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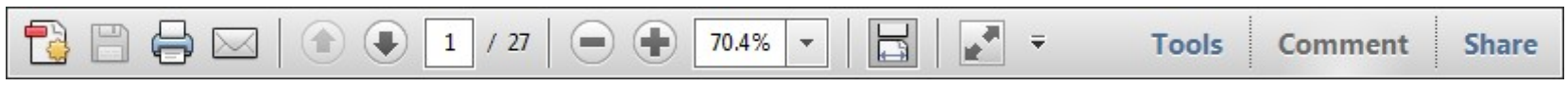


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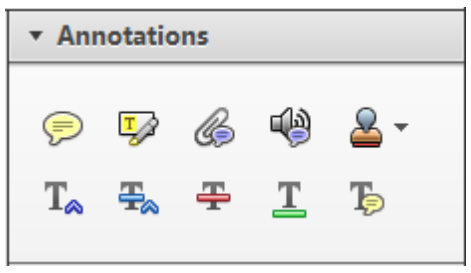


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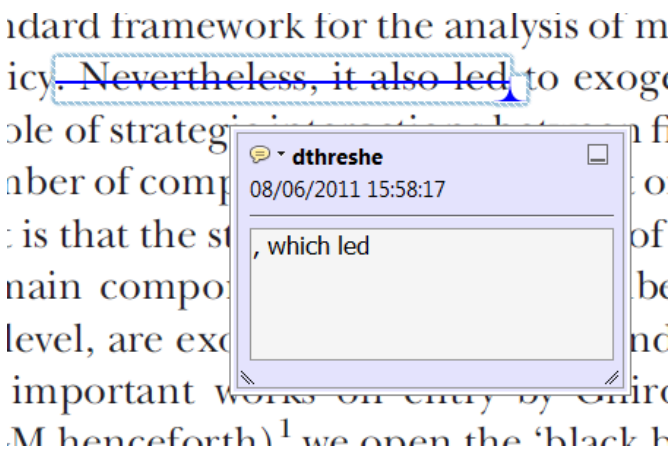


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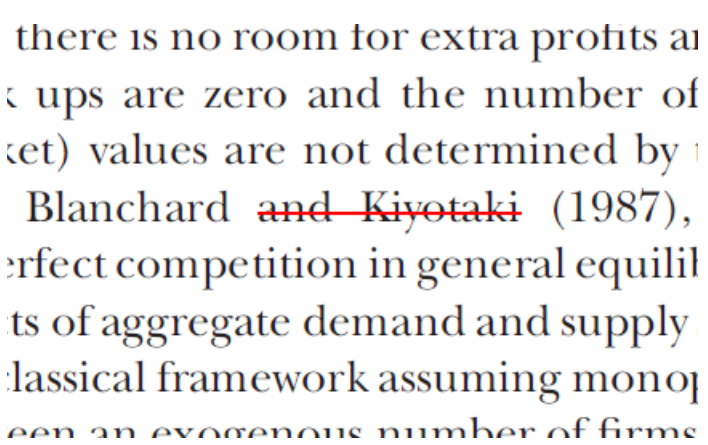

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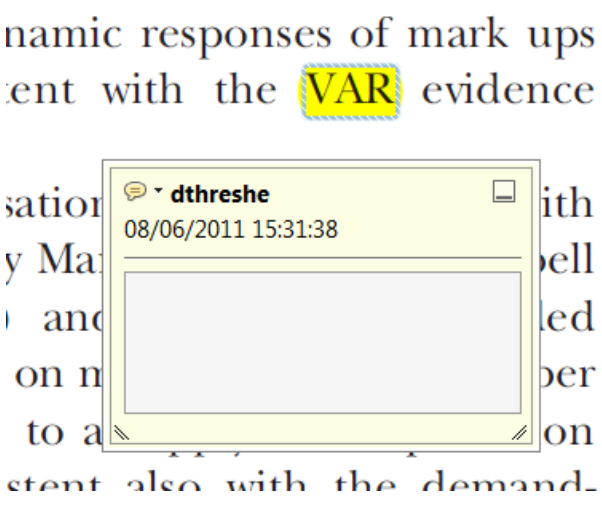


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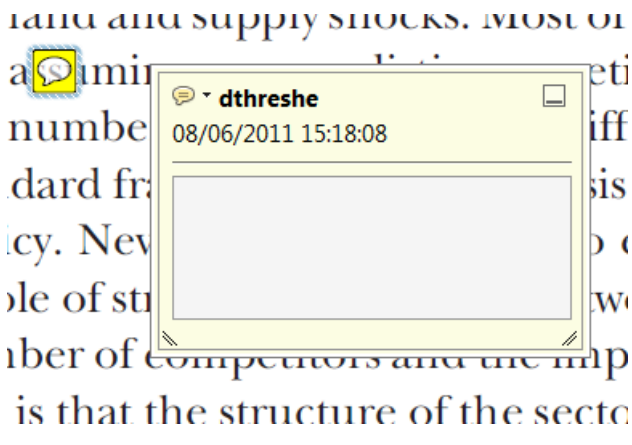

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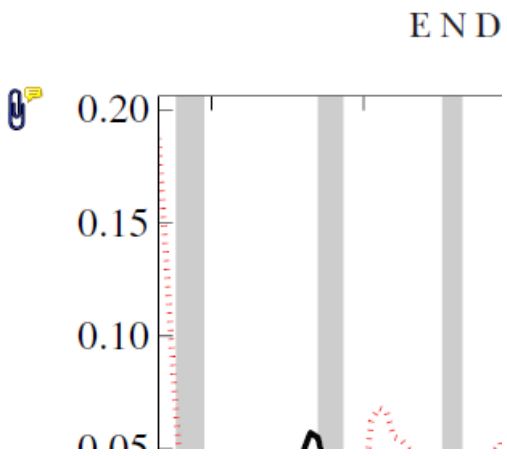


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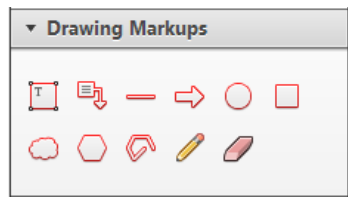


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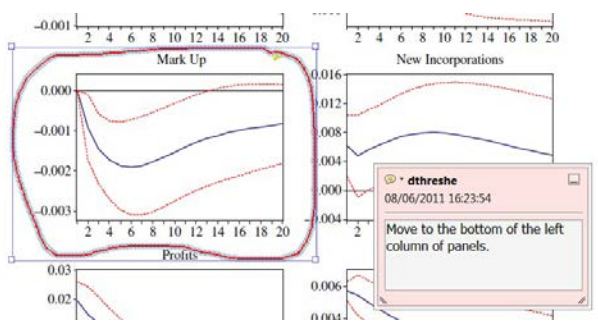

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RESEARCH ARTICLE

# Combined Effects of Transferrin and Thyroid Hormone During Oligodendrogenesis *In Vitro*

AQ6 AQ1 L. N. Marziali,[1] J. Correale,[2] C. I. Garcia,[3] and J. M. Pasquini[1]

Thyroid hormones (THs) and transferrin (Tf) are factors capable of favoring myelination due to their positive effects on oligodendroglial cell (OLG) differentiation. The first notion of a combined effect of apotransferrin (aTf) and TH emerged from experiments conducted in young hyperthyroid animals, which showed a seven-fold increase in the expression of Tf mRNA and precocious myelination when compared with control animals. The mechanism underlying this phenomenon in young hyperthyroid rats could consist of an increase in Tf synthesis, which in the CNS is almost exclusively produced by OLG. Overall, our results show that, during the initial stages of OLG differentiation, Tf synthesis triggers thyroid hormone receptor alpha 1 (TR$\alpha$1) expression in the subventricular zone (SVZ) and promotes proliferating cells to become responsive to this trophic factor. Exposure to TH could then regulate Tf expression through TR$\alpha$1 and promote the induction of thyroid hormone receptor beta (TR$\beta$) expression, which mediates TH effects on myelination through the activation of final OLG differentiation. This regulation of the combined effects of Tf and THs implies that both factors are fundamental actors during oligodendrogenesis.

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**Key words:** oligodendrocytes, myelin, transferrin, thyroid hormones, thyroid hormone receptors

## Introduction

The development of the vertebrate CNS involves a succession of finely coordinated events taking place on a time course pattern (Nicolay et al., 2007). Initially, neural stem cells (NSCs) differentiate first into neurons, then into astrocytes and, finally, into oligodendrocyte precursors (OPCs). Later on, OPCs mature to render myelinating oligodendrocytes (OLGs), which will extend and wrap their membranes around axons in order to originate myelin, a process that in rodents takes place post natally (Rowitch and Kriegstein, 2010). After birth, NSCs remain in restricted regions of the CNS such as the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus, niches which serve as a source of new OPCs that migrate along the CNS and mature to produce myelin (Garzón-Muvdi et al., 2010; Takebayashi and Ikenaka, 2015).

Thyroid hormones (THs) and the glycoprotein transferrin (Tf) are factors capable of favoring myelination on the basis of their positive effects on oligodendrogenesis and the maturation of OPCs, extensively reviewed (Bernal, 2005; Franco et al., 2015; Leitner and Connor, 2012; Todorich et al., 2009). The first hint of a combined effect of apotransferrin (aTf) and THs was revealed by studies carried out on young hyperthyroid animals, which rendered a seven-fold increase in the expression of Tf mRNA and accelerated myelination when compared with euthyroid animals (Marta et al., 1998). This increase in the synthesis of Tf, which in the CNS is almost exclusively produced by OLGs (Bloch et al., 1985; Espinosa de los Monteros et al., 1988), could be one of the mechanisms triggered by THs to induce the accelerated myelination process observed in young hyperthyroid rats. Such changes also seem to stress the importance of Tf in the biology of myelin and OLG maturation, an important fact that has been strengthened by studies of our group related to the action of a single intracerebral injection of this glycoprotein in young rats (Escobar Cabrera et al., 1994, 1997).

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It is clear that THs contribute to the timing of OLG development *in vitro* and *in vivo* (Ahlgren et al., 1997; Barres et al., 1994). THs actions are mediated by nuclear hormone receptors alpha and beta (TR$\alpha$ and TR$\beta$), whose importance is well established. Studies performed on TR-knockout animals have shown that OPC maturation is slightly delayed in THRA$^{-/-}$ but not in THRB$^{-/-}$ mice (Billon et al., 2001, 2002), whereas a population of slow cycling OPCs persists in the optic nerve of mice deficient in all receptors (Bass, 2002). Our group has demonstrated that only the more mature N20.1 oligodendroglial cell line responds to aTf, increasing both TR$\alpha$ and TR$\beta$ expression and their DNA-binding capacity (Paez et al., 2004, 2006). However, when myelin basic protein (MBP) expression was evaluated in these cells in a medium containing high amounts of THs, no changes were detected in the absence or presence of aTf. It is well known that the MBP promoter is activated by THs via a TH-response element (TRE) (Farsetti et al., 1991) and that TRs bind THs with high affinity and recognize identical TREs (Lazar, 1993). Therefore, by inducing TRs, aTf may participate in the cell differentiation process but not in MBP synthesis regulation.

Baxi et al. (2014) has shown that a TR$\beta$ agonist promotes oligodendrogenesis in rodents as well as in human OPCs *in vitro. In vivo*, this thyromimetic compound increases the oligodendrogenesis and the expression of myelin proteins such as MBP, 2′,3′cyclic nucleotide phosphohydrolase (CNPase) and myelin associated glycoprotein (MAG), which indicates that beta receptors can enhance OLG differentiation and promote myelination. As demonstrated by studies from our laboratory, TRs are not expressed in the SVZ of control animals; in contrast, TR$\alpha$ is induced in the SVZ during demyelination, although no significant changes are observed between demyelinated and TH-treated animals. In contrast, TR$\beta$ expression is only faintly detected in demyelinated animals but strongly upregulated in the periventricular area of demyelinated rats treated with THs. These results suggest that the regulation of TR$\alpha$ and TR$\beta$ expression may differ (Franco et al., 2008). Accordingly, results from recent work indicate that TR$\alpha$1 is independent of TH modulation and that its expression is induced by aTf, while TR$\beta$ expression appears to respond exclusively to TH stimulus (Marziali et al., 2015).

Studies on TH effects on OPCs have demonstrated the ability of T3 to induce a variety of transcription factors (Dugas et al., 2006), among which Kruppel-Like Factor 9 (KLF9) is the most rapidly and robustly induced, and found to be capable of promoting OLG differentiation *in vitro* and reducing the impact of myelin loss during cuprizone-induced demyelination (Dugas et al., 2012). In addition, our group has recently demonstrated that, at P10, KLF9 mRNA expression increases in hyperthyroid animals and decreases in hypothyroid ones. However, the values in animals injected with Tf are similar to controls, which support the notion that KLF9 exclusively mediates TH ability to accelerate the myelination process (Marziali et al., 2015).

Taken together, our previous results so far show that both Tf and THs are necessary for proper OLG development from NSCs and, furthermore, that neither factor is capable of compensating the absence of its counterpart's effects. Also, TRs appear to be key factors to understand the cooperative effects of Tf and THs. In this context, and to further explore their impact on OLG development, the present work investigates the relationship between Tf and THs and their role during oligodendrogenesis *in vitro*.

## Materials and Methods

### Animals and Animal Treatments

All animal protocols were approved by the Institutional Review Board of the School of Pharmacy and Biochemistry, University of Buenos Aires, and animal experimentation was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Culture of SVZ-derived NSCs

Zero- to three-day-old Wistar rats were used in all experiments. The procedure used for neurosphere culture has been previously described (Silvestroff et al., 2012). After 7 days, the neurospheres were pelleted and mechanically dissociated. Cells were cultured another 7 days in the presence of bFGF (20 ng mL$^{-1}$) and EGF (20 ng mL$^{-1}$). Neurospheres were pelleted, dissociated and cell differentiation triggered by plating the single cell suspensions on glass coverslips or multiwell plates coated with poly-L-ornithine (0.1 mg mL$^{-1}$, SIGMA, poly-L-ornithine hydrobromide molecular weight 30,000–70,000). bFGF (20 ng mL$^{-1}$) and PDGF-AA (20 ng mL$^{-1}$, PeproTech, Recombinant Human PDGF-AA) were added to the culture media and removed after 2 days, and cells were allowed to differentiate during 6 more days.

NSCs were treated with aTf or T3 during differentiation. The dose of aTf used was previously described by Silvestroff et al. (2012), while the dose of T3 was determined in previous experiments according to a dose-response curve (T3 concentration vs. MBP$^+$ cells originated after 6 days of exposure).

### Production and Concentration of Retrovirus

Viral particles were produced and concentrated as indicated by Kutner et al. (2009). Retroviral particles were constructed containing shRNAs that codify for 19-nt long sequences with homology to Rattus norvegicus mRNA for transferrin (NM_001013110.1) (Seq 1 and Seq 2) or without homology to any rat mRNA (scramble-SC). The 19-nt long sequences used are: Scramble (Scr) GAAACTGCT GACCGTTAAT, Sequence 1 (Seq1) GCTCCGAACAACAGAGA GG and Sequence 2 (Seq2) GGCTGACAGGGATCAATAT. The different 19-nt sequences had been cloned into the pSUPER vector (pSUPER.retro.puro, Oligoengine®, pSUPER.retro.puro, Seattle, WA) and each shRNA containing pSUPER vector was cotransfected together with pGAG and pVSV packaging plasmids into HEK293T cells. Supernatants containing viral particles were harvested sequentially during 3 days and viral particles concentrated using ultracentrifugation to render viral stocks.





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### Retrovirus-mediated Tf Knockdown

After 7 days, spheres were dissociated, split into a six-well plate and kept proliferating with bFGF and EGF. The next day 1 μg $\mu L^{-1}$ Polybrene® (SIGMA, St. Louis, MO, 1,5-dimethyl-1,5-diaza decamethylene poly methobromide, hexadimethrinebromid) was added together with 1 μL of the corresponding retroviral stocks. On the next day, another 1 μL of viral stocks was added. The next day, retroviruses were removed and media renewed. Cells were cultured for 1 more day, after which 1 μg $mL^{-1}$ puromycin (InvivoGen, San Diego, CA) was added during 3 days. Finally, cells were dissociated, plated and allowed to differentiate. bFGF (20 ng $mL^{-1}$) and PDGF-AA (20 ng $mL^{-1}$) were added to the culture media together with triiodothyronine (T3, 50 nM, SIGMA, 3,3′,5-Triiodo-L-thyronine sodium salt), aTf (100 μg $mL^{-1}$, SIGMA, human aTF) or saline. bFGF and PDGF-AA were removed after 2 days.

### Chemical Inhibition of TRα1

After 7 days, spheres were dissociated and cell differentiation initiated by plating the single cell suspensions on glass coverslips or multiwell plates coated with poly-L-ornithine. bFGF (20 ng $mL^{-1}$) and PDGF-AA (20 ng $mL^{-1}$) were added to the culture media together with 0.05% dimethyl sulfoxide (DMSO), the vehicle of TRα1 antagonist, I-850), I-850 TRα1 antagonist (3 μM, EMD-Millipore Corporation, Darmstadt, Germany, I-850, 2-(2-(-(4-nitrophenyl)−4-piperidinylidene)acetyl-*N*-(3-(trifluoromethyl)phenyl)−1-hydrazine carboxamide), aTf (100 μg $mL^{-1}$) −0.05%DMSO or the combination of the past two. bFGF and PDGF-AA were removed after 2 days and the cells were allowed to differentiate during 6 days.

### Immunocytochemistry

After differentiation, cells were fixed during 20 min at room temperature with PBS-buffered 2% paraformaldehyde (PFA). Cells were rinsed twice and blocked during 2 h at room temperature with 5% fetal calf serum (FCS)—0.3% Triton-X100—1X PBS. All the antibodies were diluted with 2% FCS—0.1% Triton-X100—1X PBS. Primary antibodies were incubated overnight at 4°C. The following primary antibodies were used: rabbit anti-MBP (1:400) (kind gift from Dr. Campagnoni, UCLA, USA), goat anti Platelet-Derived Growth Factor Receptor alpha (PDGF R Alpha/CD140A) (1:200, Neuromics), mouse anti-APC antibody (CC-1) (1:200, abcam®). After three washes, the cells were incubated with Hoechst-33258 (1 μg $mL^{-1}$) and the corresponding secondary antibodies conjugated to Alexa Fluor® (1:500, Jackson Immuno Research Laboratories) during 2 h at room temperature. Finally, cells were washed three times and mounted on slides with Mowiol® 4–88 anti-fade mounting solution (Calbiochem®). Images were acquired with an Olympus BX50 epifluorescence microscope (Olympus Corporation) and analyzed using ImageJ software.

### Protein Extraction and Western Blot

Proteins were solved in TOTEX buffer (20 nM HEPES pH 7.9, 350nM NaCl, 20% Glycerol, 1% Igepal, 1 mM $MgCl_2$ and 0.5 nM EGTA) containing complete protease inhibitor cocktail (SIGMA, complete™). Protein extracts were incubated on ice during 30 min and then centrifuged for 10 min at 10,000 rpm. Protein concentration was determined using Bradford's assay and 10 μg protein were subjected to SDS-PAGE in a 10 or 15% polyacrylamide gel. Proteins were transferred onto PVDF membranes and blocked during 2 h at room temperature with 5% non-fat milk—TBS-T 1×. All the antibodies were diluted with 2% non-fat milk—TBS-T 1×. Primary antibodies were incubated overnight at 4°C. The following primary antibodies were used: anti-Tf (1:1000, GeneTex, Serotransferrin antibody) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000, abcam®). After being washed, the membranes were incubated with the corresponding horse radish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Jackson Immuno Research Laboratories) during 2 h at room temperature. Membranes were developed by colorimetric assay using 0.1% 3,3′-diaminobenzidine (DAB), 0.1% $NiSO_4$, 0.1 M sodium acetate buffered solution, pH 5, and freshly added 0.01% hydrogen peroxide. Densitometric analyses were performed using Gel Pro 4 software (Media Cybernetic, Bethesda, MD).

### RNA Extraction and RT-PCR

Cell RNA was extracted using Trizol reagent according to the manufacturer's instructions. RNA integrity was checked and cDNA synthesis done using 1 μg of total RNA, Oligo d(T) adaptors and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase according to the manufacturer's instructions (Promega, Madison, WI). After retro-transcription, RNA was degraded and cDNA precipitated. Briefly, NaOH to a final concentration of 0.5 M was added to the samples and incubated at 65°C for 30 min; the solution was neutralized by the addition of HEPES and cDNA was precipitated with ethanol.

For PCRs 30 ng cDNA were used for each PCR reaction by means of an Eppendorf Mastercycler Personal 5332.PCRs were performed as follows: 0.5 μM of the corresponding pair of primers, 1.25U GoTaq® DNA Polymerase (Promega, Madison, WI), 0.2 mM dNTPs mix and 1× GoTaq® DNA Polymerase Reaction Buffer (1.5 mM $MgCl_2$) with the following reaction cycling parameters: an initial 4-min denaturation step at 94°C, and cycles consisting of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C. After reactions were performed, PCR products were electrophoresed on 2% agarose gels stained with 0.5 μg $mL^{-1}$ ethidium bromide. Gels were photographed and densitometric analyses performed using Gel Pro 4 software (Media Cybernetic, Bethesda, MD).

The following primers were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH. NM_017008.4) Forward: CTTACTCCTT GGAGGCCATG, Reverse: TTAGCCCCCCTGGCCAAGG. Kruppel-like factor 9 (Klf9, NM_057211.1) described previously (Dugas et al., 2012): Forward TCCGGAACTTTCAAACCTTG; Reverse GTGTGCCAAACAGAATGTCG. Tf (NM_001013110.1): Forward CCTCAAAGTGGCTCAGGAACA; Reverse AGGAGAG CCGAACAGTTGGA; Thyroid hormone receptor alpha 1 (TRα1, NM_001017960.1): Forward TTCAGCGAGTTTACCAAGATCAT CAC; Reverse TTAGACTTCCTGATCCTCAAAGACCT. Thyroid hormone receptor beta1/2 (TR*β*1/TR*β*2, NM_012672.3/ NM_001270854.1): Forward AAGTTGCCCATGTTTTGTGAG; Reverse TCACTGCCATTTCCCCATTC.

### MTT Assay

MTT assay was performed according to a previous published method (Hansen et al., 1989) and absorbance measured with an Amersham Biotrack TM II Visible Plate Readerat 570-nm wave length.





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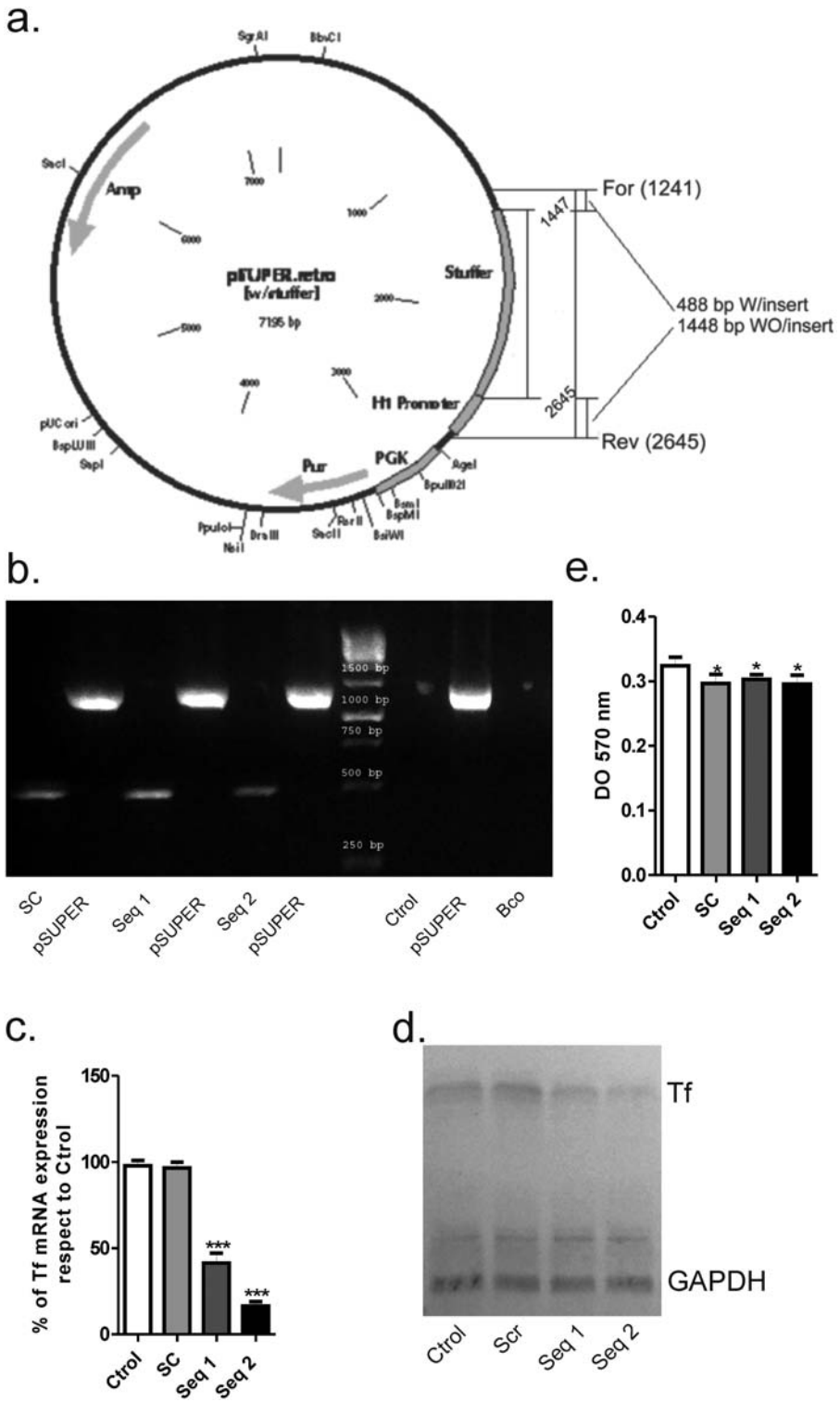


**FIGURE 1: Downregulation of Tf by shRNA. (a, b) Retroviruses were constructed with pSUPER vectors coding for two specific Tf shRNAs and NSCs transduced with these viral particles. (c, d) Knockdown efficiency was determined by RT-qPCR and Western blot and a 70% decrease in Tf expression was observed. (e) Cell viability was assayed through MTT and no differences were found among transduced cells containing shRNA with no homology to any rat mRNA (Scr) and cells transduced with Tf shRNAs (Seq 1 and Seq 2).**

### *Statistical Analysis*

Data analysis was conducted with Graph-Pad Prism 5.0 (GraphPad Software, La Jolla, United States) Software and results were expressed as the mean ± standard deviation (SD). Three independent tests were performed for each experiment. To compare experimental groups, one-way ANOVA followed by Newman–Keuls post-hoc

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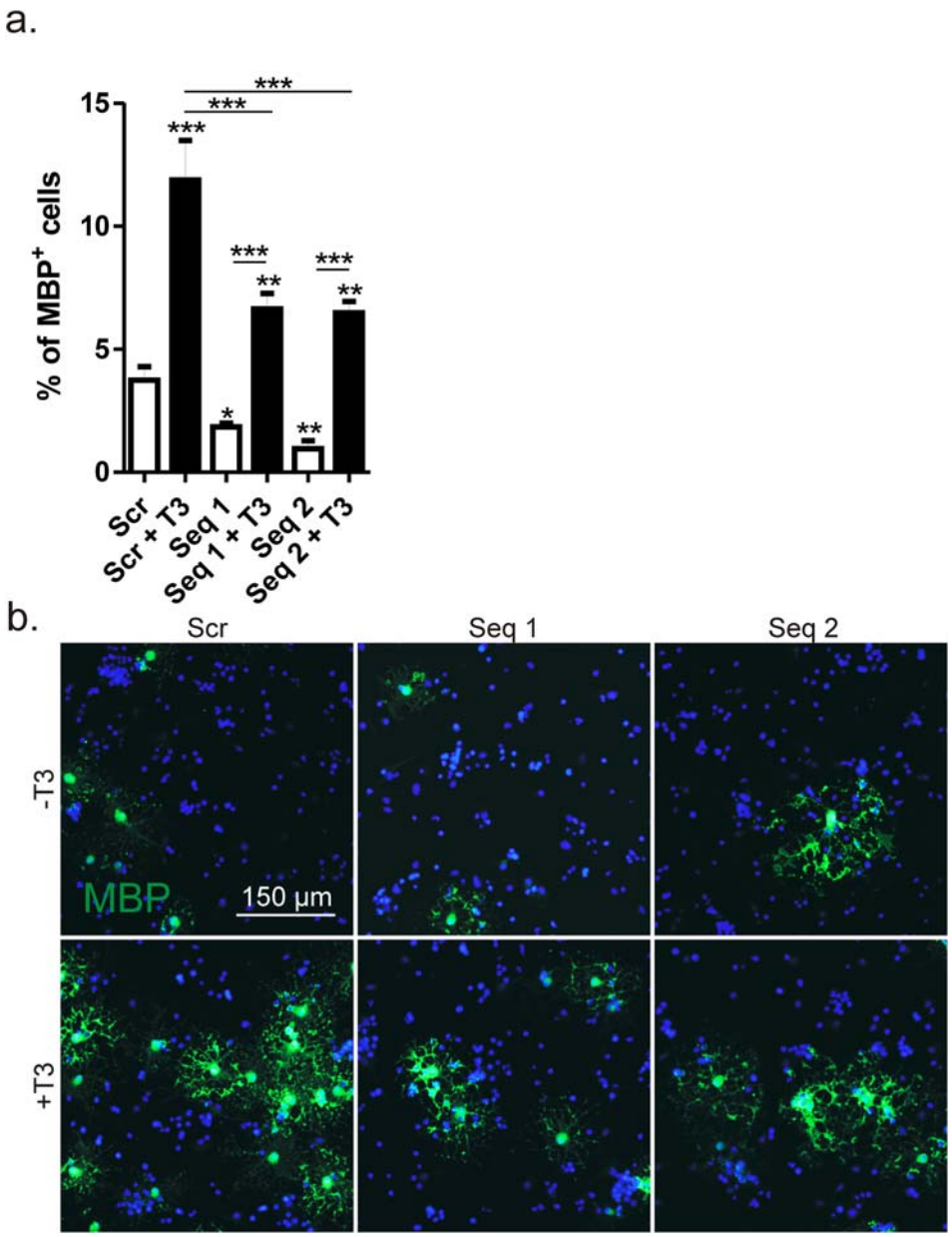


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**FIGURE 2: Pro-oligodendrogenic effect of T3 under Tf silencing conditions. (a) Quantitation of the effect of Tf knockdown (Seq 1 and Seq 2) on MBP$^+$ OLG population and the partial reversion caused by the administration of 50 nM T3 (Seq 1 + T3 and Seq 2 + T3). (b) Representative images of each experimental condition. Statistical analysis was performed using one way ANOVA followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance respect to Scramble (Scr); when comparisons were made among other groups, line bars indicate the groups involved. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.**

tests, two-way ANOVA followed by Bonferroni post-hoc tests or Student's *t* tests were used. Statistical significance indicated with asterisks: *$P < 0.05$; **$P < 0.01$, and ***$P < 0.001$.

## Results

### *Tf Knockdown Decreases OPC Maturation*

Experiments were performed on Tf silencing mediated by shRNA. Knockdown efficiency was checked by Western blot and RT-qPCR, resulting in an average of 70% decrease in Tf expression. Cell viability was checked through MTT assays. No differences were found between cells transduced with viral particles containing shRNA with scramble mRNA and cells transduced viral particles containing shRNAs with homology to Tf mRNA (Seq 1 and Seq 2) (Fig. 1). F1

To test the combined effects of Tf and THs on NSC oligodendrogenesis, transduced cells were treated with T3 and allowed to differentiate during 3 days. OLGs were then immunocytochemically analysed using anti-MBP antibody, with results showing that, when Tf expression is down regulated, THs only partially favour the capacity of NSCs to generate OLGs (Fig. 2). F2



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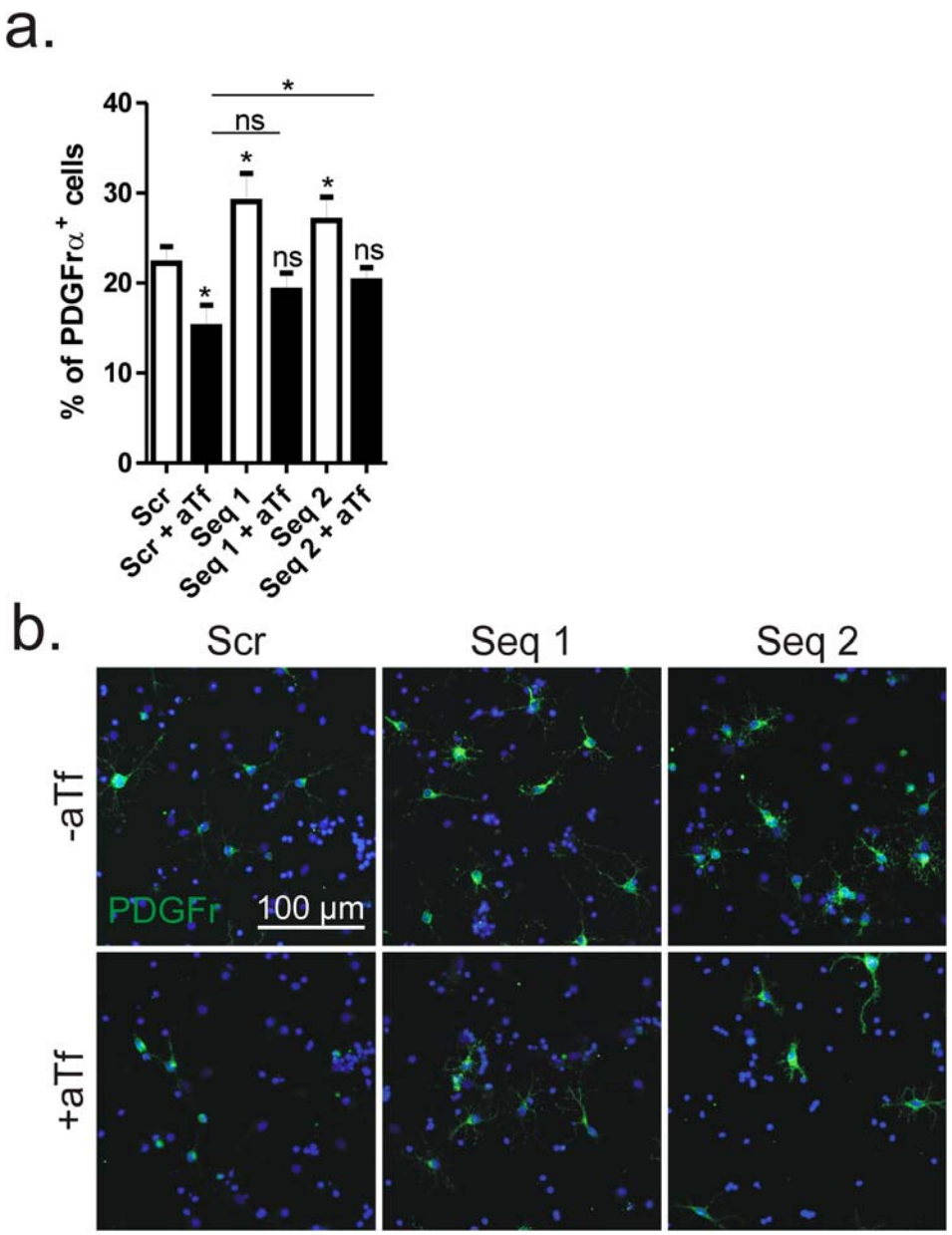


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**FIGURE 3: Effect of Tf silencing on PDGFrα$^+$ OPC population. (a) Quantitation of the effect of Tf knockdown (Seq 1 and Seq 2) on PDGFrα$^+$ OPC population and the reversion caused by the administration of 100 μg μL$^{-1}$ exogenous aTf (Seq 1 + aTf and Seq 2 + aTf). (b) Representative images of each experimental condition. Statistical analysis was performed using one way ANOVA followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance respect to Scramble (Scr); when comparisons were made among other groups, line bars indicate the groups involved. *$P \leq 0.05$.**

The maturation of NSC-derived OLGs was further evaluated by immunocytochemistry using antibodies against PDGFRα for OPCs and MBP/CC1 for mature OLGs, after NSCs were allowed to differentiate during 6 days. The amount PDGFRα$^+$ OPCs was higher when cells were transduced with Seq 1 and Seq 2 as compared with Scr, while treatment with 100 μg mL$^{-1}$ aTf brought the amount of PDGFRα$^+$ cells near Scr values. In turn, the comparison of Scr cells and Scr cells treated with aTf (Scr + aTf) showed aTf ability to reduce the number of PDGFRα$^+$ cells (Fig. 3). Tests using MBP and CC1 antibodies in Scr + aTf cells showed an expected increase in the number of mature OLGs, in accordance with previous results from our laboratory (Silvestroff et al., 2012).

In contrast, Tf-knockdown cells had the opposite effect on the mature OLG population, evidenced by fewer MBP$^+$ and CC1$^+$ cells in both in Seq 1 and Seq 2 cells as compared with Scr cells. Finally, the addition of 100 μg mL$^{-1}$ aTf completely restored the number of mature OLGs, now comparable to the amount observed in Scr + aTf cells. Altogether,



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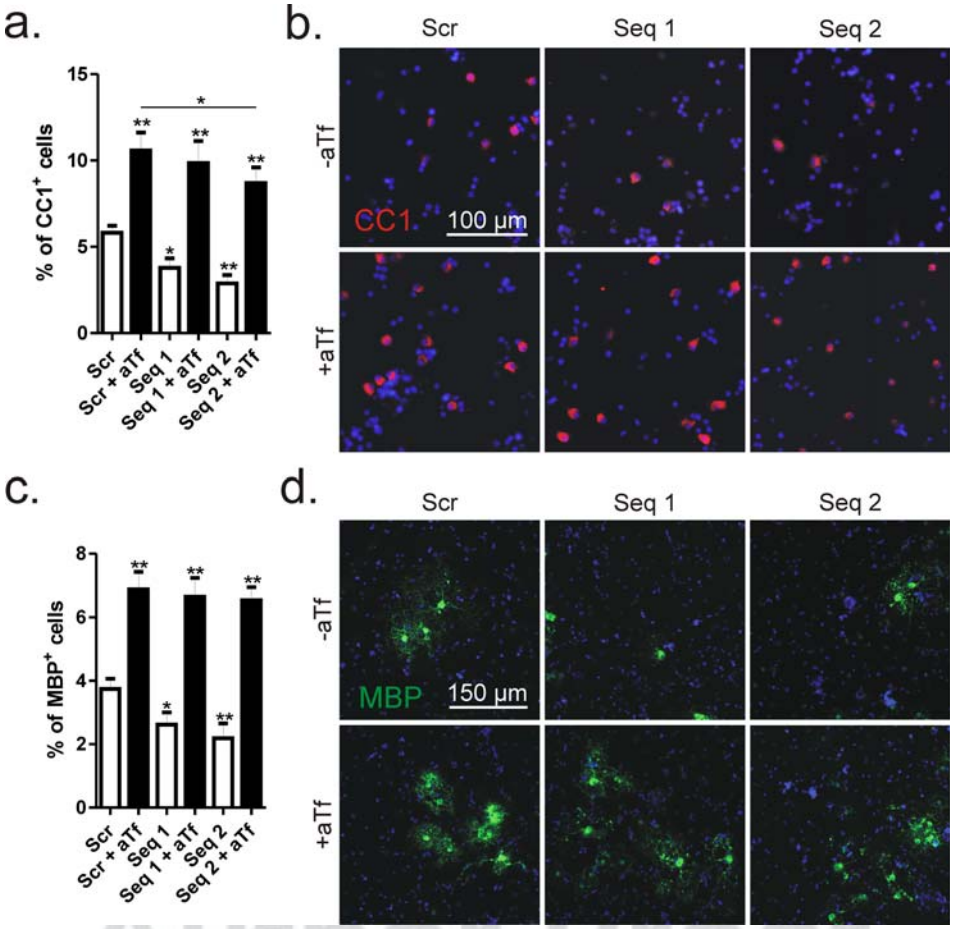


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**FIGURE 4: Effect of Tf silencing on CC1$^+$ and MBP$^+$ OLG population. (a) Quantitation of the effect of Tf knockdown (Seq 1 and Seq 2) on CC1$^+$ OLG population and the reversion caused by the administration of 100 µg µL$^{-1}$ exogenous aTf (Seq 1 + aTf and Seq 2 + aTf). (b) Representative images of each experimental condition. (c) Quantitation of the effect of Tf knockdown (Seq 1 and Seq 2) on MBP$^+$ OLG population and the reversion caused by the administration of 100 µg µL$^{-1}$ exogenous aTf (Seq 1 + aTf and Seq 2 + aTf). (d) Representative images of each experimental condition. Statistical analysis was performed using one way ANOVA followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance respect to Scramble (Scr); when comparisons were made among other groups, line bars indicate the groups involved. *$P \leq 0.05$, **$P \leq 0.01$.**

these results show that Tf down regulation impairs OPC maturation (Fig. 4). F4

### *TRα1 Inhibition Limits OPC Maturation*

Control NSCs were used to test the expression of Tf, TH regulators such as KLF9 and TRα1 and TR$\beta$, during *in vitro* OLG differentiation. Tf and TRα1 were found to constantly increase from DIV0 to DIV6, while TR$\beta$ increased up to DIV4 and decreased thereafter. KLF9 expression did not change along the experimental timeline (Fig. 5). F5

To test the effects of Tf gene silencing on the different TH modulators and Tf expression in the presence of T3, transduced NSCs were analysed after differentiating during 3 days.

PCR analyses show an increase in Tf expression in control cells after T3 treatment and a down regulation in Seq 1 and Seq 2 cells, unchanged by T3. Notably, when cells were treated with T3 during differentiation, the amount of Tf in the culture media decreased, indicating the absence of Tf secretion during differentiation (unpublished results). Regarding TRs, TRα1 decreased in Tf-knockdown cells and was unaffected by the addition of T3, while TR$\beta$ remained unaltered by Tf silencing but increased with the addition of T3. Similar results were observed when KLF9 was analyzed in the same experimental conditions (Fig. 6). F6

To study the effect of TRα1 on oligodendrogenesis, its antagonist was added to the culture and immunocytochemical studies were performed in NSCs under differentiation using antibodies against PDGFRα for OPCs and MBP/CC1 for mature OLGs during 6 days. Results show that treatment with the TRα1 antagonist I-850 increased the number of PGDFRα$^+$ cells when compared with vehicle (DMSO) treatment, an effect partially compensated upon aTf administration. In turn, aTf-DMSO treatment resulted in a lower number of PGDFRα$^+$ cells compared with DMSO (Fig. 7). F7



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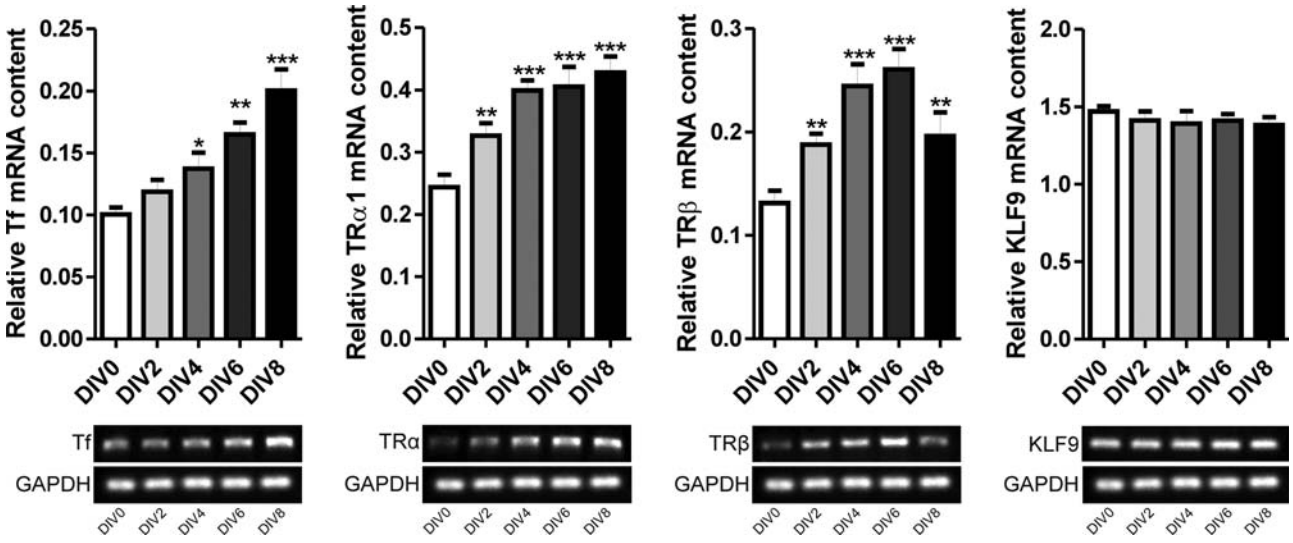


**FIGURE 5: Expression of Tf, TRα, TR*β*, and KLF9 during NSC differentiation. The analysis was performed by RT-PCR and GAPDH used as housekeeping gene. Samples were obtained every 2 days during an 8-day-long protocol (DIV2, 4, 6, and 8). IODs were determined with Gel-Pro Analyzer 4.0 and IOD of every gene analyzed was normalized to GAPDH. Results are expressed as relative to the housekeeping gene. Statistical analysis was performed using one way ANOVA followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance respect to controls (Ctrol); when comparisons were made among other groups, line bars indicate the groups involved. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.**

By contrast, treatment with TRα1 antagonist reduced the number of mature OLGs, as evidenced by fewer $MBP^+$ and $CC1^+$ cells than in DMSO treatment conditions, while aTf-DMSO treatment rendered a higher number of $MBP^+$ and $CC1^+$ cells. The capacity of aTf to favour the production of mature OLGs cells was also observed when cells were double-treated with TRα1 antagonist and aTf. Nevertheless, aTf effects appeared to be weaker in this case, as the number of $MBP^+$ and $CC1^+$ cells after combined treatment did not reach the values of aTf-DMSO-treated cells (Fig. 8). F8

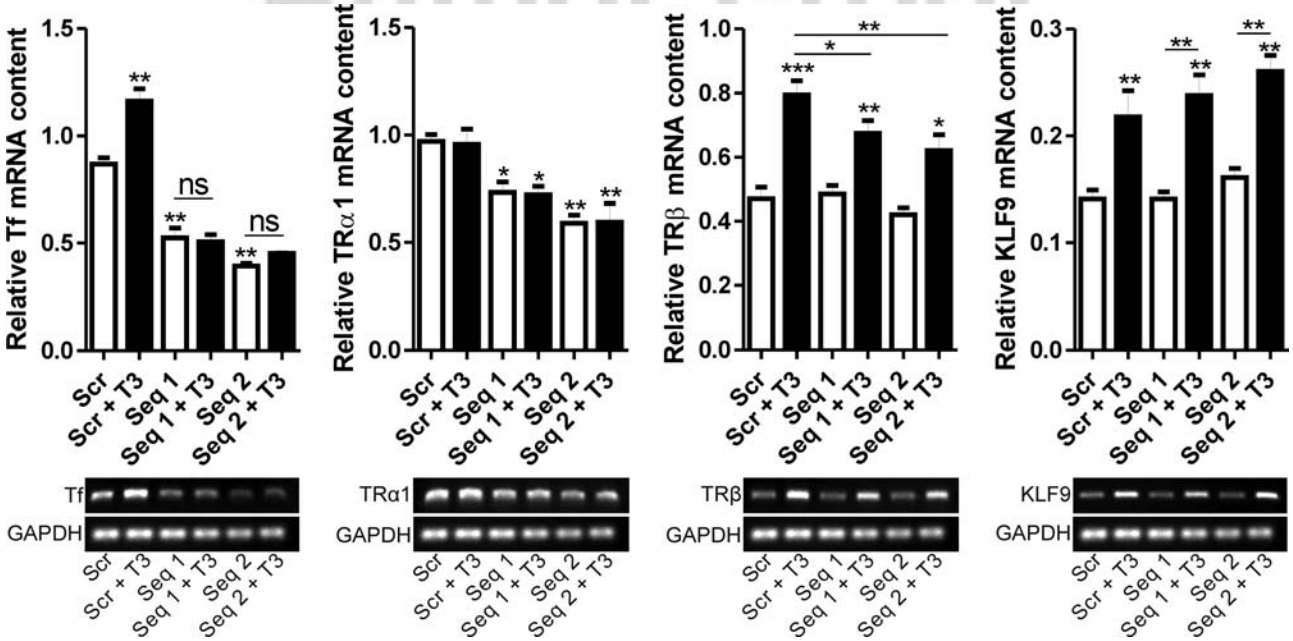


**FIGURE 6: Effect of T3 on the expression of Tf, TRα, TR*β*, and KLF9 during NSC differentiation under Tf silencing conditions (Seq 1 and Seq 2). The analysis was performed by RT-PCR and GAPDH used as housekeeping gene. Samples were obtained after 3 days of differentiation (DIV3). IODs were determined with Gel-Pro Analyzer 4.0 and IOD of every gene analyzed was normalized to GAPDH. Results are expressed as relative to the housekeeping gene. Statistical analysis was performed using one way ANOVA followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance respect to Scramble (Scr); when comparisons were made among other groups, line bars indicate the groups involved.*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.**

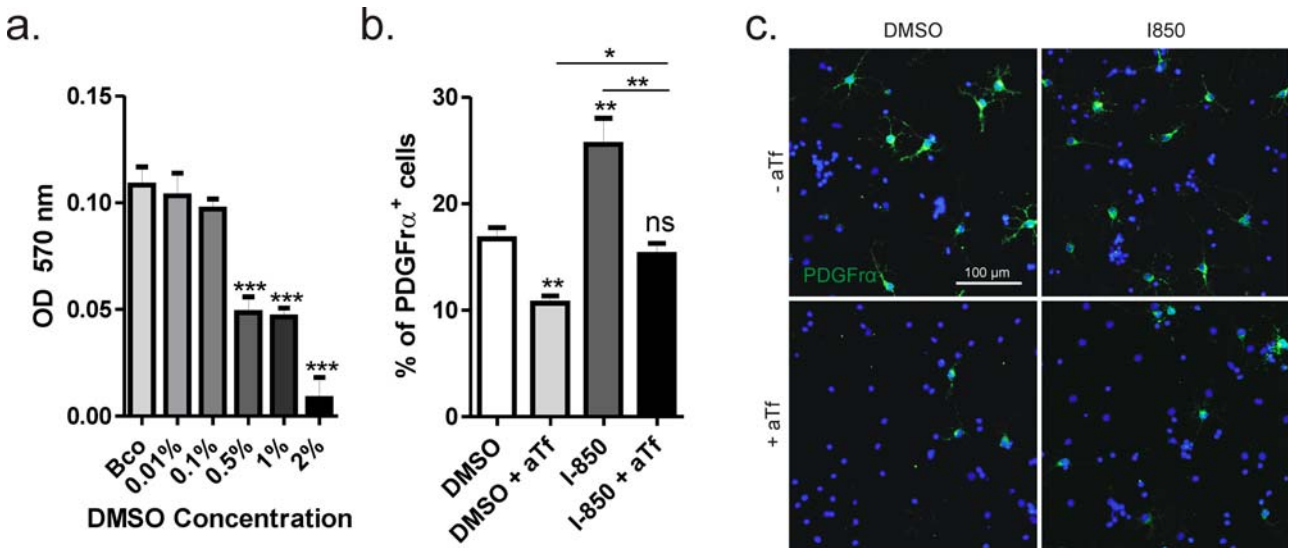


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**FIGURE 7: Effect of TRα1 antagonist on PDGFrα$^+$ OPC population. (a) Cell viability was assayed through MTT of DMSO (TRα1 antagonist vehicle). (b) Quantitation of the effect of TRα1 antagonist I-850 on PDGFrα$^+$ OPC population and the reversion caused by the administration of 100 μg μL$^{-1}$ exogenous aTf (I-850 + aTf). (c) Representative images of each experimental condition. Statistical analysis was performed using one way ANOVA followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance respect to non-treated cells (DMSO); when comparisons were made among other groups, line bars indicate the groups involved.*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.**

NSCs were used to study the effects of aTf administration in the presence of TRα1 inhibitor over the expression of the mRNAs of Tf, TH receptors and KLF9 all in the presence of Tf. The addition of 100 $\mu$g mL$^{-1}$ Tf to NSC cultures significantly decreased the expression of endogenous Tf. This decrease was more evident when the cells were cultivated in the presence of the inhibitor. The coincubation of Tf along with I-850 did not produce additive effects in the regulation of Tf mRNA, since the values observed were similar to those with only Tf (Fig. 9a). A likely behavior was obtained for the regulation of TRα1 (Fig. 9b). Both Tf and I-850 increase the receptor expression individually. However, the presence of the two factors in the culture medium renders similar results that Tf alone. The relative content of TR$\beta$ was similar in the presence and absence of Tf and decreased when the inhibitor was present, without changes upon Tf addition (Fig. 9c). Regarding KFL9, no changes were observed under any condition in its relative content (Fig. 9d).

Finally, effects of the TRα1 antagonist on the OPC and mature OLG populations were not reversed by T3 (Fig. 10).

## Discussion

A potential role for TH in myelination was initially suggested by clinical studies of infants with congenital hypothyroidism, where a major feature of the neuropathology was a paucity of myelin (Rosman, 1972). Notably these deficits were partially reversed by parental or breast milk administration of T4 (Noguchi et al., 1985). Conversely, when hyperthyroidism is induced in young animals, a significant increase in both myelin proteins gene expression and myelination was observed together with a dramatic increase in Tf mRNA (Marta et al., 1998). Increase in Tf synthesis could be one of the mechanisms triggered by THs which might be involved in the accelerated myelination observed in young hyperthyroid rats. It is clear that TH contributes to OLG maturation both *in vitro* and *in vivo* (Ahlgren et al., 1997; Barres et al., 1994), although the precise mechanism involved in this process remains undefined. Furthermore, the association between Tf and THs during oligodendrogenesis is far from being elucidated.

Previous results from our laboratory indicate a relationship between THs and Tf (Marta et al., 1998; Paez et al., 2004, 2006) however; a direct link between TR induction and MBP activation induced by aTf has not been clearly established yet. The increase in Tf mRNA levels reported above in young hyperthyroid animals and the fact these levels remain stable when maturation is complete both emphasize the role of Tf as a putative trophic factor and a key participant in myelin biology and OLG maturation. A recent *in vivo* study from our group established a possible relationship between THs and Tf through TH receptors and its implications in OLG development (Marziali et al., 2015). To further understand this interplay, the present work analyzes the effect of THs and Tf on Tf-silenced NSCs cultures and presents evidence that, when the Tf gene is knocked down in a neurosphere culture system, OPCs remain in an undifferentiated state until the addition of Tf allows them to differentiate. Moreover, T3 alone was unable to induce NSCs to

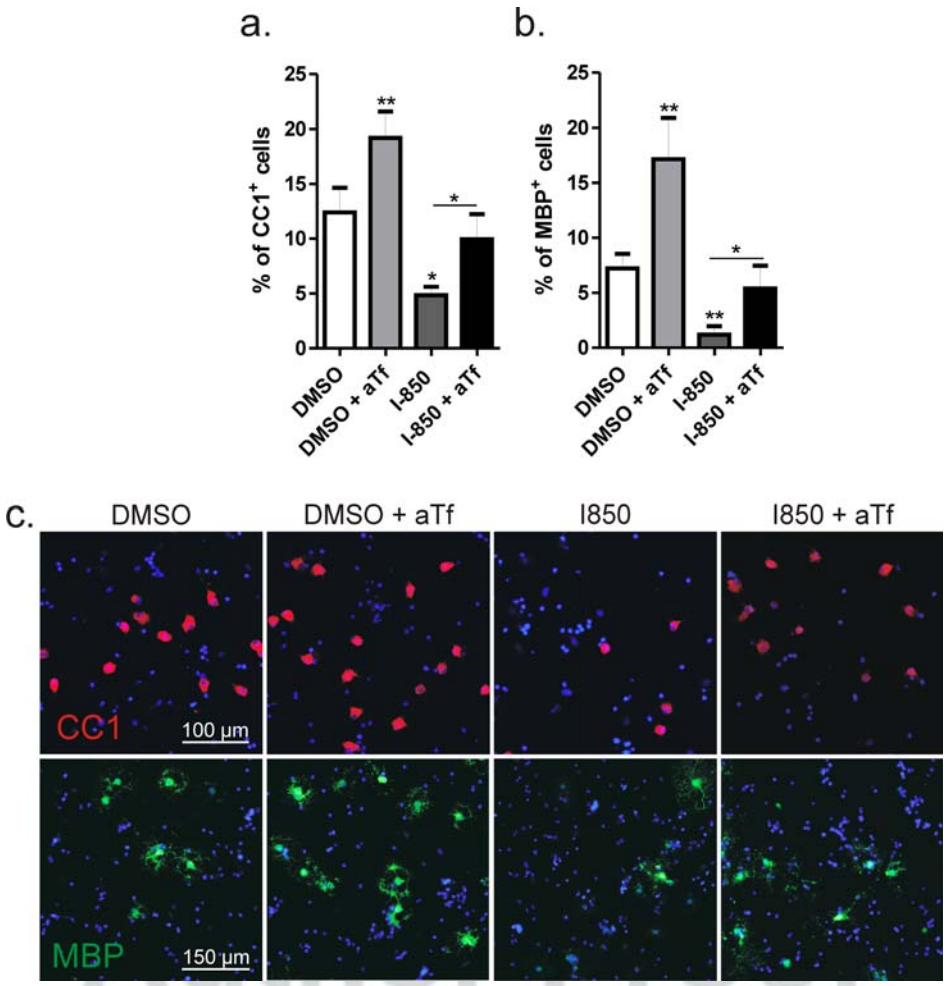


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**FIGURE 8: Effect of TRα1 antagonist on $CC1^+$ and $MBP^+$ OLG population. (a) Quantitation of the effect of TRα1 antagonist I-850 on $CC1^+$ OPC population and the partial reversion caused by the administration of 100 µg $µL^{-1}$ exogenous aTf (I-850 + aTf). (b) Quantitation of the effect of TRα1 antagonist I-850 on $MBP^+$ OLG population and the partial reversion caused by the administration of 100 µg $µL^{-1}$ exogenous aTf (I-850 + aTf). (d) Representative images of each experimental condition. Statistical analysis was performed using one way ANOVA followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance respect to non-treated cells (DMSO); when comparisons were made among other groups, line bars indicate the groups involved.*$P \leq 0.05$, **$P \leq 0.01$.**

differentiate into mature OLG, which indicates that the concomitant effects of both Tf and T3 are necessary and relevant for oligodendrogenesis.

There appears to be general consensus that both OPCs and OLGs express TRα and that OLGs express TR$\beta$ (Carré et al., 1998; Fierro-Renoy, 1995), in agreement with Bury et al. (2002), who showed that TRα and TR$\beta$ colocalize in OLGs before CNPase is expressed. Very recently, Baxi et al. (2014) showed that a TR$\beta$ agonist promotes oligodendrogenesis in rodents and in human OPCs *in vitro*. Results from another laboratory, however, suggest that OPC maturation is slightly delayed in $THRA^{-/-}$ but not in $THRB^{-/-}$ mice (Billon et al., 2001, 2002). These results could be explained by the activity of TRs in the absence of ligand, considering in this regard that the effects of THs on OPC maturation can be regulated in a simplified way as a combination of a negative stimulus (in the absence of ligand) and a positive stimulus (in the presence of ligand) mediated by TRα and a positive stimulus mediated by TR$\beta$ (Bernal and Morte, 2013).

A study by Lin (2003) using HepG2, a well differentiated hepatocellular carcinoma cell line without detectable TR protein expression, showed that TH treatment induced an increase in Tf mRNA and protein expression in a time- and dose-dependent manner in HepG2-TRα1 (cells overexpressing functional TRα1), but not in HepG2-Neo cells (control cells). This TH-dependent regulation of Tf takes place at a transcriptional level, as demonstrated by nuclear run-on experiments conducted by the above mentioned group. The authors also suggest that cells overexpressing TRα1 have lower levels of Tf mRNA and protein, indicating a possible negative effect of apo-TRα1 on Tf expression. The results by Lin (2003) imply that the induction of Tf by THs is direct and may in fact be

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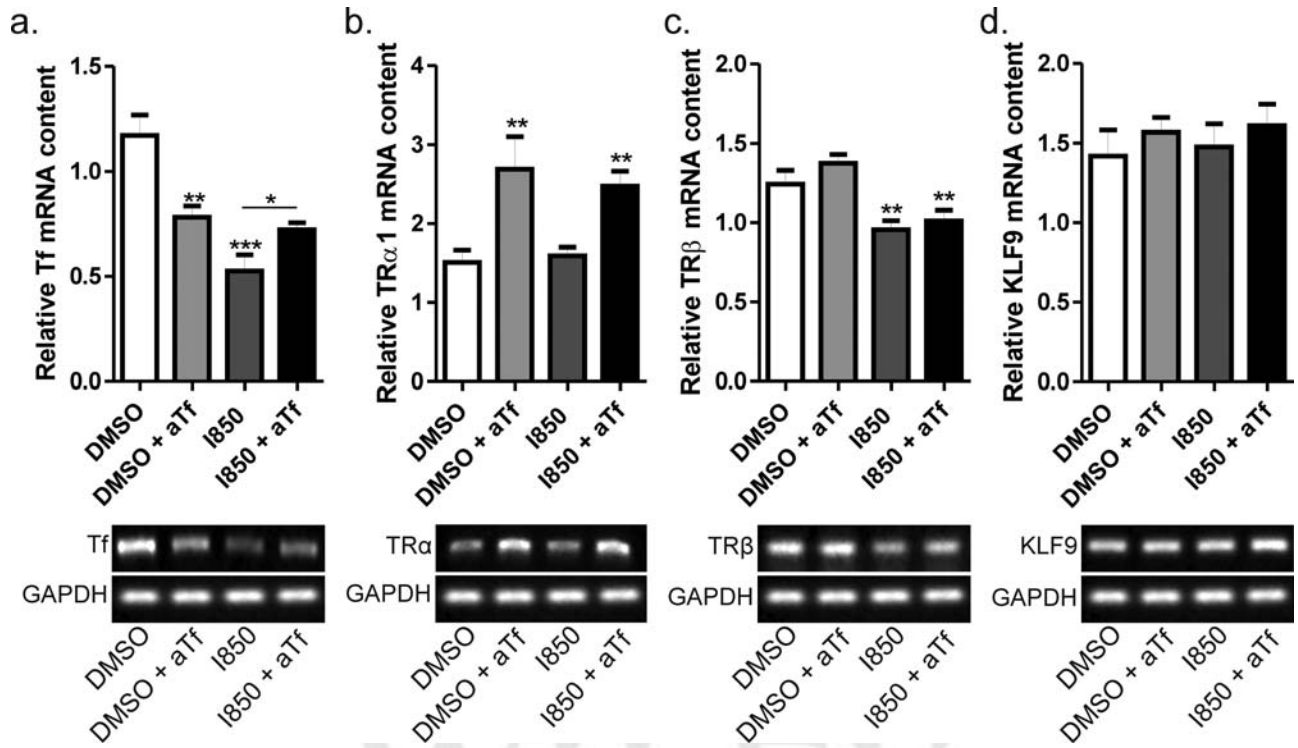


**FIGURE 9: Effect of aTf administration on the expression of Tf (a), TRα (b), TR*β* (c), and KLF9 (d) during NSC differentiation when treated with TRα1 antagonist I-850. The analysis was performed by RT-PCR and GAPDH used as housekeeping gene. Samples were obtained after 6 days of differentiation (DIV6). IODs were determined with Gel-Pro Analyzer 4.0 and IOD of every gene analyzed was normalized to GAPDH. Results are expressed as relative to the housekeeping gene. Statistical analysis was performed using one way ANOVA followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance respect to nontreated cells (DMSO); when comparisons were made among other groups, line bars indicate the groups involved.*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.**



mediated by a TRE in the promoter region. Given that TRα1 is needed to achieve proper oligodendroglial maturation and has emerged as a key factor in the regulation of Tf expression, this work tested the effects of I-850, a TRα1 selective antagonist, on OLG maturation. Results show that, in the presence of TRα1 antagonist, OLG differentiation was diminished and was subsequently and partially reversed by aTf administration.

TRα is ubiquitously expressed as from early developmental stages whereas TR*β* is expressed much later, which suggests different receptor functions (Bradley et al., 1992). Studies by our group show that TR expression is not detected in the SVZ of control animals. In contrast, TRα is induced in the demyelinated SVZ, with no significant changes between demyelinated and TH-treated animals. In turn, TR*β* is only slightly detected in demyelinated animals and strongly upregulated in the periventricular area of TH-treated demyelinated animals. These results suggest a differential regulation of TRα and TR*β* (Franco et al., 2008). Furthermore, our group has recently reported that, during myelination, TRα expression is independent of TH modulation and regulated by aTf, while TR*β* expression appears to respond exclusively to TH stimulus (Marziali et al., 2015).

Dugas et al. (2012) have shown KLF9 to be a transcription factor early induced by T3 administration and both necessary and sufficient to stop the proliferation of OPC and as a consequence to promote OLG differentiation *in vitro*. However, it is worth nothing that KLF9 loss, not perfectly recapitulate hypothiroisdism *in vivo*, because KLF9 is only one of the several genes induced by T3 in OPC. These other genes may contribute to promoting OLG differentiation and, if so, they could help to compensate for KLF9 loss (Dugas et al., 2012). In addition, our current studies demonstrate that aTf does not influence KLF9 expression, a finding which reinforces the hypothesis that aTf exerts a positive influence on OLG maturation independently of TH effects and that the absence of either factor hampers a proper final OLG maturation.

Overall, the present work confirms our *in vivo* results and sheds additional light on the mechanisms of OLG maturation during oligodendrogenesis, proving that TRα1 expression is independent of TH but completely dependent on aTf administration and that TR*β* is independent of aTf and only regulated by the presence of TH. It may be hence hypothesized that, during the initial stages of OLG differentiation, TH increases Tf, which in turns triggers TRα expression in the SVZ and promotes undifferentiated proliferating cells to become responsive to this trophic factor. Under these



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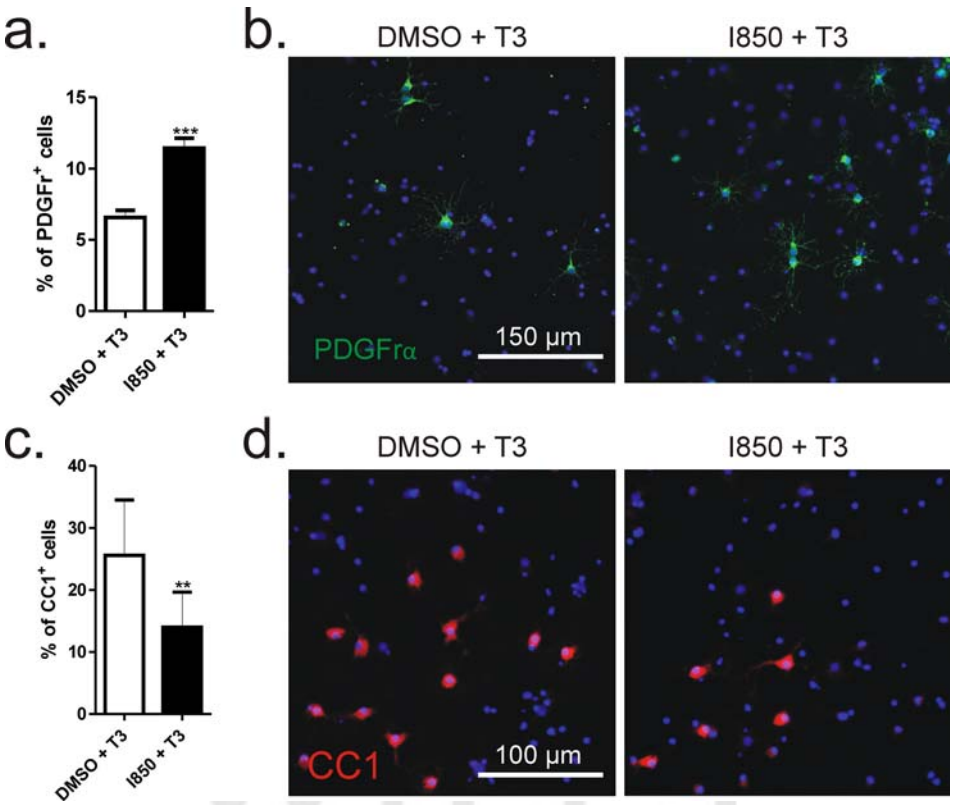


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**FIGURE 10: Effect of TRα1 antagonist on PDGFrα$^+$ OPCs and CC1+ OLGs despite the administration of T3. (a) Quantitation of the effect of TRα1 antagonist I-850 on PDGFrα+ OPC population and the absence of reversion by 50 nM T3 (I-850 + T3). (b) Representative images of each experimental condition. (c) Quantitation of the effect of TRα1 antagonist I-850 on CC1$^+$ OLG population and the absence of reversion by 50 nM T3 (I-850 + T3). (d) Representative images of each experimental condition. Statistical analysis was performed using Student's *t* test. *$P \leq 0.05$, **$P \leq 0.01$.**



circumstances, exposure to TH could, on the one hand, regulate Tf expression through TRα and, on the other hand, promote the induction of TR$\beta$ expression, which mediates TH effects on myelination through the activation of OLG differentiation. This elegantly tight regulation of responsiveness of Tf and TH implies a causal relationship showing that both factors are fundamental actors during the process of oligodendrogenesis in a unique interplay of the two signaling systems.

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## References

Ahlgren SC, Wallace H, Bishop J, Neophytou C, Raff MC. 1997. Effects of thyroid hormone on embryonic oligodendrocyte precursor cell development in vivo and in vitro. Mol Cell Neurosci 9:420–432.

Baas D, Legrand C, Samarut J, Flamant F. 2002. Persistence of oligodendrocyte precursor cells and altered myelination in optic nerve associated to retina degeneration in mice devoid of all thyroid hormone receptors. Proc Natl Acad Sci USA 99:2907–2911.

Barres BA, Lazar MA, Raff MC. 1994. A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. Development 120:1097–1108.

Baxi EG, Schott JT, Fairchild AN, Kirby LA, Karani R, Uapinyoying P, Pardo-Villamizar C, Rothstein JR, Bergles DE, Calabresi PA. 2014. A selective thyroid hormone $\beta$ receptor agonist enhances human and rodent oligodendrocyte differentiation. Glia 62:1513–1529. doi: 10.1002/glia.22697. Epub 2014 May 24.

Bernal J. 2005. Thyroid hormones and brain development. Vitam Horm 71: 95–122.

Bernal J, Morte B. 2013. Thyroid hormone receptor activity in the absence of ligand: Physiological and developmental implications. Biochim Biophys Acta 1830:3893–3899. doi: 10.1016/j.bbagen.2012.04.014. Epub 2012 Apr 24.

Billon N, Jolicoeur C, Tokumoto Y, Vennström B, Raff M. 2002. Normal timing of oligodendrocyte development depends on thyroid hormone receptor alpha 1 (TRalpha1). EMBO J 21:6452–6460.

Billon N, Tokumoto Y, Forrest D, Raff M. 2001. Role of thyroid hormone receptors in timing oligodendrocyte differentiation. Dev Biol 235:110–120.

Bloch B, Popovici T, Levin M-J, Tuil D, Kahn A. 1985. Transferrin gene expression visualized in oligodendrocytes of the rat brain by using in situ hybridization and immunohistochemistry. Proc Natl Acad Sci USA 82:6706–6710.

Bradley DJ, Towle HC, Young WS III. 1992. Spatial and temporal expression of alpha- and beta-thyroid hormone receptor mRNAs, including the beta 2-subtype, in the developing mammalian nervous system. J Neurosci 12:2288–2302.

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Bury F, Carré JL, Vega S, Ghandour MS, Rodriguez-Peña A, Langley K, Sarlième LL. 2002. Coexpression of thyroid hormone receptor isoforms in mouse oligodendrocytes. J Neurosci Res 67:106–113.

Carbajal KS, Miranda JL, Tsukamoto MR, Lane TE. 2011. CXCR4 signaling regulates remyelination by endogenous oligodendrocyte progenitor cells in a viral model of demyelination. Glia 59:1813–1821. doi: 10.1002/glia.21225.

Carré JL, Demerens C, Rodríguez-Peña A, Floch HH, Vincendon G, Sarlième LL. 1998. Thyroid hormone receptor isoforms are sequentially expressed in oligodendrocyte lineage cells during rat cerebral development. J Neurosci Res 54:584–594.

Dugas JC, Ibrahim A, Barres BA. 2012. The T3-induced gene KLF9 regulates oligodendrocyte differentiation and myelin regeneration. Mol Cell Neurosci 50:45–57. doi: 10.1016/j.mcn.2012.03.007. Epub 2012 Mar 24.

Dugas JC, Tai YC, Speed TP, Ngai J, Barres BA. 2006. Functional genomic analysis of oligodendrocyte differentiation. J Neurosci 26:10967–10983.

Escobar Cabrera OE, Bongarzone ER, Soto EF, Pasquini JM. 1994. Single intracerebral injection of apotransferrin in young rats induces increased myelination. Dev Neurosci 16:248–254.

Escobar Cabrera OE, Zakin MM, Soto EF, Pasquini JM. 1997. Single intracranial injection of apotransferrin in young rats increases the expression of specific myelin protein RNA. J Neurosci Res 47:603–608.

Espinosa de los Monteros A, Chiapelli F, Fisher RS, de Vellis J. 1988. Transferrin: An early marker of oligodendrocytes in culture. Int J Dev Neurosci 6: 167–175.

Farsetti A, Mitsuhashi T, Desvergne B, Robbins J, Nikodem VM. 1991. Molecular basis of thyroid hormone regulation of myelin basic protein gene expression in rodent brain. J Biol Chem 266:23226–23232.

Franco PG, Pasquini LA, Pérez MJ, Rosato-Siri MV, Silvestroff L, Pasquini JM. 2015. Paving the way for adequate myelination: The contribution of galectin-3, transferrin and iron. FEBS Lett 589:3388–3395. 2015 Nov 14; doi: 10.1016/j.febslet.2015.08.001. Epub 2015 Aug 18. Review.

Franco PG, Silvestroff L, Soto EF, Pasquini JM. 2008. Thyroid hormones promote differentiation of oligodendrocyte progenitor cells and improve remyelination after cuprizone-induced demyelination. Exp Neurol 212:458–467. doi: 10.1016/j.expneurol.2008.04.039.

Garzón-Muvdi T, Quiñones-Hinojosa A. 2010. Neural stem cell niches and homing: Recruitment and integration into functional tissues. Ilar J 51:3–23.

Hansen MB, Nielsen SE, Berg K. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 119:203–210.

Kutner RH, Zhang XY, Reiser J. 2009. Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. Nat Protoc 4:495–505. doi: 10.1038/nprot.2009.22.

Lazar MA. 1993. Thyroid hormone receptors: Multiple forms, multiple possibilities. Endocr Rev 14:184–193.

Leitner DF, Connor JR. 2012. Functional roles of transferrin in the brain. Biochim Biophys Acta 1820:393–402. doi: 10.1016/j.bbagen.2011.10.016. Epub 2011 Nov 21.

Lin KH. 2003. Plasma protein regulation by thyroid hormone. J Endocrinol 179:367–377.

Marta CB, Adamo AM, Soto EF, Pasquini JM. 1998. Sustained neonatal hyperthyroidism in the rat affects myelination in the central nervous system. J Neurosci Res 53:251–259.

Marziali LN, Garcia CI, Pasquini JM. 2015. Transferrin and thyroid hormone converge in the control of myelinogenesis. Exp Neurol 265:129–141. doi: 10.1016/j.expneurol.2014.12.021. Epub 2015 Jan 14.

Nicolay DJ, Doucette JR, Nazarali AJ. 2007. Transcriptional control of oligodendrogenesis. Glia 55:1287–1299.

Noguchi T, Sugisaki T, Satoh I, Kudo M. 1985. Partial restoration of cerebral myelination of the congenitally hypothyroid mouse by parenteral or breast milk administration of thyroxine. J Neurochem 45:1419–1426.

Paez PM, García CI, Davio C, Campagnoni AT, Soto EF, Pasquini JM. 2004. Apotransferrin promotes the differentiation of two oligodendroglial cell lines. Glia 46:207–217.

Paez PM, García CI, Pasquini JM. 2006. Expression of myelin basic protein in two oligodendroglial cell lines is modulated by apotransferrin through different transcription factors. J Neurosci Res 83:606–618.

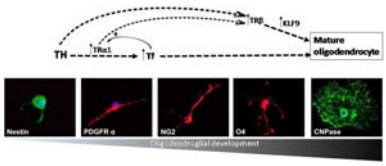
Rosman NP. 1972. The neuropathology of congenital hypothyroidism. Adv Exp Med Biol 30:337–366.

Rowitch DH, Kriegstein AR. 2010. Developmental genetics of vertebrate glial-cell specification. Nature 468:214–222. doi: 10.1038/nature09611.

Silvestroff L, Franco PG, Pasquini JM. 2012. ApoTransferrin: Dual role on adult subventricular zone-derived neurospheres. PLoS One 7:e33937. doi: 10.1371/journal.pone.0033937.

Takebayashi H, Ikenaka K. 2015. Oligodendrocyte generation during mouse development. Glia 63:1350–1356. doi: 10.1002/glia.22863. Epub 2015 May 25.

Todorich B, Pasquini JM, Garcia CI, Paez PM, Connor JR. 2009. Oligodendrocytes and myelination: The role of iron. Glia 57:467–478. doi: 10.1002/glia.20784.




- Thyroid hormone (TH) induces Transferrin (Tf) expression.
- Tf and TH interact promoting oligodendrogenesis
- Tf induces THR$\alpha$ expression
- TH induces THR$\beta$ expression
- THR$\beta$ expression mediates TH effects on myelination

AQ1: Please confirm that all author names are OK and are set with first name first, surname last.

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AQ3: Kindly provide the department name for affiliation 3.

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