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RESEARCH ARTICLE

Inhibition of endogenous phosphodiesterase 7 promotes oligodendrocyte precursor differentiation and survival

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Abstract During the development of the central nervous system (CNS), oligodendrocyte precursors (OPCs) are generated in specific sites within the neural tube and then migrate to colonize the entire CNS, where they differentiate into myelin-forming oligodendrocytes. Demyelinating diseases such as multiple sclerosis (MS) are characterized by the death of these cells. The CNS reacts to demyelination and by promoting spontaneous remyelination, an effect mediated by endogenous OPCs, cells that represent approximately 5–7 % of the cells in the adult brain. Numerous factors influence oligodendrogliogenesis and oligodendrocyte differentiation, including morphogens, growth factors, chemotropic molecules, extracellular matrix proteins, and intracellular cAMP levels. Here, we show that during development and in early adulthood, OPCs in the murine cerebral cortex contain phosphodiesterase-7 (PDE7) that metabolizes cAMP. We investigated the effects of different PDE7 inhibitors (the well-known BRL-50481 and two new ones, TC3.6 and VP1.15) on OPC proliferation, survival, and differentiation. While none of the PDE7 inhibitors analyzed altered OPC proliferation, TC3.6 and VP1.15 enhanced OPC survival and differentiation, processes in which ERK intracellular signaling played a key role. PDE7 expression was also observed in OPCs isolated from adult human brains and the differentiation of these OPCs into more mature oligodendroglial phenotypes was accelerated by treatment with both new PDE7 inhibitors. These findings reveal new roles for PDE7 in regulating OPC survival and differentiation during brain development and in adulthood, and they may further our understanding of myelination and facilitate the development of therapeutic remyelination strategies for the treatment of MS.

Keywords PDE7 inhibitors · Oligodendrocyte differentiation · Multiple sclerosis

Abbreviations

CNS	Central nervous system
OPCs	Oligodendrocyte precursor cells
MS	Multiple sclerosis
PDE	Phosphodiesterase
cAMP	3’-5’-cyclic adenosine monophosphate
PKA	Protein kinase A
CREB	cAMP response element-binding protein
DIV	Days in vitro

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Introduction

Multiple sclerosis (MS) is a chronic autoimmune disorder of the central nervous system (CNS) that is characterized by inflammation. As MS progresses, it produces axonal demyelination due to oligodendrocyte loss, which is followed by astrogliosis and eventual axonal degeneration [1, 2]. MS is a major cause of disability, affecting over 2.5 million people worldwide and it is the most common neurological disease among young and middle-aged adults. The etiology of MS is poorly understood and as yet there is no effective, although several therapies can ameliorate or delay the characteristic outbreaks/relapses. Currently available MS treatments rely on an immunomodulatory mechanism of action but they do not promote remyelination [3, 4], highlighting the urgent need for the development of novel neuroreparative treatments [5].

During embryonic development, oligodendrocyte precursor cells (OPCs) migrate to their final destination where they differentiate into myelin-forming oligodendrocytes [6, 7]. OPCs represent 5–7 % of the total cells in the mature CNS of both healthy and sick individuals, making this cell type an interesting target for therapies that aim to produce tissue repair in demyelinating diseases such as MS [8, 9].

Several studies suggest that 3'-5'-cyclic adenosine monophosphate (cAMP) plays an important role in neuroprotection and in the neuroinflammatory response. Accordingly, controlling the levels of this nucleotide could regulate the pathological neuroinflammatory process and decelerate the progression of MS [10–12]. Intracellular cAMP levels depend on its synthesis by adenylyl cyclases and its degradation by cyclic nucleotide 3'-5'-phosphodiesterases (PDEs). PDE7, one of the 11 PDEs isoenzymes identified to date, is a cAMP-specific enzyme that is insensitive to rolipram (a PDE4 inhibitor), and that is expressed in T cells and in multiple brain structures [13–15]. PDE7 has emerged as a novel therapeutic target to alleviate chronic inflammation in a variety of immunological conditions and conditions of immunodeficiency, as well as in neurodegenerative disorders like MS in which both the immune system and CNS are implicated [12, 16, 17].

There is little information regarding the physiological activities regulated by PDE7, although it has been implicated in pro-inflammatory processes and it is necessary to induce T-cell proliferation [18]. Moreover, specific inhibitors of PDE7 have recently been proposed as potential treatments for neurological disorders due to their modulatory effects on inflammation, a well-established neuroprotective strategy [12, 19, 20]. These data suggest that this class of PDE7 inhibitors is a potential source candidate drugs to treat MS. The effectiveness of current pharmacological treatments for MS is limited by several severe drawbacks, including poor efficacy and pharmacokinetic properties, and the search for new treatments can be considered as a rapidly growing field of research.

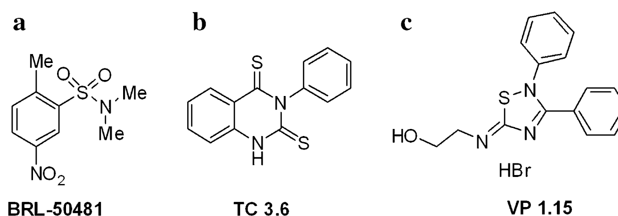


Fig. 1 PDE7 inhibitors used as chemical probes. **a** Structure of commercial PDE7 inhibitor BRL-50481. **b** Structure of the quinazoline TC3.6 **c** Structure of the 5-imino-1,2,4-thiadiazole VP1.15

In the present study, we investigated the effects of PDE7 inhibitors on oligodendrocyte precursor cells (OPCs). Inhibition of PDE7 activity accelerated the differentiation of newborn cortical OPCs and increased their survival in culture via a pathway dependent on PKA-cAMP. These effects were reproduced in OPCs from P15 mouse brain and in OPCs isolated from biopsies of adult individuals that did not suffer MS. Indeed, this is one of the first studies to perform a parallel analysis of OPCs from mice (P0 and P15) and human. To modulate PDE7 activity in OPCs, we used small molecules to characterize the biological mechanism [21, 22]. The well-known PDE7 inhibitor BRL-50481 (BRL; Fig. 1a) and two newly discovered PDE7 inhibitors, the TC3.6 quinazoline (Fig. 1b) and the 5-imino-1,2,4-thiadiazole derivative, VP1.15 (Fig. 1c). As a result, we reveal novel roles for PDE7 in regulating OPC survival and differentiation in the brain, which may further our understanding of myelination and improve the possibility of defining adequate remyelination strategies to treat diseases such as MS.

Materials and methods

Animals

Postnatal P0 and P15 mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and maintained in the animal facilities at the Hospital Nacional de Parapléjicos (Toledo, Spain). All animal experiments were carried out in accordance with Spanish (RD223/88) and European (2010/63/EU) regulations, and they were approved by the Animal Review Board at the Hospital Nacional de Parapléjicos (SAPA001).

Human samples

Human biopsies were obtained from major surgery of non-responders to drug treatment adult temporal epilepsy patients (29–59 years old) performed by the Neurosurgery

Service at the Hospital Universitario de La Princesa (Madrid, Spain). All experiments involving human samples were carried out in accordance with the guidelines of the Research Ethics Committee of Toledo (Spain) and all subjects provided informed consent.

PDE7 inhibitors

BRL-50481 (BRL; Fig. 1a) was supplied by Calbiochem, while Quinazoline TC3.6 (Fig. 1b [23]) and 5-imino-1,2,4-thiadiazole, VP1.15 (Fig. 1c [24]), were synthesized at the Instituto de Química Médica-CSIC. Both compounds were discovered by our research group by employing various medicinal chemistry strategies in the search of new PDE7 inhibitors. The quinazolines were identified via ligand-based virtual screening using CODES descriptors [25] while 5-imino-1,2,4-thiadizoles were discovered using a specific artificial neuronal network [26]. Hit-to-lead programs led to the identification of TC3.6 and VP1.15 based on the optimization of biological activity and drug-like properties, such as blood–brain barrier penetration. These compounds have IC_{50} values for PDE7 inhibition of 1.04 ± 0.08 and 1.1 ± 0.17 μM, respectively [26, 27]. Both compounds have demonstrated significant therapeutic potential for the treatment of neurological disorders [28, 29].

Cell culture

To isolate OPCs, primary mixed glial cultures were prepared as described previously for the rat forebrain [30, 31] using the modified technique of [32]. Briefly, the cerebral cortex of P0 and P15 mice was dissected out and dissociated enzymatically for 15 min at 37 °C in HBSS (Invitrogen) containing papain (0.9 mg/ml; Worthington Biochemical), L-cysteine (0.2 mg/ml; Sigma), and EDTA (0.2 mg/ml; Sigma). The digested tissue was then filtered through a 100-μm nylon mesh strainer (BD Biosciences) and seeded on poly-L-ornithine-coated 75-cm^2 flasks in DMEM medium containing 10 % fetal bovine serum (FBS; BioWhittaker) and a 1 % antibiotic anti-mycotic solution (Sigma). The cultures were maintained at 37 °C and 5 % CO_2, and the medium was changed every 4 days, adding 10 ng/ml human PDGF-AA (Millipore) fromP15 (for 2 weeks). When the cultures reached confluence, they were shaken overnight at 230 rpm and at 37 °C to detach the remaining oligodendrocyte progenitors located on top of the confluent astrocyte monolayer. The medium was then filtered through a 40-μm nylon mesh strainer (BD Biosciences) and centrifuged at 900 rpm. The cells were seeded twice (45 min each) in bacterial grade Petri dishes (Sterilin) to remove the loosely adherent microglia. After another round of centrifugation, the resulting enriched oligodendrocyte progenitor cell suspension was counted and seeded. After 2 days in culture the PDGFRα-positive oligodendrocyte precursors represented 87 ± 0.2 % of the total cells (mean ± SEM; $n = 50$ independent cultures) at P0 and 82 ± 0.7 % ($n = 30$ independent cultures) at P15 (Suppl. Fig. 1j).

For cells obtained from human biopsies, the same protocol was used except that the medium was supplemented with 10 ng/ml human PDGF-AA (Millipore) for 2 weeks, and then incubated for 2 weeks without growth factors before shaking. After 2 days in culture the PDGFRα-positive oligodendrocyte precursors represented 70 ± 0.04 % ($n = 20$ independent cultures; Suppl. Fig. 1).

Differentiation assay

Purified OPCs were placed on coverslips coated with poly-L-lysine and laminin in 24-well tissue culture dishes at a density of 2×10^4 cells/well. To promote differentiation, the cells were maintained in serum-free differentiation medium, as described previously [33], consisting of BME:F12 (1:1) supplemented with 100 μg/ml transferrin, 20 μg/ml putrescine, 12.8 ng/ml progesterone, 10.4 ng/ml selenium, 25 μg/ml insulin, 0.8 μg/ml thyroxine, 0.6 % glucose, and 6.6 mM glutamine. The PDE7 inhibitors (TC3.6, VP1.15 or BRL; 1 μM) were added to the culture medium to study their effects: and after 5 (for P0 and human cultures) or 7 DIV (for P15 cultures), the cells were fixed with 4 % PFA for further immunocytochemical analyses.

For the kinetics assays, OPCs were maintained for 2, 3, 5, or 7 DIV, to compare their rate of differentiation. Oligodendroglial cultures were examined by phase-contrast microscopy and differentiation was confirmed by immunostaining with antibodies against oligodendroglial cell-specific markers (CNPase and MBP), as described below.

To determine whether the cAMP/PKA pathway was involved in differentiation, cultures were treated with the cAMP antagonist Rp-cAMP (100 μM, BIOMOL Research Laboratories) to block PKA activation.

Proliferation assay

Purified OPCs were placed on coverslips coated with poly-L-lysine and laminin (2×10^4 cells/well) and they were incubated with the PDE7 inhibitors TC3.6, VP1.15 or BRL (1 μM) in differentiation medium. After 42 h in culture, BrdU (50 μM; Sigma) was added for 6 h and after a total of 72 h in culture, the cells were fixed and BrdU incorporation was detected by immunocytochemistry (1:20, G3G4 Hybridoma Bank) combined with the detection of the oligodendroglial markers described above.

Cell viability

OPCs were cultured with PDE7 inhibitors (TC3.6, VP1.15 or BRL; 1 μM) in differentiation medium and after 2 DIV, they were fixed with 4 % PFA and subjected to immunocytochemistry for active caspase 3 (1:200, Abcam) and the oligodendroglial markers described above.

Immunocytochemistry

OPCs were characterized as described previously [25, 26]. The different stages of oligodendroglial differentiation were detected using the following markers: PDGFRα (anti-PDGFRα, 1:200; Santa Cruz Biotechnology), NG2 (anti-NG2, 1:200; Millipore), or A2B5 (anti-A2B5, 1:10; Hybridoma Bank) for OPCs; CNPase (anti-CNPase, 1:200; Covance) for pre-oligodendrocytes; MBP (anti-MBP, 1:100; Abcam) or PLP (anti-PLP, 1:200; Abcam) for mature oligodendrocytes; and Olig2 (anti-Olig2, 1:250; Millipore) as a marker for all oligodendroglial stages. Cultures were co-stained with anti-PDE7A (1:50; Santa Cruz Biotechnology) or anti-PDE7B (1:50; Santa Cruz Biotechnology). The secondary antibodies used were donkey anti-goat Alexa 594, donkey anti-mouse Alexa 488, donkey anti-rat Alexa 594 and donkey anti-rabbit Alexa 488 (all diluted 1:1,000).

Western blotting

ERK signaling was analyzed in 96-well tissue culture plates seeded with 5×10^4 cells per well. Cells plated in OPC medium were maintained at 37 °C in a 5 % CO_2 atmosphere at 95 % relative humidity. After 1 DIV, the cells were deprived of serum for 24 h and subsequently stimulated with PDE7 inhibitors (30 μM). After 10 min the cells were fixed for a further 10 min at RT with 4 % PFA. An In-Cell Western blot was carried out using the Odyssey Infrared Imaging System and the OPCs were immunostained with anti-pERK (1:250; Santa Cruz) and anti-ERK (1:300; Santa Cruz) following the manufacturer's instructions. Antibody binding was assessed using secondary antibodies conjugated to 680 or 800 IRDye and the 96-well tissue culture plates were scanned using the Odyssey Infrared Imaging System (LICOR). ERK and pERK expression were analyzed according to the Odyssey LICOR instruction manual and the amount of pERK relative to that of total ERK was normalized to the pERK/ERK value in control samples. Values are represented as the mean ± SEM.

To quantify pCREB and CREB, we performed a quantitative Western blot using the Odyssey Infrared Imaging System (LICOR) for simultaneous analysis of two different proteins in separate fluorescent channels. The membranes were incubated with secondary antibodies conjugated to 680 or 800 IRDye and scanned using the Odyssey infrared scanner (LICOR). The bands corresponding to pCREB and CREB were analyzed according to the Odyssey LICOR instruction manual. The amount of pCREB relative to total CREB was normalized against pCREB/CREB values in control samples and presented as the mean ± SEM.

To quantify the expression of PDE7A/B, and PDE4B/D, at different stages we also carried out an In-Cell Western blot using the Odyssey Infrared Imaged System, as described above. In this case, OPCs were immunostained with anti-PDE7A (1:50), anti-PDE7B (1:50), anti-PDE4B (1:50), anti-PDE4D (1:50; all from Santa Cruz Biotechnology) and with anti-tubulin (1:80,000; Sigma), following the manufacturer's instructions. OPC cultures were analyzed by immunocytochemistry after 1, 3, and 5 DIV in differentiation medium to compare PDEs expression at different stages of oligodendrocyte differentiation. The expression of PDE7 and PDE4 isoforms was analyzed as indicated in the Odyssey LICOR instruction manual, and the relative phosphodiesterase levels were normalized against the corresponding tubulin levels and expressed as the mean ± SEM.

Imaging analysis

Digital fluorescence images were obtained with a DFC480 FX digital camera (Leica) coupled to a Leica DM5000B microscope or using a SP5 resonant scanner (Leica Microsystems).

Statistical analysis

The data is shown as the ratio to the oligodendroglial lineage cells, and were analyzed using SigmaPlot software (Jandel Scientific). A comparative analysis was performed using a Student's *t* test (or Mann–Whitney rank-sum test) or one-way ANOVA. Statistical significance was set at $p < 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

Phosphodiesterase 7 is expressed by oligodendrocyte precursor cells

To analyze the expression of both PDE7 isoforms (PDE7A and PDE7B) in rodent OPCs, we cultured isolated cortical OPCs from P0 postnatal mouse brains in vitro and performed dual immunocytochemistry for PDE7A and PDE7B together with the well-characterized OPC markers: NG2 (not shown) and PDGFRα (Fig. 2a–f). Double immunocytochemistry studies clearly revealed that all OPCs expressed both PDE7A and PDE7B, isoforms which were also expressed by all OPCs isolated from the cortex at P15 (Fig. 2g-l and Suppl. Fig. 2).

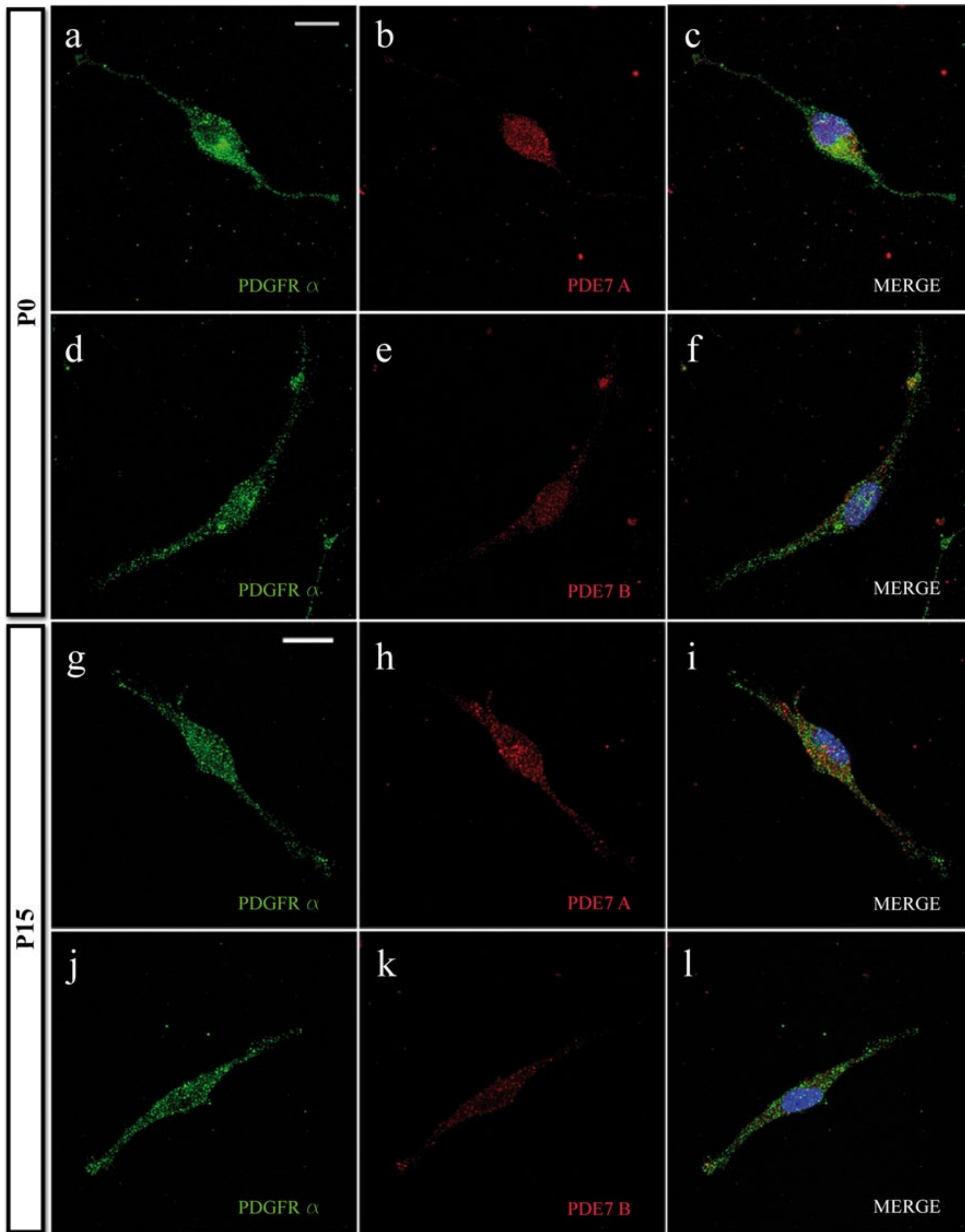


Fig. 2 OPCs express PDE7. **a–f** High-magnification immunofluorescence images of PDE7A/PDGFRα **a–c** or PDE7B/PDGFRα **d-f** double-labeled P0-derived OPCs. Both PDE7 isoforms were expressed by almost every OPC (see text) identified with the OPC molecular marker PDGFRα, after 1 DIV in differentiation medium. **g–l** High-magnification immunofluorescence images of PDE7A/PDGFRα **g–i** or PDE7B/PDGFRα **j–l** double-labeled P15-derived OPCs in similar conditions as P0 OPC cultures, with similar results. *Scale bars* are 10 μm in **a–f** and 7.5 μm in **g–l**

PDE7 inhibitors promote the differentiation of P0 cortical-derived OPCs

We investigated the effects of the PDE7 inhibitors, TC3.6 and VP1.15, on cultures of P0 cortical OPCs (see Materials and methods), using the commercial PDE7 inhibitor BRL to set a threshold for further comparison with the new inhibitors. We first carried out experiments using OPCs cultured for 5 DIV in differentiation medium (see Materials and methods) in the presence of both inhibitors and the commercial one: TC3.6, VP1.15, BRL, and their corresponding control. While BRL had no effect on OPC differentiation, exposure to 1 μM of TC3.6 produced a significant increase in the number of OPCs that became pre-oligodendrocyte (CNPase^{+}/Olig2^{+} cells) relative to the total number of Olig2 cells in the cultures, compared with the controls (Fig. 3a–f, y) and the increase produced by VP1.15 was stronger (Fig. 3m–r, y). When the number of OPCs that formed mature oligodendrocytes (MBP^{+}/Olig2^{+} cells) was assessed after 5DIV in differentiation medium, and relative to the total number of oligodendrocytes (Olig2^{+}) similar increases were observed to those detected for CNPase^{+}/Olig2^{+} cells (Fig. 3g–l, s–x, z): again, TC3.6 and VP1.15 exerted more potent and significant effects than BRL.

A more detailed analysis of the differentiation rate in the presence of PDE7 inhibitors at different time points (2, 3, 5, and 7 DIV in differentiation medium) revealed that PDE7 inhibition induced OPCs differentiation earlier than in control conditions, producing more CNPase-positive cells from 2 DIV until 5 DIV (Suppl Fig. 3a). This difference only disappeared at the seventh day (Suppl Fig. 3a). To rule out the possibility that the differences in differentiation were due to changes in PDE7 expression along the oligodendrocyte lineage, we analyzed the expression of both isoforms (PDE7A and PDE7B) in our cultures at 1, 3, and 5 DIV. In the In-Cell Western blot (see Methods), there were no changes in PDE7A and PDE7B protein at different stages of oligodendrocyte differentiation (Suppl. Fig. 3b). However, when we analyzed the expression kinetics of different isoforms of another well-known PDE, as PDE4 (PDE4B and PDE4D), the OPCs increasingly expressed more PDE4B as they were cultured for more days, but differences were not significant for PDE4D (Suppl. Fig. 3b).

PDE7 inhibition exerts a neuroprotective effect on cultured OPCs

Analysis of OPC cultures incubated in the presence of PDE7 inhibitors revealed that the number of oligodendroglial cells, identified as Olig2^{+} cells, increased compared with the corresponding controls. This effect was observed for all three PDE7 inhibitors used in this study (% Olig2^{+} cells versus CT: BRL: $125 \pm 6, p = 0.002$; TC3.6: 130 ± 8, $p = 0.023$ and VP1.15: 179 ± 12, $p < 0.001$), being this increase higher when the inhibitor was VP1.15.

To determine whether this increase was due to an increase in OPC proliferation or survival, we analyzed BrdU incorporation and the number of caspase3^{+} cells in the cultures. The analysis of BrdU incorporation in P0 OPC cultures revealed that none the PDE7 inhibitors had any effect on oligodendrocyte proliferation (Fig. 4a). However, in survival assays, we found that there were fewer dying OPCs (expressing caspase3) in the presence of the PDE7 inhibitors than in control cultures (Fig. 4b). Remarkably, fewer dying OPCs were observed in cultures treated with the new PDE7 inhibitors than with the commercial inhibitor (BRL; Fig. 4b). To define the signaling pathways underlying the effects of PDE7 inhibitors on oligodendrocyte maturation and survival, we first investigated the contribution of ERK activation since this kinase has been implicated in OPC biology and it plays a key role in triggering the switch between OPC proliferation and differentiation [34]. OPC cultures from P0 mice were serum deprived for 24 h and treated for 10 min with PDE7 inhibitors, after which pERK/ERK was analyzed. All three inhibitors significantly increased the pERK/ERK levels (Fig. 4c), suggesting that PDE7 inhibition involves the activation of this signaling pathway.

PDE7 is a cAMP-specific enzyme and the most common intracellular target for cAMP is protein kinase A (PKA), whose activation is responsible for many cAMP-related effects [35]. Thus, we assessed whether PKA activation was required for the pro-differentiation effects of TC3.6 and VP1.15 in OPCs. Treatment of OPC cultures with the cAMP antagonist Rp-cAMP (which blocks cAMP/PKA activation) prevented the increase in oligodendroglial differentiation induced by the PDE7 inhibitors (Fig. 4d), suggesting that the cAMP/PKA pathway mediates the effects of these inhibitors. We next analyzed the phosphorylation state of the cAMP response element-binding protein (CREB), a known target of the cAMP/PKA signaling pathway. At P0, the pCREB/CREB ratio increased in the presence of PDE7 inhibitors compared with the corresponding controls (Fig. 4e), suggesting that the new PDE7 inhibitors acted through the PKA/CREB pathway. Notably, the effect of TC3.6 was significantly weaker than that of the other two inhibitors studied.

PDE7 inhibitors promote the differentiation and survival of cortical-derived P15 OPCs

Based on the studies on OPCs isolated from P0 mouse brains, we investigated the effects of our PDE7 inhibitors

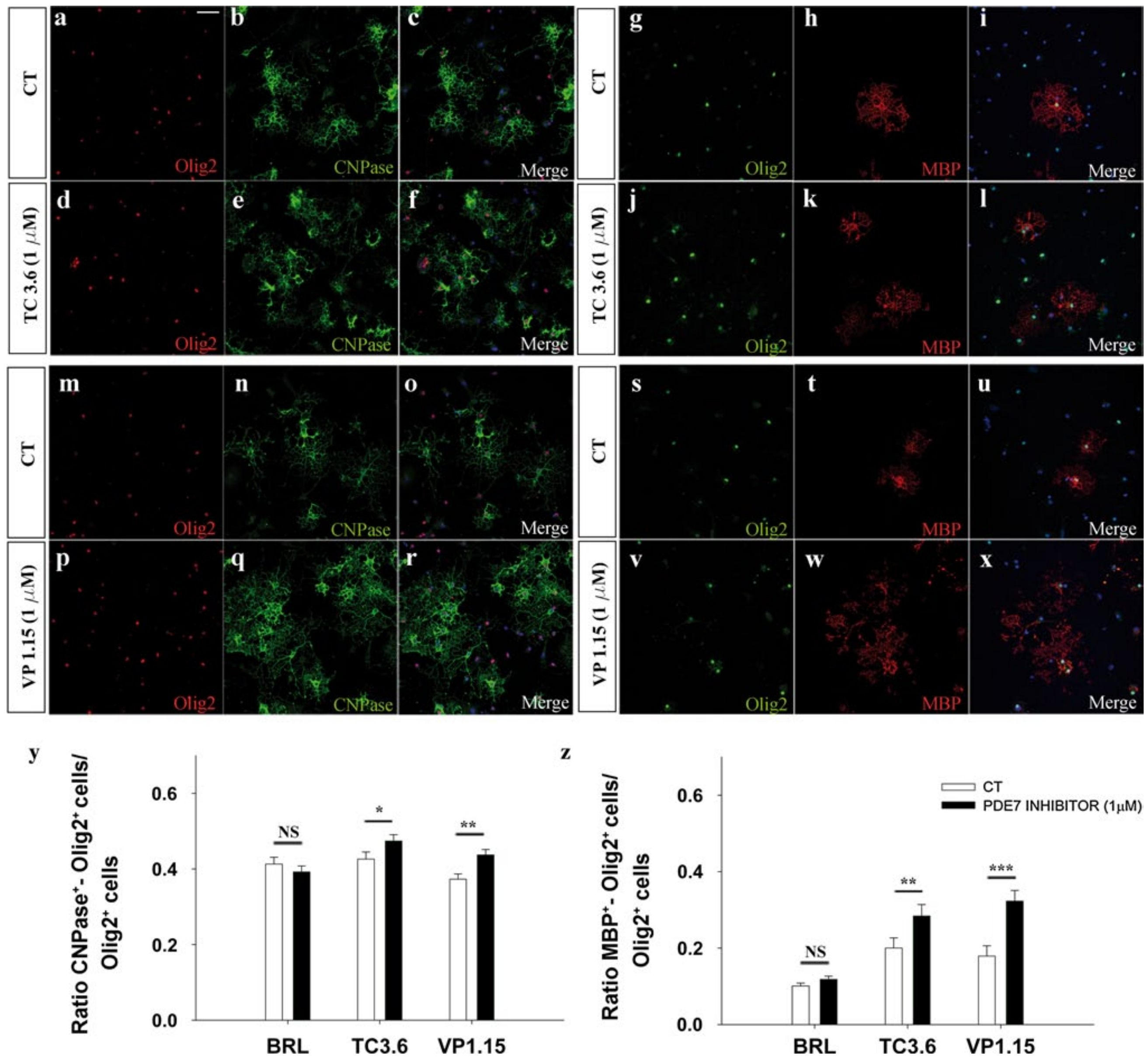


Fig. 3 Both TC3.6 and VP 1.15 PDE7 inhibitors promote the differentiation of OPCs from P0 mice brain cortices. **a–l** Immunofluorescence images of Olig2 and CNPase (**a–f**) or Olig2 and MBP (**g–l**) expression in OPCs isolated from P0 brains grown for 5 DIV in control differentiation medium (**a–c, g–i**) and in the presence of 1 μM of TC3.6 (**d–f, j–l**). Cultures were counterstained with DAPI. **m–x** Images shown CNPase (**m–r**) or Olig2 and MBP (**s–x**) expression when differentiation was studied in control conditions (**m–o, s–t**) versus. In the presence of 1 μM of VP 1.15 (**p–r, v–x**) (*y*) the plot represents the number of pre-oligodendrocytes ($CNPase^+/Olig2^+$) in respect to the total number of oligodendrocyte ($Olig2^+$). In the presence of both PDE7 inhibitors, the percentage of differentiated cell was significantly higher than in control conditions, except for BRL. (*z*) Quantification of mature oligodendrocytes ($MBP/Olig2^+$) differentiated in respect to the total number of oligodendrocyte ($Olig2^+$). Both new PDE7inhibitors (TC3.6 and VP1.15) were more efficient than commercial BRL. *Scale bar* represents 50 μm for **a–x**. Values are given as mean ± SEM and the results of Student's *t* test are represented as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

on OPCs isolated from cortices of older mice, purifying OPCs from the cortex of P15 mice when a peak of PLP mRNA expression occurs [36].

Both new PDE7 inhibitors accelerated the differentiation of P15 OPCs to pre-oligodendrocyte while the commercial one did not show a significant effect (Fig. 5a–l, y) and, moreover, we observed an increase in the number of mature oligodendrocytes after 7 DIV in differentiation medium (Fig. 5m–x, z). As seen in OPCs isolated at P0, exposure to PDE7 inhibitors had no effect on the number

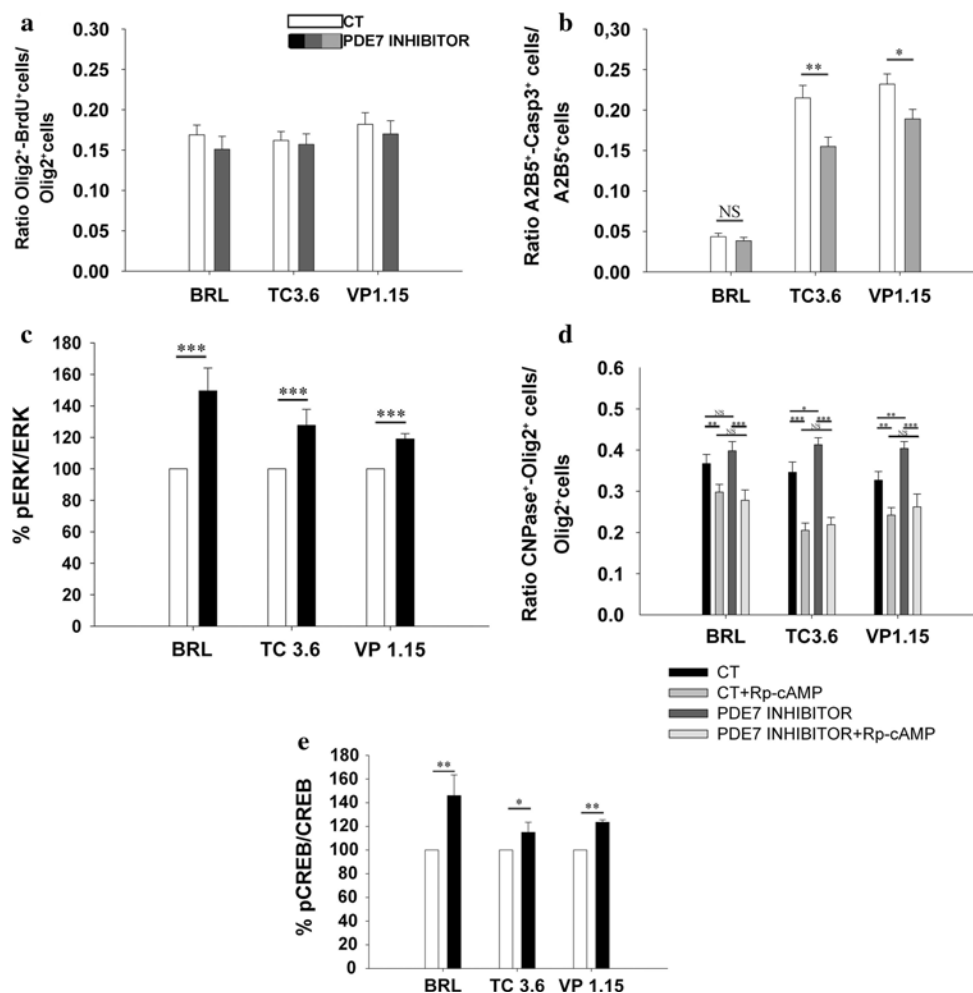


Fig. 4 TC3.6 and VP 1.15 do not affect OPC proliferation but increase their survival at P0, by activating ERK, PKA, and CREB intracellular pathways. **a** Quantification of BrdU incorporation by P0 OPCs characterized with the oligodendroglial marker Olig2. The presence of BRL, TC3.6, or VP1.15 (1 μM) did not modify the number of P0 $BrdU^+$/$Olig2^+$ OPCs after 3 DIV in comparison to their respective controls. **b** Determination of dead OPCs (active $caspase3^+$/$A2B5^+$) versus the total number of OPCs ($A2B5^+$cells) after 2 DIV in the presence of PDE7 inhibitors. With both new PDE7 inhibitors survival of OPCs was increased, while the commercial BRL, did not have any effect. **c** Estimation of p-ERK/ERK quotient by measurement of fluorescence intensity in purified OPCs cultures by immunostaining with anti-pERK and anti-ERK. ERK was significantly more activated when OPCs were exposed to BRL, TC3.6, and VP1.15 (30 μM). **d** Detection and quantification of $CNPase^+$/$Olig2^+$ pre-oligodendrocytes in purified cultures treated with PDE7 inhibitors plus the cAMP antagonist, Rp-cAMP (100 μM), which inhibits PKA. The effects observed after PDE7 inhibition on OPCs differentiation disappeared when PKA pathway was inhibited, which strongly suggest the implication of cAMP/PKA pathway in OPC differentiation. **e** Determination of pCREB/CREB quotient by measurement of fluorescence intensity. An increase of pCREB/CREB was also observed in the isolated OPCs under the presence of the three PDE7 inhibitors studied but, in this case, commercial BRL and VP1.15 showed stronger effects than the other new inhibitor, TC3.6. Values are given as mean ± SEM and the results of Student's *t* test are represented as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

of P15 oligodendrocytes that incorporated BrdU (Fig. 6a) while they enhanced OPC survival (Fig. 6b). As P0 and P15 OPCs responded similarly to these PDE7 inhibitors, we investigated the role of the pERK/ERK signaling pathway in the former. As seen in younger OPCs, all three inhibitors induced an increase in pERK/ERK activity (Fig. 6c), suggesting that the PDE7 inhibitors employ a similar mechanism of action in P0 and P15 OPCs, although the increase in pERK/ERK activity observed in P0 OPCs was greater than that observed in P15 OPCs (Fig. 4c).

Effects in isolated human OPCs

We next confirmed that PDE7A and PDE7B were expressed by all OPCs in cultures of OPCs isolated from adult human biopsies (Fig. 7a–f). In the presence of VP1.15 and TC3.6, there were more pre-oligodendrocytes in human

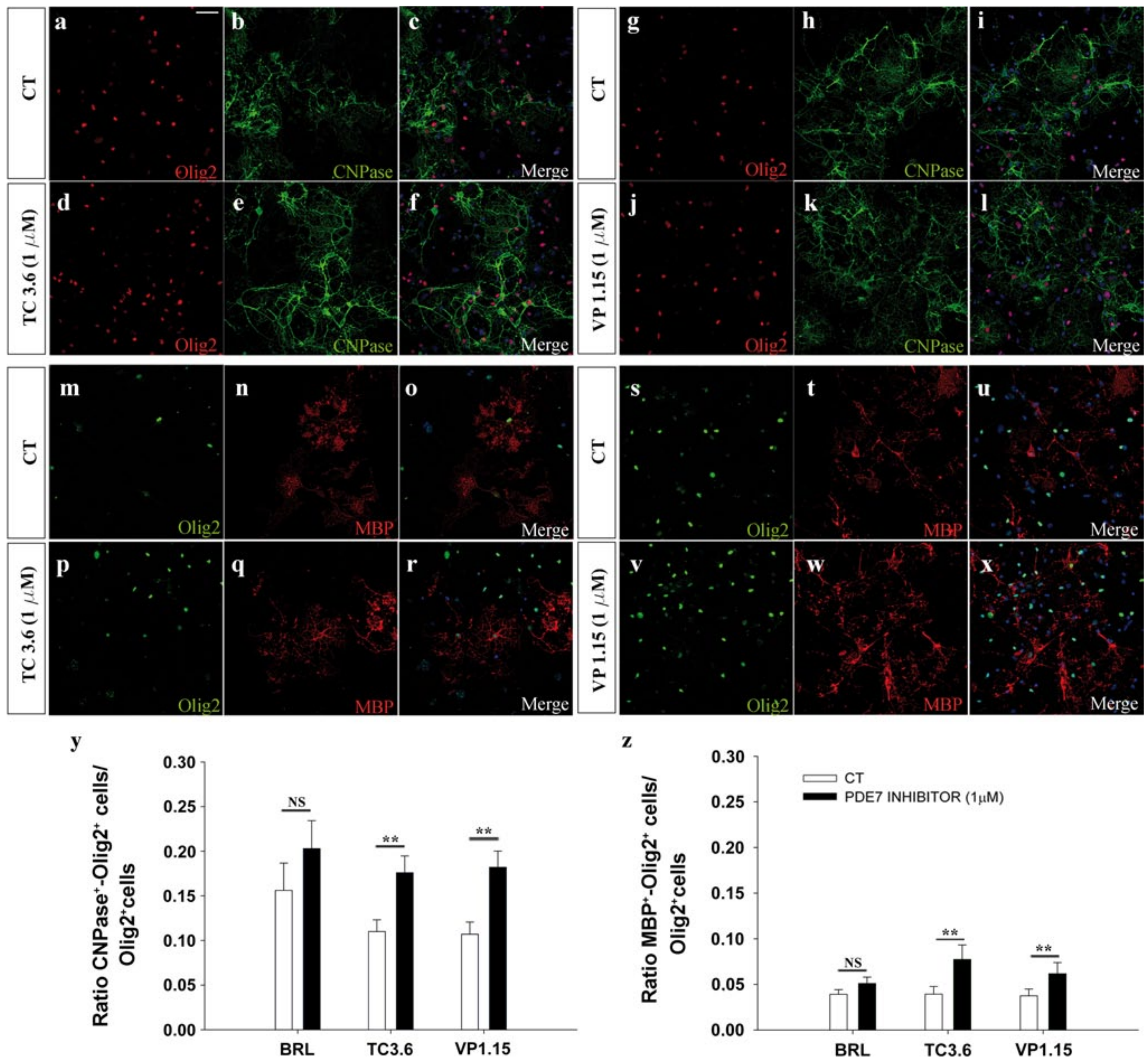


Fig. 5 TC3.6 and VP 1.15 PDE7 inhibitors increased the differentiation of OPCs at P15. **a–l** Immunofluorescence images of Olig2 and CNPase expression in P15-derived OPCs grown for 7 DIV in control differentiation medium (**a–c, g–i**) or in the presence of 1 μM of TC3.6 (**d–f**) or 1 μM of VP1.15 (**j–l**). Cultures were counterstained with DAPI. **m–x** Images show mature oligodendrocytes differentiated after 7 DIV in differentiation medium of P15-derived OPCs cultures identified as MBP/Olig2$^+$ cells in control medium (**m–o, s–u**) and in the presence of 1 μM of TC3.6 (**p–r**) or 1 μM of VP1.15 (**v–x**). Cultures were counterstained with DAPI. **y** Total number of differentiated pre-oligodendrocytes (CNPase$^+$/Olig2$^+$) in respect to the total number of oligodendrocyte cells (Olig2$^+$). In the presence of both new PDE7 inhibitors, the number of differentiated cell was significantly higher than in control conditions. **z** Quantification of mature oligodendrocytes (MBP/Olig2$^+$) differentiated. In the presence of TC3.6 and VP1.15 PDE7 inhibitors, the number of mature oligodendrocytes was significantly higher than in control conditions. Both TC3.6 and VP1.15 were more efficient than commercial BRL. *Scale bar* represents 50 μm for **a–x**. Values are given as mean ± SEM and the results of Student's *t* test are represented as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

OPC cultures exposed to the inhibitors than in their corresponding controls (Fig. 7g). These findings suggest that PDE7 activity in adult human OPCs is likely to be similar to that observed in mouse OPCs.

Discussion

In the present study, we demonstrate that both PDE7 isoforms (PDE7A and PDE7B) are expressed by murine and

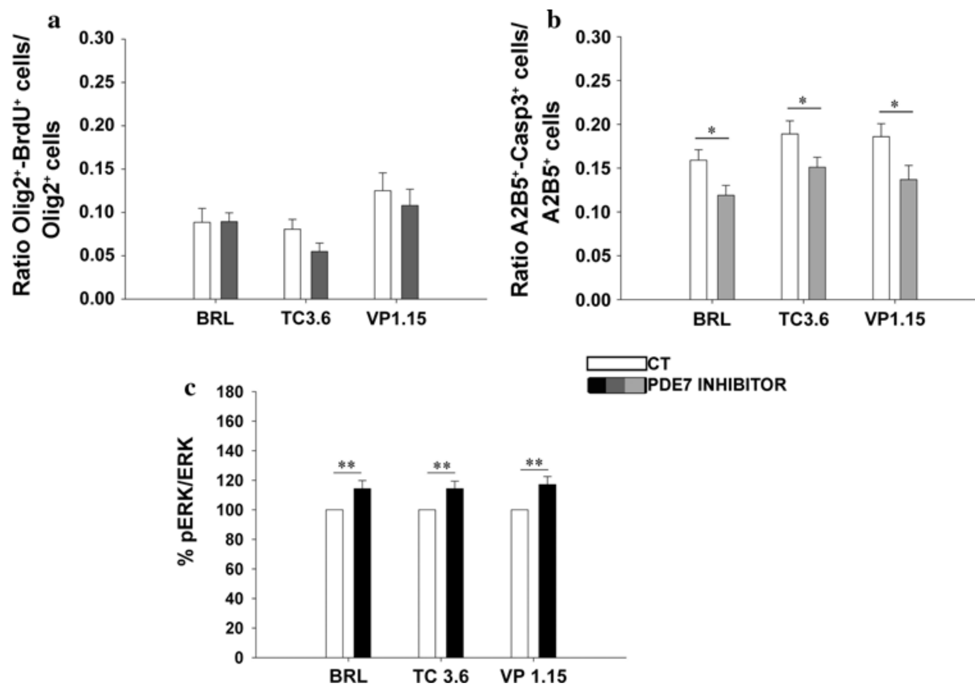


Fig. 6 TC3.6 and VP 1.15 do not affect OPC proliferation but favor OPC survival at P15. *a* Quantification of BrdU incorporation by double immunocytochemistry in P15 OPCs characterized with the oligodendroglial marker Olig2. Like at P0, the presence of TC3.6, VP1.15, or BRL (1 μM) did not modify the number of $BrdU^{+}/Olig2^{+}$ OPCs after 3DIV versus the total number of oligodendrocytes ($Olig2^{+}$). *b* Determination of dead OPCs percentage (active caspase $3^{+}/A2B5^{+}$) versus the total number of OPCs ($A2B5^{+}$) after 2 DIV. OPC survival increased in the presence of PDE7 inhibitors (1 μM). *c* Estimation of p-ERK/ERK quotient by measurement of fluorescence intensity in purified P15 OPCs cultures by immunostaining with anti-pERK and anti-ERK. p-ERK/ERK was equally activated in these cells when exposed to BRL, TC3.6, and VP 1.15 (30 μM). Values are given as mean ± SEM and the results of Student's *t* test are represented as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

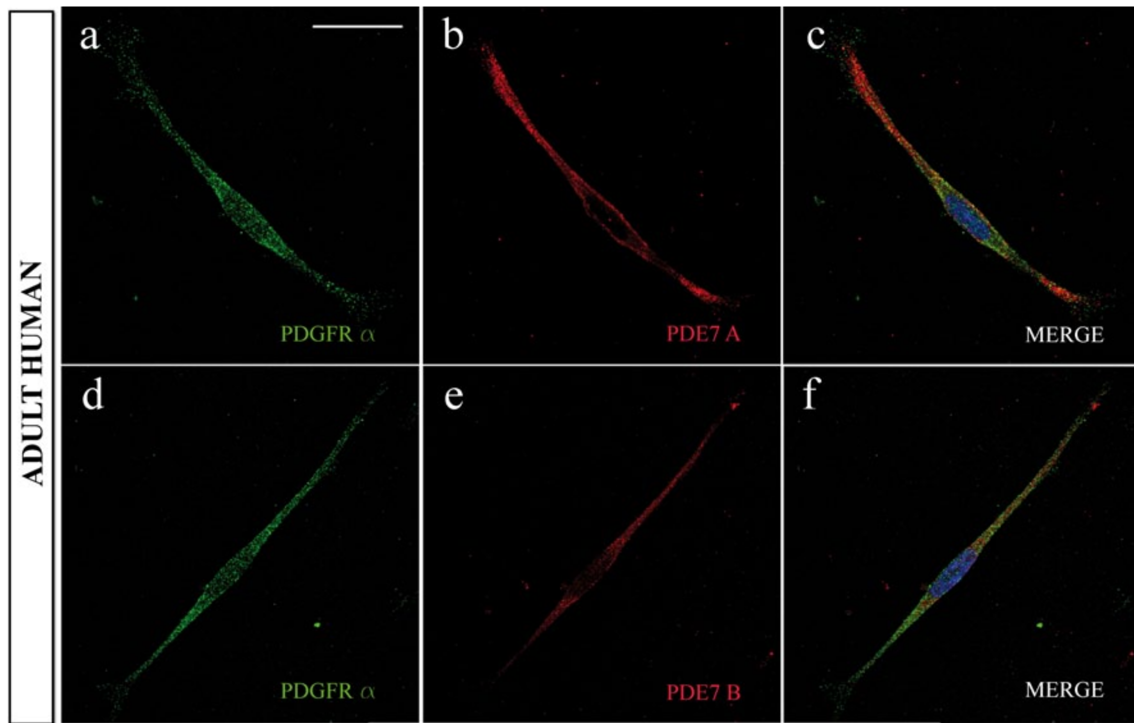
human OPCs in culture. We also show that two new PDE7-inhibitors (TC3.6 and VP1.15) promote OPCs survival and differentiation towards myelin-forming phenotypes in both murine and human OPCs.

Although PDE expression has been analyzed in different tissues, the expression of the different PDE isoforms remains to be fully characterized [37, 38]. Multiple PDE isoforms have been identified in neural populations, including PDE3, 4, 7, and 9a [15, 38–40] and PDE9a expression has also been described in a subpopulation of astrocytes [41]. PDE expression was first reported in myelin-forming cells over two decades ago [42], and since then, only one further study has examined PDE4 expression in Schwann cells [43], although alterations in PDE expression following CNS injury have also been reported [17, 44, 45], Thus, the present study is the first to demonstrate PDE7 expression and activity in oligodendroglial cells, and common enzymatic activity in OPCs from different neuronal types [15].

Increase in intracellular levels of cAMP appears to favor the differentiation of oligodendroglial and Schwann cells towards myelin-forming phenotypes [46, 47]. This suggests that inhibition of PDE7 activity would be a more effective remyelination strategy than the use of olesoxime [48] or FTY720 (Fingolimod), for example, which have proven effects on adult human oligodendrocytes [49]. LINGO-1 antagonists [50, 51], one of the best studied as a potential treatment, also displays significant therapeutic potential.

Myelination appears to be mediated by a limited number of molecules that vary widely in their function, including chemokines [52, 53], kinases like Cdk2 (only after demyelination) [54] and MAPK [55, 56], semaphorins [57, 58], transferrin [59], and the Na^{+}/Ca^{2+} exchanger-3 [60]. Other molecules are implicated in myelination, such as retinoic acid [61], although to date this has only been demonstrated in Schwann cells. To the best of our knowledge, of all the factors that promote oligodendrocyte differentiation, only Fingolimod [49] and PDE7 inhibitors (present work) have been shown to exhibit such effects in human oligodendroglia.

While the thyroid hormone T3 is classically considered fundamental for OPC survival, proliferation, and maturation/myelin gene expression [62], a panoply of other molecules subsequently promote oligodendroglial survival and protection in animal models of demyelination, including: neurotrophins (CNTF, PDGF, IGF-1), neuregulin-1, and interleukins [63]. Many of these effects are combined with an influence on proliferation and/or differentiation. Indeed, the neurotrophin BDNF was recently shown to exert potent and selective



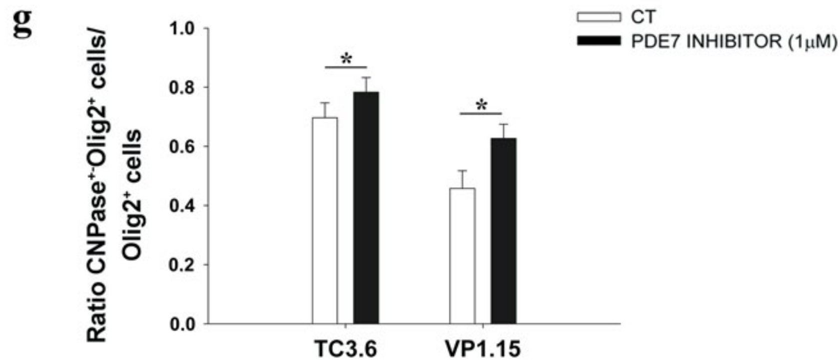


Fig. 7 PDE7 inhibition on adult human OPCs potentiates their differentiation process to form myelin. **a–f** High-magnification immunofluorescence images of PDE7A/PDGFRα (**a–c**) or PDE7B/PDGFRα (**d–f**) double-labeled OPCs derived from adult human brain biopsies. Both isoforms was expressed on adult human OPCs. (*g*) The number of pre-oligodendrocytes (CNPase$^+$/Olig2$^+$) respect total number of Olig2 cells in comparison to control conditions in the presence of VP1.15 (1 μM) for 5 DIV was significantly higher than in control conditions. With TC3.6, there is also a significant increase in the number of pre-oligodendrocytes. *Scale bar* represents 25 μm in **a–f**. Values are given as mean ± SEM and the results of Student's *t* test are represented as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

neuroprotective effects on oligodendrocytes in murine EAE, without affecting maturation [64, 65]. Both BDNF and FGF are components of the neurotrophic soup produced by the endothelium to give rise to the so called "oligovascular" niche in the CNS, which protects OPCs during development [66]. The tetracycline antibiotic minocycline was recently proposed as an effective neuroprotector of oligodendrocytes in conditions of oxidative stress [68]. In the present study, the effect of PDE7 inhibitors on OPC survival (~20–40 %) was more marked than that previously reported for minocycline (~10 %; [67]), and similar to that effect described for the endothelium-produced neurotrophic soup (~40 %; [66]).

Unlike cell survival and differentiation, oligodendroglial proliferation was not affected when PDE7 activity was blocked in OPCs. OPC proliferation is strongly promoted by sonic hedgehog (both during development and in adulthood), HGF and, to a lesser extent, other growth factors like PDGF-AA, IGF neurotrophin-3, and some chemokines. By contrast, the role of mitogen FGF-2 in this process remains to be fully clarified [6, 68–72].

PDE7 inhibitors produce therapeutic affects in in vitro and in vivo models of MS, Parkinson disease, spinal cord injury, and stroke [12, 17, 19, 28]. For example, the commercial PDE4-inhibitor rolipram protects oligodendrocytes

from cell death secondary to spinal cord injury, promoting their survival and eventually increasing their size and number in rodent models, establishing axonal conduction across the lesion [73, 74]. Rolipram also promotes remyelination possibly via the MEK-ERK pathway, in the cuprizone-induced mouse demyelination model [75], and its combination with lovastatin promotes neurorepair in an animal model of experimental autoimmune encephalomyelitis (EAE; [76]). The prevention of demyelination by rolipram has also been described in primates [77]. Moreover, the PDE1-inhibitor vinpocetine also exerts an effect on oligodendroglial differentiation and myelin expression [78].

The non-selective PDE-inhibitor Ibudilast exerts protective effects against hypoxic white-matter lesions [79] and exhibits some neuroprotective effects in MS patients, possibly by regulating the Th1/Th2 cytokine balance [80]. The immunomodulatory effects of both PDE3 and PDE4-inhibitors have potential applications in the treatment of Th1 autoimmune disorders, like MS [81, 82]. Indeed, PDE4B2 activity is dramatically increased around vessels in the EAE model of demyelination [83]. The administration of the PDE7-inhibitors studied in the present work significantly attenuates clinical symptoms in a mouse model of EAE in vivo, demonstrating the potential of these compounds in the treatment of MS [12, 20]. Together with the data presented here, these findings highlight the need to advance the study of TC3.6 and VP1.15 towards pre-clinical trials in order for these new therapies to eventually benefit MS patients.

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Conflict of interest The authors declare no conflicts of interest.

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