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Thyroid hormones promote differentiation of oligodendrocyte progenitor cells and improve remyelination after cuprizone-induced demyelination

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ABSTRACT

In the present work we analyzed the capacity of thyroid hormones (THs) to improve remyelination using a rat model of cuprizone-induced demyelination previously described in our laboratories. Twenty one days old Wistar rats were fed a diet containing 0.6% cuprizone for two weeks to induce demyelination. After cuprizone withdrawal, rats were injected with triiodothyronine (T3). Histological studies carried out in these animals revealed that remyelination in the corpus callosum (CC) of T3-treated rats improved markedly when compared to saline treated animals. The cellular events occurring in the CC and in the subventricular zone (SVZ) during the first week of remyelination were analyzed using specific oligodendroglial cell (OLGc) markers. In the CC of saline treated demyelinated animals, mature OLGcs decreased and oligodendroglial precursor cells (OPCs) increased after one week of spontaneous remyelination. Furthermore, the SVZ of these animals showed an increase in early progenitor cell numbers, dispersion of OPCs and inhibition of Olig and Shh expression compared to non-demyelinated animals. The changes triggered by demyelination were reverted after T3 administration, suggesting that THs could be regulating the emergence of remyelinating oligodendrocytes from the pool of proliferating cells residing in the SVZ. Our results also suggest that THs receptor β mediates T3 effects on remyelination. These results support a potential role for THs in the remyelination process that could be used to develop new therapeutic approaches for demyelinating diseases.

Introduction

The oligodendroglial cell (OLGc) lineage arises from undifferentiated precursor cells that progress to mature myelinating oligodendrocytes mainly during central nervous system (CNS) development. Identification of each sequential phase of maturation is possible, based on cell morphology and expression of different cell markers characteristic of the various differentiation stages (Baumann and Pham-Dinh, 2001). The subventricular zone (SVZ) of the lateral ventricle is an important germinal layer formed during development that persists in the mature brain and contains multipotential progenitor cells (Doetsch et al., 1997). Other sources of progenitor cells have been identified in different areas of the brain including the subcortical white matter (Gensert and Goldman, 1997; Goldman, 2003). Evidence clearly proves that these endogenous progenitors react to demyelinating lesions and actively participate in the process by generating remyelinating oligodendrocytes (Gensert and Goldman, 1997; Carroll et al., 1998; Nait-Oumesmar et al., 1999).

There is strong evidence supporting that thyroid hormones (THs) act directly on OLGc differentiation and maturation processes (Marta et al., 1998; Rodríguez- Peña, 1999; Billon et al., 2001). Triiodothyronine (T3) is the active hormone that regulates gene expression after binding to specific intracellular TH receptors (TR). THs are required *in vitro* for the normal timing of oligodendrocyte differentiation (Bhat et al., 1979; Barres et al., 1994; Younes-Rapozo et al., 2006), and regulate myelinogenesis and expression of oligodendrocyte specific genes *in vivo* (Walters and Morell, 1981; Adamo et al., 1990). Although the influence of THs on OLGc differentiation has been extensively studied during brain development, there is limited information regarding the effect of these hormones on the adult rat brain, particularly on multipotent stem cells that persist in the brain long after myelination is complete. Exogenous administration of thyroid hormone induces cell proliferation and Nestin expression in the SVZ of adult rats (Giardino et al., 2000), suggesting that oligodendroglial precursor cells (OPCs) in adult brain are still sensitive to signalling molecules known to regulate oligodendrogenesis during development.

Even less information is available regarding T3 regulation of OLGc differentiation during the process of remyelination, one of the few regenerative events occurring in the adult CNS after different injuries. Experimental allergic encephalomyelitis (EAE) is an experimental model of multiple sclerosis which displays variable and disseminated

Abbreviations: aTf, apotransferrin; bHLH, basic helix loop helix; CC, corpus callosum; CNPase, 2′3′-cyclic nucleotide 3′-phosphodiesterase; CPZ, cuprizone; CTL, control; CPZ-C, cuprizone control; CPZ-T3, cuprizone T3 treated; EAE, Experimental autoimmune encephalomyelitis; IOD, Integrated optical density; IR, immunoreactivity; MBP, myelin basic protein; OLGc, oligodendroglial cell; OPC, oligodendroglial precursor cell; PLP, proteolipid protein; Shh, Sonic hedgehog; SVZ, subventricular zone; Tf, transferrin; TH, thyroid hormone; T3, triiodothyronine; TR, Thyroid hormone receptor.

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demyelination areas in the brain and spinal cord. In the spinal cord of EAE rats, TH treatment reduces the number of proliferating cells and triggers OLGc differentiation, which in turn leads to remyelination (Calzà et al., 2002, 2005; Fernandez et al., 2004; Calzà et al., 2005).

The cuprizone model in mice is a widely used protocol for toxic demyelination that induces oligodendrocyte degeneration, disruption of myelin sheaths and demyelination mainly localized in the medial CC (Matsushima and Morell, 2001a,b; Stidworthy et al., 2003). At variance with the results obtained by other investigators (Love, 1988), in our laboratories Adamo et al. (2006) have recently found that adjusting the cuprizone doses and the intoxication time frame, similar demyelinating effects to those induced in mice can be obtained in Wistar rats. Furthermore, similar to what occurs in mice, in rat models spontaneous remyelination takes place after cuprizone withdrawal from the diet and this process could be used to test the effect of different factors on myelin restoration.

In the present work we used the experimental approach mentioned above and investigated the *in vivo* effects of triiodothyronine (T3) on remyelination and on precursor cells and OLGc populations during the recovery stages that follow cuprizone withdrawal. In the CC we found histological evidences showing that T3 improves remyelination. Immunohistochemical studies showed that cells expressing molecular markers of maturity increased while those expressing molecular markers of immature OLGc decreased. Albeit OLGc differentiation was clearly induced by T3 treatment, expression of TRs was not detected in the CC. However both TRs were expressed in the SVZ of T3 treated animals.

In addition, we studied cell dynamics in the SVZ in response to cuprizone-induced demyelination and the effect of T3 on this process. We demonstrated that in the SVZ, demyelination induced by cuprizone intoxication increased neuroepithelial precursor cell numbers, down regulated the expression of signalling molecules which participate in early OLGc specification and promoted the dispersion of OPCs. These changes were not observed in the T3 treated animals in which the hormone promoted the differentiation of multipotent adult progenitor OLGs into mature oligodendrocytes.

Materials and methods

Reagents

Cuprizone (bis cyclohexanone oxaldihydrazone), paraformaldehyde, Höechst 33258 and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). A2B5 antibody was kindly provided by Dr Bansal (U. of Connecticut) and anti myelin basic protein (MBP) antibody was a generous gift from Dr. A.T. Campagnoni (UCLA). Anti NG2 antibody and mouse anti oligodendrocytes monoclonal antibody RIP were from Chemicon International (Temecula, CA); anti proteolipid protein (PLP) and anti Olig 1-2-3 antibodies were from Neuromics Antibodies (Edina, MN); O4 and anti APC (also known as CC1) antibodies were purchased from Calbiochem, San Diego, CA; anti GFAP, anti Sonic hedgehog (Shh), anti Id4, anti thyroid receptors (TR) antibodies (anti TRα and anti TRβ antibodies) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The following secondary antibodies were used: goat anti-rabbit IgG, goat anti-Mouse IgG and donkey anti-Mouse IgM, obtained from Jackson Immuno Research Co. Laboratories (West Grove, PA) and goat anti-Chick Igγ purchased from Neuromics Antibodies (Edina, MN). For tissue freezing we used Freez-it 2000 from ITW Chemtronics (Kennesaw, GA). Fluorescent mounting medium was from Dako North America Inc. (Carpinteria, CA). Triiodothyronine (T3) was a generous gift from Glaxo, (Argentina). All other chemicals were of analytical grade.

Animal treatment

Wistar rats were housed under standard light conditions (12 h light/dark cycle). To induce demyelination, a randomly selected group of 21 days old rats was fed a standard powdered rodent chow carefully

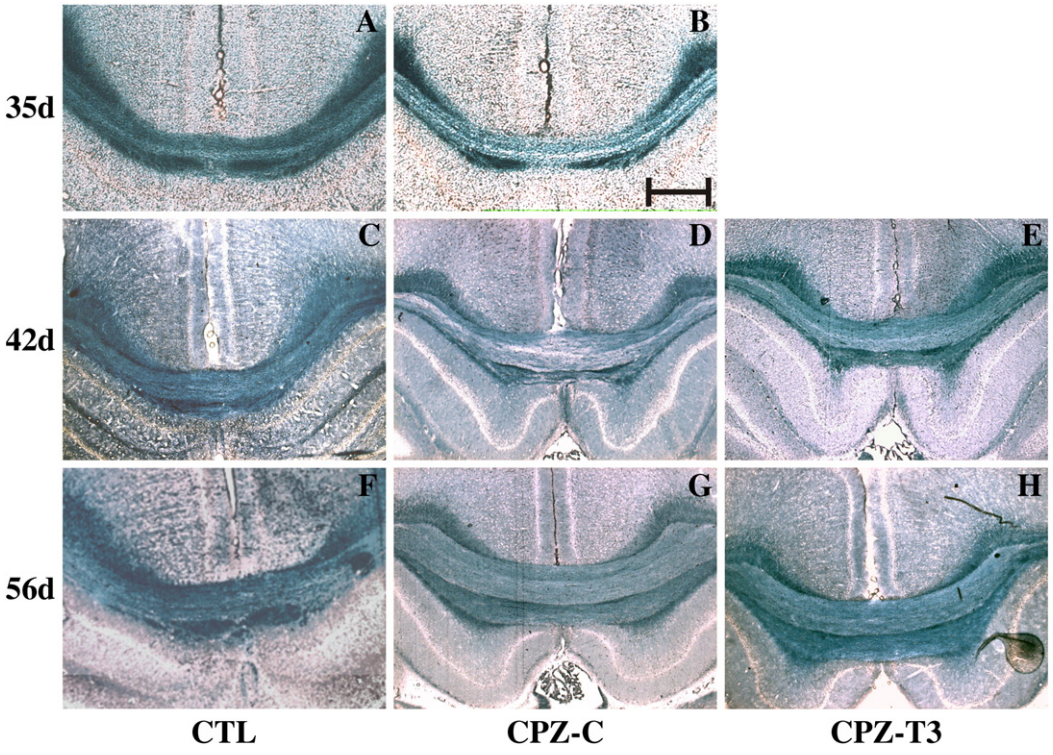

Fig. 1. Time course of demyelination–remyelination in the corpus callosum after CPZ-induced demyelination. Rat brain coronal sections were stained with Sudan Black to analyze demyelination/remyelination in the CC after cuprizone intoxication. Brains from control (A) and CPZ (B) 35 days old rats evidence cuprizone-induced demyelination. Time course of remyelination at 42 (D,E) and 56 (G, H) days of age in CPZ-C (D,G) and CPZ-T3 rats (E,H) shows improved myelin staining in T3 treated animals. Brain sections from normal CTL animals of the same age shown in C and F. Scale bar in B=500 μm. All images are representative of 5 independent experiments.

mixed with cuprizone 0.6% (w/w) during two weeks (CPZ). Aged matched normal controls received a standard powdered chow without cuprizone (CTL). Both groups of rats were weighed daily during cuprizone intoxication and then weekly until they were killed. At 35 days of age, the cuprizone fed animals were returned to a normal diet with standard rodent chow pellets for up to three further weeks. To evaluate the effect of THs on remyelination, cuprizone treated rats were subcutaneously injected on days 35, 37 and 39 of age with 20 μg of triiodothyronine (T3)/100 g body weight (CPZ-T3) or with the same volume of saline solution (CPZ-C). The whole protocol is summarized in Fig. 1A.

Tissue preparation

Animals were anesthetized with 75 mg/kg ketamine and 10 mg/kg xilazine and perfused through the left heart ventricle with PBS followed by paraformaldehyde (PFA) 4% in PBS. The brains were carefully removed and postfixed overnight in PFA 4% at 4 °C. Brains were then washed in PBS and cryoprotected by extensive immersion in 15% and 30% sucrose in PBS at 4 °C. Finally, tissues were frozen with freezing spray and stored at −80 °C till processed.

Immunohistochemistry

For immunohistochemical studies, 20 μm cryostat brain tissue sections, corresponding to 0.48 mm from bregma according to Paxinos and Watson's stereotaxic atlas (Paxinos and Watson, 1986), were conserved in 50% glycerol at −20 °C until processed. These sections include the dorso-lateral angle of the lateral ventricle, an area with a high cell proliferation rate. For the immunological detection we performed floating immunohistochemistry. Briefly, sections were washed in PBS, permeabilized with 0.1% Triton X-100 in PBS and blocked with 5% fetal calf serum in PBS. For indirect immunofluorescence, sections were incubated o/n at 4 °C with the following antibodies: anti NG2, an antigen expressed by OLGs at early stages of development; anti Shh, a factor known to induce two bHLH transcription factors (Olig 1 and Olig 2) that play a key role in the specification of oligodendrocyte precursors; an antibody to Olig 1-2-3 was also tested; anti Id4 a differentiation inhibitor expressed by progenitor cells and anti Nestin, a maker of neuroepithelial stem cells; A2B5, an antibody that marks OPCs and O4, an antibody marking intermediate/late stages of development of OLGcs (pre-oligodendrocytes) were also used. Identification of mature/adult OLGcs

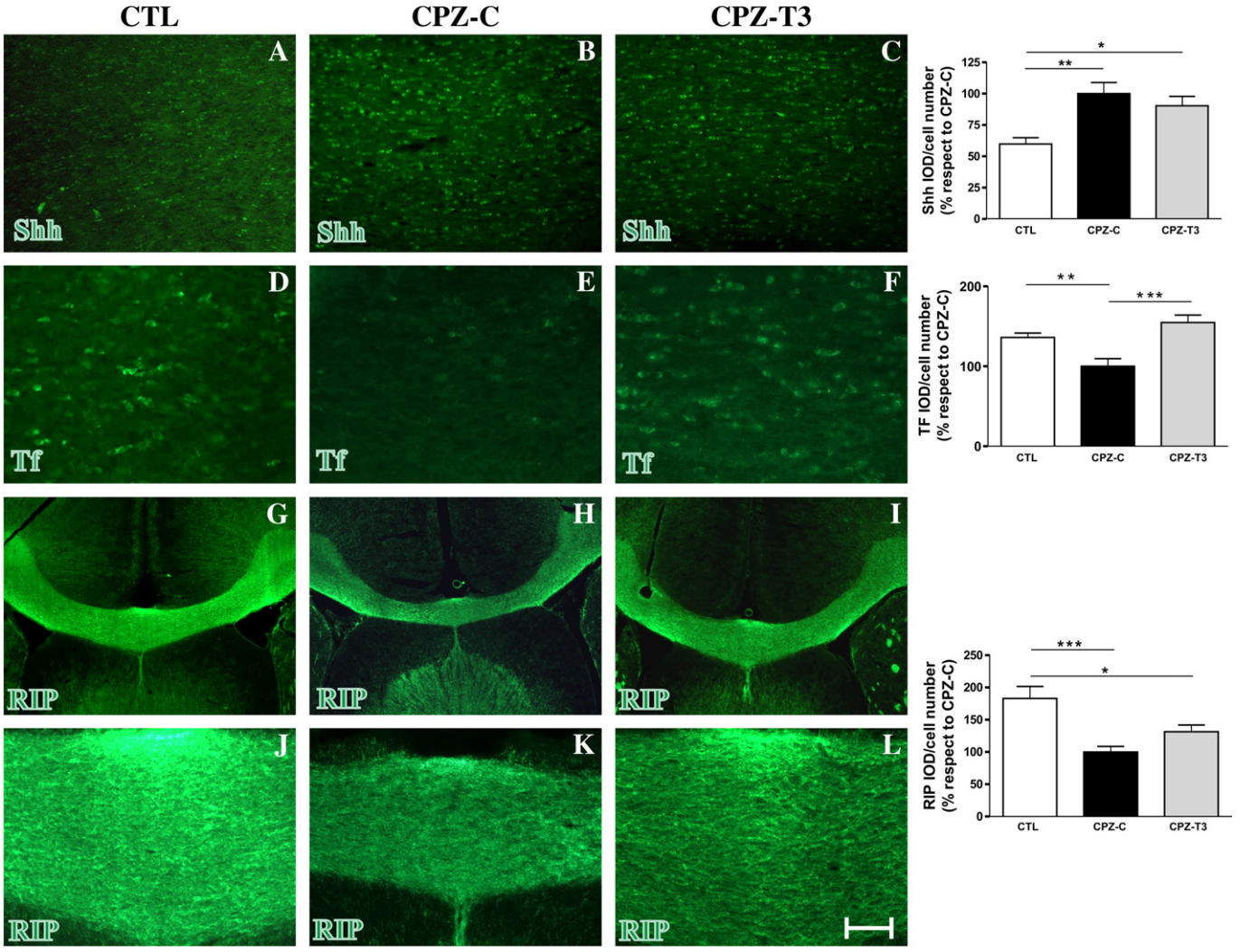

Fig. 2. Detection of Shh, Tf, and RIP immunoreactivity in the corpus callosum of 42 days old rat brains in different experimental situations. Shh positive cells were weakly detected in CTL brains (A). Increase in Shh immunoreactive cells was found in the CC in both CPZ-C and CPZ-T3 demyelinated animals (B, C). Tf IR in the CC of CPZ-C rats (E) decreased substantially when compared to CTL rats (D). In CPZ-T3 rats, (F) Tf IR was restored to values similar to CTL situation. RIP labelled brain coronal sections showed that the width of the medial area of the CC was noticeably reduced in the CPZ-C (H, K) demyelinated animals in comparison to CTL rats (G, J). RIP IR was higher in CTL and CPZ-T3 rats than in CPZ-C animals (G–L). Scale bar in L = 50 μm for D–F; 100 μm for A–C, J–L and 500 μm for G–I. Quantification of each marker was performed as described in Materials and methods and is expressed as percentage relative to the CPZ-C condition. Bars represent the mean ± SEM of at least three independent experiments, each performed in triplicate. Significant differences are indicated by asterisks (*$p<0.05$; **$p<0.01$, ***$p<0.001$).

was done using anti MBP, anti APC (CC1), anti PLP, and anti RIP antibodies. Antibodies against thyroid receptors TRα and TRβ and anti Tf antibody were also assayed. Tissue sections were then rinsed with PBS and incubated for 2 h at room temperature with the appropriate secondary antibody and 5 μM Höechst dye. Sections were rinsed again with PBS, carefully placed on glass slides, dried overnight and mounted with a fluorescence mounting medium for epifluorescence microscopy. Additionally, 30 μm brain sections, corresponding to −3.80 mm from bregma, were obtained and placed directly on gelatinized glass slides. After air drying, sections were soaked in PBS, dehydrated in 70% ethanol, stained with 0.5% Sudan Black in 70% ethanol for 30 min and finally rinsed with water. Samples were observed under fluorescence or light microscopy in an Olympus BX50 microscope. Photography was carried out with a CoolSnap digital camera and the Image Pro Plus software (version 5.5) was used for image analysis. Immunohistochemistry for each marker was performed in triplicate from at least 3 independent experiments to verify accuracy of results.

Quantitative and statistical analysis

Quantitative analysis of the immunohistochemical preparations was performed using the Image Pro plus software (version 5.1). In the CC, the Integrated Optical Density (IOD) was measured for each condition in six randomly selected fields of the medial area of this structure (100×100 micrometers each). Cell number was also determined in these sectors by counting Hoechst positive nuclei. Immunohistochemistry for the different markers was performed in triplicate from a variable number of independent experiments ($n=6$ for MBP, $n=3$ for O4, RIP, PLP, CC1, Shh, Tf and A2B5). The ratio IOD/cell number was calculated and data were expressed as percentage respect to the CPZ-C condition. For similar purposes in the SVZ, the area including positive immunoreactive cells was manually selected with the appropriated software tool and IOD or area size were determined and expressed as percentage relative to the CPZ-C condition. Statistical analysis between multiple groups was carried out using two-way ANOVA followed by Newman–Keuls post test with the Graph Pad Prism 4.0 software.

Results

After two weeks of cuprizone administration, rats exhibited a decrease in growth rate compared to control littermates. While CTL animals continued to gain weight throughout the experimental period, CPZ animals maintained a constant body weight during the

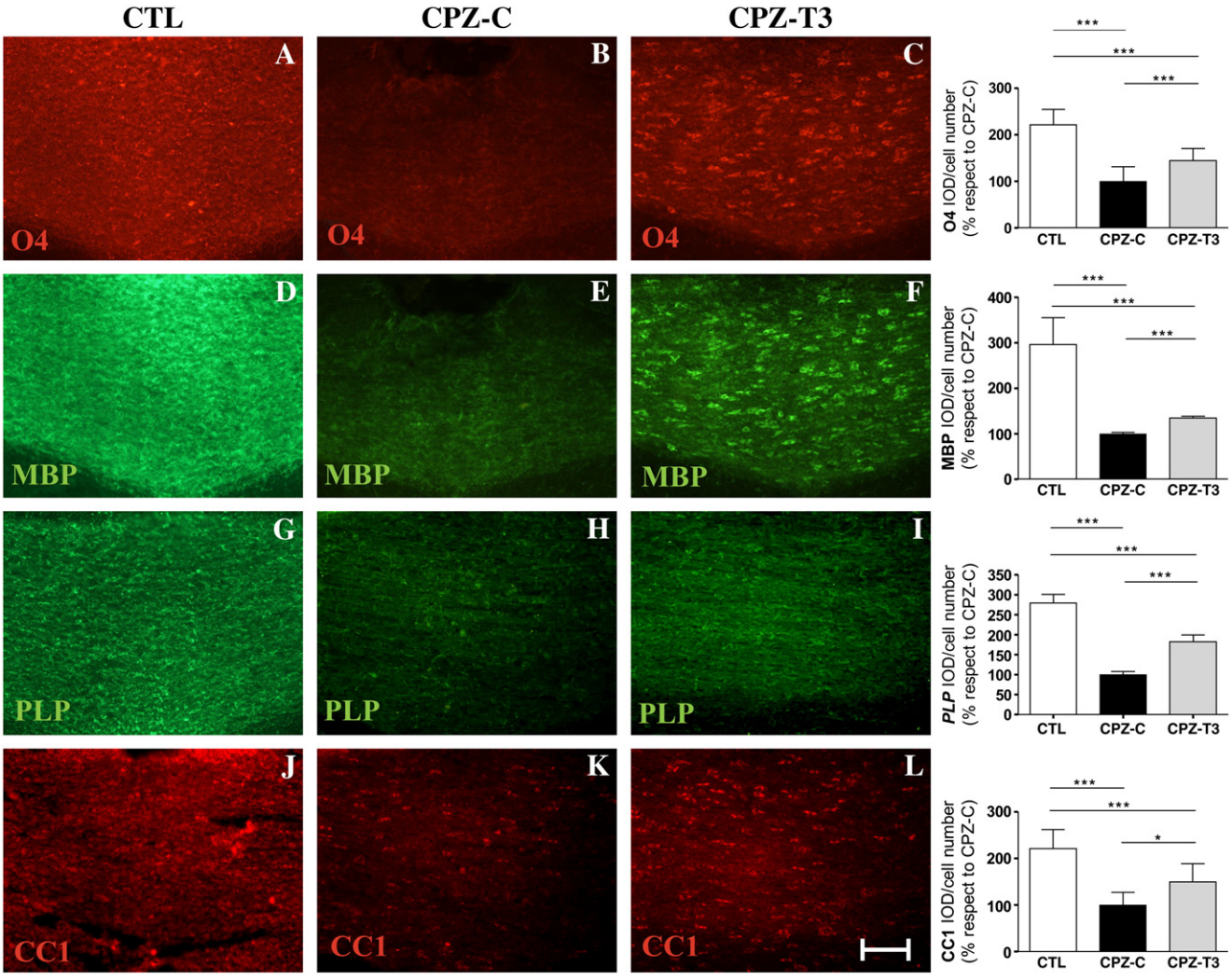

Fig. 3. O4, MBP, PLP and CC1 immunoreactive structures in the corpus callosum of 42 days old rats in different experimental situations. Immunoreactivity of markers of maturity in CTL animal brain sections (A, D, G and J) decreased markedly in CPZ-C demyelinated rats (B, E, H and K). Reactivity of these markers of OLGc differentiation was significantly increased, particularly for O4, MBP and CC1 in demyelinated rats treated with T3 (CPZ-T3) (C, F, I and L). Scale bar in L = 100 μm. Quantification of each marker was performed as described in Materials and methods and is expressed as percentage of the CPZ-C condition. Bars represent the mean ± SEM of at least three independent experiments, each performed in triplicate. Significant differences are indicated by asterisks (*$p<0.05$; ***$p<0.001$).

intoxication period. However, after cuprizone removal from the diet, CPZ animals reached growth rates equivalent to controls.

Sudan Black was used to stain myelin tracts in brain sections obtained from the various groups of animals. In 35 days old cuprizone fed rats (Fig. 1B), the CC showed clear signs of demyelination when compared to normal controls (Fig. 1A). At 37 days, despite the fact that cuprizone had been removed from the diet, severe demyelination was still present (not shown). Maximum demyelination was histologically detected at 42 days of age (Fig. 1D). Histological signs of remyelination were first observed at 49 days of age (not shown) and progressive recovery was observed till 56 days of age (Fig. 1G), but remyelination did not reach the staining level observed in control animals at the same age (Figs. 1C, F). To determine the effects of THs on remyelination, we administered T3 during the first week following cuprizone removal according to the schedule mentioned above and analyzed brain sections from 42 days old rats by Sudan Black staining. Remyelination was markedly improved in T3 treated rats (Figs. 1E, H) compared to cuprizone control animals (Figs. 1D, G).

The expression of different OPC and OLGc markers in CTL and CPZ animals was studied in the medial region of the CC and in the subventricular zone (SVZ) and surrounding areas at 42 days of age, when maximal demyelination was detected histologically in CPZ-C animals.

In the CC, Shh immunoreactive cells were detected in CTL rats (Fig. 2A) and in CPZ-C and CPZ-T3 rats, Shh positive cells increased (Figs. 2B–C). A2B5 immunoreactivity (IR) was faintly detected in CTL rats and was slightly induced in CPZ-C animals (not shown). In some sections we found that after T3 treatment A2B5 IR was somewhat reduced when compared to CPZ-C, resembling the CTL condition (not shown), although differences were not significant due to the heterogeneity found in the expression of this marker. Tf immunoreactivity was evaluated since previous studies have shown that sustained neonatal hyperthyroidism in the rat induces a marked increase in the expression of the Tf-mRNA in the brain (Marta et al., 1998) and because it has been found to induce OLGc differentiation (Paez et al., 2002, 2004). Numerous cells expressing Tf were detected in CTL rats (Fig. 2D). Tf positive cells decreased around 35% in CPZ-C animals (Fig. 2E) and after T3 treatment we observed a 55% increment in Tf expression respect to CPZ-C (Fig. 2F) to values similar to those seen in CTL. The analysis of RIP expression revealed strong and dense IR in the CC. As expected, a significant decrease in RIP IR (80%) was observed in CPZ-C animals in comparison to CTL rats (Figs. 2G, J, H, K). It is interesting to point out that the CC, clearly immunostained by this antibody, was markedly thinner in the CPZ-C than in CTL animals as a consequence of the demyelination produced by the toxic insult. After T3 treatment, CPZ-T3 rats showed a slight induction in RIP IR (30%) that did not reach CTL levels (Figs. 2I, L). Although differences in the ratio IOD/cell number between CPZ-C and CPZ-T3 conditions were not significant, the CC of CPZ-T3 rats was markedly thicker when compared to CPZ-C. Fig. 4 shows O4 IR and expression of differentiated OLGc and myelin markers in the CC in the different experimental situations. As expected, control rats of this age showed the presence of a high number of O4, MBP, PLP and CC1 positive cells (Figs. 3A, D, G and J). MBP expression in CPZ-C rats was significantly lower (Fig. 3E) in coincidence with the

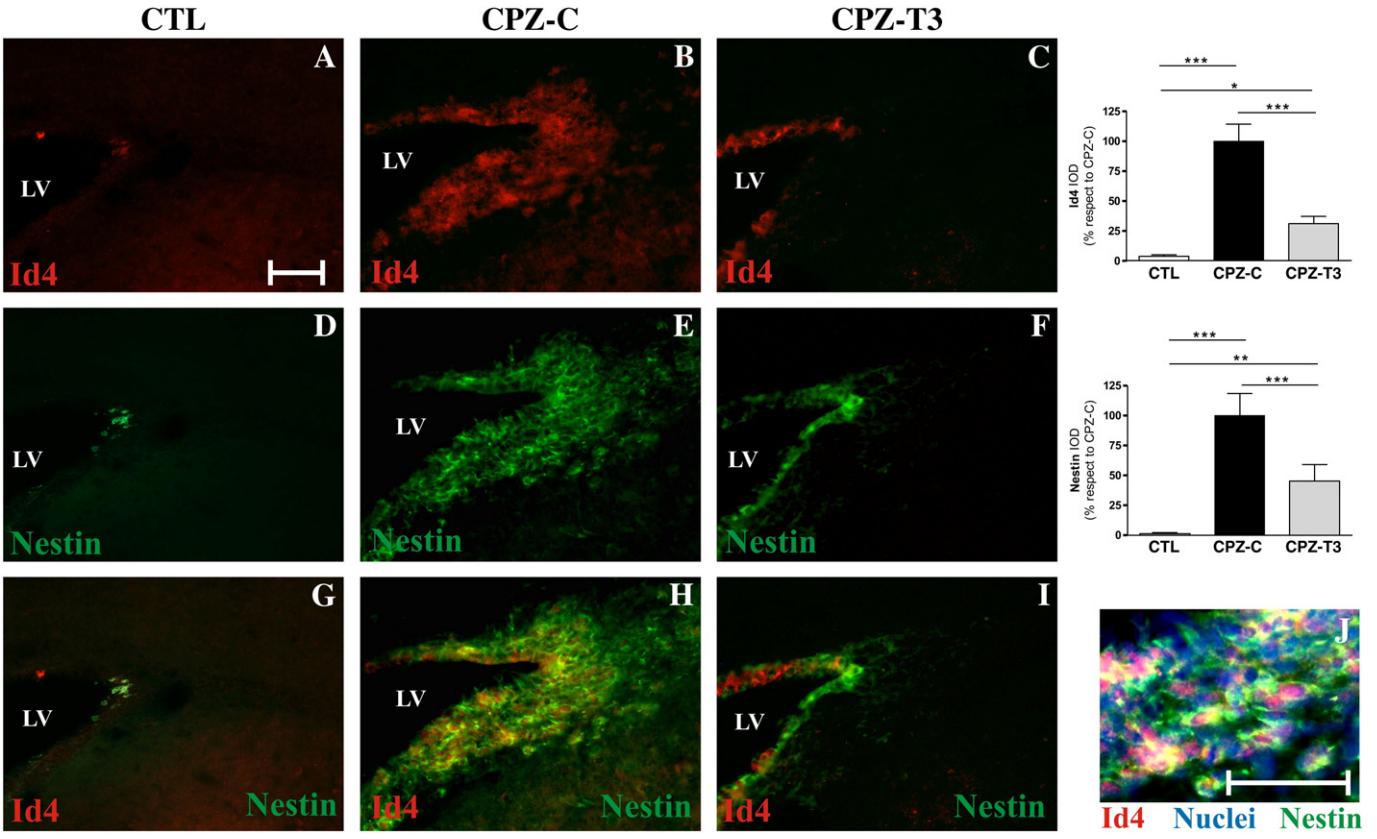

Fig. 4. Effect of CPZ intoxication and thyroid hormone treatment on Id4 and Nestin immunoreactivity in the SVZ of 42 days old rat brain. Coronal sections show the SVZ and surrounding areas. CTL rats, (A, D) showed limited IR for these markers. In CPZ-C animals (B, E), a significant increase in immunopositive cells was detected in the wall of the ventricle and particularly in the SVZ (B, E). After T3 treatment, IR of these markers of undifferentiated progenitor cells decreased markedly (C, F). Merged images of Nestin and Id4 in the SVZ (G–I). Quantification of each marker was performed as described in Materials and methods and is expressed as percentage of the CPZ-C condition. Bars represent the mean ± SEM of at least three independent experiments, each performed in triplicate. Significant differences are indicated by asterisks (*$p<0.05$; **$p<0.01$, ***$p<0.001$). Scale bar in A = 100 μm. (J) Merged image at higher magnification of 4H with nuclear Hoechst staining (blue) showing that Nestin immunoreaction is mainly found outside the nucleus, while Id4 expression (red) predominantly overlaps nuclei staining (magenta). Scale bar = 100 μm.

marked demyelination observed in these animals with Sudan Black staining (Fig. 1D). T3 treatment resulted in an early increase in MBP expression suggesting an accelerated OLGc maturation in these animals (Fig. 3F). O4 IR behaved in a similar way (Figs. 3B, C) and co-localized with MBP expression (not shown). Similar results were found with the expression of PLP and CC1, which were also up regulated in

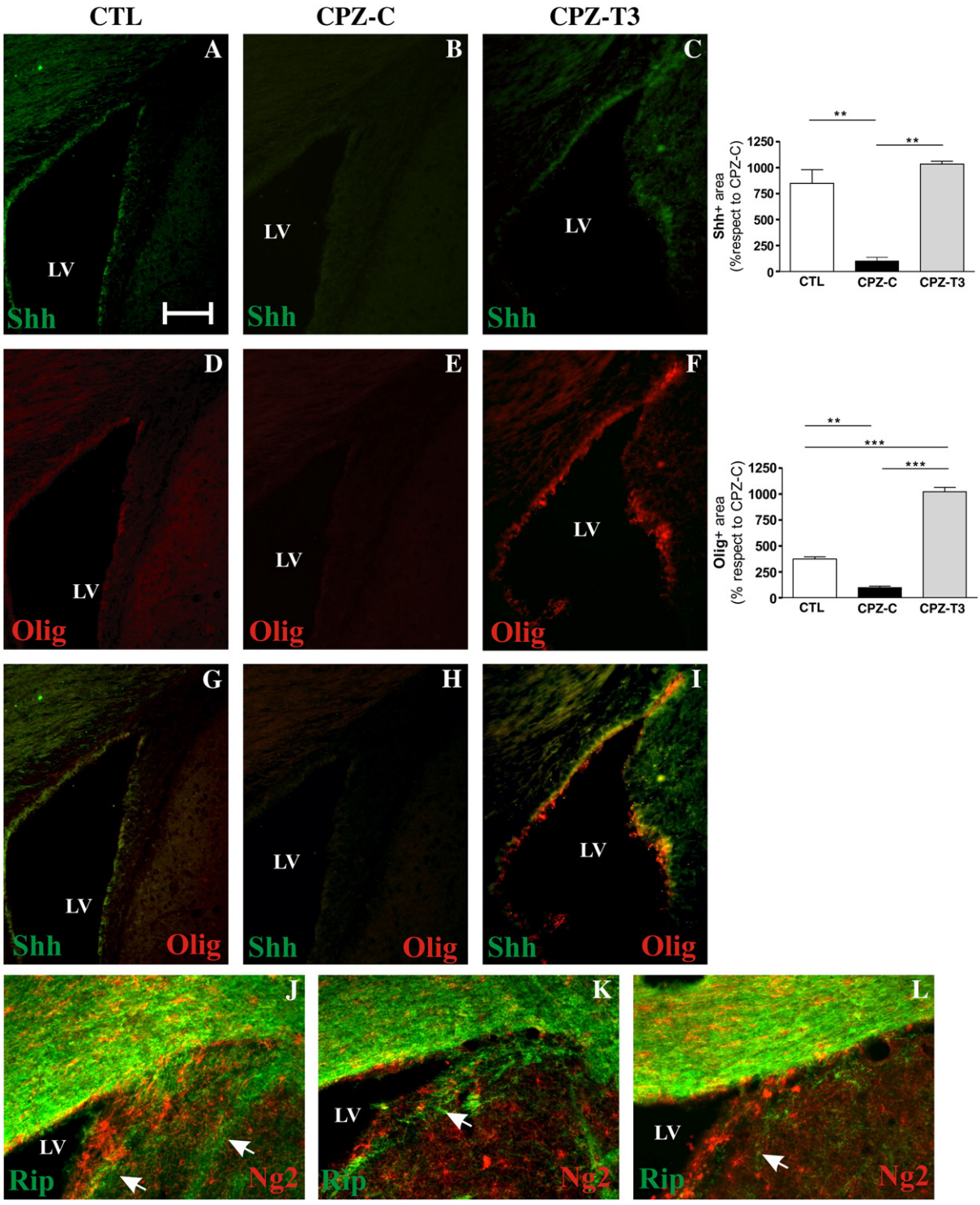

Fig. 5. Expression of different OLGc markers in the SVZ of 42 days old rats in different experimental situations. Positive Shh and Olig1 IR in CTL rats (A, D) decreased markedly and became barely detectable in CPZ-C (B, E). A slight increase in reactivity, particularly for Olig 1 was detected in CPZ-T3 rats (C, F). Merged images (G, H, I). NG2 expression in CTL sections was detected periventricularly and extending beyond the dorso-lateral corner into the SVZ (red, J). IR to this marker appeared to be more dispersed in CPZ-C, suggesting migration of the reactive cells away from the SVZ (K), with no major changes after T3 treatment, except for a slight concentration of the immunoreactive cells as a thin rim located near the ventricle wall (L). RIP immunoreaction was found in two bands of cells surrounding the ventricular wall and the SVZ (green, J, arrows). In CPZ-C animals RIP positive cells were confined to the ventricular wall (K, arrow) and only a few RIP positive cells were detected in CPZ-T3 rats (L, arrow). Scale bar in A = 100 μm. Quantification of each marker was performed as described in Materials and methods and is expressed as percentage of CPZ-C condition. Bars represent the mean ± SEM of at least three independent experiments, each performed in triplicate. Significant differences are indicated by asterisks (**$p<0.01$, ***$p<0.001$).

the CC of CPZ-T3 animals in comparison to CPZ-C rats (Figs. 3H, I, K and L). The expression of all these myelin markers was induced between 35% and 80% by T3 treatment when compared to CPZ-C animals.

Immunohistochemical analysis of different OLGc markers in the SVZ and surrounding areas in CTL animals showed positive IR for the differentiation inhibitor Id4 and the neuroepithelial stem cell marker Nestin in a few cells located in the dorso-lateral angle of the lateral ventricle (Figs. 4A and D). A marked increase in the area showing positive cells for both markers was detected in the dorsal area and dorso-lateral angle of the ventricle as well as in the SVZ in CPZ-C rats (Figs. 4B and E). This enhanced expression decreased around 60% after T3 treatment, although it did not reach the levels observed in the CTL animals (Figs. 4C and F). These results suggested that the density of progenitor cells in the SVZ was markedly reduced after T3 treatment. Co-localization studies revealed overlapped expression of both markers in the periventricular area (Figs. 4G, H and I). In immunohistochemical studies performed to detect the expression of Shh and Olig proteins, a compact area of intensely Shh positive cells was found in the SVZ of CTL animals (Fig. 5A) while Olig expressing cells were also detected in a similar position, though more restricted to the external angle of the ventricle (Fig. 5D). No expression of Shh or Olig was detected in the SVZ of CPZ-C rats (Figs. 5B, E, H) but positive cells for both markers, particularly for Olig, were observed once more in the SVZ and in the upper wall of the lateral ventricle after T3 administration (Figs. 5C, F, I). We found that cells located near the ventricle were only Olig positive, those further away from this area were Shh positive, while both markers were expressed in between these two areas (Fig. 5I). A few A2B5 positive cells, which probably represent OPCs, were present in a compact area of the external angle of the lateral ventricle in CTL rats (not shown). In CPZ-C rats, A2B5 expression in this area decreased, but many A2B5 positive cells were still found dispersed in its vicinity while in the CPZ-T3 condition, A2B5 positive cells were detected again in a compact area of the external angle of the lateral ventricle and in the SVZ as well (not shown). NG2 IR was detected in the SVZ in CTL animals (Fig. 5J). The cells were particularly concentrated in a rather thick area located near the ventricle wall. In CPZ-C animals there was a reduction in the density of NG2 positive cells in the ventricular wall (Fig. 5K) and the IR of this marker appeared to be more dispersed suggesting migration of the reactive cells away from the SVZ. After T3 treatment, NG2 IR became concentrated again as a thin rim located near the ventricle wall (Fig. 5L). Despite the strong RIP IR in the CC, we detected some positive cells delimiting the ventricular walls and particularly located in the SVZ and its vicinity in CTL rats (arrows in Fig. 5J). In the CPZ-C animals, RIP immunoreactive cells appeared to concentrate in a thin area of the SVZ and decreased in nearby structures (arrow in Fig. 5K), while after T3 treatment, RIP immunoreactive cells almost disappeared from the SVZ and its vicinity (arrow in Fig. 5L).

In an analysis of the expression of thyroid hormone receptors (TR) in different brain areas we found that there was no expression of TRα or TRβ in the CC in any experimental situation (not shown). TRs were not expressed in the SVZ of control brains (Figs. 6A and D). However, in both groups of cuprizone-intoxicated animals, positive IR of TRs was found in the ventricle walls (Figs. 6B, C, E and F). In the CPZ-C brains, TRα expression was present along the entire wall of the lateral ventricle and in the SVZ (Fig. 6B), while TRβ was confined to two small cell patches located in the medial and lateral angles of the ventricle wall (Fig. 6E).

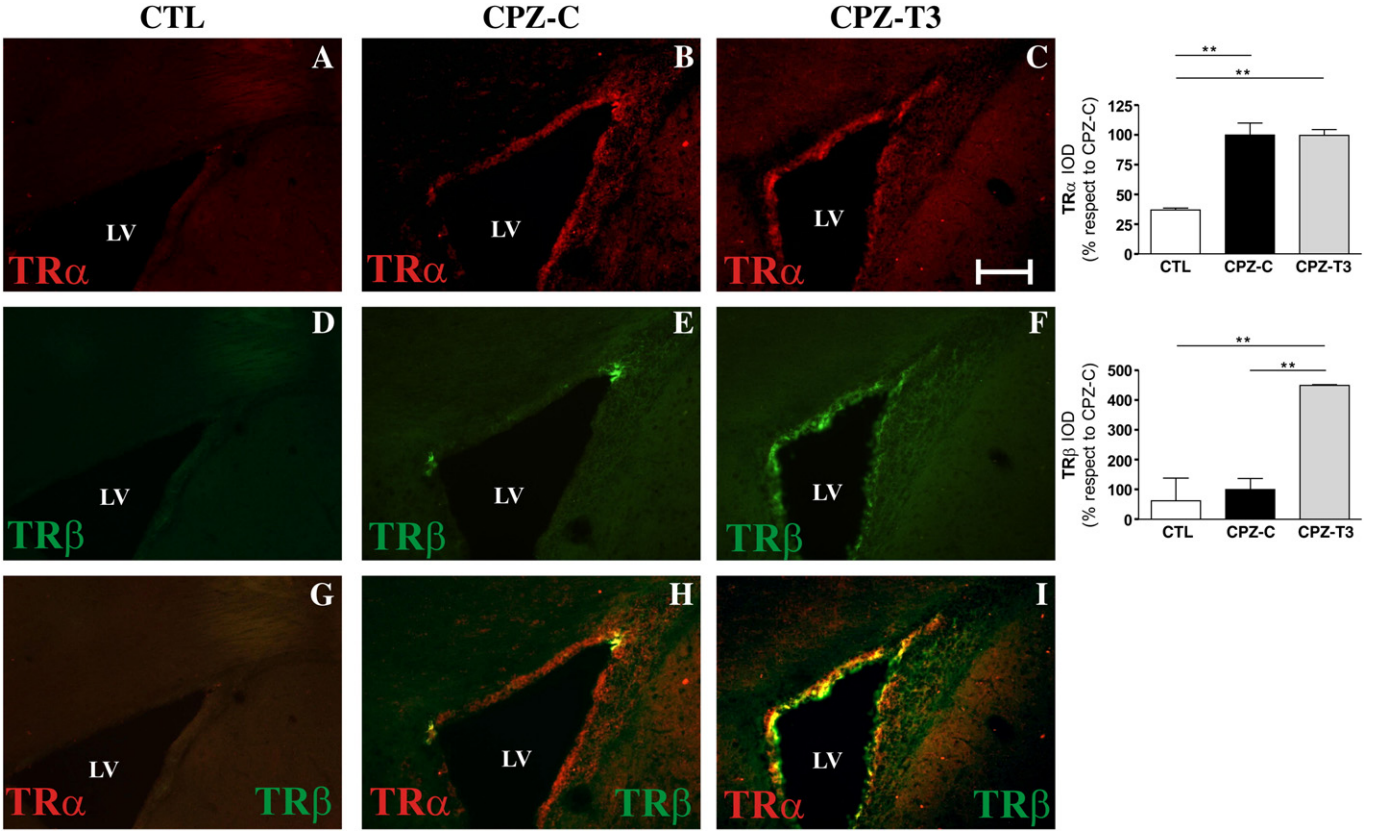

Fig. 6. Detection of thyroid hormone receptors (TRs) in the SVZ of 42 days old animals. Expression of TRα and TRβ in non-demyelinated CTL animals was undetectable (A, D). TRα expression increased markedly in the ventricle wall in CPZ demyelinated rats (B). T3 treatment induced a further increase in expression, particularly in the SVZ (C). TRβ in the SVZ of CPZ-C rats (E) was barely detectable. Strong activation of TRβ expression was observed in CPZ-T3 animals (F). Merged images of TRα (red) and TRβ (green in G–I) show overlapped expression of both receptors (yellow) in cells of the ventricular wall. Scale bar in C = 100 μm. Quantification of each marker was performed as described in Materials and methods and is expressed as percentage of the CPZ-C condition. Bars represent the mean ± SEM of at least three independent experiments, each performed in triplicate. Significant differences are indicated by asterisks (**$p<0.01$).

After T3 administration, the distribution pattern of TRα positive cells in the areas mentioned above persisted (Fig. 6C), while TRβ IR was strongly induced along the walls of the lateral ventricle but faintly in the SVZ (Fig. 6F). Co-expression analysis (Figs. 6G, H and I) revealed areas showing overlapped expression of both receptors in the ventricle wall.

Discussion

In previous studies we have developed a cuprizone-induced rat demyelination model and have successfully used it to study remyelination after an aTf intracranial injection (Adamo et al., 2006). In the present paper we have used the same cuprizone demyelination model to study the effect of T3 on remyelination in the CC and on OLGc dynamics in the SVZ. We have found that in the SVZ, the strong up regulation of neuroepithelial stem cell markers observed in demyelinated animals was clearly reduced after T3 treatment while in the CC, TH treatment accelerated the remyelination process due to an increase in the differentiated OLGcs population.

Thyroid hormones have been implicated in OPCs differentiation based on their effects on postnatal myelination (Rodríguez- Peña, 1999). In our laboratories Marta et al. (1998) found that in 10 days old hyperthyroid animals, expression of the mRNAs of MBP, PLP, 2′3-cyclic nucleotide 3′-phosphodiesterase (CNPase) and Tf were markedly higher than in euthyroid controls, while at 17 days of age the levels of the previously mentioned mRNAs decreased, indicating an important effect of T3 before the myelination peak.

Our histological studies using Sudan Black staining revealed clear signs of demyelination in the CC of the animals fed with cuprizone in agreement with previous results from our laboratories (Adamo et al., 2006). They also showed remyelination was markedly improved after T3 treatment when compared to spontaneously remyelinated animals, a result consistent with the idea that THs have similar effects, both on normal myelination and on the remyelination processes that follows myelin damage. Fernandez et al., (2004) and Calzà et al. (2002, 2005) have shown that THs stimulate remyelination in the spinal cord of EAE rats. Although these studies were done in an autoimmune inflammatory demyelination model, lacking anatomical reproducibility amongst animals, with the disadvantage of being sporadic and asynchronous, a fact that greatly hampers accurate and reliable assessment of changes in cell population and quantification of demyelination, (Matsushima and Morell, 2001a,b) their results agree with our own.

As expected, CPZ-C animals displayed few cells expressing mature OLGc markers in the CC such as MBP, PLP and CC1 indicating they were at the very early stages of spontaneous remyelination. The strong increase of these OLGc maturity markers observed in the CC of CPZ-T3, in coincidence with the decrease in A2B5 immunoreactive cells, would indicate that differentiation of these precursor cells to mature OLGcs takes place in response to TH treatment. Although in CPZ-T3 treated rats many cells displayed O4 positive immunoreactions, they also showed MBP expression, indicating that they were mainly mature OLGcs, rather than pre-OLGcs or immature oligodendrocytes, which are also O4 positive. Analysis of RIP expression, a marker of oligodendrocytes and myelin sheaths, confirmed the above mentioned results.

Analysis of Tf expression is consistent with those previously published by our laboratories showing that sustained hyperthyroidism in neonatal rats accelerates myelination and is accompanied by a significant increase in Tf-mRNA (Marta et al., 1998). Since aTf has been shown to accelerate remyelination in cuprizone demyelinated rats (Adamo et al., 2006), these findings suggest that this putative trophic factor might contribute in mediating THs action.

Although Shh expression in the CC was activated in response to the demyelinating insult, we did not find differences between CPZ-C and CPZ-T3 treated rats. This allows us to assume that Shh signalling in the CC does not mediate TH action on OLGc maturation. This was not the case however with reference to our findings in the SVZ (see below).

There is growing consensus indicating that the remyelination process initiates in the SVZ, where OPCs are induced to proliferate, migrate toward demyelinated areas and differentiate into mature myelinating oligodendrocytes (Picard-Riera et al., 2002; Nait-Oumesmar et al., 1999). Additional progenitor cell reservoirs have been found dispersed in other areas of the adult brain that serve as a source of remyelinating oligodendrocytes (Gensert and Goldman, 1997, Goldman, 2003). Nevertheless, their contribution to the remyelination process seems to be spatially restricted (Carroll et al., 1998). Evidence supports progenitor and OPCs as TH targets during remyelination. TRs expression was neither detected in the CC of control or demyelinated animals nor in the SVZ of control animals, while the SVZ in demyelinated rats showed TRα induction. TRα expression was activated in the ventricular wall in response to the cuprizone insult but no significant changes were seen between CPZ-C and CPZ-T3 treated rats. An interesting finding however was that TRβ expression was only faintly detected in the CPZ-C animals but was strongly up regulated in the periventricular area of CPZ-T3 treated rats. These results suggested that regulation of TRα and TRβ expression may differ: while TRα is induced by demyelination and seems to be independent of T3 modulation, TRβ expression appears to respond to T3 stimulus. The TRα gene is ubiquitously expressed from early stages during development, whereas the TRβ gene is expressed much later, suggesting both receptors may have different functions (Bradley et al., 1992). A general consensus stipulates that both OPCs and OLGcs express TRα and that OLGcs express TRβ (Carre et al., 1998). According to Bury et al. (2002), both TRα and TRβ are co-localized in OLGcs before CNPase is expressed. It is tempting to speculate that during the initial stages, demyelination activates TRα in the SVZ and makes undifferentiated proliferating cells responsive to T3. Under these circumstances, exposure to T3 could promote the induction of TRβ expression, which mediates T3 effects on remyelination through the activation of OLGc differentiation.

While demyelination strongly augments the number of Nestin and Id4 positive cells in the SVZ, a decrease in the number of these progenitor cells was observed in T3 treated animals, probably due to migration of precursor cells to demyelinated areas undergoing remyelination. Taken together, these findings indicate that on the one hand, T3 treatment reduces the number of proliferating progenitor cells in the SVZ, while on the other it induces OLGc differentiation in the CC.

Sonic hedgehog (Shh) is an early secreted protein required for OLGc specification during myelination (Alberta et al., 2001) and exogenous Shh delivery into the lateral ventricle of adult mice increases the number of proliferating cells expressing the OLGc marker DM20 in the CC (Loulier et al., 2006). An increase in positive Shh immunoreactive cells was detected in the ventricular wall after T3 treatment and similar results were found with the expression of Olig, a transcription factor of the bHLH family, which is directly associated with OLGc differentiation (Machold et al., 2003). These results are in agreement with previous findings showing that Shh acts on OLGc specification through the induction of Olig1 and Olig2 (Lu et al., 2000; Alberta et al., 2001; Rowitch, 2004; Briscoe and Therond, 2005). Our findings provide data supporting the involvement of Shh-Olig signalling during remyelination by the activation of Shh expression in the lateral ventricle wall upon T3 treatment. It should be mentioned however that recently, Fancy et al. (2004) were unable to detect Shh expression in areas of demyelination induced by ethidium bromide, suggesting that in this situation, Olig signalling is independent of Shh.

The disperse aspect of A2B5 and NG2 immunoreactive cells detected in the SVZ in CPZ-C animals is a probable effect of cell migration from the SVZ toward demyelinated areas. The slight increase in these cells observed in the SVZ of CPZ-T3 animals suggests an additional role for T3 in the control of OPC proliferation. It has been suggested that THs have mitogenic activity on early precursor cells through the activation of TRα (Ben-Hur et al., 1998). Our results agree with this point of view since precursor cells are the main adult SVZ proliferating cells. Within this context it should be mentioned that

in vivo experiments of Giardino et al., (2000) have shown that the administration of TH to control rats increases the expression of Ki67, a nuclear antigen associated with cell proliferation.

The mature OLGc markers IR found in CPZ-T3 animals did not reach the levels found in 42 days old CTL animals, suggesting the remyelination process in the former case was not yet complete. At this time point, CPZ-T3 rats displayed the first molecular evidences of remyelination, in contrast to CPZ-C animals that did not seem to have initiated this process. Our results also suggest that mature oligodendrocytes found in CPZ-T3 animals were not remanent cells resistant to the demyelination since CPZ-C animals showed little to no immunoreaction for these markers.

Thyroid hormones administration did not produce changes in the expression of the various markers analyzed in control animals (not shown), indicating that the demyelinating insult is needed to generate a pool of T3 responsive cells. A possible explanation for this could be the lack of TRα expression, which appears necessary to mediate thyroid hormone's effects in cells of the adult control SVZ.

THs appear to have multiple stimulating effects on the transcription of the major myelin protein coding genes, such as MBP, PLP, MAG and CNP (Tosic et al., 1992; Marta et al., 1998). In particular, the MBP gene promoter contains a TH responsive element (TRE) that confers an efficient transactivation by TRβ1 but a poor transactivation by TRα1, indicating that MBP is a direct target of TRβ1 (Jeannin et al., 1998). Despite this direct regulation at the transcriptional level, our data support the idea that T3 exerts an additional effect on the generation of newly formed oligodendrocytes arising from the SVZ undifferentiated progenitors. However, these T3 effects were only observed in the demyelinated brain. In fact, we did not observed the same T3 effects in control animals. Besides myelin damage, demyelination insult produces the SVZ undifferentiated cell pool expansion and the activation in the expression of TRα. Our findings strongly support the idea that SVZ cells could be the cell targets mediating T3 action on remyelination. Within this demyelinating context we hypothesize exogenous T3 administration is able to accelerate the remyelination process, by first activating TRβ expression in undifferentiated cells of the ventricular walls, and then re-activating shh/Olig pathway involved in oligodendroglial linage specification. As a result of more cells undergoing differentiation, we detect a consequent reduction in the pool of SVZ undifferentiated cells. All these changes triggering oligodendroglial differentiation finally generate progenitors with migrating properties that reach the CC to restore myelin sheaths.

Taken together our results indicate that thyroid hormones and their receptors act as a complex system that regulates emergence of remyelinating oligodendrocytes from the proliferative progenitor cell pool residing in the SVZ of adult rats, through a balanced control of cell proliferation and differentiation. Manipulation of this system could serve as an interesting tool to develop new therapeutical approaches for demyelinating diseases.

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