Normal timing of oligodendrocyte development depends on thyroid hormone receptor alpha 1 (TR α 1)

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The timing of oligodendrocyte development is regulated by thyroid hormone (TH) *in vitro* and *in vivo*, but it is still uncertain which TH receptors mediate this regulation. TH acts through nuclear receptors that are encoded by two genes, TR α and TR β . Here, we provide direct evidence for the involvement of the TR α 1 receptor isoform *in vivo*, by showing that the number of oligodendrocytes in the postnatal day 7 (P7) and P14 optic nerve of TR α 1 $^{-/-}$ mice is decreased compared with normal. We demonstrate that TR α 1 mediates the normal differentiation-promoting effect of TH on oligodendrocyte precursor cells (OPCs): unlike wild-type OPCs, postnatal TR α 1 $^{-/-}$ OPCs fail to stop dividing and differentiate in response to TH in culture. We also show that overexpression of TR α 1 accelerates oligodendrocyte differentiation in culture, suggesting that the level of TR α 1 expression is normally limiting for TH-dependent OPC differentiation. Finally, we provide evidence that the inhibitory isoforms of TR α are unlikely to play a part in the timing of OPC differentiation.

Keywords: differentiation/oligodendrocyte/thyroid hormone receptors

Introduction

In many vertebrate cell lineages, precursor cells divide a limited number of times before they stop proliferating and terminally differentiate. It is not known what limits cell proliferation and causes the cells to stop dividing and differentiate when they do. The stopping mechanisms are important because they influence both the timing of cell differentiation and the number of differentiated cells generated.

We have been studying the stopping mechanism in the oligodendrocyte cell lineage in the rodent optic nerve. Oligodendrocyte precursor cells (OPCs) migrate from the brain into the developing rat optic nerve just before birth (Small *et al.*, 1987). After a period of proliferation, most stop dividing and terminally differentiate into oligoden-

drocytes (Temple and Raff, 1986), which then myelinate the axons in the nerve. The first oligodendrocytes appear in the rat optic nerve around birth and then increase in number for the next 6 weeks (Skooff *et al.*, 1976; Miller *et al.*, 1985; Barres *et al.*, 1992).

The normal timing of oligodendrocyte differentiation can be reconstituted in cultures of perinatal rat optic nerve cells (Raff *et al.*, 1985). This requires that the OPCs are stimulated to proliferate by either astrocytes or platelet-derived growth factor (PDGF) (Raff *et al.*, 1988). It also requires the presence of hydrophobic signals such as thyroid hormone (TH) or retinoic acid (RA) (Barres *et al.*, 1994; Ahlgren *et al.*, 1997; Gao *et al.*, 1998). Clonal analyses in such cultures show that the progeny of an individual OPC stop dividing and differentiate at about the same time, even if separated and cultured in different microwells, indicating that an intrinsic mechanism operates in the OPCs to limit their proliferation and initiate differentiation after a certain period of time or number of cell divisions (Temple and Raff, 1986; Barres *et al.*, 1994). The finding that OPCs cultured at 33°C divide more slowly but stop dividing and differentiate sooner, after fewer divisions, than when they are cultured at 37°C suggests that the intrinsic mechanism does not operate by counting cell divisions but instead measures time in some other way (Gao *et al.*, 1997).

Although the timing mechanism is apparently built into each OPC, it depends on extracellular signals, such as PDGF and TH, to operate normally. If OPCs are cultured in the absence of PDGF, they stop dividing and differentiate prematurely, regardless of their maturation state or the presence or absence of hydrophobic signals (Barres *et al.*, 1994). In the presence of PDGF but in the absence of hydrophobic signals such as TH, most OPCs keep dividing and do not differentiate (Barres *et al.*, 1994; Ahlgren *et al.*, 1997; Tang *et al.*, 2001); if TH is introduced into these cultures after 8 days, most of the OPC stop dividing and differentiate within 4 days (Barres *et al.*, 1994). Together, these findings and others suggest that the intrinsic timer consists of at least two components: a timing component, which depends on PDGF and measures time independently of TH; and an effector component, which is regulated by TH and stops cell division and initiates differentiation when the timing component indicates it is time (Barres *et al.*, 1994; Bogler and Noble, 1994). Even in the absence of TH, however, some OPCs cultured in PDGF spontaneously stop dividing and differentiate (Barres *et al.*, 1994; Ahlgren *et al.*, 1997; Tang *et al.*, 2001). Thus, whereas TH is required for the normal timing of OPC differentiation, it is not required for oligodendrocyte development *per se*.

There is also abundant evidence that TH plays an important part in regulating the development of the oligodendrocyte cell lineage *in vivo*. Myelination is delayed in hypothyroid animals (Dussault and Ruel,

Fig. 1. Comparison of oligodendrocyte development in the optic nerve of WT and *TRα1*^{-/-} mice. For each time point, optic nerve cells were isolated, counted and cultured on polylysine-coated coverslips for 2 h. The cells were then fixed and stained for GC to identify oligodendrocytes. Between 5 and 15 pups were analysed for each time point and genotype. Each pair of optic nerves was processed separately, and 2 coverslips were counted for each pair. The total number of cells isolated per nerve was not significantly different in the two genotypes (data not shown). The results are expressed as the mean $\pm$ SD. The differences between the *TRα1*^{-/-} and WT cells at both P7 and P14 are statistically significant when analysed by Student's t-test ($p < 0.02$).

1987; Rodriguez-Pena *et al.*, 1993) and accelerated in hyperthyroid animals (Walters and Morell, 1981; Marta *et al.*, 1998). Perinatal hypothyroidism decreases the number of oligodendrocytes in the optic nerve of the rat (Ibarrola *et al.*, 1996) and mouse (Ahlgren *et al.*, 1997), and TH coordinates the onset of myelination in the central and peripheral parts of the auditory nerve (Knipper *et al.*, 1998). Moreover, *in vitro*, TH promotes the development of oligodendrocytes in cultures of developing brain cells (Almazan *et al.*, 1985; Koper *et al.*, 1986), and it may promote the commitment of cultured central nervous system (CNS) stem cells to the oligodendrocyte lineage (Johe *et al.*, 1996).

While it is clear that TH contributes to timing oligodendrocyte development *in vitro* and *in vivo*, it is still uncertain how it does so. The effects of TH are mediated by ligand-regulated transcription factors that belong to the superfamily of nuclear receptors. Thyroid hormone receptors (TRs) are the products of two genes, *TRα* and *TRβ*. The *TRβ* gene encodes at least three N-terminal variants, TRβ1–3, which all bind TH and regulate gene transcription (Koenig *et al.*, 1988; Murray *et al.*, 1988; Hodin *et al.*, 1989; Wood *et al.*, 1991; Williams, 2000). The *TRα* gene encodes a functional TH receptor, TRα1, and two C-terminal variants, TRα2 and TRα3, which bind weakly to DNA but do not bind TH and have been suggested to act as dominant-negative antagonists of TH signalling (Sap *et al.*, 1986; Weinberger *et al.*, 1986; Koenig *et al.*, 1988; Lazar *et al.*, 1988; Mitsuhashi *et al.*, 1988; Koenig *et al.*, 1989). The *TRα* gene also encodes two N-terminally truncated isoforms of TRα1 and TRα2 called TRΔα1 and TRΔα2, which lack both an intact TH-binding domain and a DNA-binding domain. They too have been suggested to act as dominant-negative antagonists (Chassande *et al.*, 1997; Plateroti *et al.*, 2001).

The *TRα* gene is ubiquitously expressed from early in development, whereas the *TRβ* gene is expressed in a more

restricted range of tissues and later in development, suggesting that the α and β receptors may have different functions (Forrest *et al.*, 1990; Strait *et al.*, 1992; Bradley *et al.*, 1992). This suggestion is supported by gene inactivation studies in mice; *TRα1*^{-/-} mice have an abnormal heart rate and a reduced body temperature, whereas *TRβ*^{-/-} mice have impaired hearing and pituitary function (Forrest *et al.*, 1996a,b; Wikström *et al.*, 1998). Mice deficient in both α and β TRs, however, show more abnormalities than the sum of abnormalities seen in *TRα*^{-/-} and *TRβ*^{-/-} mice, suggesting that the two classes of receptors either cooperate in some of their functions, can substitute for each other, or both (Gauthier *et al.*, 1999; Göthe *et al.*, 1999). Inactivation of both *TRα* and *TRβ* genes, for example, is necessary to reproduce the cochlear developmental defects seen in hypothyroidism (Rüsch *et al.*, 2001).

TRs bind TH with high affinity and recognise identical TH response elements (TREs) in DNA (Lazar, 1993; Glass, 1994), although they may preferentially regulate different target genes (Lezoualc'h *et al.*, 1992; Strait *et al.*, 1992; Zavacki *et al.*, 1993; Denver *et al.*, 1999). They regulate transcription both in the absence and in the presence of TH and can either repress or activate transcription. Some target genes are repressed by empty TRs and activated in the presence of TH (Nagy *et al.*, 1999; Hermanson *et al.*, 2002). Others are activated by empty TRs and repressed in the presence of TH (Love *et al.*, 2000). Abnormal regulation of transcription by empty TRs is thought to explain why hypothyroidism results in more severe defects in postnatal CNS development (Hashimoto *et al.*, 2001; Flamant *et al.*, 2002; Morte *et al.*, 2002) than does the inactivation of all TRs (Gauthier *et al.*, 1999; Göthe *et al.*, 1999).

Although there is general agreement that both OPCs and oligodendrocytes express *TRα* and that oligodendrocytes express *TRβ* (Baas *et al.*, 1994a,b; Carlson *et al.*, 1994; Fierro-Renoy *et al.*, 1995; Carre *et al.*, 1998; Billon *et al.*, 2001), there has been controversy about whether OPCs express *TRβ*. Some studies reported the presence of TRβ1 and TRβ2 proteins in rat OPCs (Barres *et al.*, 1994; Gao *et al.*, 1998; Kondo and Raff, 2000a). Other studies, however, failed to detect *TRβ* mRNAs in OPCs; TRβ1 could not be detected in cultures of cells from developing rat brain, which would be expected to contain OPCs (Baas *et al.*, 1994a,b; Carre *et al.*, 1998), and neither *TRβ1* nor *TRβ2* mRNA could be detected in cultures of purified rat optic nerve OPCs (Billon *et al.*, 2001). Moreover, newborn *TRβ*^{-/-} mice seem to have normal numbers of oligodendrocytes in their optic nerves, and *TRβ*^{-/-} OPCs stop dividing and differentiate normally in response to TH *in vitro* (Billon *et al.*, 2001). Taken together, these findings suggest that TRα receptors mediate the effect of TH on the timing of OPC differentiation. In the present study, we have used *TRα1*^{-/-} mice, retrovirus-mediated gene transfer and RT-PCR to investigate the role of TRα isoforms in OPC development. Our findings establish a role for TRα1 in timing OPC differentiation and suggest that the number of TRα1 receptors is normally limiting for TH-dependent OPC differentiation. They also suggest that the inhibitory isoforms of TRα are unlikely to influence the timing of OPC differentiation.

Fig. 2. Effect of TH or RA on OPC differentiation in mixed optic nerve cell cultures from WT and *TRα1*^{-/-} mice. Cells were dissociated from P7 optic nerve and cultured in slide flasks for 3 days in PDGF without TH or RA. They were then either kept in PDGF or switched to PDGF + TH or PDGF + RA for 5 days, before they were stained for GC. In this and the following three figures, the results are expressed as the mean $\pm$ SD of at least three experiments. The difference between the WT and *TRα1*^{-/-} cells in PDGF + TH is statistically significant when analysed by Student's t-test ($p < 0.03$); by contrast, the difference in the results for *TRα1*^{-/-} cells in PDGF versus in PDGF + TH is not statistically significant using the same test ($p = 0.15$).

Results

Oligodendrocyte development is delayed in TRα1^{-/-} mouse optic nerve

If the timing of OPC differentiation normally depends on TRα1, one would expect it to be delayed in *TRα1*^{-/-} mice. We therefore compared the number of oligodendrocytes in the optic nerves of wild-type (WT) and *TRα1*^{-/-} mice at birth (P0), P4, P7 and P14. We dissociated optic nerve cells at these times and counted both the total number of cells and the proportion of differentiated oligodendrocytes, identified by staining with an anti-galactocerebroside (GC) antibody (Raff *et al.*, 1978; Ranscht *et al.*, 1982). At each time point, there was no significant difference in the total number of cells isolated from WT and *TRα1*^{-/-} optic nerves (data not shown). The proportions of GC-positive oligodendrocytes, however, were significantly reduced at P7 and P14 in *TRα1*^{-/-} optic nerves compared with WT, although the proportions were similar at P0 and P4 (Figure 1). Thus, in the absence of TRα1, the number of oligodendrocytes in the optic nerve is decreased during the second postnatal week, when the vast majority of oligodendrocytes are produced in the normal rodent optic nerve (Skoff *et al.*, 1976; Barres *et al.*, 1992).

TRα1^{-/-} OPCs fail to differentiate in response to TH

To confirm the role of TRα1 in the timing of oligodendrocyte differentiation, we assessed the response of WT and *TRα1*^{-/-} mouse OPCs to TH *in vitro*. We cultured mixed P7 mouse optic nerve cells in the presence of PDGF and the absence of TH for 3 days and then changed the medium to one containing PDGF, PDGF and TH, or PDGF and RA. After 5 days, we stained the cultures for GC to determine the proportion of oligodendrocytes. As shown in Figure 2, both TH and RA induced oligodendrocyte differentiation in WT cultures, but only RA significantly did so in *TRα1*^{-/-} cultures. Thus, TRα1 seems to be

Fig. 3. Effect of the expression of a *TRα1* transgene on rat OPC differentiation *in vitro*. P7 rat OPCs were purified and cultured for 2 days in PDGF without TH or RA. They were then infected with either the pBird control or pBird-TRα1 retroviral vector for 3 h. After a further day in PDGF without TH or RA, the cells were either left in PDGF without TH or RA (A), or switched to either PDGF and TH (B), or PDGF and RA (C) to induce differentiation. The percentage of infected (GFP+) cells that had acquired the characteristic morphology of oligodendrocytes was scored in an inverted fluorescence microscope at various times. Similar results were obtained when oligodendrocytes were identified by staining with anti-GC antibody (data not shown).

required for OPCs to differentiate in response to TH *in vitro*.

Expression of a TRα1 transgene in OPCs accelerates TH-dependent differentiation

To test whether an increase in the expression of *TRα1* accelerates the timing of OPC differentiation in the presence of PDGF and TH, we cultured purified P7 rat OPCs in PDGF without TH for 2 days and then infected them with either a control retroviral vector (pBird), which encoded GFP only, or a retroviral vector, which encoded both GFP and TRα1 (pBird-TRα1). After a further day in culture, we either maintained the cells in PDGF alone or switched them to either PDGF plus TH, or PDGF plus RA

Fig. 4. Effect of the expression of a *TRα2* transgene on rat OPC differentiation *in vitro*. P7 rat OPCs were purified, cultured and analysed as in Figure 3, except that they were infected with either pBird or pBird-*TRα2* retroviral vectors. Note that these experiments and those in Figure 5 were performed at the same time as those in Figure 3, and so the results with the pBird vector are the same in all three figures. The difference between the pBird-infected cells and the *TRα2*-infected cells at 7 days in (B) is not statistically significant when analysed by Student's *t*-test ($p > 0.07$). (D) RT-PCR analysis of *TRα2* mRNA in OPCs infected with pBird or pBird-*TRα2* retroviral vectors; as only about 20% of the cells at the time of harvest were infected in both cases, the increase in *TRα2* mRNA in the pBird-*TRα2*-infected cells is an underestimate. PCR cycle numbers were 30 for *TRα2* and 22 for *G3PDH*.

to induce their differentiation. After various periods of time, we determined the percentage of GFP-positive cells with morphological features characteristic of oligoden-

Fig. 5. Analysis of *TRΔα* mRNAs and effect of the expression of *TRΔα1* or *TRΔα2* transgenes on rat OPC differentiation *in vitro*. (A) P7 OPCs were purified and cultured in slide flasks for 4, 6, 8 or 10 days in PDGF without TH or RA. The spontaneously differentiated oligodendrocytes were removed by immunopanning on an anti-GC antibody plate before mRNA was extracted and analysed by RT-PCR (lanes 1–4). In lanes 5 and 6, P7 OPCs were purified, infected with pBird-*TRΔα1* or pBird-*TRΔα2* retroviral vectors, respectively, and cultured in PDGF for 3 days before they were analysed as above. PCR cycle numbers were 38 for *TRΔα1*, 38 for *TRΔα2* and 22 for *G3PDH*. (B and C) OPCs were purified, cultured and analysed as in Figure 3, except that they were infected with either pBird, pBird-*TRΔα1* or pBird-*TRΔα2* retroviral vectors. The cells were either left in PDGF without TH or RA (B) or switched to PDGF and TH to induce differentiation (C).

drocytes (Temple and Raff, 1986). In some experiments, we confirmed the proportion of oligodendrocytes by staining for GC (data not shown).

Expression of the *TRα1* transgene did not significantly affect the spontaneous differentiation rate of OPCs in the absence of TH (Figure 3A). In the presence of TH, however, *TRα1* greatly accelerated oligodendrocyte differentiation; after 3 days in the presence of TH, for example, only about 35% of control cells had differentiated into oligodendrocytes, whereas >85% of the *TRα1*-

overexpressing cells had done so (Figure 3B). Thus, the level of *TR α 1* expression is apparently normally limiting for TH-dependent OPC differentiation. In contrast, overexpression of *TR α 1* delayed oligodendrocyte differentiation induced by RA (Figure 3C), perhaps because the extra TR α 1 receptors competed with RA receptors for RXR subunits or other transcriptional cofactors.

Expression of transgenes encoding inhibitory isoforms of TR α in OPCs has little effect on TH-dependent differentiation

We previously found that the expression of *TR α 2* mRNA decreased as rat OPCs proliferate *in vitro* and *in vivo*, raising the possibility that this decrease may at least partially account for the increased sensitivity of OPCs to the differentiation-promoting action of TH with maturation (Billon *et al.*, 2001). To test whether the expression of a *TR α 2* transgene would delay the differentiation of OPCs in the presence of PDGF and TH, we infected purified P7 rat OPCs with either the control vector (pBird) or a vector that encoded both GFP and TR α 2 (pBird-TR α 2) and then cultured the cells as described in Figure 3. As shown in Figure 4, the expression of the *TR α 2* transgene did not significantly affect either the spontaneous differentiation of OPCs cultured in PDGF without TH (Figure 4A), or the differentiation induced by either TH (Figure 4B) or RA (Figure 4C). Although for technical reasons we were unable to determine whether the *TR α 2* transgene led to the expression of TR α 2 protein in the infected OPCs, it did increase the level of *TR α 2* mRNA in these cells, as assessed by semi-quantitative RT-PCR (Figure 4D). These findings argue against the possibility that the previously reported fall in *TR α 2* mRNA as OPCs mature (Billon *et al.*, 2001) contributes to the normal timing of TH-dependent OPC differentiation.

TR $\Delta\alpha$ 1 and TR $\Delta\alpha$ 2 are two N-terminally truncated isoforms of TR α 1 and TR α 2, respectively, which are generated from an internal promoter located within intron 7 of the *TR α* gene. They lack an intact TH-binding domain and have been proposed to antagonize the TH-induced effects mediated by the TH-binding TRs, at least *in vitro* (Chassande *et al.*, 1997; Plateroti *et al.*, 2001). As shown in Figure 5A, neither TR $\Delta\alpha$ 1 nor TR $\Delta\alpha$ 2 mRNAs could be detected in rat OPCs cultured in PDGF without TH for various periods of time, although they were readily detected in OPCs infected with retroviral vectors encoding them. Moreover, expression of transgenes encoding TR $\Delta\alpha$ 1 or TR $\Delta\alpha$ 2 proteins did not significantly affect either the spontaneous differentiation of OPCs (Figure 5B) or the differentiation of OPCs induced by TH (Figure 5C).

Discussion

In the present study, we provide direct evidence that TR α 1 is required for the normal timing of oligodendrocyte development. We show that there are decreased numbers of oligodendrocytes in the P7 and P14 optic nerve of *TR α 1*^{-/-} mice and that *TR α 1*^{-/-} OPCs fail to respond to the differentiation-promoting effects of TH in culture. Moreover, we show that expression of a *TR α 1* transgene in purified rat OPCs in culture causes precocious differentiation in the presence of PDGF and TH, suggesting that the number of TR α 1 receptors in developing OPCs normally

plays a part in timing TH-dependent OPC differentiation. In contrast, expression of a *TR α 1* transgene in OPCs delayed RA-induced oligodendrocyte differentiation, attesting to the TH specificity of the acceleration effect. The delay in RA-induced differentiation is perhaps not surprising, as both TH and RA receptors (RARs) dimerize with the same partners, RXRs, so that overexpression of TR α 1 may decrease the level of RAR–RXR heterodimers or sequester other transcriptional cofactors.

There is a remarkable concordance between our present results on *TR α 1* mice and previous results on hypothyroid mice and rats. In the present study, we find a 52% decrease of oligodendrocytes in the optic nerves of P7 *TR α 1* mice compared with controls (see Figure 1), whereas previous studies found a reduction of 57% in the optic nerves of P7 hypothyroid mice (Ahlgren *et al.*, 1997) and of 50% in P8 hypothyroid rats (Ibarrola *et al.*, 1996). These findings suggest that the TH regulation of oligodendrocyte accumulation in the postnatal rodent optic nerve depends exclusively on TR α 1.

Although developing rat OPCs seem not to express the *TR β* gene (Baas *et al.*, 1994a,b; Carre *et al.*, 1998), we previously showed that overexpression of TR β 1 in these cells accelerated their differentiation in the presence of PDGF and TH (Billon *et al.*, 2001). These findings are consistent with our present results, as TR β 1 and TR α 1 can bind to the same TREs and recruit some of the same co-regulatory proteins. Just as *TR α 1*, when expressed at sufficiently high levels, can substitute for the *TR β* gene to regulate cochlear development and TH homeostasis in the pituitary in *TR β* ^{-/-} mice (Ng *et al.*, 2001), it seems likely that developing rat OPCs could use TR β receptors to help time their differentiation if they expressed these receptors. These findings further strengthen the emerging view that TRs are largely, but not entirely, interchangeable and that it is the total level of TR expression in a cell that determines its response to TH (Forrest, 2002).

Although the sensitivity of developing OPCs to the differentiation-inducing effect of TH increases with OPC maturation (Barres *et al.*, 1994; Gao *et al.*, 1998), the level of *TR α 1* mRNA in OPCs does not increase (Billon *et al.*, 2001). We showed previously, however, that the level of *TR α 2* mRNA progressively decreases in OPCs as they proliferate *in vitro* and *in vivo* (Billon *et al.*, 2001). Since TR α 2 was suggested to inhibit TH signalling (Katz and Lazar, 1993; Burgos-Trinidad and Koenig, 1999; Macchia *et al.*, 2001), we previously proposed that the progressive decrease in TR α 2 may help to explain the increasing sensitivity of OPCs to TH-induced differentiation with OPC maturation (Billon *et al.*, 2001). Our present findings argue against this proposal. First, as described above, a deficiency in *TR α 1* has virtually the same effect on oligodendrocyte development in the P7 mouse optic nerve as a deficiency in TH, suggesting that TR α 1 alone is responsible for the TH-dependent timing of OPC development in the nerve. Secondly, expression of a *TR α 2* transgene does not significantly decrease TH-induced differentiation of rat OPCs in culture.

Two other TR α isoforms have been shown to inhibit TH signalling in overexpression experiments—TR $\Delta\alpha$ 1 and TR $\Delta\alpha$ 2 (Chassande *et al.*, 1997; Plateroti *et al.*, 2001). Is it possible that the increased sensitivity of OPCs to the differentiation effects of TH during development results

from a decrease in these two isoforms? Our failure to detect *TRΔα1* and *TRΔα2* mRNAs in OPCs regardless of their maturation state would seem to exclude this possibility. Moreover, we find that the expression of a *TRΔα1* or a *TRΔα2* transgene in OPCs does not delay TH-induced differentiation. Taken together, our findings make it very unlikely that inhibitory isoforms of TRα normally play a part in timing oligodendrocyte development.

The roles of *TRΔα1* and *TRΔα2* in general are still unclear. Mice that lack all TRα isoforms are viable and fertile but have defects in bone and intestine development (Gauthier *et al.*, 2001; Plateroti *et al.*, 2001). In contrast, mice that lack both TRα1 and TRα2 but still express *TRΔα1* and *TRΔα2* stop growing by 2 weeks after birth and die within 5 weeks (Fraichard *et al.*, 1997), suggesting that expression of *TRΔα1* and *TRΔα2* on their own are lethal.

Although TH deficiency has been shown to delay both CNS myelination and OPC development in the rodent optic nerve, inactivation of *TRα* and *TRβ* genes, either alone or together, has been reported to affect neither myelination nor the number of OPCs in the P15 mouse optic nerve (Baas *et al.*, 2002). This was proposed to be another example of TR deficiency producing a milder CNS phenotype than TH deficiency (Gauthier *et al.*, 1999; Göthe *et al.*, 1999), suggesting that unliganded TRs are responsible for many of the CNS defects in hypothyroid animals (Morte *et al.*, 2002), including the defects in oligodendrocyte development. As discussed above, however, our present findings indicate that a deficiency in *TRα1* is sufficient to produce a decrease in oligodendrocyte development in the P7 optic nerve that is very similar to that seen in hypothyroidism, suggesting that the ligand-bound TR is required for promoting oligodendrocyte development. It is not clear if there is a real discrepancy between our results and those of Baas *et al.* (2002). While they identified oligodendrocytes by staining sections of P15 optic nerve for myelin basic protein (MBP), we stained freshly dissociated cells from P7 and P14 optic nerve. As the difference we found between WT and *TRα1*^{−/−} nerves at P14 was much less than at P7, it is possible that the difference would be even less at P15 and would be missed by staining sections for MBP, where individual oligodendrocytes cannot easily be identified and quantified.

It is known that some OPCs persist in the adult rat optic nerve (Ffrench-Constant and Raff, 1986; Wolswijk and Noble, 1989). Interestingly, the adult optic nerves in *TRα*^{−/−}*TRβ*^{−/−} mice, and to a lesser extent in *TRβ*^{−/−} mice, were reported to contain an increased number of proliferating OPCs compared to WT adult nerves (Baas *et al.*, 2002). This finding raises the possibility that *TRβ* may normally be expressed in adult OPCs and play a part in OPC differentiation in the adult optic nerve, a possibility that would be worth testing.

It is still unknown how TH promotes OPC differentiation. It has recently been found that it rapidly inhibits the expression of *E2F-1* in purified rat OPCs; the *E2F-1* promoter contains a negative TRE (called a Z-element) that binds TRs, which directly activate *E2F-1* transcription in the absence of TH and repress it in the presence of TH (Nygård *et al.*, 2003). As *E2F-1* promotes progression from G₁ into S phase of the cell cycle (Helin, 1998), its

repression by TH is likely to contribute to the cell cycle withdrawal and differentiation of OPCs in response to TH (Nygård *et al.*, 2003). On a slower time-scale, TH also influences the expression of other genes that probably help induce OPC to exit cell cycle and differentiate; by 16 h, for example, it stimulates an increase in mRNAs that encodes various cyclin-dependent protein kinase inhibitors, and, by 24 h, it decreases the level of cyclin D1 and D2 proteins (Tokumoto *et al.*, 2001). Moreover, the differentiation-inducing effect of TH is blocked by the expression of a dominant-negative form of p53, which inhibits both p53 and other members of the p53 family (Tokumoto *et al.*, 2001), although it is still uncertain which family members are important for OPC differentiation and how they act.

In conclusion, we have provided the first direct evidence that *TRα1* mediates the differentiation-promoting effect of TH on OPCs. It remains a major challenge to identify all of the target genes regulated by *TRα1* in this response.

Materials and methods

Purification and culture of rat OPCs

Sprague/Dawley rats were obtained from the breeding colony of University College London. All chemicals were from Sigma unless otherwise indicated. Optic nerves were removed from postnatal day 7 (P7) rats and dissociated as described previously (Barres *et al.*, 1992). OPCs were purified to >99% purity by sequential immunopanning, as described previously (Barres *et al.*, 1992). The purified cells were plated on poly-D-lysine (PDL)-coated tissue culture flasks (Falcon) and cultured in 8% CO₂ at 37°C in a modified Bottenstein–Sato medium (Bottenstein *et al.*, 1979), containing insulin (10 µg/ml), PDGF-AA (Preprotech, 10 ng/ml), NT3 (5 ng/ml), human transferrin (100 µg/ml), BSA (100 µg/ml), progesterone (60 ng/ml), sodium selenite (40 ng/ml), N-acetyl-cysteine (60 µg/ml), putrescine (16 µg/ml), forskolin (5 µM), biotin (10 ng/ml) and penicillin–streptomycin–glutamine (Gibco–BRL).

TRα retrovirus vectors

To identify transfected cells, we used the pBird vector, which encodes enhanced GFP, driven by the CMV promoter (Tokumoto *et al.*, 2001). To overexpress *TRα1*, *TRα2*, *TRΔα1* or *TRΔα2*, we cloned the respective mouse cDNAs (Chassande *et al.*, 1997) into the pBird vector to create pBird-*TRα1*, pBird-*TRα2*, pBird-*TRΔα1* and pBird-*TRΔα2* retroviral vectors, which all encode the transgenes under the control of the Moloney murine leukemia virus long terminal repeat promoter. Recombinant retroviruses were produced and concentrated as described previously (Kondo and Raff, 2000b).

Infection of rat OPCs with retroviral vectors

After 2 days in culture in PDGF without TH, purified rat OPCs were infected with concentrated retroviral supernatant for 3 h, washed and removed from the culture flask with trypsin. The cells were then replated at clonal density in the presence of PDGF and the absence of TH. After 1 day, TH (triiodothyronine, 40 ng/ml) or RA (all-*trans*, 1 µM) was added, and the percentage of GFP+ cells that had differentiated into oligodendrocytes was determined at different time points using a Leica inverted fluorescence microscope and morphological criteria (Temple and Raff, 1986). In some experiments, the percentage of oligodendrocytes was confirmed by staining the cells with a monoclonal anti-GC antibody, as described below.

Mouse optic nerve cell cultures

BALB/c wild-type and *TRα1*^{−/−} mice were produced as described previously (Wikström *et al.*, 1998) and bred at the animal facility at University College London. Optic nerves were removed from P0 to P12 mice, and the cells were dissociated with papain. The cells were cultured on either PDL-coated glass coverslips or PDL-coated Nunc slide flasks in the same medium as described above, except that forskolin was used at 15 µM and the medium was supplemented with the non-hydrolysable cyclic-AMP analogue CPT (200 µM) and the cyclic nucleotide phosphodiesterase inhibitor IBMX (100 µM) to increase intracellular cyclic-AMP levels and, thereby, cell survival. The cells on coverslips were stained for GC after 2 h, as described below. The cells in slide flasks

were cultured for 3 days in the presence of PDGF, without TH. They were then treated with either PDGF, PDGF and TH, or PDGF and RA for an additional 5 days. Subsequently they were stained for GC, as described below.

Immunocytochemistry

Cells were fixed in 2% paraformaldehyde in PBS for 5 min at room temperature. After washing with PBS, they were incubated for 30 min in normal goat serum to block non-specific staining. They were then incubated for 1 h in monoclonal anti-GC antibody (Raff *et al.*, 1978; Ranscht *et al.*, 1982) (supernatant, diluted 1/5), washed in PBS, and incubated for 1 h in a mixture of fluorescein- or Texas Red-coupled goat anti-mouse IgG3 antibodies (Nordic; diluted 1/100) and bisbenzamide (Sigma; 5 ng/ml). Coverslips were mounted in Citifluor mounting medium (CitiFluor, UK) and examined with a Zeiss Axioskop fluorescence microscope.

RT-PCR analysis

Purified P7 OPCs were cultured in PDL-coated Falcon tissue culture flasks for different times in PDGF without TH. They were then re-purified by removing any spontaneously differentiated oligodendrocytes on an anti-GC antibody panning dish (Billon *et al.*, 2001). Poly(A)⁺ mRNA was prepared using a QuickPrep Micro mRNA Purification kit (Pharmacia Biotech), and cDNA synthesis was carried out using a SMART PCR cDNA Synthesis kit (Clontech Laboratories), each according to the manufacturer's instructions. The PCR-amplified total cDNA was used as the template for the RT-PCR reaction. Before performing the PCR reaction, the amplified cDNA samples were all electrophoresed on the same 1% agarose gel, stained with ethidium bromide (EtBr; 0.4 µg/ml), and normalized according to the intensity of staining.

The following oligonucleotide DNA primers were synthesized. For *TRα* gene variants, the common 5' primer was 5'-TGGGCAAGTC-ACTCTCTGC-3'. The *TRα1*-specific 3' primer was 5'-TCCTGAT-CCTCAAAGACCTC-3', and the *TRα2*-specific 3' primer was 5'-CAA-CTGCTGCTCAAGCTGC-3'. For *TRΔα1*, the 5' primer was 5'-CTC-TGTGATCCTGCTGTTCCACAG-3', and the 3' primer was 5'-CGAC-TTTCATGTGGAGGAAG-3'. For *TRΔα2*, the 5' primer was 5'-CTC-TGTGATCCTGCTGTTCCACAG-3', and the 3' primer was 5'-CCTGA-ACAACATGCATTCCGA-3'. For *glyceraldehyde-3-phosphate dehydrogenase (G3PDH)*, the 5' primer was 5'-ACCACAGTCCATGCCA-TCAC-3', and the 3' primer was 5'-TCCACCACCCTGTTGCTGTA-3'. All primers were dissolved in water. The RT-PCR reaction conditions were carried out in 25 µl of reaction mixture containing 200 pg of PCR-amplified total cDNA as template, 300 nM 5' PCR primer and 300 nM 3' PCR primer, 0.2 mM dNTP, 2 mM MgSO₄, 60 mM Tris-SO₄ (pH 8.9), 18 mM ammonium sulfate and 2.5 U of Platinum High fidelity Taq DNA polymerase (Invitrogen). The reaction mixture was denatured for 2 min at 94°C. The PCR reaction was then started, using 94°C for 20 s for the denaturing step, 58°C for 20 s for the annealing step and 72°C for 40 s for the elongation step. The PCR products were electrophoresed in 2% agarose gel and stained with EtBr. The numbers of PCR cycles are given in the legends to Figures 4 and 5.

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