells in a dose dependent manner during differentiation with no modification of the number of NG2$^+$ cells and inducing only a slight decrease in the number of DAPI$^+$ cells at 10 μM (Fig. 2e,f). This effect on cell density revealed an onset of cytotoxicity of the WNT agonist II at the highest dose used, consistent with the pro-apoptotic effects of WNT signaling (Ma et al., 2023). The reduction in the number of GPR17$^+$ cells triggered by WNT, already significant at the subcytotoxic concentration of 3 μM and exacerbated at 10 μM, was associated with a significant and dose-dependent decrease of MBP expression in WNT agonist II-treated cells compared to vehicle-treated cells (Fig. 2g). Thus, *in vitro* analysis of OPC differentiation suggested that forced WNT/β-CATENIN activation could induce a block of the cells at a very early stage of maturation as demonstrated by the reduction in GPR17 and MBP expression.

3.3. Pharmacological interference with WNT pathway modulates Gpr17 and Mbp mRNA expression in differentiating Oli-neu cells

To advance our understanding of the molecular interaction between GPR17 and WNT in the differentiation of OL, we evaluated the effect of different pharmacologically active compounds modulating the WNT pathway on the differentiation of Oli-neu, a stable mouse oligodendrocyte precursor cell line. We first demonstrated that *Gpr17* mRNA level peaked around four days of differentiation (triggered by 1 μM PD174265), to decline at Day 7 while *Mbp* mRNA persisted at high level (Fig. 3a). After 48 h in differentiating medium (differentiating cells) containing WNT agonist II (1 and 3 μM), Oli-neu differentiation arrest was demonstrated by dose-dependent and significant reduction of *Mbp, Cnp* and *Gpr17* mRNA and concomitant overexpression of *Lef1* mRNA compared to vehicle-treated differentiating cells (Fig. 3b). 10 uM of WNT agonist II demonstrated strong toxicity to Oli-neu cells. In agreement with the data obtained in primary OPCs, *Tcf7l1* and *2* mRNA were significantly down-regulated while *Id2* mRNA was significantly up-regulated in WNT agonist II-treated differentiating cells compared to vehicle-treated differentiating cells (Fig. 3c).

To confirm that canonical WNT pathway modulates *Gpr17* expression, we next exposed Oli-neu to differentiation medium containing CT99021 (1 to 10 μM, 48 h of exposure), another highly specific inhibitor of glycogen synthase kinase-3 (GSK-3) used as a WNT/ β-CATENIN signaling pathway activator (Ring et al., 2003). CT99021 induced a significant reduction of *Gpr17* and *Mbp* mRNA when compared to vehicle-treated differentiating Oli-neu cells (Fig. 3d). We next used XAV939 (1 to 3 μM), a tankyrase inhibitor that stabilizes the multiprotein β-CATENIN destruction complex composed by AXIN2, GSK-3 and APC (Adenomatous polyposis coli) (Fancy et al., 2011). Thus, XAV939 treatment results in an inhibition of the WNT/β-CATENIN pathway. In differentiated Oli-neu cells, 48 h of exposure to XAV939 induced a significant increase of *Mbp* and *Gpr17* mRNA when compared to vehicle-treated differentiating Oli-neu cells (Fig. 3e). Altogether, pharmacological approaches suggested that interference with WNT activity induced transcriptional modification of *Gpr17* expression.

3.4. High WNT activity represses human and mouse GPR17 promoters in differentiating Oli-neu cells

A human *GPR17* promoter of 1 kb length was previously cloned into a luciferase reporter vector and its activity characterized in Oli-neu cells (Fratangeli et al., 2013). Human *GPR17* promoter was found to be activated in Oli-neu after 48 h of differentiation, in agreement with increased *Gpr17* expression during the onset of differentiation (Fratangeli et al., 2013). To gain insights into the molecular mechanisms involved in WNT inhibitory effect on mouse *Gpr17* expression, we cloned a homologous 1099 bp mouse genomic fragment (−2460/−1362) into the same luciferase reporter vector (*MouseGpr17-Luc*). We compared the activity of human (referred here as *HumanGpr17-Luc*) and mouse promoters in proliferating and differentiating conditions after transient transfection in Oli-neu cells. After 24 h of differentiation, human *GPR17* and mouse *Gpr17* promoter activities were strongly increased (see black bars demonstrating tenfold activity) as compared to proliferating conditions (striped bars) (Fig. 4a). Moreover, in differentiating cells, WNT agonist II (3 μM) significantly decreased activities of human and mouse promoters (Fig. 4a). Thus, human *GPR17* and mouse *Gpr17* promoters displayed high and comparable activities in differentiating Oli-neu cells. The 1 kb DNA regions driving luciferase expression were sufficient to reproduce WNT inhibitory effect exerted on endogenous *Gpr17* expression.

3.5. High WNT activity inhibits mouse Gpr17 promoter activity through redundant promoter regions, including WRE motifs

We next analyzed the DNA sequence of the mouse *Gpr17* promoter to localize elements possibly involved in the inhibitory effect of WNT. Two core TCF/LEF-binding elements (CTTTG), (Medina et al., 2018), corresponding to potential WNT response elements (WRE), were identified and mutated separately (WRE1, WRE2) or together (WRE1/2) (Fig. 4b). Oli-neu cells transfected with the different mutated constructs were treated by 3 μM WNT agonist II (white bars) or vehicle (black bars) during 24 h in differentiating condition. As observed previously, WNT agonist II induced a 50% reduction of *MouseGpr17-Luc* activity. The three mutated constructs (WRE1, WRE2, WRE1/2) were also significantly inhibited by WNT agonist II. However, this inhibitory effect was significantly reduced for the double mutation WRE1/2 (25% inhibition). In addition, WRE mutations did not alter significantly promoter activity

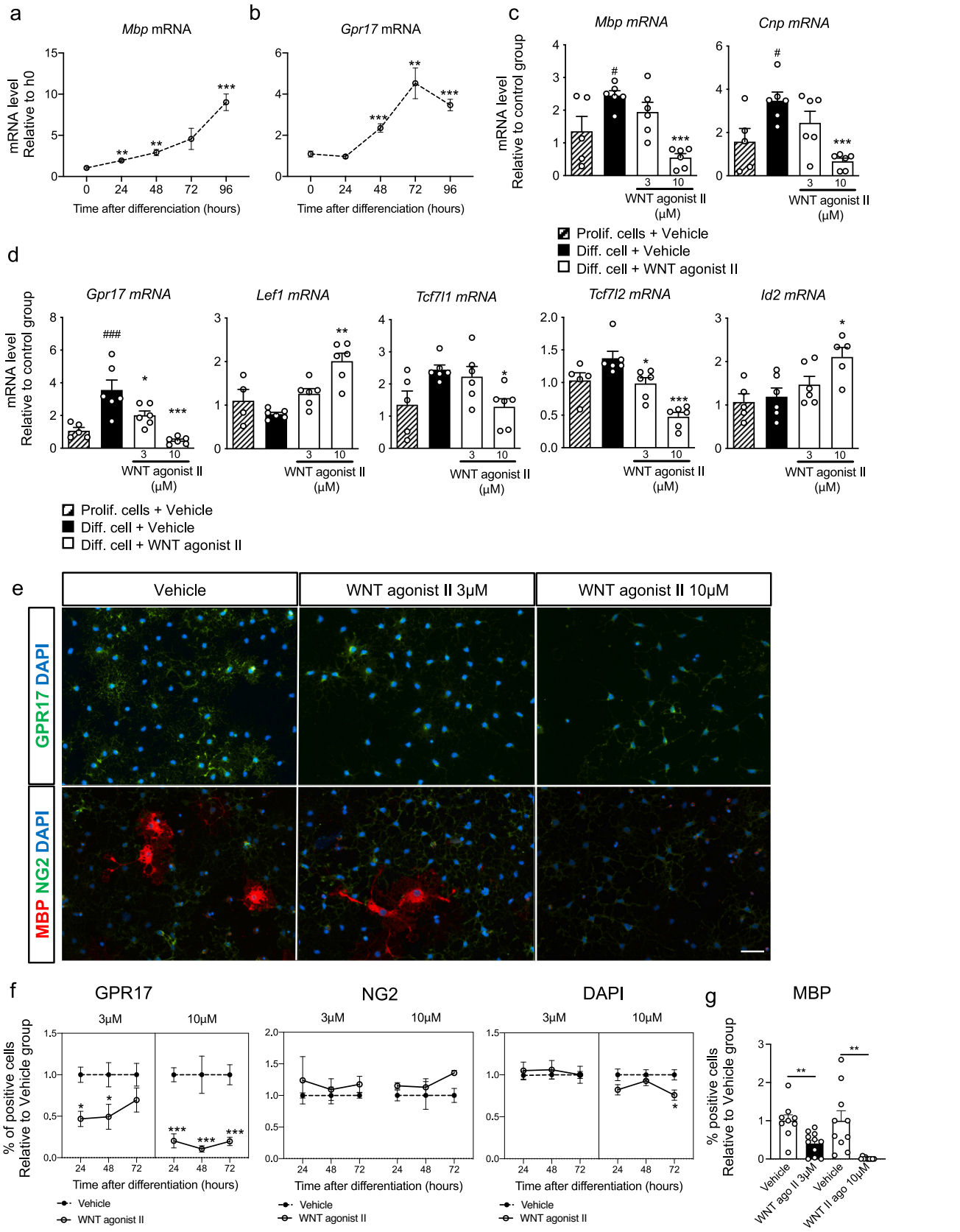

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Fig. 2. WNT activity inhibits GPR17 expression and OPC differentiation in primary PDGFRα$^+$ cultures.
(**a**,**b**) RT-qPCR analysis of *Mbp* (n = 9–20/group) and *Gpr17* (n = 9–21/group) mRNA in differentiating OPC cell cultures during 96 h. Relative expression to control group (H0). Mean+/-SEM. One way ANOVA with Dunnett's post-hoc test: (#) means a significant difference with H0, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$. (**c**) *Mbp* and *Cnp* mRNA and (**d**) *Gpr17*, *Lef1*, *Tcf7l1*, *Tcf7l2* and *Id2* mRNA in differentiated OPCs (Diff. cells) during 48 h and treated with 3 or 10 μM of WNT agonist II compared to Diff. cells receiving vehicle. Relative expression to control group (Prolif. cells + Vehicle). N = 5–6/group, mean+/-SEM, One way ANOVA with Bonferroni's post-hoc test: (#) means a significant difference with Prolif. Cells + Vehicle, #$p < 0.05$, (*) means a significant difference between WNT agonist II-treated Diff. cells and vehicle-treated Diff. cells ***$p < 0.001$. (**e**) Representative picture of GPR17 immunoreactivity (IR) (Top panel) and NG2-IR and MBP-IR (Bottom panel) in differentiated OPCs (Diff. cells) during 72 h and treated with 3 or 10 μM of WNT agonist II compared to Diff. cells receiving vehicle. Nuclei were labelled using DAPI. Scale bar = 70 μm (**f**) Percentage of GPR17$^+$, NG2$^+$ and DAPI$^+$ cells in primary OPCs at 24, 48 and 72 h of differentiation treated with 3 or 10 μM of WNT agonist II or with vehicle. Relative to control group (Vehicle-treated cells at each dose and stage of differentiation), n = 6–8/group, mean+/-SEM, Two-way ANOVA with Bonferroni's post-hoc test, (*) means a significant difference between WNT agonist II-treated OPCs and vehicle-treated OPCs, *$p < 0.05$, ***$p < 0.001$. (**g**) Percentage of MBP$^+$ cells in primary OPCs at 72 h of differentiation treated with 3 or 10 μM of WNT agonist II or with vehicle. Relative to control group (Vehicle-treated cells at each dose), n = 6–8/group, mean+/-SEM, Unpaired *t*-test, **$p < 0.01$.

in control conditions (Supplementary Fig. 1).

3.6. ID2 participates in the WNT-induced Gpr17 expression downregulation

Because none of these mutations in *MouseGpr17* promoter was sufficient to totally suppress WNT inhibitory effect, we next hypothesized that WNT could exert indirect effects through up-regulation of inhibitory factors. We investigated the involvement of ID2, known to inhibit oligodendrocyte differentiation and myelination (Samanta and Kessler, 2004). In differentiating Oli-neu cells, *Id2* was significantly up-regulated by WNT agonist II in a dose-dependent manner (Fig. 3c). LEF1 was previously shown to bind to the ID2 locus (GSE100872.LEF1.back-skin, mm10 coordinates chr12:25095341–25,095,557) in skin cells by chromatin immunoprecipitation (Adam et al., 2018). We thus postulated that WNT agonist II-inducing overexpression of *Lef1* inhibited *Gpr17* expression through *Id2* up-regulation. To test this hypothesis, we used post-transcriptional gene silencing of *Id2* mRNA by transfection of anti-*Id2*-siRNA (small interfering RNA targeting *Id2* mRNA) in Oli-neu cells. We first demonstrated that increasing concentrations of siRNA induced a dose-dependent and significant decrease of *Id2* mRNA expression (Fig. 5a, black bars). In addition, all concentrations of anti-*Id2*-siRNA significantly reduced the up-regulation of *Id2* expression induced by WNT agonist II stimulation (Fig. 5a, white bars), the concentration of 25 nM bringing its expression back at its control level without WNT agonist II. We next analyzed the impact of anti-*Id2*-siRNA on *Gpr17* mRNA expression upon WNT agonist II stimulation (Fig. 5b). In Oli-neu cells transfected with scramble siRNA (used as negative control siRNA), WNT agonist II induced a significant down-regulation of *Gpr17* mRNA expression by 40%. Anti-*Id2*-siRNA (5, 10, 25 nM) impaired WNT inhibitory effect on *Gpr17* mRNA in a dose-dependent manner (Fig. 5b). At 25 nM, anti-*Id2*-siRNA demonstrated the efficiency to abrogate the inhibitory effect of WNT agonist II (Fig. 5b). Finally, the impact of anti-*Id2*-siRNA was evaluated on the inhibitory effect exerted by WNT agonist II on *MouseGpr17-Luc* activity (Fig. 5c). Increasing concentrations of anti-*Id2*-siRNA induced a progressive reduction of WNT inhibitory effect on *MouseGpr17* promoter activity, which was totally suppressed by the highest concentration of 25 nM. Altogether, these results suggest that WNT inhibits *Gpr17* expression through *Id2* up-regulation, and that this effect is mediated by one or several elements enclosed in the 1.1 kb promoter region of the *MouseGpr17-Luc* construct.

3.7. Overexpression of Gpr17 limited differentiation arrest induced by the high WNT activity

We finally hypothesized that overexpression of GPR17 could counteract the WNT inhibitory effect on Oli-neu differentiation. After 48 h of transfection with a mouse *Gpr17* overexpression plasmid (*Gpr17* plasmid) or control plasmid, Oli-neu cells were exposed 48 h to differentiation medium containing vehicle or WNT II agonist. As illustrated in Fig. 6a, *Gpr17* plasmid transfection increased the GPR17-IR of Oli-neu compared to the control plasmid. We next analyzed CNPase-IR as a marker of Oli-neu differentiation (Fig. 6b and c). GPR17 plasmid induced a significant increase of CNPase.

WNT II agonist significantly reduced CNPase-IR in both control and GPR17 plasmid groups as demonstrated by the significant decrease of CNPase mean grey density. CNPase-IR analysis also revealed that overexpression of GPR17 in Oli-neu cells limited the WNT inhibitory effect on Oli-neu differentiation as demonstrated by the significant increase of CNPase mean grey density in *Gpr17* plasmid group compared to control plasmid group.

4. Discussion

Oligodendrocyte differentiation is regulated by a complex interplay of intrinsic, epigenetic and extrinsic factors, including WNT and the G protein-coupled receptor GPR17 (Lecca et al., 2020; Guo et al., 2015; van Tilborg et al., 2016; van Tilborg et al., 2018). GPR17 responds to both extracellular nucleotides (UDP, UDP-glucose) and cysteinyl-leukotrienes (Ciana et al., 2006), endogenous signaling molecules involved in inflammatory response and in the repair of brain lesions. After the original report describing GPR17 (Ciana et al., 2006), this receptor has been widely recognized as an important regulator of OPC maturation, both during early development and at adult stages. GPR17 is highly expressed in OPCs during the transition to immature OLs and is then down-regulated in mature cells (Chen et al., 2009; Fumagalli et al., 2015). Any alteration in this peculiar expression pattern results in myelination defects; marked GPR17 upregulation and/or accumulation of GPR17-expressing cells at the border of demyelinated lesions have been observed in a wide variety of pathological conditions associated with dysmyelination, including patients affected by MS (Chen et al., 2009; Angelini et al., 2021), traumatic brain injury (Franke et al., 2013), congenital leukoencephalopathy (Satoh et al., 2017), and neurodevelopmental disorders (Meraviglia et al., 2016; Trangle et al., 2023; Boccazzi et al., 2023). Understanding the signaling pathways modulating GPR17 expression is of utmost importance to fully understand the myelination process under normal and pathological conditions. Given this, very little is known regarding its transcriptional modulation.

Here we analyzed the previously unexplored interplay between the activation of the canonical WNT pathway and GPR17 expression. By using both primary oligodendrocyte cultures and the immortalized Oli-neu cell line, we demonstrate for the first time a functional link between the canonical WNT pathway and GPR17 in regulating the progression of oligodendrocyte precursor cells to mature cells. Specifically, we show that: (i) a lower WNT activation tone is necessary when *Gpr17* expression is sustained whereas higher WNT signaling results in decreased GPR17 expression during development; (ii) in primary OPCs, forced WNT/β-CATENIN activation at an early stage of differentiation strongly inhibits GPR17 expression and disrupts the achievement of the mature phenotype; (iii) the WNT pathway could reduce *Gpr17* expression by a dual mechanism: directly through the interaction with WREs located on its promoter, and by inducing *Id2* up-regulation which, in turn, down-regulates the receptor expression.

These findings set the basis for unveiling further pathogenic

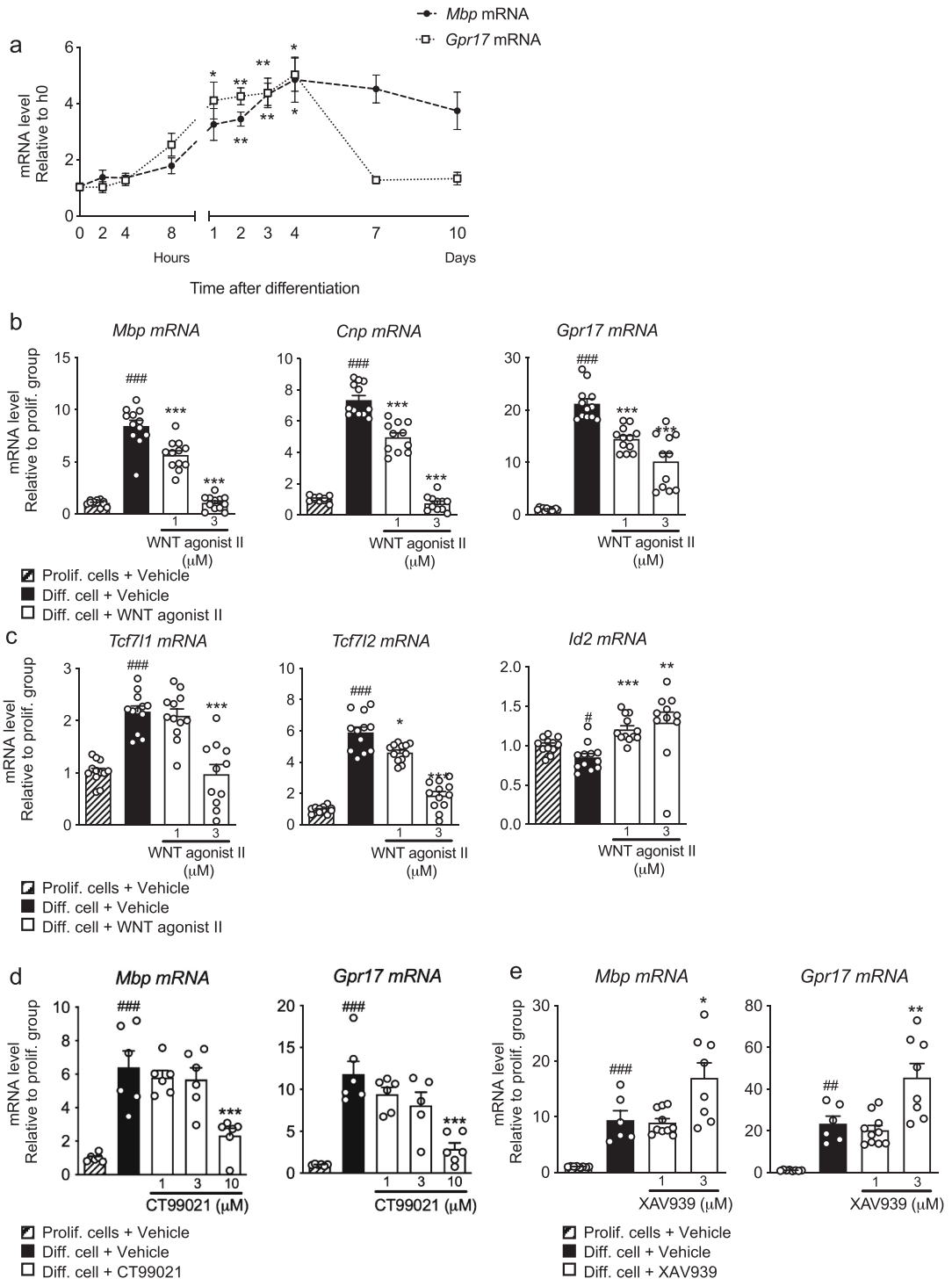

Fig. 3. WNT modulates Gpr17 and differentiation of Oli-neu cells.

(**a**) RT-qPCR analysis of *Mbp* and *Gpr17* mRNA in differentiating Oli-neu cells during 10 days. Relative expression to control group (H0), n = 4–6/group, mean+/-SEM, One way ANOVA with Dunnett's post-hoc test: (*) means a significant difference with H0, $*p < 0.05$, $**p < 0.01$. (**b**) *Mbp*, *Cnp*, *Gpr17*, *Lef1* and (**c**) *Tcf7l1*, *Tcf7l2*, *Id2* mRNA in Oli-neu after 48 h of differentiation (Diff. cells) and treated with 1 or 3 μM of WNT agonist II compared to Diff. cells receiving vehicle. Relative expression to control group (Prolif. cells + Vehicle), n = 5–6/group, mean+/-SEM, One way ANOVA with Bonferroni's post-hoc test: (#) means a significant difference with Prolif. cells + Vehicle, $###p < 0.001$, (*) means a significant difference between WNT agonist II -treated Diff. cells and vehicle-treated Diff. cells,$*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

(**d**) *Mbp* and *Gpr17* mRNA in Oli-neu after 48 h of differentiation (Diff. cells) and treated with 1, 3 and 10 μM of CT99021 (WNT pathway activator) compared to Diff. cells receiving vehicle. Relative expression to control group (Prolif. cells + Vehicle), n = 6/group, mean+/-SEM, One way ANOVA with Bonferroni's post-hoc test: (#) means a significant difference with Prolif. cells + Vehicle, $##p < 0.01$, $###p < 0.001$, (*) means a significant difference between CT99021-treated Diff. cells and vehicle-treated Diff. cells $**p < 0.01$, $***p < 0.001$. (**e**) *Mbp* and *Gpr17* mRNA in Oli-neu after 48 h of differentiation (Diff. cells) and treated with 1 and 3 μM of XAV939 (WNT pathway inhibitor) compared to Diff. cells receiving vehicle. Relative expression to control group (Prolif. cells + Vehicle), n = 6–10/group, mean+/-SEM, One way ANOVA with Bonferroni's post-hoc test: (#) means a significant difference with Prolif. cells + Vehicle, $##p < 0.01$, $###p < 0.001$, (*) means a significant difference between XAV939-treated Diff. cells and vehicle-treated Diff. cells $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

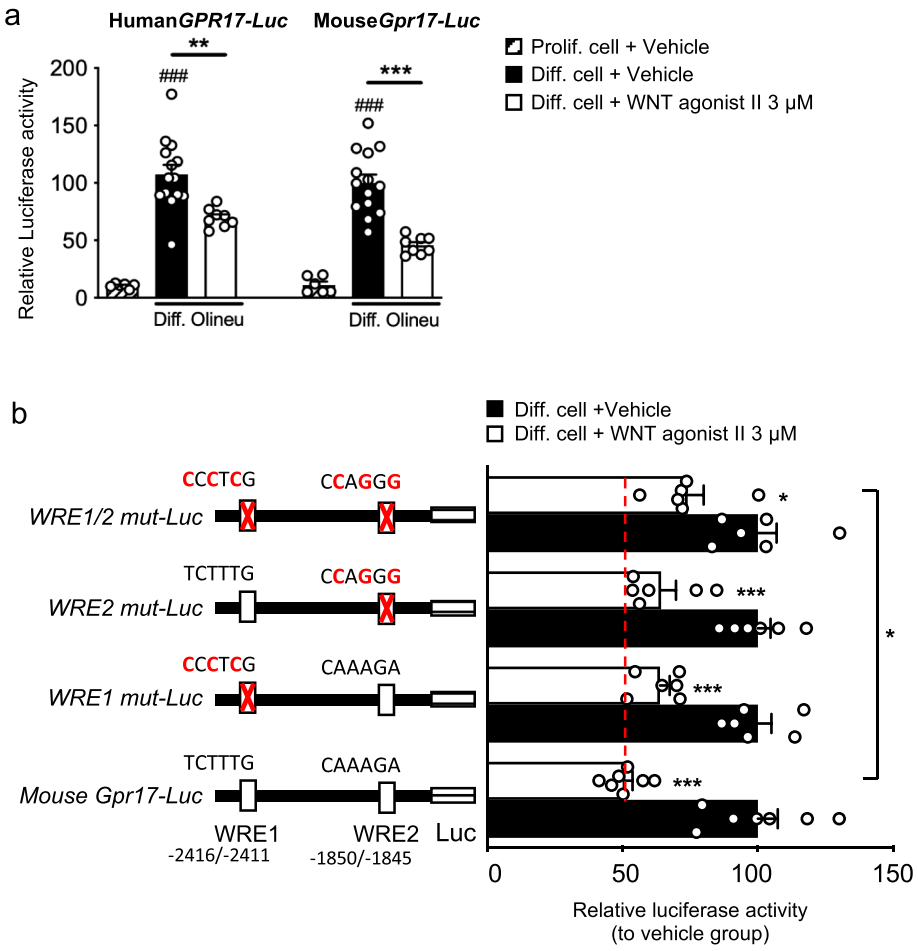

Fig. 4. Characterization of mouse *Gpr17* promoter activity and response to WNT agonist II in Oli-neu cells.
(**a**) Relative luciferase activity of two constructs driven by the human (*HumanGpr17-Luc)* and mouse (*MouseGpr17-Luc) Gpr17* promoters in Oli-neu cells after 24 h of proliferation or 24 h of differentiation without or with 3 µM WNT agonist II. Relative expression to *MouseGpr17-Luc* Diff. control group (+Vehicle), n = 6–14/group, mean+/-SEM, One way ANOVA with Bonferroni's post-hoc test: (#) means, for each construct, a significant difference with Prolif. cells, ###$p < 0.001$, (*) means a significant difference between WNT agonist II-treated Diff. cells and vehicle-treated Diff. cells, **$p < 0.01$, ***$p < 0.001$. (**b**) Inhibitory effect of WNT agonist II on Mouse *Gpr17* promoter activity after 24 of differentiation, and impact of targeted mutations of two putative WNT response elements (WRE) located in 2416/−2411 and − 1850/−1845 positions, alone or in combination. Relative expression to control group (Diff. cells + Vehicle), n = 6–7/group, mean+/-SEM, One-way ANOVA with Bonferroni's post-hoc test *$p < 0.05$, ***$p < 0.001$.

mechanisms of dysregulated myelination and for the identification of novel targets for promoting myelin repair.

Activation of the canonical WNT pathway induces nuclear translocation of β-CATENIN, which, in turn, binds TCF/LEF proteins. TCF/LEF proteins are a group of transcription factors which contain an HMG -DNA binding domain recognizing WNT responsive elements (WRE). There are four members in this family: TCF7, TCF7L1, TCF7L2, and LEF1 (also named TCF7L3).

Interestingly, we observed that both *Gpr17* and members of the *Tcf/Lef* family were differentially expressed in PDGFRα$^+$ OPCs and O4$^+$ OLs during mouse brain development. Indeed, consistently with a previous publication (Boda et al., 2011), *Gpr17* mRNA level peaked in PDGFRα$^+$ and O4$^+$ cells respectively at P7 and P14 and then decreased whereas *Lef1* was maintained in both populations at low and constant level until P10 and then gradually increased with development. In O4$^+$ cells, we also clearly detected *Tcf7l1* and *Tcf7l2* expressions that were significantly increased at P10. Only few data are available about the expression of *Tcf7l1* mRNA in oligodendrocytes. RNAseq database (brainrnaseq.org and proteinatlas.org) revealed a well detectable expression of *Tcf7l1* mRNA in oligodendrocyte lineage in human and mice, encouraging an in-depth analysis of the role of TCF7L1 in oligodendroglia. A stage-specific role of WNT signaling in OL lineage progression has also been recently described by Wang and colleagues (Wang et al., 2023): higher expression of nuclear β-catenin in the corpus callosum at P21 compared to earlier stage (P7) suggests stronger endogenous WNT activity in late stage of OL maturation. Overall, our data might suggest that, at the initial phase of oligodendrogenesis, low level of WNT activity allows *Gpr17* expression, whereas at later stage, higher WNT activity participates to the downregulation of the receptor necessary for the proper differentiation of OLs. We hypothesize that any disruption of this mutual balance may affect the proper oligodendrocyte differentiation.

Moreover, our pharmacological experiments demonstrated that sustained activation of the WNT pathway induced oligodendrocyte maturation arrest characterized by the overexpression of *Lef1* mRNA and a strong decrease of *Tcf7l1* and *2* mRNA expressions both in differentiating PDGFRα$^+$ primary OPCs and Oli-neu cells. These data are consistent with studies demonstrating that TCF7L2 is an intrinsic positive regulator of OL differentiation which is required for proper terminal differentiation but could act independently of the WNT/β-CATENIN signaling pathway. Indeed, ablation of the WNT repressor APC in OPCs in mice triggered unrestricted WNT signaling which was accompanied by the blockade of differentiation and the concomitant downregulation of the WNT effector TCF7L2 at both mRNA and protein levels (Hammond et al., 2015; Lang et al., 2013). A further study has also demonstrated that high WNT tone-inducing blockage of OPC maturation leads to an overexpression of LEF1 (Fancy et al., 2014). In the same study

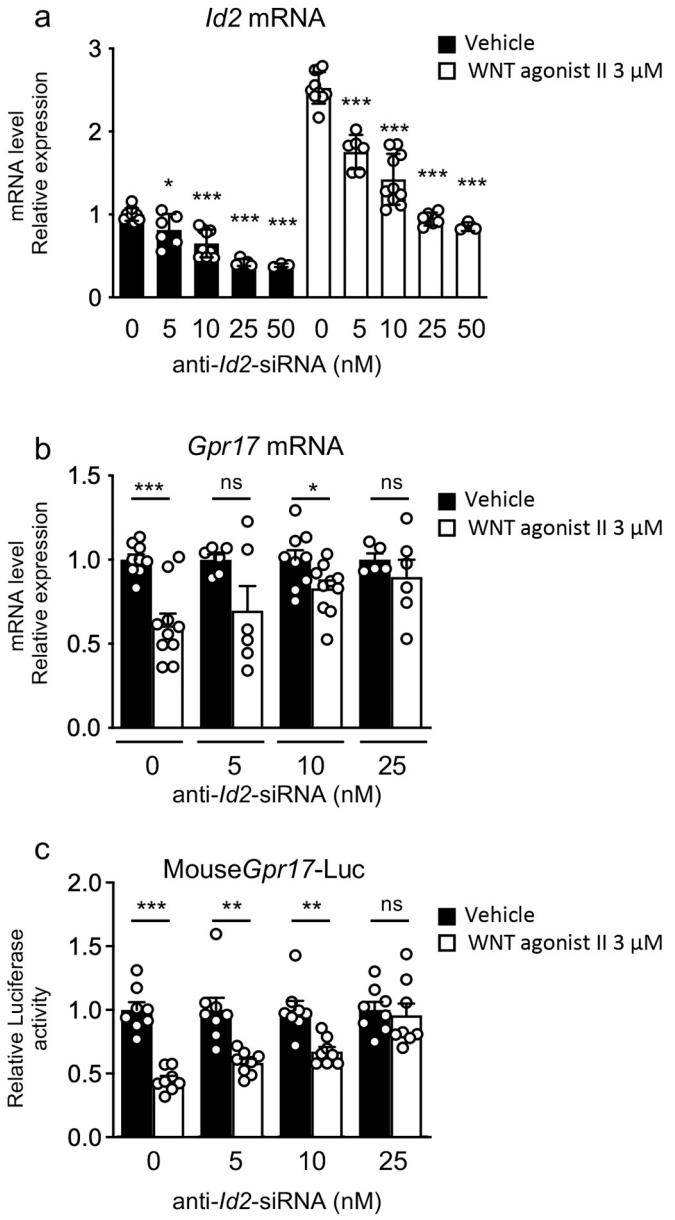

Fig. 5. *Id2* silencing impairs WNT-induced downregulation of *Gpr17* expression and inhibition of *Gpr17* promoter activity in Oli-neu cells.
(**a**) *Id2* mRNA level after 24 h of differentiation in presence of 3 μM WNT agonist II (white bars) or vehicle (black bars) in cells previously transfected with a siRNA mix containing anti-*Id2*-siRNA and Scramble siRNA to obtain 0, 5, 10, 25 or 50 nM of anti-*Id2*-siRNA and 50 nM of total siRNA in each condition. Relative expression to WNT-exposed control group (0 nM of anti-Id2-siRNA), n = 3–10/group, mean+/-SEM, One way ANOVA with Bonferroni's post-hoc test (*) means a significant difference between 5, 10, 25 or 50 nM of anti-Id2-siRNA and control cells (0 nM anti-Id2-siRNA) in each condition (with or without WNT agonist II), *p < 0.05, ***p < 0.001. (**b**) Effect of WNT agonist II after 24 h of differentiation on *Gpr17* mRNA level in cells previously transfected with 0, 5, 10 and 25 nM of anti-Id2-siRNA. Relative expression to control group (Diff. cells + Vehicle), n = 6–10/group, mean+/-SEM, Student t-test (*) means a significant difference between WNT agonist II-treated Diff. cells and vehicle-treated Diff. cells, *p < 0.05, ***p < 0.001, ns, not significant. (**c**) Effect of WNT agonist II after 24 h of differentiation on *Mouse Gpr17* activity in cells previously cotransfected with 0, 5, 10 and 25 nM of anti-Id2-siRNA and *MouseGpr17-Luc*. Relative expression to control group (Diff. cells + Vehicle), n = 8/group, mean+/-SEM, Student t-test (*) means a significant difference between WNT agonist II-treated Diff. cells and vehicle-treated Diff. cells, **p < 0.01, ***p < 0.001, ns, not significant.

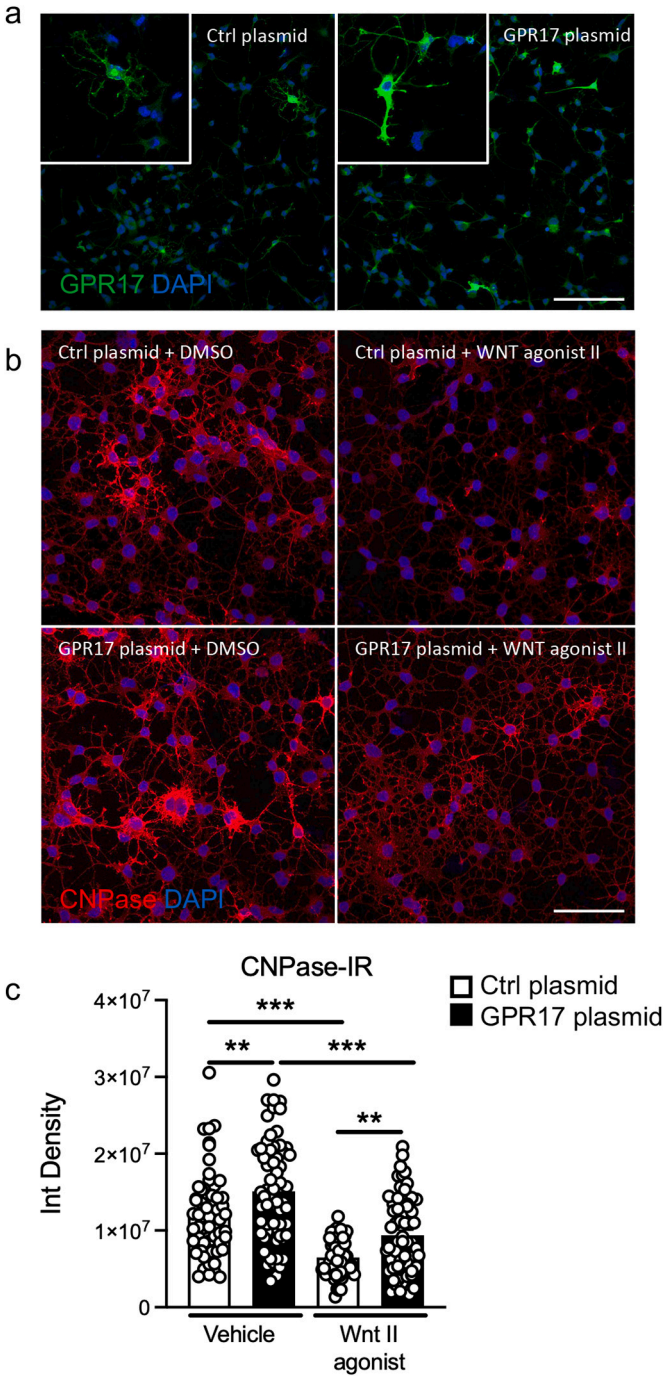

Fig. 6. Overexpression of *Gpr17* limited differentiation arrest induced by WNT agonist II.
(**a**) Illustrative picture of GPR17-IR in Oli-neu cells receiving control (Ctrl) plasmid (Left panel) and Gpr17 overexpression plasmid (Gpr17 plasmid) (Right panel). Scale bar = 140 μm (**b**) Representative picture of CNPase-IR in Vehicle- or WNT agonist II-treated differentiating Oli-neu cells receiving Ctrl plasmid (white bars) or *Gpr17* plasmid (black bars). Scale bar = 70 μm. Mean Density of CNPase-IR in Vehicle- or WNT agonist II-treated differentiating Oli-neu cells receiving Ctrl plasmid or *Gpr17* plasmid. N = 60–62 measures from 2 independent experiments, mean+/-SEM, Two-way ANOVA with Bonferroni's post-hoc test, **p < 0.01,***p < 0,001.

authors demonstrated that mice with APC deficiency restricted to OPCs showed an irreversible maturation arrest and an overexpression of LEF1 in the corpus callosum. Of note, immature oligodendrocyte cells in human neonates suffering from neonatal white matter injury also

expressed markers of high WNT tone including LEF1 (Fancy et al., 2014).

We then show for the first time that high WNT tone drastically reduces *Gpr17* mRNA level whereas low WNT tone induced by the WNT inhibitor XAV939 induced *Gpr17* mRNA overexpression. Since interactions between WNT pathway and GPR17 have never been described, we examined here the regulatory mechanisms linking WNT signaling to GPR17 expression. *GPR17* promoter activity is sensitive to extracellular stimuli, thus determining maturation timing in response to physiological or pathological signals coming from neuronal or glial cells. A previous study showed that soluble factors released by cortical neurons *in vitro* induce *GPR17* gene expression, a discovery made in Oli-neu cells using a 1 kb human *GPR17* promoter that displays a high degree of conservation among vertebrate species, suggesting a high functional significance (Fratangeli et al., 2013). However, the specific extracellular signals modulating *GPR17* gene activation, and their downstream pathways, were not investigated. In this study, we have cloned the homologous mouse DNA region and, by analyzing its activity in Oli-neu cells, we observed that human and mouse promoters were both: i) strongly activated in differentiating Oli-neu cells; and ii) inhibited by WNT II agonist. These modulations reflected the developmental and WNT-induced regulations observed for the endogenous *Gpr17* gene. We then used the *MouseGpr17-Luc* system to investigate the mechanisms involved in WNT repression. In a first place, our results indicate that WNT effect is mediated through response elements located within this 1099 bp promoter region. The similarities observed between mouse and human promoter activities and regulation also suggest that mechanisms described herein concerning mouse *Gpr17* might also be true for human *GPR17*.

WNT effects are mainly mediated by canonical pathway, involving β-CATENIN nuclear translocation and its subsequent recruitment to WRE (Kim et al., 2017). In Oli-neu, we have evidenced that WNT agonist II induced the activation of the canonical pathway, notably by triggering the expression of *Lef1* mRNA whose promoter has a WRE (Driskell et al., 2004). In order to unravel a possible direct role for this pathway in the regulation of *Gpr17* expression, we have identified and mutated two putative WRE motifs in the mouse *Gpr17* promoter. The double mutation only partially attenuated WNT repressive effect. Thus, these putative WRE motifs may participate in *Gpr17* promoter repression, together with other promoter elements. The possible redundancy of cis-regulatory elements mediating WNT repressing effect might contribute to the robustness of this repressive mechanism.

If activation of the WNT/β-CATENIN/TCF/LEF/WRE pathway is more often associated with transcriptional activation than repression of target genes (Vlad et al., 2008), our study indicates that WNT does not classically regulates *Gpr17* through WRE-mediated gene activation. Rather, WNT repressed *Gpr17* gene expression by an atypical mechanism which, at least partially, involves WRE.

In the current study, we also tested an alternative hypothesis, in which WNT canonical pathway would indeed lead to transcriptional activation of target genes including one or several transcriptional repressors. In turn, this repressor would indirectly mediate WNT repressive effect on *Gpr17* expression. In this context, we focused on ID transcriptional regulators. In particular, ID2 and ID4 are special members among basic helix-loop-helix (bHLH) transcription factors as they do not contain the DNA-binding basic region, and thus function as dominant negative transcription factors (Chen et al., 2012). While a recent paper showed that *Id2* and *Id4* deficiencies had little or no effect on OPC differentiation respectively, the same authors also demonstrated that their overexpression inhibited OL differentiation and myelin gene expression in developing neural tissue, highlighting the need for further research into their roles in the OL maturation process (Huang et al., 2022). In line, we observed that stimulation of WNT pathway by WNT agonist II induced an overexpression of *Id2*. Moreover, using a siRNA-mediated gene silencing strategy, we showed that *Id2* played a role in WNT-induced down-regulation of *Gpr17* expression. A possible link between *Id2* and *Gpr17* was previously investigated, showing that sustained GPR17 overexpression and its consequent downstream signaling induced ID2 translocation into the nucleus (Chen et al., 2009). However, given the biphasic expression pattern of GPR17 during differentiation, we can hypothesize that the reciprocal regulation between these two players changes over time based on the maturation stage.

Here, we demonstrated that overexpression of mouse GPR17 during initiation of Oli-neu differentiation induced increased expression of CNPase, corroborating previous findings highlighting a pivotal role of GPR17 in the initial phase of the differentiation process (Lecca et al., 2020). We also showed that overexpression of GPR17 allowed a partial rescue of Oli-neu differentiation under WNT II agonist treatment. This suggests that early disruption of GPR17 expression triggered by high WNT tone constitutes a downstream mechanism of WNT pathway activation which participates in the OL differentiation arrest. However, a full understanding of the link between the WNT pathway and the GPR17 receptor in oligodendrocyte maturation requires further investigation. Studying these mechanisms is particularly complex given the subtle and dual functions of these proteins, which are both essential to specific stages of OL development and maturation. Overall, our study provides a valuable contribution to GPR17 biology by describing a new interaction between two major regulators of OL maturation playing key roles in physiological and pathological conditions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2023.106315.

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Ethics approvals

Experimental protocols were approved by the institutional guidelines of the Institut National de la Santé et de la Recherche Medicale (Inserm, France), the Ethics Committee and the services of the French Ministry in charge of Higher Education and Research according to the directive 2010/63/EU of the European Parliament.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Marta Boccazzi: Conceptualization, Investigation, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Giulia Macchiarulo:** Investigation, Writing – original draft, Visualization. **Sophie Lebon:** Methodology, Investigation. **Justyna Janowska:** Investigation, Validation. **Tifenn Le Charpentier:** Investigation, Validation. **Valérie Faivre:** Methodology, Investigation, Formal analysis, Validation. **Jennifer Hua:** Investigation, Validation. **Davide Marangon:** Resources, Writing – original draft. **Davide Lecca:** Resources, Writing – original draft. **Marta Fumagalli:** Funding acquisition, Resources, Writing – original draft, Writing – review & editing. **Shyamala Mani:** Resources, Writing – original draft. **Maria P.**

Abbracchio: Resources, Writing – original draft. **Pierre Gressens:** Conceptualization, Funding acquisition, Resources, Supervision. **Anne-Laure Schang:** Conceptualization, Investigation, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Juliette Van Steenwinckel:** Conceptualization, Investigation, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors have no relevant financial or non-financial interests to disclose.

Data availability

Data will be made available on request.

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