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Regular Article

Transferrin and thyroid hormone converge in the control of myelinogenesis

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ABSTRACT

Myelination is a concerted mechanism tightly regulated in the brain. Although several factors are known to participate during this process, the complete sequence of events is far from being fully elucidated. Separate effects of apotransferrin (aTf) and thyroid hormone (TH) are well documented on rat myelin formation. TH promotes the maturation of oligodendrocyte progenitors (OPCs) into myelinating oligodendrocytes (OLGs), while aTf is able to induce the commitment of neural stem cells (NSCs) toward the oligodendroglial linage and favors OLG maturation. We have also demonstrated that Tf mRNA exhibited a seven-fold increase in hyperthyroid animals. These observations have led us to hypothesize that both factors may interplay during oligodendrogenesis. To assess the combined effects of aTf and TH on proper myelination in the rat brain, Tf expression and oligodendroglial maturation were evaluated at postnatal days 10 (P10) and 20 (P20) in several experimental groups. At P10, an up-regulation of both Tf mRNA and protein, as well as myelination, was found in hyperthyroid animals, while a decrease in Tf mRNA levels and myelin formation was detected in the hypothyroid group. At P20, no differences were found either in Tf mRNA or protein levels between hyperthyroid and control (Ctrol) rats, although differences in OLG differentiation remained. Also at P20, hypothyroid animals showed decreased Tf mRNA and protein levels accompanied with a less mature myelinating phenotype. Moreover, TH and aTf differentially regulate the expression of KLF9 transcription factor as well as TRα and TRβ at P10 and P20.

Our results suggest that TH is necessary early in OLG development for aTf action, as exogenous aTf administration was unable to counteract the effect of low TH levels in the hypothyroid state in all the time points analyzed. Furthermore, the fact that hyperthyroidism induced an increase in Tf expression and aTf-dependent regulation of TRα strongly suggests that Tf could be involved in some of TH later effects on OLG maturation. Here we describe the possible relationship between TH and aTf and its implication in oligodendrogenesis.

Introduction

During development, different types of cells populate the central nervous system (CNS). Among them, oligodendrocytes (OLGs) are in charge of myelinating axons and providing trophic support for axonal survival (Nave, 2010). The myelination process requires mature myelinating OLGs to extend processes and contact axons, wrapping them and finally compacting their membranes (Sherman and Brophy, 2005). Mature OLGs arise from immature oligodendrocyte progenitor cells (OPCs), which are mitotically active cells with migratory capacity originated from progenitor cells located at germinal niches during embryonic development (Rowitch, 2004) and postnatal life (Gonzalez-Perez and Alvarez-Buylla, 2011; Levison and Goldman, 1993).

It is well established that thyroid hormone (TH) deeply influences brain development at the embryonic and postnatal stages promoting cell migration, neuro/glial differentiation and myelinogenesis (Bernal, 2005). TH is a particularly well characterized OPC maturation factor as well as a myelination inducer (Barres et al., 1994; Marta et al., 1998; Walters and Morell, 1981). Nicholson and Altman (1972) have postulated that TH affects cell proliferation, while Balázs et al. (1971) have suggested that TH decreases the number of OLGs. However, results by Tosic et al. (1992) indicate that TH appears to favor OLG differentiation rather than affect their proliferation. This controversial issue has been the subject of quite extensive studies, and Barres et al. (1994) have even suggested that OLG proliferation and differentiation are probably regulated by a "clock mechanism" with two clear components, one of

Abbreviations: aTf, apotransferrin; CC, corpus callosum; E 14–17, embryonic days 14–17; GH, growth hormone; GHR, growth hormone receptor; ICI, intracranial injection; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; IGF-IR, insulin-like growth factor I receptor; KLF9, Kruppel-like factor 9; NSC, neural stem cell; NPC, neural progenitor cells; OPC, oligodendrocyte progenitor; OLG, oligodendrocyte; P 1-2-3-5-7-10-15-P20, postnatal days 1-2-3-5-7-10-15-20; PTU, 6-propyl-2-thiouracil; SVZ, subventricular zone; T3, triiodothyronine; Tf, transferrin; TH, thyroid hormone; THRA, thyroid hormone receptor alpha gene; THRB, thyroid hormone receptor beta gene; TR, thyroid hormone receptor; TRE, thyroid hormone response element.

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which, the effector component that stops proliferation, is controlled by TH.

TH effects are mediated by TH receptors (TRs), which belong to the family of nuclear receptors and are encoded by two genes, THRA/THRB, which show both specific spatial and temporal expression patterns (Bradley et al., 1992; Lazar, 1993). TRs bind to DNA sequences called TH response elements (TREs), located in the regulatory regions of target genes. After binding to TREs, TRs act as transcription factors capable of regulating gene expression positively or negatively (Bernal, 2007) in a variety of genes, including several transcription factors. Among TH-directly-induced factors, Dugas et al. (2012) have shown that Kruppel-like factor 9 (KLF9) is necessary for OLG maturation and myelin regeneration. Mapping experiments of the Myelin Basic Protein (MBP) gene showed that it has a functional TRE (Farsetti et al., 1992); however, TREs have not been described in other myelin-related genes, which indicates that TH effects on these genes seem to be a combination of direct transcriptional regulation and downstream transcriptional regulation (Ibarrola and Rodríguez-Peña, 1997; Strait et al., 1997). Work from our laboratory demonstrated that neonatal hyperthyroidism in the rat, sustained up to 17 days of age, produces accelerated myelination (Adamo et al., 1990; Marta et al., 1998). These results suggest that the production of myelin by OLGs starts much earlier in hyperthyroid animals than in normal controls, probably because TH induces the early maturation of OPCs. The marked increase in the levels of MBP, PLP and CNPase mRNAs at 10 days of age, followed by an increase in the amount of their protein products at 17 days of age (Adamo et al., 1990), indicates that sustained high levels of TH since birth produce a premature differentiation of OPCs, an event that could be preceded by an important arrest in their proliferation. Our studies have also shown that this increase in mRNA levels ceases shortly after as a consequence of TH excess – as evidenced by a sharp drop in mRNA levels in treated animals at 17 days of age – , which in turn suggests that the number of differentiating cells diminishes substantially. It has also been observed that transferrin (Tf) mRNA levels increase nine times with reference to controls at 10 days, and decrease to levels lower than those observed in controls at 17 days (Marta et al., 1998).

Within the CNS, Tf is almost exclusively produced by OLGs (Espinosa de los Monteros et al., 1988; Espinosa de los Monteros and de Vellis, 1988). Therefore, the dramatic variation in the levels of Tf mRNA described by Marta et al. (1998) should be particularly highlighted, as this increase could be one of the mechanisms triggered by TH and involved in the accelerated myelination observed in young hyperthyroid rats. Such changes also seem to stress the importance of Tf as a putative trophic factor in the biology of myelin and OLG maturation. This fact 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). The *in vivo* effects of aTf have also been reproduced in OLG primary cultures (García et al., 2003, 2004, 2007; Perez et al., 2009, 2013) and in the N19 and N20.1 oligodendroglial cell lines (Paez et al., 2004, 2005, 2006). More recent results suggest that aTf participates in the control of OLG differentiation by two converging regulatory mechanisms: in the presence of mitogens, aTf promotes the commitment of undifferentiated NPCs to OLG lineage and, after mitogen withdrawal, aTf seems to promote OLG terminal maturation (Silvestroff et al., 2012).

In the present study we demonstrate an interaction between TH and aTf which is independent of the growth hormone (GH)–IGF-I axis. Using *in vivo* assays, we show that TH is involved in Tf expression, as Tf levels are higher in hyperthyroid rats and lower in hypothyroid ones. Immunohistochemical analyses in the two different models further support these data, with a more mature myelinating phenotype in the hyperthyroid group and more immature OLGs in the hypothyroid one. This latter phenotype is not reversed by the administration of exogenous aTf, which indicates that TH might play a dual role in OLG maturation at different stages in the process and in combination with aTf. At early stages, TH seems to induce Tf expression, as the absence of TH in hypothyroidism does not promote Tf expression and a lack of OLGs is consequently observed. Later on, TH maturational effects are probably mediated by increased aTf, given that aTf is partially capable of accelerating OLG differentiation. Altogether, these results appear to reveal a crosstalk between TH and aTf during oligodendrogenesis.

Materials and methods

Animals and animal treatments

All animal protocols were approved by the Institutional Review Board of the 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.

Either male or female ten- (P10) or twenty- (P20) day-old Wistar rats were used. Perinatal hypothyroidism was induced by the administration of 15 ppm 6-propyl-2-thiouracil (PTU, Sigma Cat# P3755) in the mothers' drinking water from gestational day 18 and during lactation, with PTU renewed every 2/3 days (Gilbert and Paczkowski, 2003). Half the pups rendered hypothyroid received an intracranial injection (ICI) of 350 ng of rat aTf (Hypo + aTf_{ic}, n = 8) in 5 μl saline at P3. aTf injection was administered slightly above and between the eyes; the solution was slowly injected to avoid overflows and the syringe was kept in place for 1 min after being empty (Escobar Cabrera et al., 1994). The other half received a saline injection (Hypo, n = 8). Perinatal hyperthyroidism was induced according to Adamo et al. (1990) (Hyper, n = 8). Briefly, triiodothyronine (T3) was dissolved in saline, pH 7.4, and injected subcutaneously each day as follows: 5 μg at P1, 2.5 μg at P2, 0.5 μg on odd days after P2, and 1.5 μg on even days after P3 until the end of the experiment. Another group of animals received an ICI of 350 ng aTf at P3 (aTf_{ic}, n = 6). The control group (Ctrol, n = 8) received an ICI of saline at P3 and was daily administered subcutaneous saline.

For studies of Tf expression on oligodendroglial cell linage, the B6; FVB-Tg(Cnp-EGFP/Rpl10a)JD368Htz/J mouse strain was used.

At the end of experiments, animals received 6000 UI/kg of sodium heparin subcutaneously and were anesthetized through the intraperitoneal administration of a mixture of ketamine (200 mg/kg)/xylazine (2 mg/kg). After perfusion through the left ventricle with PBS 1X, brains were excised and weighed. The two hemispheres were separated; one of them was used for RNA extraction and the other for protein extraction. For immunohistochemistry, animals were sequentially perfused with PBS 1X and 4% paraformaldehyde (PFA) in PBS 1X.

Protein extraction and Western blot

Proteins were obtained from one cerebral hemisphere using TOTEX buffer containing protease inhibitors (20 nM HEPES pH 7.9, 350 nM NaCl, 20% Glycerol, 1% Igepal, 1 nM $MgCl_2$, 0.5 nM EGTA, 0.1 nM, 10 mg/ml leupeptin, 10 mg/ml pepstatin, 0.5 nM DTT, 0.5 nM PMSF). Extracts were incubated on ice for 30 min and then centrifuged for 10 min at 10,000 rpm. Protein concentration was determined using the Bradford assay and 40 μg protein was subjected to SDS-PAGE in a 10% or 15% polyacrylamide gel, after which proteins were transferred onto PVDF membranes. Membranes were blocked for 1 h at room temperature and then incubated overnight at 4 °C with the corresponding antibodies: anti-Tf (1:2500, a gift from Dr. Zakin, Institut Pasteur, France), anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (1:5000, Abcam) or anti-MBP (1:4000, a generous gift from Dr. Campagnoni, UCLA, USA). After being washed, membranes were incubated with the corresponding HRP-conjugated secondary antibodies (1:5000, Jackson Immuno Research Laboratories, Inc.) for 2 h at room temperature, 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, USA).

Table 1
T3 serum content, body and brain weights.

Group	Total serum T3 (ng/ml)		Body weight (gr)		Brain weight (gr)	
	P10	P20	P10	P20	P10	P20
Ctrol	121.8 ± 1.7	176.8 ± 2.6	20.1 ± 1.7	37.9 ± 2.4	0.878 ± 0.039	1.090 ± 0.043
Hyper	600***	600***	19.4 ± 2.4	30.9 ± 1.7***	0.772 ± 0.048**	0.932 ± 0.032***
Hypo	52.7 ± 2.2***	53.3 ± 1.7***	20.0 ± 0,7	33.0 ± 2.0***	1.044 ± 0.033***	1.196 ± 0.025***
Hypo + aTf_{ic}			19.7 ± 1.5	31.7 ± 1.7***	1.028 ± 0.066***	1.136 ± 0.037**

Hyper: animals rendered Hyperthyroid by T3 administration (see Materials and methods). Hypo: animals rendered Hypothyroid by PTU administration (see Materials and methods). aTf_{ic}: animals intracranially injected with aTf (see Materials and methods). Hypo + aTf_{ic}: Hypothyroid animals intracranially injected with aTf (see Materials and methods).
*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

RNA extraction and quantitative RT-PCR

RNA was extracted from one cerebral hemisphere using Trizol Reagent according to the manufacturer's instructions. cDNA synthesis was performed using 1 μg of total RNA and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase according to the manufacturer's instructions (Promega). 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 precipitated with ethanol.

cDNA was quantified with the Quant-iT™ OliGreen® ssDNA Assay Kit according to the manufacturer's instructions. Using specific primers, dynamic ranges were performed starting with 80 ng cDNA covering 1:2 serial dilutions up to 5 ng. The following specific primers were used: Tf (NM_001013110.1): Forward CCTCAAAGTG GCTCAGGAACA; Reverse AGGAGAGCCGAACAGTTGGA; Hypoxanthine Phosphoribosyltransferase 1 (HPRT1, NM_012583.2): Forward TTCCTCCTCAGACCGCTTTTC; Reverse GGACTGAGGGTCGACATGACA; and Kruppel-like factor 9 (Klf9, NM_057211.1) described previously (Dugas et al., 2012): Forward TCCGGAACTTTCAAACCTTG; Reverse GTGTGCCAAACAGAATGTCG.

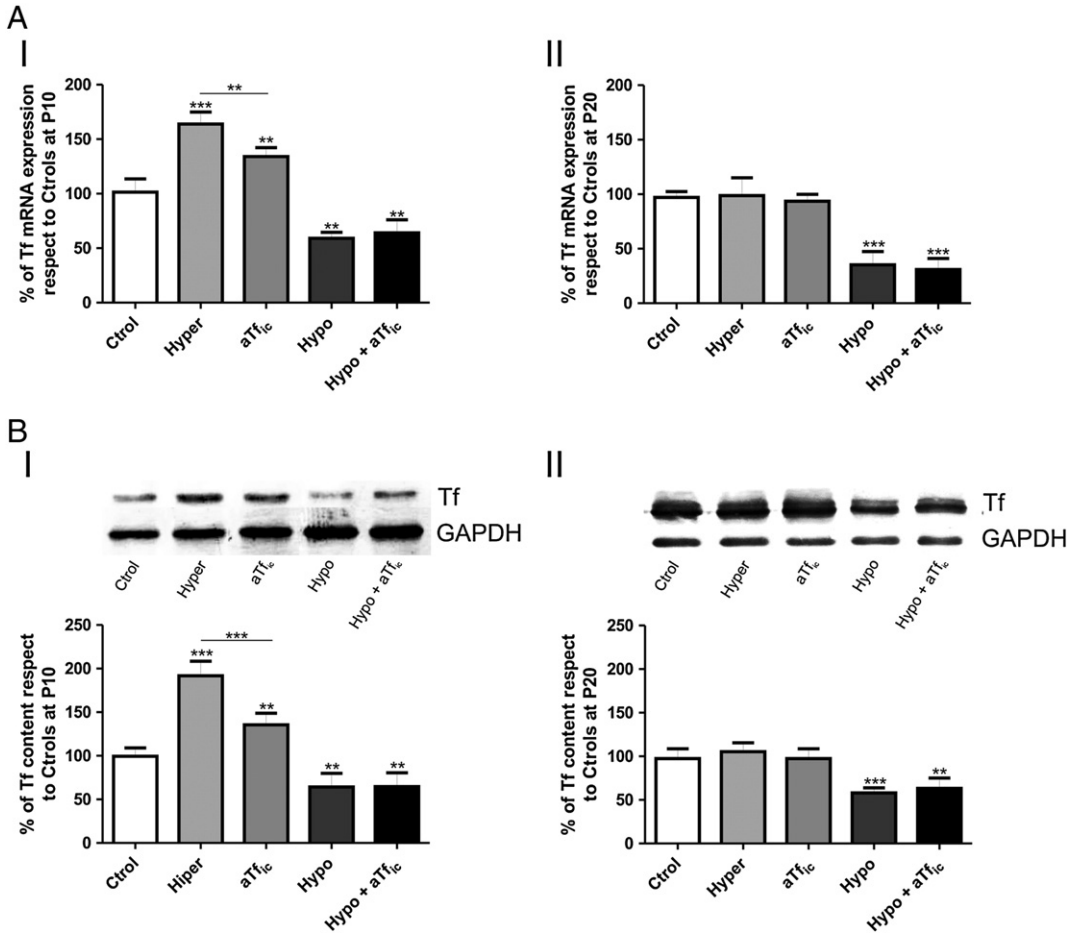
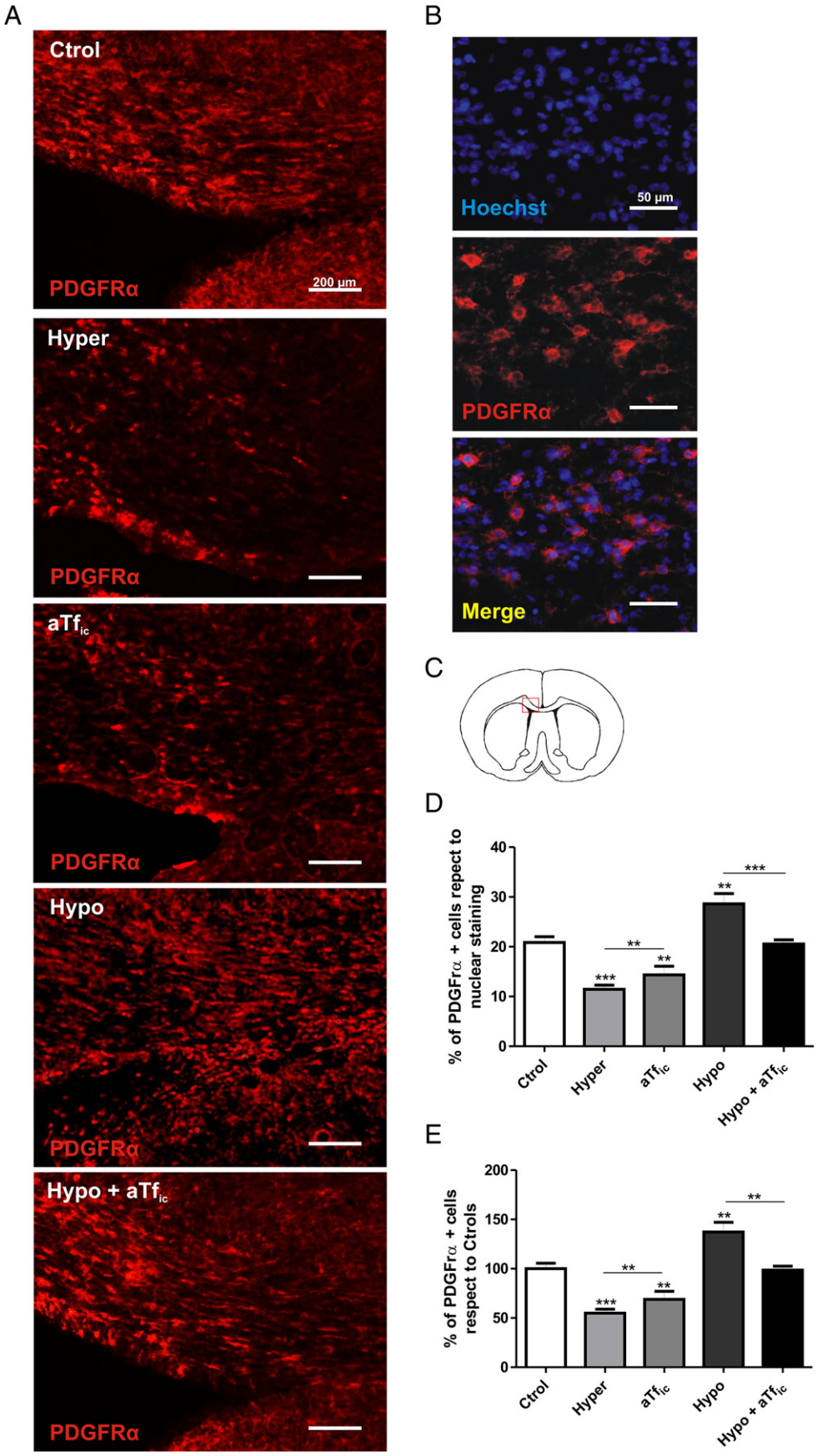


Fig. 1. Hyperthyroidism accelerates the normal pattern of Tf expression during myelination. (A) Analysis of Tf mRNA expression by RT-qPCR: Tf and HPRT housekeeping genes were amplified and the comparative C_T method was used to compare Tf mRNA expression across groups. Results are expressed as a percentage of the average expression of Ctrol animals at each age (considered 100%) (I and II). (B) Analysis of Tf content by Western blot; Tf and GAPDH immunoblotting: IODs were determined with Gel-Pro Analyzer 4.0 and Tf IOD was normalized to GAPDH. Results are expressed as a percentage of the average expression of Ctrol animals at each age (considered 100%). Graphics represent relativized Tf mRNA expression ± SD for P10 (I) and P20 animals (II). Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc tests and Student's *t* test. Asterisks represent statistical significance with respect to Ctrol; when comparisons were made among other groups, line bars indicate the groups involved. **$p \leq 0.01$, ***$p \leq 0.001$.


A
Ctrol
PDGFRα
200 μm
Hyper
PDGFRα
aTf$_{ic}$
PDGFRα
Hypo
PDGFRα
Hypo + aTf$_{ic}$
PDGFRα
B
Hoechst
50 μm
PDGFRα
Merge
C
D
% of PDGFrα + cells repect to nuclear staining
40
30
20
10
0
***
**
**
**
***
Ctrol
Hyper
aTf$_{ic}$
Hypo
Hypo + aTf$_{ic}$
E
% of PDGFrα + cells respect to Ctrols
200
150
100
50
0
***
**
**
**
**
Ctrol
Hyper
aTf$_{ic}$
Hypo
Hypo + aTf$_{ic}$

Insulin-like-growth factor I (IGF-I, NM_001082477.2, NM_178866.4, NM_001082478.1, NM_001082479.1): Forward TGTTCCTCGGGAGGCT CCTCCTA; Reverse CGCTCTTCAGTTCGTGTGTGGACC. Insulin-like-growth factor I receptor (IGF-IR, NM_052807.2): Forward CCAGGGCCTG TCCAACGAGC; Reverse CGGAGAACCAGCACGCCAGG. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH. NM_017008.4) Forward: CTTACT CCTTGGAGGCCATG, Reverse: TTAGCCCCCCTGGCCAAGG. Thyroid hormone receptor alpha 1 (TRα1, NM_001017960.1): Forward TTCAGCGA GTTTACCAAGATCATCAC; Reverse TTAGACTTCCTGATCCTCAAAGACCT. Thyroid hormone receptor beta1/2 (TRβ1/TRβ2, NM_012672.3/ NM_001270854.1): Forward AAGTTGCCCATGTTTTGTGAG; Reverse TCACTGCCATTTCCCCATTC.

Ten nanog of cDNA were used for each PCR reaction by means of a Stratagene Mx3005P qPCR System. PCRs were performed as follows: 0.4 μM of the corresponding pair of primers, 1.25 U Platinum® Taq DNA polymerase (Invitrogen), 0.3X SYBR® Green I Nucleic Acid Gel Stain (Invitrogen), 0.05X ROX Reference Dye (Invitrogen), 0.3 mM dNTP mix and 2 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. Results were analyzed using the comparative C_T method using Mx Pro-Mx3005P v 4.10 associated software.

Immunohistochemistry

After dissection and post-perfusion in 4% PFA, brains were fixed in 4% PFA overnight at 4 °C and sequentially cryoprotected by immersion in 15% and 30% sucrose. Coronal slices (40 μm) corresponding to 0.84 mm from the bregma according to Paxinos and Watson's stereotaxic atlas (Paxinos and Watson, 1986) were obtained using a Leica CM1850 cryostat.

Immunohistochemistry was conducted as follows: slices were blocked for 2 h at room temperature and incubated overnight at 4 °C with the corresponding antibodies: anti-MBP (1:400), anti-Carbonic Anhydrase Type II (CAII) (1:500, a gift from Dr. W. Cammer), anti-Platelet-Derived Growth Factor Receptor alpha (PDGFRα) (1:200, Neuromics), and Anti-Oligodendrocyte Antibody (RIP) (1:200, Millipore). After being washed, slices were incubated with Hoechst-33258 (1 μg/ml) and the corresponding secondary antibodies conjugated to Alexa Fluor® (1:500, Jackson Immuno Research Laboratories, Inc.) for 2 h at room temperature, and then mounted with Mowiol® 4-88 anti-fade mounting solution (Calbiochem®).

Image acquisition was assessed with an Olympus BX50 epifluorescence microscope (Olympus, Japan) and an Olympus FluoView™ FV1000 Confocal Microscope (Olympus, Japan), and analyzed using ImageJ software.

Statistical analysis

Data analysis was conducted with Graph-Pad Prism Software and results were expressed as the mean ± standard deviation (SD). Three independent tests were performed for each experiment. In order to compare experimental groups, one-way ANOVA was used and followed by the Newman–Keuls post-hoc tests or Student's *t* test. Asterisks represent statistical significance with respect to Ctrol; when comparisons were made with other groups, line bars indicate which groups were involved. Statistical significance is indicated with asterisks: $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$.

Results

TH influences brain weight prior to body weight

TH was immunoassayed and changes were detected for both the Hyper and Hypo groups (Table 1). Hyper rats showed a 4.9-fold increase in T3 levels at P10 and a 3.4-fold increase at P20, whereas Hypo animals showed a 2.3-fold decrease at P10 and a 3.3-fold decrease at P20 when compared to Ctrol. At P10, body weight was similar across all groups; however, at P20, there was an 18% decrease in Hyper rats and a 13% decrease in Hypo ones compared to Ctrol. When injected intracranially, aTf had no effect on Hypo animals' weight (Table 1). While body weights were not affected by thyroid status at P10, brain weights showed changes in the Hypo (19% increase) and Hyper (12% decrease) groups when compared to Ctrol. Again, the ICI of aTf had no compensatory effects either at P10 or P20 (Table 1).

TH effect on Tf expression at P10 and P20

At P10, Hyper animals showed a 55% increase in Tf mRNA and an 87% increase in Tf protein levels when compared to Ctrol (Fig. 1A), while the Hypo group presented a 25% decrease in both Tf mRNA and protein levels with respect to Ctrol (Fig. 1A). This decrease was not reversed by exogenous aTf administration in Hypo animals, although aTf_{ic} animals showed a 32% increase in Tf mRNA levels (Fig. 1A).

At P20, we found no differences among Hyper, aTf_{ic} and Ctrol animals in Tf mRNA or protein levels (Fig. 1B). In contrast, Hypo animals showed a 25% decrease in Tf mRNA levels and a 40% decrease in Tf content in comparison to Ctrol. Again, aTf administration failed to correct this decrease in Hypo animals (Fig. 1B).

TH and aTf effects on OLG differentiation and myelination

TH and aTf are two well-known factors participating in OLG maturation (Almazan et al., 1985; Baas et al., 1997; Espinosa de los Monteros et al., 1999; Paez et al., 2006). In order to explore the possible crosstalk between them in OLG differentiation and myelination, these two processes were analyzed in the following experimental conditions: Hyper, aTf_{ic}, Hypo, Hypo + aTf_{ic} and Ctrol animals. For this purpose, we performed immunohistochemical studies with several OLG and myelin markers on coronal brain sections from P10 and P20 animals of each group.

We first evaluated the expression of OPC marker PDGFRα in the corpus callosum of P10 animals (Fig. 2). Both Hyper and aTf_{ic} rats exhibited a significantly reduced number of $PDGFR\alpha^+$ cells compared to Ctrol (40% and 25% decrease, respectively). In contrast, Hypo animals showed increased numbers of $PDGFR\alpha^+$ cells (25%) when compared to Ctrol animals. Interestingly, in Hypo + aTf_{ic}, aTf subdued the effect previously observed and no differences were found in the number of PDGFRα-stained cells compared to Ctrol (Fig. 2).

We next examined the occurrence of more mature OLGs at P10 using anti-RIP antibody. As shown in Fig. 3, immunohistochemical analyses of RIP staining showed a 132% increase in Hyper animals and a 35% rise in the aTf_{ic} group when compared to Ctrol. As regards hypothyroid conditions, Hypo rats exhibited an 80% decrease in RIP labeling when compared to Ctrol, while Hypo + aTf_{ic} animals showed a 56% decrease in RIP regarding Ctrol animals. Although the exogenous administration of aTf was unable to completely counteract deficiencies in the Hypo group, it significantly increased RIP staining (24%) with respect to

Fig. 2. aTf and TH are able to modify $PDGFR\alpha^+$ cell population at P10. (A) PDGFRα immunostaining of corpus callosum (CC) of P10 animals. (B) $PDGFR\alpha^+$ cells at high magnification. (C) Schematic drawing of a coronal brain slice highlights the area shown in the photographs. $PDGFR\alpha^+$ cells were counted in the areas of the CC between cingulum bundles. Results are expressed as a percentage of positive cells with respect to nuclear Hoechst staining (D) or as a percentage with respect to Ctrol (E) ± SD. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance with respect to Ctrol; when comparisons were made among other groups, line bars indicate the groups involved. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

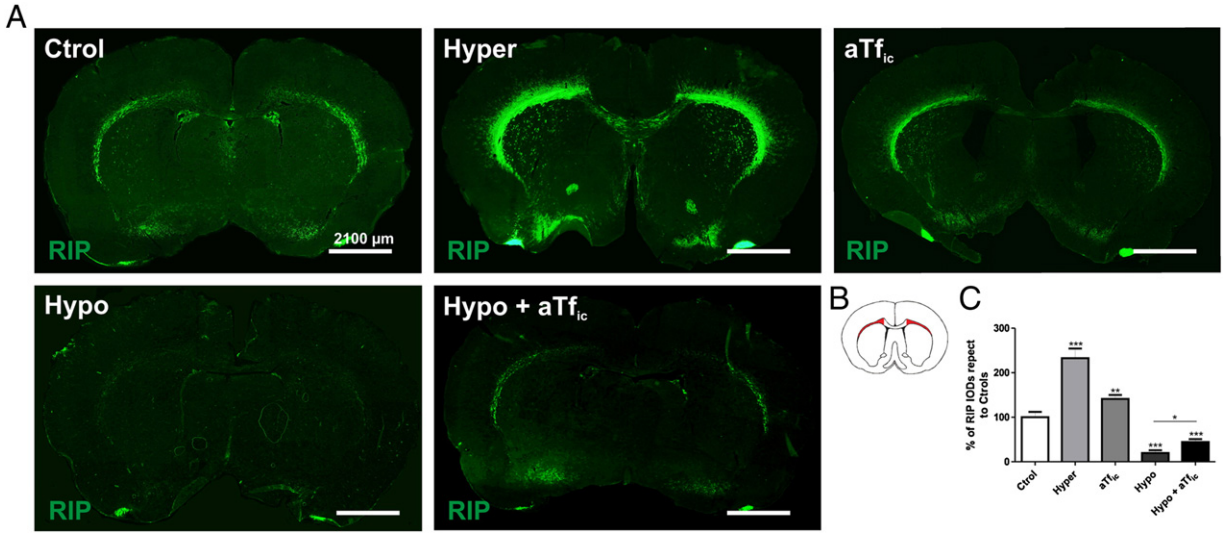


Fig. 3. aTf and TH are able to modify RIP$^+$ cell population at P10. (A) RIP immunostaining of coronal brain slices of P10 animals. IOD of RIP staining was determined with ImageJ software, at least 10 areas of interest (AOIs) were selected in the CC and mean IOD was obtained for every specimen. (B) The analysis was performed on the area highlighted in the schematic drawing. (C) Results are expressed as a percentage of the mean IOD of Ctrol animals (considered 100%). Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance with respect to Ctrol; when comparisons were made among other groups, line bars indicate the groups involved. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

non-injected Hypo animals. These results suggest that thyroid levels are crucial in early as well as in later stages of OLG development, and that TH presence is required for complete aTf effect. In turn, aTf has the potential to partially compensate the lack of TH in promoting OLG maturation. Overall, these data hint at a tightly coordinated expression of both factors for proper myelin formation.

We next conducted studies focusing on myelination status at P20, starting with RIP staining in all groups. Once again, as described for P10, Hyper and aTf$_{ic}$ animals displayed increased labeling when compared to Ctrol (124% and 48%, respectively) (Fig. 4), while Hypo and Hypo + aTf$_{ic}$ animals showed a 61% decrease in RIP staining when compared to Ctrol. However, at this time point and in contrast with P10, aTf did not even partially reverse the phenotype change (Fig. 3).

We then examined MBP, a more mature marker of the myelin sheath. Fig. 5 shows MBP results similar to those previously observed for RIP. Hyper and aTf$_{ic}$ animals exhibited higher MBP staining as compared to Ctrol (114% and 38%, respectively). In contrast, Hypo animals showed a 64% decrease in MBP labeling which was not modified by aTf administration. Western blot analyses of MBP content in total brain further corroborated immunohistochemistry, as Hyper animals presented a 61% increase in MBP; in contrast, protein levels decreased by 58% in Hypo animals as compared to Ctrol.

The presence of mature OLGs was further assessed by measuring the percentage of CAII$^+$ cells in the central segment of the corpus callosum (Fig. 6). Pro-oligodendrogenic effects on Hyper and aTf$_{ic}$ animals were evidenced by an increase in CAII$^+$ cells (65% and 30%, respectively) compared to Ctrol. In contrast, Hypo animals showed a 38% decrease in the number of CAII$^+$ cells as compared to Ctrol, which was not reversed by aTf administration.

Is Tf present in all oligodendroglial cells?

Since OLG maturation is modulated by Tf, we evaluated whether Tf expression was restricted to OLGs during normal postnatal development. Using a transgenic mouse that drives EGFP expression under the control of the CNPase promoter, immunohistochemical analyses of Tf expression were performed at P5, P7, P10 and P15. At P5, P7 and P10, Tf expression was low and ubiquitous in the brain (data not shown). Nonetheless, at P15, confocal microscopy in the corpus callosum detected EGFP$^+$–Tf$^+$ cells (Fig. 7A). Using the Manders Coefficient and fractional overlap analysis, we evaluated the proportion of co-localization of Tf and EGFP staining. Results ranged from 0.877 to 0.994, indicating that not all Tf staining co-localizes with EGFP fluorescence. However, through high magnification analysis, we found both low- and high-EGFP-expressing OLGs which were also Tf$^+$, which indicates that all Tf$^+$ cells are also positive for EGFP (Fig. 7B). Immunohistochemical analysis with RIP antibody confirmed the fact that low-EGFP expressing cells are OLGs (Fig. 7C).

Circulating levels of TH do not influence the GH–IGF-I axis

The relationship between GH and TH has been widely characterized (Wolf et al., 1989), and evidence suggests that IGF-I is the principal mediator of GH action during early postnatal development, as well as a potent regulator of OLG maturation, development and myelination (Hsieh et al., 2004; Fernandez and Torres-Alemán, 2012). Likewise, Pascual-Leone et al. (2003) have demonstrated that TH is capable of inducing IGF-I mRNA expression in hepatic tissue. Considering this bibliographical background, and as our paradigm modifies thyroid status, we evaluated the effect of these changes on IGF-I and IGF-I receptor (IGF-IR) expression. The analysis was performed by semi-quantitative PCR. Linearity of the reaction was checked and found to be lineal between 5 and 80 ng of cDNA (data not shown). Neither IGF-I nor IGF-IR expression was affected in groups studied at P10 or P20 (Fig. 8), when comparing IGF-I and IGF-IR brain levels in Ctrol animals.

TH exerts its effects through KLF9 independently of Tf

KLF9 has been previously characterized as a transcription factor early induced by TH during brain development (Thompson and Potter, 2000). Recently, Dugas et al. (2012) demonstrated that TH induces KLF9 in a dose-dependent manner, and that this factor is in turn capable of increasing the expression of several myelin genes. As our hypothesis proposes the existence of a crosstalk between TH and aTf, and considering their different roles during OLG maturation and myelination, we tested KLF9 expression by RT-qPCR at P10 and P20.

At P10, TH levels modulate KLF9 expression, as Hyper animals showed a 90% increase in KLF9 expression regarding Ctrol, while Hypo animals showed a 57% decrease. aTf$_{ic}$ animals showed no differences

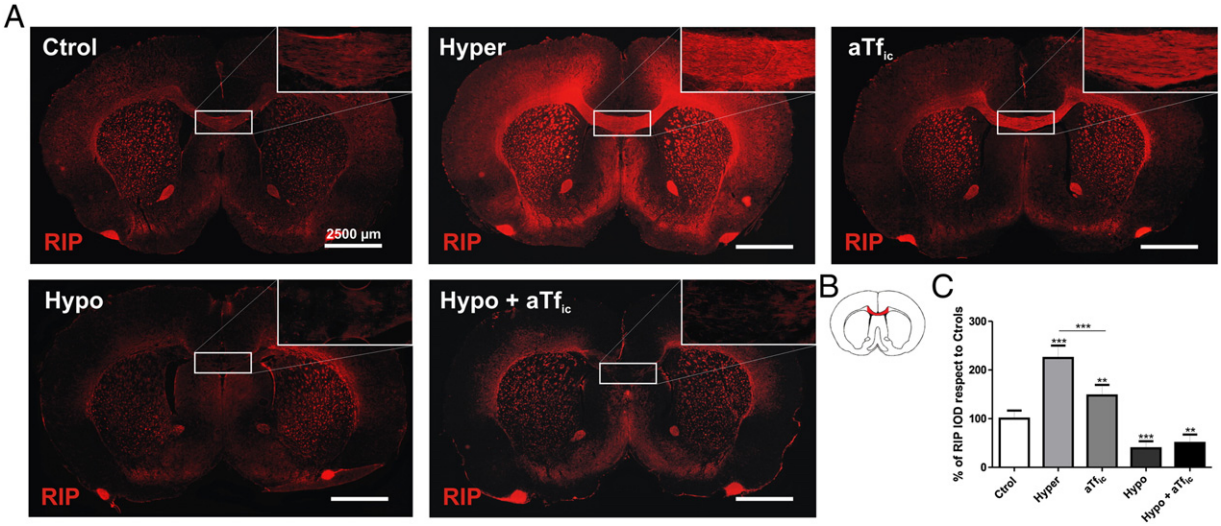


Fig. 4. aTf and TH are able to modify RIP$^+$ cell population at P20. (A) RIP immunostaining of coronal brain slices of P20 animals. IOD of RIP staining at CC was determined with ImageJ software, at least 10 areas of interest (AOIs) were selected and mean IOD was obtained for every specimen. Insets show higher magnification of the highlighted area. (B) Schematic drawing of a coronal brain slice highlights the area analyzed in the photographs. (C) Results are expressed as a percentage of the mean IOD of Ctrol animals (considered 100%). Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance with respect to Ctrol; when comparisons were made among other groups, line bars indicate the groups involved. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

with Ctrol, and aTf was unable to solve the deficits in Hypo animals (Fig. 9A). At P20, neither Hyper nor aTf$_{ic}$ showed differences with Ctrol. KLF9 remained low in Hypo animals (45% decrease), and aTf administration had still no effects (Fig. 9B).

TH receptors during myelination

As TH effects are mediated by its receptors (Bernal, 2007), and considering the promyelinogenic effects of TH and aTf, we tested the

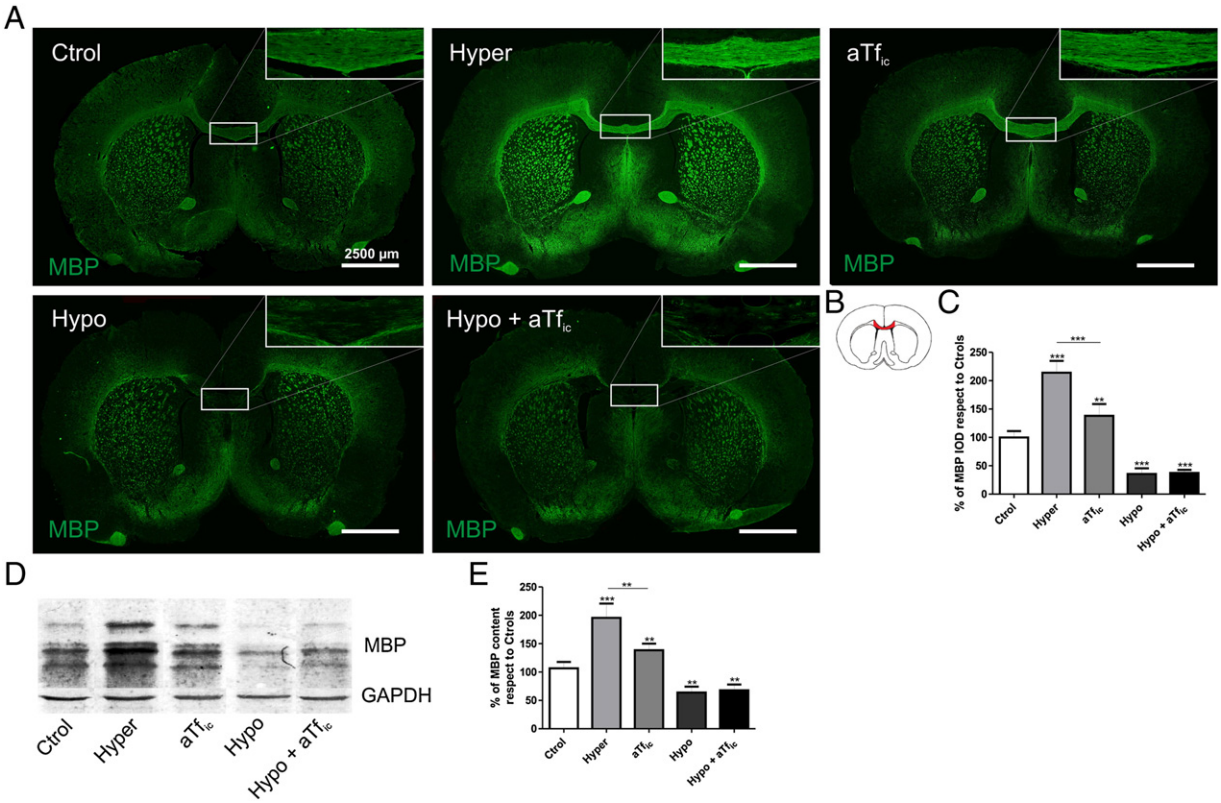


Fig. 5. aTf and TH are able to modify MBP expression at P20. MBP immunostaining of coronal brain slices of P20 animals. IOD of MBP staining at CC was determined with ImageJ software, at least 10 areas of interest (AOIs) were settled and mean IOD was obtained for every specimen. Insets show higher magnification of the highlighted area. (B) Schematic drawing of a coronal brain slice highlights the area analyzed in the photographs. (C) Results are expressed as a percentage of the mean IOD of Ctrol animals (considered 100%) ± SD. (D) Western blot analysis of MBP and GAPDH. IODs were determined with Gel-Pro Analyzer 4.0 and IOD of all MBPs isoforms were normalized to GAPDH. Results are expressed as a percentage of the average expression of Ctrol animals at each age (considered 100%) ± SD. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance with respect to Ctrol; when comparisons were made among other groups, line bars indicate the groups involved. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

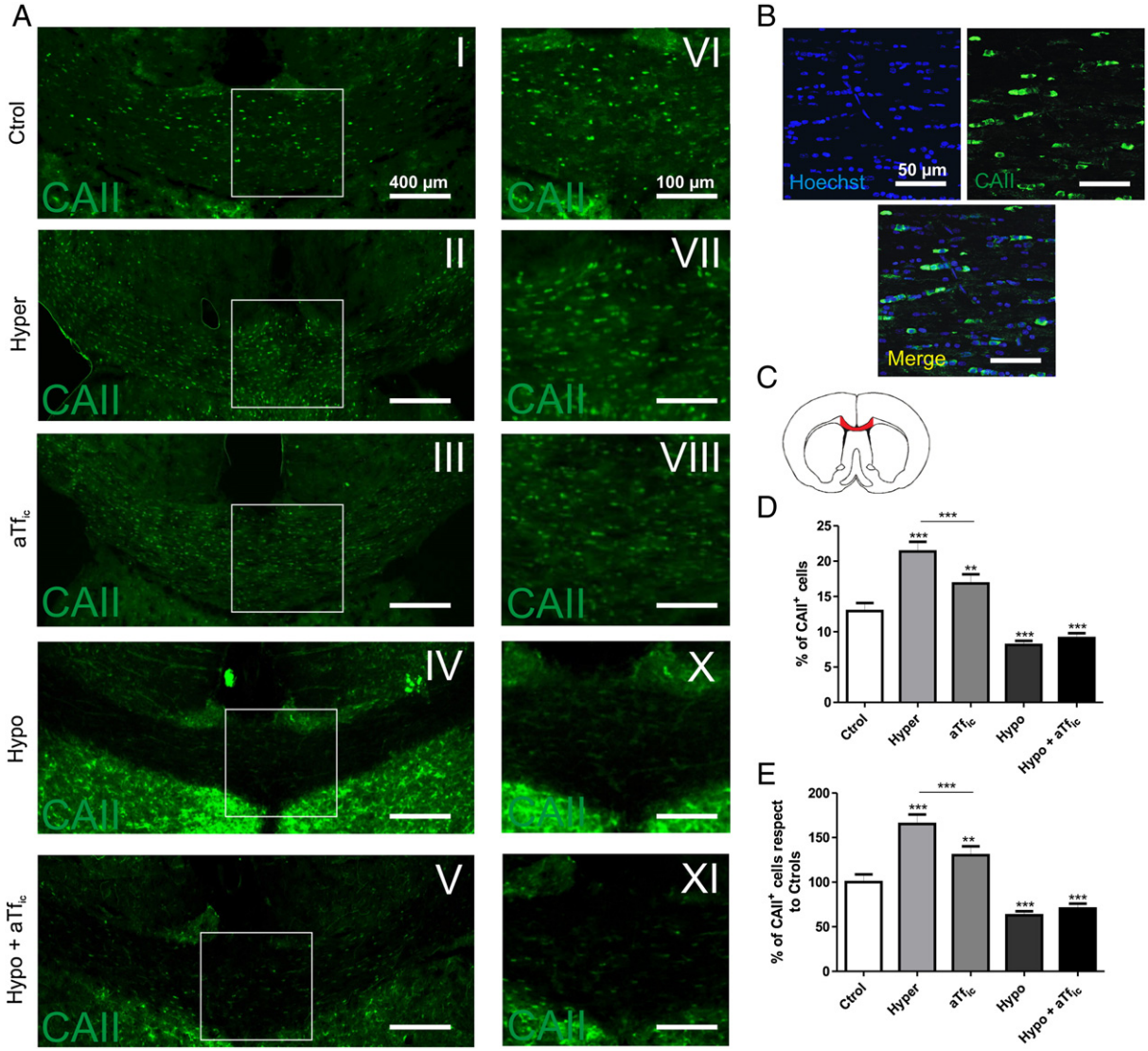


Fig. 6. aTf and TH are able to modify CAII$^+$ cell population at P20. (A) CAII immunostaining of CC of P20 animals. Photographs VI–X show high magnification of the areas highlighted in photographs I to V. (B) CAII$^+$ cells at high magnification. (C) Schematic drawing of a coronal brain slice highlights the area shown in the photographs. CAII$^+$ cells were counted in the areas of the CC between cingulum bundles. Results are expressed as a percentage of positive cells with respect to nuclear Hoechst staining (D) or as a percentage with respect to Ctrol (E) ± SD. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc tests and Student's *t* test. Asterisks represent statistical significance with respect to Ctrol; when comparisons were made among other groups, line bars indicate the groups involved. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

expression of TRα and TRβ to further characterize the contribution of TH and aTf to the process of myelin synthesis. Analysis was performed by RT-qPCR at P10 and P20 in all experimental groups.

Analysis of TRα mRNA expression at P10 showed no differences between Hyper and Ctrol, while aTf animals showed a 53% increase of TRα levels regarding Ctrol conditions. Hypo animals showed a 100% increase in TRα levels, and receptor expression was unaffected by aTf administration. At P20, Hyper and aTf$_{ic}$ animals showed no differences with Ctrol, while Hypo animals still exhibited a 50% decrease in TRα levels, which was again unaffected by aTf administration (Fig. 10A).

Analysis of TRβ mRNA expression at P10 showed a 43% increase in Hyper animals and an 80% decrease in aTf animals, both regarding Ctrol. In turn, Hypo animals showed a 70% decrease in TRβ levels, and receptor expression remained unchanged when aTf was administered. At P20, Hyper animals exhibited a 68% increase in TRβ levels when compared to Ctrol, while aTf-treated animals showed a 70% decrease in TRβ levels regarding Ctrol. In Hypo animals, TRβ levels suffered a 63% decrease which aTf administration was unable to solve (Fig. 10B).

Discussion

It is well known that differentiation is driven by changes in the gene expression program, which finally lead to the emergence of specific cell types. In the vertebrate CNS, OLGs synthesize myelin and develop from OPCs, which undergo terminal differentiation at the postnatal stages (Li et al., 2009; He and Lu, 2013; Richardson et al., 2006). Growth factors such as PDGF, FGF-2 and IGF-I are known to promote OPC migration, survival and proliferation (Furusho et al., 2011; Cui et al., 2005; Calver et al., 1998), whereas T3 and TGF-β1, among others, have been found to promote OLG differentiation (Barres et al., 1994; Böttner et al., 2000; Dugas et al., 2006; Lopes-Cardozo et al., 1989). According to results from our laboratory, aTf favors both differentiation toward oligodendroglial fate and proliferation of progenitor cells (Guardia Clausi et al., 2010; Paez et al., 2006; Silvestroff et al., 2013). Previous studies establish a significant role for TH in myelin formation (Adamo et al., 1990; Ibarrola and Rodríguez-Peña, 1997; Rodriguez-Peña et al., 1993), and reports from our laboratory demonstrate that sustained neonatal hyperthyroidism induces a rise in myelin components MBP and PLP, as well as in c-Jun, CNPase and Tf (Marta et al., 1998).

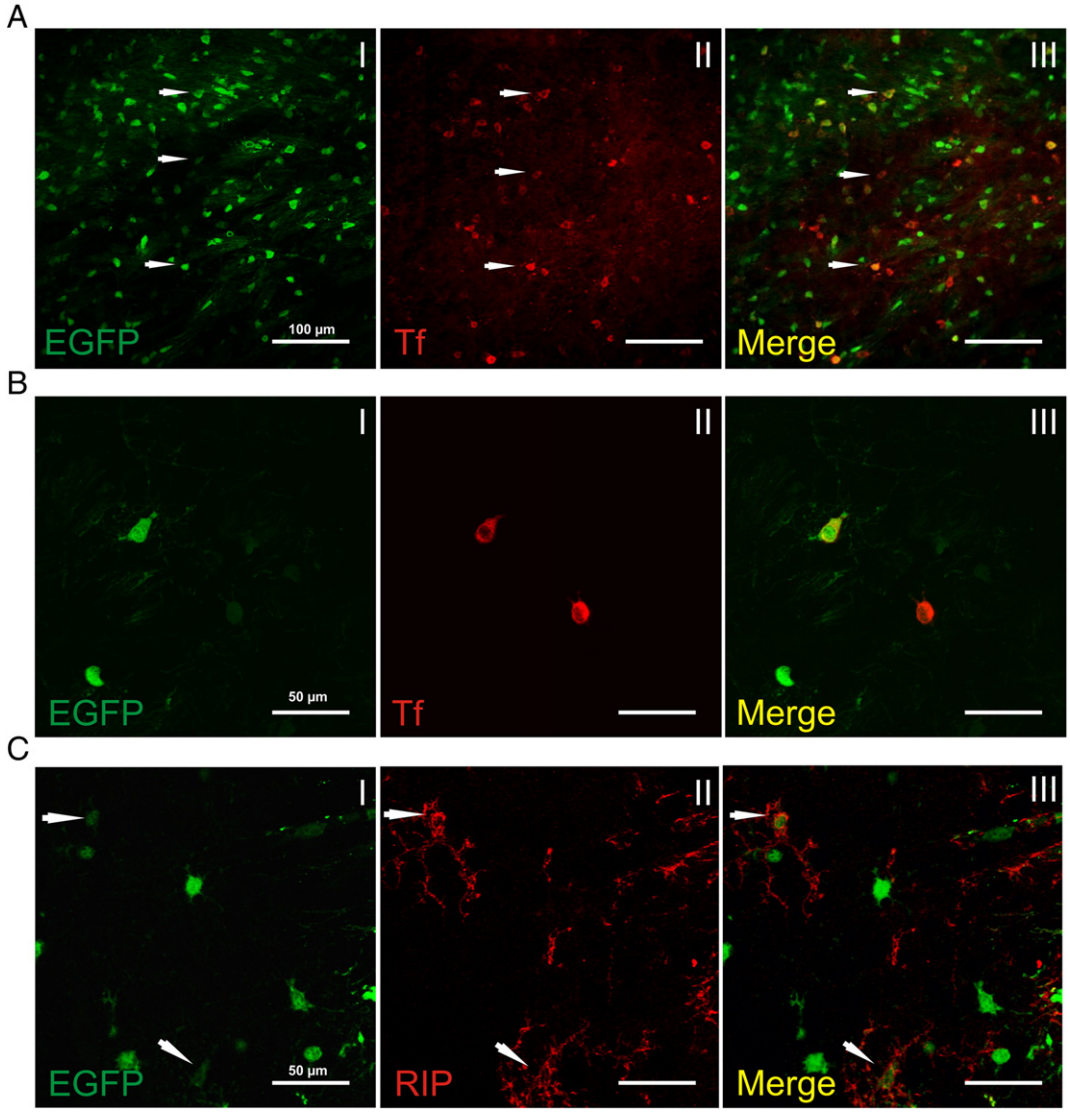


Fig. 7. High levels of Tf are detectable in OLGs. Analysis of endogenous Tf expression in oligodendroglial cell linage by Tf immunostaining on CNP::EGFP mice at P15. Photographs were obtained with confocal microscope, 25/30 z slices were collected and z stacks created with ImageJ software. (A) Shows 20× photographs of CC staining for EGFP (I), Tf (II) and merge. Arrowheads indicate positive cells for the indicated markers. (B) Higher magnification of CC showing that cells expressing high levels of Tf (II) are oligodendrocytes expressing high or low levels of EGFP (I–III). (C) Low-level EGFP-expressing cells were also stained with OLG marker RIP (II). (III) Colocalization of RIP^+ cells with low-level EGPF (arrowheads).

Tf is a plasma glycoprotein for iron transport which is expressed in all mammals and is synthesized exclusively in OLGs in the CNS (Connor and Fine, 1987; Espinosa de los Monteros et al., 1988; Espinosa de los Monteros and de Vellis, 1988). Therefore, the 9-fold increase in Tf mRNA suggests that Tf might be one of the mediators involved in the accelerated myelination observed in young hyperthyroid rats. These results indicate that Tf mRNA expression is necessary for the progression of myelination. In this context, young Hyper rats showed a premature increase in Tf mRNA, which suggests TH's ability to accelerate the myelination process. Later on, Tf mRNA levels do not show differences, probably because maturation is complete and Tf is no longer necessary. These findings emphasize Tf as a putative trophic factor, fundamental in myelin biology and OLG maturation. In close agreement with our results, a recent study by Lin et al. (2003) showed that TH induced an abundance of Tf mRNA and protein expression in a time- and dose-dependent manner in HepG2-TRα1 but not in HepG2-Neo cells, which do not express TRα. This TH-dependent regulation of Tf takes place at the transcriptional level, as determined by nuclear run-on experiments. The results by Lin et al. (2003) imply that the induction of Tf by TH is direct and may in fact be mediated by a TRE in the promoter region.

Our *in vivo* studies reveal that, at P10, Hyper rats show a significant increase not only in Tf mRNA but also in protein levels; conversely, Hypo animals show decreased levels of Tf mRNA and protein. When Hypo animals were intracranially injected with aTf; neither mRNA nor protein levels were affected, which suggests a pivotal role for TH in Tf expression. Immunohistochemical analyses revealed a decrease in $PDGFR\alpha^+$ OPCs and an increase in $CAII^+$, RIP^+ and MBP^+ cells at P20. Similar to Hyper animals but to a lesser extent, aTf_{ic} animals showed an early reduction in the number of OPCs and signs of premature myelination when compared to Ctrol. TH's stronger effects support the notion that it might induce Tf to achieve an adequate myelination. At P20, no differences were observed in Tf mRNA or content levels across groups, except for Hypo rats, whose Tf mRNA and protein levels remained low. In addition, the lack of response to aTf administration observed in these animals at P10 persisted at P20, indicating that both the presence of TH and Tf and their combined effects during myelination are necessary for OLG maturation.

In view of immunohistochemical results, as well as the finding that TH induced an increase in Tf mRNA, we studied the potential effectors regulated by TH and/or Tf involved in their actions. IGF-IR is a well-known mediator of GH signaling. The GH–IGF-I axis has been shown

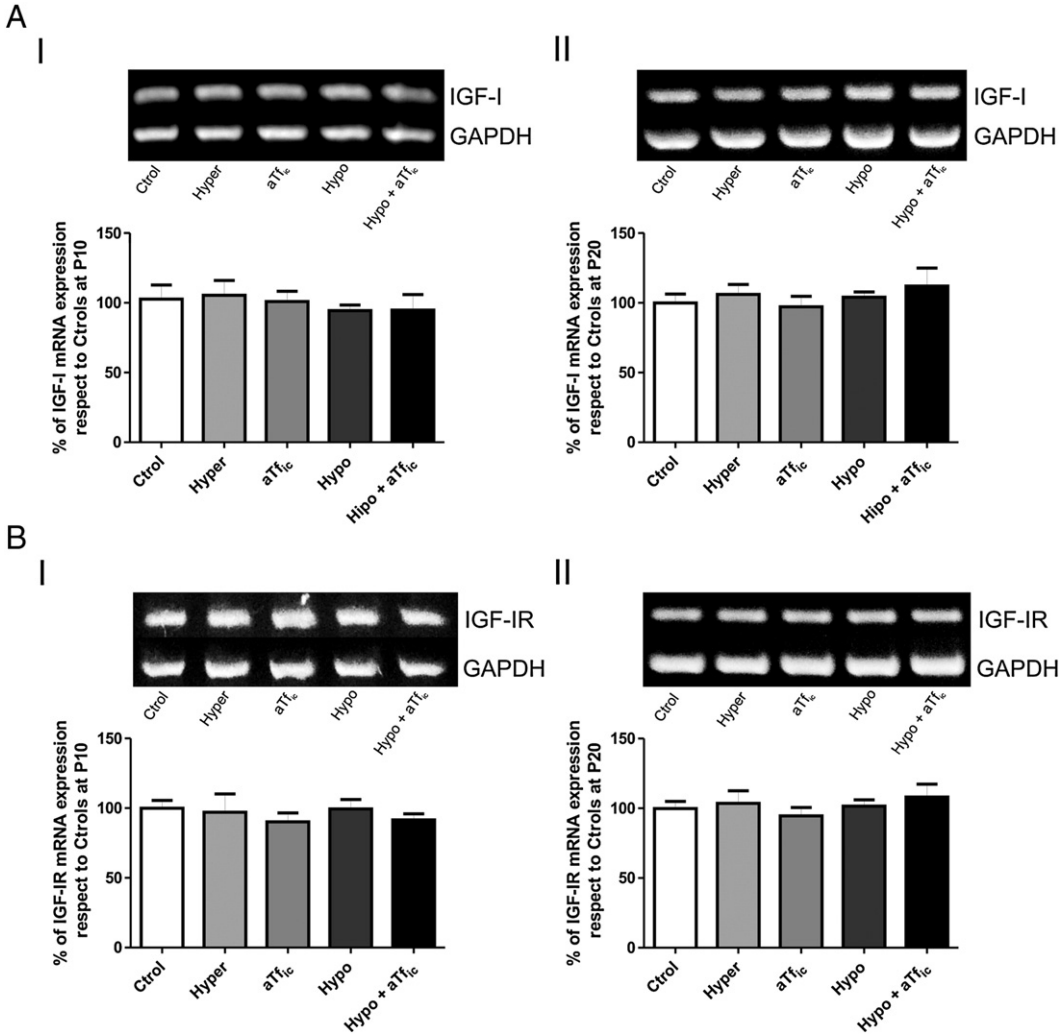


Fig. 8. aTf and/or TH levels do not affect IGF-I or IGF-IR brain expression. (A). Analysis of IGF-I mRNA expression by RT-PCR: IGF-I mRNA was co-amplified with GAPDH housekeeping gene and evaluation of reaction linearity was performed (data not shown). IODs were determined with Gel-Pro Analyzer 4.0 and IOD of IGF-I was normalized to GAPDH. Results are expressed as a percentage of the average expression of Ctrol animals at each age (considered 100%). Graphics represent relativized IGF-I mRNA expression ± SD for P10 animals (I) and P20 animals (II). (B). Analysis of IGF-IR mRNA expression by RT-PCR: IGF-IR mRNA was co-amplified with GAPDH housekeeping gene and evaluation of reaction linearity was performed (data not shown). IODs were determined with Gel-Pro Analyzer 4.0 and IOD of IGF-IR was normalized to GAPDH. Results are expressed as a percentage of the average expression of Ctrol animals at each age (considered 100%). Graphics represent relativized IGF-IR mRNA expression ± SD for P10 animals (I) and P20 animals (II). Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc tests and Student's *t* test.

to play a role in growth and differentiation in development (Anderson et al., 2002), and several studies show that, like TH, the growth hormone (GH) plays a critical role in brain development. Although it has been demonstrated that TH is a regulator for GH synthesis and secretion in the periphery (Silva et al., 2009), whether this permissive effect of TH on GH takes place in the CNS remains unknown. Tang et al. (2011)

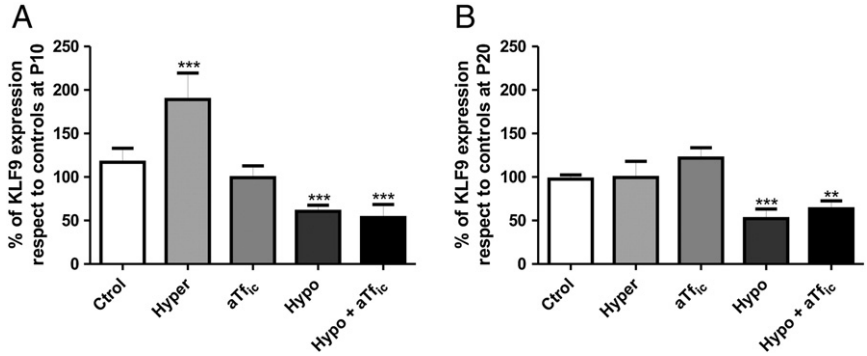


Fig. 9. KLF9 is regulated only by TH administration. Analysis of KLF9 mRNA expression by RT-qPCR: KLF9 and HPRT housekeeping gene were amplified and the C_T method was used to compare KLF9 mRNA expression across groups. Results are expressed as a percentage of the average expression of Ctrol animals (considered 100%) at P10 (A) and P20 (B). Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance with respect to Ctrol; when comparisons between other groups were done line bars indicate which ones are involved. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

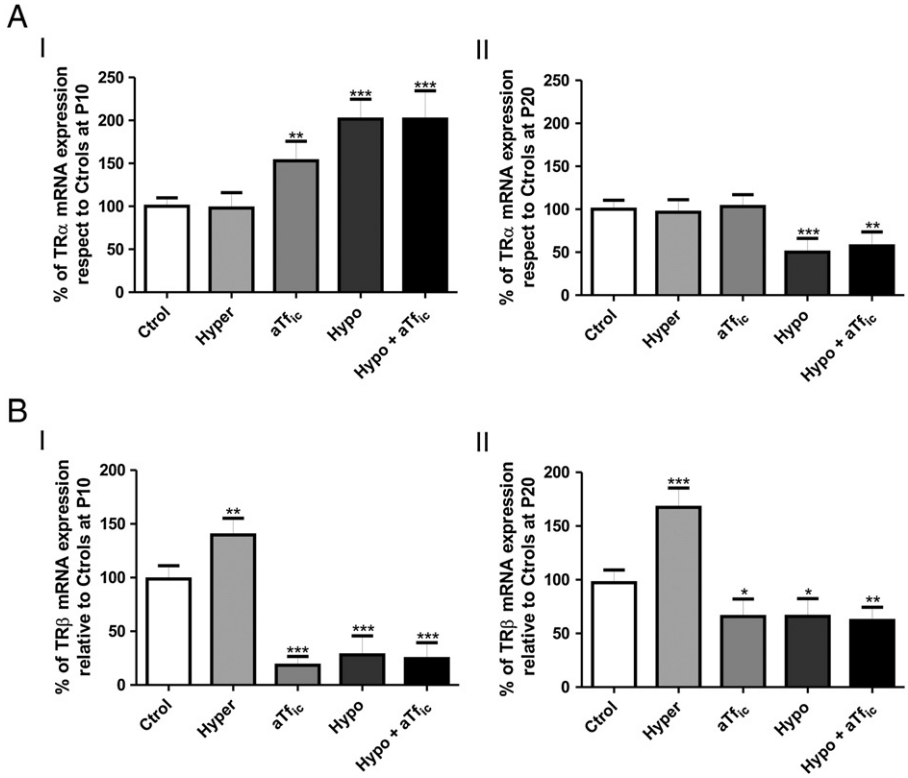


Fig. 10. Differential effect of aTf and TH in TRα and TRβ mRNA expression. Analysis of TRα and TRβ mRNA expression by RT-qPCR: TRα/TRβ and HPRT housekeeping genes were amplified and the C_T method was used to compare TRs' mRNA expression across groups. Results are expressed as a percentage of the average expression of Ctrol animals (considered 100%) at P10 (I) and P20 (II) for TRα (A) and TRβ (B). Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc tests. Asterisks represent statistical significance with respect to Ctrol; when comparisons between other groups were done line bars indicate which ones are involved. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

showed an important decrease in the expression of GH and GH receptor (GHR) in the hippocampus of hypothyroid rats, which suggests the presence of such effect in the CNS; however, in the present work we demonstrate an interaction between TH and aTf which is independent of the GH–IGF-I axis.

OPC maturation depends on the chronologically orchestrated induction of a plethora of genes (Swiss et al., 2011). TH has been shown to induce a variety of transcription factors (Dugas et al., 2006), among which KLF9 is capable of regulating OLG differentiation and myelin regeneration (Dugas et al., 2012). In our experimental groups, KLF9 assessment revealed that mRNA expression was increased in Hyper animals and decreased in Hypo at P10. At P20, Hyper animals showed no differences with Ctrol and Hypo animals evidenced a persistence of the decrease observed at P10. These data support the notion that KLF9 exclusively mediates TH ability to accelerate the myelination process, as aTf administration is not capable of modifying KLF9 expression.

The rodent thyroid gland begins to function after E17, while TH receptors (TRα/TRβ) are present from E14 and are expressed in OLGs and their precursors (Carlson et al., 1994; Barres et al., 1994). The ability of TH to trigger the effector mechanism that stops cell division and initiates differentiation becomes important around the time of birth. OPC maturation is slightly delayed in $THRA^{-/-}$ but not in $THRB^{-/-}$ mice (Billon et al., 2002), whereas a population of slow cycling OPCs persists in the adult optic nerve in mice deficient in all receptors (Baas et al., 2002). TH actions are mediated by nuclear hormone receptors whose importance is well established but whose specific functions during oligodendrogenesis are still unclear. In a very recent paper, Baxi et al. (2014) showed that a THRβ agonist promotes oligodendrogenesis in rodents as well as in human OPCs *in vitro*. *In vivo*, thyromimetics increases oligodendrogenesis and the expression of myelin proteins such as MBP, CNPase and MAG, which indicates that beta receptors can enhance OLG differentiation and promote myelination. Studies from our lab show that TR expression is not detected in the SVZ of control animals; in contrast, TRα is induced in the SVZ during demyelination, but no significant changes are observed between demyelinated and TH-treated animals. On the other hand, TRβ expression is only faintly detected in demyelinated animals but strongly upregulated in the periventricular area of demyelinated rats treated with TH. These results suggest that the regulation of TRα and TRβ expression may differ (Franco et al., 2008). Accordingly results in this work indicate that TRα is independent of TH modulation and that its expression is induced by aTf, while TRβ expression appears to respond exclusively to TH stimulus. The TRα gene is ubiquitously expressed as from early developmental stages, whereas the TRβ gene is expressed much later, which suggests different receptor functions (Bradley et al., 1992). There appears to be general consensus that both OPCs and OLGs express TRα and that OLGs express TRβ (Carré et al., 1998). According to Bury et al. (2002), TRα and TRβ colocalize in OLGs before CNPase is expressed. Thus, it might be speculated that, during the initial stages of OLG differentiation, Tf triggers TRα expression in the SVZ and promotes undifferentiated proliferating cells to become responsive to this trophic factor. Under these circumstances, exposure to TH could promote the induction of TRβ expression, which mediates TH effects on myelination through the activation of OLG differentiation.

These results unveil the interplay between TH and aTf during oligodendrogenesis, as fundamental factors for proper myelin formation. This interplay appears to take place at the level of TH receptors, as both molecules exhibit apparent effects at this point. Given that TH exerts more marked results, and considering that aTf has the potential to decrease the number of precursor cells even when TH levels have decreased, TH's role in reducing the number of precursor cells might be the consequence of the self-dependent and additive actions of aTf. Experiments are currently in progress to assess the contribution of Tf to TH effects.

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