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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).