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RESEARCH ARTICLE

Purkinje cells and Bergmann glia are primary targets of the TRα1 thyroid hormone receptor during mouse cerebellum postnatal development

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ABSTRACT

Thyroid hormone is necessary for normal development of the central nervous system, as shown by the severe mental retardation syndrome affecting hypothyroid patients with low levels of active thyroid hormone. The postnatal defects observed in hypothyroid mouse cerebellum are recapitulated in mice heterozygous for a dominant-negative mutation of *Thra*, the gene encoding the ubiquitous TRα1 receptor. Using CRE/*loxP*-mediated conditional expression approach, we found that this mutation primarily alters the differentiation of Purkinje cells and Bergmann glia, two cerebellum-specific cell types. These primary defects indirectly affect cerebellum development in a global manner. Notably, the inward migration and terminal differentiation of granule cell precursors is impaired. Therefore, despite the broad distribution of its receptors, thyroid hormone targets few cell types that exert a predominant role in the network of cellular interactions that govern normal cerebellum maturation.

KEY WORDS: Thyroid hormone receptor, Cerebellum, Mouse

INTRODUCTION

Thyroid hormone (3,3′,5-triiodo-L-thyronine, or T3) is essential for proper neurodevelopment. Deficiency in thyroid hormone activity during the perinatal period causes a syndrome known as cretinism in humans and results in severe mental retardation. Despite decades of investigations, the cellular mechanisms underlying the T3 neurodevelopmental function remain unclear. The postnatal development of the rodent cerebellar cortex has emerged as a preferred model, because of its sensitivity to T3 deficiency (Bernal, 2007; Koibuchi, 2008) and its relatively simple cytoarchitecture divided into three layers, i.e. the outermost molecular layer, the Purkinje cell (PC) layer and the inner granular layer (IGL).

Mouse cerebellum development continues during 3 weeks after birth. During this postnatal period, a fourth outermost cellular layer called the external granular layer (EGL) is present. The EGL is populated by proliferating granule cell (GC) precursors, which randomly exit the cell cycle and undergo radial migration toward the IGL along the radial fibers of specialized astrocytes called Bergmann glia (BG), to ultimately differentiate into GCs (Chédotal, 2010). At the same time, GABAergic interneuron (GI) progenitors and oligodendrocyte precursor cells migrate in the opposite direction from the white matter, GIs undergoing terminal differentiation in the molecular layer to become stellate or basket cells, or in the IGL to become Golgi cells.

T3 deficiency impairs the differentiation of most cerebellar cell types. GC precursor proliferation is slower and radial migration impaired, resulting in the persistence of the EGL beyond postnatal day (P) 21 (Lauder, 1977). GI maturation is also retarded (Manzano et al., 2007b; Wallis et al., 2008). BG radial fiber development is altered (Morte et al., 2004; Manzano et al., 2007a) and PC dendritic arborization dramatically reduced. These multiple alterations ultimately compromise synaptogenesis in the molecular layer (Nicholson and Altman, 1972b; Vincent et al., 1982-1983), a process involving close contacts between different various neuronal processes and BG (Ango et al., 2008).

The T3 response is mediated by nuclear receptors (TRα1 or TRβ1/2) encoded by *Thra* and *Thrb* genes. In the brain, *Thra* expression is ubiquitous but higher in postmitotic neurons (Mellström et al., 1991; Bradley et al., 1992; Wallis et al., 2010), whereas *Thrb* expression is restricted to few cell types and mostly occurs when the cerebellum is mature (Bradley et al., 1992). Although introducing a mutation in the mouse *Thrb* gene can alter cerebellum development (Portella et al., 2010), we previously found that all features of congenital hypothyroidism can be phenocopied by a *Thra* knock-in mutation, introducing the TRα1^{L400R} amino-acid substitution (Quignodon et al., 2007b; Fauquier et al., 2011). Accordingly, the first germline mutations of the human *THRA* gene, which were recently reported, caused a significant cognitive impairment (Bochukova et al., 2012; van Mullem et al., 2012) and supported a major role for TRα1 in the regulation of neurodevelopment by thyroid hormone.

Whether T3 acts directly or indirectly on all cellular differentiation processes that take place postnatally in the cerebellum development of rodents is currently unclear. As expected from the ubiquitous *Thra* expression, primary cultures enriched in GCs (Thompson, 1996; Quignodon et al., 2007a; Chatonnet et al., 2012), PCs (Kimura-Kuroda et al., 2002; Heuer and Mason, 2003; Boukhtouche et al., 2010) or astrocytes (Mendes-de-Aguiar et al., 2008) display various T3 responses *in vitro*. This suggests that T3 exerts an independent influence on each cerebellar cell type. However, cerebellum development is coordinated by permanent interactions between cell types of glial and neuronal origin,

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mediated by cells contacts or diffusion of neurotrophins and growth factors. T3 is required for the production of insulin-like growth factor 1 (Elder et al., 2000), nerve growth factor (Clos and Legrand, 1990), neurotrophin 3 (Lindholm et al., 1993) and brain-derived neurotrophic factor (Neveu and Arenas, 1996). Therefore, some of the cytological alterations observed in T3-deficient cerebellum might indirectly result from a defect in neurotrophin production (Gomes et al., 1999).

To unravel the direct and indirect influence of T3 during development, we previously generated a mouse strain carrying the *TRα*^{AMI} allele (Quignodon et al., 2007b). In this genetic setting, the expression pattern of the dominant-negative TRα1^{L400R} receptor is dependent on CRE-mediated recombination, which eliminates an upstream cassette stopping transcription, flanked by *loxP* sequences. As a consequence, the TRα1-mediated response is inhibited in all cells derived from *Cre*-expressing cells, in which the dominant-negative receptor is expressed. By analyzing a collection of transgenic mice differing by the *Cre* transgene expression pattern, we can thus separate cell-type autonomous versus indirect consequences of TRα1^{L400R} expression on cerebellar development. We previously demonstrated that TRα1^{L400R} expression impairs early oligodendrocyte precursor cells differentiation and myelin formation indirectly, by altering the ability of PCs, GCs and astrocytes to sustain these processes (Picou et al., 2012). Here, we present a detailed analysis of the blockade of TRα1-mediated T3 signaling on GC, PC, BG and GI development, which indicates that cell-type autonomous influence of TRα1^{L400R} is limited to a small fraction (less than 5%) of the cerebellum postmitotic cells. We conclude that in wild-type animals, primary function of the liganded TRα1 receptor during cerebellum development is restricted mainly to PCs and BG. T3 stimulation of these cells indirectly influences cerebellum development in a global manner, by defining a microenvironment suitable for proper neuronal differentiation and synapse formation.

RESULTS

A collection of Cre mice to restrict TRα1^{L400R} mutation to defined cell types

Different well-characterized transgenic mouse strains expressing CRE recombinase, or its tamoxifen-inducible version *Cre-ER*^{T2}, were crossed with mice carrying the *TRα*^{AMI} allele to induce TRα1^{L400R} expression in specific cell populations. As the mutant allele cannot be recognized by specific antibodies in heterozygous cells, we also introduced the *Rosa-loxP-stop-loxP-EYFP (ROSAYFP)* reporter transgene, to monitor CRE recombination pattern and efficacy (supplementary material Fig. S1). Although not mentioned in the following, yellow fluorescent protein (YFP) immunostaining was used systematically to identify possible individual variability in the efficacy of spontaneous or tamoxifen-induced recombination. Table 1 reports all the mouse strains that were used, the simplified nomenclature employed thereafter, and quantification of the recombination efficacy.

Nestin-Cre was used as a positive control, enabling TRα1^{L400R} expression in all neural cell types, but not in peripheral organs (Tronche et al., 2004). Tamoxifen treatment of *GlastCre-ER*^{T2} mice triggered *loxP* recombination in all astrocyte types, including BG (Slezak et al., 2007). *Otx2Cre-ER*^{T2} is also tamoxifen inducible and recombination was specific for posterior lobe GCs and their precursors (Fossat et al., 2006). *Ptf1a-Cre* (Pascual et al., 2007) provided a prenatal recombination in GABAergic neurons (GIs and PCs), whereas *L7-Cre* recombination occurred only in PCs at a later stage (around P8). This collection allowed us to address the cell type-autonomous consequences of TRα1^{L400R} expression on all the major cell types and their precursors previously reported to respond to T3 in the postnatal cerebellum (GCs, PCs, GIs, BG), excluding oligodendrocyte precursor cells, which were analyzed in a separate study (Picou et al., 2012). All mice were viable and apparently healthy, except for *TRα*^{AMI}*/N*, with pan-neural expression of TRα1^{L400R}, which usually did not survive beyond P25. As expected (Quignodon et al., 2007b) serum T3 level, measured at P15, were not changed in these mice (data not shown).

Secondary effects of TRα1^{L400R} expression on GC precursors proliferation, migration and maturation

Broad expression of TRα1^{L400R} in *TRα*^{AMI}*/N* mice resulted in the thickening of the EGL and its persistence beyond P21, which is typical of a defect in T3 signaling (Fig. 1A). During normal cerebellum development, GC precursors in the EGL exit the cell cycle, and then express *Pax6*, which is required for proper radial migration (Swanson and Goldowitz, 2011). *Pax6* expression is only transient and is downregulated when cells undergo terminal differentiation in IGL to become immunoreactive for NeuN. Combining 5-ethynyl-2′-deoxyuridine (EdU) incorporation with PAX6 immunostaining, we showed that in *TRα*^{AMI}*/N* mice, GC precursor proliferation was higher in the mutant at P15 (Table 2), persisting beyond P21. The accumulation of PAX6^{+} GC precursors both at P15 and P21 suggested that radial migration was affected too. Many PAX6^{+} cells were observed in the IGL (Fig. 2), indicating that terminal maturation of GCs was also impaired.

To define the direct influence of TRα1^{L400R} on GC precursors and their progeny, we treated with tamoxifen *TRα*^{AMI}*/O*^{T2} mice, and control littermates (without the *Otx2Cre-ER*^{T2} transgene), to define the direct influence of TRα1^{L400R} in GC precursors and their progeny. Quantitative polymerase chain reaction (qRT-PCR) was used to measure the expression level of the *Hairless* gene (*Hr*), a well-characterized TRα1 target gene expressed in GCs (Thompson,

Table 1. Genotypes, mice and nomenclature

Promoter	Nomenclature	Granular neurons	GABAergic neurons		Astrocytes/ Bergmann glia
			PC	GI	
Nes (Nestin)	TRα^{AMI}/N (*n*=6)	100	100	100	100
Ptf1a (pancreas transcription factor 1a)	TRα^{AMI}/P (*n*=10)	<0.1	71±6	94±4	<0.1
Slc1a3 (Glast, solute carrier 1a3), P1 tamoxifen injection	TRα^{AMI}/G^{T2} (*n*=8)	0	0	0	88±6
Pcp2 (L7, purkinje cell protein 2)*	TRα^{AMI}/L (*n*=6)	<0.1	96±5	0	0
Otx2‡	TRα^{AMI}/O^{T2} (*n*=3)	44±11 (EGL, p15) 4±3 (IGL, p21)	0	0	0
Ptf1a and Slc1a3, P1 tamoxifen injection	TRα^{AMI}/PG^{T2} (*n*=5)	<0.1	74±10	93±7	<0.1

Percentage of YFP+ cells observed at P15 in TRα^{AMI}/ROSAYFP/Cre cerebellum.
*Starting at P8 only.
‡*Otx2* is only expressed in posterior lobes.

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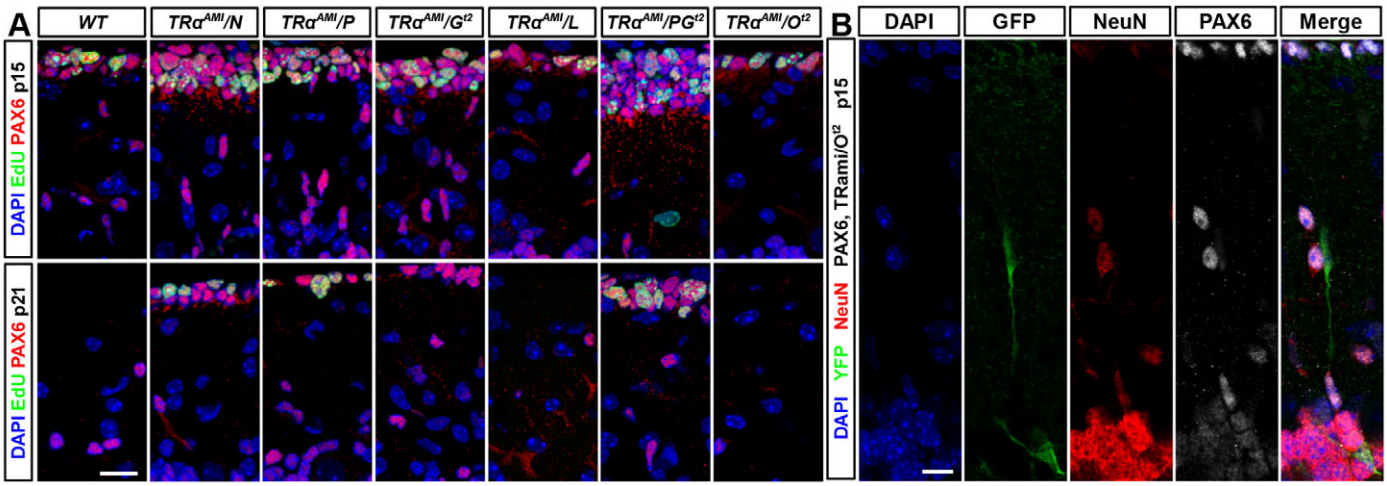

Fig. 1. TRα1-mediated signaling in PCs and BG influence GC precursor inward migration and cell-cycle exit. (A) Top row: P15 cerebellum sections show the presence of PAX6$^+$ (red) GC precursors, some of which have undergone cell division in the last 24 hours, as shown by EdU incorporation (green). The EGL is significantly thicker in *TRα^{AMI}/N/P/G^{T2}/PG^{T2}*. Bottom row: at P21, the EGL has disappeared in wild-type animals. However, it is still persistent in *TRα^{AMI}/N/P/PG^{T2}* animals, with numerous proliferating cells. In *TRα^{AMI}/G^{T2}*, PAX6$^+$ GC precursors are also present, but have stopped proliferating. (B) *Targeting TRα1^{L400R}* to GC precursors does not impair cell migration. In P15 cerebellar sections from *TRα^{AMI}/O^{T2}* animals, PAX6$^+$ (gray) GC precursors that had undergone recombination, as shown by YFP expression (green), were still able to migrate towards the NeuN$^+$ (red) IGL. WT, wild type. Scale bars: 10 µm.

1996). As expected, *Hr* expression was reduced in tamoxifen-treated animals (measured from whole cerebellum; 61.2±13% of wild-type littermate expression, $P<0.05$, $n=6$), confirming that TRα1-mediated signaling was inhibited in posterior lobe GCs. However, histological examination failed to indicate any defect in GC precursors proliferation and migration in the *TRα^{AMI}/O^{T2}* mice. YFP$^+$ cells, expressing TRα1^{L400R}, displayed typical figures of migration along the BG fibers (Fig. 1B) and underwent normal differentiation in the IGL (Fig. 2B). This allowed us to conclude that the persistence of the EGL, due to a defect in radial migration of GC precursors, as observed in *TRα^{AMI}/N* mice, is not a cell-autonomous consequence of TRα1^{L400R} expression, but results from an alteration of the GC precursor environment.

We thus analyzed in more detail the behavior of GC precursors in mice expressing TRα1^{L400R}, either in BGs (*TRα^{AMI}/G^{T2}*) or in PCs (*TRα^{AMI}/P*). Both of these mutant strains showed a persistence of the EGL until P21 (Fig. 1A), although this EGL was not continuous along the pial surface. EdU incorporation revealed two different situations, with the increase in cell proliferation at P15 and the maintenance of cell-cycling at P21 only observed in *TRα^{AMI}/P* mice. Terminal maturation of GCs in IGL was impaired in both *TRα^{AMI}/G^{T2}* and *TRα^{AMI}/P* (Fig. 2), as shown by the presence of PAX6$^+$ cells in the IGL. Interestingly, no alteration was observed when TRα1^{L400R} was expressed in PCs from P8 (*TRα^{AMI}/L*). In this case, the EGL was unaffected at P15, and normally disappeared before P21. These observations suggest that the inability of PCs to sustain GC precursor cell-cycle exit and their migration at later stages is a consequence of an early defect in the PC maturation process.

Finally, when we combined TRα1^{L400R} expression in PCs and BG (*TRα^{AMI}/PG^{T2}*), the accumulation of GC precursors in the EGL was much more pronounced than when these cell types were targeted separately (Fig. 1A; Table 2), thus confirming that the two cell types exert different influences on GC precursors.

TRα1^{L400R} prevents PCs development in a cell type-autonomous manner

PC postnatal growth was found to be affected by the blockade of TRα1-mediated T3 signaling (Fig. 3). Broad expression of TRα1^{L400R} in the brain (*TRα^{AMI}/N*) or early expression in both PCs and GIs (*TRα^{AMI}/P*) deeply altered PC arborization. This was often accompanied by a misalignment of the cell bodies. No sign of recovery was observed at P21, suggesting permanent and irreversible damage. These defects were absent when expression of TRα1^{L400R} started in PCs after P8 (*TRα^{AMI}/L*) or was targeted to glial cells (*TRα^{AMI}/G^{T2}*). To ascertain that defect in PC differentiation was

Table 2. Granule cell precursors and Bergmann glia differentiation

P15

Genotype	Control (n=6)	TRα^{AMI}/N (n=5)	TRα^{AMI}/P (n=3)	TRα^{AMI}/G^{T2} (n=4)	TRα^{AMI}/L (n=3)	TRα^{AMI}/PG^{T2} (n=3)	TRα^{AMI}/O (n=3)
PAX6$^+$ cell/100 µm EGL	22±7	46±11**	41±3**	40±3**	18±5 (NS)	60±6**	23±1 (NS)
% EdU+ cells	40±8	51±5*	61±3*	45±7 (NS)	35±4 (NS)	58±2*	ND
% mislocalized BG	2±1	29±6**	2±3 (NS)	41±11**	1±1 (NS)	43±11**	ND

P21

Genotype	Control (n=5)	TRα^{AMI}/N (n=5)	TRα^{AMI}/P (n=4)	TRα^{AMI}/G^{T2} (n=4)	TRα^{AMI}/L (n=3)	TRα^{AMI}/PG^{T2} (n=3)	TRα^{AMI}/O (n=3)
PAX6$^+$ cell/100 µm EGL	1±1	24±6**	14±3**	8±2**	0±0(NS)	23±3**	1±1 (NS)
% EdU+ cells	0±0	38±11**	49±8**	2±4 (NS)	0±0 (NS)	34±11**	ND
% mislocalized BG	1±1	21±4**	0±0 (NS)	34±5**	1±1 (NS)	30±2**	ND

Data are mean ± s.d.; *P<0.05; **P<0.005 compared with control; NS, not significant; ND, not determined.

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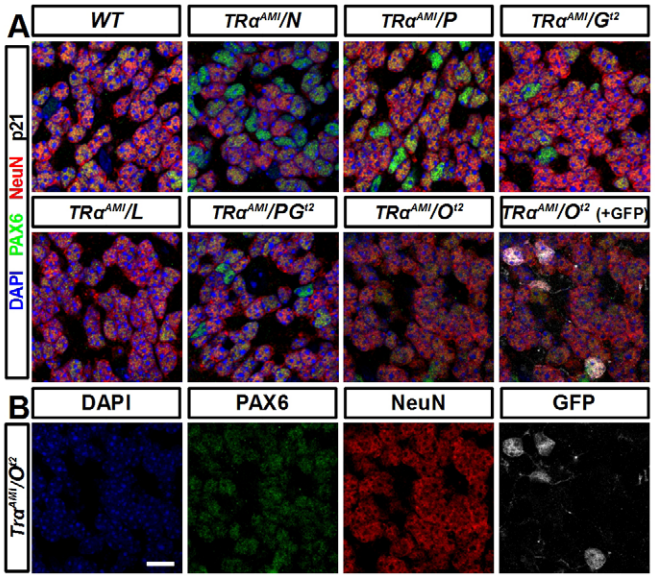

Fig. 2. Impaired granule cell differentiation in the IGL at P21. (A) At P21, downregulation of PAX6 (green) and upregulation of NeuN (red) in GCs of the IGL is impaired upon pan-neural TRα1^{L400R} expression (*TRα^{AMI}/N*). A similar defect is observed in sections from mice expressing TRα1^{L400R} early in PCs and/or BG (*TRα^{AMI}/P*, *TRα^{AMI}/G^{T2}*,*TRα^{AMI}/PG^{T2}*) but not if expression starts in PCs after P8 (*TRα^{AMI}/L*) or is restricted to the GC lineage (*TRα^{AMI}/O^{T2}*). (B) YFP immunostaining in *TRα^{AMI}/O^{T2}* (white) shows that cells that underwent CRE-mediated recombination, and are thus expected to express TRα1^{L400R}, can achieve terminal differentiation. WT, wild type. Scale bar: 10 µm.

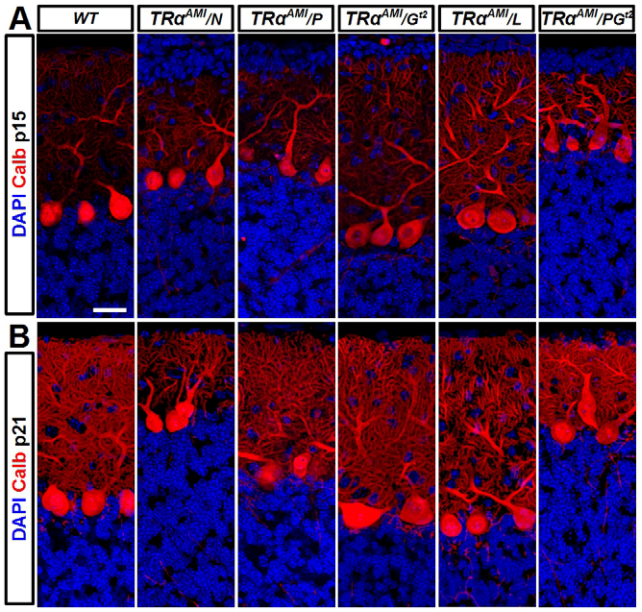

Fig. 3. Early TRα1^{L400R} expression cell type-autonomously affects Purkinje cell growth. Anti-Calbindin28K antibody was used to stain PCs during (P15; A) or at the end (P21; B) of their dendritic arborization. This process is impaired when TRα1^{L400R} is expressed early in PCs (*TRα^{AMI}/N*, *TRα^{AMI}/P*, *TRα^{AMI}/PG^{T2}*) but not if it is expressed in BG only (*TRα^{AMI}/G^{T2}*) or if expression in PCs started after P8 (*TRα^{AMI}/L*). WT, wild type. Scale bar: 20 µm.

a cell type-autonomous consequence of expressing TRα1^{L400R} before P8, we measured the size of the dendritic trees and confirmed that significant and persistent reduction was limited to *TRα^{AMI}/N*, *TRα^{AMI}/P* and *TRα^{AMI}/PG^{T2}* (Table 3).

TRα1^{L400R} prevents Bergmann glia maturation in a cell type-autonomous manner

Maturation of BG normally leads to the elaboration of strait radial fibers stretching from the cell body, located in the PC layer, towards the pial surface. In *TRα^{AMI}/N* and *TRα^{AMI}/G^{T2}*, some BG cells were found within the molecular layer (Fig. 4A). This mislocalization did not occur when the mutant was expressed in PCs and GIs only (*TRα^{AMI}/P*) (Table 2). S100β immunostaining was less abundant, and showed a peculiar distribution, with a preferred association with BG fibers. The morphology of these fibers was also affected, as shown by glial fibrillary acidic protein (GFAP) immunostaining (Fig. 4B). The presence of radial fibers with a crooked morphology, failing to reach the pial surface, was observed only in mice expressing TRα1^{L400R} in BG (*TRα^{AMI}/N and TRα^{AMI}/G^{T2}*), indicating a clear cell type-autonomous effect of the mutation.

Table 3. GABAergic neuron differentiation and synaptogenesis

P15

Genotype	Control (*n*=26)	TRα^{AMI}/N (*n*=5)	TRα^{AMI}/P (*n*=8)	TRα^{AMI}/G^{T2} (*n*=5)	TRα^{AMI}/L (*n*=4)	TRα^{AMI}/PG^{T2} (*n*=4)
PC size	100±3	61±3**	86±6*	96±6 (NS)	101±1 (NS)	84±5*
PAX2$^+$, ML	100±6	220±41**	143±25*	122±19 (NS)	98±27 (NS)	141±8*
PV$^+$, ML	100±5	28±4**	39±5**	89±18 (NS)	113±16 (NS)	35±8**
Total GI, ML	100±3	87±9*	96±13 (NS)	101±8 (NS)	105±20 (NS)	79±14 (NS)
GAD65 syn.	100±10	41±20**	72±16*	83±10 (NS)	72±7**	41±7**

P21

Genotype	Control (*n*=28)	TRα^{AMI}/N (*n*=8)	TRα^{AMI}/P (*n*=7)	TRα^{AMI}/G^{T2} (*n*=4)	TRα^{AMI}/L (*n*=5)	TRα^{AMI}/PG^{T2} (*n*=4)
PC size	100±3	64±5**	87±7**	107±5 (NS)	104±4 (NS)	86±4**
PAX2$^+$, ML	100±15	3291±2070**	573±144**	168±59*	88±108 (NS)	547±40**
PV$^+$, ML	100±5	31±4**	57±9**	86±4 (NS)	103±8 (NS)	41±3**
Total GI, ML	100±3	83±7**	112±41 (NS)	94±9 (NS)	103±10 (NS)	81±5 (NS)
GAD65 syn	100±8	24±12**	43±8**	62±4**	70±8**	44±5*
Pinceau syn.	100±4	0±0**	2±1**	89±4 (NS)	94±3 (NS)	0.3±0.3**

Data are the relative density of neurons on histological sections expressed as the mean percentage of those in control littermates ±s.d.
In the control column, the s.d. is calculated on the entire wild-type population. In other columns, mean and s.d. are calculated on wild-type and mutant littermates only. PV, parvalbumin. *P<0.05; **P<0.005; NS, not significant.

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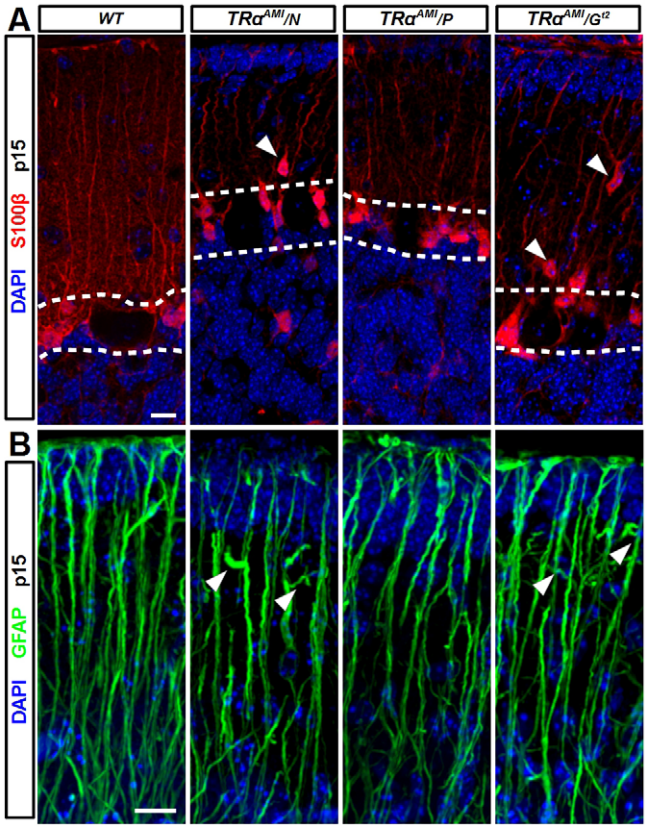

Fig. 4. Defect in Bergmann glia. (A) In wild type, S100β immunostaining at P15 (red) is homogeneously distributed in the cytoplasm of BG, and cell bodies are within the PC layer (between dashed lines). Pan-neural or BG-restricted TRα1^{L400R} expression (*TRα^{AMI}/N*, *TRα^{AMI}/G^{T2}*) leads to a close association of S100β protein with BG processes, and abnormal localization of cell bodies to the molecular layer (arrowheads). (B) GFAP immunostaining (green) shows abnormal morphology and incomplete extension in the EGL (arrowheads) of BG processes upon TRα1^{L400R} expression in BG (*TRα^{AMI}/N*, *TRα^{AMI}/G^{T2}*). Expression of the mutant in PCs (*TRα^{AMI}/P*) did not affect radial glia. WT, wild type. Scale bars: 10 µm.

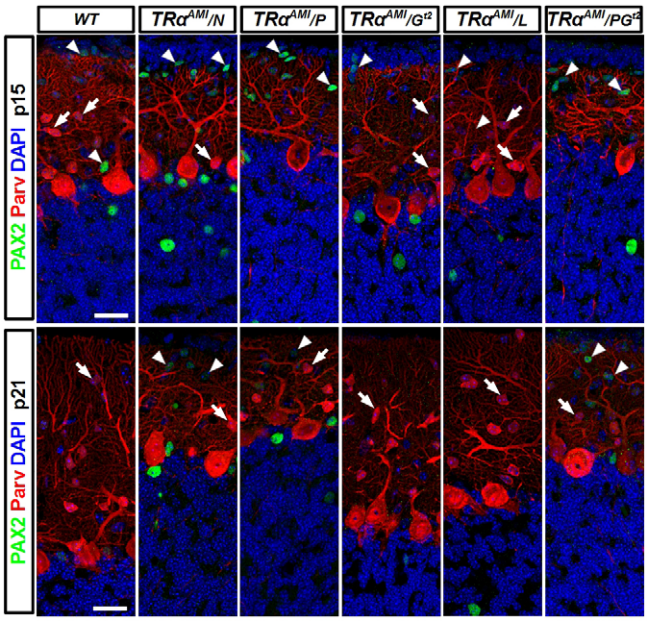

Fig. 5. Defect in GABAergic progenitor differentiation. In the molecular layer of P15 animals (top row), PAX2 (green) is expressed in immature GIs (arrowheads). Parvalbumin (red) stains Purkinje cells and mature GIs (arrows). TRα1^{L400R} expression in GABAergic neurons (PC+GI) increases the number of PAX2$^+$ nuclei, and decreases the number of parvalbumin$^+$ GI. By P21, in wild-type animals, PAX2 has stopped being expressed, and all GIs in the molecular layer express parvalbumin$^+$, whereas GIs in the IGL remain PAX2$^+$. Targeting of TRα1^{L400R} expression to GABAergic neurons impairs differentiation and leads to the persistence of PAX2$^+$ nuclei, and a decreased number of parvalbumin$^+$ interneurons. WT, wild type. Scale bars: 20 µm.

TRα1^{L400R} expression affects the maturation of GIs and the formation of synapses between PCs and GIs in the molecular layer

Our collection of *TRα^{AMI}/Cre* mice did not allow the blockade of TRα1-mediated T3 signaling in GIs only. Therefore, we cannot conclude whether this cell population is directly affected by TRα1^{L400R} expression. Nevertheless, PAX2$^+$ immunostaining of immature GIs and parvalbumin staining of differentiated GABAergic neurons (Fig. 5) revealed a defect in GI maturation in some of the mutant mice. At P15, TRα1^{L400R} expression in *TRα^{AMI}/N*, *TRα^{AMI}/P* an, *TRα^{AMI}/PG^{T2}*, induced an increase in the number of immature GIs and a decrease in the number of their mature form. Only *TRα^{AMI}/N* mice showed a marginal reduction in the total number of GIs. *TRα^{AMI}/L* and, *TRα^{AMI}/G^{T2}* mutants did not show any delay in the maturation of GIs. However, by P21, a stage at which PAX2 immunostaining is virtually absent in the molecular layer, the presence of immature neurons was observed in all but one line (*TRα^{AMI}/L*). This was concomitant with a decrease in the number of parvalbumin$^+$ GIs, but only in mice expressing TRα1^{L400R} in these interneurons, not in *TRα^{AMI}/G^{T2}*. This suggests that before P15, GI differentiation necessitates a functional TRα1, either in this cell type, or in PCs. From P15 onwards, other mechanisms involving T3 action on BG may be involved in GI maturation. Lack of change in the global number of GIs observed in most lines indicates that impairment of T3 signaling mostly acts on late steps of GI development (maturation, synaptogenesis), rather than early events (pool size, migration). The slight decrease observed in *TRα^{AMI}/N* may be a consequence of the general weakness of these mutants.

TRα1-mediated T3 signaling in GIs, PCs and BG is essential for GABAergic synaptogenesis

A number of synapses are established between PCs, GIs, GCs and climbing fibers coming from deep layers. This T3-dependent process (Nicholson and Altman, 1972a) has a major influence on cerebellum function. First, we evaluated GABAergic synaptic density in molecular layer using GAD65 immunostaining to visualize the synapses formed by stellate interneurons on PC dendritic arborization (Fig. 6A). At P15, a strong reduction in number of synapses was observed in *TRα^{AMI}/N* mutants (Table 3). TRα1^{L400R} expression in PCs and immature GIs (*TRα^{AMI}/P*), or PCs only, (*TRα^{AMI}/L*) had a milder effect ($P<0.05$; *TRα^{AMI}/N* versus *TRα^{AMI}/P* and *TRα^{AMI}/L*). Restricted expression in BG (*TRα^{AMI}/G^{T2}*) had no consequence by itself but increased the impact of expressing the dominant-negative receptor in GIs and PCs (*TRα^{AMI}/PG^{T2}*), towards levels similar to those found in *TRα^{AMI}/N* ($P>0.9$; *TRα^{AMI}/N* versus *TRα^{AMI}/PG^{T2}*). At P21, all mutants showed a decreased number of GABAergic synapses, showing that functional TRα1-mediated T3 signaling in GIs, PCs and BGs is crucial for synaptogenesis. Interestingly, reduced synaptic density was the only significant change observed in *TRα^{AMI}/L* mice, showing that

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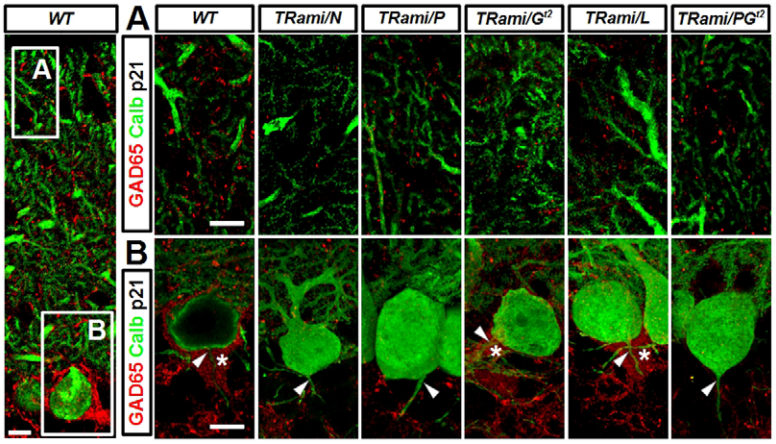

Fig. 6. Reduction in density of GABAergic synapses in the molecular layer. (A,B) The picture on the left shows the location where GABAergic synapses from stellate interneurons (A) and 'pinceau synapses' (B) were imaged for further quantification. (A) GAD65 immunostaining (red) identifies GABAergic synapses made by stellate interneurons on PC dendrites (calbindin, green). At P21, the number of GABAergic synapses is decreased when TRα1^{L400R} is expressed, whatever the expression pattern. (B) In wild-type animals, GAD65 immunostaining (red) shows pinceau synapses (asterisk) made by basket interneurons on PC axon initial segment (arrowhead). TRα1^{L400R} expression in GABAergic neurons only (*TRα^{AMI}/P*), or concomitantly with BG expression (*TRα^{AMI}/N*, *TRα^{AMI}/PG^{T2}*) impairs the formation of these synapses. Expression in BG or PC only has no effect. WT, wild type. Scale bars: 10 µm.

impaired synaptogenesis is a defect that can happen independently of a deficiency in PC arborization. Antibody against GAD65 also stained pinceau synapses, formed by descending basket cell axonal terminals converging on the axonal initial segment of PCs. Pinceau synapses were nearly absent on PCs of *TRα^{AMI}/N*, *TRα^{AMI}/P* and *TRα^{AMI}/PG^{T2}*. As, CRE recombinase is active in *TRα^{AMI}/P* mice in virtually all interneurons but only 70% of PCs, this observation is strongly in favor of a cell type-autonomous effect.

Alteration in growth factor supply by PCs

PCs act as organizers of cerebellar ontogenesis by producing diffusible factors, required in particular for proper GC precursor cell-cycle exit and inward migration. The phenotype of *TRα^{AMI}/P* mice is thus highly suggestive for a defect in the secretion of neurotrophin or growth factors by PCs. From a previous study (Picou et al., 2012) we already know that in these mice mRNA levels are not changed for *Pdgfa*, *Ngf* and *Igf1*, as well as for markers of the sonic hedgehog pathway (*Shh*, *Gli2* and *Ptch1*) ruling out an intervention of several pathways known to participate in cellular interactions during cerebellum development. In an attempt to perform an unbiased search of the defective signaling, we performed a pilot RNA sequencing analysis, comparing whole-cerebellum RNA of two *TRα^{AMI}/P* mice and one control mouse at P8. Although of insufficient sequencing depth (data not shown), this experiment suggested significant changes for few genes (at most 42; data not shown). We used qRT-PCR to measure the mRNA level for some of these in other *TRα^{AMI}/P* and control littermates at several development stages (Fig. 7). This confirmed the slight downregulation of *Etv1* and *Megf10*, a retarded reduction in *Slc17a8* mRNA level (alias *VGLUT3*) and a persistent reduction in *Fgf7* expression. According to published *in situ* hybridization data (Allen Brain Atlas), *Etv1* and *Megf10* are expressed only in the molecular layer, presumably in GIs, whereas the cerebellar expression of *Slc17a8* and *Fgf7* is restricted to the PC layer.

DISCUSSION

For years, the mechanisms by which T3 promotes cerebellar development have remained unclear. We found previously that ubiquitous TRα1^{L400R} expression produces the same defects in cerebellum development than T3 deficiency (Fauquier et al., 2011). As expected, the recently discovered human *THRA* germline mutations have irreversible consequences on neurodevelopment, reminiscent of congenital hypothyroidism (Bochukova et al., 2012; van Mullem et al., 2012; Moran et al., 2013). We now show that these neurodevelopmental alterations can also be phenocopied when the expression of TRα1^{L400R} is restricted to neural tissues: in *TRα^{AMI}/N* mice, GC precursor cell-cycle exit, inward migration and terminal maturation are impaired, PC dendritic growth is reduced, BG is disorganized, GI terminal maturation is retarded and the density of GABAergic synapses is reduced. These observations rule

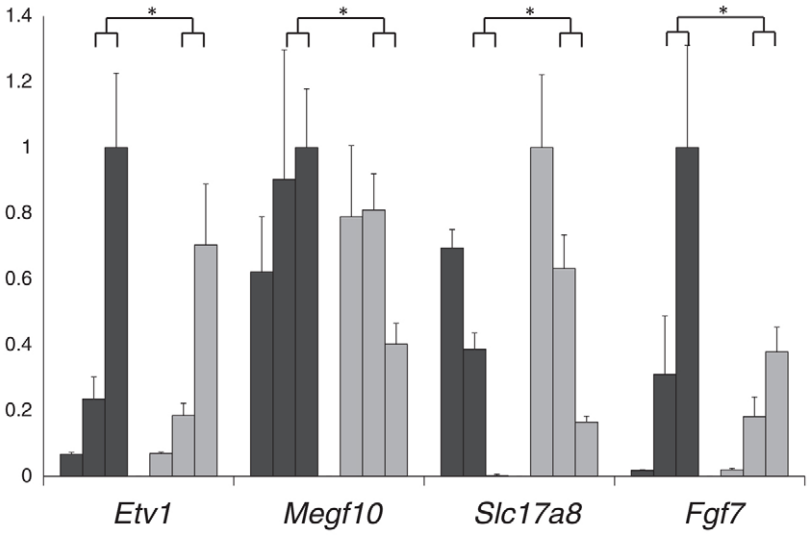

Fig. 7. Gene deregulation in *TRα^{AMI}/P* cerebellum. qRT-PCR was used to measure mRNA level on whole cerebellum at P4, P8 and P15 for *TRα^{AMI}/P* (gray; *n*=3/time point) and control littermates (black; *n*=3/time point) (scale: values are relative to the highest value measured for the gene in control mice; error bars are standard deviations). *Fgf7* encodes a growth factor produced by Purkinje cells. *Etv1* encodes an ETS-related transcription factor, *Megf10* a transmembrane protein with EGF-like domains, *Slc17a8* (alias *VGLUT3*, the vesicular glutamate transporter 3). Data were analyzed by two-way ANOVA and identified an interaction between age and genotype, corresponding to the absence of significant changes in gene expression level at P4 only. Using a paired Student's *t*-test, expression was found to differ significantly for the four genes in *TRα^{AMI}/P* mice compared with age-matched control littermates (*P<0.05) at P8 and P15.

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out the possibility that the previously observed phenotypes were mostly secondary to peripheral defects and metabolic disorders, as reported in other systems (Krysko et al., 2007). The fact that $TR\alpha^{AMI}/N$ do not usually survive beyond P21 also reveals the utmost importance of the neurodevelopmental function of TRα1 for postnatal development.

Although these data confirm the important neurodevelopmental function of the TRα1 receptor, they do not rule out the intervention of TRβ1, which is expressed in the developing cerebellum but only in PCs (Mellström et al., 1991; Bradley et al., 1992) and which dominant-negative mutation also alters their terminal differentiation (Portella et al., 2010). By recruiting transcription co-repressors despite the presence of T3, $TR\alpha1^{L400R}$ behaves as an unliganded TRα1 receptor and impacts neurodevelopment much more than a *Thra* knockout, by exerting a dominant-negative effect on the remaining intact TRα1. It may also to some extend impairs TRβ1 function. However, a previous systematic survey of TRβ1/2-dependent functions argued against this possibility (Quignodon et al., 2007b). Accordingly, *in vitro* differentiation of PCs is altered only after TRα1 knockout (Heuer and Mason, 2003). As TRβ1 mRNA level increases over time, whereas TRα1 expression decreases (Wallis et al., 2010), we favor the possibility that TRα1 and TRβ1 act sequentially during PC maturation. In other cerebellum cell types, such as BG, TRα1 remains the predominant isoform.

More importantly, we show here that two cell types, PCs and BG, play a pivotal role in the neurodevelopmental function of T3. Their proper differentiation is strictly dependent on TRα1-mediated T3 signaling. A complex combination of direct and indirect effects subsequently broadens T3 influence to all the major cell types (summarized in Fig. 8). Our collection of mouse models allows us to recognize the cell type-autonomous consequences of $TR\alpha1^{L400R}$ expression, and to identify secondary consequences, with some limitations. For example, we cannot distinguish between the indirect consequences of $TR\alpha1^{L400R}$ expression in BG and other types of astrocytes present in the IGL and white matter, as all of them express the *Glast-Cre-ER*^{T2} transgene. Also, we do not have a transgene specific for the GI lineage. We can, however, ascertain that $TR\alpha1^{L400R}$ expression has cell type-autonomous consequences in both PCs and BG: dendritic arborization of PCs is reduced in $TR\alpha^{AMI}/P$ mice, whereas the development of radial fibers and alignment of cell bodies of BG is altered in $TR\alpha^{AMI}/G^{T2}$ mice. The fact that T3 promotes an early step (before P8) of PC differentiation is in agreement with previous studies, which used primary cell cultures to minimize cellular interactions (Heuer and Mason, 2003; Boukhtouche et al., 2010). Impairing PC differentiation should also delay two important developmental transitions, known to be T3 dependent: the loss of the ability of PCs to regenerate an axon upon axotomy (Avci et al., 2012) and the elimination of afferent climbing fibers synapses to ensure PC mono-innervation (Clos et al., 1974; Crepel et al., 1981).

As a result of these cell type-autonomous effects, other cell types are indirectly affected. Disruption of T3 signaling in both early PCs and BGs ($TR\alpha^{AMI/}PG^{T2}$) is sufficient to produce a maximal effect and produce a phenotype resembling hypothyroidism. One of our main conclusions is that the persistence of the EGL beyond P21 is not a cell type-autonomous consequence of $TR\alpha1^{L400R}$ expression. This probably explains why transcriptome analyses, performed on whole cerebellum or enriched preparations of granule cells, identified a very limited number of putative T3 responsive genes (Poguet et al., 2003; Quignodon et al., 2007a; Takahashi et al., 2008; Dong et al., 2009; Chatonnet et al., 2012). The indirect mechanism underlying the control of GC precursor cell-cycle exit by T3 is thus very different from the one observed in other cell types, in which T3 seems to exert a direct control on differentiation, as in adult oligodendrocyte precursor cells (Durand and Raff, 2000; Picou et al., 2012), erythrocyte progenitors (Gandrillon et al., 1994), astrocytes (Trentin et al., 1998; Trentin et al., 2001) and intestinal crypt cells (Plateroti et al., 2001). Interestingly, PCs and BG act synergistically to control GC development. If PC differentiation is impaired, the cell cycle of GC precursors is indirectly affected, favoring their accumulation in EGL. Concomitantly, disturbance of BG organization impairs their ability to guide the inward migration of committed GC precursors and further contribute to EGL thickening. Ultimately, the presence of immature $PAX6^{+}$ GCs in the IGL could be the consequence of GCs inability to establish proper synaptic connections due to their delayed migration ($TR\alpha^{AMI}/G^{T2}$), the impaired development of their target ($TR\alpha^{AMI}/P$), or both ($TR\alpha^{AMI}/N$ and $/PG^{T2}$).

Loss of TRα1-dependent T3 signaling has a strong impact on GI maturation and synaptogenesis, but unraveling direct and indirect influences on these processes remains difficult. PCs certainly have a major influence on GABAergic synaptogenesis, because a loss of

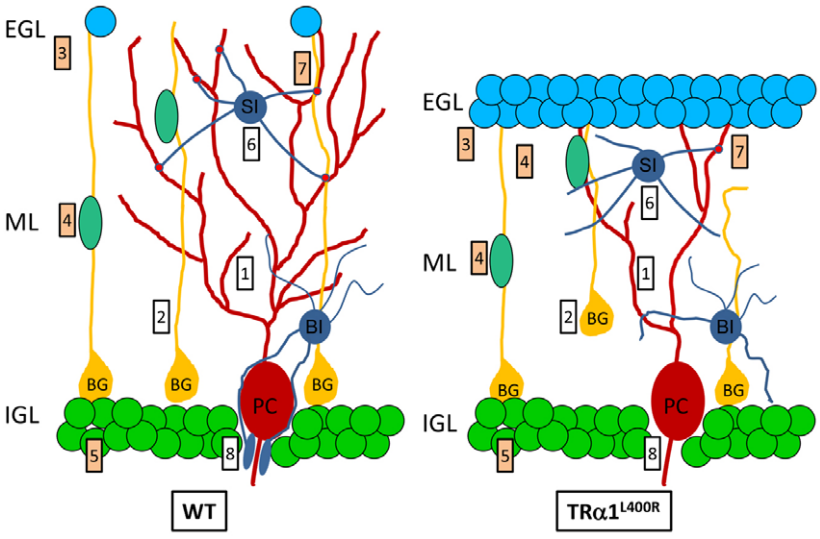

Fig. 8. Summarized scheme for the function of the T3/TRα1 pathway during cerebellar cortex development. Upon T3 stimulation, TRα1 exerts two cell type-autonomous effects on cell differentiation: in PCs (1), this is required before P8 to promote the development of a dendritic tree, and to promote the secretion of unidentified neurotrophic factors. In BG (2), it favors the anchoring of the cell bodies in the PC layer and the radial organization of fibers extending to the pial surface. By conditioning the later secretion of growth factors, T3 stimulation on early PCs indirectly controls the dynamic behavior of the population of GC precursors present in EGL (blue), ensuring cell-cycle exit of all cells before P21 (3). The proper elaboration of BG radial fibers is required for efficient inward migration (4) of postmitotic GC precursors (green), which contributes to the timely differentiation of GCs in IGL (5). The timely differentiation of stellate interneurons (SIs) (6) is also conditioned, directly or indirectly, by T3 stimulation. The formation of stable synapses in the molecular layer (7) requires the proper differentiation of PCs, GIs and BG, whereas the formation of pinceau synapses (8) by basket interneurons (BIs) on PCs axon initial segment is most likely to be a cell type-autonomous process.

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30% of stellate interneuron synapses is observed in *TRα^{AMI}/L* mice, which express TRα1^{L400R} only in PCs, after P8. In this case, no delay in GI maturation is observed. Therefore, even if their synaptic activity may play a causal role in Pax2 downregulation (Simat et al., 2007), an impairment in synapse formation does not necessarily delay GI terminal maturation. This situation contrasts with the one of *TRα^{AMI}/P* mice, in which both processes are altered. The difference observed between the *TRα^{AMI}/P* and *TRα^{AMI}/L* mice could be explained in two ways: the first possibility would be that early TRα1^{L400R} expression in PCs not only impairs synapse formation, but also blocks the synthesis by PCs of diffusible factors acting on GI maturation. The alternative would be that T3 has a cell type-autonomous effect on GIs, to control their maturation, and acts simultaneously on their target to control synaptogenesis. As expression of TRα1^{L400R} in BG also impairs stellate interneuron synaptogenesis, this process may rely on the proper expression of TRα1 target genes in three cell types: stellate interneurons, PCs and BG. Almost all pinceau synapses formed by basket interneurons on PCs axons also disappear in *TRα^{AMI}/P* but not in *TRα^{AMI}/L* mice. Again, this does not undoubtedly establish a direct influence of TRα1^{L400R} in GIs but the recombination rates observed in *TRα^{AMI}/P* observed in GIs (94% of GIs versus 75% of PCs) strongly support this possibility.

Because a number of studies pointed out that PCs orchestrate cerebellum cortex development by producing diffusible factors, it is logical to suppose that the indirect consequences of mutating TRα1 in PCs results from an impaired secretion of some of these factors. We detected four changes in gene expression in *TRα^{AMI}/P* mice, starting from P8, two being in PCs. Although change in *Slc17a8* expression might have consequences on synaptic activity (Crépel et al., 2011), it is not expected to influence PC secretory activity. By contrast, it is tempting to speculate that impaired production of fibroblast growth factor 7 (FGF7) by PCs is a key element, determining the microenvironment for other cell-type differentiation. FGF7 exerts pleiotropic paracrine influence during development, by binding to FGFR2 receptor present in both Bergmann glia and neighboring neurons (Umemori et al., 2004). Together with other FGFs, it participates to the local network of cellular interactions (Müller Smith et al., 2012) and synaptogenesis (Umemori et al., 2004). Recent analysis in hippocampus revealed a crucial intervention on the differential formation of glutamatergic and GABAergic synapses (Terauchi et al., 2010). However, further work is required to precisely assess the involvement of *Fgf7* regulation in the neurodevelopmental function of T3.

The level of T3 in mice serum peaks during the first postnatal week (Hadj-Sahraoui et al., 2000), providing temporal information that is important to synchronize cellular interactions during the maturation of neuronal circuits. Any deficiency occurring during these crucial stages has irreversible consequences for later brain functions. This synchronization of a developmental process by T3 appears to be somewhat reminiscent of T3-induced metamorphosis, as observed in frogs and fishes (Dusart and Flamant, 2012). The present data suggest that rather than exerting an independent control in each neural cell type, T3 acts in the cerebellum on a small fraction of the cell population, which has a central role in coordinating neurodevelopment. Understanding how germline mutations or environmental chemicals (Zoeller et al., 2002) alter these initial processes and indirectly influence the complex network of cellular interactions that sustains proper neurodevelopment may help propose new preventive or therapeutic strategies. This would also open an original route to explore the basic mechanisms of neurodevelopment.

MATERIALS AND METHODS

Animals

All animals used in this study were housed, raised, bred and euthanized in accordance with European directive 86/609/EEC and in compliance with national and international rules and laws on animal welfare. All mice were in C57/Bl6 genetic background, with a 129Sv contribution. *ROSA26-lox-STOP-lox-EYFP* (*R26YFP*) (Srinivas et al., 2001) was introduced in TRα^{AMI}/Cre mice to define recombination patterns (Table 1). YFP immunostaining was systematically used to quantify CRE recombination efficacy to rule out any variation between animals. Mice were genotyped by PCR for *Cre*, *R26YFP* and *TRα^{AMI}* alleles using specific primers. Tamoxifen (1 mg per 40 g of body weight in 100 µl of sunflower oil) and EdU (25 mg per kg) were injected intraperitoneally, respectively at P1 or 24 hours pre-sacrifice. For immunohistochemistry, animals were first anesthetized by intraperitoneal injection of a lethal dose of ketamine/xylazine, and perfused with 4% paraformaldehyde in PBS. Cerebella were dissected out, post-fixed for 2 hours, washed in PBS, and sections (50 µm) were performed with a vibratome (Campden Instruments, Loughborough, UK). The number of mice in each experiment was at least four per genotype, originating from two different litters, each litter providing both mutant and control mice. All experiments and statistical analyses were performed after pairing mutant and control littermates.

Antibodies for immunocytochemistry

The following antibodies were used: rabbit anti-Calbindin-D-28k (1:2000, Swant); rabbit anti-GFAP (1:2000, Dako); rabbit anti-GFP (1:1000, Invitrogen), sheep anti-GFP (1:750, AbD Serotec), mouse anti-parvalbumin (1:10,000, Sigma-Aldrich), rabbit anti-PAX2 (1:750, Zymed); rabbit anti-PAX6 (1:500, Millipore); mouse anti-S100β (1:1000, Sigma-Aldrich). All secondary antibodies were raised in donkey (Jackson ImmunoResearch Laboratories) and used at a 1:1000 dilution.

Immunofluorescence, confocal imaging and quantification

Histological analysis was performed on lobule VIII at P15 and P21. Slices were blocked in 10% normal donkey serum, 1% cold water fish skin gelatin, 0.2% Triton X-100, before primary antibody incubation [overnight, 4°C, in PBS containing 0.2% Triton X-100, 1% normal donkey serum, 1% cold water fish skin gelatin and 1% dimethyl sulfoxide (DMSO)] and rinsed before application of the secondary antibodies. Sections were mounted in Prolong Gold (Invitrogen) and imaged with 63× oil immersion (NA 1.4) or 40× oil immersion (NA 1.25) objectives using a confocal microscope (SP5 Leica). Channels were acquired sequentially to avoid bleed-through between 4′,6-diamidino-2-phenylindole (DAPI), DyLight488, DyLight549 and DyLight633. Image quantification was performed as previously described (Fauquier et al., 2011).

Cell counting and statistics

For each experiment, four cerebellum slices per animal were picked at random, processed for immunostaining and imaged as described above. For EGL and GI analysis, the entire lobule VIII was imaged, whereas for GABAergic synapses, four different fields per slice were imaged in this same lobule. EGL cells were identified on the basis of their PAX6 immunoreactivity. The cells were counted manually using ImageJ, and the total EGL length was measured, in order to calculate the average number of GC precursors per mm of EGL. For GI analysis, PAX2- and parvalbumin-expressing cells were counted manually. In the rare case in which cells were co-expressing both proteins, they were considered to have undergone terminal maturation, and therefore were counted as parvalbumin-positive only. As the changes in the size of PC dendritic arborization we observed in some mutants greatly modified the surface area of the molecular layer, we used a ratio (number of cells/total molecular layer length) in order to compare wild-type and mutant animals. Cell density for each mutant was calculated as a percentage of the average calculated for control littermates. Values for mutants of matching age and genotype where then averaged. Unpaired Student's *t*-test was used to compare animals of matching age and genotype to their wild-type littermates, or to compare mutants of different genotypes.

RNA analysis

RNA was extracted from whole cerebellum using Macherey-Nagel NucleoSpin RNA II kit. cDNAs were prepared from 1 µg of RNA using M-MuLV reverse transcriptase (Promega) and random 6-mer primers in 20 µl. One nanogram of cDNA was used for quantitative PCR, using SYBR Green (Qiagen). Quantification was performed in triplicate using HPRT as internal standard and the $2^{-\Delta\Delta Ct}$ method for analysis.

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Competing interests

The authors declare no competing financial interests.

Authors contributions

T.F. conceived, performed and analyzed all microscopy experiments, and participated in manuscript preparation. F.C. performed and analyzed gene expression analysis experiments. F.P. and S.R. performed genetic crosses genotyping and tissue preparations, and participated in manuscript preparation. N.F. performed some of the microscopy experiments and genetic crosses. N.A. performed mouse breeding, genetic crosses and genotyping. T.L. supervised experiments and edited the manuscript. F.F. conceived, supervised and analyzed experiments and participated in manuscript preparation.

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Supplementary material

Supplementary material available online at
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References

Ango, F., Wu, C., Van der Want, J. J., Wu, P., Schachner, M. and Huang, Z. J. (2008). Bergmann glia and the recognition molecule CHL1 organize GABAergic axons and direct innervation of Purkinje cell dendrites. *PLoS Biol.* **6**, e103.

Avci, H. X., Lebrun, C., Wehrlé, R., Doulazmi, M., Chatonnet, F., Morel, M. P., Ema, M., Vodjdani, G., Sotelo, C., Flamant, F. et al. (2012). Thyroid hormone triggers the developmental loss of axonal regenerative capacity via thyroid hormone receptor α1 and krüppel-like factor 9 in Purkinje cells. *Proc. Natl. Acad. Sci. USA* **109**, 14206-14211.

Bernal, J. (2007). Thyroid hormone receptors in brain development and function. *Nat. Clin. Pract. Endocrinol. Metab.* **3**, 249-259.

Bochukova, E., Schoenmakers, N., Agostini, M., Schoenmakers, E., Rajanayagam, O., Keogh, J. M., Henning, E., Reinemund, J., Gevers, E., Sarri, M. et al. (2012). A mutation in the thyroid hormone receptor alpha gene. *N. Engl. J. Med.* **366**, 243-249.

Boukhtouche, F., Brugg, B., Wehrlé, R., Bois-Joyeux, B., Danan, J. L., Dusart, I. and Mariani, J. (2010). Induction of early Purkinje cell dendritic differentiation by thyroid hormone requires RORα. *Neural Dev.* **5**, 18.

Bradley, D. J., Towle, H. C. and Young, W. S., 3rd (1992). Spatial and temporal expression of alpha- and beta-thyroid hormone receptor mRNAs, including the beta 2-subtype, in the developing mammalian nervous system. *J. Neurosci.* **12**, 2288-2302.

Chatonnet, F., Guyot, R., Picou, F., Bondesson, M. and Flamant, F. (2012). Genome-wide search reveals the existence of a limited number of thyroid hormone receptor alpha target genes in cerebellar neurons. *PLoS ONE* **7**, e30703.

Chédotal, A. (2010). Should I stay or should I go? Becoming a granule cell. *Trends Neurosci.* **33**, 163-172.

Clos, J. and Legrand, C. (1990). An interaction between thyroid hormone and nerve growth factor promotes the development of hippocampus, olfactory bulbs and cerebellum: a comparative biochemical study of normal and hypothyroid rats. *Growth Factors* **3**, 205-220.

Clos, J., Crépel, F., Legrand, C., Legrand, J., Rabié, A. and Vigouroux, E. (1974). Thyroid physiology during the postnatal period in the rat: a study of the development of thyroid function and of the morphogenetic effects of thyroxine with special reference to cerebellar maturation. *Gen. Comp. Endocrinol.* **23**, 178-192.

Crepel, F., Delhaye-Bouchaud, N. and Dupont, J. L. (1981). Fate of the multiple innervation of cerebellar Purkinje cells by climbing fibers in immature control, x-irradiated and hypothyroid rats. *Brain Res.* **227**, 59-71.

Crépel, F., Galante, M., Habbas, S., McLean, H. and Daniel, H. (2011). Role of the vesicular transporter VGLUT3 in retrograde release of glutamate by cerebellar Purkinje cells. *J. Neurophysiol.* **105**, 1023-1032.

Dong, H., Yauk, C. L., Rowan-Carroll, A., You, S. H., Zoeller, R. T., Lambert, I. and Wade, M. G. (2009). Identification of thyroid hormone receptor binding sites and target genes using ChIP-on-chip in developing mouse cerebellum. *PLoS ONE* **4**, e4610.

Durand, B. and Raff, M. (2000). A cell-intrinsic timer that operates during oligodendrocyte development. *Bioessays* **22**, 64-71.

Dusart, I. and Flamant, F. (2012). Profound morphological and functional changes of rodent Purkinje cells between the first and the second postnatal weeks: a metamorphosis? *Front Neuroanat* **6**, 11.

Elder, D. A., Karayal, A. F., D'Ercole, A. J. and Calikoglu, A. S. (2000). Effects of hypothyroidism on insulin-like growth factor-I expression during brain development in mice. *Neurosci. Lett.* **293**, 99-102.

Fauquier, T., Romero, E., Picou, F., Chatonnet, F., Nguyen, X. N., Quignodon, L. and Flamant, F. (2011). Severe impairment of cerebellum development in mice expressing a dominant-negative mutation inactivating thyroid hormone receptor alpha1 isoform. *Dev. Biol.* **356**, 350-358.

Fossat, N., Chatelain, G., Brun, G. and Lamonerie, T. (2006). Temporal and spatial delineation of mouse Otx2 functions by conditional self-knockout. *EMBO Rep.* **7**, 824-830.

Gandrillon, O., Ferrand, N., Michaille, J. J., Roze, L., Zile, M. H. and Samarut, J. (1994). c-erbA alpha/T3R and RARs control commitment of hematopoietic self-renewing progenitor cells to apoptosis or differentiation and are antagonized by the v-erbA oncogene. *Oncogene* **9**, 749-758.

Gomes, F. C., Maia, C. G., de Menezes, J. R. and Neto, V. M. (1999). Cerebellar astrocytes treated by thyroid hormone modulate neuronal proliferation. *Glia* **25**, 247-255.

Hadj-Sahraoui, N., Seugnet, I., Ghorbel, M. T. and Demeneix, B. (2000). Hypothyroidism prolongs mitotic activity in the post-natal mouse brain. *Neurosci. Lett.* **280**, 79-82.

Heuer, H. and Mason, C. A. (2003). Thyroid hormone induces cerebellar Purkinje cell dendritic development via the thyroid hormone receptor alpha1. *J. Neurosci.* **23**, 10604-10612.

Kimura-Kuroda, J., Nagata, I., Negishi-Kato, M. and Kuroda, Y. (2002). Thyroid hormone-dependent development of mouse cerebellar Purkinje cells in vitro. *Brain Res. Dev. Brain Res.* **137**, 55-65.

Koibuchi, N. (2008). The role of thyroid hormone on cerebellar development. *Cerebellum* **7**, 530-533.

Krysko, O., Hulshagen, L., Janssen, A., Schütz, G., Klein, R., De Bruycker, M., Espeel, M., Gressens, P. and Baes, M. (2007). Neocortical and cerebellar developmental abnormalities in conditions of selective elimination of peroxisomes from brain or from liver. *J. Neurosci. Res.* **85**, 58-72.

Lauder, J. M. (1977). The effects of early hypo- and hyperthyroidism on the development of rat cerebellar cortex. III. Kinetics of cell proliferation in the external granular layer. *Brain Res.* **126**, 31-51.

Lindholm, D., Castrén, E., Tsoulfas, P., Kolbeck, R., Berzaghi, M. P., Leingärtner, A., Heisenberg, C. P., Tessarollo, L., Parada, L. F. and Thoenen, H. (1993). Neurotrophin-3 induced by tri-iodothyronine in cerebellar granule cells promotes Purkinje cell differentiation. *J. Cell Biol.* **122**, 443-450.

Manzano, J., Bernal, J. and Morte, B. (2007a). Influence of thyroid hormones on maturation of rat cerebellar astrocytes. *Int. J. Dev. Neurosci.* **25**, 171-179.

Manzano, J., Cuadrado, M., Morte, B. and Bernal, J. (2007b). Influence of thyroid hormone and thyroid hormone receptors in the generation of cerebellar gamma-aminobutyric acid-ergic interneurons from precursor cells. *Endocrinology* **148**, 5746-5751.

Mellström, B., Naranjo, J. R., Santos, A., Gonzalez, A. M. and Bernal, J. (1991). Independent expression of the alpha and beta c-erbA genes in developing rat brain. *Mol. Endocrinol.* **5**, 1339-1350.

Mendes-de-Aguiar, C. B., Alchini, R., Decker, H., Alvarez-Silva, M., Tasca, C. I. and Trentin, A. G. (2008). Thyroid hormone increases astrocytic glutamate uptake and protects astrocytes and neurons against glutamate toxicity. *J. Neurosci. Res.* **86**, 3117-3125.

Moran, C., Schoenmakers, N., Agostini, M., Schoenmakers, E., Offiah, A., Kydd, A., Kahaly, G., Mohr-Kahaly, S., Rajanayagam, O., Lyons, G. et al. (2013). An adult female with resistance to thyroid hormone mediated by defective thyroid hormone receptor alpha. *J. Clin. Endocrinol. Metab.*

Morte, B., Manzano, J., Scanlan, T. S., Vennström, B. and Bernal, J. (2004). Aberrant maturation of astrocytes in thyroid hormone receptor alpha 1 knockout mice reveals an interplay between thyroid hormone receptor isoforms. *Endocrinology* **145**, 1386-1391.

Müller Smith, K., Williamson, T. L., Schwartz, M. L. and Vaccarino, F. M. (2012). Impaired motor coordination and disrupted cerebellar architecture in Fgfr1 and Fgfr2 double knockout mice. *Brain Res.* **1460**, 12-24.

Neveu, I. and Arenas, E. (1996). Neurotrophins promote the survival and development of neurons in the cerebellum of hypothyroid rats in vivo. *J. Cell Biol.* **133**, 631-646.

Nicholson, J. L. and Altman, J. (1972a). The effects of early hypo- and hyperthyroidism on the development of the rat cerebellar cortex. II. Synaptogenesis in the molecular layer. *Brain Res.* **44**, 25-36.

Nicholson, J. L. and Altman, J. (1972b). Synaptogenesis in the rat cerebellum: effects of early hypo- and hyperthyroidism. *Science* **176**, 530-532.

Pascual, M., Abasolo, I., Mingorance-Le Meur, A., Martínez, A., Del Rio, J. A., Wright, C. V., Real, F. X. and Soriano, E. (2007). Cerebellar GABAergic progenitors adopt an external granule cell-like phenotype in the absence of Ptf1a transcription factor expression. *Proc. Natl. Acad. Sci. USA* **104**, 5193-5198.

Picou, F., Fauquier, T., Chatonnet, F. and Flamant, F. (2012). A bimodal influence of thyroid hormone on cerebellum oligodendrocyte differentiation. *Mol. Endocrinol.* **26**, 608-618.

Plateroti, M., Gauthier, K., Domon-Dell, C., Freund, J. N., Samarut, J. and Chassande, O. (2001). Functional interference between thyroid hormone receptor alpha (TRalpha) and natural truncated TRDeltaalpha isoforms in the control of intestine development. *Mol. Cell. Biol.* **21**, 4761-4772.

Poguet, A. L., Legrand, C., Feng, X., Yen, P. M., Meltzer, P., Samarut, J. and Flamant, F. (2003). Microarray analysis of knockout mice identifies cyclin D2 as a possible mediator for the action of thyroid hormone during the postnatal development of the cerebellum. *Dev. Biol.* **254**, 188-199.

Portella, A. C., Carvalho, F., Faustino, L., Wondisford, F. E., Ortiga-Carvalho, T. M. and Gomes, F. C. (2010). Thyroid hormone receptor beta mutation causes severe impairment of cerebellar development. *Mol. Cell. Neurosci.* **44**, 68-77.

Quignodon, L., Grijota-Martinez, C., Compe, E., Guyot, R., Allioli, N., Laperrière, D., Walker, R., Meltzer, P., Mader, S., Samarut, J. et al. (2007a). A combined approach identifies a limited number of new thyroid hormone target genes in post-natal mouse cerebellum. *J. Mol. Endocrinol.* **39**, 17-28.

Quignodon, L., Vincent, S., Winter, H., Samarut, J. and Flamant, F. (2007b). A point mutation in the activation function 2 domain of thyroid hormone receptor alpha1 expressed after CRE-mediated recombination partially recapitulates hypothyroidism. *Mol. Endocrinol.* **21**, 2350-2360.

Simat, M., Ambrosetti, L., Lardi-Studler, B. and Fritschy, J. M. (2007). GABAergic synaptogenesis marks the onset of differentiation of basket and stellate cells in mouse cerebellum. *Eur. J. Neurosci.* **26**, 2239-2256.

Slezak, M., Göritz, C., Niemiec, A., Frisén, J., Chambon, P., Metzger, D. and Pfrieger, F. W. (2007). Transgenic mice for conditional gene manipulation in astroglial cells. *Glia* **55**, 1565-1576.

Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M. and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev. Biol.* **1**, 4.

Swanson, D. J. and Goldowitz, D. (2011). Experimental Sey mouse chimeras reveal the developmental deficiencies of Pax6-null granule cells in the postnatal cerebellum. *Dev. Biol.* **351**, 1-12.

Takahashi, M., Negishi, T. and Tashiro, T. (2008). Identification of genes mediating thyroid hormone action in the developing mouse cerebellum. *J. Neurochem.* **104**, 640-652.

Terauchi, A., Johnson-Venkatesh, E. M., Toth, A. B., Javed, D., Sutton, M. A. and Umemori, H. (2010). Distinct FGFs promote differentiation of excitatory and inhibitory synapses. *Nature* **465**, 783-787.

Thompson, C. C. (1996). Thyroid hormone-responsive genes in developing cerebellum include a novel synaptotagmin and a hairless homolog. *J. Neurosci.* **16**, 7832-7840.

Trentin, A. G., Gomes, F. C., Lima, F. R. and Neto, V. M. (1998). Thyroid hormone acting on astrocytes in culture. *In Vitro Cell. Dev. Biol. Anim.* **34**, 280-282.

Trentin, A. G., Alvarez-Silva, M. and Moura Neto, V. (2001). Thyroid hormone induces cerebellar astrocytes and C6 glioma cells to secrete mitogenic growth factors. *Am. J. Physiol.* **281**, E1088-E1094.

Tronche, F., Opherk, C., Moriggl, R., Kellendonk, C., Reimann, A., Schwake, L., Reichardt, H. M., Stangl, K., Gau, D., Hoeflich, A. et al. (2004). Glucocorticoid receptor function in hepatocytes is essential to promote postnatal body growth. *Genes Dev.* **18**, 492-497.

Umemori, H., Linhoff, M. W., Ornitz, D. M. and Sanes, J. R. (2004). FGF22 and its close relatives are presynaptic organizing molecules in the mammalian brain. *Cell* **118**, 257-270.

van Mullem, A., van Heerebeek, R., Chrysis, D., Visser, E., Medici, M., Andrikoula, M., Tsatsoulis, A., Peeters, R. and Visser, T. J. (2012). Clinical phenotype and mutant TRα1. *N. Engl. J. Med.* **366**, 1451-1453.

Vincent, J., Legrand, C., Rabié, A. and Legrand, J. (1982-1983). Effects of thyroid hormone on synaptogenesis in the molecular layer of the developing rat cerebellum. *J. Physiol.* (*Paris*) **78**, 729-738.

Wallis, K., Sjögren, M., van Hogerlinden, M., Silberberg, G., Fisahn, A., Nordström, K., Larsson, L., Westerblad, H., Morreale de Escobar, G., Shupliakov, O. et al. (2008). Locomotor deficiencies and aberrant development of subtype-specific GABAergic interneurons caused by an unliganded thyroid hormone receptor alpha1. *J. Neurosci.* **28**, 1904-1915.

Wallis, K., Dudazy, S., van Hogerlinden, M., Nordström, K., Mittag, J. and Vennström, B. (2010). The thyroid hormone receptor alpha1 protein is expressed in embryonic postmitotic neurons and persists in most adult neurons. *Mol. Endocrinol.* **24**, 1904-1916.

Zoeller, T. R., Dowling, A. L., Herzig, C. T., Iannacone, E. A., Gauger, K. J. and Bansal, R. (2002). Thyroid hormone, brain development, and the environment. *Environ. Health Perspect.* **110 Suppl. 3**, 355-361.