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Running Title: COUP-TFI in Axon Myelination

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Abstract

We report here that a member of the nuclear hormone receptor superfamily, chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TFI), plays a critical role in glial cell development and subsequent central nervous system myelination. We demonstrate that COUP-TF1 is expressed in cells of oligodendrocyte lineage. Furthermore, we demonstrate that COUP-TF1 null mutant mice exhibit delayed axon myelination and increased dysmyelination in the central nervous system. Using *in vitro* differentiation assays, we show that these myelination defects are due to delays in oligodendrocyte differentiation. Finally, *in situ* hybridization and transfection analysis suggests that COUP-TF1 acts as an upstream regulator of SCIP/Oct-6/Tst-1, a transcription factor involved in axon myelination. Taken together, these results suggest that COUP-TF1 is an important regulator of oligodendrocyte differentiation

Introduction

Both cell-intrinsic programs and extracellular signals play important roles in determining the differentiation of oligodendrocyte progenitor cells (OPCs) in optic nerves (Barres et al., 1994; Temple and Raff, 1986). In rat optic nerves, the timing of oligodendrocyte differentiation and myelination is controlled by signals including PDGF, thyroid hormone and Notch pathway (Wang et al., 1998). In addition, a number of transcription factors are involved in axon myelination (Wegner, 2000). In the CNS, SCIP/Oct-6/Tst-1, a POU-domain transcription factor, is expressed at high levels in the cerebral cortex, in oligodendrocyte progenitor cells of the developing CNS and the astrocytes (Collarini et al., 1992; Collarini et al., 1991). In the PNS, SCIP is highly expressed in Schwann cells and its expression gradually decreases with time (Monuki et al., 1990). SCIP null mice show a delayed Schwann cell differentiation (Bermingham et al., 1996; Jaegle et al., 1996). SCIP mutants do not show detectable defect in the myelination of spinal cord axons but transgenic mice overexpressing SCIP in oligodendrocytes developed severe neurological symptoms and defects in axon myelination (Jensen et al., 1998), suggesting that SCIP is also involved in axon myelination in the CNS.

Chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) are members of the nuclear receptor superfamily (Tsai and Tsai, 1997). COUP-TFs have two homologues, COUP-TFI and COUP-TFII in the mouse (Jonk et al., 1994; Qiu et al., 1994) and they share a very high degree of amino acid sequence conservation. Expression of COUP-TFs starts around embryonic day 7.5 (E7.5), increases dramatically by E9.5, peaks around E12-13, and declines before birth. In addition, COUP-TFs are differentially expressed during organogenesis suggesting that they have different physiological functions (Qiu et al., 1994). The importance of COUP-TFI in the developmental process has been demonstrated by the use of knockout models. The majority of COUP-TFI mutants die perinatally, while 1-2% of COUP-TFI mutants survive until 3-4 weeks of age. COUP-TFI null mutants display abnormal morphogenesis of ninth ganglionic neurons due to excessive cell death in neural crest ganglionic precursor cells, defects in axonal guidance (Qiu et al., 1997). In the CNS of COUP-TFI mutants, cortical layer IV is absent, a consequence of the disruption of thalamocortical projections (Zhou et al., 1999). More recently, COUP-TFI has been shown to be one of the intrinsic factors controlling early neocortical regionalization (Zhou et al.,

2001).

In this paper, we examined the role of the COUP-TFI in oligodendrocyte development. We demonstrated that COUP-TFI mutants display defects in myelination and increased numbers of dysmyelinated axons in the developing optic nerves. Delayed axon myelination is due to delayed differentiation of oligodendrocytes resulting from down regulation of the expression of SCIP/Oct-6/Tst-1in COUP-TFI mutants. These results indicate that COUP-TFI is an important regulator of oligodendrocyte differentiation, which is crucial for axon myelination.

Experimental Procedures

Mice

COUP-TFI mouse colonies were maintained in a pure 129Sv background. Since COUP-TFI heterozygotes are phenotypically normal, the control mice in the experiments include both wild type and heterozygotes. Generally, three pairs of mice were used in each experiment except those involving electron microscopic analysis of the optic nerves.

Histochemistry

Brains, optic nerves and sciatic nerves were dissected out, cleaned in phosphate buffer saline (PBS), and fixed in 4% (w/v) paraformaldehyde (PFA) in PBS overnight at 4°C. Tissues were paraffin embedded and sectioned at 7 μm thickness in the coronal plane for forebrain, and the longitudinal plane for optic and sciatic nerves. For the immunostaining of myelin basic protein (MBP), primary antibodies were used at the following dilutions: polyclonal anti-MBP antibody (Zymed, Southern San Francisco, CA)**.** Sections were incubated in primary antibodies overnight at 4°C. After washing with PBS, sections were incubated with Cy3-conjugated goat anti-rabbit antibodies (1:400, Jackson ImmunoResearch laboratories, West Grove, PA) for 1 hr at room temperature. After washing, slides were mounted using Vectashield mounting medium (Vector laboratories, Burlingame, CA).

In situ hybridization

In situ hybridization was performed as previously described by Wilkinson et al (Wilkinson et al.,

1987). For detecting cell types expressing COUP-TFI, fluorescein-labeled probes were used. Riboprobe hybrids were visualized in situ by immunohistochemistry with alkaline phosphatase-conjugated fluorescein antibody (Boehringer Mannheim) according to the manufacturer's instructions.

Electron microscopy

Optic nerves with eye-balls were dissected out from 3 mice of each genotypes (-/, +/-and +/+) at P8, P17, and P25 and 2 mice at P10, P16, P18, P22 and P27 and fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylated buffer (pH7.2) at 4°C. The tissues were washed in 0.1M cacodylate buffer (pH7.2), postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr at room temperature, washed with H_2O, dehydrated in graded alcohol and propylene oxide, and embedded in Epon. Sections of the mid-portions of optic nerves were cut perpendicular to the axis of the optic nerves with a diamond knife. Ultrathin sections were mounted on mesh grids and stained with uranyl acetate and lead acetate. Eight to ten electron micrographs of random field were taken at 5,000X magnification for the optic nerves, and at 1,500X for the sciatic nerves. Axons in electron micrographs were identified, numbered and categorized as either unmyelinated axons (including wrapped and no compact myelin encircling the axons) or myelinated axons. When microfilament and microtubule profile indicated that axons were not cut perpendicular, the axons were excluded from analysis.

Optic nerve cultures

The optic nerves at postnatal day 0 were removed, cut into fragments, and treated with trypsin (0.05%, Roche, Indianapolis, IN) in Earle's Balanced Salt Solution (Gibco, Rockville, ML). Cells were dissociated by passing the nerve fragments through a 21, 23 and 26 gauge needle in Dulbecco's modified Eagle's medium containing 30%(v/v) fetal bovine serum and DNaseI (0.04%). The cells were plated onto poly-D-lysine-coated glass coverslips (3,000-5,000 cells per coverslip) and grown in 5% CO2 at 37°C in modified Bottenstein-Sato (B-S) medium (Lillien and Raff, 1990) for 2 days. Forskolin and PDGF-AA (Peprotech, Rocky Hill, N.J.) were added to final concentration of 15μg/ml and 10ng/ml, respectively. After 2 days, medium was changed to B-S medium without PDGF-AA to let cells differentiate for 48 or 72 hr. Half of the medium and additives were subsequently changed every 2 days. Three independent in vitro differentiation

assay were carried out for both wild type and COUP-TFI mutant and analyzed by student t-test.

Immunofluorescence staining

For immunostaining, cells were fixed in 4% PFA in 0.1 M PBS (pH 7.2) for 5 min at room temperature. After washing, the cells were incubated in 50% normal goat serum solution to block non-specific staining. They were then incubated in rabbit anti-NG2 serum (gift of Dr. Bill Stallcup, diluted 1:100), followed by biotinylated goat anti-rabbit IgG antiserum (Jackson ImmunoResearch laboratories, West Grove, PA) and FITC-coupled streptavidin (Jackson ImmunoResearch laboratories, diluted 1:200). The cells were then incubated in anti-O4 antibodies (Chemicon, Temecula, CA, diluted 1:3), followed by Cy3-coupled goat anti-mouse IgM antibody (Jackson ImmunoResearch laboratories, diluted 1:400). All incubations were done for 30 min at room temperature, and the dilutions were done in Tris-buffered saline (pH 7.2) containing 1% bovine serum albumin and 10mM L-lysine.

Transfections and luciferase assays

The mouse SCIP/Oct-6/Tst-1 reporter plasmid, p2Kluc, having approximately 2 kb (-2 K to +49) of the 5' flanking region cloned into the luciferase plasmid pGL2 basic (Promega), was kindly provided by Dr. John R. Bermingham, Jr. (Renner et al., 1996). The plasmid pCXN2-mCI was used for the expression of mouse COUP-TFI cDNA in transfection assays. SK-N-SH neuroblastoma and P19 embryonic carcinoma cells were grown in Dulbecco's modified medium (DMEM) supplemented with 10% fetal bovine serum (FBS). One day prior to transfection, SK-N-SH and P19 cells were plated at $1X10^5$ and $5x10^4$ per well in 6-well plate, respectively. Cells were transfected by the Lipofactamine Plus reagent (In Vitrogen) according to manufacturer's instructions with 0.3 μg of luciferase reporter plasmids and 0.2 μg plasmid for the mCOUP-TFI cDNA. The total amount of plasmids was maintained constant. Forty hours later, cells were harvested, and extracts were assayed for luciferase activity.

Results

COUP-TFI expression in developing optic nerves

Previously, we have shown that COUP-TFI is important for neuronal cell survival and differentiation (Qiu et al., 1997; Zhou et al., 1999). Neurons and glia in the CNS form a functional unit and neuronal-glial interactions are important for neuronal migration and survival, neurite outgrowth and axonal guidance during neuronal development (Banker, 1980; Gasser and Hatten, 1990; Hatten et al., 1988; Lindsay, 1979). To assess if neuronal cell survival and differentiation defects observed in the COUP-TFI null mutants are contributed by glial cells, we examined the pattern of COUP-TFI expression in the developing optic nerves by *in situ* hybridization. Optic nerves do not contain any neuronal cell bodies but the nerve processes of retinal ganglion neurons and glial cells, oligodendrocytes and astrocytes. From E17 to P21 the optic nerves contain increasing amount of oligodendrocyte precursor cells migrating from the brain (Raff et al., 1983). We found that COUP-TFI was highly expressed in optic nerves and its expression levels seemed to be higher at P5 (Figure 1A) than at P15 (Figure 1B). No signals were found in sections incubated with sense riboprobes (data not shown).

To further identify the cells expressing COUP-TFI in the optic nerves, we performed *in situ* hybridization in mixed glial cell cultures derived from dissociation of optic nerves at P5 using a fluorescein-conjugated COUP-TFI antisense riboprobe (Figures 1E-H). Oligodendrocyte lineage cells, precursors and oligodendrocytes, express stage-specific surface antigens and have characteristic morphology (Baumann and Pham-Dinh, 2001). In dissociated mixed glial cell cultures it is therefore possible to identify which cell type(s) express COUP-TFI. We used Glial fibrillary acidic protein (GFAP) as a marker for astrocytes, and NG2 as a marker for oligodendrocyte progenitor cells (OPCs), O4 for pro-oligodendrocytes, GalC (GC) for immature oligodendoryctes, and myelin basic protein (MBP) and myelin associated glycoprotein (MAG) for mature oligodendrocytes (Baumann and Pham-Dinh, 2001). Our studies demonstrated that COUP-TFI was expressed in both bipolar oligodendrocyte progenitor cells as confirmed by the expression of NG2 but not GC and GFAP surface markers ($NG2^+GC^-GFAP^-$), and multi-process bearing oligodendrocytes which are $NG2^-GC^+$ (data not shown). COUP-TFI was also expressed in type-2 astrocytes that display characteristic morphology and are $GFAP^+NG2^+GC^-$ (data not shown).

Hypomyelination and dysmyelination in the CNS of COUP-TFI mutants

To investigate whether COUP-TFI is important for oligodendrocyte development, we analyzed axon myelination in COUP-TFI mutants using Luxol Fast Blue staining which identifies myelinated fibers. Luxol Fast Blue staining was greatly reduced in the white matter of COUP-TFI mutants as compared to controls (Figures 2A-F). To further confirm that axon myelination is reduced in COUP-TFI mutants, immunostaining with myelin basic protein (MBP), one of the major myelin proteins of the CNS, was examined. MBP- positive fibers were significantly decreased in the white matter and striatum of COUP-TFI mutants at P21 (Figures 2G-J). However, the numbers of axon bundles in the white matter and striatum of COUP-TFI mutants and control mice were similar, implying that hypomyelination of the CNS is not a consequence of a reduction in the number of axon bundles. Taken together, these results indicate that the lack of COUP-TFI function results in hypomyelination in the CNS.

To analyze the quantitative and qualitative defects in axon myelination in COUP-TFI mutants, we examined the optic nerves of COUP-TFI mutants by electron microscopy at different postnatal ages. Myelin is first detected around P6, 1-2 days after oligodendrocytes begin to undergo their final cell division at P5 (Skoff et al., 1976). The myelination then progresses in a retinal-to-chiasmal pattern (Colello et al., 1995; Skoff et al., 1980). We examined three parts of the optic nerves, retinal, middle, and chiasmal thirds, in COUP-TFI mutants at P8 to follow the onset of axon myelination. Myelin could be seen in littermate controls (+/+ and +/-) in a retinal-to-chiasmal gradient (Figure 3A and data not shown), but myelin could not be seen in any part of the optic nerves of COUP-TFI mutants (Figures 3D). These data suggest that the onset of myelinogenesis is delayed in COUP-TFI mutants. Myelination increases rapidly over the ensuing three weeks and by P28, 85% of the axons are myelinated in the optic nerves of control mice (Tennekoon et al., 1977). Electron microscopic studies revealed a large increase in unmyelinated axons in COUP-TFI mutants, especially at P17 (Figures 3B and E), but the average number of axons per electron micrograph field and the number of the retinal ganglion cells in retina remained unaltered in COUP-TFI mutants at P17 (data not shown). As the animals develop, the ratio of unmyelinated-to-myelinated axons decreased in both COUP-TFI mutants and controls. Although the axons were still significantly hypomyelinated in COUP-TFI mutants at P27, the difference between the mutants and the controls, in terms of the unmyelinated-to-myelinated

ratio, was dramatically reduced (Figures 3C,F and G). These results indicate that the onset of myelination is delayed in COUP-TFI mutants, resulting in a significant reduction in myelination in the developing optic nerves.

In addition to the delay in myelination, instances of aberrant myelinated axons, redundant myelin and hypermyelin, were also observed in the optic nerves of COUP-TFI mutants. The term redundant myelin describes myelin processes that appear in the absence of definitive axonal association (Figures 4A and B), while hypermyelin describes axons surrounded by two or more compact myelin sheaths (Figures 4C and D). Examination of the axons in the optic nerves of three pairs of mice revealed that the incidence of redundant myelin is higher in COUP-TFI mutants than in the littermate controls (Table 1). The hypermyelin was not observed in controls where a total of 3490 myelinated optic nerve axons were examined. In contrast, axons with aberrant myelinated units were observed in COUP-TFI mutants (Figure 4D). In addition to hypermyelin, we occasionally encountered other myelin defects in the mutants, such as intermyelin or periaxonal deposits (data not shown), which were not seen in controls. These results indicate that dysmyelination is increased in COUP-TFI mutants.

COUP-TFI is required for proper differentiation of oligodendrocytes

Hypomyelination in the CNS of COUP-TFI mutants could originate from defects of oligodendrocytes, which affect their (1) migration, (2) proliferation, (3) differentiation, and/or (4) survival. Studies conducted in the rat, have shown that during oligodendrocyte development, OPCs enter the optic nerve before birth and colonize the nerve in a chiasmal-to-retinal pattern during the first postnatal week (Small et al., 1987). The number of oligodendrocytes then increases for 6 weeks postnatal (Barres et al., 1992). To evaluate the number of oligodendrocytes in the developing optic nerves of COUP-TFI mutants, we performed *in situ* hybridization to study the expression patterns of Olig2, a basic helix-loop-helix protein (Lu et al., 2000; Zhou et al., 2000). Expression of Olig2 is restricted to oligodendrocyte lineage cells and its expression is observed in all stages of oligodendrocyte differentiation. As shown in Figures 5A-L, Olig2 expression levels were similar between wild type and COUP-TFI mutants at P5, P10, and P17, suggesting that the density of oligodendrocytes is not altered in optic nerves of COUP-TFI mutants. In addition TUNEL assay on of optical nerves at different postnatal ages showed no

increase of apoptosis in COUP-TFI mutants (data not shown). Taken together, these results suggest that migration, proliferation and survival of oligodendrocytes at early postnatal stages are not affected in COUP-TFI mutants.

Since a comparable number of oligodendrocytes are present in the optic nerves of COUP-TFI mutants and controls, delayed axon myelination may be explained by the delayed differentiation of the oligodendrocytes. To explore this possibility, we used immunohistochemistry to assess the state of oligdendrocytes. It has been shown, using anti-myelin basic protein (MBP) antibody, that MBP immunoreactivity is lost in the cell body of oligodendrocytes by P10 and the staining is mainly confined to myelinated fibers at later stages (Casaccia-Bonnefil et al., 1999). Since MBP-positive fibers of white matter and striatum are decreased in COUP-TFI mutants (Figures 2G-J), the differentiation of oligodendrocytes is most likely defective *in vivo*. To confirm this assertion, we took advantage of an *in vitro* oligodendrocyte differentiation system. Developmental stages of oligodendrocyte lineage cells were identified by both morphology and the sequential expression of developmental surface antigens (Woodruff et al., 2001). We chose NG2 proteoglycan as an early marker of oligodendrocyte lineage cells, and O4 as a later marker of oligodendrocyte lineage cells. Although NG2 is also expressed in meningeal cells, meningeal cells have fibroblast-like morphology, which is easily distinguished from the bipolar morphology of oligodendrocyte progenitor cells. To determine whether the differentiation of oligodendrocytes in mutants is defective, we harvested cells from the optic nerves of wild type or COUP-TF defective mice at P0 and cultured them in Bottenstein-Sato (B-S) complemented medium. In the absence of PDGF, the mitogen for oligodendrocyte precursor cells (OPC), the OPCs will differentiate spontaneously in 72hours (Durand et al., 1997). The timing of differentiation can be followed through the expression of membrane markers. Wild type cells lose the expression of NG2 in 24-48 hours and start to express O4 and the anti-galactocerebroside (GC) antibody in 24 to 72 hours. Removal of PDGF from the culture medium induces the synchronized differentiation of these cells. After withdrawal of PDGF, we fixed these cells at either 48 or 72 hours, immunostained them for NG2 and O4 markers, and counted the proportion of bipolar OPCs that are only positive for NG2 ($O4^-/NG2^+$) and multi-process bearing oligodendrocytes that are only stained by O4 antibody ($O4^+/NG2^-$). In cultures of P0 optic nerve cells grown in Bottenstein-Sato (B-S) medium in the presence of PDGF, most of

oligodendrocyte lineage cells were NG2 single positive bipolar OPCs (data not shown). However in the absence of PDGF, after 48 or 72hours, the proportion of $O4^-/NG2^+$ bipolar OPCs is higher in cultures derived from COUP-TFI mutants as compared to wild type controls (Figure 6A). In contrast, the ratio of $O4^+/NG2^-$ multi-process bearing oligodendrocytes is lower in COUP-TFI mutants as compared to control cells (Figure 6B). These results further support the notion that oligodendrocytes in COUP-TFI mutants differentiate slower. Therefore, COUP-TFI is required for proper timing of oligodendrocyte differentiation.

Expression of SCIP/Oct-6/Tst-I is down regulated in the CNS of COUP-TFI mutants

To explore the underlying mechanism of COUP-TFI modulation of axon myelination, we investigated the expression of SCIP/Oct-6/Tst-1, a member of the POU domain family of transcriptional regulators, which has been shown to play a role in axon myelination using *in situ* hybridization. We found that the expression of SCIP/Oct-6/Tst-1 is down regulated in the brains of COUP-TFI mutants at P1 (Figures 7A-D). SCIP/Oct-6/Tst-1 is expressed in the cerebral cortex, especially in layers II/III and V neurons (Frantz et al., 1994). Our early studies indicated that these layers of cerebral cortex in COUP-TFI mutants are largely normal and this was further confirmed by *in situ* hybridization with Otx-1, a marker for layer V neurons (data not shown). As shown in Figure 7, the expression of SCIP/Oct-6/Tst-1 in neuronal cells is greatly reduced in COUP-TFI mutants. Similarly, the expression of SCIP/Oct-6/Tst-1 is also decreased in white matter, where oligodendrocytes are abundant, suggesting that SCIP/Oct-6/Tst-1 expression in glial cells might be affected in COUP-TFI mutants.

In the optic nerves, astrocytes and OPCs progenitors express high levels of SCIP mRNA and protein. SCIP mRNA is rapidly down regulated, followed by a decline in SCIP protein and the sequential activation of myelin-specific genes during oligodendrocyte differentiation. In the optic nerves, the expression of SCIP/Oct-6/Tst-1 peaks at postnatal day 14 to 21 in the rat (Monuki et al., 1989). To determine whether SCIP/Oct-6/Tst-1 expression is also decreased in the optical nerves of COUP-TF mutants, *in situ* hybridization was performed. Expression of SCIP/Oct-6/Tst-1 in optic nerves at P15 was decreased in COUP-TFI mutants (Figures 7E-H). Since the density of oligodendrocytes in optic nerves of COUP-TFI mutants is comparable to that of controls as indicated by Olig2 expression (data not shown), down-regulated SCIP/Oct-6/Tst-1

expression in COUP-TFI mutants suggests that decreased SCIP/Oct-6/Tst-1 expression is not due to the loss of oligodendrocyte cells. Therefore, it seems that COUP-TFI is an upstream regulator of the POU domain transcription factor, SCIP/Oct-6/Tst-1.

Promoter activity of SCIP/Oct-6/Tst-1 is regulated by COUP-TFI

To investigate the possibility of a transcriptional regulation by COUP-TFI, we determined whether the promoter, containing the 5' flanking region of the mouse SCIP/Oct-6/Tst-1 gene fused to a luciferase reporter, would respond to COUP-TFI regulation in neuroblastoma cells as well as embryonic carcinoma P19 cells. When the reporter containing ~2 kb of 5' flanking region from the mouse SCIP/Oct-6/Tst-1 gene was transfected into SK-N-SH cells, the activity of this reporter construct was stimulated significantly in the presence of COUP-TFI as compared to its absence (Figure 8A). Furthermore, a similar enhancement of promoter activity was observed when this reporter construct and COUP-TFI were co-transfected into P19 cells (Figure 8B). These results demonstrated that COUP-TFI could modulate the transcriptional activity of the POU domain protein SCIP/Oct-6/Tst-1.

Normal axon myelination in the sciatic nerves of COUP-TFI mutants

It has been shown that SCIP/Oct-6/Tst-I is important for axon myelination of the sciatic nerves and for the development of Schwann cells (Lemke et al., 1990; Monuki et al., 1989). Results of SCIP/Oct-6/Tst-1 knockout mice studies indicate that the postnatal development of Schwann cells is delayed (Bermingham et al., 1996; Jaegle et al., 1996), but the development of oligodendrocytes in the spinal cord is normal. Also, study of the ultrastructure of sciatic nerves of SCIP/Oct-6/Tst-1 knockout mice showed that myelination of sciatic nerves is not seen until the second postnatal week (Bermingham et al., 1996). To explore the possibility that COUP-TFI might control the SCIP/Oct-6/Tst-1 expression in Schwann cells, we examined axon myelination in the PNS of COUP-TFI mutants. We first studied the ultrastructure of sciatic nerves at different postnatal ages in COUP-TFI mutants. However, no major delay in myelination was seen in electron micrographs of sciatic nerves at P4, P10 and P21 in COUP-TFI mutants (Figures 9A-F), suggesting that PNS myelination in COUP-TFI mutants is not defective. Dysmyelination, such as redundant myelin and hypermyelin, was not detected in the sciatic nerves of COUP-TFI mutants (data not shown). To further elucidate why myelination is not affected in developing sciatic

nerves of COUP-TFI mutants, we examined the expression of COUP-TFI and SCIP/Oct-6/Tst-1 in developing sciatic nerves using *in situ* hybridization. Although COUP-TFI is expressed in sciatic nerves at P5 and P15 (Figures 10A-D), the expression of SCIP/Oct-6/Tst-1 is not altered in the sciatic nerves of COUP-TFI mutants (Figures 10E-H). These results could explain why myelination defects are not seen in sciatic nerves of COUP-TFI mutants.

Discussion

COUP-TFI is required for proper axon myelination in the CNS

In addition to its role in PNS development reported previously (Qiu et al., 1997), in this paper we have shown that COUP-TFI is important for axon myelination in the CNS. One of the most striking observations in our study was the significant reduction of myelinated axons and an increase in the proportion of unmyelinated axons in the developing optic nerves of COUP-TFI mutants. Moreover, a higher proportion of axon dysmyelination was observed in optic nerves of COUP-TFI mutants as compared to the control littermates. We showed that delayed myelination of optic nerves is due to delayed differentiation of oligodendrocytes in COUP-TFI mutants and is not due to defects in migration or proliferation, or increased cell death of oligodendrocytes. This delayed differentiation of oligodendrocytes leads to hypomyelination in COUP-TFI mutants.

The lack of severe defect in oligodendrocyte differentiation in COUP-TFI mutant can be due to the nature of this mutant or it is possible due to the redundancy of other genes in these cells. Recent work using *LacZ* knockin in the *COUP-TFII* locus, we found that COUP-TFII is also expressed in the glia cells of the optical nerves (unpublished results). Since COUP-TFII is highly homologous to COUP-TFI in both protein sequences and in their molecular functions, it is likely that COUP-TFII may compensate some of the functions of COUP-TFI in oligodendrocyte differentiation.

Mechanisms of hypomyelination of developing optic nerves in COUP-TFI mutants

Oligodendrocyte precursor cells (OPCs) enter the rat optic nerves before birth, proliferate during the first postnatal week and are able to migrate a long distances in the order of several millimeters during development (Ono et al., 1997). To estimate the number of oligodendrocyte

lineage cells in optic nerves, we examined the expression of Olig2 which is an excellent marker for all cells of the oligodendrocyte lineage (Lu et al., 2000; Woodruff et al., 2001; Zhou et al., 2000). The expression of Olig2 in the optic nerves of COUP-TFI mutants is comparable to that of littermate controls at different postnatal ages. An *in vitro* assay of oligodendrocyte proliferation using BrdU incorporation showed that PDGF stimulated proliferation of oligodendrocytes is not defective in COUP-TFI mutants (data not shown). These data suggest that neither migration nor proliferation of OPCs is compromised in COUP-TFI mutants.

To investigate potential defects in oligodendrocyte differentiation, we studied the expression of MBP, a major myelin protein in the CNS, by immunohistochemistry. MBP immunoreactivity in the white matter and striatum of COUP-TFI mutants is greatly reduced as compared to that of littermate controls suggesting that oligodendrocyte differentiation is defective in COUP-TFI mutants. To support this notion, we performed an *in vitro* differentiation assay of oligodendrocyte precursor cells. This study clearly indicated that *in vitro* differentiation of oligodendrocytes was delayed in COUP-TFI mutants (Figures 6A and B). Taken together, these results strongly support the hypothesis that defects in axonal myelination in the CNS of COUP-TFI mutants is mainly due to defects in oligodendrocyte differentiation instead of defects in migration, proliferation or survival of these cells.

SCIP/Oct-6/Tst-1 is a target gene of COUP-TFI in the CNS

Regulation of axon myelination involves many transcriptional factors (Wegner, 2000). SCIP/Oct-6/Tst-1, a POU domain transcription factor, is expressed in neuronal and glial cells. After establishing a one to one relationship between Schwann cells and axons, SCIP/Oct-6/Tst-1 knockout mice show a delayed myelination of the sciatic nerves, suggesting that SCIP/Oct-6/Tst-1 is required for an inductive event in Schwann cell development (Bermingham et al., 1996; Jaegle et al., 1996). Contrary to its role in the PNS, it has been reported that SCIP/Oct-6/Tst-1 is not required for myelination of the ventral funiculus, relatively large axons of the CNS. Study of myelination in MAG/Fyn double knockout mice showed that impaired formation of oligodendrocyte processes might cause more hypomyelination in tracts with smaller axons (i.e., optic nerves and corticospinal tract) than in tracts with larger axons (i.e., fasciculus gracilis and ventral funiculus) (Biffiger et al., 2000). Therefore, it is possible that myelination of smaller

axons (optic nerves) might be affected in SCIP/Oct-6/Tst-1 knockout mice. Moreover, transgenic animals overexpressing SCIP/Oct-6/Tst-1 in oligodendrocytes develop severe neurological syndromes and premature myelin formation in the CNS (Jensen et al., 1998). However, Monuki et al., (Monuki et al., 1990) demonstrated that SCIP/Oct-6/Tst-1 is a negative regulator of myelin gene expression in Schwann cell in an in vitro assay. This observation is in contrast with the delayed axon myelination in Sciatic nerve of SCIP/Oct-6/Tst-1 mutants and in optic nerve of COUP-TFI mutants. The reason for this discrepancy is not clear and might indicate that proper level of SCIP/Oct-6/Tst-1 expression is required for proper differentiation of oligodendrocytes or Schwann cells.

In any event, we have shown that SCIP/Oct-6/Tst-I is down regulated in neuronal cells and oligodendrocytes of the COUP-TFI mutant CNS. In addition, we have shown that the reporter construct of the SCIP/Oct-6/Tst-1 gene promoter can be up regulated by COUP-TFI in a transient transfection assay suggesting that COUP-TFI is an upstream regulator of SCIP/Oct-6/Tst-1 in the CNS. On the other hand, SCIP expression is not altered in the PNS of COUP-TFI mutants. It is possible that other genes, such as COUP-TFII, which is also expressed in the PNS, compensate for the function of COUP-TFI in COUP-TFI mutants. The evidence that hypomyelination in the CNS occurs in COUP-TFI mutants, but not in SCIP null mice implies that COUP-TFI may also control an unknown gene which compensates for the function of SCIP/Oct-6/Tst-1 in SCIP/Oct6/Tst-1 mutants.

We should emphasize that our conclusion that COUP-TF is an upstream regulator of SCIP/Oct-6/Tst-I does not mean that this regulation is through a direct mechanism. We have carried out extensive studies to determine whether COUP-TF regulation of the SCIP/Oct-6/Tst-I promoter is direct. Using DNA biding (Gel mobility shift) and chromatin immunoprecipitation (ChIP) assays we were not able to demonstrate that COUP-TF binds to or recruited to the promoter sequences used here in the transfection assay. Therefore, we believe that this regulation may through an indirect mechanism.

Dysmyelination is increased in COUP-TFI mutants

Electron micrographs of COUP-TFI mutants showed that, in addition to hypomyelination of

optic nerves, the number of dysmyelinated axons is also increased. Notable features of the myelin defects in COUP-TFI mutants are redundant myelin (serpentine myelin) and hypermyelin. Redundant myelin is occasionally found in the CNS of normal animals during myelinogenesis and represents immature myelinated fibers (Li et al., 1998). The increased occurrence of redundant myelin in COUP-TFI mutants indicates that premature formation of myelination occurred before establishment of the axon-glial interaction. Hypermyelin was also observed in COUP-TFI mutants, but not in littermate controls. The existence of hypermyelination in the optic nerves suggests oligodendrocytes fail to distinguish myelinated axons from unmyelinated axons. The decreased myelinogenesis might result from compromised glial-axon recognition. However, most myelinated axons are morphologically intact in COUP-TFI mutants. One possible explanation is that a compensatory mechanism could be in place, such as myelin-associated glycoprotein (MAG). MAG is a myelin-specific protein that has been proposed to function in glial-axon recognition and to play an adhesion role during myelinogenesis (Li et al., 1994; Montag et al., 1994). MAG null mice also have an increased number of dysmyelinated axons in their optic nerves (Li et al., 1998), as found in COUP-TFI mutants. Knockout mice for Fyn, a receptor for the large isoform of MAG, showed hypomyelination in their optic nerves (Umemori et al., 1994). MAG/Fyn double knockout mice show more severe hypomyelination than either single knockout (Schachner and Bartsch, 2000). These observations prompted us to investigate the expression pattern of Fyn and MAG in COUP-TFI mutants. However, *in situ* hybridization analysis revealed that the expression of Fyn and MAG in COUP-TFI mutants was not altered (data not shown). These results suggest that Fyn and MAG are not involved in mediating the dysmyelination in COUP-TFI mutants. Although COUP-TFI is not essential for myelin formation, COUP-TFI may be involved in proper glial-axon recognition *in vivo*.

Taken together, this study shows that COUP-TFI is important for differentiation of oligodendrocytes. Inherited myelin diseases in humans called leukodystrophies, have dysmyelination and hypermyelination due to the failure of proper myelination during fetal life or early infancy (Kolodny, 1993). The increased dysmyelination observed in COUP-TFI mutants may provide a clue as to the molecular foundation of dysmyelinating disease. It would be of great interest to examine whether or not COUP-TFI is involved in leukodystrophies.

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	Control	Mutant	*P*
Redundant myelin	38/3770 (1.01%)	117/3416 (3.43%)	0.007[a]
Hypermyelin	0/3770 (0%)	58/3416 (1.70%)	0.006[a]

Table 1. Dysmyelination in optic nerves of COUP-TFI mutants (P25, n=3)

Each number represents the number of dysmyelinated axons/ total number of axons counted, with percentages in brackets. [a] One tailed *t*-test.

Figure 1. Expression of COUP-TFI in the developing optic nerves

(A-D) Optic nerves at P5 (A) and P15 (B) were hybridized with antisense riboprobe for COUP-TFI, and counterstained with Hematoxylin (C and D). COUP-TFI was found to be expressed in developing optic nerves. Scale bar, 150μm. (E-H) Expression of COUP-TFI in oligodendrocyte lineage cells was demonstrated by *in situ* hybridization in primary mixed glial cell culture from optic nerves (E). Signals were visualized with alkaline phosphatase. Brown colors represent COUP-TFI mRNA. COUP-TFI is expressed in bipolar oligodendrocyte progenitor cells (F), type-2 astrocytes (G) and differentiated multi-process bearing oligodendrocytes (H), which were distinguished by their morphology and surface antigens. Sense-strand COUP-TFI riboprobes yield no specific signals. Scale bar, 15μm.

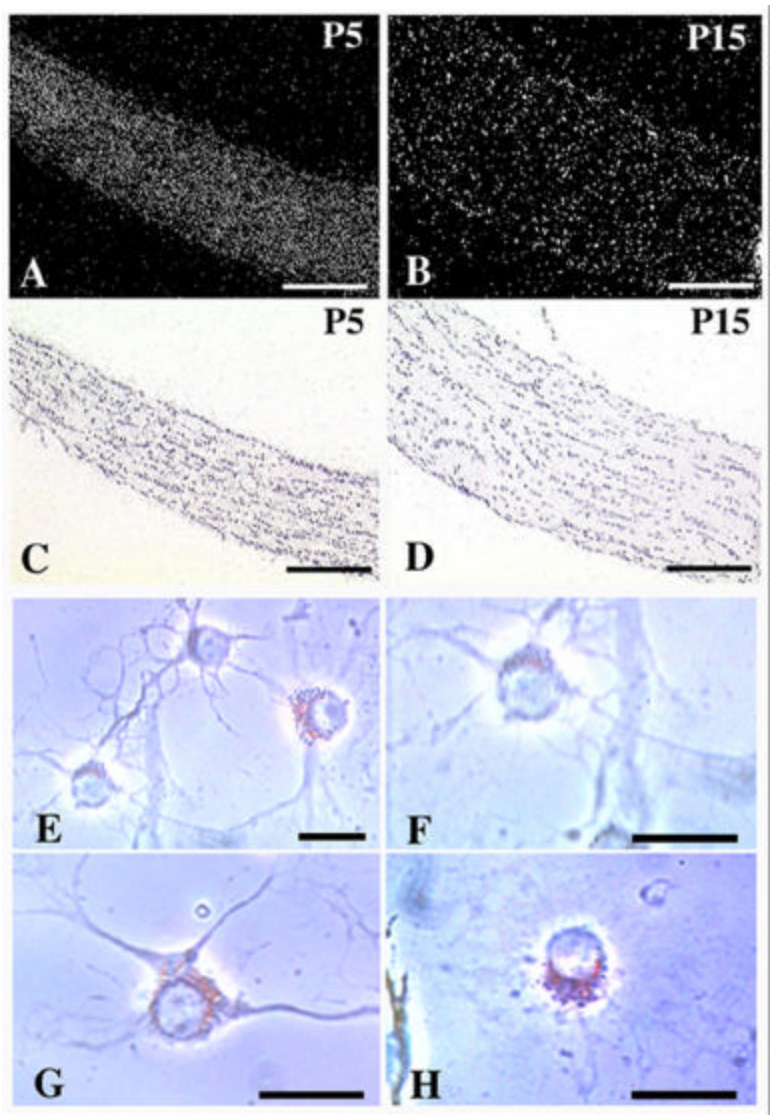


Figure 2. Hypomyelination of the CNS in COUP-TFI mutants

(A-F) Coronal sections of 3-week-old control (A, C, and E) and COUP-TFI mutant littermate (B, D and F) stained with Luxol Fast Blue. The lower panels are higher magnifications of the white matter (C and D) and caudate pallidum (E and F) shown in the upper panels. Luxol Fast Blue staining was decreased in the white matter and the striatum of COUP-TFI mutants. COR, cortex; WM, white matter; and CP, caudate putamen. (G-J) Coronal section of 3-week old control (G and I) and its COUP-TFI mutant littermate (H, J) were immunostained with MBP antibody. MBP immunostaining in white matter (G and H) and caudate pallidum (I and J) was decreased in COUP-TFI mutants. Scale bar, 500μm (A and B) and 100 μm (C-F). The lower panels (I and J) are higher magnification pictures of the upper panels (G and H). Scale bar, 200μm (G and H) and 400 μm (I and J).

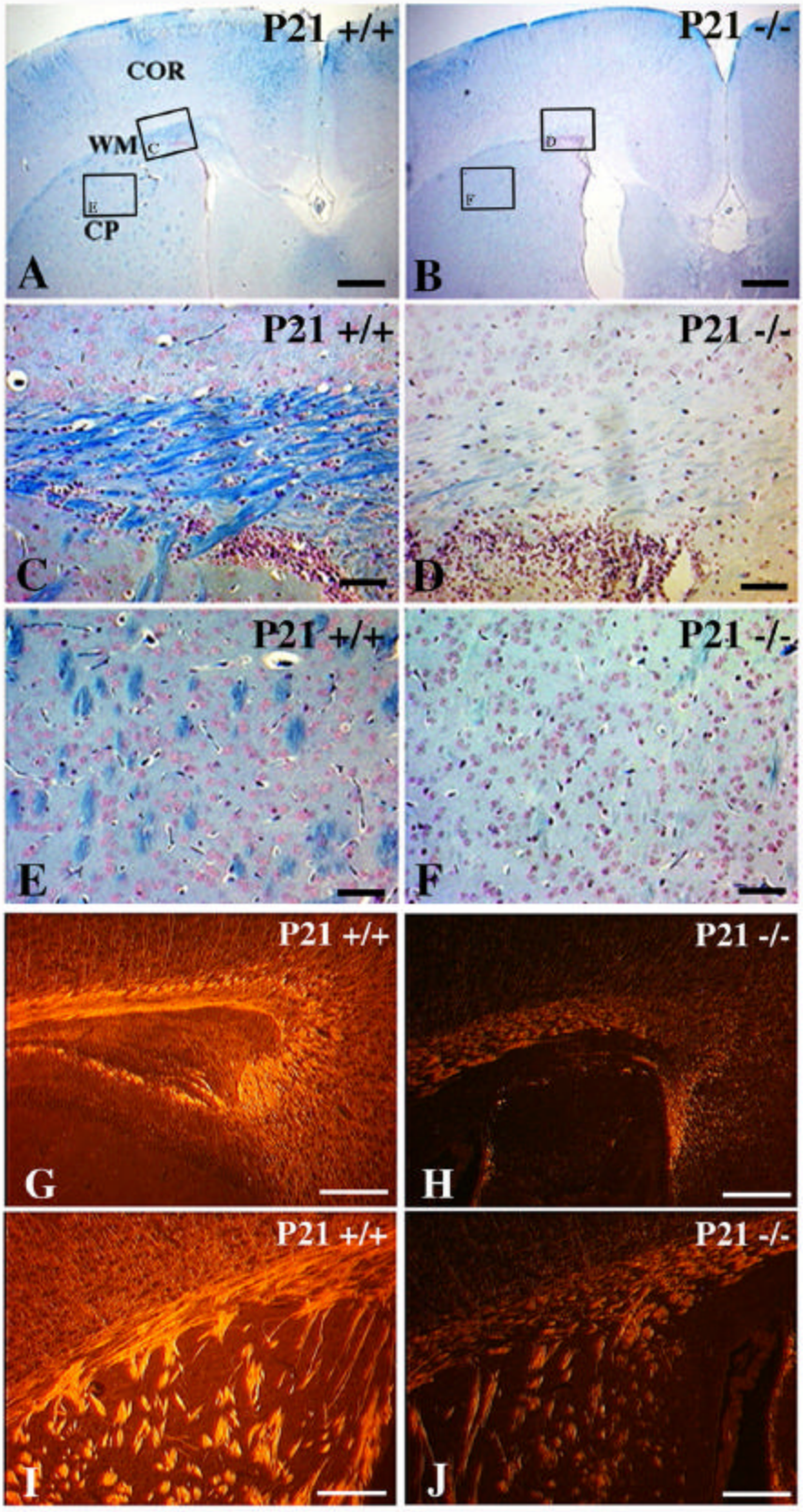
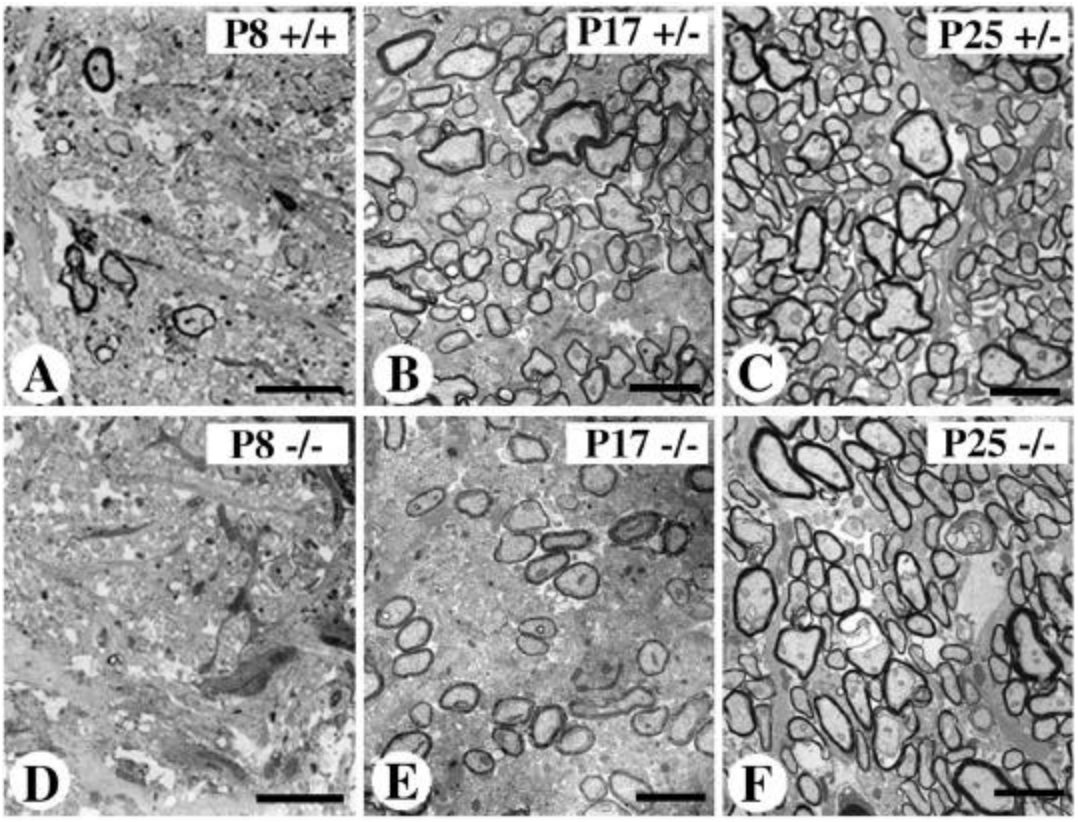

P21 +/+
COR
WM
C
E
CP
A
P21 -/-
D
F
B
P21 +/+
C
P21 -/-
D
P21 +/+
E
P21 -/-
F
P21 +/+
G
P21 -/-
H
P21 +/+
I
P21 -/-
J

Figure 3. Delay in the start of axon myelination and increased unmyelinated axons in COUP-TFI mutants

(A-F) Electron micrographs of optic nerves are shown. The age of mice is indicated on the top. First appearance of myelinated axons was found to be delayed in mutants at P8 (D) compared to littermate control (A). Unmyelinated axons were increased at P17 in mutants (E). Axon myelination in mutants at P25 (F) was slightly lower than that of littermate control (C. Original magnification, ×5,000. Scale bar, 2μm. (G) Quantitative analysis of the number of myelinated axons. Axon myelination is delayed in mutants (dash line) as compared to controls (solid line). Sections of optic nerves are photographed at ×5,000 magnification from three independent fields, and the number of myelinated axons/total axons (%) was counted (means ± S.D.).


P8 +/+
P17 +/-
P25 +/-
A
B
C
P8 -/-
P17 -/-
P25 -/-
D
E
F

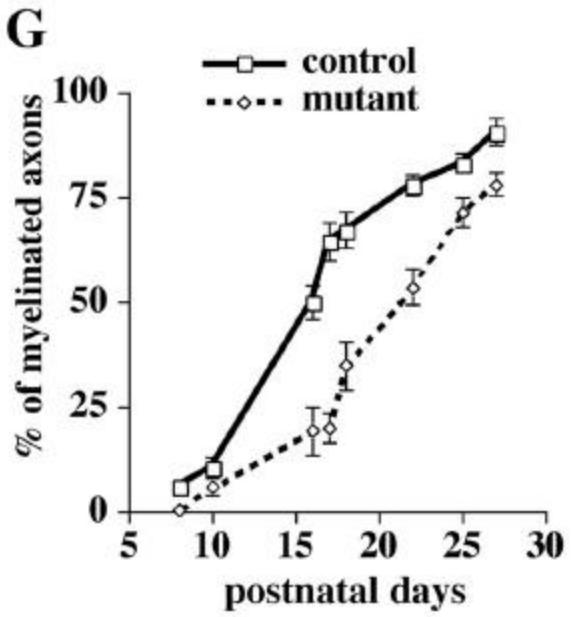

G
control
mutant
% of myelinated axons
100
75
50
25
0
5
10
15
20
25
30
postnatal days

Figure 4. Dysmyelination in COUP-TFI mutants

(A-D) Electron micrographs of optic nerves at P25. Redundant myelin (arrowhead in A and B) and hypermyelin (arrow in C and D) in mutants is shown. Original magnification, ×5,000. Scale bar, 0.8μm.

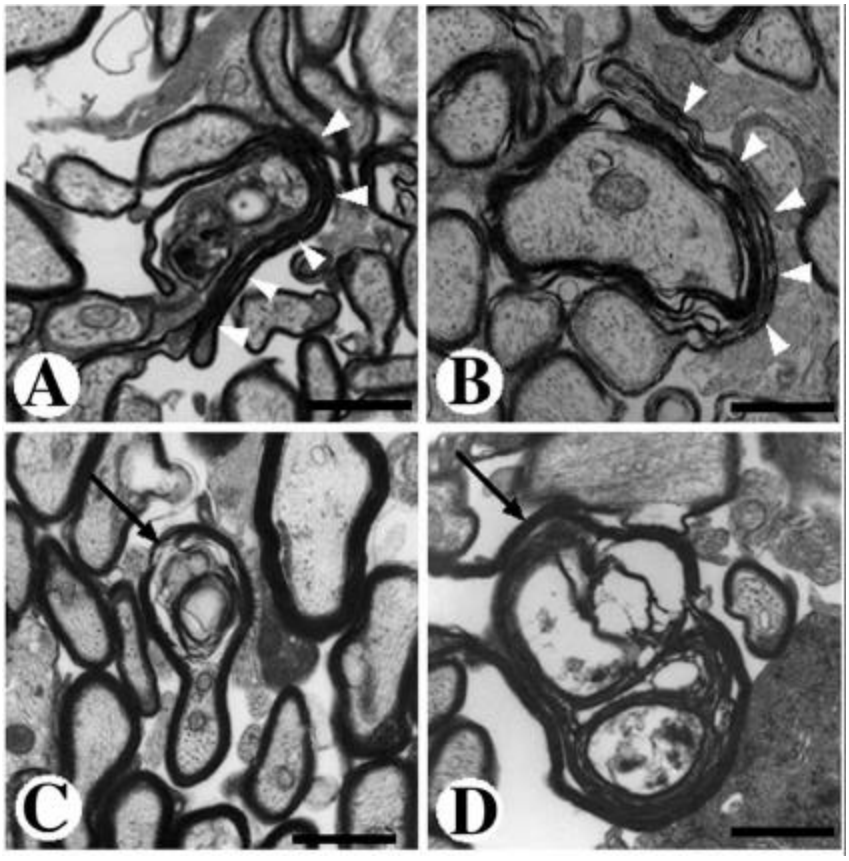


Figure 5. Normal density of oligodendrocyte lineage cells of optic nerves in COUP-TFI mutants (A-L) *In situ* hybridization of Olig2 in optic nerves at different postnatal ages, and Hematoxylin counterstain (A-C and J-L). Olig2, a basic helix-loop-helix protein, is expressed in both the earliest stage of OPCs and mature oligodendrocytes and thus serves as a specific marker for oligodendrocytes. The ages of mice are indicated on the top. The Olig2 signals in mutants are comparable to those of littermate controls (D-I). Scale bar, 150μm.

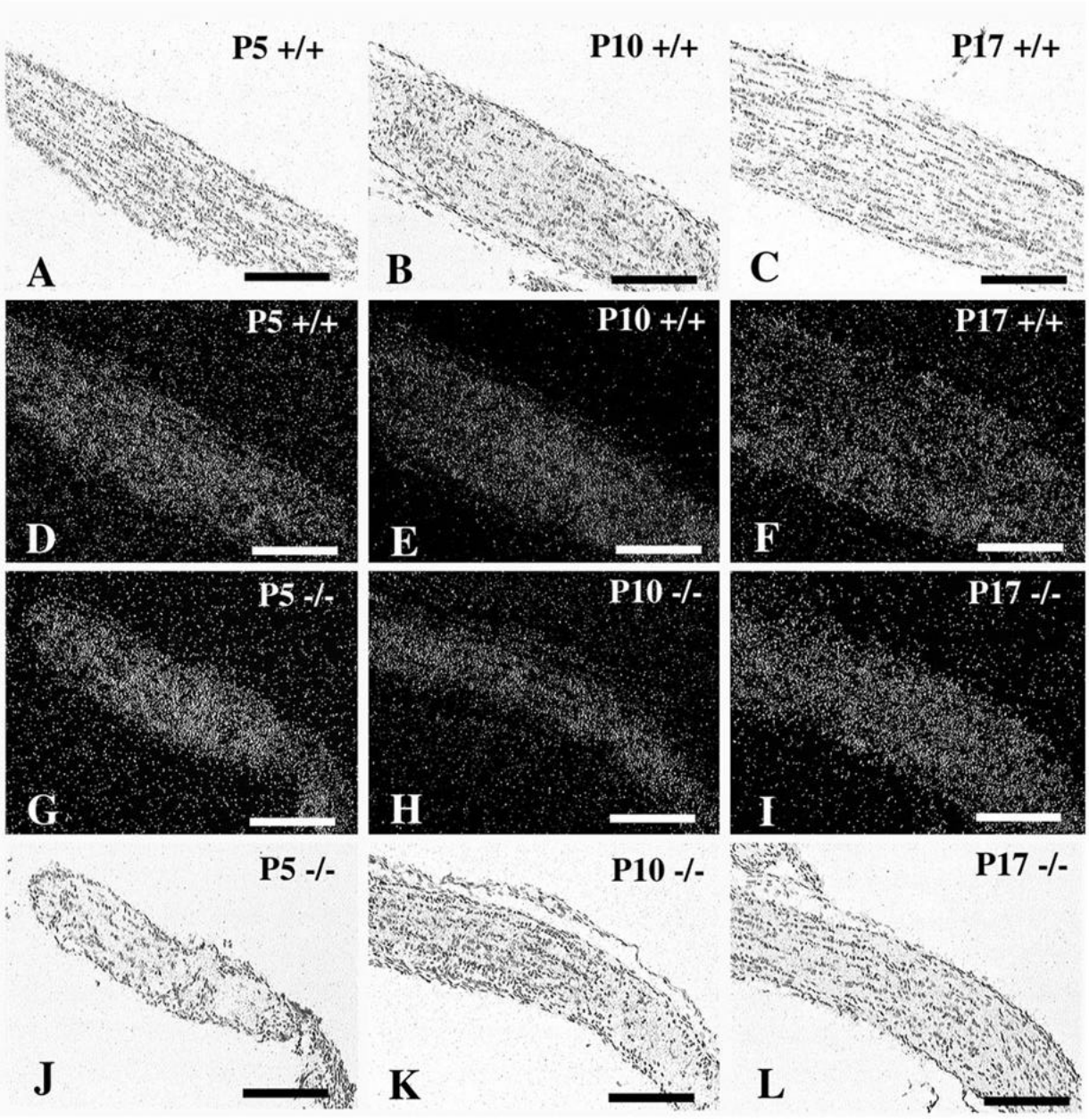


Figure 6. Delayed *in vitro* differentiation of oligodendrocytes in COUP-TFI mutants

(A) The ratios of $NG2^{+}O4^{-}$ bipolar oligodendrocyte progenitor cells (OPCs) to total oligodendrocyte lineage cells ($O4^{+}$ cells and $NG2^{+}O4^{-}$ process-bearing cells) are shown. (B) The ratio of $NG2^{-}O4^{+}$ multi-process bearing oligodendrocytes to total oligodendrocyte lineage cells. The results are shown as means ± SD. **P*<0.05 for mutant vs. control.

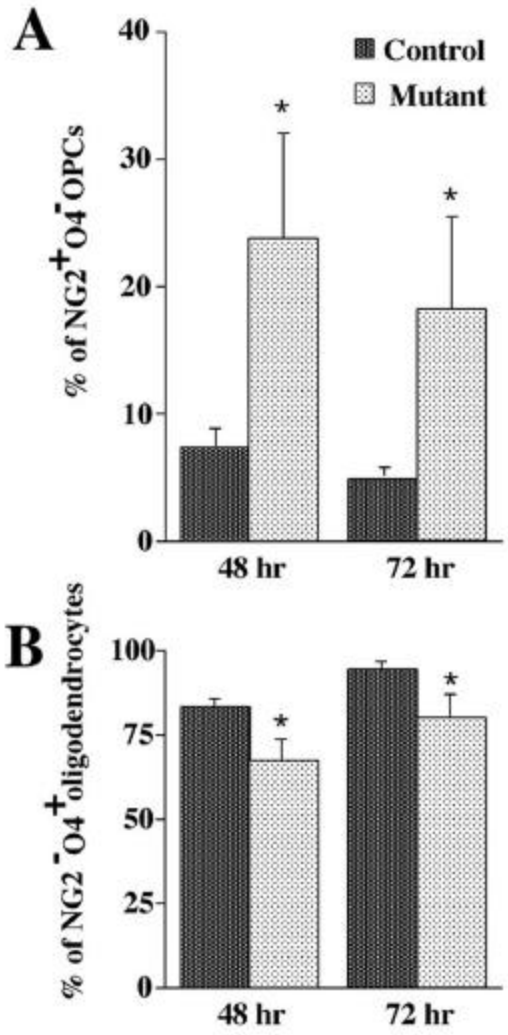


Figure 7. Down-regulation of SCIP/Oct-6/Tst-1 in the CNS of COUP-TFI mutants.
(A-D) *In situ* hybridization on coronal sections of P1 control and mutant brain using antisense SCIP/Oct-6/Tst-1 probe are shown (A and B). The Bright field images are the sections counterstained with hematoxylin (C and D). Scale bar, 800μm. (E-H). *In situ* hybridizations of optic nerves in control and littermate mutant at P15 using antisense SCIP/Oct-6/Tst-1 probe are shown in panels E and F. The Bright field images are sections counterstained with hematoxylin (G and H). Scale bar, 150μm.

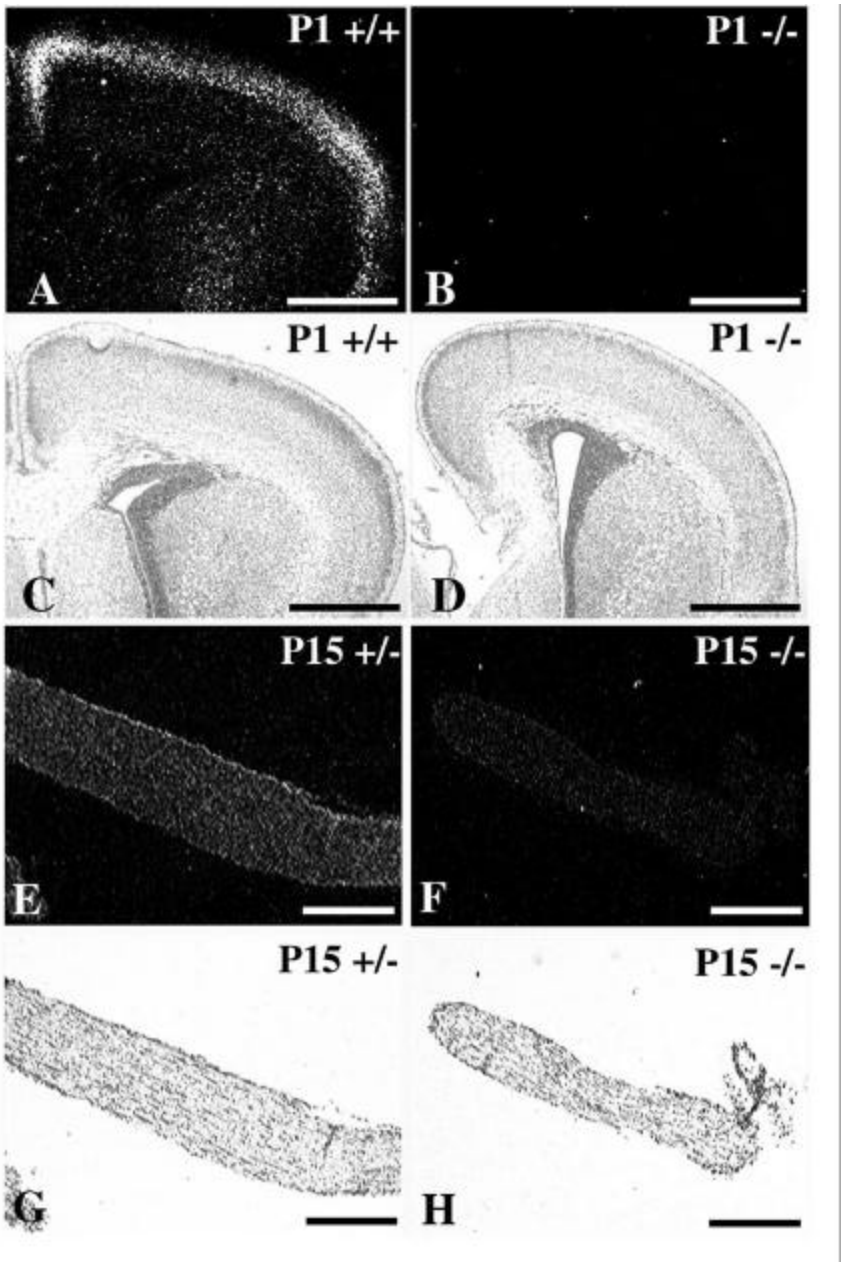


Figure 8. COUP-TFI stimulates the promoter activity of the SCIP/Oct-6/Tst-1.

Luciferase reporter plasmids containing approximate 2 kb of the 5' flanking region of the mouse SCIP/Oct-6/Tst-1 gene was transfected into SK-N-SH (panel A) and P19 (panel B) cells in the absence or presence of the COUP-TFI expression plasmid, pCXN2-mCI. Luciferase activities in extracts from transfected cells were determined in six independent experiments for SK-N-SH cells and three for P19 cells. A representative experiment is shown above. Data presented are the average relative luciferase unit, normalized to the amount of protein in the extracts, with its standard deviation.

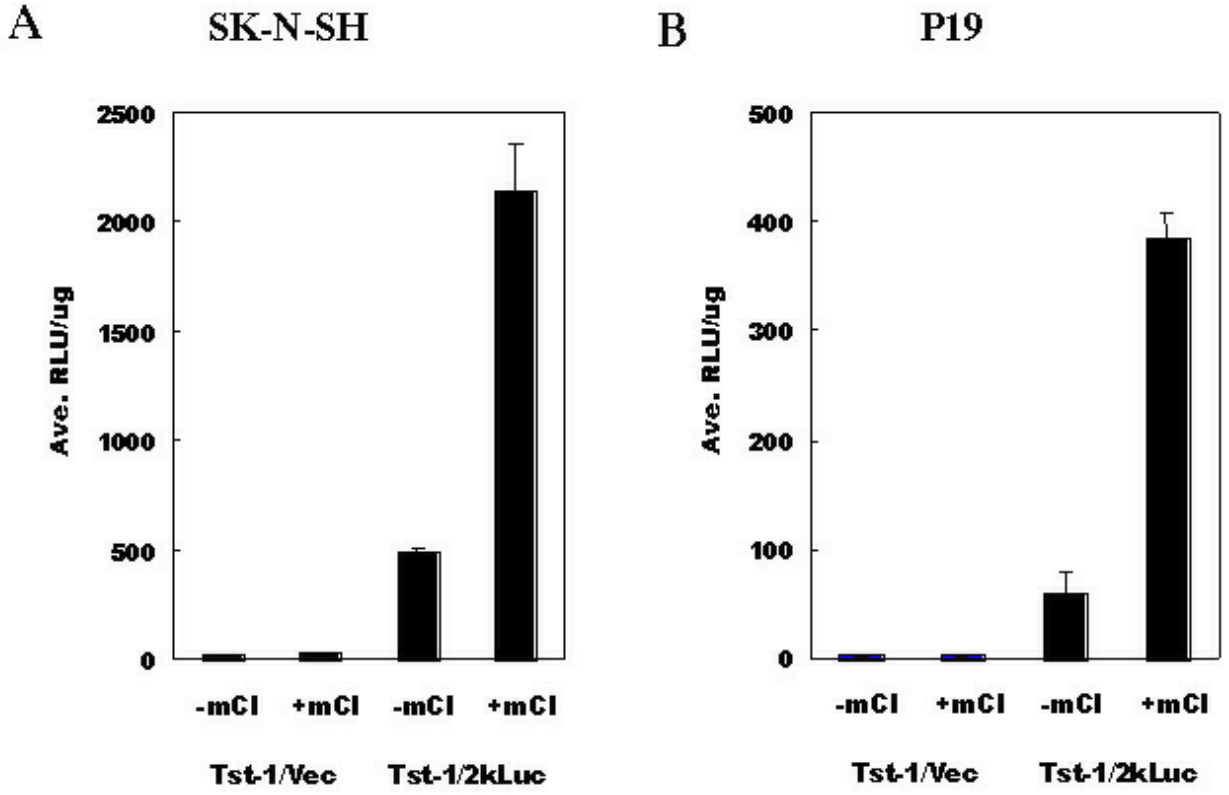


Figure 9. No gross defects of myelination in the PNS of COUP-TFI mutants.

Ultrastructure of cross-sections of sciatic nerves is shown from controls (A-C) and mutant littermates (D-F) at different postnatal ages. The age of the mice is indicated on the top. Myelination of sciatic nerves was not impaired in mutants. Original magnification, ×1,500. Scale bar, 2μm.

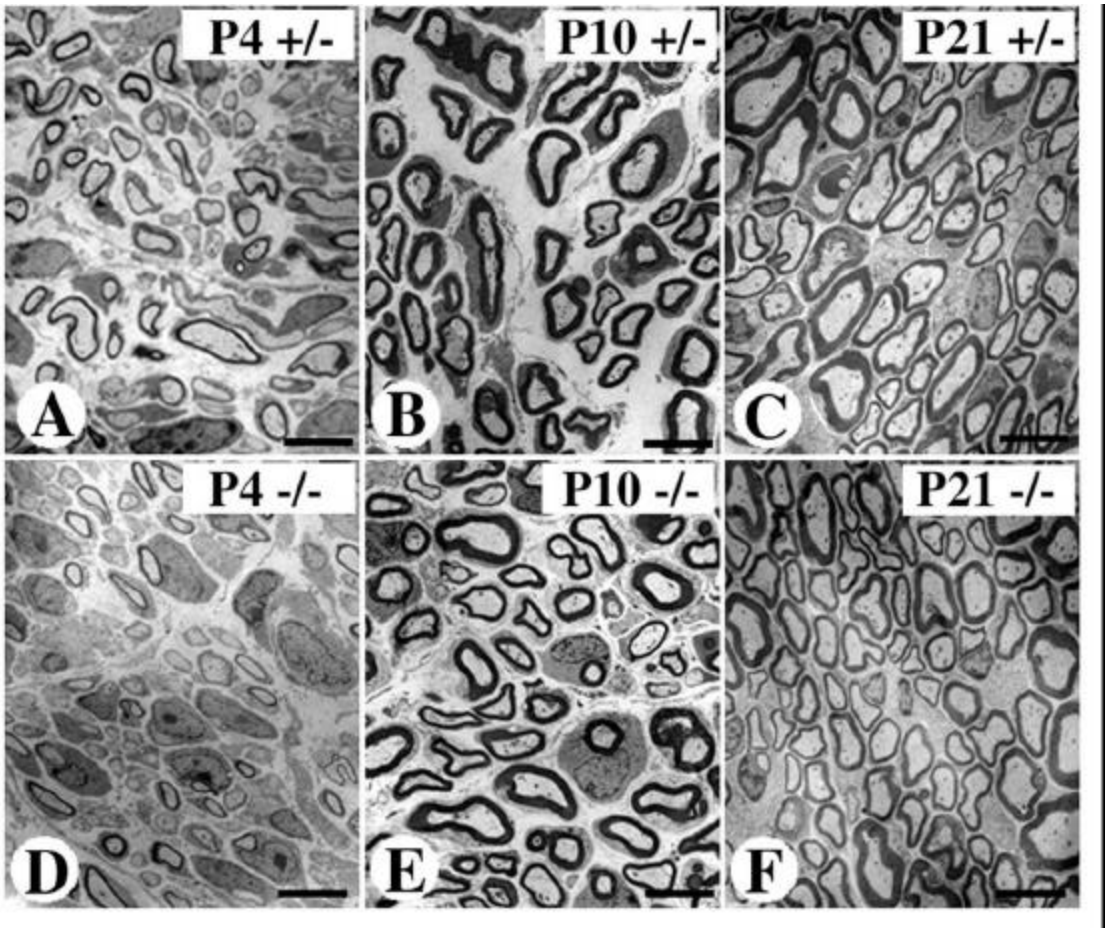


Figure 10. Expression of COUP-TFI in sciatic nerves and normal expression of SCIP/Oct-6/Tst-1 in sciatic nerves of COUP-TFI mutants.

Sciatic nerves at P5 (A) and P15 (B) were hybridized with an antisense riboprobe for COUP-TFI, and counterstained with hematoxylin (C and D). Scale bar, 150μm. (E-H) Normal expression level of SCIP/Oct-6/Tst-1 in sciatic nerves of COUP-TFI mutants. *In situ* hybridizations were performed on coronal sections of P5 control (E) and littermate mutant (F) sciatic nerves using antisense SCIP/Oct-6/Tst-1 probe. The Bright fields images (G and H) are the sections counterstained with hematoxylin. Scale bar, 150μm.