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The roles of testicular orphan nuclear receptor 4 (TR4) in cerebellar development

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Abstract
Since Testicular Receptor 4 (TR4) was cloned, efforts have been made to elucidate its physiological function. To examine the putative functions of TR4, the conventional TR4 knockout ($TR4^{-/-}$) mouse model was generated. Throughout postnatal and adult stages, $TR4^{-/-}$ mice exhibited behavioral deficits in motor coordination, suggesting impaired cerebellar function. Histological examination of the postnatal and adult $TR4^{-/-}$ cerebellum revealed gross abnormalities in foliation. Further analyses demonstrated changes in the lamination of the $TR4^{-/-}$ cerebellar cortex, including reduction in the thickness of both the molecular layer (ML) and the internal granule layer (IGL). Analyses of the developing $TR4^{-/-}$ cerebellum indicate that the lamination irregularities observed may result from disrupted granule cell proliferation within the external granule cell layer (EGL), delayed inward migration of post-mitotic granule cells, and increased apoptosis during cerebellar development. In addition, abnormal development of Purkinje cells was observed in the postnatal $TR4^{-/-}$ cerebellum, as indicated by aberrant dendritic arborization. In postnatal, neuronal-specific TR4 knockout mice, architectural changes in the cerebellum were similar to those seen in $TR4^{-/-}$ animals, suggesting that TR4 function in neuronal lineages might be important for cerebellar morphogenesis, and that the effect on Purkinje cell development is likely mediated by changes elsewhere, such as in granule cells, or is highly dependent on developmental stage. Together, our findings from various TR4 knockout mouse models suggest that TR4 is required for normal cerebellar development and that failure to establish proper cytoarchitecture results in dysfunction of the cerebellum and leads to abnormal behavior.

Key words: *Testicular Receptor 4, TR4 knockout mice, cerebellar development*

Introduction

The nuclear receptor superfamily includes a group of transcription factors that are highly homologous in sequence and structural components. Nuclear receptors contain six independent functional domains, A-F, with each domain governing distinct molecular functions, including transactivation, dimerization, DNA binding, and ligand binding (1,2). Functionally, nuclear receptors are known to initiate recruitment of transcriptional machinery and subsequently modulate target gene expression. With this unique transactivational ability, nuclear receptors are believed to be important in directing organogenesis, maintaining homeostasis, and even in tumorigenesis and the development of disease (3–11). In 1988, with the advantage of molecular biology techniques, the first cDNA clones encoding steroid hormone receptors were identified (12,13). Classic nuclear receptors were also known as steroid hormone receptors, so named because they were initially identified as receptors for steroid hormones, including thyroid hormone, retinoids, estrogen, and androgen. The receptors for these steroid hormones exhibit similar characteristics, and are primarily present in the cytoplasm of the cell. After binding their ligands, the activated ligand-receptor complexes translocate into the nucleus. After translocation, the complexes recognize specific DNA sequences, hormone response elements that exist in gene promoter regions, and then recruit other factors to the transcription start site to initiate gene transcription (2). In addition to having the same activating mechanism, these classic nuclear receptors share high homology in the sequence encoding the DNA-binding domain. Based on this characteristic, receptors for Vitamin D, glucocorticoids and peroxisome proliferator were identified (1,14–17). Identification of the partnership between nuclear

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receptors and their ligands not only enriches our understanding of signaling pathways governed by those steroids, but also sheds some light on putative cures for numerous diseases caused by disrupted steroid signaling. Interestingly, as gene cloning became more common, many putative nuclear receptor genes, which share similar sequence and structural identity with classic nuclear receptors, were identified without associated ligands. Because these putative nuclear receptors have similar protein structures and exert transcriptional effects through functional mechanisms similar to those used by other nuclear receptors, but do not yet have identified ligands, they are categorized as orphan nuclear receptors. Growing evidence has suggested that these orphan nuclear receptors, like other nuclear receptors with known ligands, play important roles in organogenesis, steroidogenesis, and neurogenesis, as well as in the development of reproductive organs and lymphoid maturation (5,8,18–21). In 1994, the orphan nuclear receptor, Testicular Receptor 4 (TR4), was identified, and so named because its amino acid sequence shares high homology with the previously cloned orphan nuclear receptor, Testicular Receptor 2 (22–24). In the rodent, TR4 expression can be detected in brain and peripheral organs, including the adrenal gland, spleen, testis, thyroid and pituitary glands, and prostate. The extensive expression of TR4 in mammalian tissues suggests putative roles in particular physiological functions (22,23), such as neurogenesis and spermatogenesis. Over the last decade, numerous molecular studies have demonstrated the putative function of TR4 in modulating intracellular signaling pathways of several nuclear receptors. However, due to the lack of an identified ligand, the role of TR4 in mammals remains a relative mystery. The major challenge in elucidating the function of TR4 is the lack of a molecule that is able to modulate its physiological function. To address this issue, a mouse model in which TR4 is functionally ablated, the conventional TR4 knockout mouse, was generated (25). Consistent with prior predictions, multiple abnormalities were observed in TR4 knockout mice, particularly in neurological function. Subsequently, conditional TR4 knockout animals were produced, targeting the absence of TR4 to specific neuronal lineages. In this review, we revisit previous findings and discuss evidence obtained from conventional and tissue-specific TR4 knockout mouse models, which have enhanced our understanding of TR4 in the central nervous system.

The discovery of orphan nuclear receptor TR4

Full-length cDNAs for human and rat TR4 were identified in the products generated using reverse transcription-PCR of rat hypothalamic RNA, with degenerate primers recognizing the conserved DNA binding domain of nuclear receptors, and by rapid amplification of cDNA ends-PCR (RACE-PCR) methods. The open reading frames of the human and rat TR4 genes encode 615 and 596 amino acids, respectively. The molecular mass of human TR4 is 67.3 kDa, and of rat TR4 is 65.4 kDa (23). This newly identified gene was named TR4 due to high homology (65%) with another orphan nuclear receptor, TR2 (24). At about the same time, a gene named TAK1 was also described as a newly identified orphan nuclear receptor (22,26). Later, this gene was found to be identical to TR4. The official, systematic designation for TR4, or TAK1, then became nuclear receptor subfamily 2, group C, member 2 (NR2C2). In *Homo sapiens,* this gene is located on chromosome 3 p25. Structurally, like other nuclear receptors, TR4 contains an amino-terminal transactivation domain, a central DNA-binding domain with 2 zinc finger motifs, and a ligand-binding domain near the carboxyl-terminus. The difference in amino acid sequence between human and rat TR4 is minor, with an overall sequence homology of 98%.

The distribution of TR4 in the nervous system

In mammals, TR4 is widely expressed in central and peripheral organs, such as brain, liver, lung, skeletal muscle, and testes, as well as in prostate, suggesting it is important in maintaining certain physiological functions (23). Interestingly, the expression of TR4 is highest in the central nervous system (CNS). Brain regions with particularly high TR4 expression include the hypothalamus, hippocampus, and cerebellum (23). In rodents, one study demonstrated that the expression of TR4 correlates with the onset of neurogenesis. By *in situ* hybridization, the authors demonstrated that TR4 was expressed in the mesencephelon, the cerebellar primordium, during embryonic stages, and in the cerebellum during postnatal stages (27). This expression pattern suggests a putative role for TR4 in the nervous system during development, particularly in the cerebellum. In addition, the expression of TR4 at embryonic day 16 extends to the spinal cord and motor neurons (28). Numerous neuronal nuclei that are involved in sensory organ development have been found to express TR4 as well. Specific TR4-expressing neuronal nuclei include the dorsal root ganglia, superior cervical ganglia, sympathetic ganglia, and the trigeminal ganglia. In the adult mouse brain, TR4 is predominantly expressed in regions of high neuronal density, such as hippocampal regions CA1, CA2, and CA3, as well as in the granule cells of the dentate gyrus, the cerebellar cortex, the habenular nuclei, and the piriform cortex (28).

Putative regulatory effects of TR4

Since TR4 was cloned, its physiological function has remained largely unknown. For the past decade, numerous molecular approaches have been used to address this issue, and information helpful in understanding signaling mechanisms affected by TR4 has been obtained. Currently, it is believed that the binding of activated nuclear receptor complexes (monomers, homodimers and heterodimers), via DNA-binding domains with direct repeat response elements, is one of the steps essential for the initiation of transcription. Like other nuclear receptors, TR4 has a highly conserved DNA-binding domain containing two zinc-finger motifs, which indicate its ability to bind to specific DNA sequences. For several years, scientists worked to determine which DNA sequence(s) would be a binding target for TR4. The DNA-binding domain of TR4 shares high homology with that of TR2, RAR, and RXR. Therefore, it is possible that these receptors also target the same DNA sequences (for example, direct repeat 1, AGGTCAAAGGTCA), or hormone response elements, in gene promoters. In one study, the TR4 homodimer (TR4-TR4) was shown to compete, for binding to direct repeat 1 (DR1) sequences, with RAR-RXR heterodimers and RXR homodimers, successfully suppressing the signaling mediated by those nuclear receptors (29,30). Likewise, direct repeat response elements for TR4 were identified in the promoter regions of peroxisome proliferator-activated receptor alpha (PPARα) target genes (31), thyroid hormone target genes (32), the ciliary neurotrophic factor receptor (CNTFR) gene (28,33), and in the promoter of the apolipoprotein E (ApoE) gene (34). Additionally, direct repeat sites recognized by TR4 were found in the SV40 major late promoter and the 25-hydroxyvitamin D3 24-hydroxylase (p450cc24) promoter (35,36). In the promoter of human luteinizing hormone receptor (LHR), an imperfect direct repeat site was identified, which was then demonstrated to be recognized by TR4 (37). Recently, an imperfect DR1 element was found in the promoter of the hepatitis B virus (HBV), and although this direct repeat site is not identical to the consensus DR1 sequence, a reporter gene assay has demonstrated that TR4 can suppress HBV core promoter transcriptional activity (38). In addition to binding to direct repeat elements, TR4 was shown to bind to the monomeric AGGTCA motif and subsequently suppress the expression of steroid 21-hydroxylase and oxytocin (39,40). As shown in Table I, a summary of putative TR4 target genes, numerous genes were discovered to be regulated by TR4 in recent years. In addition to directly regulating gene expression via binding to direct repeat sites, and subsequently recruiting other transcription factors onto the transcription start codon, TR4 was shown to modulate the signaling of several nuclear receptors by promoting or attenuating the binding of those receptors to direct repeat sites. For instance, one study demonstrated that TR4 promotes the binding of several nuclear receptors, including the thyroid hormone (T_3) receptor and the chicken ovalbumin upstream promoter-transcription factor (COUPTF) receptors, to direct repeat sites in the promoter regions of target genes (36,41). Through crosstalk with the COUPTFR and the T_3R, TR4 was able to increase transcriptional activity of the human immunodeficiency virus type 1 (HIV1) long terminal repeat region (41). Moreover, TR4 was found to attenuate the transcriptional ability of the estrogen and androgen receptors by forming heterodimeric complexes with them (42,43). The ability of TR4 to crosstalk with other nuclear receptors is particularly interesting, because this phenomenon indicates that the function of TR4 might not be restricted to the traditional nuclear receptor pathways, which require the binding of an activated nuclear receptor to direct repeat response elements in order to influence target gene expression. Alternatively, TR4 may extend its capacity to affect signaling pathways through influencing the DNA-binding affinity of other nuclear receptors.

Table I. TR4 target genes.

Target gene	Direct repeat response element	Regulation
P450cc24	DR3	Repression
CRBPII	DR1	Repression
RARb	DR5	Repression
SV40	DR2	Repression
PPARα target gene	DR1	Repression
Steroid 21-hydroxylase	Monomeric	Repression
Human ε & γ globin	DR1	Repression
$T_3R\alpha$ target genes	DR4	Activation
CNTFRα	DR1	Activation
HPV-16	DR4	Activation
HIV1	Indirect	Activation
hLHR	DR0	Activation
HBV	DR1	Activation
Oxytocin	Monomeric	Activation

Given that the signaling pathways governed by COUPTFR, T_3R, as well as by RAR and RXR, were demonstrated to be important in neurogenesis (8,20,44–49), the ability of TR4 to modulate the signaling mediated by these nuclear receptors indicates that TR4 may also participate in regulating related events. Regulation, by TR4, of signaling that is important in nervous system development and degeneration, as well as abundant expression of TR4 in multiple brain regions, indicates an important role for this orphan nuclear receptor in the CNS.

Indications from various TR4 knockout mouse models

For a decade, the putative function of TR4 has been explored extensively using various molecular

biological methods; however, many of its physiological functions remain unknown. To study the physiological roles of TR4 in an animal model, with particular attention to its function in the CNS, conventional TR4 knockout ($TR4^{-/-}$) mice were generated by homologous recombination in embryonic stem cells (25). In addition, neuronal and Purkinje cell-specific TR4 knockout mouse models were established to further investigate the role of TR4 from tissue and cell-type specific perspectives.

Conventional TR4 knockout ($TR4^{-/-}$) mice

Behavioral abnormalities in $TR4^{-/-}$ mice. $TR4^{-/-}$ mice were viable and fertile, however, they displayed reduced body weight and suffered from high mortality before 2 months of age (25). Behaviorally, $TR4^{-/-}$ mice display several phenotypes that typically indicate impaired motor coordination, including unsteady gait, failure to maintain balance on a ledge, as well as sudden jerks and tremors. Further, increased spontaneous locomotor activity was observed in $TR4^{-/-}$ mice when they were introduced to an unfamiliar environment, with this phenomenon persisting in subsequent days, while locomotor activity gradually declined in subsequent testing days among wildtype controls. In contrast, rearing and vertical movement counts were not different between $TR4^{-/-}$ mice and controls, indicating that exploratory behavior is intact in $TR4^{-/-}$ mice (Chen et al., unpublished observations). One of the phenotypes of $TR4^{-/-}$ mice is hyperkinetic response following physical stimulation, manipulation, or handling, as revealed by increased horizontal ambulation and stereotypic counts during the first 5 minutes of each open-field trial. Moreover, this phenotype persists in subsequent tasks, as would be expected if it was a response to physical handling (Chen et al., unpublished observations).

In addition to hyperkinetic response to physical manipulation, $TR4^{-/-}$ mice lack nest building ability, both in home and new cages. Nest building behavior is normally performed by both male and female mice, and has been suggested to be correlated with thermoregulation and with the function of the hippocampus (50–52). In $TR4^{-/-}$ mice, no difference was found either in body temperature or in hippocampal architecture, suggesting that lack of functional TR4 may interfere with other unknown mechanisms that govern this behavior. A possible explanation for this behavioral deficiency is the disturbance of cerebellar function in the $TR4^{-/-}$ mice, leading to the inability to coordinate multiple fine movements, including holding and tearing cotton pieces needed for nest building. Since no significant architectural alterations were observed in regions other than the cerebellum in the $TR4^{-/-}$ brain, our findings suggest that dysfunction of the cerebellum may affect the execution of this behavior (Chen et al., unpublished observations).

Histological alterations in the adult $TR4^{-/-}$ brain. Histological analyses of different areas of the adult $TR4^{-/-}$ brain revealed regionally restricted cerebellar hypoplasia. In $TR4^{-/-}$ mice, the cerebellum was significantly reduced in size, as observed in whole brain preparations, and showed diminished fissure structure; notably, cerebellar lobules VI and VII failed to develop (Figure 1). In the $TR4^{-/-}$ cerebellar cortex, the density of granule cells was reduced when compared with controls. The change in cell proportion was accompanied by a decrease in the density of parallel fiber-Purkinje cell dendritic boutons, as revealed by Electron Microscopy. Interestingly, the parallel fiber boutons also showed significant enlargement, possibly to compensate for attenuated synaptogenesis (Chen et al., unpublished observations). These alterations in principal cell number and bouton density were not mere reflections of the smaller $TR4^{-/-}$ cerebellum, because similar changes in other cell populations, such as interneurons, were not observed. Taken together, the decrease in the number of granule cells, and in their parallel fiber boutons, provide indirect evidence that the excitatory inputs from granule cells to Purkinje cells may be compromised in the $TR4^{-/-}$ cerebellum.

In addition to the abnormalities in cerebellar architecture, the levels of the major inhibitory neurotransmitter GABA and its synthetic enzyme, GAD, were found to be diminished in the soma of Purkinje cells in the $TR4^{-/-}$ cerebellum. Intriguingly, GABA and GAD labeling intensities were also diminished in the inhibitory interneurons in the ML, namely the stellate and basket cells (Chen et al., unpublished observations). Previous studies in the cerebellum have shown that the dendrites of stellate and basket cells, like Purkinje cells, receive excitatory afferents predominately from parallel fibers (53,54). Thus, the diminished GABA

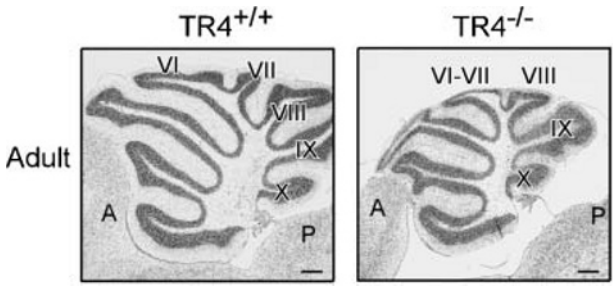

Figure 1. Cerebellar morphology in adult $TR4^{+/+}$ and $TR4^{-/-}$ mice. Paraffin-embedded, sagittal sections revealed aberrant folia arrangement in the $TR4^{-/-}$ cerebellum. Failure in establishing folia VI and VII was observed in the $TR4^{-/-}$ cerebellum when compared to corresponding regions in $TR4^{+/+}$ controls. Mouse genotypes are indicated. Cerebellar lobules are indicated by Roman numerals; A, anterior portion; P, posterior portion of cerebellum. Scale bars, 250 μm.

synthesis in interneurons and Purkinje cells further indicates that the excitatory input from parallel fibers may not be able to trigger changes in membrane potential, in these GABAergic cells, that are adequate to stimulate the normal production of GABA, causing subsequent disruption of the intrinsic inhibitory circuitry of the $TR4^{-/-}$ cerebellar cortex. Thus, the diminished GABA synthesis in the inhibitory neurons of the $TR4^{-/-}$ cerebellum may reflect reduced excitatory afferents to these cells, leading to a decrease in neuronal activity.

Given that Purkinje cells provide the only output from the cerebellum, and mainly through secretion of GABA from inhibitory pre-synaptic terminals (53), the reductions in GABA and GAD amount, specifically in Purkinje cells in the $TR4^{-/-}$ cerebellum, suggest that the inhibitory output from the cerebellar cortex might be altered. The diminished amount of GABA in Purkinje cells further suggests that signaling in the $TR4^{-/-}$cerebellum may not provide sufficient inhibitory signal to the cerebellar nuclei, which may then lead to the inability to terminate motor commands once initiated.

In agreement with the idea that the activity of Purkinje cells is reduced in the $TR4^{-/-}$ cerebellar cortex, the immunoreactivity of AMPA type glutamate receptor(s) was found to be decreased in Purkinje cell bodies and dendrites (Chen et al., unpublished observations). The reduction of AMPA receptors in GABAergic neurons, which has been correlated with the neuronal activity of these cells, combined with the observed decreases in GABA/GAD and GluR2/3 levels in the Purkinje cells of the $TR4^{-/-}$ cerebellum, strongly indicate that the activity of Purkinje cells in the $TR4^{-/-}$ cerebellum is appreciably lessened. Although electrophysiological studies were not conducted to directly assess synaptic transmission or cell activity, a reduced excitatory drive from the parallel fibers is a very likely consequence of the decrease in granule cell number in the $TR4^{-/-}$ cerebellum. It is known that cerebellar granule cells mediate the excitatory afferents from mossy fibers, which provide the major excitatory input toward Purkinje cell dendrites, thus fewer mediators (granule cells) in the $TR4^{-/-}$ mice are available to directly influence the magnitude of the neurotransmission, and subsequently alter the excitation status of Purkinje cells. Indeed, the ultrastructural changes in the parallel fiber-Purkinje cell dendrite boutons in the $TR4^{-/-}$ cerebellar cortex further support this hypothesis: the reduced bouton density may reflect the decrease in granule cell number, and the increased bouton size may reflect compensation by the pre-synaptic terminals to overcome the loss of excitatory drive.

Current data from analyses of the $TR4^{-/-}$ brain provide evidence that abnormal behaviors observed in $TR4^{-/-}$ mice may be due to dysfunction of the cerebellum. Since further architectural degeneration was not found in the $TR4^{-/-}$ cerebellum at any of the ages examined, the malfunction of the adult $TR4^{-/-}$ cerebellum most likely originates from a failure to establish proper cell-cell signaling, and subsequently results in disruption of motor coordination.

Abnormal cerebellar development in $TR4^{-/-}$ mice. In addition to general differences in physical appearance, such as reduced body size and weight (25), developing $TR4^{-/-}$ mice also exhibited a variety of behavioral abnormalities, including mild trembling, unsteady gait, and hyper-reactivity upon manipulation, that were similar to the behavioral phenotypes shown in adult mice (after puberty). As revealed by a simple task, tail suspension, $TR4^{-/-}$ mice at postnatal day 21 (P21) clasped their limbs, whereas control mice extended their legs to maintain a balanced posture in the air (55). This behavioral phenotype indicates that motor coordination is impaired earlier, in postnatal $TR4^{-/-}$ mice (55).

Analysis of the developing $TR4^{-/-}$ cerebellum further demonstrated that impaired motor coordination may have resulted from alterations in cerebellar cytoarchitecture that occurred during development. Throughout postnatal stages, the foliation of the cerebellum was stunted in $TR4^{-/-}$ mice (Figure 2), and further examination indicated that the reduction of granule cell density and internal granule cell layer (IGL) size may have resulted from a combination of effects, including disturbances in the proliferation of granule cell progenitors, delayed inward migration of post-mitotic granule cells, and increased apoptosis (55). Intriguingly, proliferation of granule cell progenitors in the $TR4^{-/-}$ external granule cell layer (EGL) occurred in a pattern opposite to that in the EGL of control cerebellum during postnatal development. In the normal EGL, the proliferation of granule cell progenitors increases during the first postnatal week and then declines to undetectable levels during the second and third postnatal weeks.

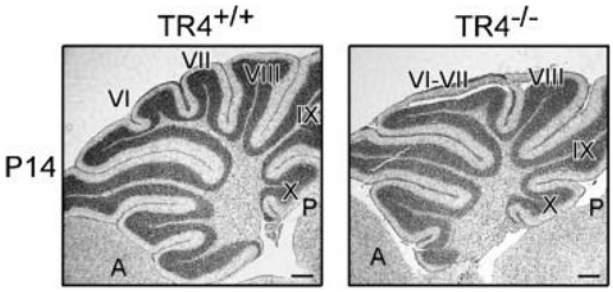

Figure 2. Cerebellar morphology in developing $TR4^{+/+}$ and $TR4^{-/-}$ mice. Paraffin-embedded, midsagittal sections revealed abnormal cerebellar structure at postnatal day 14 (P14) in $TR4^{-/-}$ mice. Development of folia VI and VII is stunted in $TR4^{-/-}$ mice in comparison to $TR4^{+/+}$ controls. Age of mice and genotypes are as indicated. Cerebellar lobules are indicated by Roman numerals; A, anterior portion; P, posterior portion of cerebellum. Scale bars, 200 μm.

Interestingly, in the TR4$^{-/-}$ EGL, fewer proliferation signals could be detected at P0 and P7 when compared to the control EGL. In contrast, a prolonged granule cell proliferation was observed at P14, and even as late as P18, in TR4$^{-/-}$ mice (55). Such alterations in the granule cell proliferation profile suggest that TR4 may not simply regulate the proliferation of granule cell progenitors, but may also promote exit of the cell cycle at late developmental stages. A possible mechanism controlling these effects involves changes in the presence or levels of participating co-regulators or other nuclear receptors at different postnatal stages. This assumption is partly supported by previous *in vitro* studies that demonstrated the ability of TR4 to crosstalk with numerous nuclear receptor signaling pathways (30,41). In addition to the abnormal proliferation pattern of granule cells in the TR4$^{-/-}$ EGL, delayed migration of post-mitotic granule cells and higher incidence of apoptosis in the cortex were observed in the TR4$^{-/-}$ cerebellum (55). Although granule cell proliferation is prolonged in the TR4$^{-/-}$ EGL during late postnatal stages, those postmitotic granule cells may not be able to migrate into the IGL due to increased apoptosis, which may explain the reductions in granule cell density and IGL size in the developing TR4$^{-/-}$ cerebellum. This interpretation is further supported by the reduction in BrdU-labeled cells in the TR4$^{-/-}$ IGL and ML, as demonstrated by long-term BrdU incorporation (55).

In addition to the abnormalities observed in granule cells, dendritic arborization of Purkinje cells in the TR4$^{-/-}$ cerebellum is stunted, and the immunoreactivity of calbindin is reduced during the first two postnatal weeks (55). Calbindin is a known calcium-binding protein, and has been suggested to be important in maintaining intracellular calcium homeostasis (56–59). The reduction of calbindin immunoreactivity in the TR4$^{-/-}$ cerebellum suggests that the function of Purkinje cells may be disrupted. It has been demonstrated that when post-mitotic granule cells depart from the inner portion of the EGL and migrate radially into the IGL, their axons extend horizontally to establish synapses with the dendrites of Purkinje cells. During this process, the ML grows considerably in size to accommodate the extensions of neurites of both cell types. Thus, the stunted formation of dendritic trees of Purkinje cells may also affect the extension of neurites of granule cells, and may account for the reduced width of the ML in the TR4$^{-/-}$ cerebellar cortex. These findings in Purkinje cells not only reveal abnormal dendritic extension, but also provide evidence that the differentiation or function of these cells may be compromised in the developing TR4$^{-/-}$ cerebellum.

Since cerebellar development requires proper interaction between granule and Purkinje cells, and since TR4 was found to be expressed in these two principal cell types during development, it is possible that TR4 plays an important role in the differentiation of neuronal progenitors at the onset of cerebellar morphogenesis. This suggestion was supported by the observations of thinner germinal zones in the cerebellar primordium of the TR4$^{-/-}$ mouse embryo (55). In the TR4$^{-/-}$ cerebellar primordium, the sizes of the primary germinal zone for granule cell progenitors, the rhombic lip, and the germinal zone for Purkinje cell progenitors, the neuroepithelium, were reduced when compared with age-matched controls. This result provides evidence that, prior to postnatal stages, the differentiation of principal neurons of the cerebellum was disrupted. This disruption may then account for the abnormal development of the TR4$^{-/-}$ cerebellum.

Tissue/cell-specific TR4 knockout mice

In conventional TR4 knockout mice, profound cerebellar architectural alterations during development are accompanied by substantial behavioral phenotypes in the adult. These findings indicate the importance of TR4 in cerebellar morphogenesis and, consequently, function. The expression of TR4 is not limited to cerebellar lineages, such as granule cells and Purkinje cells, in the developing cerebellum. Therefore, whether aberrant cerebellar development in TR4 null mice results from cell autonomous changes in those cerebellar neurons and/or in glial cells, or from other developmental defects, remain unclear. To address this issue, tissue/cell-specific TR4 knockout mouse models were established by crossing the Nestin-cre and Pcp2-cre strains with the floxed TR4 strain, a mouse model generated by Drs. Engel and Tanabe at University of Michigan, to eliminate TR4 specifically in developing CNS precursors and Purkinje cells, respectively. In postnatal, neuron-specific TR4

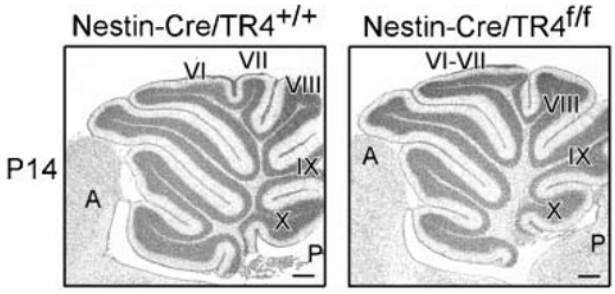

Figure 3. Cerebellar morphology in postnatal neuron-specific TR4 knockout mice. Paraffin-embedded, midsagittal sections revealed abnormal cerebellar structure at postnatal day 14 (P14) in Nestin-Cre/TR4$^{loxP/loxP}$ mice. Aberrant development in Nestin-Cre/TR4$^{loxP/loxP}$ cerebellar folia VI and VII is apparent when compared to sections of cerebella from control littermates. Age of mice and genotypes are as indicated. Cerebellar lobules are indicated by Roman numerals; A, anterior portion; P, posterior portion of cerebellum. Scale bars, 200 μm.

knockout mice, architectural changes in the cerebellum (Figure 3), sharing some similarities with those found in the conventional TR4 knockout model, were observed. These alterations include reduced thickness of the cerebellar cortex and prolonged existence of the EGL, as well as delayed migration of post-mitotic granule cells and stunted Purkinje cell dendritic arborization (Chen et al., unpublished observations). The results from neuron-specific TR4 knockout mice suggest that the abnormalities observed in the conventional $TR4^{-/-}$ cerebellum could be due to the ablation of TR4 in neuronal lineages.

In contrast to the neuron-specific TR4 knockout mice, no significant morphological alterations were observed in the cerebella of the Purkinje cell-specific knockout mice (Figure 4). One explanation for the lack of abnormality in the Purkinje cell-specific knockout cerebellum could be that the Pcp2-Cre driven target gene deletion is not fully established until postnatal day 10 (60), when the signaling cascade for Purkinje cell development and dendritic extension has already been initiated. It is also possible that the function of TR4 in Purkinje cells may be affecting particular processes, such as differentiation. In fact, in the conventional TR4 knockout cerebellum, at late developmental stages, the dendritic trees of Purkinje cells are fully extended into the ML, although the width of the ML was reduced when compared with age-matched controls. Thus, results from Purkinje cell-specific and conventional TR4 knockout mice suggest that alternative signaling mechanisms control the development of Purkinje cells at late postnatal stages.

Together, current findings from different TR4 knockout mouse models suggest that TR4 function in neuronal lineages is important for cerebellar morphogenesis, and that effects on Purkinje cell development and function may be mediated by changes elsewhere, such as in granule cells, or may be highly dependent on developmental stage.

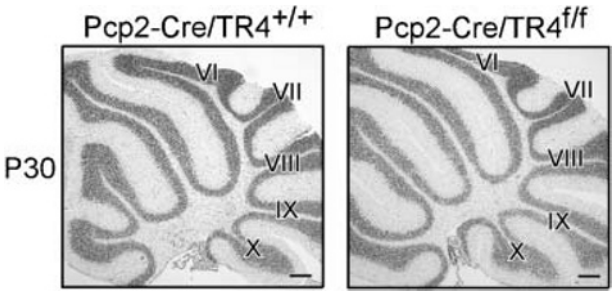

Figure 4. Cerebellar morphology in Purkinje cell-specific TR4 knockout mice. Paraffin-embedded, midsagittal sections revealed normal cerebellar structure at postnatal day 30 (P30) in Pcp2-Cre/$TR4^{loxP/loxP}$ mice. Normal folia development in Pcp2-Cre/$TR4^{loxP/loxP}$ cerebellar sections was observed when compared with sections from control littermates. Cerebellar lobules are indicated by Roman numerals. Scale bars, 200 μm.

Other deficiencies observed in the conventional TR4 knockout nervous system

In addition to profound architectural alterations in the $TR4^{-/-}$ cerebellum, the differentiation of glial cells, particularly oligodendrocytes, was abnormal, and these defects occurred in a regional and stage-specific manner. In $TR4^{-/-}$ mice, myelination of the forebrain region was disrupted specifically in early developmental stages, and this abnormality may result from deficits in oligodendrocyte differentiation (Zhang et al., unpublished observations). Although the phenomenon of hypomyelination in the $TR4^{-/-}$ forebrain becomes marginal at later stages, this stage-specific defect in mice lacking functional TR4 further supports the idea that the role of TR4 in mammalian development and physiology may vary, and may rely on the existence of other molecules, such as cofactors and other nuclear receptors. TR4 may only participate at a specific step in a complex cascade controlling myelination, and other mechanisms may compensate for the deficit caused by ablation of TR4.

Concluding remarks

Since TR4 was cloned a decade ago, evidence indicates its importance in maintaining proper physiological function, particularly in the nervous system. The exact signaling mechanisms directed by TR4 remain to be elucidated, however, the profound neurological abnormalities and significant architectural alterations in the cerebella of conventional and tissue-specific TR4 knockout mice have provided significant clues and directions for further study aimed toward increasing our understanding of the roles of TR4 in the brain.

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