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Abnormal Cerebellar Cytoarchitecture and Impaired Inhibitory Signaling in Adult Mice Lacking Testicular Orphan Nuclear Receptor 4

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Abstract

Since Testicular orphan nuclear receptor 4 (TR4) was cloned, its physiological functions remain largely unknown. In this study, the TR4 knockout (TR4^{-/-}) mouse model was used to investigate the role of TR4 in the adult cerebellum. Behaviorally, these null mice exhibit unsteady gait, as well as involuntary postural and kinetic movements, indicating a disturbance of cerebellar function. In the TR4^{-/-} brain, cerebellar restricted hypoplasia is severe and cerebellar vermal lobules VI and VII are underdeveloped, while no structural alterations in the cerebral cortex are observed. Histological analysis of the TR4^{-/-} cerebellar cortex reveals reductions in granule cell density, as well as a decreased number of parallel fiber boutons that are enlarged in size. Further analyses reveal that the levels of GABA and GAD are decreased in both Purkinje cells and interneurons of the TR4^{-/-} cerebellum, suggesting that the inhibitory circuits signaling within and from the cerebellum may be perturbed. In addition, in the TR4^{-/-} cerebellum, immunoreactivity of GluR2/3 was reduced in Purkinje cells, but increased in the deep cerebellar nuclei. Together, these results suggest that the behavioral phenotype of TR4^{-/-} mice may result from disrupted inhibitory pathways in the cerebellum. No progressive atrophy was observed at various adult stages in the TR4^{-/-} brain, therefore the disturbances most likely originate from a failure to establish proper connections between principal neurons in the cerebellum during development.

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Keywords

Testicular orphan nuclear receptor 4; cerebellar atrophy; locomotor

1. Introduction

Testicular orphan nuclear receptor 4 (TR4) is a member of the nuclear receptor superfamily and has been classified as an orphan receptor because its ligand has not been identified (Chang et al., 1994; Hirose et al., 1994; Law et al., 1994). Currently, the physiological role of TR4 remains unclear; however, the presence of a homologous DNA binding domain suggests that, functionally, TR4 works as a transcription factor (Chang et al., 1994; Lee et al., 2002). Molecular studies using *in vitro* expression systems have demonstrated that TR4 could either enhance or diminish the ability of other nuclear receptors to bind direct repeat sites (AGGTCA) by forming heterodimeric complexes with them (Lee et al., 1995; Young et al., 1997) or through competition for direct repeat sites with various nuclear receptors (Hwang et al., 1998; Inui et al., 2003; Young et al., 1998). These unique molecular characteristics may endow TR4 with the ability to modulate various nuclear receptor-driven signaling pathways.

During the past few decades, by using knockout mouse models, the roles of dozens of genes with previously unknown physiological function have been explored. To further investigate the role of TR4 *in vivo*, a TR4 knockout mouse model was created (Collins et al., 2004). TR4^{-/-} mice display profound phenotypes, including growth retardation, impaired spermatogenesis, and various behavioral abnormalities (Chen et al., 2005b; Collins et al., 2004; Mu et al., 2004), suggesting multiple functions. Intriguingly, TR4 has been shown to be highly expressed in the central nervous system (CNS) of embryonic rodents and is thus believed to play an important role in CNS development (van Schaick et al., 2000). Indeed, in embryonic and postnatal mice lacking functional TR4 protein, profound deficits in neuronal proliferation and migration were observed in the cerebellum (Chen et al., 2005b). Although the developmental abnormalities in the immature TR4^{-/-} cerebellum suggest a role for TR4 during cerebellar development, the abundant expression of TR4 in the adult CNS, including the hypothalamus, hippocampus, and cerebellum (Chang et al., 1994; Lopes da Silva et al., 1995), raises the possibility that TR4 may also be important for mature brain function, particularly in the cerebellum.

The cerebellum is an unique region of the CNS; it only represents a small portion of the whole brain in volume, but contains more than half of the neurons in the nervous system and consists of a relatively simple cellular circuitry compared to other brain regions (Herrup and Kuemerle, 1997). In past decades, numerous anatomic and physiological studies have suggested involvement of the cerebellum in motor coordination, as well as in higher-order processing (Desmond and Fiez, 1998). Thus, deficits in the function of the cerebellum, in the human particularly, may not only result in impaired movement, but it could also contribute to developmental and psychological disorders, such as autism and schizophrenia (Schmahmann, 1998; Schmahmann and Caplan, 2006). With the advance of modern technologies, such as electrophysiology, target gene manipulations, as well as mutant mouse models, the underlying mechanisms controlling the function and development of the cerebellum are being determined. Among these technologies, the genetic mutant mouse provides a model of ubiquitous loss through which to interpret the relevance of one particular gene within the cerebellum (Chizhikov and Millen, 2003; Heintz and Zoghbi, 2000). Several genes reported to be important in cerebellar development have also been found to be essential for maintaining neuronal plasticity beyond developmental stages. Some examples include the effect of the Retinoid-related Orphan Receptor Alpha (ROR α) on dendritic plasticity of Purkinje cells (Boukhtouche et al., 2006), Cyclin-dependent kinase 5 (Cdk5) in neurotransmitter transport and

neurodegeneration (Tanaka et al., 2001; Tomizawa et al., 2002), and Reelin in synaptic transmission (Beffert et al., 2004). These genes are of particular interest because some of the histological and behavioral phenotypes of developing TR4^{-/-} mice are similar to those of RORα^{-/-} mice (*Staggerer*), Reelin deficient mice (*reeler*), and Cdk5^{-/-} mice (Gold et al., 2006; Herrup and Mullen, 1979; Trenkner, 1979; Yoon, 1972; Yuasa et al., 1993).

To examine the possibility that the absence of functional TR4 would result in defects in cerebellar structure and function at adult stages, TR4^{-/-} brain samples from adult mice were analyzed in the present study. Histological analysis indicates that the production of the major inhibitory neurotransmitter gamma-amino butyric acid (GABA) was diminished in the adult TR4^{-/-} cerebellum. This decrease in GABA content may be a consequence of alterations in principal cell populations during postnatal development. Given that no further cellular atrophy or loss of laminar organization was found in the TR4^{-/-} cerebellum at various adult stages, the disturbance of motor coordination in adult TR4^{-/-} mice most likely results from abnormal cerebellar development.

2. Results

2.1 Cerebellar hypoplasia in adult TR4^{-/-} mice

We previously demonstrated profound abnormalities in cerebellar development in TR4^{-/-} mice during embryonic and postnatal stages, which may be correlated with behavioral deficits observed in adult TR4^{-/-} mice (Chen et al., 2005b). To determine whether the lack of TR4 beyond developmental stages would lead to further degeneration, particularly in the cerebellum, the adult TR4^{-/-} brain was analyzed.

At 12 months of age, the TR4^{-/-} brain was slightly reduced in size, a phenotype apparent in gross appearance, at the levels of both the intact whole brain (Fig. 1A) and at midsagittal section (Fig. 1B), when compared with littermate TR4^{+/+} controls; the midsagittal size of the TR4^{-/-} brain was reduced to 83% of TR4^{+/+} controls (Fig. 1D). However, TR4^{-/-} mice were also smaller than littermate TR4^{+/+} mice in both trunk length and body weight (76.5%; 60% respectively, compared to littermate TR4^{+/+} controls) (Collins et al., 2004). Therefore, when taking this decrease into account, the reduction in overall brain size of TR4^{-/-} mice is modest. Histologically, in the TR4^{-/-} cerebrum, no significant differences in neuronal arrangement within the cortical layers were observed (Fig. 2B, insets). In addition, no abnormality was seen in the zonal arrangement of TR4^{-/-} cortical grey matter when compared with TR4^{+/+} controls. The differences in neuronal density and zonal arrangement of the hippocampal regions, CA1, CA3 and dentate gyrus were also marginal between TR4^{+/+} and TR4^{-/-} mice (Fig. 2B and C), despite abundant TR4 expression reported in this region (Chang et al., 1994; Lopes da Silva et al., 1995). The only difference observed in the TR4^{-/-} cerebrum was the reduction of Luxol fast blue staining in the corpus callosum, suggesting that the myelination of neurites may be disrupted (Fig. 2B).

In contrast to the subtle differences observed in the cerebrum, the size of the cerebellum was markedly reduced, even after accounting for the overall reduced size of the TR4^{-/-} brain. The TR4^{+/+} cerebellum was 15.83% of the size of the entire brain whereas the TR4^{-/-} cerebellum was 10.45% of the entire brain ($P < 0.05$, Student's t-test) (Fig. 1D). Gross appearance of the whole brain shows no clear demarcation between folia VI and VII in the dorsal-posterior aspect of the TR4^{-/-} cerebellum (Fig. 1C, arrowhead). Although the palm structure was retained in the cerebellum, the branch on the tip of each folium was stunted, and the fissure between vermal lobules VI and VII failed to develop (Fig. 2D and E). Histologically, substantial hypoplasia was observed in the TR4^{-/-} cerebellum when compared with littermate TR4^{+/+} controls (Fig. 2D). Additionally, the region of posterior/superior folia was stunted in the TR4^{-/-} cerebellum compared to the same region in littermate TR4^{+/+} controls (Fig. 2D, arrowhead).

Under higher magnification, reductions in the sizes of the molecular layer (ML) and the granule cell layer (GCL) were found in the TR4^{-/-} cerebellar cortex, in contrast with comparable lobules in the TR4^{+/+} cerebellum (Fig. 2F, 3A). Additionally, the density of granule cells in the GCL was reduced by 26% in the TR4^{-/-} cerebellum (Fig. 3B). Ultrastructurally, granule cell-Purkinje cell synaptic boutons in the TR4^{-/-} cerebellar cortex were reduced in number by approximately 45% (Fig. 4A and B), but increased by 40% in size (Fig. 4C), compared to those found in littermate TR4^{+/+} controls. Together, these data show cytoarchitectural changes in the TR4^{-/-} brain that are restricted to the cerebellum.

2.2 Disrupted cerebellar circuitry in TR4^{-/-} mice

Histological analyses in the TR4^{-/-} brain reveal significant atrophy in the cerebellar region with a proportional change in the numbers of principal neurons (granule and Purkinje cells), suggesting that the neuronal circuitry of the TR4^{-/-} cerebellum might be altered. To examine this possibility, immunohistochemical analysis of GABA, the major inhibitory neurotransmitter in the cerebellum, was conducted. In the adult TR4^{-/-} cerebellar cortex, the intensity of GABA-positive signal was reduced specifically in the somata of Purkinje cells (Fig. 5A, arrowheads). Moreover, this phenomenon was also observed in inhibitory interneurons, the stellate cells and basket cells, in the ML, when compared with littermate TR4^{+/+} controls (Fig. 5A, arrows). Further analysis of the amount of glutamic acid decarboxylase (GAD), the synthetic enzyme for GABA, also shows diminished immunoreactivity in the TR4^{-/-} cerebellum, particularly in GABAergic neurons, Purkinje cells and interneurons (Fig. 5B). In contrast to the significant reduction in the immunoreactivities of GABA and GAD, comparable glutamate levels were observed in both TR4^{-/-} and TR4^{+/+} cerebellar cortices (Fig. 5C). This finding suggests that the reduction in GABA levels in Purkinje cells of the TR4^{-/-} cerebellum does not result from the depletion of substrate content, but is likely related to mechanisms controlling GAD amount.

The reduced levels of GABA and GAD in GABAergic neurons in the TR4^{-/-} cerebellum suggest that neuronal activity might be altered. To assess this possibility, the level of the AMPA type glutamate receptor was examined. The immunoreactivity of GluR2/3 in the TR4^{+/+} cerebellar cortex was prevalent in the somata of Purkinje cells, while slight staining was observed in the dendrites; however, in the TR4^{-/-} cerebellum, the intensity of GluR2/3 staining was reduced in the Purkinje cell bodies, and no immunoreactivity was detected in the dendrites (Fig. 6A). Alternatively, GluR2/3 immunoreactivity was elevated in the deep cerebellar nuclei of TR4^{-/-} mice when compared with TR4^{+/+} controls (Fig. 6B).

2.3 Increased locomotor activity of TR4^{-/-} mice

Previously, TR4^{-/-} mice have been reported to exhibit an unsteady gait and failure to maintain balance on a horizontal rod, suggesting deficiencies in motor coordination (Chen et al., 2005b). In the home cage, TR4^{-/-} mice tend to prefer cage corners; also, tremors were frequently observed at the end of motor episodes, such as grooming or ambulatory activity. This phenotype, in conjunction with histological abnormalities in the cerebellum, suggest that although TR4^{-/-} mice can process locomotor commands from the cortical motor region, inhibitory modulation from the cerebellum may not be sufficient to adjust/cease the movement once initiated. To test the hypothesis that cerebellar inhibition of motor function is impaired in TR4^{-/-} mice, spontaneous locomotor activity was monitored during 30-minute periods over three consecutive days. In TR4^{+/+} mice, the ambulatory distance declined progressively over a 30-minute period in a single day, as seen when the session was divided into six 5-minute segments (Fig. 7A, B and C, black bars). The average cumulative ambulatory distance each day also progressively decreased (Fig. 7A, B and C, insets, black lines), consistent with the normal exploratory pattern in these animals (Voikar et al., 2001). In TR4^{-/-} mice, the ambulatory distances declined over time in a single day and in consecutive days, suggesting

that spatial learning may be intact in these mice. However, there were significant differences in the pattern of acclimation. The average ambulatory distances in each segment were higher in TR4^{-/-} mice than those in controls over the 3 days (Fig. 7 A, B, C). In addition, TR4^{-/-} mice showed increased locomotor activity in the first 5 minutes of being introduced into a new environment on the first day (Fig. 7A, section 1). This difference became more significant when animals were re-exposed to the same chambers on the second and third days (Fig. 7, section 1). One plausible explanation for the increased locomotor activity in TR4^{-/-} mice right after being manually transferred from the home cage to the testing chamber is that voluntary movement, such as escape or avoidance behaviors, triggered hyperactivity. Consistent with this assumption, higher stereotypic counts were observed in TR4^{-/-} mice compared with littermate TR4^{+/+} controls in the first segment of each task (Fig. 7D). By visual observation of animals during testing sessions, a pronounced tremor was noted in the TR4^{-/-} mice, but obsessive grooming was not observed. Thus, the pronounced increase in locomotor activity of TR4^{-/-} mice is likely to reflect a deficiency in the ability of the cerebellum to modulate voluntary movement rather than sensorimotor impairment.

2.4 Absence of nest building activity in TR4^{-/-} mice

During our observations of TR4^{-/-} mice in their home cages, one distinguishable phenotype appeared consistently; TR4^{-/-} mice did not use provided cotton squares for nesting building. None of the TR4^{-/-} mice ($n > 20$) built a nest during 7 consecutive days of monitoring without physical interruption. Occasionally, a few separated pieces of cotton could be found in the home cage. In contrast, TR4^{+/+} mice built nests within 2 hours following the introduction of a new cotton square. As shown in the nest building task, in TR4^{+/+} mice, newly built nests were observed as early as 2 hours after the task began (2 out of 4); 24 hours later, each TR4^{+/+} mouse had built a nest in the corner of the cage. In contrast, no nests were built in the cages that hosted TR4^{-/-} mice, although the cotton squares had all been moved from the center of each cage (Fig. 8A). Interestingly, while monitoring the nesting behavior of TR4^{+/+} mice, we found that nest building requires coordination of mouth and limbs, and the execution of various movements, such as holding and tearing. Thus, the lack of motor coordination and impaired regulation of fine movements may account for, at least in part, the failure of TR4^{-/-} mice to perform this particular task.

In addition to the abnormal behavioral phenotypes in TR4^{-/-} mice, long toenails were found on both the front and hind paws when compared with littermate TR4^{+/+} controls (Fig. 8B). This phenotype suggests that grooming might be impaired in these animals. In agreement with this assumption, the fur of all TR4^{-/-} mice appeared less smooth in comparison to TR4^{+/+} controls (data not shown). Thus, our findings indicate defects in fine motor skills which require limb coordination, such as grooming, digging, and tearing, in TR4^{-/-} mice.

3. Discussion

In this study, histological analyses of different areas of the adult TR4^{-/-} brain revealed hypoplasia restricted to the cerebellum. In TR4^{-/-} mice, the cerebellum was significantly reduced in size and showed diminished fissure structure; notably, cerebellar lobules VI and VII failed to develop. Previous studies using methods of inbreeding have revealed several quantitative trait loci that might be involved in modulating the size and structure of the cerebellum. Furthermore, the authors also demonstrated that the proportional size of the cerebellum compared to the whole brain is relatively conserved between strains despite alterations in brain size and weight, as well as in body weight (Airey et al., 2001; Williams, 2000). Structural and functional analyses have identified TR4 as a transcription factor, therefore, it is highly possible that TR4 may directly or indirectly regulate the expression of genes that are involved in determining brain size. In the TR4^{-/-} cerebellar cortex, the density

of granule cells, as well as the size of the IGL, were reduced when compared with TR4^{+/+} controls. The change in cell proportions in the TR4^{-/-} cerebellum was accompanied by a decrease in the density of parallel fiber-Purkinje cell dendritic boutons. Interestingly, the parallel fiber boutons also showed significant enlargement, possibly to compensate for attenuated synaptogenesis. These alterations in principal cell number and bouton density were not simply reflections of the smaller TR4^{-/-} cerebellum because similar changes in other cell populations, such as interneurons, were not observed (data not shown). Taken together, the decrease in the number of granule cells and in the number of parallel fiber boutons suggest that excitatory input to the Purkinje cells may be reduced 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. Further examination of glutamate content in the cerebella of mice of both genotypes indicates that the reduction of GABA amount may not be a consequence of the depletion of GAD substrate. Intriguingly, GABA and GAD labeling intensities were also diminished in the inhibitory interneurons in the ML, namely the stellate and basket cells. 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 (Herrup and Kuemerle, 1997; Voogd and Glickstein, 1998). Thus, the diminished GABA synthesis in interneurons and Purkinje cells raises the possibility that the reduced excitatory input from parallel fibers may not be able to trigger the generation of GABA, and subsequently interfere with the intrinsic inhibitory circuitry of the TR4^{-/-} cerebellar cortex. Several studies have provided substantial evidence that the levels of GABA and GAD in GABAergic neurons are correlated with neuronal activity (Hendry et al., 1988; Izzo et al., 2001). A recent study, combining the use of patch clamp electrophysiology and immunocytochemistry in primary cultured GABAergic neurons, showed that the amplitude of miniature inhibitory postsynaptic currents (mIPSCs) was reduced when neuronal activity was blocked with antagonists for NMDA or AMPA receptors (Swanwick et al., 2006). Moreover, the effects on mIPSCs were traced to lower GABA content in these cells. Thus, the diminished GABA synthesis in the inhibitory neurons of the TR4^{-/-} cerebellum may reflect reduced excitatory input to these cells that leads to a subsequent 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 (Voogd and Glickstein, 1998), the reductions in GABA and GAD amount, specifically in Purkinje cells in the TR4^{-/-} cerebellum suggest that the inhibitory output from cerebellar cortex might be impaired. In the cerebellum, the deep cerebellar nuclei (DCN) are the primary targets of Purkinje cell axons. These inhibitory afferents are believed to modulate the excitatory input to the DCN from the collaterals of mossy fibers and climbing fibers. After integrating all of this information, the DCN transmit excitatory signals to the thalamic nuclei and intralaminar thalamic nuclei, which subsequently send out excitatory projections to various motor function related regions in the cerebral cortex or limbic cortices (Apps and Garwicz, 2005). Thus, the diminished amount of GABA in the Purkinje cells further suggests that signaling in the TR4^{-/-} cerebellum may not provide sufficient inhibition to the DCN, which may then lead to the inability to terminate motor commands once initiated.

Studies using cannabinoid and Δ^9 -tetrahydrocannabinol application to suppress glutamatergic synaptic transmission in the cerebellum provided direct evidence that diminished excitatory input could result in the reduction of GluR2/3 expression in Purkinje cells, and lead to dysfunction of the cerebellum (Suarez et al., 2004; Szabo et al., 2000). Given that the amount of AMPA receptors in GABAergic neurons has been correlated with the neuronal activity of these cells (Suarez et al., 2004), the observed decreases in GABA/GAD and GluR2/3 amounts in the Purkinje cells of the TR4^{-/-} cerebellum suggest 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 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 to Purkinje cell dendrites. Therefore, 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 ultra-architectural 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.

Consistent with the profound abnormalities found cytoarchitecturally and immunohistochemically in the TR4^{-/-} cerebellum, mice lacking functional TR4 show several typical behavioral phenotypes indicative of cerebellar dysfunction. In this study, increased spontaneous locomotor activity was observed in TR4^{-/-} mice when introduced to an unfamiliar environment, and this phenomenon persisted in subsequent days of testing (Fig. 7). In contrast, the rearing or vertical movement counts were not different between TR4^{-/-} mice and TR4^{+/+} controls (data not shown), suggesting that exploratory behavior might be intact in the TR4^{-/-} mice. One of the phenotypes of TR4^{-/-} mice is hyperkinetic response following physical stimulation/manipulation or handling, as revealed by increased ambulation and stereotypic counts during the first 5 minutes of each trial. Moreover, this phenotype persisted in days 2 and 3 (Fig. 7), as would be expected if it were a response to physical handling. It is known that one of the functions of the cerebellum in motor control is adjusting and ceasing subsequent movement accordingly by integrating information received from the sensory system and motor cortex (Voogd and Glickstein, 1998). Therefore, the increased locomotor activity in TR4^{-/-} mice may result from the disturbance of cerebellar function, or TR4 may be involved in modulating the underlying mechanisms that control habituation and anxiety, such as the function of the hypothalamo-pituitary-adrenal axis or the production of corticotrophin-releasing factor (Kasahara et al., 2006; Marin et al., 2007).

Interestingly, TR4^{-/-} mice do not demonstrate nest building ability, and this abnormal behavior was found 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 the thermo-regulation of mice and with the function of the hippocampus (Bhatia et al., 1995; Deacon et al., 2002; Woodside and Leon, 1980). In one study in mice, damage of the hippocampal dorsal region, but not the cortical or thalamic regions, impairs nest construction (Deacon et al., 2002). In TR4^{-/-} mice, however, no difference was found in either body temperature or hippocampal architecture, suggesting that the lack of TR4 function may interfere with other unknown mechanisms that govern this behavior. Another 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. This inference is partly supported by the absence of nest building behavior in two cerebellar mutant mice, staggerer and weaver (Bulloch et al., 1982). Although the authors suggested that this behavioral deficiency may be caused by a global effect of the gene mutation in other systems, such as the endocrine system or the function of the suprachiasmatic nucleus (Bult et al., 2001), our findings in the TR4^{-/-} mice suggest that dysfunction of the cerebellum may be involved in the execution of this behavior. Indeed, the TR4^{-/-} mice display deficits in other tasks that require fine motor control, such as grooming.

Recently, several psychiatric diseases such as fragile X syndrome (fra X), autism, and attention-deficit hyperactivity disorder (ADHD), which were previously recognized as resulting from deficiencies in the cerebral cortex, have also now been linked to malfunction of the cerebellum

(Berquin et al., 1998; Courchesne et al., 1988; Huber, 2006; Mostofsky et al., 1998; Palmen et al., 2004; Schmahmann, 1998). Several lines of evidence from analysis of fra X and autistic brains, have suggested that the diminished posterior superior lobe in these patients results from defective development rather than degeneration after the formation of the mature cerebellum (Mostofsky et al., 1998; Reiss et al., 1988). In addition, cerebellar abnormalities were also observed in patients with ADHD, obsessive-compulsive disorders, and schizophrenia, via profound anatomical evidence. Specifically, in schizophrenia, deficiency in the prefronto-thalamic-cerebellar circuit was suggested by a positron-emission tomography study (Andreasen et al., 1996). In TR4^{-/-} mice, hypoplasia was specific to the cerebellum and was initially observed during postnatal development. Given the fact that there is no significant progressive structural degeneration found at subsequent ages, it is likely that the cerebellar abnormalities found in TR4^{-/-} mice result primarily from developmental deficiencies, as reported previously (Chen et al., 2005b). Some of the characteristics of the TR4^{-/-} cerebellum are comparable to those found in the cerebella of autism and fra X patients, suggesting that the TR4^{-/-} cerebellum may serve as a model to explore the relevance of deficiencies in cerebellar function in those neurodevelopmental disorders.

In adult TR4^{-/-} mice, in addition to lack of motor coordination, frequent tremors were observed following intentional movements, such as grooming or ambulatory behavior. Further, hyperkinetic responses were triggered when the TR4^{-/-} mice were lifted by the tail or simply held in place on a horizontal surface. The tremor in TR4^{-/-} mice that is evoked by voluntary movement shares characteristics with the involuntary movement disorder essential tremor. Although the underlying mechanisms of essential tremor remains a mystery, accumulating studies suggest that deficits in the cerebello-thalamo cortical pathway may play a significant role (Chen et al., 2005a). The postural and kinetic tremors observed in TR4^{-/-} mice have similarity to behavioral symptoms present in essential tremor patients, suggesting that the TR4^{-/-} mouse may be a good animal model for evaluating the effectiveness of pharmaceutical intervention, or for exploring the underlying mechanisms of essential tremor.

Current data from studies of the TR4^{-/-} brain provide evidence that abnormal behaviors observed in TR4^{-/-} mice may be due to dysfunction of the cerebellum. Since neurodegeneration was not found in the TR4^{-/-} cerebellum at any of the ages examined, the malfunction of the adult TR4^{-/-} cerebellum most likely originates from developmental deficits rather than from adult-stage effects of the loss of TR4 function. Together, our results suggest that, in the cerebellum, TR4 may play a critical role during developmental stages, but may be less critical in the mature animal. However, given its function as a transcription factor and its potential for modulating gene expression through DNA binding and transactivation, the possibility of a direct role of TR4 in the function of mature neurons needs further investigation.

4. Experimental Procedures

4.1 Animals

TR4^{+/+} and age-matched TR4^{-/-} mice for this study were produced from heterozygous breeding pairs, which were obtained from Lexicon Genetics Incorporated (The Woodlands, Texas), having been generated as described (Collins et al., 2004). Genotypes of the mice were determined by PCR analysis of tail genomic DNA as described previously (Collins et al., 2004). Mice were housed in the Vivarium of the University of Rochester Medical Center, provided a standard diet with constant access to food and water, and maintained on a 12-hour light/dark cycle. All mice used in this study were of a hybrid C57BL/6 and 129/SvEv background, and all experimental protocols were approved by the University Committee on Animal Resources (UCAR).

4.2 Histological analysis and immunohistochemistry

Mice were sacrificed using a lethal dose of sodium pentobarbital (250 mg/kg) in accordance with UCAR guidelines. For tissue processing, in brief, cerebella of TR4^{+/+} and TR4^{-/-} mice were fixed overnight, or longer, in fresh 10% buffered paraformaldehyde, then dehydrated through a series of graded alcohols before being embedded in paraffin. Sagittal sections from TR4^{+/+} and TR4^{-/-} cerebella were cut at 4 μ m and placed on slides for staining.

For immunohistochemical staining, sections were first incubated with anti-GABA (1:300; Sigma, St. Louis, MO), anti-GAD (1:250; Chemicon, Temecula, CA), anti-Glutamate (1:250; Sigma, St. Louis, MO), or anti-GluR2/3 (1:250; SpringBio, Fremont, CA) antibodies. After incubation with primary antibodies, sections were washed 3 times with PBS and incubated with corresponding biotin-conjugated secondary antibodies (1:200). To visualize biotin-conjugated staining under bright field microscopy, the avidin-biotin-immunoperoxidase complex method (ABC) (Vector, Burlingame, CA), and the diaminobenzidine substrate (DAB) (Vector, Burlingame, CA) were used. All immunostaining results were replicated in 6 pairs of mice, and at least 3 sagittal-spinocerebellar sections were obtained from each animal. After visualization by using ABC and DAB methods, TIFF images were captured, without genotype labeling, using the SPOT imaging system, and images were further analyzed using the image analysis program Scion Image. Finally, the relative immunoreactivities, based on labeling intensity, were translated to pixel values and compared between genotypes using the Student's t-test. The terms "increased" or "reduced" were used when differences between genotypes were statistically significant ($p < 0.05$).

4.3 Calculations of cerebellar cortical area

For brain morphometry, coronal sections of the cerebrum were cut, based on markers on the ventral aspect of each brain, to obtain comparable brain regions for analysis. A coronal cut was made at the anterior edge of the optic chiasm to obtain comparable regions of the front parietal cortex, and a coronal cut was made at the center of the median eminence (below the hypothalamus and 3rd ventricle) to obtain comparable regions of the dorsal lobes of the hippocampus and the corpus callosum. The sizes of the ML and GCL in the midsagittal cerebellar cortex of TR4^{+/+} and TR4^{-/-} mice were determined by photographing Nissl-stained transverse sections, using a digital camera (Diagnostic Instruments, Inc.) mounted on a Nikon microscope. Randomly selected regions, 10 per sample, in the center of lobules at identical anteroposterior and mediolateral coordinates, were measured. SPOT software (Diagnostic Instruments, Inc.) was used to examine the sizes of cerebellar layers. Quantitative data were obtained from 4 sections per mouse and 8 mice per genotype.

Electron microscopic analysis of granule/Purkinje cell synapses in the cerebellar cortex was carried out using a JEOL microscope, according to previously described methods (Meshul et al., 1994; Sirvanci et al., 2005). For quantification, electron micrographs were imported into ImagePro Plus software, granule cell terminal boutons were traced, and area measurements were obtained via conversion of pixel counts.

4.4 Behavioral analysis

Locomotor activity—Locomotor activity was measured by using the DIG-729 photo-beam system (Med Associates Inc., Albans, VT). Each testing chamber (ENV-510, 27 $\times$ 27 $\times$ 20.3 cm) contained horizontal infrared (I/R) sources and sensors. The subject location was tracked by 16 evenly spaced I/R beams on each axis (X, Y). To monitor spontaneous locomotor activity, each mouse was housed separately for 48 hours prior to behavioral testing. Each mouse was tested individually (6 TR4^{+/+} mice; 6 TR4^{-/-} mice) for 30 minutes, for 3 consecutive days, during daytime hours. Each testing chamber was cleaned after each individual test was performed to prevent disturbance from the odors left by the previous test subject. Ambulatory

distance was measured as the distance the mouse travels from the end of the last ambulatory episode to the end of the subsequent episode. Data are expressed as mean + S.E.M ambulatory distance in 5 minute periods, or as cumulative distance over the entire 30 minute session. The stereotypic count was defined as the number of times the surrounding light beams were broken when the mouse was in resting status. Data are expressed as mean + S.E.M.

Nest building task—For the nest building task, 4 TR4^{+/+} and 4 TR4^{-/-} mice were introduced into new cages, individually, each was provided with one cotton square (5 cm²) in the middle of the cage, and mice were monitored for 24 hours. Each cage was photographed at 1, 2, and 24 hours after the task began.

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Fig. 1. Gross appearance of the TR4^{-/-} brain

Dorsal view (A) and a midsagittal view (B) of whole brains from TR4^{+/+} and TR4^{-/-} mice. Dorsal posterior view of the cerebellum from TR4^{+/+} and TR4^{-/-} mice; arrowhead points to vermal lobules VI and VII (C). Quantitative result of Midsagittal size of TR4^{+/+} and TR4^{-/-} whole brain sections. The data shown are means + S.E.M. Gray and black bars represent of cerebella and cerebral cortex portions, respectively. Mouse genotypes as indicated. Cerebellar lobules are indicated by Roman numerals in C. Cx, cerebral cortex; Cb, cerebellum; P, pons; M, medulla; Bs, brain stem. Scale bar in A and B, 5 mm.

Fig. 2. Cerebral and Cerebellar morphology in TR4^{+/+} and TR4^{-/-} mice
 Appearance of sagittal brain sections from TR4^{+/+} and TR4^{-/-} mice (A). Comparable regions of the hippocampus and the corpus callosum (open arrowheads) from Luxol fast blue stained TR4^{+/+} and TR4^{-/-} cerebral cortex coronal sections (B). Closed arrowheads point to the 3rd ventricle as an indication of comparable brain regions. Insets in B represent cerebral cortical layers from both genotypes; layers are indicated by Roman numerals. Nissel stained, midsagittal cerebral cortical sections revealed similar architecture between TR4^{+/+} and TR4^{-/-} mice (C). Aberrant folia arrangement in TR4^{-/-} cerebellar sections is indicated by arrowheads (D). Magnified views of folia VI and VII from both genotypes (E); asterisks indicate abnormal fissure structure in the TR4^{-/-} cerebellum. Nissle-stained sections from comparable lobules of TR4^{+/+} and TR4^{-/-} cerebella (F). Mouse genotypes are indicated. Cerebellar lobules are indicated by Roman numerals in D. Cx, cerebral cortex; Cb, cerebellum; A, anterior portion; P, posterior portion of cerebellum; ML, molecular layer; PCL, Purkinje cell layer; GCL, internal granule cell layer. Scale bars in (C) and (E), 250 μ m; in (D), 400 μ m.; in (F), 20 μ m.

Fig. 3. Differences in cortical layer thickness, and granule cells density in TR4^{+/+} and TR4^{-/-} cerebella

Size of cerebellar cortical layers (A) and granule cells density (B) in TR4^{+/+} and TR4^{-/-} mice. In (A), gray bars represent GCL, open bars represent ML. In B, gray and open bars represent TR4^{+/+} and TR4^{-/-} mice, respectively. ML, molecular layer; GCL, granule cell layer. The data shown are means + S.E.M. * P < 0.05, student's t-tests; n = 6.

Fig. 4. Parallel fiber synaptic defects in the TR4^{-/-} mouse cerebellum

Differences in parallel fiber synaptic boutons between TR4^{+/+} and TR4^{-/-} cerebella (A) Pf, indicate the boutons; S, Purkinje cell dendritic spine; d, Purkinje cell dendrite. Ultrastructurally, the number of synaptic terminal boutons of granule cell parallel fibers in the cerebella of TR4^{-/-} mice was reduced by approximately 45% (B), but the size of individual boutons was increased by 40% (C), compared to those found in TR4^{+/+} mice. Scale bar, 0.5 μm .

Fig. 5. Expression levels of GABA, GAD, and glutamate in the $TR4^{-/-}$ cerebellar cortex
 Immunostaining with anti-GABA (A), anti-GAD (B), and anti-glutamate (C) antibodies was performed on sagittal cerebellar sections from $TR4^{+/+}$ and $TR4^{-/-}$ mice, and arrows point to interneurons, arrowheads indicate Purkinje cells. ML, molecular layer; PCL, Purkinje cell layer; GCL, granule cell layer. Scale bar: 20 μ m. Representative of 3 samples of each genotype.

Fig. 6. GluR2/3 immunoreactivity is decreased in Purkinje cells and increased in deep cerebellar nuclei in the $TR4^{-/-}$ cerebellum

Immunostaining with an anti-GluR2/3 antibody was performed on sagittal cerebellar sections from $TR4^{+/+}$ and $TR4^{-/-}$ mice. Cerebellar cortex (A), and deep cerebellar nuclei (B); genotypes as indicated. Arrows in (A) indicate Purkinje cells, and in (B) point to deep cerebellar nuclei. ML, molecular layer; PCL, Purkinje cell layer; GCL, internal granule cell layer. Scale bar: 20 μ m. Representative of 3 samples from each genotype.

Fig. 7. Increased locomotor activity of TR4^{-/-} mice in the open field test

Open field activity of TR4^{+/+} (n=6) and TR4^{-/-} (n=6) mice was measured by automatic tracking software across 3 days of testing. Data are presented as means + S.E.M. of distance traveled per timeblock (section, 5 min) and cumulative distance across 30 minutes of the task (insets). TR4^{-/-} mice showed higher activity than TR4^{+/+} mice during each task (A), (B), (C). Greater differences appeared during task 2 (B) and 3 (C), and more activity was shown by TR4^{-/-} mice than TR4^{+/+} controls, both in the first 2 sections and in cumulative distance through out the 30 min trials. Stereotypic counts within the first 5 minutes of each task (D). The data shown are means + S.E.M. * P < 0.05, student's t-test; n = 6.

Fig. 8. Impaired nest building behaviors and prolonged nail length in $TR4^{-/-}$ mice
 (A), photographs of nests built by a $TR4^{+/+}$ mouse and $TR4^{-/-}$ mouse at 1, 2, 24 hours after introduction into a new cage with a cotton square placed in the center. All $TR4^{+/+}$ mice tested ($n=4$) were able to build nests in the corner of hosted cages, while no nests were built in $TR4^{-/-}$ mice cages. (B), photographs of long-nail phenotype of $TR4^{-/-}$ mice front and hind paws, arrow points to the prolonged nail.