Loss of Testicular Orphan Receptor 4 Impairs Normal Myelination in Mouse Forebrain

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Testicular orphan nuclear receptor 4 (TR4) has been suggested to play important roles in the development and functioning of the central nervous system (CNS). We find reduced myelination in TR4 knockout (TR4\(^{-/-}\)) mice, which is particularly obvious in forebrains and in early developmental stages. Further analysis reveals that CC-1-positive (CC-1\(^+\)) oligodendrocytes are decreased in TR4\(^{-/-}\) forebrains. The O4\(^+\) signals are also reduced in TR4\(^{-/-}\) forebrains when examined at postnatal d7. However, the number and proliferation rate of platelet-derived growth factor receptor \(\alpha\)-positive (PDGF\(\alpha R^+\)) oligodendrocyte precursor cells (OPCs) remain unaffected in these regions, suggesting that loss of TR4 interrupts oligodendrocyte differentiation. This is further supported by the observation that CC-1\(^+\) oligodendrocytes derived from 5-bromo-2\(^\prime\)-deoxyuridine incorporating OPCs are significantly reduced in TR4\(^{-/-}\) forebrains. We also find higher Jagged1 expression levels in axon fiber-enriched regions in TR4\(^{-/-}\) forebrains, suggesting a more activated Notch signaling in these regions that correlates with previous reports showing that Notch activation inhibits oligodendrocyte differentiation. Together, our results suggest that TR4 is required for proper myelination in the CNS and is particularly important for oligodendrocyte differentiation and maturation in the forebrain regions. The altered Jagged1-Notch signaling in TR4\(^{-/-}\) forebrain underlies a potential mechanism that contributes to the reduced myelination in the forebrain. (Molecular Endocrinology 21: 908–920, 2007)

The formation of myelin in the vertebrate nervous system ensures rapid and efficient conduction of nerve impulses along myelinated axons, which is essential for the proper functioning of the nervous system. Oligodendrocytes are the myelinating glia in the central nervous system (CNS). They are generated from oligodendrocyte precursor cells (OPCs) through a number of sequential differentiation steps accompanied by coordinated changes in the expression of cell surface antigens (1). The expression of genes during development and differentiation are controlled by transcription factors. Several transcription factors have been described to be involved in myelination in the CNS, such as homeodomain proteins NKx2.2 (2), basic helix-loop-helix transcription factor olig2 (3–5), Hes5 and Mash1 (6), and Sox proteins (7). Nuclear receptors define a unique family of transcription factors whose activities are regulated by lipophilic ligands (8). Thyroid hormone, glucocorticoid hormone, and retinoid acid have been shown to regulate oligodendrocyte differentiation (9–11), and their actions are mediated through their cognate nuclear receptors. The mechanisms of regulation involve direct control of myelin-related gene expression (12–14) or function as part of the cell intrinsic timer as has been described for thyroid hormone receptor \(\beta\) (15). In a recent report, chicken ovalbumin upstream promoter transcription factor 1 (COUP-TFI), an orphan nuclear receptor has been shown to play a role in oligodendrocyte differentiation potentially through acting as an upstream regulator of SCIP/Oct-6/Tst-1 (16).

TR4 is an orphan nuclear receptor whose ligand is currently unidentified (17). TR4 forms homodimers or heterodimerizes with TR2, and binds to the direct repeat DNA sequence with the consensus half-site of AGGTCA in its target genes (18–21) and regulates the expression of its target genes. TR4 transcripts have been detected in multiple tissues and organs with particularly high levels in the CNS during embryonic development and in certain restricted areas of the adult brains (22–24), suggesting that TR4 might play an important role in the development and maturation...
of the CNS. TR4−/− mice display profound neural defects such as a jerking head, coordination problems, hypersensitivity to stimuli, and lack of maternal behavior (25, 26). Abnormal cerebellum development in TR4−/− mice has been characterized (25).

In this report, we assessed the myelin defects in TR4−/− mice and provided evidences showing that TR4 plays an important role in differentiation and maturation of oligodendrocytes, especially those in the forebrain regions. The potential mechanism might involve regulation of the Jagged1-Notch signaling pathway.

RESULTS

A Temporal and Spatial Pattern of Reduced Myelination in TR4−/− Mice

Mice at different ages were checked for their myelination status in different regions including forebrain (Fig. 1A), cerebellum (Fig. 1B), and spinal cord (Fig. 1C) by immunohistochemistry staining of the myelin marker myelin basic protein (MBP) in paired wild-type TR4 (TR4+/+) and TR4−/− mice tissue sections. In the forebrain (Fig. 1A), the MBP staining intensity was decreased in TR4−/− mice when examined at postnatal d 14 (P14), 4 wk (4w), and 6 wk (6w). At P14, MBP-immunoreactivity (ir) was apparent in nerve fiber-enriched regions (e.g. corpus callosum and striatum) in TR4+/+ forebrains, however the intensity and extent of MBP-ir were significantly reduced in TR4−/− forebrains. As the mice grow older, the differences tend to become less robust. At 6w, no significant differences were observed in corpus callosum region; however, reduced MBP intensity was still clear in cerebral cortex region in TR4−/− forebrains. Similar results were also obtained when myelination was examined by luxol fast blue staining method (not shown). No MBP staining signal was detected in the forebrains sections of P7 TR4+/+ and TR4−/− mice (Fig. 1A, a1 and a1'). In the

Fig. 1. Expression of MBP in Forebrains, Cerebella, and Spinal Cords of TR4+/+ and TR4−/− Mice

Similar sections from paired TR4+/+ and TR4−/− mice were compared at different ages as indicated for their myelination status by MBP staining (brown color). In forebrain (A), decreased MBP staining intensity was observed in P14, 4w, and 6w sections from TR4−/− mice. MBP staining signal was not observed in P7 sections from either TR4+/+ or TR4−/− mice. The sections were counterstained with hematoxylin to outline the brain structure (a1 and a1'). In cerebella (B), reduced MBP staining intensity was more obvious in P7 TR4−/− mice (b1 vs. b1'), and the differences between TR4+/+ and TR4−/− mice diminished gradually as the age of the mice increased. In spinal cords (C), no obvious differences were observed between TR4+/+ and TR4−/− mice in all the ages tested. All sections in panel A and paired sections in panels B and C used the same scale. Scale bars: a4', b1', b2', b3', b4', c3', and c4', 400 μm; c1' and c2', 200 μm.
cerebellum (Fig. 1B), the decreased MBP intensity in TR4−/− mice was more obvious in P7. However, the difference was minimized and was not readily observed in 4w and older mice. In spinal cord sections (Fig. 1C), no obvious differences between TR4+/+ and TR4−/− mice were observed at P7 and older ages. To address the finding quantitatively, Western blot was carried out to check the MBP levels in different regions at different ages. As shown in Fig. 2A, at P14, MBP levels were decreased in cerebral cortex and cerebellum of TR4−/− mice as compared with those of TR4+/+ mice, whereas differences in spinal cord were not obvious. At 3 wk (3w) postnatally, MBP levels in cerebral cortex region in TR4−/− mice were still lower than that in TR4+/+ mice. However, in cerebellum (Fig. 2B), no significant difference was detectable. These results suggest that TR4 is particularly important for proper myelination in forebrain during early postnatal development.

**Decreased Myelination in TR4−/− Forebrain Correlates with a Reduction in CC-1+ Oligodendrocytes**

Because we observed decreased myelination in TR4−/− mice, we asked whether the decrease was due to reduction in mature oligodendrocyte number or defect(s) in myelin production, whereas the normal number of oligodendrocytes is retained. A mature oligodendrocyte marker CC-1 (27) was used to identify oligodendrocytes. The forebrain regions of P14 mice were examined because the difference in myelination between TR4+/+ and TR4−/− mice was most obvious at this stage (Fig. 1A). CC-1+ signals were mostly located in fiber tract accumulating regions, such as corpus callosum, correlating the pattern of myelination in forebrains. We observed a significant reduction in CC-1+ oligodendrocytes in TR4−/− mice as compared with the TR4+/+ mice (Fig. 3). Figure 3A showed the sample differences in corpus callosum regions (a and b) and cingulum regions (c and d). Three red-boxed areas in a and b were enlarged and presented as a1–a3 and b1–b3. The CC-1+ cell bodies were visualized as brown color. High-magnification images of single cells were shown as inserts in c and d. We didn’t observe a difference in CC-1 staining intensity between TR4+/+ and TR4−/− mice. The numbers of CC-1+ cells were counted in sampling regions (including corpus callosum, cingulum, external capsule, caudate putamen, and anterior commissure) as illustrated in the cartoon drawing (Fig. 3B). The numbers were summed for each section and averaged from three consecutive sections for each animal. Seven pairs of TR4+/+ and TR4−/− mice were compared. The average numbers of CC-1+ cells for each genotype are shown as a bar graph in Fig. 3B. CC-1+ cells were significantly decreased in TR4−/− forebrain (P < 0.002). These results suggest that reduction in mature oligodendrocyte number contributes to the decreased myelination in TR4−/− mice, although defects in myelin production cannot be excluded.

**OPC Status in TR4−/− Forebrain**

Developmentally, oligodendrocytes originate from neuroepithelial stem cells through multiple steps of fate determination, lineage specification, and maturation (28). We further examined the status of OPCs to

**Fig. 2.** MBP Levels in Different Regions of the CNS of TR4+/+ and TR4−/− Mice

Protein extracts of different regions of the CNS from P14 (A), and 3w-old mice (B) were subjected to Western blot (WB) analysis for MBP expression. β-Actin was used as loading control. Intensities of MBP bands were quantified and normalized to loading control. Values are presented as relative intensity to TR4+/+ samples (set as 1) in the bar graph to the left of the figures. The results were summarized from three to four pairs of TR4+/+ and TR4−/− mouse samples for each region at each stage. Student’s t test was employed to examine the statistical significance. *, P < 0.05. Cb, Cerebellum; Cx, cerebral cortex; Sp, spinal cord.
see whether the generation of oligodendrocyte lineage cells is interrupted in TR4−/− forebrains. The forebrain sections were immunostained for the OPC marker PDGFRα as shown in Fig. 4A. No obvious difference was observed in PDGFRα staining intensity between TR4+/+ and TR4−/− in similar regions. The number of OPCs was counted from boxed sampling regions (Fig. 4B). Our results revealed that in P7 and P14 mouse forebrains the number of PDGFRα+ cells does not differ significantly between TR4+/+ and TR4−/− (Fig. 4B) and, as expected, less PDGFRα+ cells were present in P14 forebrains than in P7 forebrains (Fig. 4B). We also stained the forebrain sections with another OPC marker, NG2, and got similar results (data not shown). Thus, in early postnatal development, PDGFRα and NG2 both label early stages of OPCs, which are highly proliferative. As OPCs develop further, just before entering into the postmitotic stage, they start to express cell surface antigens that are recognized by O4 antibody (29, 30). We examined O4 immunoreactivity in P7 forebrain sections and found that the staining intensity was significantly reduced in TR4−/− forebrains (Fig. 4C), suggesting the progression of OPCs to more mature stages is interrupted.
Oligodendrocyte Differentiation Is Affected in TR4−/− Forebrains

To further explore the hypothesis that TR4 might play a role in the differentiation and maturation of oligodendrocytes, we injected TR4+/+ and TR4−/− mice with 5-bromo-2′-deoxyuridine (BrdU) at P7 and examined the BrdU-positive (BrdU+) oligodendrocytes at P14. BrdU is incorporated into the genomic DNA during the S phase of a cell cycle and is widely used as a marker for cell proliferation. Because oligodendrocytes are postmitotic, the BrdU+ oligodendrocytes represent the cells derived from proliferating OPCs at the time of BrdU injection. Our results from six pairs of TR4+/+ and TR4−/− mice (Fig. 5A) show that BrdU+ oligodendrocytes were significantly reduced in TR4−/− forebrains (P < 0.01). To exclude the possibility that the above observed reduction in BrdU+ oligodendrocytes is due to a decrease in proliferation rate of TR4−/− OPCs, we examined the BrdU incorporation rate of
OPCs in the forebrains of P7 TR4^{+/+} and TR4^{−/−} mice. Our result reveals that about 13% OPCs were actively incorporating BrdU at P7 and there is no significant difference between TR4^{+/+} and TR4^{−/−} mice (Fig. 5B).

We then examined the apoptosis status in the forebrain of P14 TR4^{+/+} and TR4^{−/−} mice using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay. We found that the apoptotic signals are mainly located in the periventricular regions in both types of mice and there is no significant difference in the number of apoptotic cells between TR4^{+/+} and TR4^{−/−} forebrains (P > 0.1) (Fig. 5C). Thus, the decreased oligodendrocyte number observed in TR4^{−/−} forebrains is unlikely to have resulted from an increase in oligodendrocyte death. The above findings support our hypothesis that oligodendrocyte differentiation is affected in TR4^{−/−} forebrain.

Neuronal Status in TR4^{+/+} and TR4^{−/−} Brains

The differentiation and maturation of oligodendrocytes are critically influenced by adjacent axons (31). To test the possibility that decreased myelination might be due to inadequate neuronal development, we examined the neuronal status in TR4^{+/+} and TR4^{−/−} forebrains using a neuron-specific antibody, βIII-tubulin. βIII-tubulin antibody stains the neuronal cell body, axons, and dendrites (32). In both P7 and P14 animals, no obvious differences were observed in staining pattern and intensity between TR4^{+/+} and TR4^{−/−} forebrains (Fig. 6). Whereas in P7 forebrains the staining intensity is relatively even across the whole section (Fig. 6A), in P14 forebrains the axon fiber tracts showed stronger immunoreactivity to βIII-tubulin than other regions (Fig. 6B). Particularly, there was no significant difference in staining intensities in the fiber tract regions between TR4^{+/+} and TR4^{−/−} mice (Fig. 6B), suggesting that the reduced myelination in TR4^{−/−} mice is not due to insufficient axon fiber formation, although perturbation of signaling between axons and oligodendrocyte lineage cells cannot be excluded. In addition, no obvious differences were observed in the cytoarchitectural arrangement of the cortical layers between TR4^{−/−} and TR4^{+/+} forebrains (Fig. 6C).

Glial Fibrillary Acidic Protein (GFAP)+ Astrocytes Are Increased in TR4^{−/−} Forebrains

We also investigated whether there were accompanying changes in astrocyte status in hypomyelinated TR4^{−/−} forebrains because reactive gliosis has been observed in various demyelinating pathologies such as multiple sclerosis and in jimpy mice (33–35). Immunoreactivity to GFAP, an astrocyte-specific intermediate filament protein, was examined. GFAP-ir was significantly increased in P14 TR4^{−/−} forebrains (Fig. 7A, b vs. a), particularly, in cerebral cortex regions (Fig. 7A, d vs. c). As shown, whereas little GFAP+ signals were detected in TR4^{+/+} cortex (Fig. 7A-c), GFAP+ signals were readily detected in similar regions in TR4^{−/−} cortex (Fig. 7A-d). When comparing GFAP+ individual cells at similar regions, the immunoreactive intensity to GFAP was similar between TR4^{+/+} and TR4^{−/−} mice (Fig. 7A, e vs. f). To further verify the results seen in tissue sections, GFAP expression levels were checked in different regions by Western blotting. At P7, no significant differences in GFAP levels were observed in any of the regions tested between TR4^{+/+} and TR4^{−/−} mice (Fig. 7B). Increased GFAP levels were observed in cerebral cortex and cerebellum lysates of TR4^{−/−} mice at the ages of P14 and 3w, whereas no differences were detected in spinal cord lysates (Fig. 7, C and D). The most prominent difference was observed in lysates from cerebral cortices.

Notch Signaling Is Altered in TR4^{−/−} Forebrain

OPCs express Notch1 receptor and neuronal axons express the Notch1 ligand Jagged1 (36). Contact-me-
mediated activation of Notch1 receptor by Jagged1 has been suggested to inhibit oligodendrocyte differentiation (36). Our finding that the differentiation of oligodendrocytes in TR4−/− forebrains is impaired led us to examine whether Notch signaling is altered in these regions. We compared the expression of Jagged1 in...
P14 TR4\(^{+/+}\) and TR4\(^{-/-}\) forebrain sections by immunostaining and found that the intensity of Jagged1 signal in axon fibers is obviously higher in TR4\(^{-/-}\) than in TR4\(^{+/+}\) sections as exemplified in Fig. 8A. At P14, decreased Jagged1 signal (brown) was observed in the axon fiber-enriched regions in TR4\(^{-/-}\) forebrain, for example, the external capsule and the fiber tracts in striatum (pointed out by black arrows) (Fig. 8A-a). Although Jagged1-ir also tends to decrease in axon fiber-enriched regions in TR4\(^{-/-}\) forebrains (Fig. 8A-b), the decrease is much less obvious than that in TR4\(^{+/+}\) forebrains. With high-power view of the staining in external capsule regions (Fig. 8A-c and A-d), the fibrous staining of Jagged1 is seen in both TR4\(^{+/+}\) and TR4\(^{-/-}\) sections, but the signals are much more intense in TR4\(^{-/-}\) sections. Some glial cells along the axon fibers are also positive for Jagged1, which is consistent with previous report (36). For comparison, spinal cord sections were also stained for Jagged1, but no obvious difference was observed between TR4\(^{+/+}\) (Fig. 8A-f) and TR4\(^{-/-}\) mice (Fig. 8A-g). The control staining, incubated with Jagged1 antibody in the absence of a Jagged1-specific blocking peptide, did not have positive staining (Fig. 8A-e), suggesting that the Jagged1 signals detected are specific. These findings indicate that Notch signaling in the OPCs in these regions might be more active in TR4\(^{-/-}\) mice.

We further examined the expression of Hes1 and Hes5 genes, which are Notch1 downstream targets (37), by real-time PCR. Our results (Fig. 8B) showed that Hes5 transcripts were 4- to 13-fold (9-fold on average) more abundant in TR4\(^{-/-}\) brains than in TR4\(^{+/+}\) brains, supporting the hypothesis that Notch signaling is more active in TR4\(^{-/-}\) brains. The Hes1 levels were not significantly different between TR4\(^{-/-}\) and TR4\(^{+/+}\) brains. The sample results from RT-PCR are shown on the right, and they are consistent with the real-time PCR results.

![Fig. 8. Expression of Jagged1 and Hes Genes Are Altered in P14 TR4\(^{-/-}\) Forebrains](image-url)
DISCUSSION

The Spatial and Temporal Pattern of Hypomyelination in TR4−/− Mice

We found hypomyelination is most prominent in the forebrains and in early developmental stages. A large variation in the timing of gliogenesis is one of the striking features in CNS development. It is known that myelination progresses in a caudal-rostral direction, beginning significantly earlier in the spinal cord than in the brain (38, 39). Even within the brain, a wide range of timing for myelination is observed in different regions (38, 39). Studies have shown that OPCs isolated from different regions of the brain have marked differences in self-renewal and differentiation characteristics (40), suggesting the existence of progenitor cell populations with different properties and contributions to the diverse time course of myelination in the CNS. How could TR4 contribute to the regional differences in myelination? We checked the expression of TR4 in brain and spinal cord of P7 and P14 TR4−/− mice by RT-PCR and Western blot, but no significant difference was found between the two regions (data not shown), suggesting that differential expression of TR4 in these regions might not be a major contributor to the regional differences. Regional factors directly or indirectly regulated by TR4 might help to explain this phenomenon and further exploration of this issue is needed.

We also observed significant catching up in myelination in older TR4−/− mice. In cerebellum, the decreased myelination is rather transient in TR4−/− mice. By the end of wk 3, the MBP level is comparable to that in TR4+/− mice. The transiently delayed myelination correlates with the abnormal development of Purkinje cells and lamination irregularities in TR4−/− cerebellar cortex in the early postnatal stages (25), and might be subsequent to the perturbed neuronal development in TR4−/− cerebellum. However, in TR4−/− forebrain, the axon fiber arrangement and the zonal organization of cortical gray matter appears normal postnatally, and the catching-up in myelination proceeds gradually and the reduced myelination is still detectable at wk 6. A different scenario might apply to the forebrains. We postulate that TR2, an orphan nuclear receptor closely related to TR4 (41), might functionally compensate the loss of TR4 and alleviate the defects in TR4−/− mice at later developmental stages. There are several pieces of evidence: the tissue expression pattern of TR2 mostly overlaps with TR4 (18); TR2−/− mice do not have serious developmental defects (42); however, TR2 and TR4 double-knockout leads to embryonic lethality (unpublished lab observation); and TR2 and TR4 can induce transcriptional activity of the same gene through the same hormone response element (18). Interestingly, significant reduced myelination was observed in the corpus callosum of adult TR4−/− brain (Chen, Y.-T., L. L. Collins, H. Uno, S. M. Chou, C. K. Meshul, S.-S. Chang, and C. Chang, submitted for publication), which is quite different from that of 6w forebrains. It is currently unclear whether this might indicate that TR4 plays different roles in the myelination process during early development and in adult stage or that the potential functional compensation by TR2 is only transient.

TR4 Regulates Oligodendrocyte Differentiation

We found that hypomyelination in TR4−/− forebrains was associated with a decrease in mature oligodendrocyte number. Several possibilities might contribute to this abnormality. One consideration is that oligodendrocyte lineage entry might be affected by losing TR4 function. However, the normal number of PDGFαR+ OPCs in TR4−/− forebrains (Fig. 4B) nullifies this hypothesis. Another possibility is that oligodendrocyte differentiation is interrupted in the TR4−/− forebrain. This hypothesis is supported by several findings. 1) The number of BrdU+ mature oligodendrocytes (CC-1+), which are derived from BrdU incorporating OPCs, is reduced in TR4−/− mice (Fig. 5A). This is not due to decreased BrdU incorporation by OPCs (Fig. 5B). It is also unlikely due to increased oligodendrocyte apoptosis at the time rapid myelination is occurring (Fig. 5C). 2) O4+ signals were decreased in P7 TR4−/− forebrains. O4 recognizes surface antigens that are present in preoligodendrocytes and immature and mature oligodendrocytes (29, 30, 43). This indicates that the transition from highly proliferative and PDGF-responsive OPCs to less proliferative and postmitotic cells is interrupted. All of these evidences indicate that TR4 regulates oligodendrocyte differentiation. It is not clear whether the interruption starts at the preoligodendrocyte stage or later in TR4−/− forebrains. Because little MBP+ signal was detected at P7 forebrain, O4+ signals in this region might represent cells comprising preoligodendrocytes and immature oligodendrocytes. Thus, the interruption in differentiation in TR4−/− forebrain is likely to happen before mature oligodendrocytes are formed. How TR4 contributes to this process is currently unclear. It may act intrinsically on OPCs and regulate the expression of genes that are important for the determination of the intrinsic timing process of OPCs. It might also act on neurons or other types of glial cells to produce signals that might modulate OPC differentiation. We observed higher Jagged1 expression in axon fiber-enriched regions in the developing TR4−/− forebrains (Fig. 8A) correlating with decreased myelination in these regions (Fig. 1A). It has been shown that Jagged1 expressed on axon surface activates Notch signaling in OPCs contacting the axons and inhibits differentiation of the OPCs (36). The higher expression of Jagged1 on axons of TR4−/− forebrains indicates Notch signaling is more active in OPCs contacting these axons. This provides a plausible explanation to the abnormal differentiation and maturation of oligodendrocytes and hypomyelination in these mice. It is possible that other
mechanisms, which are currently unclear, might also contribute to the hypomyelination in TR4−/− mice.

GFAP Overexpression in TR4−/− Forebrain

Reactive astrogliosis is a characteristic phenomenon observed in response to injury and inflammation of the CNS, as well as in various types of dysmyelinating diseases (33, 34), characterized by hypertrophy of astrocytes and increased expression of GFAP. We found that overexpression of GFAP was particularly obvious in the cerebral cortex. It has been reported that a pool of astrocytes exist that express little or undetectable levels of GFAP protein and these astrocytes are particularly enriched in gray matters such as the cerebral cortex (44, 45). The observed overexpression of GFAP in TR4−/− forebrain can be an indication for either overproliferation of astrocytes or activation of GFAP expression in astrocytes that originally express little GFAP. How does TR4 influence GFAP expression? Direct regulation of the GFAP promoter by TR4 is unlikely to be a universal mechanism because overexpression of GFAP presents only in forebrain gray matter (e.g. cerebral cortex), but not white matter (e.g. corpus callosum). Furthermore, no difference in GFAP expression was observed in the spinal cord, where myelination proceeds normally in TR4−/− mice. Differences in GFAP expression are present only in regions where hypomyelination was detected (Fig. 7, C and D). Thus, TR4-regulated signaling might involve a complex network that is important for coordinated development of oligodendrocytes and astrocytes.

Notch1 influences the differentiation of both oligodendrocytes and astrocytes, but in opposite directions. Activated Notch1 signaling inhibits oligodendrocyte maturation (36), whereas it promotes astrocyte generation (46). Studies have shown that Notch signaling can enhance GFAP expression by direct CBF1/Su(H)/LAG1 (CSL)-mediated transactivation on GFAP promoter (47). Whereas Hes5 mediates inhibition of oligodendrocyte differentiation by Notch activation (6, 36), Hes1 is more likely to mediate the proliferation effect on astrocytes (46). Although our results (Fig. 8) suggest Notch signaling is more activated in TR4−/− brains, we did not observe a significant change in Hes1 expression level in TR4−/− brains (Fig. 8B). This indicates that Hes1 is not a major effector if Notch signaling is involved.

TR4 and Notch Signaling

As discussed above, TR4 might regulate glial cell development through modulating the Notch signaling pathway. Notch receptors play crucial roles in various cellular differentiation programs and have been extensively studied and reviewed (48–50). However, how their upstream ligands are regulated remains unclear. Specifically, no studies have been reported relating to what signals instruct Jagged1 to be down-regulated on the axons to permit myelination. In the TR4−/− forebrains, the timely down-regulation of Jagged1 on axons is disrupted, suggesting that TR4 is involved in such regulation. How this regulation happens is still a mystery, and it may involve either direct transcriptional regulation or indirect mechanisms with multiple intermediates. In addition, although the increased Hes5 level in TR4−/− brains may result from activation of Notch1 by Jagged1, regulation of Hes5 by TR4 through non-Notch-related pathway is also possible. The cross talk between TR4 and Notch signaling is an interesting area to be further investigated.

In summary, by analyzing the myelination defects in TR4−/− mice, we provide evidences showing that TR4 plays an important role in the differentiation and maturation of oligodendrocytes, particularly in the forebrain. The altered Notch signaling underlies a potential mechanism that contributes to the reduced myelination in TR4−/− forebrain. Future studies to understand the detailed mechanisms of how TR4 regulates myelination may provide insight into the demyelinating disorders such as multiple sclerosis, and therefore, will be of substantial interest.

MATERIALS AND METHODS

Experimental Animals and Tissue Preparation

All animal experimentation described in this manuscript was conducted in accord with accepted standards of humane animal care, as outlined in Ethical Guidelines. TR4+/− mice were generated from heterozygous to heterozygous breeding of sexually mature male and female mice. The TR4 heterozygous mice were originally obtained from Lexicon Genetics Inc. (26). The day of birth is postnatal d 0 (P0). Brains and spinal cords from three to seven paired (littermates) TR4+/− and TR4+/+ mice were compared for each stage analyzed. To prepare the tissue samples, animals were deeply anesthetized and perfused through the left ventricle with 4% paraformaldehyde. After dissection, brains and spinal cords were postfixed in the same fixative. The tissues were either paraffin-embedded or cryoprotected with 20–30% sucrose and then embedded in optimal cutting temperature embedding medium and stored in −80 C. When protein or RNA extraction was required, animals were deeply anesthetized, brains and spinal cords were quickly dissected and stored in −80 C until the experiments were performed.

BrdU Injection

For assaying the proliferation rate of OPCs, paired P7 mice were injected peritoneally with BrdU (100 μg/g body weight) twice at 1-h intervals and killed 1 h after the second injection. For assaying the maturation rate of OPCs, paired P7 mice were injected with BrdU (100 μg/g body weight) and killed 7 d later. Mature oligodendrocytes detected at P14 as BrdU positive (BrdU+) were considered to be derived from proliferating OPCs that incorporated BrdU.

Immunohistological Analyses

For paraffin-embedded tissues, sections were cut at 4 μm. Sections were dewaxed and rehydrated through a xylene and ethanol series. The quenching of endogenous peroxidase activity was carried out by immersing the sections in 0.3% H2O2 at 80 C. When protein or RNA extraction was required, animals were deeply anesthetized, brains and spinal cords were quickly dissected and stored in −80 C until the experiments were performed.

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Immunohistological Analyses

For paraffin-embedded tissues, sections were cut at 4 μm. Sections were dewaxed and rehydrated through a xylene and ethanol series. The quenching of endogenous peroxidase activity was carried out by immersing the sections in 0.3% H2O2 at 80 C. When protein or RNA extraction was required, animals were deeply anesthetized, brains and spinal cords were quickly dissected and stored in −80 C until the experiments were performed.
H$_2$O$_2$ in 1× PBS for 30 min or 1% H$_2$O$_2$ for 10 min. For immunostaining with antibodies that require antigen retrieval, sections were heated in 10 mM sodium citrate at 98°C for 15–20 min. Then, sections were blocked in 10% normal serum for 30 min, followed by incubation of proper dilution of the primary antibody for 1–2 h at room temperature or overnight at 4°C. The immunocomplex was either detected by incubating the sections with biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA), followed by streptavidin-horseradish peroxidase incubation (ABC kit; Vector Laboratories) and color developing with diaminobenzidine tetrahydrochloride (DAB) substrate (Vector Laboratories), or detected with fluorescein- or Texas Red-conjugated secondary antibodies (1:200, Vector Laboratories; 1:100, MP Biomedicals, Solon, OH). The primary antibodies used were: anti-MBP antibody 1:100-200 (Lab Vision Corp., Fremont, CA), anti-GFAP antibody 1:200 (Lab Vision Corp.), anti-adenomatous polyposis coli antibody (CC-1) 1:100 (EMD Biosciences, San Diego, CA), anti-O4 antibody 1:200 (R&D Systems, Minneapolis, MN), anti-jellil-tubulin antibody 1:2000–3000 (Covance Research Products, Denver, PA), and anti-Jagged1 antibody 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA). In the case of adenomatous polyposis coli antibody staining, MOM kit (Vector Laboratories) was used following the manufacturer’s instruction. For staining with anti-PDGF-R antibody (1:300; BD Biosciences Pharmingen, San Jose, CA) or the number (10 μm) were treated with 20 μg/ml proteinase K (in 10 mM Tris, pH 8) for 2 min followed by immersing into cold methanol (−20°C) for 20 min before applying the primary antibody. For double staining (BrdU and cell-specific markers), sections were stained with anti-BrdU antibody (BrdU staining kit; Zymed Laboratories, South San Francisco, CA) and first color developed using DAB-Ni (Vector Laboratories) as substrate. After a biotin-blocking step (avidin/biotin blocking kit; Vector Laboratories), the sections were incubated with the secondary primary antibody (PDGF-R or CC-1) and color developed using DAB as substrate.

**Western Blot Analysis**

Proteins from the spinal cord and each brain region were extracted as follows. Tissues were homogenized and lysed in RIPA buffer [1× PBS, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate, 1 μM phenylmethylsulfonyl fluoride, 1× protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN)]. Supernatants were collected by centrifuge at 12,000 rpm for 20 min at 4°C and aliquots were stored at −80°C until use. Proteins were separated on 8–17% SDS-PAGE according to the size of the proteins to be assayed and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in TBST buffer [150 mM NaCl, 10 mM Tris (pH 8.0), and 0.5% Tween 20] at room temperature for 1 h. Then the membranes were immunoblotted with primary antibodies for 2 h at room temperature or overnight at 4°C, followed by incubation with secondary antibodies for 1 h at room temperature. Blots were developed with the alkaline phosphastase color developing reagents (Bio-Rad Laboratories, Hercules, CA) or the ECL-Plus reagent (Amersham Biosciences, Piscataway, NJ). The dilutions of the first antibodies are as follows: anti-MBP antibody 1:10,000, anti-GFAP antibody 1:2000, anti-glyceraldehyde-3-phosphate dehydrogenase antibody 1:2000 (Abcam Inc., Cambridge, MA), and anti-actin antibody 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA). Western blot results were quantified using Image J software. The relative intensity of MBP and GFAP bands was calculated by normalizing with the loading control protein. Results from three to four pairs of TR4+/− and TR4−/− samples were summarized and the differences were analyzed for statistical significance using Student’s t test.

**Detection of Apoptotic Cell Death**

Apoptotic cells were detected by using TUNEL assay (fluorescein-FragEL DNA fragmentation detection kit; EMD Biosciences, San Diego, CA), following the manufacturer’s protocol. Briefly, paraffin-embedded tissue sections were treated with 20 μg/ml proteinase K (in 10 mM Tris, pH 8) for 10 min and washed. After 20 min incubation with the equilibrium solution (provided by the kit), the sections were incubated with the labeling solution for 1 h at 37°C. Then the sections were washed in 1× PBS and mounted with 4′,6-diamidino-2-phenylindole (DAPI)-containing mounting media. The TUNEL-positive (TUNEL+) cells were identified under a fluorescence microscope as green signals in the nuclei (blue DAPI staining).

**RT-PCR and Real-Time PCR**

Total RNA was extracted from the cerebral cortices of P14 TR4−/− and TR4+/− mice using the Trizol (Invitrogen, Carlsbad, CA) reagent, according to the manufacturer’s instructions, and 5 μg of RNA were subjected to reverse transcription using Superscript III (Invitrogen). The first strand cDNA was diluted 10-fold and stored in −80°C in aliquots until use. The sequences for specific primers were as follows: Hes1, 5’-GCCATTTTGGCTTCCTTCGC-3’ (forward) and 5’-CGCCAGGGTCTCCACATG-3’ (reverse); Hes5, 5’-CGCATTACAAGCACAGGATAC-3’ (forward) and 5’-GCAG GCACAGGAGTACG-3’ (reverse); 18S rRNA primers, 5’-TGCCCTCTTGGATGTG TGAT-3’ (forward) and 5’-CTGCTG GCCATGCAATTCCTGG-3’ (reverse). 18S rRNA served as internal control. We used 5 μl cDNA for Hes1 gene, 10 μl cDNA for Hes5 gene, and 2 μl cDNA for 18S rRNA. The PCR was performed in the following conditions: 1) 2 min at 94°C and 2) 35 cycles, with 1 cycle consisting of 30 sec at 94°C, 30 sec at 63°C, and 30 sec at 72°C. Real-time PCR was performed with the above reverse transcription products on an iCycler iQ multicolor real-time PCR detection system (Bio-Rad Laboratories) as follows: 1) 3 min at 94°C and 2) 40 cycles, with 1 cycle consisting of 15 sec at 94°C, 30 sec at 63°C, and 30 sec at 72°C. Each sample was run in triplicate. Quantitative analysis was performed using iCycler iQ software (Bio-Rad Laboratories). The quantification of each sample relative to the control was calculated using the 2ΔΔCT method (51). Paired TR4+/− and TR4−/− samples (from litters) were compared. The final results were summarized from three pairs (for Hes1) and four pairs (for Hes5) of samples.

**Cell Quantification and Statistical Analysis**

Brain samples were collected in pairs (from TR4−/− and TR4−/− littermates) to control for environmental and polycenic effects. Each set of paired samples were processed and analyzed simultaneously to minimize the systematic experimental errors. Three to seven pairs of samples were collected for experiments. Similar forebrain sections (by matching the morphological characteristics, e.g. the shape of lateral ventricles and the position and shape of anterior comissure) were compared. Immunostained cells were counted in selected sampling regions from each section and the numbers were summed for each section. A mean cell count was obtained for each tissue sample by averaging the total numbers from three consecutive sections. Student’s t test was used to test the differences of the number of counted cells between TR4+/− and TR4−/− mice. A two-sided P value of 0.05 or less was considered statistically different.

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